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# Inhibitory role of neem seed kernel extracts and terpenoids on growth and aflatoxin production by *Aspergillus parasiticus* (NRRL 2999)

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The effect of neem seed kernel extracts and purified terpenoids on *Aspergillus parasiticus* in relation to fungal growth, ergosterol synthesis and aflatoxin elaboration was investigated in yeast extract sucrose (YES) liquid medium. Aqueous (0.05, 0.25, 0.5 and 1.0 %), hexane, methanolic, ethyl acetate and acid-methanolic extracts (0.01, 0.1, 0.5 and 1.0 %) of neem seed kernel were tested at 3, 5, 7, 9 and 12 days of incubation. Fungal growth was not affected by aqueous and hexane extracts, while it was inhibited by the methanolic, ethyl acetate and acid-methanolic extracts. The inhibition of ergosterol biosynthesis was 73 and 82 % by the aqueous extract at 0.5 and 1.0 % conc, while hexane and ethyl acetate inhibited in a dose dependent manner on day 7. The $AFB_1$/total toxin altered significantly ($p<0.01$) in the presence of various neem seed kernel extracts, except that of hexane extract. Purified terpenoids, azadirachtin, nimbin and salannin had no inhibitory effect on fungal biomass and aflatoxin elaboration at 0.01, 0.1, 0.25 and 0.5 % levels. Azadirachtin at 0.25 and 0.5 % level inhibited ergosterol biosynthesis by 30 and 34 %, respectively. Neem seed kernel extracts were inhibitive to the fungal growth, ergosterol biosynthesis and toxin elaboration as compared to purified neem terpenoids, suggesting anti-fungal and anti-aflatoxigenic properties.

**Keywords:** Neem seed kernel extracts, Terpenoids, *Aspergillus parasiticus,* Aflatoxin production, Ergosterol synthesis.

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## Introduction

Aflatoxins are toxic secondary metabolites produced by toxigenic strains of *Aspergillus* group of fungi, and have gained worldwide importance due to their hazardous biological effects in both humans and animals[1,2]. The biological effects include carcinogenic, mutagenic, immunosuppressive, dermatotoxic and teratogenic effects[3]. Among the major naturally occurring aflatoxins: $B_1$, $B_2$, $G_1$, $G_2$, and $M_1$, $AFB_1$ is the most potent[4]. Prevention of toxigenic mould contamination in agricultural commodities involves the use of natural or synthetic compounds[5-7]. Naturally occurring plant derived fungicidal compounds offer relatively lower incidence of adverse reactions and reduced cost over modern synthetic pharmaceuticals[8].

Neem tree (*Azadirachta indica* A. Juss.) has gained worldwide importance due to wide use of neem derived products in agriculture, health, medicine, toiletries, cosmetics and live stock production[9,10]. Further, the neem and its bioactive components are well known for their anti-inflammatory, anti-malarial, anti-microbial[11,12], insecticidal[13], and anti-conceptional properties[14,15] in medicine. Singh *et al*[16] have reported inhibition of fungal growth in *Fusarium oxyspurm, F. ciceri, Rhizoctonia solani* and *Sclerotium rolfsii* in the liquid medium containing extracts of leaf, trunk, bark, fruit pulp and neem oil. Leaf extracts of *A. indica* were found to inhibit the radial growth, sporulation and spore germination of *Curvularia lunata*[17]. In addition, sulphurous compounds present in *A. indica* leaves were found to possess fungicidal properties[18].

Among the various parts of the neem tree, neem kernels have gained more prominence due to the presence of bioactive compounds such as, terpenoids[19,20]. These terpenoids include azadirachtin, salannin, meliantriol, deacetyl azadirachtinol, vepol, isovepol, nimbidin and 7-deacetyl-17-hydroxy azadiradions[21-23]. Among these terpenoids, the mode of action of 'azadirachtin' has been well studied and it has been established that it interferes with the neuroendocrine regulation of ecdysteroid hormone[24]. Kaaya *et al*[25] suggested that neem seed kernel extracts may exhibit greater inhibitory effect on the growth of *A. flavus* and aflatoxin production, than the intact kernels or kernel fragments.

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Earlier studies have reported the fungicidal effects of extracts of neem leaf, neem oil and neem cake on various groups of fungi[16,26,27] whereas, the effect of various neem seed kernel extracts on toxigenic fungi *A. parasiticus* (NRRL 2999) has not been studied so far. In the recent past we have reported neem seed kernel as a poor substrate for aflatoxin elaboration under controlled experimental conditions, suggesting the possible role of terpenoids on anti-aflatoxigenic potential[28]. Hence, a detailed experimental investigation was undertaken to evaluate the fungistatic/fungicidal effect of various neem seed kernels extracts (aqueous, hexane, methanolic, ethyl acetate and acid methanolic) and purified bioactive terpenoids (azadirachtin, nimbin and salannin) on growth, aflatoxin elaboration and ergosterol content (an index of fungal biomass) in Yeast Extract Sucrose (YES) liquid medium, employing an established highly toxigenic strain of *A. parasiticus* (NRRL 2999).

## Materials and Methods

### Chemicals

Fresh neem seeds were obtained from a local market in Hyderabad. Reference standards for aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$) and ergosterol were obtained from Sigma Chemicals Co., St. Louis, USA. Azadirachtin, salannin, nimbin (each with 98% purity) were provided as gratis by the Southern Petrochemicals Industries Corporation (SPIC; Madras, India). All the other chemicals and solvents used were of analytical grade and purchased locally.

### Strain

A highly toxigenic fungus strain, *A. parasiticus* (NRRL 2999) was obtained from United State Department of Agriculture (USDA), Peoria, Illinois, USA. Cultures were maintained on potato dextrose agar (PDA) slants for eight days at 28.0 ± 1.0°C in a cooling incubator (Kalorstat, Dwaraka equipment (P) Ltd., Mumbai, India).

### YES liquid medium

Yeast extract sucrose (YES) broth with 2% (w/v) yeast extract and 15% (w/v) sucrose was prepared and the pH was adjusted to 6.0. Aliquots of 20 mL were dispensed into 100 mL Erlenmeyer flasks, plugged with cotton and autoclaved at 1.05 kg $cm^{-2}$ (103 kPa) pressure at 121°C for 15 min.

### Preparation and incorporation of different neem seed kernel extracts in YES liquid medium

Neem seed kernels were ground in a laboratory mechanical grinder (Sumeet, Mumbai, India) to a fine powder. The preparation of the various extracts of the neem seed kernel powder, containing various types of bioactive components was based on the method described by Schroeder and Nakanishi[29].

#### *Aqueous extracts*

The neem kernel powder (50 g) was mixed with one litre of sterile potassium phosphate buffer (10 mM, pH 7.0) and was left, overnight at 4°C until further use.

#### *Hexane extracts*

The neem kernel powder (250 g) was defatted with n-hexane by using Soxhlet apparatus[30]. Hexane was evaporated using flash evaporator (Superfit, Mumbai, India) at 45-50°C to obtain brown viscous oil. Four millilitres (equivalent to 4.0 g) of oil was dissolved in 2.0 mL volume of petroleum ether and was mixed thoroughly. To this total volume of the mixture, equal volume (6.0 mL) of sterile distilled water was added and shaken vigorously.

