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ORIGINAL ARTICLE

Effect of aqueous extracts of *Mentha arvensis* (mint) and *Piper betle* (betel) on growth and citrinin production from toxigenic *Penicillium citrinum*

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Abstract Due to growing concern of consumers about chemical residues in food products, the demand for safe and natural food is increasing greatly. The use of natural additives such as spices and herbal oil as seasoning agents for their antimicrobial activity has been extensively investigated. This paper discusses the efficacy of the aqueous extract of mint (*Mentha arvensis*) and betel (*Piper betle*) on the mycelial growth and citrinin production of *Penicillium citrinum*. The present investigation revealed that mint extract inhibited citrinin production up to 73 % without inhibiting the mycelium growth. The citrinin production decreased with increase in the concentration of mint extract as observed from the data obtained from High pressure liquid chromatography. The samples also showed reduced cytotoxicity on HeLa cells. On the other hand betel extract resulted in stimulatory effect on citrinin production and mycelial growth. The study showed that mint extract has the potential to be used safely for restraining citrinin contamination.

Keywords Penicillium citrinum · Mycotoxin · Citrinin · Mentha arvensis · Inhibitory activity · Cytotoxicity

Introduction

Microbial contamination and occurrence of mycotoxins in food and feed is a major concern during storage. Mycotoxins are common natural contaminants. Removal or the detoxification of these mycotoxins is a pre-requisite to make food safe for consumption and save the economic losses. Most of the mycotoxins are thermally stable. So, normal cooking and processing are ineffective in the degradation of these toxins. On the other hand the biological sources seem to be quite promising and less hazardous in controlling mycotoxins. The common mycotoxins include aflatoxin, ochratoxin, fusarium toxins and citrinin.

Citrinin is a mycotoxin produced by fungi belonging to the species *Penicillium*, *Aspergillus* and *Monascus*. It is a toxic secondary metabolite and was first detected in *P. citrinum* by Hetherington and Raistrick (1931). It was recognized as an antibiotic having inhibitory activity for gram positive bacteria. However due to its toxic nature its use as an antibiotic remains prohibited. Citrinin is a common contaminant of the agricultural crops and animal feed. It mostly occurs in maize (Janardhana et al. 1999), corn (Boca et al. 2002) and barley (Abramson et al. 1999). It is also frequently found in rice grains causing yellow rice (Tanaka et al. 2007). Apart from grains it is often reported in high concentrations in fruits such as apple (Martins et al. 2002).

Consumption of citrinin contaminated food poses severe health hazards. Studies have found it to be a potential nephrotoxic and hepatotoxic agent (Flajs and Peraica 2009). It is reported to be involved in Balkan nepropathy (Frank 1992). In-vitro studies showed LD^{50} of citrinin to HeLa cells is 19 ppm and similar toxicity to various cell lines (Kitabatake et al. 1993). But citrinin is less toxic compared to other known mycotoxins such as aflatoxin and ochratoxin. Lower toxicity of citrinin and inconclusive carcinogenicity appeared to be the main reasons for absence of legislative regulations for its contamination. However the toxigenic strain *P. citrinum* is wide spread. In the present study during isolation of natural contaminants, toxigenic *P. citrinum* producing high amount of citrinin was obtained.

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To prevent the harmful effects of citrinin, it is important to develop detoxification strategies. A number of physical, chemical and biological methods are available for citrinin degradation (Xu et al. 2006). Citrinin is more thermally labile compared to other mycotoxins like aflatoxin and ochratoxin. Citrinin can be degraded at 130 °C in the presence of water (Kitabatake et al. 1991). However the water content is crucial as in the presence of less water it produces a new compound citrinin H1 which is more toxic than citrinin (Mehta et al. 1993).

Several medicinal herbs and aromatic plants which are harmless and traditionally consumed by human beings and animals have been investigated for their antimicrobial and antifungal properties. These herbs and plants can be safely used against fungal contamination of food and also as antimycotoxigenic agents. For instance biosynthesis of aflatoxin by *A. flavus* is inhibited by extract of *Azadirachta indica* (Bhatnagar et al. 1990) and *Allium sativum* (Sandosskumar et al. 2007). The essential oils eugenol, anethol and thymol extracted from clove, star anise and thyme respectively inhibit the growth of *A. flavus* and aflatoxin production (Hitokoto et al. 1980). The essential oils of lemon and orange showed 90 % reduction in aflatoxin production (Hasan 2000).

