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ORIGINAL ARTICLE

Antifungal and antimycotoxigenic potency of *Solanum torvum* Swartz. leaf extract: isolation and identification of compound active against mycotoxigenic strains of *Aspergillus flavus* and *Fusarium verticillioides*

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Keywords
aflatoxin B1, antifungal, antimycotoxigenic, *Aspergillus flavus*, fumonisin B1, *Fusarium verticillioides*, *Solanum torvum*, torvoside K.

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Abstract

Aims: The main objective of this study was to investigate the antifungal effect of *Solanum torvum* leaves against different field and storage fungi, and to identify its active compound. In addition, to evaluate *in vitro* and *in vivo* inhibitory efficacy on toxigenic strains of *Aspergillus flavus* and *Fusarium verticillioides*.

Methods and Results: Leaves of *S. torvum* were sequentially extracted with petroleum ether, toluene, chloroform, methanol and ethanol. The antifungal compound isolated from chloroform extract was identified as torvoside K based on spectral analysis. The antifungal activity of chloroform extract and torvoside K was determined by broth microdilution and poisoned food techniques. The minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and zone of inhibition (ZOI) were recorded. Further, inhibitory effects of chloroform extract and torvoside K on growth of *A. flavus* and *F. verticillioides*, and their toxin productions were evaluated using *in vitro* and *in vivo* assays. Torvoside K showed the significant activity against tested fungi with ZOIs and MICs ranging from 33·4 to 87·4% and 31·25–250 μg ml^{-1}, respectively. Further, torvoside K showed concentration-dependent antimycotoxigenic activity against aflatoxin B1 and fumonisin B1 production by *A. flavus* and *F. verticillioides*, respectively.

Conclusions: It was observed that the compound torvoside K significantly inhibited the growth of all fungi tested. Growth of *A. flavus* and *F. verticillioides*, and aflatoxin B1 and fumonisin B1 productions were completely inhibited *in vitro* and *in vivo* by torvoside K with increasing concentration.

Significance and Impact of the Study: Control of mycotoxigenic fungi requires compounds that able to inhibit both fungal growth and mycotoxin production. The antimycotoxigenic potential of torvoside K of *S. torvum* is described in this study for the first time. The results indicate the possible use of *S. torvum* as source of antifungal agents against postharvest fungal infestation of food commodities and mycotoxin contaminations.

Introduction

Fungi have been reported to cause damage to grains and other foodstuffs (>12%) during pre- and post-harvest processing and storage (Al-Reza *et al.* 2010). Mycotoxin contamination of various foodstuffs and agricultural commodities is a major problem in the tropics and subtropics, where climatic conditions and agricultural and storage practices are favourable to fungal growth and toxin production (Kumar *et al.* 2008). Approximately

25–40% of foodstuffs worldwide are contaminated with mycotoxins, which are well known to have hazardous effects on human beings and animals (Lutfullah and Hussain 2012). Many species of *Aspergillus*, *Fusarium* and *Penicillium* are not only recognized plant pathogens but are also sources of the important mycotoxins of concern in animal and human health (Placinta *et al.* 1999). Among the mycotoxins, aflatoxins and fumonisins are the most toxic secondary metabolites mainly produced by species of *Aspergillus* and *Fusarium*. *Aspergillus flavus* and *Fusarium verticillioides* are two important mycotoxigenic moulds that colonize different kinds of food grains. They spoil various types of foods *viz*., cereals, legumes, spices, vegetables, fruits *etc.* and also produce mycotoxins that can be mutagenic, teratogenic, carcinogenic causing feed refusal and emesis in humans or animals (Nguefack *et al.* 2009; Shukla *et al.* 2012a). Aflatoxins are highly toxic polyketide secondary metabolites produced mainly by *A. flavus*. The human health impact of aflatoxin B1 (AFB1) exposure is widespread in developing countries. It is known that AFB1 causes teratogenicity, immunotoxicity, hepatotoxicity and even death in humans and farm animals (Reddy *et al.* 2010; Rosas-Taraco *et al.* 2011). Exposure to fumonisins, mainly produced by *F. verticillioides*, has been associated with several diseases in animals including leucoencephalomalacia in equines, pulmonary oedema in swine, liver cancer in rats and immunosuppression in poultry (Ficoseco *et al.* 2014). Both aflatoxins and fumonisins are relevant in food and feedstuffs due to their widespread occurrence and co-occurrence (Chulze 2010).

To prevent mycotoxin contamination in grain-based foods and feeds, control of growth of mycotoxigenic moulds is necessary. Management of fungal contamination, biodeterioration and mycotoxin accumulation in foodstuffs is generally achieved using synthetic chemicals (Tripathi *et al.* 2004; Garcia *et al.* 2012). However, residues of these chemicals in agricultural produce and by-products cause damage to animal and human health, further, continuous and indiscriminate use of chemical preservatives can lead to the development of resistance in micro-organisms (Al-Reza *et al.* 2010; Shukla *et al.* 2012b). Botanicals, being the natural derivatives, are biodegradable and do not leave toxic residues or by-products to contaminate the environment, hence gaining attention as alternative chemical control measures (Tripathi *et al.* 2004; Marin *et al.* 2011). Natural antimicrobials have also shown important antifungal properties, the identification of antifungal compounds from plants is one of the promising and alternative strategy for preventing fungal-deterioration and mycotoxin contaminations (Al-Reza *et al.* 2010; Marin *et al.* 2011; Shukla *et al.* 2012a).