#### *Methanolic extracts*

Hundred grams of defatted powder was extracted thrice with 50 mL portions of the 95 % ethanol. The ethanol layer was separated by a sintered funnel and dried by flash evaporation. The resulting thick residue (46.0 g) obtained was subjected to two quick partitioning steps between petroleum ether and 95 % aqueous methanol. The petroleum ether layer was discarded and the methanolic fraction was dried by flash evaporation. The resultant methanolic extract was dried under nitrogen and 4.0 g was dissolved in 8.0 mL of sterile distilled water.

#### *Ethyl acetate extracts*

Ten grams of methanolic extract was dissolved in ethyl acetate (50 mL) and was partitioned with equal volume of double distilled water (50 mL). The ethyl acetate portion was separated and dried in a flash evaporator. The resultant thick brown residue (4.3 g) was again dried under nitrogen. Four grams of this dried material was dissolved in 2.0 mL of ethyl acetate and 2.0 mL of methanol. This mixture was shaken vigorously and was mixed with 4.0 mL sterile distilled water.

#### *Acid-methanolic extracts*

Defatted neem kernel powder (500 g) was extracted with 300 mL of 1.0 % (v/v) HCl in methanol for 1 h in a mechanical shaker. The methanolic portion was flash evaporated at 45-50°C to remove methanol and HCl. The residue (4.0 g equivalent) was then re-dissolved in 20 mL sterile water.

**Incorporation of different neem seed kernel extracts and terpenoids into YES medium**

All the extracts were filtered by using sintered funnel and then pH was adjusted to 6.0 with sodium hydroxide (0.1M). The extracts were then filter sterilized by passing them through a 0.45 µm membrane filter unit (Millipore products divisions, MA, USA) under aseptic conditions. Appropriate respective volumes of each extract were added to YES liquid media to get 0.05, 0.25, 0.5 and 1.0 % (w/v) of aqueous and 0.01, 0.1, 0.5 and 1.0 % (w/v) concentrations for hexane, methanol, ethyl acetate and acid methanol extracts. The flasks were incubated at 28.0 ± 1.0°C in a cooling incubator for 3, 5, 7, 9 and 12 days after inoculation of fungal spores. Appropriate controls were also prepared without neem seed kernel extracts.

The pure terpenoids (azadirachtin, salannin and nimbin) were dissolved in minimum amount of methanol and dispensed as aliquots of known concentrations into sterile glass stoppered tubes. Later the solvent was evaporated using sterile nitrogen. Four different concentrations (0.01, 0.1, 0.5 and 1.0% w/v) of terpenoids were prepared and incorporated in to sterile YES medium along with anionic detergent, sodium dodecyl sulphate (SDS) at 0.001% (w/v) concentration. In the present study, SDS (0.001%) in YES media was maintained for solubility of hydrophobic compounds of neem seed extracts, as our earlier studies revealed no effect on growth of *A. parasiticus* and aflatoxin elaboration at this concentration[31].

Controls (without the test compounds) were also prepared along with the experimental flasks. The flasks were incubated at 28.0 ± 1.0°C in cooling incubator for 7 days after inoculation of fungal spores.

**Inoculation of YES liquid media**

Aliquots of 5 x$10^5$ spores in a volume of 50 µL were used to inoculate flasks containing 20 mL of pre-sterilized YES broth under aseptic conditions. The flasks were maintained under stationary conditions at 28.0 ± 1°C in a cooling incubator. Fungus was allowed to grow for a period of 12 days, while monitoring growth and aflatoxin production at regular intervals of 3, 5, 7, 9 and 12 days. Duplicate flasks were maintained for each time point. At the end of the incubation period, the biomass was collected and thoroughly washed with quartz distilled water and later blotted with filter paper and kept at 70°C for drying. The weights of the dried biomass were recorded by using an analytical balance (Afcoset, Mumbai, India).

**Extraction and quantification of aflatoxins**

To the YES medium containing the mycelial mat, 20 mL extraction solvent (methanol:water, 55:45) was added and homogenized at 15000 rpm using a high speed mechanical blender (Boss, Mumbai, India). The homogenate was latter filtered through siliconized glass wool and was washed twice with 10 mL distilled water. Aflatoxin was extracted from the filtrate by solvent extraction procedure. The filtrate containing aflatoxins was initially defatted with 25 mL n-hexane in the presence of sodium chloride (80 mg/mL), followed by extraction with 10 mL chloroform, twice. The pooled chloroform extracts containing aflatoxin was passed through anhydrous sodium sulphate and flash evaporated to dryness at 45°C. The residue was reconstituted in benzene: acetonitrile (98:2).

Aflatoxins were estimated by the thin layer chromatography (TLC)/flourodensitometric method[32]. The TLC plates were developed in toluene: ethyl acetate: formic acid (6:3:1) solvent system and visualized under long-wave ultraviolet light (365 nm). Annotated digital density images of the spots were recorded by CCD camera (UVItec, Cambridge, UK) and saved in PC-compatible file format (tif. file) on a compact disk. Later, the digital density images of different toxins were analyzed by the software for determining the net density, which was measured as peak volume. Reference standards $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ were used for calculating the aflatoxin content in the samples.

**Extraction and quantification of ergosterol**

Ergosterol was extracted by the method described by Seitz *et al*[33]. The fungal biomass (15 mg, wet weight) was soaked in 5.0 mL 95% ethanol for 10-15 min and extracted for five min using a hand held high-speed mechanical blender (Boss, Mumbai, India) at 15000 rpm. The extract was centrifuged at 900×g for 10 min. The supernatant was separated and transferred into screw-cap tubes containing 0.152% butylated hydroxyl toluene and 8% potassium hydroxide. The pellet was re-extracted with four mL methanol, centrifuged and supernatants were pooled. The mixture was refluxed at 80°C for 15 min using a heating mantle and allowed to cool. Petroleum ether (15 mL) followed by double distilled water (20 mL) was added, vigorously shaken and allowed to separate. The aqueous phase was discarded and petroleum ether layer was passed through anhydrous sodium sulphate and evaporated to dryness. The residue was re-dissolved in 250 µL of benzene: acetonitrile (98:2).

Ergosterol was estimated by using the method reported by Sashidhar *et al*[34]. Ergosterol standard in the range of 0.1-2.0 µg in 10 µL were spotted onto an activated silica gel-G TLC plate. The plate was developed in a toluene:acetone (9:1) solvent system. The air-dried plate was exposed to iodine vapours in a pre-saturated glass chamber for less than 2 min. Presence of ergosterol was indicated by the development of yellow spots. The density of the yellow spots was scanned under ultra violet light at 365 nm using a TLC digital image based analysis system (UVItec, Cambridge, UK). Annotated digital density images of the yellow spots were recorded by the CCD (Charge Coupled Device) camera and saved in PC-compatible file format (tif. file) on a compact disk. Later, the digital density images of ergosterol were analysed by the software for determining the net density, which was measured as peak volume. A calibration curve, based on the area under the curve versus the concentration of ergosterol (reference standard), was used for calculating the ergosterol content in the samples.

