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Effect of oil and aqueous extract of *Neem* (*Azadirachta indica*) seeds on growth of *Aspergillus* species and biosynthesis of aflatoxin

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SUMMARY

Aflatoxin contamination is a major problem in several food crops. Aflatoxin, a mycotoxin, produced by *Aspergillus flavus* has gained immense concern in the scientific world because of its tremendous harmful effects. The study was focused to see the effect of oil and aqueous extract of *neem* (*Azadirachta indica*) seeds on the growth of *Aspergillus* and production of aflatoxin by the mold. Various amounts of *neem* oil (5 - 50 μl/ml) and aqueous extract of *neem* (5 - 50 mg/ml) were used both in the broth as well as the solid medium. Fungistatic (MIC) and minimal fungicidal concentrations (MFC) were found to be 10 μl/ml and 50 μl/ml respectively for *neem* seed oil. At the concentration of 5 μl/ml *neem* oil and 5 mg/ml of aqueous extract, a significant decrease in the aflatoxin content was found in broth medium. Aflatoxin production was totally inhibited at 50 μl/ml and 50 mg/ml for *neem* oil and aqueous extract of *neem* respectively, in both treatments. There was significant inhibition of mycelium dry weight by the *neem* seed oil. Mycelial growth was totally inhibited at 20 μl/ml of *neem* seed oil concentration in broth, whereas it was not affected at all by aqueous extract. It can therefore be inferred that the oil and extract from the *neem* seed leads to inhibition of aflatoxin production while *neem* seed oil also significantly inhibits the mycelial growth. *Neem* seed oil thus can be used as potent, natural and easily available anti-aflatoxigenic agent.

Key words: Seeds oil; Aqueous extract; *Aspergillus species*; Aflatoxin; Antifungal; *Azadirachta indica*

INTRODUCTION

Aflatoxins are secondary metabolites produced by aflatoxigenic strains of *Aspergillus*, they are known to be hepatotoxic, carcinogenic and teratogenic (Stoloff, 1977). Aflatoxin causes acute liver damage, liver cirrhosis, induction of tumors, skin disorders and hormonal imbalance (Pitt, 1989). The problem of aflatoxin contamination has been commonly associated with numerous crops including peanuts, cereals etc. The fungi producing aflatoxin infects a variety of the food articles and consumption of such contaminated food by human and animals can pose serious health hazards (Dominguez-Malagon *et al.*, 2001). There are 14 known naturally occurring aflatoxins, out of them aflatoxin B1 and B2 are highly toxic and carcinogenic secondary metabolites, produced by *Aspergillus* species (Stoloff, 1977). Formation of these toxins is linked to fungal growth and environment in which the grain or cereals are

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stored. Protection of stored grain currently relies on synthetic pesticides such as the fumigant phsophine or dust formulation primiphos methyl. However, the widespread use of these pesticides have significant drawbacks including increased cost, handling hazards, concern about pesticide residues on grains, and threat to human health and environment (Paster and Bullerman, 1988). Public awareness of these risks has increased interest in finding safer insecticides or alternatives for protection of stored food products, to replace synthetic chemical preservatives and pesticides. One such alternative is to use for natural plant protection having pesticidal activity, and at the same time having low mammalian toxicity, less environmental pollution and wide public acceptance (Hemilton-Kemp *et al.*, 1995; Paster *et al.*, 1995; Don-Pedro, 1996). Reports by several authors (Bullerman *et al.*, 1977; Morozumi, 1978; Hitokoto *et al.*, 1980) supported that extract of certain spices and herbs of medicinal importance exhibit antifungal property. These natural antifungal agents can be potentially exploited in controlling the growth of fungi and consequently inhibiting aflatoxin formation (Yin and Cheng, 1998). Allameh *et al.* (2002) reported more than 50% inhibition of aflatoxin production at 50% (w/v) *neem* extract concentration. Allameh *et al.* studied the relationship between the activities of 3 cytosolic enzymes the isicitrate dehydrogenase (IDH), the fatty acid synthase (FAS) and the glutathione S transferase (GST) with aflatoxin biosynthesis in *Aspergillus parasiticus* cultured under different conditions to find out the role of each enzyme in aflatoxin biosynthesis. Allameh *et al.* carried out investigations to see the changes in the activities of these enzymes while the aflatoxin biosynthesis was under restraints by neem leaf extract. The results obtained by the studies conducted by Allameh *et al.* further confirms that there is a positive correlation between GST activity and aflatoxin production in fungi.