There are however very few studies on the effect of plant extracts on citrinin production. Mossini and Kemmelmeier (2008) reported that the *A. indica* is also effective against citrinin production by *P. citrinum*. To the best of the authors knowledge there is no other study which discusses the control of citrinin production by plant extracts.

Mint (*Mentha arvensis*) is widely used as condiment to impart flavor to food and mouth freshener. Betel (*Piper betle*) leaves commonly known as chewing leaf is also used as mouth freshener as well as digestive after meals in South and South East Asia. Since ancient times both these plants are being consumed without any side effects and they are generally considered safe for human and animal consumption. *M. arvensis* is an aromatic herb belonging to the family Lamiaceae. It is rich in polyphenols like menthoside, rutin, tilianine and luteolin. The essential oil of mint contains menthol, menthone, methyl esters, carvone, limonene and mycrene (Baliga and Rao 2010). *P. betle* is a common vine belonging to the family *piperaceae*. The leaf has been reported to contain polyphenols and flavonoids (Maisuthisakul et al. 2007), Safrole, chavicol (Chang et al. 2002), β carotene and α tocopherol (Azuine and Bhide 1992).

Mint's essential oil inhibits the growth of *A. flavus* and aflatoxin production on stored corn (Gibriel et al. 2011). The essential oil is also effective against *Aspergillus ochraceus* and ochratoxin production (Basilico and Basilico 1999). In another study it has been reported that the chloroform and ethanolic betel leaf extract inhibited the growth of *Aspergillus parasiticus* and aflatoxin production (Chou and Yu 1984). However the use of these plants on citrinin inhibition has not been reported so far.

In the present study the antifungal and anticitrinin properties of *M. arvensis* and *P. betle* were evaluated against *P. citrinum*. The aqueous extract of mint and betel were supplemented in culture medium and tested for growth and citrinin production from *P. citrinum*. Growth was studied in terms of dry mycelia weight produced after incubation. Citrinin was qualitatively and quantitatively analyzed by thin layer chromatography (TLC), UV-visible Spectrophotometry and High pressure liquid chromatography (HPLC). The results obtained from all the three methods were compared. Cytotoxicity assay was performed on HeLa cells using the extracted citrinin samples.

Materials and methods

Microorganism and cell line

Citrinin producing strain of *Penicillium citrinum* was isolated from locally (Vellore, India) collected sample of mulethi (a medicinal plant used as an expectorant). It was identified on the basis of colony appearance, morphology and conidial arrangement as *Penicillium citrinum* and confirmed on the basis of 100 % sequence similarity with the rDNA sequence of *Penicillium citrinum* sopp (1910), NCBI accession number HQ232482.1 by the Agharkar research institute, Pune, India. This strain was used throughout the present study. The fungi was maintained on Potato dextrose agar slants and stored at 4 °C for future use. The HeLa cell lines used in the study were purchased from NCCS, Pune, India.

Chemicals

Citrinin was purchased from Sigma chemical Co. (St. Louis, MO, USA) and the stock solution was prepared in chloroform at 0.5 mg mL^{-1} and stored at 4 °C. The medium constituents; Yeast extract from Himedia Laboratories Pvt. Ltd, Mumbai, India and sucrose (analytical grade), solvents; chloroform, methanol, DMSO (dimethyl sulfoxide), acetonitrile and benzene used in the study were of HPLC grade from Rankem (RFCL Limited), New Delhi, India. The cell culture chemicals such as Dulbecco's modified Eagle medium (DMEM) from Himedia Laboratories Pvt. Ltd, Mumbai, India, MTT from Sigma chemical Co. (St. Louis, MO, USA) and Fetal bovine serum (FBS) from Invitrogen corporation USA.

Preparation of leaf extract

Mint and betel leaves were purchased from the local market. The fresh leaves were washed in sterilized distilled water. The aqueous extract was then prepared by macerating 10 g of

leaves in 100 mL sterilized distilled water. Subsequently the extract was filtered twice with whatman filter paper 1 (Mahmoud 1999). This extract was lyophilized, weighed and used for investigations.