Solanum torvum Swartz. is a small shrub of the Solanaceae family, distributed widely in India, Malaya, China, Phillipines and tropical America. The fruits of *S. torvum* are edible and traditionally used for the treatment of abscesses, jigger wounds, skin infections and athlete's foot (Balachandran *et al.* 2012). Pharmacological studies on this plant have demonstrated antiviral (Arthan *et al.* 2002), immunosecretory (Israf *et al.* 2004), antioxidant (Sivapriya and Srinivas 2007; Loganayaki *et al*. 2010; Ramamurthy *et al.* 2012), analgesic, anti-inflammatory (Ndebia *et al.* 2007) and anti-ulcerogenic (Nguelefack *et al.* 2008) activities. *Solanum torvum* contains a number of potential pharmacologically active chemicals like isoflavonoid sulphate and steroidal glycosides (Yahara *et al.* 1996; Arthan *et al.* 2002), chlorogenone and neochlorogenone (Cuervo *et al.* 1991), triacontane derivatives (Mahmood *et al.* 1983, 1985), 22-β-*O*-spirostanol oligoglycosides (Iida *et al.* 2005) and 26-*O*-β-glucosidase (Arthan *et al.* 2006). Antimicrobial activity of the leaf and fruit of this plant have been previously reported (Chah *et al.* 2000; Balachandran *et al.* 2012; Lalitha *et al.* 2010). Balachandran *et al.* (2012) reported the antimicrobial and antimycobacterial activities of methyl caffeate isolated from *S. torvum* fruit. Although the antimicrobial activity of crude extracts of *S. torvum* leaves has been reported earlier (Bari *et al*. 2010; Lalitha *et al.* 2010), there are no reports on active compound responsible for antifungal activity of *S. torvum* leaves. In this context, an attempt has been made to identify the active compound responsible for antifungal activity as well as its effect on mycotoxin biosynthesis by *A. flavus* and *F. verticillioides*.

Materials and methods

Fungal strains

Fusarium oxysporum (NCIM 1043) was obtained from the National Chemical Laboratory, Pune (India). *Alternaria brassicicola* (Sunflower isolate) and *Penicillium expansum* (Apple isolate) were obtained from the Department of Microbiology, University of Mysore, and *Fusarium lateritium* (Mulberry isolate) was collected from the Central Sericulture Research and Training Institute, Mysore, India. Maize isolates of *Aspergillus flavus* (aflatoxigenic strain), *Aspergillus fumigatus*, *Aspergillus ochraceus*, *Aspergillus tamari, Aspergillus terreus*, *Fusarium equiseti*, *Fusarium udum, Fusarium verticillioides* (fumonisinogenic strain) and *Penicillium citrinum*, and sorghum isolates of *Alternaria geophila* and *Curvularia tetramera* were used for the study, which were reported in our previous studies (Thippeswamy *et al.* 2013, 2014). These test fungal strains were maintained on Sabouraud dextrose agar (SDA), and 7 days old cultures were used for further assays.

Plant material and preparation of plant extracts

Fresh leaves of *S. torvum* were collected from the Jnanabharathi Campus, Bangalore University, Bengaluru (India) in the month of July, 2012. The plant sample was authenticated by Prof. Sankara Rao (JCB National Herbarium, Indian Institute of Science, Bengaluru, India) by comparison with the voucher specimens already deposited in the Herbarium. The collected plant material was washed with distilled water and shade-dried at room temperature. The dried leaves were ground well and stored in airtight containers.

Fifty grams of the powdered plant sample was successively extracted in 200 ml solvent with increasing polarity *viz.*, petroleum ether, toluene, chloroform, methanol and ethanol, using a Soxhlet apparatus (Labline, Mumbai, India). All the solvent extracts were concentrated separately under reduced pressure using a rotary flash evaporator (Superfit, Mumbai, India) and stored in airtight glass tubes (Harborne 1998), then checked for antifungal activity.

Bioautographic method

In preliminary antifungal activity assay, the chloroform extract showed fungal inhibitory activity, which was further subjected to bioautographic method for identification of antifungal band following the procedure of Ficoseco *et al.* (2014). A spot of active chloroform extract was deposited on thin layer chromatography (TLC) plate (silica gel G60 F254, Merck, Darmstadt, Germany) and eluted using a solvent system of chloroform/methanol (7·5 : 2·5, v/v), to find the active band of inhibition. TLC plates were prepared in duplicate, one plate was used for bioautography assay and other was kept for comparison. In direct bioautography assay, the plate was overlaid by the SDA (HiMedia, Mumbai, India) and swabbed by the suspension of spores of *F. verticillioides* (10^5 spores ml^{-1}). The plate was incubated at 28 ± 2°C for 48 h and observation of the inhibition was based on the inhibition caused by the active band. Antifungal band was recorded by comparing with visualized bands on uninoculated TLC plate sprayed with Ehrlich reagent (2·0 g of *p*–dimethylaminobenzaldehyde in 50 ml of 95% ethanol and 50 ml of concentrated hydrochloric acid) and/or 10% H_2SO_4-acetic anhydride-chloroform (1 : 10 : 25, v/v/v) followed by further exposure to 105°C.

Separation and identification of the antifungal compound from the chloroform extract of *Solanum torvum*

The TLC band with antifungal activity identified in chloroform extract was separated by column chromatography. The column containing silica gel (mesh size: 60–120; SRL, Mumbai, India) was eluted with solvents of increasing polarity using a gradient of chloroform and methanol (10 : 0, 9 : 1 → 0 : 10, v/v). The eluates were collected in different fractions and concentrated. Based on antifungal activity and TLC analysis, the active fractions of similar profile were pooled together. The active compound was obtained as a white amorphous powder, then analysed for purity of the active compound following TLC analysis and tested with Ehrlich reagent as previously indicated. The isolated compound was subjected to Fourier transform infrared (IR), electrospray ionization-mass spectrometry (ESI-MS), ^1H and ^{13}C NMR analyses. In the negative ion mode $[M + H]^-$ of ESI-MS, active compound of *S. torvum* showed a molecular ion peak at *m/z* 740 corresponding to the molecular formula $C_{39}H_{64}O_{13}$ (calculated *m/z* 740·92). Further, the obtained data were compared with chemical database and published values in literature (Yahara *et al.* 1996; Iida *et al.* 2005; Challal *et al.* 2014).

Screening for antifungal activity against different fungi by poisoned food technique

Antifungal activity of the chloroform extract (CE) and its active constituent torvoside K (TK) were tested against 15 different storage and field fungi using poisoned food technique as described by Mohana *et al.* (2008). CE and TK were dissolved in DMSO and incorporated into SDA to achieve the media of requisite concentration 1000 $\mu g\ ml^{-1}$. The control media without test samples was added by the DMSO, the solvent which was used for dissolving the samples. The prepared media were autoclaved, 20 ml of media poured into Petri dishes and allowed to cool. Five millimetre discs of 7 day-old culture of test fungi were placed at the centre of the Petri dishes. The inoculated plates were incubated at 28 ± 2°C for 7 days. Triplicates were maintained for each concentration and control. The fungitoxicity of test samples in terms of percentage of mycelial growth inhibition was calculated as follows:

$$\text{Growth inhibition}(\%) = (C - T/C) \times 100$$

where, C is the diameter of mycelial growth in control plates, and T is the diameter of mycelial growth in treated plates.

Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC)

The MIC and MFC of CE and TK were determined by employing the broth microdilution method following standard procedures (NCCLS 2002; Khaledi *et al.* 2014). The CE and TK dissolved in 10% DMSO were first diluted to

the highest concentration (1000 μg ml^{-1}) to be tested, and then serial two-fold dilutions were made in a concentration range from 31·25 to 1000 μg ml^{-1} in 96-well microtitre plate with Sabouraud dextrose broth (SDB). The cell suspension of overnight incubated test fungi in broth culture was adjusted to 10^4 spores ml^{-1}. Each well of 96-well microtitre plate was containing 200 μl of two-fold diluted broth of different concentrations and inoculated with 15 μl of cell suspension of test fungi. The well containing DMSO without the test samples and inoculated with test fungi was used as the negative control. Synthetic fungicides copper oxychloride 50% WP (Fungicop-50, Karnatak Agrochemicals Pvt Ltd, Bengaluru, India) and zinc ethylene bisthiocarbamate 75% WP (Indifil Z-75, Indofil Chemicals Company, Mumbai, India) were used as positive controls in conditions identical to tests samples. The inoculated microtitre plate was sealed with parafilm, then agitated with a microtitre plate shaker (Bio-Rad, Hercules, CA, USA) and incubated at 28 ± 2°C for 72 h. The inoculated plates were observed for the presence or absence of fungal growth. After macroscopic observation, a 10 μl of treated broths were radially streaked onto the SDA plates and incubated at 28 ± 2°C for 72 h. After the incubation period, the lowest concentration at which the micro-organism tested did not demonstrate visible growth that value was recorded as MIC. The complete absence of growth on the agar surface in the lowest concentration of the sample tested was defined as MFC. For further confirmation, 50 μl of iodo-nitro-tetrazolium chloride (INT, SRL; 200 μg ml^{-1}) was added to each well and incubated at 30°C for 30 min. The pale yellow-coloured INT was reduced to pink colour which indicates the presence of viable microbial cells, while yellow colour remained same where the microbial growth was inhibited (Hajji *et al.* 2010).

In vitro efficacy of CE and TK on mycelial growth and mycotoxin production by *Aspergillus flavus* and *Fusarium verticillioides*

Inhibitory effects of CE and TK on growth of mycotoxigenic *A. flavus* and *F. verticillioides*, and production of their toxins aflatoxin B1 and fumonisin B1 were evaluated using an *in vitro* assay. Sucrose-magnesium sulphate-potassium nitrate- yeast extract (SMKY) liquid medium was used to determine the efficacy of CE and TK against growth of *A. flavus* and AFB_1 production (Shukla *et al.* 2012a; Thippeswamy *et al.* 2013). SMKY medium (25 ml) was taken in 100 ml flasks, to which requisite amount of test samples were added to get 62·5, 125, 250, 500 and 1000 μg ml^{-1} concentrations. The flasks were aseptically inoculated with suspension of toxigenic strain of *A. flavus* (10^4 spores ml^{-1}, 100 μl flask^{-1}) and incubated at 28°C for 10 days. The flask containing 0·5 ml of DMSO was maintained as control. The broth cultures were filtered through Whatman no. 1 filter paper, mycelia dried at 100°C for 12 h and mycelial dry weight (MDW) was recorded. The MDW of CE and TK treated samples were compared with control for their MDW losses. The filtrate was used for the extraction of AFB_1 by adding equal volume of chloroform (25 ml) and shaken well in a separating funnel. The chloroform layer was passed through anhydrous sodium sulphate (Na_2SO_4) and evaporated in dark condition at room temperature. The residue was re-dissolved in 1 ml of chloroform and 10 μl of sample was spotted onto the TLC plate adjacent to AFB_1 standard (Sigma, Steinheim, Germany). The plate was developed in chloroform-acetone (96 : 4, v/v), air-dried and visualized under ultra-violet light (365 nm; UV-cabinet, Labline). Qualitative identification of AFB1 content was done by visual comparison of intensity of fluorescence of the samples with standard spots. For quantitative estimation, the fluorescent spots were scrapped off the plates, dissolved in 5 ml cold CH_3OH, and centrifuged at 3000 ***g*** for 5 min. The absorbance of supernatant was measured at 265 nm using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) and the amount of AFB_1 content was calculated following formula:

$$\text{Aflatoxin content } (\mu\text{g kg}^{-1}) = [D \times M/E \times L] \times 1000$$

where, D is absorbance, M is molecular weight of AFB_1 (312), E is molar extinction coefficient of AFB_1 (21 800), and L is path length (1 cm).

The efficacy of CE and TK on growth of *F. verticillioides* and FB1 production was determined *in vitro* following the method of Bailly *et al.* (2005) with some modifications. Briefly, 100 μl of a spore suspension (10^4 microconidia ml^{-1}) of *F. verticillioides* was inoculated into SDB containing the requisite amount of CE and TK (62·5, 125, 250, 500 and 1000 μg ml^{-1}), and incubated at 28 ± 2°C for 10 days. Fungal biomass of *F. verticillioides* obtained after the filtration of the SDB medium was measured by weighing the mycelial mat after 48 h of freeze-drying. To estimate FB1 production, mycelial mat of each culture was ground in acetonitrile : water (1 : 1, v/v) and filtered through 0·45 μm membrane filter. The filtrate was evaporated on water bath at 60°C and the residue was redissolved in 1 ml of methanol. From this, 10 μl of sample was spotted adjacent to standard FB_1 (Sigma) on TLC plate, then eluted in a solvent system comprising of butanol : acetic acid : water (20 : 10 : 10, v/v/v) and allowed to air dry. The air dried TLC plate was sprayed with 0·5% *p*-anisaldehyde in methanol : acetic acid : H_2SO_4 (85 : 10 : 0·5, v/v/v) solution followed by heating at 110°C for 10 min. After that, the amount of FB_1 on TLC plate was estimated qualitatively and quantitatively using spectrophotodensitometer (Bio-Rad,

Universal Hood II 720BR/02170) at 600 nm by comparing with different concentrations of standard FB_1.