Use of the crude extract of seeds of *neem* to control of plant pathogenic fungi is well known (Parveen and Alam, 1993; Locke, 1995). Although a number of triterpenoids of the limonoid type have been isolated from seeds/seed oil of the Indian *neem* tree (*Azadirachta indica.*) (Devkumar and Sukh Dev, 1993) however, information is not available on their anti *Aspergillus* activities. The only references to antifungal activities of *neem* constituents relate to nimbidin (supposedly a mixture of a number of triterpenoids from seed oil) against *Rhizoctonia nodulosum, Alternaria tenuis, Fusarium oxysporum, Helminthosporium nodulosum* and *Curvularia tuberculata* (Khan *et al.*, 1974), and isomeldenin and nimonol against groundnut leaf rust (Suresh *et al.*, 1997). The fatty acid profile of the oil of *neem* has been subjected to several studies. Presence of terpenoids has been reported in the oil having antimicrobial and antifungal activity. (Govindachari *et al.*, 1998). Anti-aflatoxigenic activity of *neem* oil has previously been discussed by Sinha *et al.* (1999).

Many author reported the acute and sub-acute effect of different concentration of *Neem* (*Azadirachta indica*) leaf on the test animal Rat (Hore *et al.*, 1999; Bhanwra *et al.*, 2000), Mice (Usha *et al.*, 2001), although the effects of pure neem derived compounds on human health are not documented. Beard (1989) mentioned that azadirachtin was not toxic to humans. However, if products of this tree are to be used to treat stored seeds against insects, the mammalian consumers of these seeds ought not to be affected by residues of this treatment. Much controversy exists about the use of especially the seed oil of the neem tree. It is claimed that the oil is easily removed from seeds and leaves no negative effect on the taste of the seeds (Anonymous, 1995).

Neem seed oil has been used previously as antifungal agent. However, ample experimental proof is not available. The objective of the current study was to investigate the fungicidal and anti-aflatoxigenic effects of the oil and aqueous extract of *neem* seed against *A. flavus* and *A. parasiticus* with the aim of developing a cost effective and environment friendly protective system.

MATERIALS AND METHODS

Fungal strains

The strains of *Aspergillus* species used in this study were *Aspergillus flavus* (MTCC 277) and *Aspergillus parasiticus* (MTCC 3558), procured from Imtech, Chandigarh, India and was maintained as conidial suspension in water and tween 20 (80 : 20) at room temperature and subcultures were grown on the potato dextrose agar (PDA) (Difco, Becton Dickinson Microbiology System. USA) incubated at 28°C for 5 days. Spore population was determined using hemocytometer and a concentration of 10^5 spores/ml was used for the study. M1 broth medium was used for aflatoxin production. Aflatoxin B1, as standard, was procured from Sigma Chemical Company, USA. All other solvent and reagent were of analytical grade obtained from Merck, Germany.

Isolation of oil from *neem* seeds

Neem (*Azadirachta indica*) seeds were collected from botanical garden of Jamia Hamdard, New Delhi, India in June 2003. The seeds were air dried in shade and 200 g of dried seeds were crushed and extraction was carried out using 1 liter of hexane in a glass column at room temperature in 24 h. Hexane was evaporated at room temperature The oil was cooled to about 5°C (preferably less than about 10°C) as cooling of oil to below 10°C leads to solidification of certain waxes and fatty acids present in the oil. The solid components were filtered out to obtain a semi-solid *neem* wax fraction. The remaining liquid filtrate was retained and classified as oil of the *neem* seeds. The extractive value of *neem* oil was found to be 27% w/v in terms of dried starting material.

Aqueous extract of *neem* seeds

Dry *neem* seeds were crushed in grinder. A soxhlet extraction was carried out for two days by boiling the distilled water. After two days the extract was collected and was kept in oven at 60°C to remove the excess water. The extractive value of aqueous extract was found to be 27.5% w/w in terms of dried starting material.

