Chemoprevention by essential oil of turmeric leaves (*Curcuma longa* L.) on the growth of *Aspergillus flavus* and aflatoxin production

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A B S T R A C T

Turmeric is well known for a wide range of medicinal properties. Essential oil of turmeric leaves (*Curcuma longa* L.) were evaluated at varying concentrations of 0.01, 0.05, 0.1, 0.5, 0.75, 1.0 and 1.5% (v/v) in Yeast Extract Sucrose (YES) broth inoculated with spore suspension of *Aspergillus flavus* of 10^6 conidia/ml. These were evaluated for their potential in the control of aflatoxigenic fungus *A. flavus* and aflatoxin production. Turmeric leaf oil exhibited 95.3% and 100% inhibition of toxin production respectively at 1.0% and 1.5%. The extent of inhibition of fungal growth and aflatoxin production was dependent on the concentration of essential oil used. The oil exhibited significant inhibition of fungal growth as well as aflatoxins B_1 and G_1 production. The LD_{50} and LD_{90} were also determined. GC–MS analysis of the oil showed α-phellandrene, p-cymene and terpinolene as the major components in turmeric leaf oil. The possibility of using these phytochemical components as bio-preservatives for storage of spices is discussed.

1. Introduction

Aspergillus flavus and the closely related subspecies *parasiticus* have a worldwide distribution and normally occur as saprophytes in soil and on many kinds of decaying organic matter (Salunkhe et al., 1987; Dvorockova, 1990). The *A. flavus* and *Aspergillus parasiticus* can produce secondary metabolites called aflatoxins which are highly toxic, mutagenic and carcinogenic to animals (Anisworth and Anstwick, 1973; Hesseltine, 1965). They may also be involved to some degree in primary liver cancer in humans (Van Rensburg et al., 1985). Aflatoxins have been implicated in hepatocellular carcinoma, acute hepatitis, Reye's syndrome, cirrhosis in malnourished children and Kwashiorkor. (Jelinek et al., 1989; Gourama and Lloye Bullerman, 1995). Aflatoxin B_1 is the form most commonly found in agricultural commodities and it is the most toxic and carcinogenic aflatoxin (Keenan and Savage, 1994).

The high health risk caused by aflatoxins leads to strict concentration limits in different countries. The US Food and Drug Administration (USFDA) established 20 ppb as the minimum acceptable level of aflatoxin in all foods other than milk. The European Union has banned the import of peanut with >2 ppb of AFB_1 content and with >4 ppb of total aflatoxins in nuts prepared for human consumption. To export tree nuts to the European market, aflatoxin levels should be >3 ppb (Schatzki, 2001).

Elimination or inactivation of aflatoxin by physical or chemical methods bears many drawbacks (Park, 1993). Physical approaches involving treatment with heat, UV light or ionizing radiation are not entirely effective. Chemical degradation by the addition of chlorinating, oxidizing or hydrolytic agents, are not widely accepted, except ammoniation, which require expensive equipments and may result in loss in nutritional quality of spices.

Plants have been known for their medicinal and antimicrobial properties since ancient times (Massod and Ranjan, 1991). Plant extracts or essential oils can provide potential alternatives to the currently used toxic fungicides to control post harvest fungal deterioration. Little work is done to manage fungal deterioration of stored products by the use of plant derived bioactive compounds despite their good pharmacological actions. The biosynthesis of aflatoxin B_1 can be inhibited by extracts of certain plants that are toxic to fungi and may be useful in controlling the fungal growth and mycotoxin production (Steinhart et al., 1996). Extracts of plants, such as garlic and onion, effectively retard growth and aflatoxin production (Fan and Chen, 1999). Natural compounds such as flavonoids, stilbene, essential oils and others were also active in inhibition of aflatoxin production (Bullerman et al., 1977; Mallozzi et al., 1996; Norton, 1999; Sobolev et al., 1995; Chatterjiee, 1990; Hirasa and Takemasa, 1998). Recently, there has been increasing interest in using naturally occurring compounds, especially essential oils, to limit fungal growth and toxin production. These naturally occurring compounds are known to be quite safe for humans because they have been used not only for flavoring food

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but also for their antioxidant, preservative and medicinal properties (Hesseltine, 1965; Nakatini, 1994).