Culture conditions

Twenty milliliter of YES medium (yeast extract 2 % and sucrose 15 %) was prepared in 100 mL conical Erlenmeyer flasks. Lyophilized aqueous extract of mint or betel leaf was added to each flask at the concentration 0.21, 0.42, 0.63 and 0.84 mg mL^{-1}. Control was maintained without the addition of any plant extract. The medium in the flasks was sterilized by autoclaving. The *P. citrinum* spores were harvested from the 7 days old culture on PDA and counted using haemocytometer. Hundred micro liter of spore suspension (10^5 spores mL^{-1}) were inoculated into each flask (Aberkane et al. 2002). The flask was incubated at 27±2 °C for a period of ten days. At the end of the incubation period the fungal mass in terms of dry mycelia weight and citrinin production in spent media was determined.

Determination of dry mycelia weight

The mycelium was harvested by filtration through whatman No.1 filter paper and broth obtained was used for extraction of citrinin. The wet weight of the mycelium was recorded. The mycelium was dried at 60 °C in hot air oven till it reached a constant weight.

Citrinin extraction and determination

The spent media was extracted with equal volume of chloroform. The extraction was done thrice using a separating funnel by wrist action. The extracts were pooled and analyzed by TLC, Spectrophotometry and HPLC for citrinin.

Twenty microliter of the chloroform extract from the test and control along with citrinin standard was spotted on TLC (silica gel on aluminum foil with F 254 from Fluka, Germany) and placed in a tank saturated with the solvent (toluene: ethyl acetate: formic acid) in the volume proportion of 6:3:1. The chromatograms were visualized under UV light. Quantification of citrinin by TLC was carried by the minimum visible fluorescent spot.

For the spectrophotometric analysis extracts were suitably diluted and analyzed in ultraviolet visible spectrophotometer (Shimadzu UV 1800, Japan). The citrinin concentration was estimated by the absorbance at 329 nm. Further samples were analyzed by HPLC (waters 1525 binary, Germany) with UV detector (waters 2487, Germany) using C18 column (150 mm length×4.6 mm inner diameter, 5 μm particle size of the column material). Twenty microliter of sample in acetonitrile was injected using a syringe (SGE, Australia). Elution was done with acetonitrile: water (1:1) at flow rate of 1 mL min^{-1}. The absorbance of samples was detected at 254 nm. The peak area and height was calculated using Empower software.

The concentration of citrinin was calculated as given by Kimura and Rodriquez-Amaya (2002).

Citrinin (μg mL^{-1})=(Peak area of sample Citrinin/Peak area of standard Citrinin)×Concentration of standard

Cytotoxicity assay

The citrinin obtained from the extraction of spent media as mentioned above was subjected to cytotoxicity assay on HeLa cells. The MTT colorimetric method was used for the assay (Mosmann 1983; Trivedi et al. 1990). In brief, extracts were dissolved in DMSO and further diluted with medium. Then, 100 μL of this sample was added to the 96 well microplate. To each well 100 μL of HeLa cell suspension of 1×10^5 cells mL^{-1} was added. Plates were incubated at 37 °C under 5 % carbon dioxide and 95 % atmospheric air. The DMSO concentration was kept below 0.1 % in the samples. Observations were taken after 48 h of incubation. The cell growth was measured in terms of colour development due to formation of formazan from reduction of MTT by live cells. The absorbance was measured with microplate reader (Bio-Rad 680) at 540 nm with reference wavelength of 655 nm.

Statistics

Mean values and standard deviations were calculated from the data obtained. One way analysis of variance (ANOVA) was performed to test the significance of differences between test and control groups.

Results

In the present study the effects of different concentrations of mint and betel leaf extract on the growth of toxigenic *P. citrinum* and citrinin production were studied in YES medium. Maximum growth and citrinin production in YES medium was observed at 10 days of incubation. Incubation longer than 10 days showed decline in citrinin production and stagnation of growth. Thus all observations presented here are on 10th day of incubation. Citrinin showed linear relation between concentration range (1–50 μg mL^{-1}) and the absorbance at 329 nm as shown in Fig. 1a (Inset). This standard curve has been used for calculation of the concentration of citrinin in the samples.