Antifungal activity

To determine antifungal activities of the oil and aqueous extract of *neem* seeds, 50 ml of potato dextrose agar (PDA) medium was dispensed into 100 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. Different doses of the oil (5 - 50 μl/ml) and dried aqueous extract (5 - 50 mg/ml) were added and mixed into molten PDA. The media was than poured onto the petriplates. After solidifying the medium 100 μl of 10^5 spores/ml suspension were spread on the above plates separately. The plates were then incubated at ambient temperature (28 ± 2°C) for 3 days. Samples without any oil or aqueous extract treatment were considered as control. The minimum inhibitory concentration (MIC or the fungistatic concentration) of seeds oil and aqueous extract were taken as the lowest concentration which inhibited the growth of the test fungus in the medium. The lowest concentration of the seed oil/aqueous extract, which killed the test fungus, was considered as the minimum lethal concentration (MLC or the fungicidal concentration).

Estimation of aflatoxin

In different flasks containing 50 ml of M1 medium, different concentrations (5 - 50 μl) of *neem* seed oil and (5 - 50 mg/ml) of aqueous extract were added. To all these flasks 0.1 ml suspension of 10^5 spores/ml was mixed. The flasks were then incubated at 28°C for 5 days without shaking. After 5 days, the above culture filtrate (after separating mycelia, if any) was processed according to the protocol of de Jesus *et al.* (1988). with slight modification. Aflatoxin B1 was then analyzed by HPLC (Waters) using the following condition, flow rate of 1 ml/min, U.V spectrometric detector at 365 nm, reverse phase column C18 and mobile phase solvent containing methanol: water: acetonitrile (22.5 : 22.5 : 50). The amount of aflatoxin was calculated by Millennium 2000 software (Waters) by comparison to the

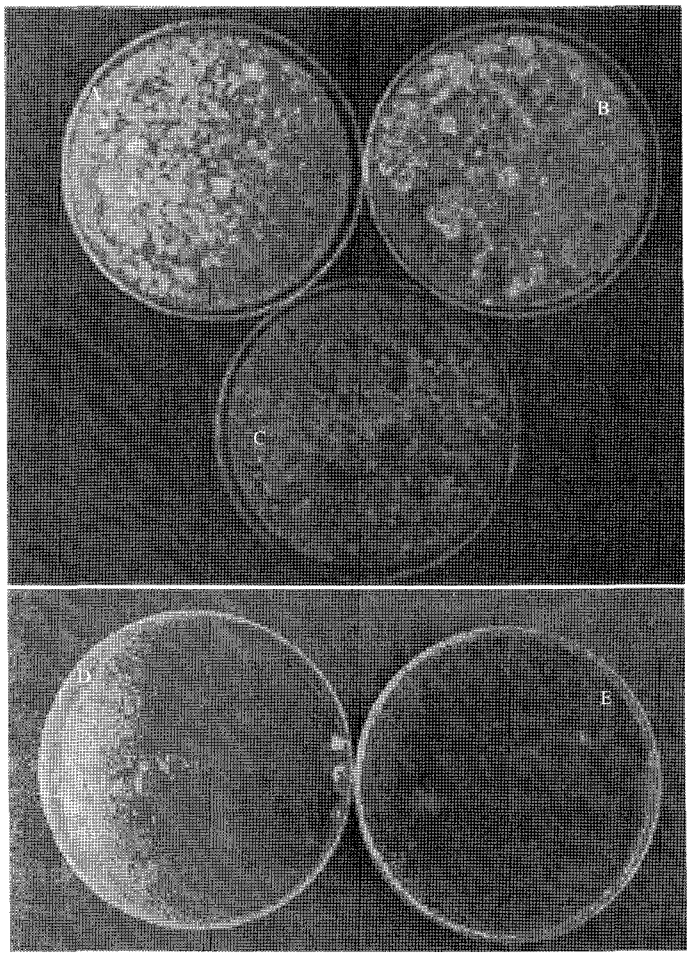

Fig. 1. Effect of neem seeds oil on the growth on *Aspergillus flavus* A: 5 μl/ml, B: 10 μl/ml, C: 20 μl/ml, D: 50 μl/ml and E: Control.

standard aflatoxin chromatogram. Aflatoxin B1 content was expressed in term of μg/ml of medium.