The objective of the present study is to evaluate the chemopreventive effect of the essential oil from turmeric leaves on the aflatoxin production of *Aspergillus* sp. *in vitro*.

2. Materials and methods

2.1. Extraction of essential oil

The essential oils from turmeric leaf were extracted by hydro distillation of the powdered samples adopting the modified Clevenger method. The oil is lighter than water and the percentage is computed as volume by weight (ASTA, 1968).

2.2. Fungal strain

Aspergillus flavus isolated from market samples of nutmeg collected during the survey (No. 76) was used as an aflatoxigenic strain for all *in vitro* experiments. The culture was identified in comparison with the authenticated cultures obtained from Microbial Type Culture Collection and Gene Bank (MTCC), a national facility housed at the Institute of Microbial Technology (IMTECH), Chandigarh, India. It was cultured using Potato Dextrose Agar (PDA) media and was incubated at 28 ± 2 °C for 7 days.

2.3. Fungal inoculum preparation

From single spore cultures of *A. flavus* grown on PDA media, the spores were harvested from the surface of the agar plates with sterile distilled water. The spore suspensions were filtered through sterile cheese cloth. The concentration of dislodged spores were determined with the aid of haemocytometer and adjusted to a final concentration of 10^6 conidia/ml. This spore suspension was used for further evaluation.

2.4. Antifungal activity of essential oils

To determine the fungistatic/fungicidal effect of the essential oils, 25 ml of YES broth synthetic media were dispensed into 100 ml Erlenmeyer's flask and autoclaved for 20 min at 121 °C. Stock solution of essential oil prepared using ethanol (1:1) was added to the flasks at various concentrations ranging from 0.01% to 1.5% (v/v) of turmeric leaf oil. The spore suspension was inoculated to all the flasks and incubated at 28 ± 2 °C for 21 days. YES broth with ethanol was used as one control, since it was used as the diluent. 0.5 ml spore suspension in broth served as the absolute control. The LD_{50} and LD_{90} were also determined. The experiments were replicated thrice.

2.5. Estimation of aflatoxin content of basal medium by HPLC

After 21 days of incubation period, the cultures present in the medium were subjected to aflatoxin analysis by HPLC using Kobra cell. 20 g of the culture medium and 5 g of sodium chloride were blended with 100 ml of 80% methanol for 1 min. After filtration, the blended contents were filtered through a fluted filter paper into a 250 ml beaker. The 25 ml of filtrate was made up to 50 ml with distilled water. The contents were allowed to pass through a glass fiber filter. About 10 ml of the filtrate was allowed to absorb on the Aflatest column used for cleanup, (Aflaprep^R R-Biopharm Rhone Ltd., Glasgow, UK, previously conditioned with 10 ml of PBS) and rinsed with 10 ml of HPLC grade water thrice. The 1 ml methanol was added to the column and the methanol eluate was collected in a vial. The 25 μl of methanol filtrate was injected into HPLC system for analysis.

2.5.1. Instrument conditions

Samples were separated by a C-18 column. Mobile phase used was a combination of deionised water with 0.1 g/l KBr, Methanol and Acetonitrile in the ratio 63:22:15 with a flow rate of 1.2 ml/min. The detection and quantification were performed with an excitation-365 nm, emission-464 nm and Kobra cell at 100 μ-amps using a fluorescence detector (RF-10AXL Shimadzu Liquid Chromatography). The retention time of the sample was compared with standard aflatoxin (Supelco). Aflatoxin was calculated from the formula:

$$\text{Aflatoxin } (\mu g/kg) = \frac{pG\ (conc) \times 1 \times 1000 \times 50 \times 100 \times 1}{20\ \mu l \times 10 \times 20 \times 25}$$