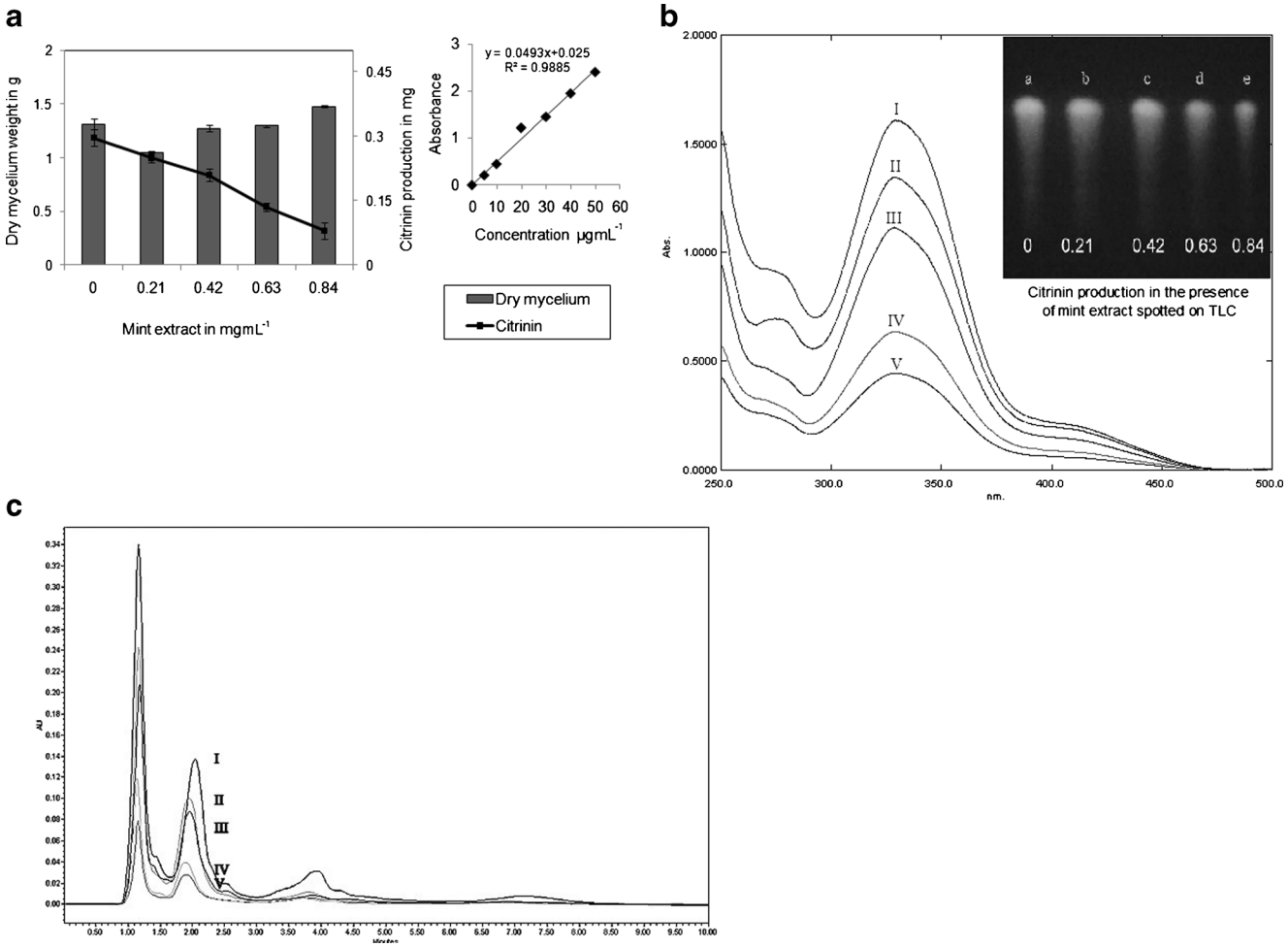

Fig. 1 Effect of mint on the growth of *P. citrinum* and citrinin production. Aqueous mint leaf extract (lyophilized) added at concentrations (I) 0, (II) 0.21, (III) 0.42, (IV) 0.63 and (V) 0.84 mg ml^{-1} of culture medium. Observations were taken after 10 days of static incubation at 27 °C. **a** Growth in terms of dry mycelium weight and citrinin concentration as calculated by absorbance at 329 nm. Top right inset shows the standard *curve* for citrinin. Data are shown as mean±standard deviation for triplicates. **b** UV spectrum of citrinin samples and top right inset shows the thin layer chromatograph of the same. **c** HPLC of citrinin samples. Citrinin eluted between 1.9 and 2 mins