Mycelial growth

The above flasks containing mycelial growth were filtered through Whatman filter paper no.1 and washed with distilled water followed by methanol. The mycelia were placed on pre weighed petri plates and were allowed to dry at 60°C overnight. The flask containing dry mycelia was weighed. Growth inhibition on the basis of dry weight was calculated by the following formula: Sample weight/ Control weight × 100.

Statistical analysis

The data were expressed as mean ± S.E.M. and analysed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. Probability level of less than 5% was considered as statistically significant.

RESULTS

Effect of oil and aqueous extract of *neem* seeds on fungi

Oil of *neem* seeds was found to be both fungistatic and fungicidal in nature. The complete inhibition was found at 50 μl/ml concentration of *neem* seed oil. *Neem* seed oil was found to be fungicidal at

50 μl/ml at which concentration no revival of spores was observed even after). MIC of *neem* seed oil was found to be 10 μl/ml at which concentration revival of spores was observed after plating on fresh PDA within 7 days (Fig. 1). Similar observations were also found in case of *Aspergillus parasiticus* by the application of *neem* seeds oil. In case of aqueous extract, no effect was seen on the growth of both fungi (*A. flavus,* and *A. parasiticus*) even at a concentration of 50 mg/ml.

Effect of oil and aqueous extract of *neem* seeds on aflatoxin biosynthesis

The study shows that oil and aqueous extract of *neem* seeds inhibited the aflatoxin production of *A. flavus* and *A. parasiticus* in a dose dependent manner. It is also found statistically significant as compared to control ($P < 0.01$). Aflatoxin production of *A. flavus* and *A. parasiticus* was completely inhibited by oil and aqueous extract of *neem* seeds at 50 μl/ml and 50 μg/ml respectively ($P < 0.01$). The results are shown in Table 1.

Effect of oil and aqueous extract of *neem* seeds on mycelial dry weight

The study shows that *neem* seeds oil significantly inhibited the dry weight of mycelia (*A. flavus* and *A. parasiticus*) in a dose dependent manner. It is also found statistically significant as compared to control ($P < 0.01$). The complete inhibition of mycelial growth of *A. flavus* and *A. parasiticus* was observed at 50 μl/ml ($P < 0.01$). The weight of mycelia (*A. flavus* and *A. parasiticus*) was not inhibited by aqueous extract of *neem* seeds and found statistically non-significant ($P > 0.05$). The results are shown in Table 2.

Table 1. Effect of oil and aqueous extract of neem seeds on aflatoxin biosynthesis

Dose	Aflatoxin production in μg/ml (Mean ± S.E.M.)			
	A. flavus		*A. parasiticus*	
	Neem oil (μl/ml)	Aqueous extract (μg/ml)	Neem oil (μl/ml)	Aqueous extract (μg/ml)
Control	291.55 ± 6.6	291.55 ± 6.6	302.45 ± 12.5	302.45 ± 12.5
5	29.30 ± 3.10**	32.00 ± 2.3**	32.86 ± 2.45**	38.09 ± 4.67**
10	22.85 ± 2.02**	28.00 ± 0.86**	26.92 ± 1.21**	29.00 ± 0.63**
20	13.30 ± 0.92**	12.5 ± 0.78**	16.32 ± 2.06**	14.00 ± 0.09**
50	0.00 ± 0.00**	0.00 ± 0.00**	0.00 ± 0.00**	0.00 ± 0.00**

(n = 3); **$P < 0.01$ as compared to control (statistically significant).

Table 2. Effect of oil and aqueous extract of neem seeds on mycelial dry weight

Dose	Mycelial dry weight in gm. (Mean ± S.E.M.)			
	A. flavus		A. parasiticus	
	Neem oil (μl/ml)	Aqueous extract (μg/ml)	Neem oil (μl/ml)	Aqueous extract (μg/ml)
Control	6.40 ± 0.63	6.40 ± 0.16	6.94 ± 0.04	6.94 ± 0.04
5	3.33 ± 0.33**	6.43 ± 0.15^{NS}	3.96 ± 0.50**	6.72 ± 0.39^{NS}
10	1.26 ± 0.11**	6.41 ± 0.06^{NS}	1.57 ± 0.14**	6.65 ± 0.63^{NS}
20	0.42 ± 0.02**	6.25 ± 0.17^{NS}	0.39 ± 0.05**	6.80 ± 0.39^{NS}
50	0.00 ± 0.00**	6.48 ± 0.09^{NS}	0.00 ± 0.00**	6.78 ± 0.38^{NS}