2.6. GC–MS profile of essential oil

The oil was analysed using a Shimadzu GC-2010 gas chromatograph equipped with QP 2010 mass spectrometer. RTX–5 column (30 m length × 0.25 mm ID) was used. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The split ratio was 1:40 and ionization energy 70 eV. The injection port temperature was maintained at 260 °C, the detector temperature was 250 °C. The 0.1 μl of sample was injected. The percentage composition of the oil was determined by area normalization. The constituents of the oil were identified by matching the mass spectral data with those stored in NIST and Wiley library.

2.7. Effect of volatiles

A. flavus was grown on PDA media as a single colony in the centre of the petri plate. Essential oil at concentrations *viz*; 1.5% for turmeric was poured in the lower lid. For test, the petri plate containing single colony was inverted over the lid containing the leaf oil, sealed and incubated at 28 °C for 21 days. For control, single colony of *A. flavus* alone was placed on the centre of PDA media and sealed.

2.8. Statistical analysis

Values taken for statistical analysis are the mean of three observations. The data were analyzed using M. Stat C package.

3. Results

3.1. Effect of turmeric leaf oil on the fungal growth

Table 1 shows the effect of turmeric leaf oil on the fungal growth and aflatoxin production. The oil completely inhibited the toxin production at 1.5% (v/v) (Fig 1).There was a drastic reduction in the aflatoxin content from 163 ppb at 0.75% of the oil to 4.3 ppb at 1.0%. The LD_{50} was found to be 0.3% and LD_{90} as 0.93%. Both aflatoxins B_1 (AFB_1) and G_1 (AFG_1) showed the same pattern. Higher content of AFB_1 was detected at all concentrations as compared to AFG_1.The percentage inhibition showed an ascending pattern with the increase in the concentrations used. The optimal protective dosage of 1.5% recorded for turmeric leaf oil *in vitro* is good in terms of the practical application. The percentage inhibition of aflatoxin production is given in Table 2. At leaf oil concentrations of 1.0% and 1.5%, the inhibition of both aflatoxins B_1 and G_1 production ranged from 95.3% to 100%.

Table 1
Effect of turmeric leaf oil on aflatoxin production.

Concentration (%)	Control (without turmeric leaf oil) in ppb			Test (with turmeric leaf oil) in ppb		
	G_1	B_1	$G_1 + B_1$	G_1	B_1	$G_1 + B_1$
0.01	156.5	284.9	441.4	142.4	257.5	399.9
0.05	151.4	262.7	414.4	62.2	245.0	307.2
0.1	120.0	226.2	346.2	41.0	190.8	231.8
0.5	80.10	238.1	318.2	18.2	199.9	218.1
0.75	71.8	234.9	305.9	5.60	157.4	163
1.0	63.23	213.8	277.0	2.97	1.31	4.28
1.5	114.8	150.4	265.2	–	–	–

Values expressed in terms of ppb.
cd at 5% for G_1-5.650.
cd at 5% for B_1-14.02.