#### Estimation of total sugars

Total sugar content was estimated by phenol-sulphuric acid method described by Dubois *et al*[35]. Aliquots of the filtrates of the culture media were taken at various time points and diluted appropriately. To 2.0 mL of the diluted filtrates, one mL of phenol reagent (5 % w/v) was added, followed by rapid addition of 5.0 mL concentrated sulphuric acid. After 30 min of incubation, the yellowish orange colour that developed was read at 490 nm (Spekol 1200, Analytik jena, Jena, Germany). As a reference standard, α-D-glucose (20-100 µg) was used to establish the calibration curve.

### Statistical analysis

The experimental data obtained in the present study was analysed by two-way analysis of variance (ANOVA), Student's *t*-test and Correlation[36]. The Sigma Stat Statistical software, version 1.0 (Jandel Corporation, San Rafael, CA, USA) software programme package was used in data analysis.

## Results

In the present study, the neem seed kernel extracts (aqueous, hexane, methanolic, ethyl acetate and acid-methanolic) were evaluated for their antifungal activity in YES liquid media inoculated with *A. parasiticus* (NRRL 2999). In the controls the fungal growth (as indicated by fungal biomass), total toxin and ergosterol content was found to increase with time, maximum being on day 7 (peak production) and thereafter it decreased till the end of the incubation period (day 12). The sugar utilization by the fungus was found to increase with the increase in the period of incubation. The $AFB_1$/total ratio for controls was calculated and used for comparison with the experimental groups.

#### Aqueous extract

The aqueous extract of neem seed kernel showed no effect on fungal growth. No statistically significant difference was observed in fungal biomass at all the concentrations (0.05, 0.25, 0.5 and 1.0%) when compared with controls (Fig. 1a) during different days of incubation. At various concentrations of aqueous extract, ergosterol content peaked on day 7 of the incubation period. The total toxin produced by the fungi was found to decrease with the increasing concentration of aqueous extract from 0.05 to 1.0%. At 1.0 % concentration, the toxin production was inhibited by 55 % on day 7 of the incubation period and it was found to be statistically significant (Table 1). The $AFB_1$/ total toxin ratio was different with respect to concentration and it was statistically significant ($p<0.05$; $p<0.01$) indicating high production of $AFB_1$ with respect to the total toxin (Table 2). Ergosterol biosynthesis was not effected at 0.05 and 0.25% level but 74 and 82% inhibition was shown at 0.5 and 1.0% concentration on day 7 respectively (Fig. 2a) and was statistically significant ($p<0.01$). Sugar utilization by the fungus during various time periods was unaffected in the presence of the aqueous extract from 0.05 to 1.0% (Table 3).

#### Hexane extract

Hexane extract of neem seed kernel from 0.01 to 1.0% conc. showed no affect on fungal biomass, toxin elaboration, $AFB_1$/total toxin ratio and sugar utilization and the difference was not statistically significant (Fig. 1b, Tables 1-3). Ergosterol synthesis was inhibited by 71 % at 0.1, 0.5 and 1.0 % conc. of neem seed kernel on day 7 of the incubation period and it was statistically significant ($p<0.01$) (Fig. 2b). At 0.01 % conc. the change in the ergosterol content during all time points was not significantly different.

#### Methanolic extract

Presence of methanolic extract in YES liquid medium has an inhibitory effect on growth of *A. parasiticus* as well as total toxin, ergosterol biosynthesis and sugar utilization at various time periods and it was statistically significant ($p<0.01$, $p<0.05$). The effect of methanolic extract on fungal

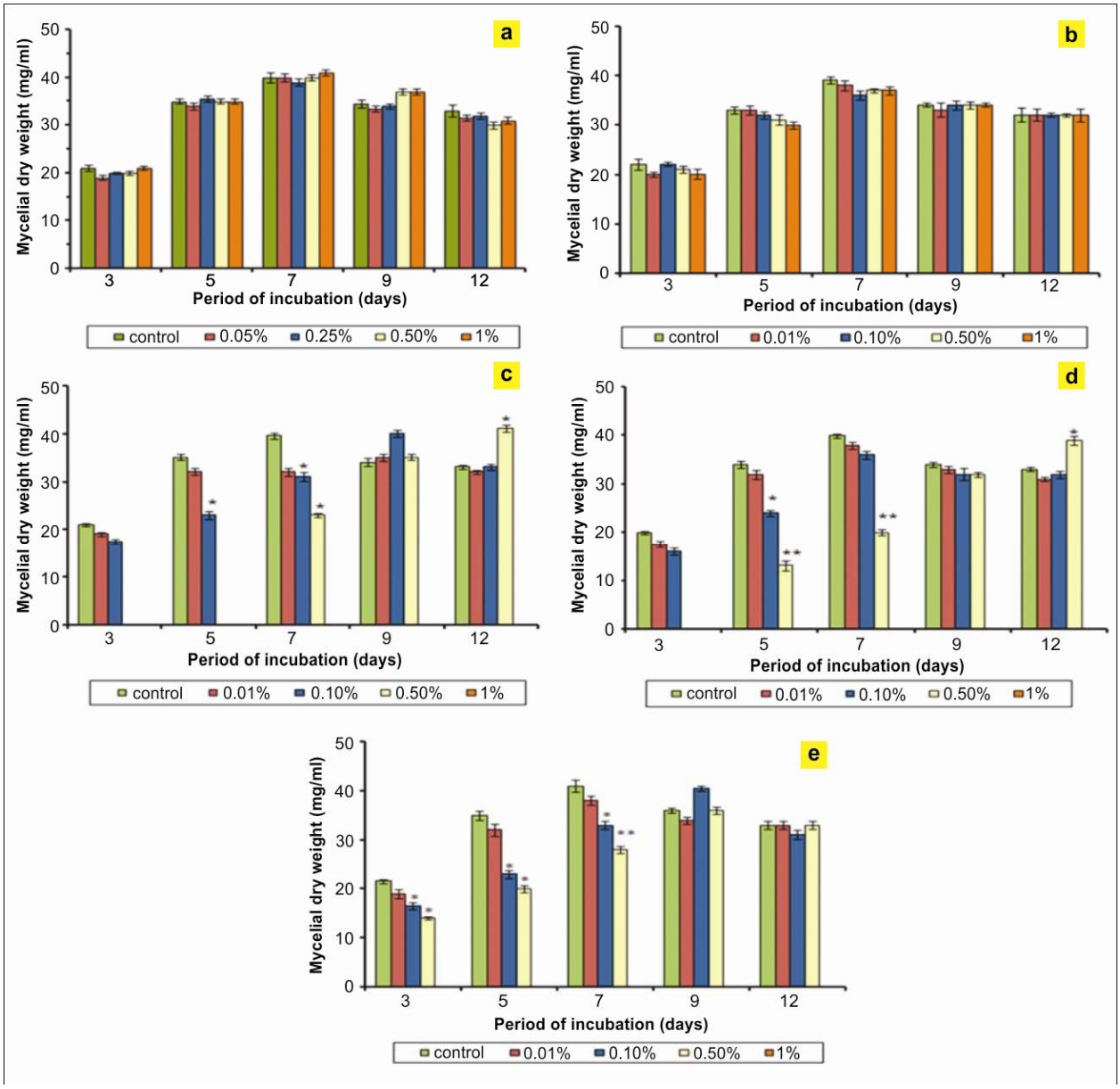


Fig.1— Effect of neem seed kernel extracts on fungal biomass: a. Aqueous extract, b. Hexane extract, c. Methanolic extract, d. Ethyl acetate extract, e. Acid-methanolic extract