(n = 3); **$P < 0.01$ as compared to control (statistically significant). $^{NS}P > 0.05$ (statistically non-significant)

DISCUSSION

Contamination of food commodities with aflatoxin resulting from fungal attack can occur before, during and after harvest and storage operations. Antifungal agents have been in use to prevent mycotoxin contamination in the field for quite long. Due to health and economic considerations, use of natural plant extracts has gained importance as a safe alternative method to protect food and feed from fungal contamination. The mode of action of the extracts prepared from neem plant i.e., Azadirachta indica on aflatoxin formation in toxigenic *Aspergillus* species is not well understood. Association of aflatoxin production with morphological changes suggest that probably integrity of the cell barriers particularly cell wall is critical in regulation of aflatoxin production and excretion. One of the characteristics of prevention of aflatoxin production by plant derivatives is that it should destroy the mycelia and spores of toxic fungi, which may proliferate under favorable conditions (Namazi *et al.*, 2002). The results of this study comply with above specified characteristics. The MIC of the *neem* seeds oil was observed to be 10 µl/ml at which concentration the spores revived in the presence of fresh PDA medium. However, MLC at which the spores could not be revived even on fresh media was found to be 50 µl/ml. *Neem* seeds oil inhibition of mycelial growth was observed to be associated with a significant decrease in the level of aflatoxin production. Surprisingly this association was not observed in case of aqueous extract where no effect was observed on mycelial growth, although, aflatoxin production was found to be significantly decreased.

Antifungal and aflatoxin inhibition efficacy of *neem* seeds oil may be attributed to its composition. The *neem* seed kernel is very rich in fatty acids, often up to 50 percent of the kernel's weight. *Neem* seed oil is quite bitter with a garlic/sulfur smell and contains vitamin E and other essential amino acids. Earlier research efforts in this as well as other labs have shown that *neem* leaf (Bhatnagar and McComick, 1988) can alter *Aspergillus* growth and consequently, aflatoxin production. *Neem* oil contain, azadirachtin (0.03%) and nimbidin (0.005%) as the major components found in crude form. It is generally accepted that the tetranotriterpenoid (also called limonoid) compound azadirachtin is responsible for the majority of biological effects observed in organisms exposed to *neem* compounds (Isman *et al.*, 1990; Mordue and Blackwell, 1993; Verkerk and Wright, 1993). However evaluation of the activity of *neem* oil (*Azadirachta indica*) and the fractions derived through solvent partitioning, against *Drechslera oryzae, Fusarium oxysporum* and *Alternaria tenuis* showed that the active antifungal fraction is a mixture of tetranortriterpenoids. Pure azadiradione, nimbidin, salannin and epoxy-azadiradione did not have appreciable activity. However when these terpenoids were mixed and bioassayed, they showed significant antifungal activity, indicating possible additive/synergistic effects (Govindachari *et al.*, 1998). *Neem* leaf extracts have also been shown to be effective against *Aspergillus* (Bhatnagar and Zeringue, 1993). Nearly 25 different biologically active compounds have so far been isolated from *neem* seeds (Lee *et al.*, 1991). Other compounds may also be present in *neem* oil that may be responsible for some of the biological activity observed and for the possible additive/synergistic effects.

Majority of food crop seeds specially the oil seeds are stored within shells e.g. groundnut, and the kernel is used after the pealing of the seeds. Therefore the neem seed oil does not come in direct contact with the kernels that are consumed by the human. The relative cost of neem oil is 50% less than any other fungicide thus making it economically viable option.

CONCLUSION

The present study has been conducted on the oil and aqueous extract of the *neem* seeds showing

significant inhibition of the growth of the *Aspergillus* and biosynthesis of aflatoxin. It can be inferred from the present data that oil and aqueous extract of the *neem* seed can be seen as a potential agent to control aflatoxin contamination in major food crops.

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