In vivo efficacy of CE and TK on growth of mycotoxigenic fungi and, aflatoxin and fumonisin production in viable maize

Inhibitory effects of CE and TK on growth of mycotoxigenic *A. flavus* and *F. verticillioides*, and production of their toxins aflatoxin B_1 and fumonisin B1 were evaluated *in vivo* using viable maize (*Zea mays* L.) as model. The efficacy of CE and TK on aflatoxin production in viable maize was determined by following the procedure of Garcia *et al.* (2012), and Probst and Cotty (2012) with slight modifications. The maize seeds were treated with different concentrations of CE and TK ranging from 62·5 to 1000 $\mu g\ g^{-1}$ of maize seeds. The maize seeds without sample treatment were maintained as control. The sample-treated and untreated maize were inoculated with 100 μl spore suspension of *A. flavus* toxigenic strain containing 10^4 spores ml^{-1}. The water activity (a_w 0·95) of maize was adjusted by aseptically adding sterile distilled water to maize kernels in sterile container as described by Garcia *et al.* (2012). The inoculated maize were kept for incubation at room temperature for 15 days. After incubation, 5 g of maize seeds were milled and extracted with 15 ml of acetonitrile-water (60 : 40, v/v) and shaken for 10 min. The extract was filtered through Whatman No. 1 filter paper and the filtrate was extracted with equal volume of chloroform. Further, the extracted AFB1 was estimated qualitatively and quantitatively as described above *in vitro* assay.

The efficacy of CE and TK on FB1 production *in vivo* was determined in viable maize seeds following the procedures of Bailly *et al.* (2005) and Thippeswamy *et al.* (2014) with minor modifications. Briefly, freshly harvested maize samples were collected and the water activity (a_w 0·95) was adjusted. The maize samples were treated with different concentrations of CE and TK separately (62·5, 125, 250, 500 and 1000 $\mu g\ ml^{-1}$) and inoculated with 100 μl of a spore suspension (10^4 microconidia ml^{-1}) of *F. verticillioides* and incubated at 25°C up to 15 days. After incubation, the maize samples were used for FB1 extraction and quantification following the procedure of Bailly *et al.* (2005). The amount of FB1 was estimated qualitatively and quantitatively using spectrophoto-densitometric method as described above *in vitro* assay.

Effect of TK on ergosterol content in the plasma membrane of toxigenic *Aspergillus flavus* and *Fusarium verticillioides*

The ergosterol content in the plasma membrane of *A. flavus* and *F. verticillioides* was detected by a method described previously by Tian *et al.* (2012) and Prakash *et al.* (2014) with slight modifications. Fifty μl spore suspension of toxigenic strains of *A. flavus* and *F. verticillioides* containing 10^6 spores ml^{-1} was inoculated into respective medium (SMKY for *A. flavus* and SDB for *F. verticillioides*) containing different concentrations of TK *viz.*, 62·5, 125, 250, 500 and 1000 $\mu g\ ml^{-1}$ and incubated at 28 ± 2°C for 4 days. A control set was kept parallel to the treatment sets without treatment. After incubation, mycelia were harvested and washed twice with distilled water. The net wet weights of the cell pellets were recorded. Five millilitre of 25% alcoholic potassium hydroxide solution (25 g KOH in 35 ml distilled water and made up to 100 ml with absolute ethanol) was added to each sample and vortex mixed for 2 min, followed by incubation at 85 ± 2°C for 4 h in water bath. Sterols were extracted from each sample by adding a mixture of 2 ml distilled water and 5 ml *n*-heptane. Then, the mixture was sufficiently mixed by vortex for 2 min allowing the layers to separate for 1 h at room temperature and *n*-heptane layer was analysed by scanning spectrophotometry (UV-1700, Shimadzu) between 230 and 300 nm. Base correction of the absorbance was done with control containing only respective concentration of test compound without inoculation of test fungi. The presence of ergosterol (at 282 nm) and the late sterol intermediate 24(28) dehydroergosterol (at 230 and 282 nm) in the *n*-heptane layer led to a characteristic curve. The ergosterol amount was calculated as a percentage of the wet weight of the fungal mycelia, was based on the absorbance and wet weight of the initial mycelial pellet. The calculated formula of the ergosterol amount is as follows:

$$\%\text{ergosterol} + \%24(28)\ \text{dehydroergosterol} = (A282/290)/\text{pellet weight},$$

$$\%24(28)\ \text{dehydroergosterol} = (A230/518)/\text{pellet weight},$$

$$\%\text{ergosterol} = (\%\text{ergosterol} + \%24(28)\ \text{dehydroergosterol}) - \%24(28)\ \text{dehydroergosterol}$$

where, 290 and 518 are the *E* values (in percentages per cm) determined for crystalline ergosterol and 24(28) dehydroergosterol, respectively, and pellet weight is the net wet weight (g).

Statistical analysis

The experiments were performed in triplicate and values were expressed as means ± standard error. Analysis of variance was conducted, and the differences between

values were tested for significance by ANOVA with the SPSS 19 (IBM, Armonk, NY, USA) programme. Differences at $P \leq 0{\cdot}05$ were considered statistically significant.

Results

Antifungal activity visualized by bioautographic assay

The chloroform extract of *S. torvum* leaves showed the highest antifungal activity than other extracts *viz.*, petroleum ether, toluene, methanol and ethanol extracts. A TLC approach was followed to identify the active compound responsible for the observed antifungal activity in chloroform extract of *S. torvum*. Bioautographic and TLC analysis indicated that the band with Rf = 0·56 showed the observed antifungal activity. The active band was visualized in TLC plate after spraying with Ehrlich reagent and/or 10% H_2SO_4-acetic anhydride-chloroform, separately. The brick-red colour confirmed that the compound belongs to glycoside group.