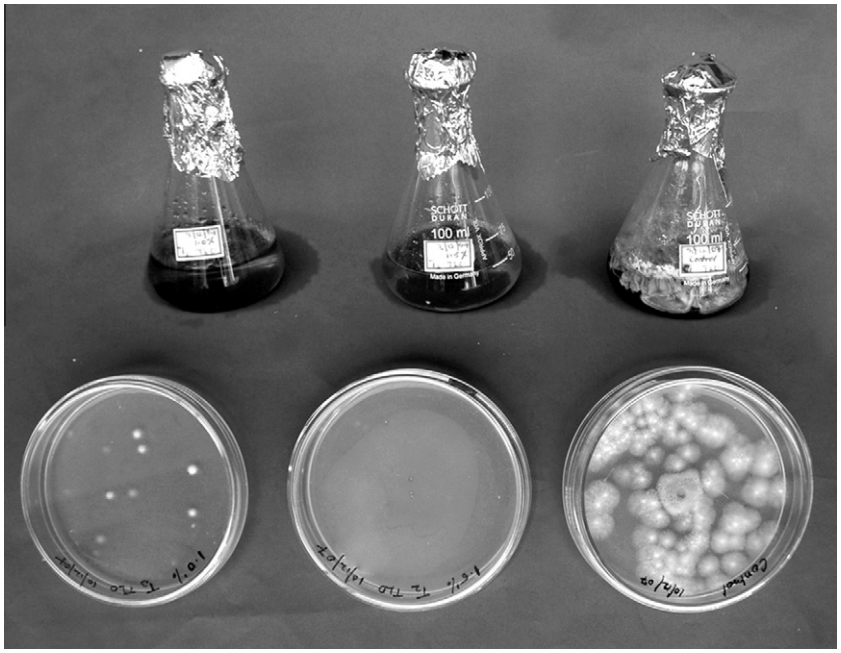

Fig 1. Effect by Turmeric leaf oil on aflatoxin production at varying concentrations. (A) 1% Turmeric leaf oil showing 98.5% (G_1 + B_1) inhibition of aflatoxin, (B) 1.5% turmeric leaf oil showing complete inhibition of aflatoxin and (C) control showing fungal growth.

Table 2
Percentage inhibition of aflatoxin production by turmeric leaf oil.

Sl. no.	Conc (%)	G_1 (%)	B_1 (%)	G_1+B_1 (%)
1	0.01	9.00	9.60	9.40
2	0.05	58.9	6.76	25.9
3	0.1	65.8	15.6	33.0
4	0.5	77.3	16.0	31.5
5	0.75	92.2	33.0	46.7
6	1.0	95.3	99.4	98.5
7	1.5	100	100	100

3.2. Effect of volatiles on aflatoxin production

The usual methods of testing the antimicrobial activity of natural products are either by supplementing growth media or by inoculation of the plant itself. However these methods do not reflect the actual activity of the volatile fraction alone. Moreover, preliminary studies on storage of spices by exposing the vapor of the essential oil (EO) were found to be highly effective in preventing fungal contamination. In this context, the experiment on vaporization of the leaf oil against *A. flavus* clearly shows the fungicidal property of the compound (Fig 2).

3.3. GC–MS pattern of turmeric leaf oil

Table 3 gives the GC–MS pattern of turmeric leaf oil, wherein the major components are α-phellandrene (24.4%), p-cymene (11.07%) and terpinolene (13.1%) as reported earlier by Ramachandraiah et al. (1998).

4. Discussion

Aromatic organic compounds of spice oils possess antifungal activity and therefore may control the production of mycotoxin by fungi (Wilson and Wisniewski, 1992; Hesseltine, 1965; Chatterjiee, 1990; Chaurasia and Kher, 1978). Recently there has been increasing interest in using naturally occurring compounds, especially essential oils, to limit fungal growth and toxin production. Theses naturally occurring compounds are known to be quite safe for humans because they have been used not only for flavoring food but also for their antioxidant preservative and medicinal properties (Bhatnagar and McCormick, 1988; Thanaboripat et al., 1997). Fungi inhibiting chemicals (mainly low molecular weight organic acids) have been used for the preservation of stored products. But there is a worldwide trend towards limiting their use in grain and foodstuffs. Natural plant products may provide an alternative to these preservatives. Many antifungal materials derived from plants have been identified (Shelef, 1984; Beuchat

Fig 2. Effect of volatiles by turmeric leaf oil. *A. flavus* was grown on PDA media as a single colony. (A) Test - Petri plate containing single colony was inverted over the lid containing 1:1 dilution of the leaf oil (1.5%), sealed and incubated at 28 °C for 21 days. (B) Control-single colony of *A. flavus* alone placed on the centre of PDA media and sealed.

Table 3
GC–MS profile of turmeric leaf oil.