growth at five different time points is shown in Fig. 1c. However, unlike the general trend observed the fungal growth (biomass) peaked on day 9 and day 12 at 0.1 and 0.5% conc., respectively. When compared to the controls, the methanolic extract at 0.01% conc. had no statistically significant inhibitory effect on fungal biomass, whereas at 0.1% level a statistically significant ($p$<0.05) decrease was observed on days 5 and 7. At 0.5% the fungal biomass production was delayed until day 5 and an increase in the biomass production was observed till day 12 and was statistically significant ($p$<0.05). The germination of *A. parasiticus* spores was completely inhibited at 1.0% conc. on all days of incubation. Increasing conc. from 0.01 to 0.5% resulted in gradual decrease in aflatoxin production by 33, 41 and 62%, respectively on day 7 and the inhibition was found to be statistically significant ($p$<0.01, $p$<0.05) and dose dependent (Table 1). The $AFB_1$/total toxin ratio at 0.1 and 0.5% conc. increased when compared to the controls indicating an increase in the $AFB_1$ production with respect to total toxin (Table 2). Ergosterol synthesis was found to be decreased at all incubation periods and it was statistically significant ($p$<0.01)

Table 1—Effect of neem seed kernel extracts on total aflatoxin elaboration (µg/mL)

| Extracts | Period of incubation (days) | Concentration levels | | | | |
|---|---|---|---|---|---|---|
| | | Control | 0.01% | 0.1% | 0.5% | 1.0% |
| Hexane | 3 | 2.0±0.04 | 2.0±0.06 | 2.0±0.06 | 2.0±0.08 | 1.9±0.05 |
| | 5 | 2.9±0.16 | 2.9±0.21 | 2.8±0.18 | 2.9±0.26 | 2.0±0.26 |
| | 7 | 3.9±0.12 | 3.4±0.25 | 3.9±0.15 | 4.0±0.21 | 3.9±0.16 |
| | 9 | 3.6±0.13 | 3.6±0.13 | 3.7±0.30 | 3.6±0.22 | 3.5±0.26 |
| | 12 | 3.4±0.18 | 3.4±0.20 | 3.1±0.10 | 3.4±0.20 | 3.3±0.16 |
| Methanolic | 3 | 1.9±0.30 | 1.6±0.40 | 1.6±0.08 | ND | ND |
| | 5 | 3.0±0.06 | 2.3±0.04 | 1.8±0.06* | 0.4±0.01** | ND |
| | 7 | 3.9±0.12 | 2.6±0.06* | 2.3±0.03* | 1.5±0.03** | ND |
| | 9 | 3.6±0.12 | 2.6±0.06* | 1.8±0.03** | 2.3±0.0* | ND |
| | 12 | 3.3±0.11 | 2.4±0.04* | 3.0±0.02* | 2.3±0.05* | ND |
| Ethyl acetate | 3 | 2.2±0.12 | 2.1±0.05 | 2.0±0.09 | ND | ND |
| | 5 | 2.9±0.16 | 2.8±0.03 | 2.6±0.04 | 2.0±0.05* | ND |
| | 7 | 3.8±0.11 | 3.5±0.06 | 3.6±0.05 | 2.2±0.04* | ND |
| | 9 | 3.5±0.28 | 3.4±0.13 | 3.2±0.15 | 3.0±0.10* | ND |
| | 12 | 3.6±0.09 | 3.4±0.07 | 3.3±0.06 | 3.0±0.04* | ND |
| Acid-methanolic | 3 | 2.2±0.14 | 1.8±0.03 | 1.9±0.11 | 0.5±0.01** | ND |
| | 5 | 2.9±0.16 | 2.7±0.08 | 1.8±0.05 | 0.4±0.02** | 0.25±0.01** |
| | 7 | 3.9±0.12 | 3.3±0.26 | 3.3±0.21* | 1.5±0.05** | 0.7±0.02** |
| | 9 | 3.6±0.13 | 2.8±0.05* | 2.7±0.03* | 2.2±0.16* | 1.3±0.01** |
| | 12 | 4.0±0.09 | 2.7±0.12 | 2.6±0.08 | 2.5±0.07* | 1.3±0.04** |
| | | Control | 0.0.5% | 0.25% | 0.5% | 1.0% |
| Aqueous | 3 | 2.0±0.08 | 1.0±0.05* | 0.8±0.03** | 0.3±0.01** | 0.1±0.02** |
| | 5 | 2.7±0.22 | 2.3±0.2* | 2.6±0.09* | 1.8±0.11* | 1.7±0.03* |
| | 7 | 3.8±0.30 | 2.4±0.2* | 2.3±0.15* | 3.3±0.29** | 1.7±0.13** |
| | 9 | 3.4±0.28 | 2.3±0.1* | 2.0±0.16* | 2.5±0.15* | 2.0±0.19* |
| | 12 | 3.4±0.29 | 1.3±0.05** | 1.4±0.09** | 2.7±0.15* | 2.0±0.17* |

Values are mean ± SD of four replications. *ND* not detected; *$p<0.05$, **$p<0.01$

Table 2 — Effect of neem seed kernel extracts on $AFB_1$/total toxin ratio

| Extracts | Period of incubation (days) | Concentration levels | | | | |
|---|---|---|---|---|---|---|
| | | Control | 0.01 % | 0.1 % | 0.5 % | 1.0 % |
| Hexane | 3 | 0.58 | 0.58 | 0.60 | 0.58 | 0.52 |
| | 5 | 0.58 | 0.58 | 0.58 | 0.51 | 0.60 |
| | 7 | 0.58 | 0.52 | 0.60 | 0.60 | 0.60 |
| | 9 | 0.44 | 0.44 | 0.46 | 0.45 | 0.43 |
| | 12 | 0.46 | 0.46 | 0.45 | 0.44 | 0.44 |
| Methanolic | 3 | 0.55 | 0.62 | 0.66 | ND | ND |
| | 5 | 0.53 | 0.53 | 1.0** | 1.0** | ND |
| | 7 | 0.50 | 0.50 | 0.57 | 0.76* | ND |
| | 9 | 0.31 | 0.48 | 0.70* | 0.68* | ND |
| | 12 | 0.39 | 0.37 | 0.37 | 0.44 | ND |
| Ethyl acetate | 3 | 0.47 | 0.47 | 0.45 | ND | ND |
| | 5 | 0.52 | 0.52 | 0.56 | 0.45 | ND |
| | 7 | 0.50 | 0.50 | 0.50 | 0.50 | ND |
| | 9 | 0.40 | 0.43 | 0.40 | 0.50* | ND |
| | 12 | 0.35 | 0.37 | 0.35 | 0.54* | ND |
| Acid-methanolic | 3 | 0.50 | 0.60 | 0.48 | 1.0** | ND |
| | 5 | 0.50 | 0.54 | 0.52 | 0.44 | 1.0** |
| | 7 | 0.45 | 0.58 | 0.6 | 0.65 | 0.76* |
| | 9 | 0.35 | 0.35 | 0.25 | 0.54** | 0.64** |
| | 12 | 0.35 | 0.50 | 0.50 | 0.3 | 0.65** |
| | | Control | 0.05% | 0.25% | 0.5% | 1.0% |