Effect of mint leaf extract

It is evident from Fig. 1a that the mint extract did not control the growth of *P. citrinum* though the citrinin production was affected drastically. The mycelium weight in the presence of various concentrations of mint extracts i.e. 0.21, 0.42 and 0.63 mg mL^{-1} was inhibited by 23.6, 6.9 and 5.5 % however, the highest concentration used i.e. 0.84 mg mL^{-1} stimulated mycelial growth by 4.6 %. All concentrations of mint extracts showed inhibitory effect for the citrinin production Fig. 1a. As the concentration of mint extract increased in the medium citrinin production was decreased. A maximum reduction of 73 % was noted at 0.84 mg mL^{-1} of mint extract. The inhibition of citrinin production at different concentration of mint extract showed statistical significance at $p<0.05$ level. None of the concentrations tested showed complete inhibition of citrinin production.

The characteristic absorption peak of citrinin at 329 nm in UV spectrophotometry decreased with the increase in concentration of mint extract Fig. 1b. Like spectral data of UV spectrophotometry the chromatogram on the TLC of same samples showed diminishing fluorescent spots of citrinin at higher concentrations of mint extract Fig. 1b inset. The quantitative analysis of samples was carried out using RP-HPLC. Citrinin showed peak at around 2 min for the solvent flow rate of 1 mL min^{-1}. The peak height reduced with increasing concentration of mint confirming the semi quantitative results obtained from TLC and spectrophotometry Fig. 1c.

Effect of betel leaf extract

In the presence of betel leaf extracts the growth of *P. citrinum* and citrinin production increased Fig. 2a. Various concentrations of betel leaf extract 0.21, 0.42, 0.63 and 0.84 mg mL^{-1}

showed stimulatory effect on the growth of *P. citrinum* from 18–35 %. Increase in mycelium weight was observed with increasing concentrations of betel leaf extract. Citrinin biosynthesis was also stimulated in the presence of betel leaf extract. At various test concentrations the citrinin production increased by 29, 35, 46 and 51 % respectively in comparison to control as measured by the absorbance of chloroform extract at 329 nm Fig. 2b. The citrinin production in the presence of betel leaf extract is significantly ($p<0.05$ level) higher than the control. The thin layer chromatogram Fig. 2b inset also showed increase in the fluorescence intensity of the citrinin spot with increasing concentrations of betel leaf extract. HPLC results were also in accordance with the UV spectrophotometry and TLC Fig. 2c.

All the samples in the present study were analyzed by three different techniques i.e. TLC, UV Spectrophotometry and HPLC for the quantification of citrinin. Results are compared in Table 1. In general the values obtained from the UV