Identification of the active compound isolated from the chloroform extract of *Solanum torvum*

The chloroform extract was subjected to column chromatography with a gradient elution of chloroform and methanol (10 : 0, 9 : 1→0 : 10, v/v) to afford 26 fractions. The 9th and 10th fractions of column chromatography showed similar chromatographic profile and bioautographic results (Fig. 1), which were pooled together. The fraction was further purified by preparative TLC, then the compound obtained as amorphous powder and subjected to spectral analysis. The compound was characterized as torvoside K based on the comparison of its spectral data with the reported values in literature, which was previously reported as torvoside C by Yahara *et al.* (1996). In the ESI-MS spectrum, the isolated compound showed a molecular ion peak ($[M + H]^-$) at *m/z* 740 ($C_{39}H_{64}O_{13}$, requires, 740·92) corresponding to the molecular formula $C_{39}H_{64}O_{13}$. The ^{13}C-NMR spectrum showed 39 carbon signals and ^{1}H-HMR spectrum showed 64 proton signals similar to those of reported values (Yahara *et al.* 1996; Iida *et al.* 2005; Challal *et al.* 2014). In an earlier report, it was reported as glycoside of neosolaspigenin having 22-α-*O*-spirostane skeleton because its ^{13}C-NMR signals due to the aglycone moiety were identical with those of neosolaspigenin (Yahara *et al.* 1996). However, the ^{13}C-NMR signals of torvoside K were coincident with those of the sapogenol moiety of this torvoside K (Iida *et al.* 2005; Challal *et al.* 2014). Therefore, torvoside K was determined to be 6-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-quinovopyranosyl (22*R*,23*S*,25*R*)-3β,6α, 23-trihydroxy-5α-spirostane (Fig. 2).

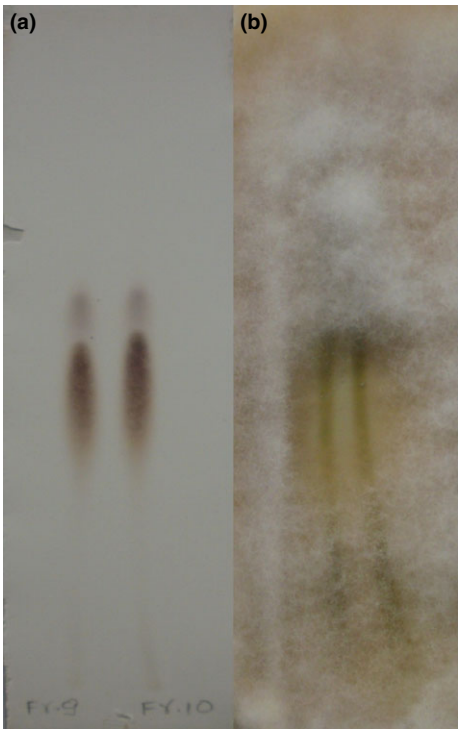

Figure 1 Silica gel chromatograms of 9th and 10th fractions obtained from column chromatography of chloroform extract of *Solanum torvum* leaves. The plates were developed in chloroform : methanol (7·5 : 2·5, v/v). Chromatograms were (a) observed after spraying with 10% H_2SO_4 : acetic anhydride : chloroform (1 : 10 : 25, v/v/v) and further exposure to 105°C (b) bioautographed against *Fusarium verticillioides*.

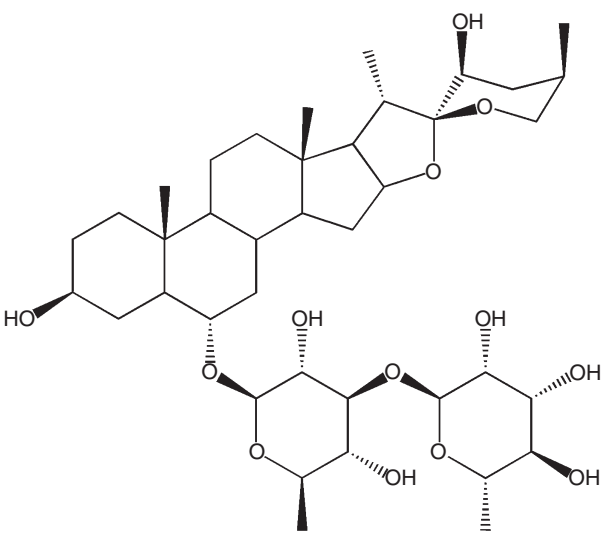

Figure 2 Structure of torvoside K isolated from *Solanum torvum* leaves.

Antifungal efficacy of CE and TK assessed using poisoned food technique and microdilution method

The growth inhibitory effects of CE and TK were screened against a panel of 15 fungal pathogens containing storage and field fungi are given in Table 1. The percentage of growth inhibition by the CE and TK was estimated by measuring the growth diameter of colony grown in the medium with treatment and control. Most of the treated field fungi were susceptible at 1000 μg ml^{-1} concentration of CE and TK with inhibition of spore germination. Among the fungi tested, *P. expansum*, *A. flavus* and *A. tamari* were found to be most resistant, with the mycelial growth inhibition 26·5, 28·0 and 30·0%, respectively. The field fungi such as *A. brassicicola* (87·4%), *F. verticillioides* (84·2%), *F. udum* (84·1%) and *C. tetramera* (82·4%), were found be most susceptible organisms at 1000 μg ml^{-1}. At the concentration of 1000 μg ml^{-1}, TK caused more than 50% mycelial inhibition of most of the fungi except *P. expansum*, *A. flavus* and *A. tamari*. However, remarkable antifungal index (50·2–87·4%) was recorded by TK against rest of the fungi at the same concentration. Among the two mycotoxigenic fungi tested, aflatoxigenic strain of *A. flavus* was found to be resistant, whereas fumonisinogenic strain of *F. verticillioides* was susceptible to TK and CE.

In broth microdilution assay, the TK showed significant inhibitory activity against most of the pathogenic fungi tested with lower MIC and MFC than that of CE and copper oxychloride 50%. The results revealed that the antifungal activity of TK was comparable to zinc ethylene bisthiocarbamate 75% which showed the significant inhibitory activity against a wide range of fungi tested. The test samples showed significant inhibitory activity against field fungi tested *viz.*, *Alternaria* spp., *Curvularia* sp. and *Fusarium* spp., with lower MIC/MFC values than those recorded for storage fungi *viz.*, *Aspergillus* spp. and *Penicillium* spp. On comparative evaluation with synthetic fungicides copper oxychloride 50% and zinc ethylene bisthiocarbamate 75%, CE and TK showed varying degrees of MIC and MFC values against different fungi tested. Among the samples tested, copper oxychloride 50% was found least effective against any of the fungi tested, most of the fungi were not completely inhibited even at concentration of 1000 μg ml^{-1}.