*contd*

Table 2 — Effect of neem seed kernel extracts on $AFB_1$/total toxin ratio—*contd*

| Extracts | Period of incubation (days) | Concentration levels | | | | |
|---|---|---|---|---|---|---|
| | | Control | 0.01 % | 0.1 % | 0.5 % | 1.0 % |
| | 5 | 0.54 | 0.52 | 0.59 | 0.70* | 0.78* |
| | 7 | 0.52 | 0.68 | 0.60* | 0.81 | 0.72* |
| | 9 | 0.34 | 0.76** | 0.68 | 0.78** | 0.78** |
| | 12 | 0.40 | 0.60* | 0.32 | 0.30 | 0.32 |

Values are mean of four replications. *ND* not detected; *$p$<0.05, **$p$<0.01

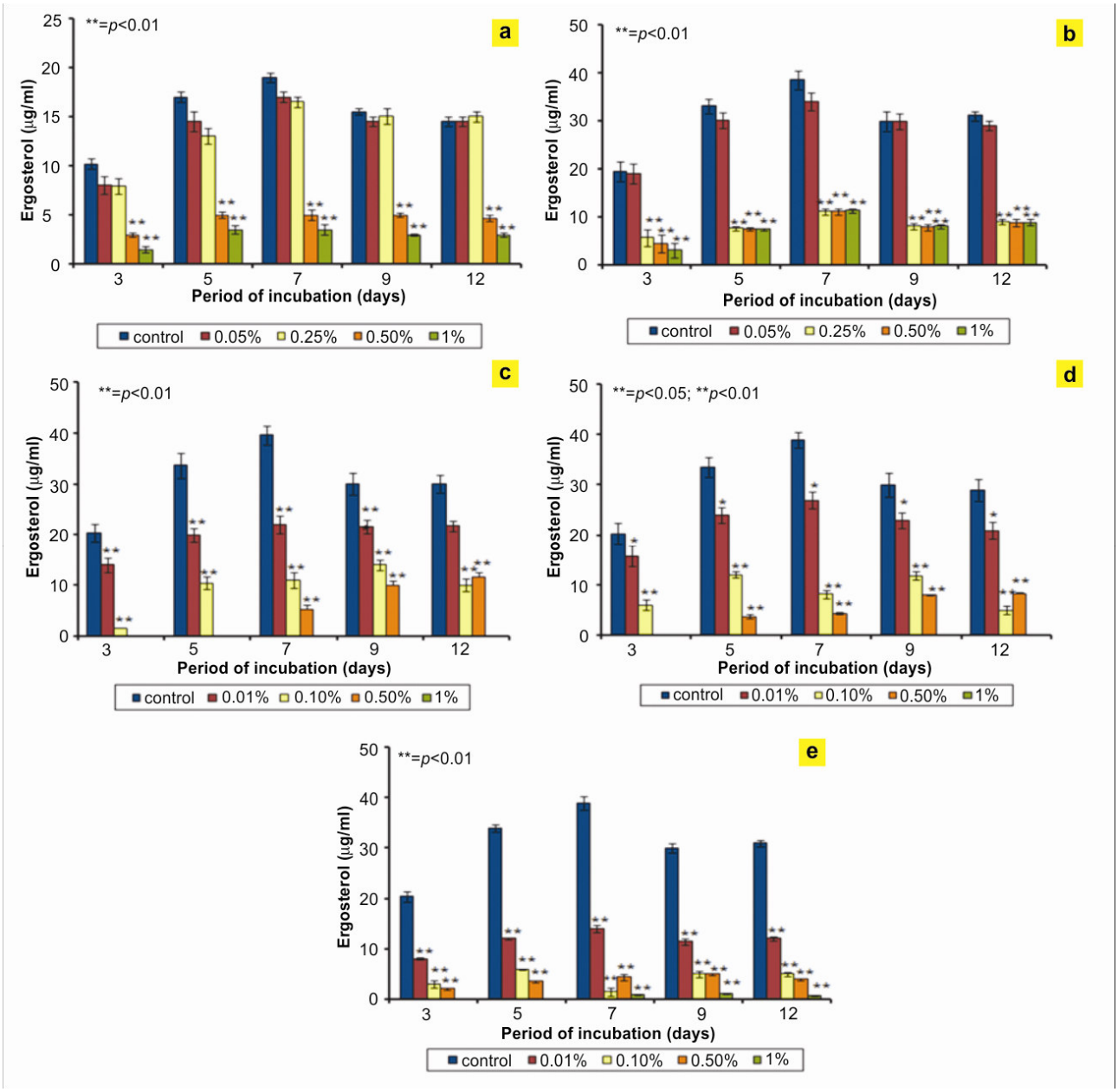


Fig. 2— Effect of neem seed kernel extracts on ergosterol biosynthesis: a. Aqueous extract, b. Hexane extract, c. Methanolic extract, d. Ethyl acetate extract, e. Acid-methanolic extract

Fig. 2c). Utilization of sugars during various time periods of fungal growth was found to reduce with increase in conc. from 0.01 to 1.0% of methanolic extract (Table 3).

**Ethyl acetate extract**

Ethyl acetate extract was found to inhibit fungal growth, ergosterol synthesis and toxin elaboration in a dose dependent manner from 0.01 to 1.0 % conc. and was statistically significant ($p<0.01$, $p<0.05$). The effect of ethyl acetate extract on fungal growth during different time points is represented in Fig. 1d. At 1.0 % conc., the germination of fungal spores was totally inhibited. At 0.5% conc., the spore germination was not observed until day 3 and was found to be maximal on day 12. No statistically significant difference in fungal biomass was observed at 0.01 % on all days. At 0.1 % conc. also no significant difference in fungal biomass was observed except on day 5 ($p<0.05$). Production of aflatoxin was completely inhibited at 1.0 %, reduced at 0.5 % and was unaffected at 0.1 and 0.01 % conc. when compared to the controls (Table 1). A significant change in the $AFB_1$/total toxin ratio was observed at 0.5 and total inhibition at 1.0% conc. of the extract (Table 2). At 0.01 and 0.1% peak synthesis of ergosterol was recorded on day 7, at 0.5 % the ergosterol synthesis was found to be initiated on day 5 and increased till day 12. At 1.0% concentration the ergosterol synthesis was completely inhibited (Fig. 2d). Both fungal biomass and ergosterol were found to be maximal on day 12 at 0.5 % concentration. At 0.01 and 0.1 % levels, sugar utilization of fungus was not affected during various time periods except on day 3 (Table 3). At 0.5 % conc. the sugar utilization by the fungus was reduced and was statistically significant ($p<0.01$) and at 1.0 % complete utilization was observed.