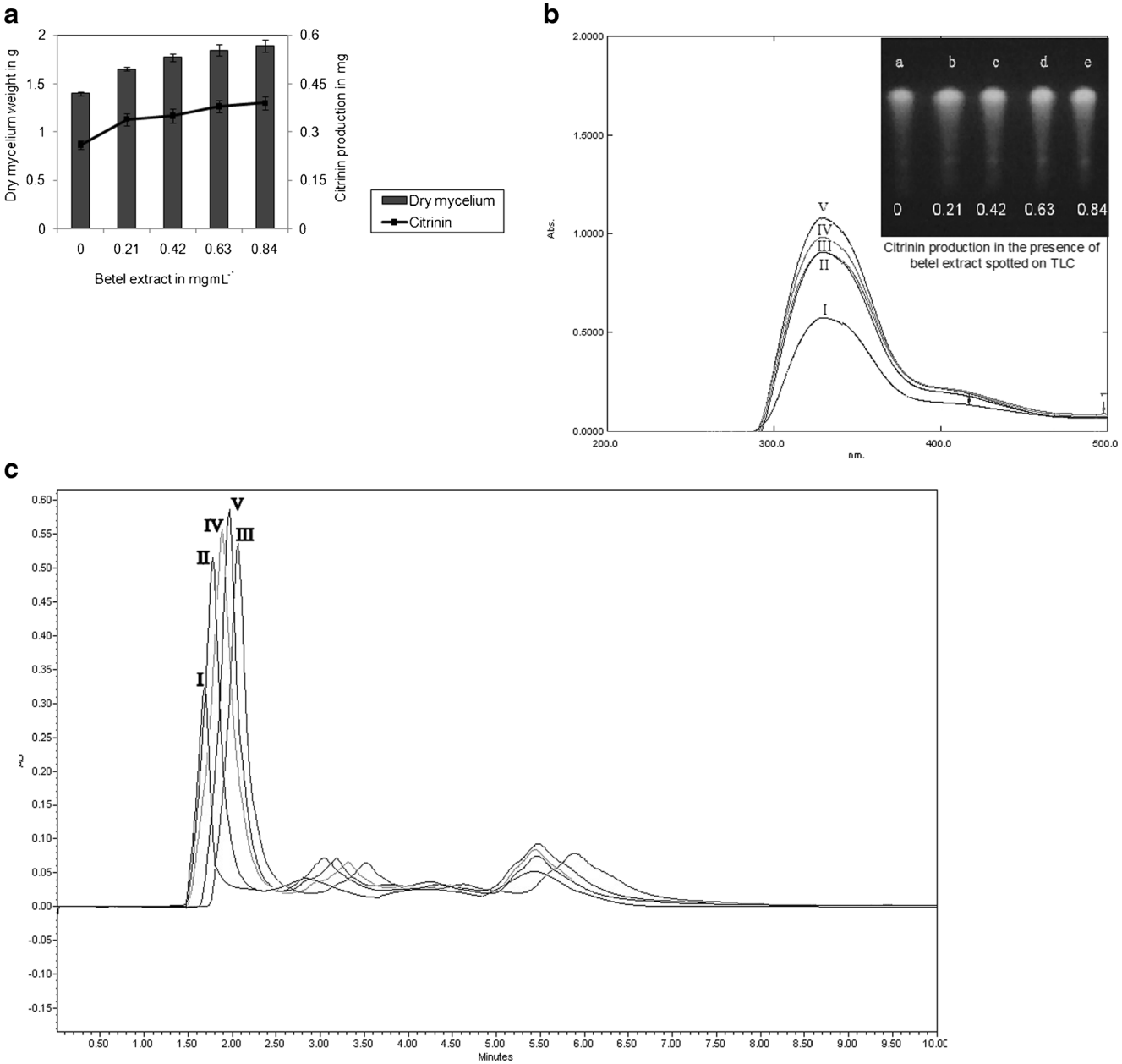

Fig. 2 Effect of betel on the growth of *P. citrinum* and citrinin production. Aqueous betel leaf extract (lyophilized) added at concentrations (I) 0, (II) 0.21, (III) 0.42, (IV) 0.63 and (V) 0.84 mg ml^{-1} of culture medium. Observations were taken after 10 days of static incubation at 27 °C. **a** Growth in terms of dry mycelium weight and citrinin concentration as calculated by absorbance at 329 nm. Data are shown as mean±standard deviation for triplicates. **b** UV spectrum of citrinin and top right inset shows the thin layer chromatograph of the same. **c** HPLC of citrinin samples. Citrinin eluted between 1.6 and 2 mins

Table 1 Comparison of Citrinin concentration determined by three analytical techniques; TLC, UV Spectrophotometry and HPLC

Leaf extract concentration in culture medium (mg mL^{-1})		Citrinin concentration determined by TLC (μg)	Citrinin concentration determined by UV Spectrophotometry (μg)	Citrinin concentration determined by HPLC (μg)
Mint	0	304	297±0.3	305
	0.21	257	250±0.7	262
	0.42	212	210±0.3	218
	0.63	142	135±0.7	148
	0.84	86	80±0.28	89
Betel	0	275	260±0.2	263
	0.21	353	340±0.2	348
	0.42	368	350±0.3	360
	0.63	393	380±0.45	387
	0.84	394	390±0.4	401

Spectrophotometry are lower than other two techniques. Values obtained from all three techniques are comparable and within the error limit of 5 %.