Inhibitory effect CE and TK on fungal growth and mycotoxin production in culture medium

Inhibitory effects of CE and TK on mycelial growth, and production of aflatoxin B1 by *A. flavus* and fumonisin B1 by *F. verticillioides* were evaluated using suitable growth media. The biomass (MDW) of *A. flavus* and

Table 1 Antifungal activity of chloroform extract of *Solanum torvum*, torvoside K and synthetic fungicides against different field and storage fungi

	Chloroform extract			Torvoside K			CO		ZEB	
Test fungi	% Mycelial inhibition*	MIC	MFC	% Mycelial inhibition*	MIC	MFC	MIC	MFC	MIC	MFC
Alternaria brassicicola	79·34 ± 0·32	125	250	87·4 ± 0·32	31·25	125	31·25	500	31·25	62·5
Alternaria geophila	60·40 ± 0·26	125	250	76·0 ± 0·26	62·5	125	125	>1000	31·25	500
Aspergillus flavus†	28·00 ± 0·14	250	>1000	33·4 ± 0·14	125	500	250	>1000	125	1000
Aspergillus fumigatus	42·78 ± 0·76	250	1000	50·2 ± 0·76	125	250	250	>1000	250	1000
Aspergillus ochraceus	49·50 ± 0·08	125	500	56·8 ± 0·08	125	250	250	500	62·5	500
Aspergillus tamari	30·00 ± 0·12	500	>1000	34·3 ± 0·12	250	1000	500	>1000	125	250
Aspergillus terreus	41·05 ± 0·45	125	500	52·0 ± 0·45	62·5	250	250	>1000	125	250
Curvularia tetramera	69·32 ± 0·76	62·5	250	82·4 ± 0·76	31·25	125	250	>1000	31·25	500
Fusarium equiseti	52·90 ± 0·33	125	250	63·5 ± 0·33	62·5	125	250	>1000	31·25	125
Fusarium lateritium	67·78 ± 0·14	125	250	79·8 ± 0·14	62·5	250	500	>1000	31·25	125
Fusarium oxysporum	68·00 ± 0·43	125	250	79·5 ± 0·43	62·5	125	500	>1000	31·25	125
Fusarium udum	73·56 ± 0·22	62·5	125	84·1 ± 0·22	31·25	62·5	250	>1000	31·25	125
Fusarium verticillioides‡	76·42 ± 0·27	125	250	84·2 ± 0·27	62·5	250	1000	>1000	62·5	125
Penicillium expansum	26·25 ± 0·11	500	>1000	29·5 ± 0·11	250	>1000	500	>1000	500	>1000
Penicillium citrinum	30·44 ± 0·00	500	>1000	33·6 ± 0·00	250	>1000	1000	>1000	500	>1000

CO: Copper oxychloride 50%; ZEB: Zinc ethylene bisthiocarbamate 75%.
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values are expressed in μg ml^{-1}.
Data given are the mean values of three replicates ± standard error ($P \leq 0·05$).
*Per cent mycelial inhibition at sample concentration of 1000 μg ml^{-1}.
†Aflatoxin B1 producing strain.
‡Fumonisin B1 producing strain.

Table 2 *In vitro* efficacy of chloroform extract of *Solanum torvum* and torvoside K on mycelial dry weight (MDW), FB1 production from *Aspergillus flavus* and FB1 production from *Fusarium verticillioides*

	Effect on growth of *A. flavus* and AFB1 production in SMKY medium				Effect on growth of *F. verticillioides* and FB1 production in SDB medium			
	Chloroform extract		Torvoside K		Chloroform extract		Torvoside K	
Concentration* (μg ml^{-1})	MDW (mg)	AFB1 content (μg l^{-1})	MDW (mg)	AFB1 content (μg l^{-1})	MDW (mg)	FB1 content (μg mg^{-1})	MDW (mg)	FB1 content (μg mg^{-1})
Control	548·0 ± 9·3	1478·4 ± 28·7	548·0 ± 9·3	1478·4 ± 28·7	140·8 ± 8·7	4·50 ± 0·04	140·8 ± 8·7	4·50 ± 0·04
62·5	473·0 ± 9·6	1331·0 ± 25·5	423·0 ± 10·2	1108·0 ± 28·9	112·0 ± 8·8	4·30 ± 0·02	94·0 ± 8·1	2·80 ± 0·03
125	394·3 ± 8·9	944·6 ± 28·6	174·6 ± 5·3	102·6 ± 21·5	98·4 ± 7·3	2·24 ± 0·03	73·0 ± 6·5	0·85 ± 0·02
250	292·3 ± 6·5	517·8 ± 25·4	78·9 ± 3·8	ND	54·3 ± 5·2	0·98 ± 0·01	32·3 ± 6·4	ND
500	135·6 ± 3·2	95·71 ± 21·4	ND	ND	37·0 ± 4·5	ND	ND	ND
1000	37·0 ± 2·7	ND	ND	ND	ND	ND	ND	ND

Data given are the mean of three replicates ± standard error ($P \leq 0{\cdot}05$).
ND, not detected; SMKY, Sucrose-magnesium sulphate-potassium nitrate- yeast extract; SDB, Sabouraud dextrose broth.
**In vitro* treatment with different concentrations of samples in μg ml^{-1} of growth medium.

F. verticillioides and mycotoxin production were significantly inhibited by CE and TK in a dose-dependent manner (Table 2). The amount of AFB_1 production in control was found to be 1478·4 ± 28·7 μg l^{-1} and FB_1 was 4·50 ± 0·04 μg mg^{-1} of fungal biomass. AFB_1 and FB_1 productions were completely inhibited by TK at concentrations higher than 250 μg ml^{-1}, while fungal biomass were completely inhibited at concentrations higher than 500 μg ml^{-1}. Similarly, in the case of CE, AFB_1 production was inhibited at 1000 μg ml^{-1} with no complete inhibition of fungal biomass, whereas FB_1 production was inhibited at concentrations higher than 500 μg ml^{-1} with complete inhibition of fungal biomass at 1000 μg ml^{-1}.

Inhibitory effect of CE and TK on aflatoxin and fumonisin production in viable maize

In vivo inhibitory efficacy of CE and TK on production of aflatoxin B1 by *A. flavus* and fumonisin B1 by *F. verticillioides* were evaluated using viable maize as model (Table 3). The amount of AFB1 and FB1 production in control were found to be 1294·0 ± 20·1 μg kg^{-1} and 2·65 ± 0·03 μg g^{-1}, respectively. AFB1 production in maize was inhibited completely by TK at 1000 μg kg^{-1}, but CE did not inhibit AFB1 production completely. Similarly, FB1 production was inhibited by TK at concentrations higher than 500 μg kg^{-1}, whereas CE inhibited at 1000 μg kg^{-1}.