**Acid-methanolic extract**

The effect of acid-methanolic extract of neem seed kernel on fungal biomass is shown in Fig. 1e. The fungal biomass was reduced and statistically significant ($p<0.05$, $p<0.01$) at all conc. except at 0.01 % concentration on day 7. At higher conc. of 1.0 %, complete inhibition of spore germination was observed during experimental period of incubation (12 days). Production of aflatoxin was found to be

Table 3 — Effect of neem seed kernel extracts on percent sugar utilization

| Extracts | Period of incubation (days) | Concentration levels | | | | |
|---|---|---|---|---|---|---|
| | | Control | 0.01 % | 0.1 % | 0.5 % | 1.0 % |
| Hexane | 3 | 32 | 28 | 29 | 29 | 29 |
| | 5 | 59 | 58 | 60 | 58 | 58 |
| | 7 | 78 | 78 | 79 | 79 | 80 |
| | 9 | 82 | 83 | 82 | 83 | 82 |
| | 12 | 90 | 89 | 90 | 90 | 90 |
| Methanolic | 3 | 31 | 25 | 20* | ND | ND |
| | 5 | 62 | 59 | 57 | ND | ND |
| | 7 | 79 | 66 | 65* | 39** | ND |
| | 9 | 82 | 79 | 76 | 57* | ND |
| | 12 | 95 | 92 | 88 | 69* | ND |
| Ethyl acetate | 3 | 30 | 23 | 17* | ND | ND |
| | 5 | 63 | 64 | 62 | ND | ND |
| | 7 | 75 | 74 | 74 | 39** | ND |
| | 9 | 80 | 78 | 80 | 59* | ND |
| | 12 | 97 | 96 | 97 | 69* | ND |
| Acid-methanolic | 3 | 32 | 29 | 19* | ND | ND |
| | 5 | 62 | 63 | 42* | 10* | ND |
| | 7 | 79 | 78 | 59** | 17** | ND |
| | 9 | 82 | 81 | 77** | 29** | ND |
| | 12 | 97 | 96 | 77* | 39** | 30** |
| | | Control | 0.05% | 0.25% | 0.5% | 1.0% |
| Aqueous | 3 | 30 | 29 | 29 | 28 | 27 |
| | 5 | 61 | 59 | 58 | 56 | 57 |
| | 7 | 69 | 68 | 69 | 69 | 67 |
| | 9 | 82 | 80 | 81 | 79 | 80 |
| | 12 | 91 | 90 | 90 | 89 | 90 |

Values are mean of four replications. *ND* not detected; *$p<0.05$, **$p<0.01$

inhibited with increasing concentrations of extract from 0.01 to 1.0 % in a dose dependent manner (Table 1). The $AFB_1$/total toxin elaboration was found to increase with increase in acid-methanolic extract levels on day 7. However, statistically significant ($p$<0.01) change in $AFB_1$/toxin ratio was observed at 1.0 % conc. of acid-methanolic extract (Table 2). Ergosterol biosynthesis was found to be reduced at all concentrations tested and was statistically significant ($p$<0.01) (Fig. 2e). Utilization of sugar during various time periods was found to decrease with increase in concentration of extract from 0.01 to 1.0 % (Table 3). The decrease in the sugar utilization was statistically significant ($p$<0.05; $p$<0.01) at 0.1, 0.5 and 1.0% levels.

The correlation ($r^2$) of aflatoxin production versus ergosterol content and sugar utilization in all the experimental groups was found to be positive and significant ($p$<0.01). The correlation ($r^2$) values of total aflatoxin production, with ergosterol content were 0.692, 0.650, 0.558, 0.591 and 0.467 and with sugar consumption were 0.944, 0.838, 0.846, 0.810 and 0.807, respectively, during different periods of incubation. The decrease in the total toxin and sugar utilization in the presence of various levels of aqueous, hexane, methanolic, ethyl acetate extract and acid-methanolic extracts followed similar pattern to that of fungal biomass indicating their interdependence.

A two-way ANOVA of total toxin at different periods of incubation (3, 5, 7, 9 and 12 days) is represented in Fig. 3a-e. The total toxin production in the presence of various neem seed kernel extracts and at various concentrations were different and was statistically significant ($p$<0.01). The interaction between neem seed kernel extracts and at various concentrations was also significantly different ($p$<0.05) during various time periods of incubation. A similar pattern was also observed for ergosterol biosynthesis (Fig. 4 a-e).

**Effect of purified bioactive terpenoids from neem seed kernel**

The effect of the selected neem seed kernel bioactive principles such as azadirachtin, nimbin and salannin were evaluated in the YES liquid medium inoculated with the *A. parasiticus* on day 7 where the maximum fungal growth and aflatoxin production had been observed in the controls. Fungal growth and aflatoxin elaboration by *A. parasiticus* were not affected by the presence of azadirachtin, nimbin and salannin from 0.01 to 0.5% concentration in the liquid YES medium (Fig. 5a & b). The effect of terpenoids (azadirachtin, salannin and nimbin) on ergosterol

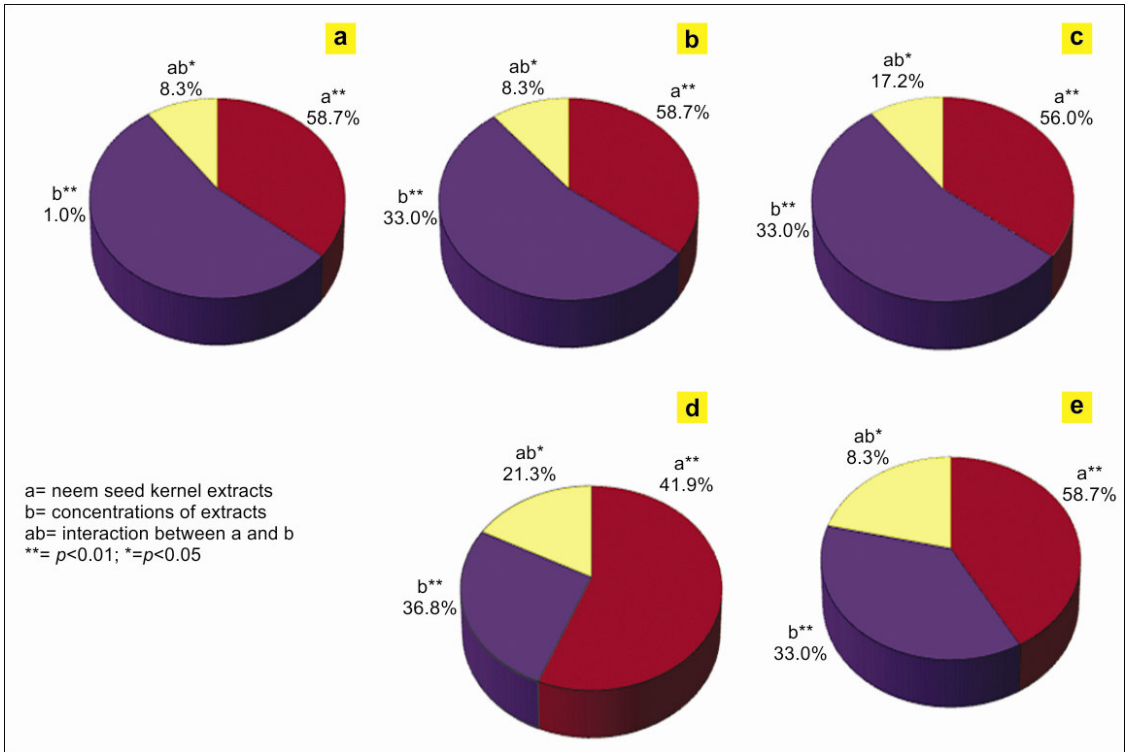


Fig. 3— Pie chart of analysis of variance of total toxin production, indicating significant ($p$<0.05) interaction between neem seed kernel extracts, at various concentrations during different time periods of incubation: a. 3 days, b. 5 days, c. 7 days, d. 9 days, e. 12 days.