From analytical observations it is evident that mint extract has inhibitory effect on the citrinin production and upto 73 % reduction in citrinin was observed in comparison to control. To find if this reduction also shows reduction in toxicity the cytotoxicity assay was performed on HeLa cells. The Fig. 3 shows that the cytotoxicity of the extracts decreased with the increasing concentration of mint extract in culture medium. In the absence of mint extracts the maximum cytotoxicity (82 %) was observed and it decreased with increasing the concentration of mint extract. These results are in accordance with analytical results. At various test concentrations of mint citrinin production decreased in comparison to control and accordingly showed decrease in cytotoxicity. Contrary to mint, the betel extract showed enhancing effect on the citrinin production from *P. citrinum*. The cytotoxicity assay also showed increase in toxicity. A maximum 51 % increase in citrinin production was observed. Similarly the cytotoxicity increased from 82 to 94 %. The cytotoxicity results were correlating well with analytical results showing reduction in citrinin production and an absence of any other toxic substance in the extract when treated with mint.

Discussion

Mycotoxins have been defined as luxury molecules, they are not a necessity for the cells, and the organism can survive without them (Bennett 1983). Mycotoxin production has been attributed to the accumulation of excessive reactive oxygen species inside the cells. Reverberi et al. (2010) suggested the oxidative stress theory of mycotoxin synthesis; the onset of secondary metabolism is under control of reactive oxygen species. A close relation between oxidative stress and aflatoxin biosynthesis has been reported (Lledias et al. 1999; Fanelli et al. 2004). Culture of *A. parasiticus* supplemented with antioxidants showed inhibition of aflatoxin synthesis by down regulating the genes involved in aflatoxin biosynthesis (Reverberi et al. 2005). Comparison of the toxigenic and non-toxigenic strains demonstrates higher accumulation of reactive oxygen species in case of the toxigenic strains (Jayashree and Subramanyam 2000).

Studies have proved that when oxidant stressors are added into culture media it stimulates the aflatoxin biosynthesis (Fanelli et al. 2004). In order to down regulate the production of aflatoxin the use of antioxidant supplements are effective (Kim et al. 2008).

Plants are abundant in antioxidants. The addition of plant derived antioxidants to culture media has inhibited the aflatoxin production by 99 %. The antioxidants were however ineffective in inhibiting the fungal growth (Mahoney et al. 2010). Apart from aflatoxin, the plant derived antioxidants have inhibited the production of other mycotoxins as well such as Nivalenol, Deoxynivalenol, Fusarenone (Chipley and Uraih 1980; Beekrum et al. 2003; Fanelli et al. 2004; Tolaini et al. 2010).

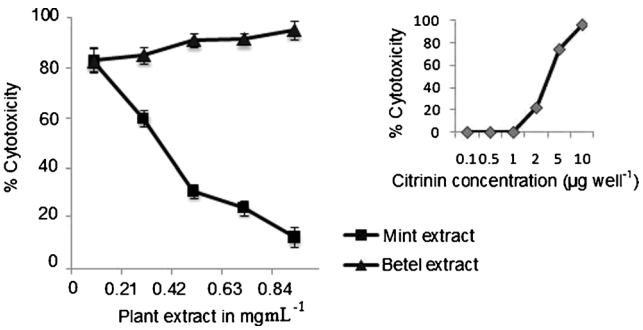

Fig. 3 Cytotoxicity of citrinin sample extracted from spent medium, supplemented with different concentration of mint and betel extract. Top right inset shows the cytotoxicity of different concentrations of standard citrinin towards HeLa cells. Data are shown as mean±standard deviation for triplicates

Table 2 Inhibition of growth and mycotoxin production by plant extracts

Mycotoxin	Plant source	Mycelial inhibition (Inhibitor concentration)	Mycotoxin inhibition (Inhibitor concentration)	Reference
Aflatoxin	Thymol	72 % (200 μg mL^{-1})	98 % (200 μg mL^{-1})	Hitokoto et al. 1980
	Piper betle essential oil	78 % (0.6 μg mL^{-1})	100 % (0.6 μg mL^{-1})	Prakash et al. 2010
	Mentha piperita L (whole)	97 % (0.5 %)	100 % (0.5 %)	Skrinjar et al. 2009
	Betel leaf extract	49 % (1,000 ppm)	Not reported	Srichana et al. 2009
Ochratoxin	Oregano essential oil	100 % (100 ppm)	100 % (100 ppm)	Basilico and Basilico 1999
Citrinin	*Azadirachta indica*	Stimulated	94.86 % (6.25 mg mL^{-1})	Mossini and Kemmelmeier 2008
	Eugenol	100 % (200 μg mL^{-1})	100 % (150 μg L^{-1})	Vazquez et al. 2001
	Mint leaf extract	Stimulated	73 % (0.84 mg mL^{-1})	Present study
	Betel leaf extract	Stimulated	Stimulated (0.84 mg mL^{-1})	Present Study