Constituents in turmeric leaf oil	Retention index	Percentage composition
α-Pinene	934	3.49
1-β-Pinene	979	4.72
β-Myrcene	993	4.03
I-Phellandrene	1011	24.35
δ-3-Carene	1015	1.45
α-Terpinene	1020	1.05
p-Cymene	1031	11.07
dl-Limonene	1035	4.61
1,8-Cineole	1038	7.04
γ-Terpinene	1062	1.83
Terpinolene	1095	13.10
Linalool	1103	1.42
Cuminol	1190	1.14

and Golden, 1989) and some have been found to inhibit mycotoxin formation (Rusal and Marth, 1988).

Several studies have shown that natural products are capable of fungitoxic activity against a good number of micro-organisms (Akgul and Kivanc, 1989; Chaurasia and Kher, 1978; Dubey and Dwivedi, 1991; Oluma and Garba, 2004). The antifungal effect of the essential oil of *Cedrus deodara* Roxb. ex Lambert G. Don, as well as some of its active components, against storage moulds of *Capsicum annuum* L. have been previously investigated (Essien and Essien, 2000). In addition to the presence of antioxidative compounds derived from plants, the ability of the essential oil to impart reduced growth conditions in substrates by retarding the availability of moisture to spoilage organisms has been reported by Nwachukwu and Umechurupa (2001). It is proposed that due to its wide range of activity, non-phytotoxicity, and long term persistence of fungitoxicity, the essential oil from turmeric leaf can be exploited as fungi toxicants against storage fungi for the preservation of spices.

The major components reported in turmeric leaf oil are α-phellandrene (24.4%), p-cymene (11.07%) and terpinolene (13.1%). Dhingra Onkar et al. (2007) reported that essential oil of turmeric rhizomes showed toxicity to seven fungi including *A. flavus* involved in deterioration of stored agricultural commodities. The effect is attributed to ar-turmerone which constitutes 87% of the fungitoxic component of the oil.

Terpinolene, which is one of the major constituents of turmeric leaf oil, is a monoterpenic constituent of other essential oils of various fir and pine species, as well as plants such as *Manilla elemi*, *Nectranda elaiophora*, and *Dacrydium colensoi* (Burdock, 1995). It displays antifungal activity against various pathogens (Himejima et al., 1992), which supports the present observation.

The fungistatic and fungicidal effects exhibited by the oil might be due to the effect of the individual constituent in the oil. It is also possible that interactive effects of other minor components may also be responsible for the antifungal effect. It seems likely that the activity of the plant extracts results from a synergistic and cumulative effect existing between the plant components, but further validation of the mode of action of essential oils is needed in order to ratify a possible synergism.

The presence of the oil in the culture medium might have induced marked alterations in the structure of the cells. Such modifications induced by essential oils may be related to the interference of essential oil components with enzymatic reactions of wall synthesis, which affects fungal morphogenesis and growth. Cell wall degradation due to a lack of polysaccharide constituents of the fungal wall has also been observed on *Candida albicans* cells treated with *Carica papaya* latex sap (Belanger et al., 1997; Giordani et al., 1996). They have noticed growth inhibition, cell wall thinning and cell membrane degradation on cells of *Candida* sp. treated with voriconazole and fluconazole. Kurita et al. (1981) suggested that the antifungal activity of essential oil components, particularly aliphatic aldehydes, might be due to their ability to form charge transfer complexes with electron donors in the fungus cell.

The results of the present study not only support the finding that the essential oils possess antifungal activity against *A. flavus*, but also give a clear indication of the extent of inhibition of toxin production. The significant reduction of aflatoxin production suggests that phytochemical compounds could be used alone or in conjunction with other substances or processes to control the presence of toxic metabolites in spices. Further studies to characterize the active compound, define toxicity and evaluate economic feasibility are being explored. However, there is need for further in depth studies to formulate cost effective and ecologically friendly biopreservatives such as α-phellandrene and terpinolene to improve the storage life of spices in general.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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