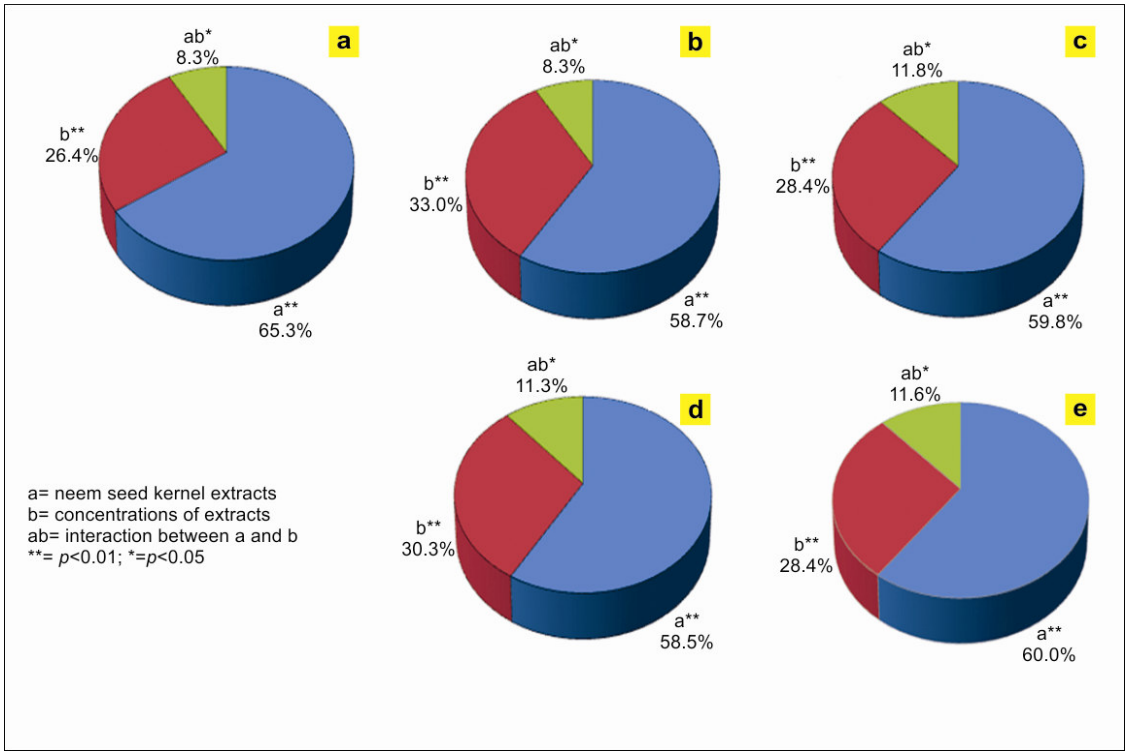


Fig. 4— Pie chart of analysis of variance of ergosterol content, indicating significant ($p$<0.05) interaction between neem seed kernel extracts, at various concentrations during different time periods of incubation: a. 3 days, b. 5 days, c. 7 days, d. 9 days, e. 12 days

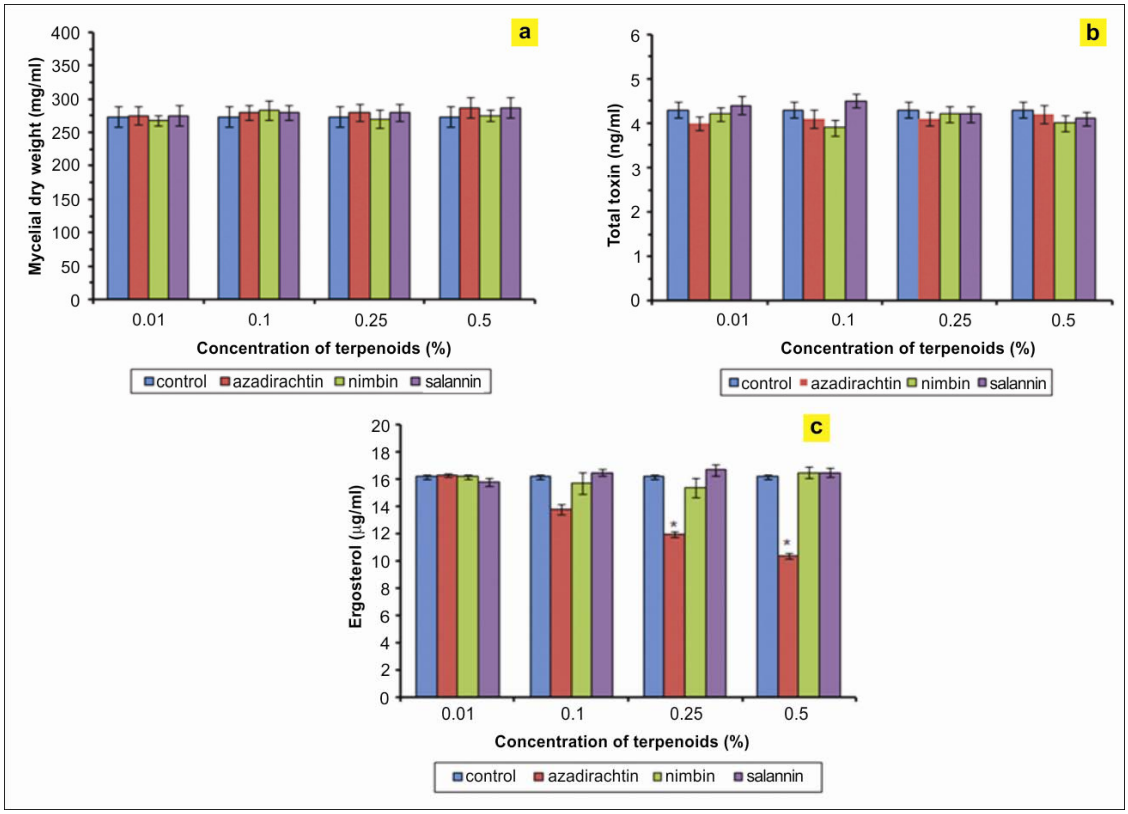


Fig.5—Effect of terpenoids on: a. Fungal biomass, b. Aflatoxin production, c. Ergosterol biosynthesis by *A. parasiticus* (NRRL 2999) in liquid YES medium.

biosynthesis is shown in Fig. 5c. Ergosterol as an index of fungal contamination was found to be un-affected by salannin and nimbin at concentration from 0.01 to 0.5 %. However, azadirachtin at 0.25 and 0.5 % conc. had reduced the ergosterol biosynthesis by 30 and 34 %, respectively on day 7 of the incubation period and was statistically significant ($p$<0.05).