Effectiveness of active compound TK on ergosterol content in the plasma membrane of toxigenic fungi

The inhibitory effects of TK on ergosterol content in the plasma membrane of *A. flavus* and *F. verticillioides* are shown in Fig. 3. When compared to control, the reduction percentage of ergosterol content in the plasma membrane of *A. flavus* by TK was recorded to be 48·67%, 57·30%, 70·43% at 62·5, 125, and 250 μg ml^{-1}, respectively (data not presented). Similarly, in *F. verticillioides*, a reduction percentage of the ergosterol content as com-

Table 3 *In vivo* efficacy of chloroform extract of *Solanum torvum* and torvoside K on AFB1 production from *Aspergillus flavus* and FB1 production from *Fusarium verticillioides* in maize

	AFB1 production (μg kg^{-1}) in maize		FB1 production (μg g^{-1}) in maize	
Concentration* (μg g^{-1})	Chloroform extract	Torvoside K	Chloroform extract	Torvoside K
Control	1294·0 ± 20·1	1294·0 ± 20·1	2·65 ± 0·03	2·65 ± 0·03
125	1191·0 ± 18·5	1008·5 ± 19·4	2·20 ± 0·04	1·56 ± 0·03
250	1014·6 ± 19·1	716·8 ± 17·5	1·24 ± 0·02	0·19 ± 0·02
500	677·8 ± 15·4	241·3 ± 12·1	0·39 ± 0·01	ND
1000	163·4 ± 13·3	ND	ND	ND

Data given are the mean of three replicates ± standard error ($P \leq 0{\cdot}05$).
ND, not detected.
**In vivo* treatment with different concentrations of samples in μg g^{-1} of viable maize.

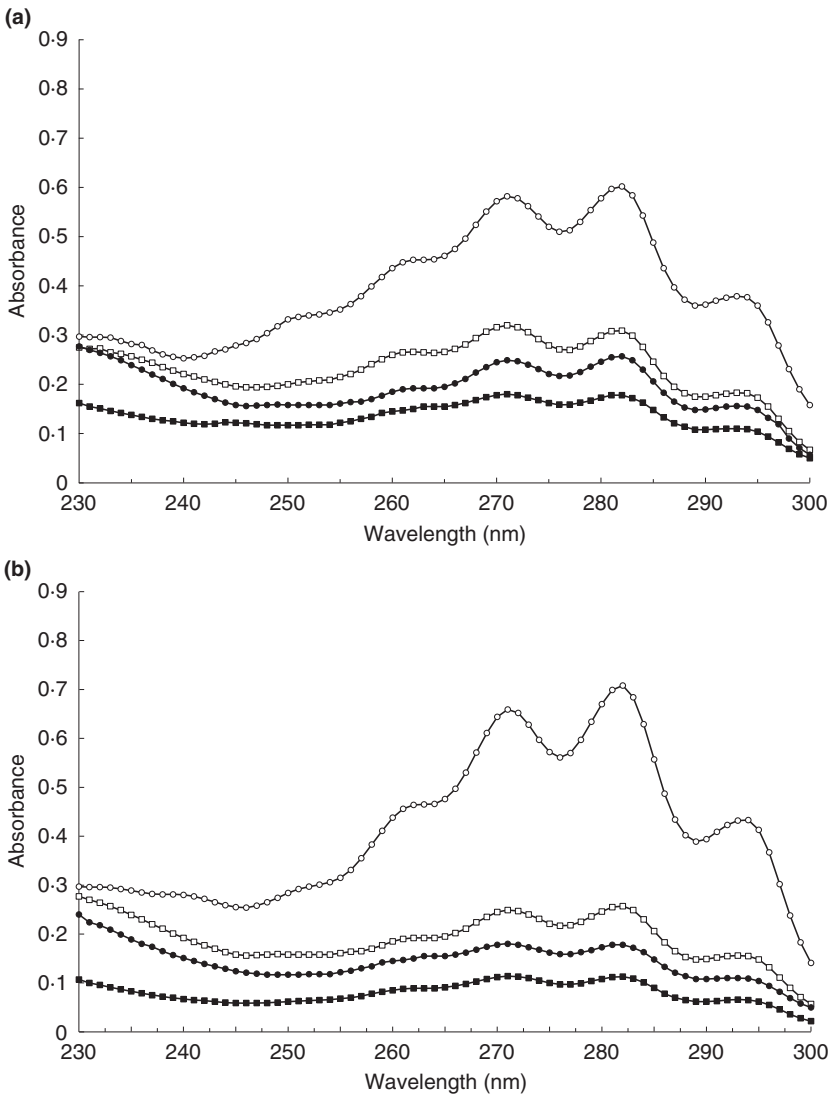

Figure 3 Effect of different concentrations of torvoside K on ergosterol content in the plasma membrane of toxigenic strains of (a) *Aspergillus flavus* and (b) *Fusarium verticillioides*. (○) Control; (□) 62·5 μg ml^{-1}; (●) 125 μg ml^{-1} and (■) 250 μg ml^{-1}.

pared with the control was observed at 63·70% for 62·5 μg ml^{-1}, 74·85% for 125 μg ml^{-1}, 84·03% for 250 μg ml^{-1}. A dose-dependent decrease in ergosterol production in both *A. flavus* and *F. verticillioides* was observed when isolates were grown in the presence of TK. At 500 and 1000 μg ml^{-1} concentrations of TK, 100% reduction in ergosterol content was observed in both *A. flavus* and *F. verticillioides*. The results demonstrated that the ergosterol content (at 282 nm) in the plasma membrane of both *A. flavus* and *F. verticillioides* was completely inhibited at concentrations higher than 500 μg ml^{-1}.

Discussion

In this study, we evaluated the antifungal and antimycotoxigenic capability of torvoside K isolated from *S. torvum* Swartz. leaf extract on growth of *A. flavus* and *F. verticillioides*, and mycotoxin production in viable maize grain at adjusted a_w and inoculum level. Conditions studied *in vivo* were close to conditions that may occur during pre- and post-harvest of cereals. Further, we also investigated the antifungal potency against a panel of 15 different field and storage fungi using *in vitro* assays. The antifungal and antimycotoxigenic properties of torvoside K isolated from *S. torvum* leaves have been reported here for the first time.