The published data about the effect of botanicals and their products; specially essential oil suggests mixed results as some of the botanicals like clove, star anise, allspices, cinnamon, garlic and carrot inhibited fungal growth as well as aflatoxin production proportional to the concentration used (Hitokoto et al. 1980; Thanaboripat et al. 1997; Tian et al. 2011). On the other hand onion, oregano and thyme did not have any inhibitory effect on the mycelial growth, sporulation and toxin synthesis (Chalfoun et al. 2004). Many of the essential oils namely eugenol and thymol as well as the essential oil from cinnamon, zataria, T*hymus vulgaris* etc. have been found effective against toxigenic fungi and aflatoxin production (Hitokoto et al. 1980; Reverberi et al. 2005; Kumar et al. 2008).

The previous studies on mint and betel leaf extract showed inhibitory effect on the growth of *Aspergillus* species and aflatoxin production (Skrinjar et al. 2009; Srichana et al. 2009). Similarly the essential oil from these plants were also found effective against fungal and aflatoxin contamination (Prakash et al. 2010).

Mossini and Kemmelmeier (2008) found the citrinin inhibition in presence of *A. Indica* without inhibition of fungal growth. Similarly in the present study the mint leaf extract at the highest concentration showed inhibition of citrinin production by 73 % without inhibition of fungal growth. Contrary to mint leaf extract the betel leaf extract showed stimulatory effect on both the fungal growth as well as citrinin production Table 2. Another work carried out using betel extract against *A. flavus* showed inhibition of fungal growth (Srichana et al. 2009). This disparity between *P. citrinum* and *A. flavus* could be attributed to variable responses towards the plant extracts used.

Though mint and betel leaf are both reported to be good antioxidants, mint inhibited the citrinin production in the present study but betel leaf could not. There are other studies also which show that irrespective of their antioxidant activity some plant extracts are unable to inhibit mycotoxin production (Meimaroglou et al. 2009). This shows that not only oxidative stress but other factors too may be responsible for the onset of secondary metabolism. Methyl jasmontes (MeJA) a volatile organic compound from plants was tested for its effect on aflatoxin biosynthesis by *A. parasiticus*. The tested concentrations of MeJA i.e. 10^{-4} and 10^{-6} M had no significant effect on the mycelia when compared with the control while it had stimulatory effect on aflatoxin production and increased the concentration of aflatoxin by 141.6–212.8 % respectively. But 10^{-2} M inhibited toxin production as well as mycelia growth (Meimaroglou et al. 2009).

The mint leaves used at the highest concentration showed inhibitory effect on the citrinin production by *P. citrinum* in YES medium however no inhibitory effect on the mycelia growth was noted in the present study. This is supported by work done by Samapundo et al. (2007). The phenolic compounds tested against *Aspergillus* isolates showed decrease in AFB1 production without influencing the fungal growth.

Betel leaf which is also reported as having strong antioxidant activity (Dasgupta and De 2004) did not show any inhibitory effect on the growth of *P. citrinum* and citrinin production.

To the best of our knowledge this is the first study on the effect of aqueous extracts of mint and betel leaves on citrinin production. The inhibitory effects of essential oils like eugenol, thymol etc. have been reported earlier (Vazquez et al. 2001). The antitoxigenic activity of mint against *P. citrinum* can be used for the preservation of food material. Further work is needed to find the efficacy of the mint extract for the prevention of citrinin production in food material.

Conclusion

In this study aqueous mint extract was found to effectively inhibit citrinin synthesis in the toxigenic strain of *P. citrinum*. Its safety was assured by toxicity assay using HeLa cells. It has a potential to be used for combating citrinin contamination

during storage. The use of natural plant products in storage will also eliminate the problem of chemical poisoning that could arise from use of synthetic chemicals in the storage of grains.

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