## Discussion

Fungicidal effect of extracts of neem leaf, neem oil and neem cake on various groups of fungi[16,26,27] are well documented. Further, inhibitory effects of aqueous extracts of neem leaves on the growth of *Pythium aphanidermatum*[37] and aflatoxin production by *A. parasiticus*[38] are also reported confirming the antifungal potential of the extracts. Our earlier studies clearly indicated that, neem seeds are poor substrate for growth of *A. parasiticus* and aflatoxin elaboration as compared to groundnut which is an established high risk agricultural commodity, even though, the proximate composition (protein, carbohydrate and fat) of neem and groundnut were observed to be similar[28]. The presence of small molecular weight terpenoids in neem seed kernel has been attributed in conferring poor substrate suitability for aflatoxin elaboration. In the present study, a detailed experimental investigation was carried out to assess the antifungal and anti-aflatoxigenic activity of neem seed kernel extracts (aqueous, hexane, methanolic, ethyl acetate and acid-methanolic) and purified terpenoids (azadirachtin, nimbin and salannin) in YES liquid medium.

The aqueous extract showed no effect on fungal biomass and sugar utilization but inhibited aflatoxin production and ergosterol synthesis. Thus the present investigation suggests that the aqueous extract has bioactive molecules which may be involved in inhibition of the enzymes responsible for the biosynthesis of aflatoxins and ergosterol. Earlier studies by Bhatnagar and Mc Cormick[38] also reported that the inhibitory effect of neem (*A. indica*) leaf extracts on aflatoxin production of *A. parasiticus* (SRRC 143) was due to inhibition of the enzymes responsible for the early stage biosynthesis of aflatoxins.

The hexane extract of neem kernel was not inhibitive to the fungal growth (fungal biomass) and toxin production but inhibited the ergosterol synthesis. Vir and Sharma[39] observed that at 10 % conc. the hexane extract of neem seeds was completely inhibitive to the growth of *Fusarium moniliforme*, *Aspergillus niger*, *Drechslera rostratum* and *Macrophomia phaseolina*. In the present study, *A. parasiticus* appears to be more resistant to hexane extract possibly due to the presence of specific bio-active compounds involved in the reduction of ergosterol content of plasma membrane, without affecting the aflatoxin biosynthesis.

As various concentrations of acid methanolic extracts in liquid medium were prepared based on the polyphenol concentration, any change on the growth of *A. parasiticus* and its toxin production can be attributed to the polyphenols of the neem seed kernels. Lansden[40] isolated three fractions of tannins from seed coats of groundnut (cultivar – 'Flournner') and observed that the growth of *A. parasiticus* on potato dextrose agar medium and production of aflatoxin in liquid culture were inhibited by these tannins. Studies by Azaizeh *et al*[41] also had shown that various phenols, tannins and related pigments present in groundnut testa to be involved in defence mechanism against *A. flavus* invasion. The results of the present study, suggests that polyphenols of neem seed kernels are inhibitive to the fungal growth as indicated by reduced fungal biomass and ergosterol synthesis during different time periods.

Antifungal drugs which are used in medicine are usually inhibitors of fungal protein, lipid and cell wall synthesis[42]. One of the important targets of most of the antifungal compounds is ergosterol synthesis[43]. Terconazole, a new broad spectrum, antimycotic triazole derivative, has been shown to have potent activity against *Candida albicans*. The compound inhibited production of 14 α-desmethylsterols (eg. ergosterol) and it was reported that there was concomitant accumulation of methylated sterols (eg. lanosterol)[44]. Possibly, the inhibition of ergosterol biosynthesis observed in the present investigation might be attributed to inhibition of enzyme (s), which is involved in the biosynthetic pathway of ergosterol biosynthesis. The azadirachtin from neem seeds was found to inhibit ecdysteroid titer in insects[45], which shares the same biopolymer chitin as that of fungal cell wall. The ergosterol biosynthesis in the present study was found to be inhibited in the presence of all five types of neem seed kernel extracts to various extents. At higher concentrations (1.0 % and 0.5 %) of neem seed kernel extracts and ethyl acetate extract was found to be more inhibitive to the fungal growth as indexed by fungal biomass and ergosterol biosynthesis. But at

lower concentrations (0.1% and 0.01%), acid-methanolic extract was more effective in inhibiting ergosterol biosynthesis. This clearly suggests that polyphenols present in the acid-methanolic extract may contribute to inhibition of ergosterol biosynthesis. The most important bioactive terpenoid azadirachtin present in the ethyl acetate extract may contribute to the inhibition of ergosterol synthesis and toxin elaboration in the fungus (*A. parasiticus*).

Ergosterol is a major sterol component of fungal plasma membranes[46] that aids in regulating fungal membrane fluidity and is a major sterol component required for the viability of all fungi. Polyene antibiotics have been reported to bind directly with ergosterol in fungal membrane, leading to membrane distortion and leakage, resulting in cell death. Anti-fungal azoles and other compounds (example allyamines) inhibit enzymes of the ergosterol biosynthesis commonly the squalene epoxidase and C-14 demethylase[47]. In the present study, inhibition of ergosterol biosynthesis may be due to antifungal compounds present in the neem seed kernel extracts. The difference in the fungicidal activity of various neem kernel extracts, which was evident from the present experimental investigations, is important in evaluating new antifungal compounds with different mode(s) of action.

The $AFB_1$/total toxin ratio was found to be significantly affected ($p<0.01$) in the presence of various neem seed kernel extracts, except in that of hexane extract, during fungal growth. In all the experimental groups, percent sugar utilization by *A. parasiticus* in liquid YES medium was found to be positively correlated with the aflatoxin production ($p<0.01$).

In the present investigation, it was observed that pure azadirachtin was not highly effective as a fungicidal agent, when compared to azadirachtin rich crude neem seed kernel extracts (Fig. 5c). It indicates that the synergetic action of the neem seed kernel bioactive compounds along with azadirachtin might be responsible for an effective fungicidal action of the neem seed extracts.

It is pertinent to point out that *A. parasiticus* (NRRL 2999) is a highly toxigenic strain and is known to produce copious amount of toxin in yeast extract sucrose medium under controlled conditions. Under such controlled conditions also, neem seed kernel extracts were highly inhibitive to the fungal growth and toxin elaboration. Further, in the natural environmental conditions, the fungal growth and toxin elaboration in agricultural commodities are influenced by biotic and abiotic factors. Possibly, in the natural environment, the neem seed kernel extracts would be much more effective as antifungal agents.

## Conclusion

Commercial formulations based on neem seed kernel extracts have been successfully used in the past as an insecticide in controlling insect pests of crops. The formulations made for the insecticidal purpose can be appropriately modified to have additional fungicidal and fungistatic action (with respect to pathogenic and mycotoxins producing fungi) based on experimental investigations carried out in the present study. Additionally, based on our earlier studies inclusion of a detergent (cationic, anionic and non-ionic) in such formulations would potentiate the fungicidal or fungi static activity of such formulations.

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