The chloroform extract of *S. torvum* yielded two active fractions- 9th and 10th of column chromatography, which were pooled together due to similar chromatographic profile and bioautographic results. The bioautographic assay showed that only one band inhibited fungal growth in each of these fractions. TLC chromatogram sprayed with 10% H_2SO_4: acetic anhydride: chloroform

(1 : 10 : 25, v/v/v) showed two brick-red coloured bands, among which the first band (R_f 0·56) showed antifungal activity. The active band was separated as white amorphous powder, which was identified as torvoside K based on spectral analysis. In an earlier report by Yahara *et al.* (1996), torvoside C has been reported from aerial parts of *S. torvum*, a glycoside of neosolaspigenin having 22-α-*O*-spirostane skeleton because its ^{13}C-NMR signals due to the aglycone moiety were identical with those of neosolaspigenin. Later, Iida *et al.* (2005) have reported the same compound from the fruits of *S. torvum*, the compound was determined to be torvoside K as the ^{13}C-NMR signals were coincident with those of the sapogenol moiety of this torvoside K. Further, Challal *et al.* (2014) reported torvoside K from aerial parts of *S. torvum*, all these data available in literature were coincident with our spectral values. *Solanum torvum* has been reported for a number of potential pharmacologically active compounds like isoflavonoid sulphate and steroidal glycosides (Yahara *et al.* 1996; Arthan *et al.* 2002), chlorogenone and neochlorogenone (Carabot *et al.* 1991), triacontane derivatives (Mahmood *et al.* 1983, 1985), 22-β-*O*-spirostanol oligoglycosides (Iida *et al.* 2005) and 26-*O*-β-glucosidase (Arthan *et al.* 2006). Torvoside K was reported as novel compound in *S. torvum* by Yahara *et al.* (1996) and Iida *et al.* (2005), and it is reported for anticonvulsuion activity (Challal *et al.* 2014). There are no reports on antifungal and antimycotoxigenic effects of torvoside K.

In antifungal assays *viz.*, poisoned food technique and broth microdilution method, the differences in the degree of fungal inhibition were evident between both storage and field fungal species tested, which showed that storage fungi *viz.*, species of *Aspergillus* and *Penicillium*, were more resistant when compared to field fungi *viz.*, species of *Alternaria*, *Curvularia* and *Fusarium*. Antifungal activity of test samples was recorded in term of MIC/MFC. The MFC of TK for complete inhibition of growth of the aflatoxigenic strain of *A. flavus* was recorded at 500 μg ml^{-1} in broth microdilution method, similarly in antiaflatoxigenic assay, mycelia was completely inhibited at 500 μg ml^{-1}. The MFC of TK for complete inhibition of fumonisinogenic strain of *F. verticillioides* was 250 μg ml^{-1}, whereas mycelia was completely inhibited at 500 μg ml^{-1} in antiaflatoxigenic assay. It was interesting to note that the minimum inhibitory concentrations for toxigenic strains of *A. flavus* and *F. verticillioides* were 125 and 62·5 μg ml^{-1}, respectively, the values were almost similar to standard fungicide zinc ethylene bisthiocarbamate. The MIC values were determined against different test fungal species using broth microdilution method. This method offers better opportunity to test samples to come in close contact with fungi as both of them are homogenously distributed inside the medium as it has been earlier reported by Prakash *et al.* (2014). MIC values of TK were comparable to synthetic fungicide zinc ethylene bisthiocarbamate; however, copper oxychloride was found lease effective than all the samples tested. The antifungal effect of both CE and TE was stronger than that observed for copper oxychloride 50%, a synthetic contact fungicide used against a wide range of plant diseases.

Antimycotoxigenic efficacy of CE and TK on inhibition of mycelial growth and production of aflatoxin B1 by *A. flavus* and fumonisin B1 by *F. verticillioides* were evaluated using *in vitro* and *in vivo* assays. During antimycotoxigenic assay in culture medium, a gradual decrease in MDW and mycotoxin production by *A. flavus* and *F. verticillioides*, was observed with increasing concentration of CE and TK. Results showed a positive correlation between the subsequent decrease in mycelial growth and mycotoxin production *in vitro* with increasing concentrations of TK and CE. The TK was more efficacious as mycotoxin suppressor in culture medium than viable maize as it caused complete inhibition of aflatoxin B1 secretion by *A. flavus* and fumonisin B1 by *F. verticillioides* at 250 μg ml^{-1}. Both aflatoxin and fumonisin productions were completely inhibited by TK in maize at higher concentrations, at 1000 and 500 μg ml^{-1}, respectively. The maize, which is susceptible to common fungal infestation, was selected as model for this study. Conditions studied *in vivo* were close to conditions that may occur during pre- and post-harvest of maize. In most of the cases, seed treatment was most effective in suppression of seed borne fungi with no effect on seedling vigour (data not shown). Maize is one of the most important crops worldwide; however, a good substrate for growth, development and activity of filamentous fungi. Maize is associated with a large number of fungal species belonging to the species of *Aspergillus*, *Fusarium* and *Penicillium*, that can cause spoilage and mycotoxin contaminations (Soares *et al.* 2013). Results of this study revealed that the TK and CE were found effective in inhibiting the growths of different test fungi belong to species of *Aspergillus*, *Fusarium* and *Penicillium*. However, *Penicillium* spp. was found more resistant among the fungi tested, but toxigenic strains of *A. flavus* and *F. verticillioides* were effectively inhibited *in vitro* and *in vivo*. Both aflatoxins and fumonisins are relevant in maize and maize-based foods and feeds due to their widespread occurrence and co-occurrence (Chulze 2010).

The results from the measurement of ergosterol content demonstrated that the TK caused adverse effect on plasma membrane of the toxigenic strains of *A. flavus* and *F. verticillioides*. The results revealed that the ergosterol content (at 282 nm) in the plasma membrane of both *A. flavus* and *F. verticillioides* was completely inhib-

ited at concentrations higher than 250 μg ml^{-1}. Ergosterol, a sterol, is specific to fungi and is the major sterol component of the fungal cell membrane. It is also responsible for maintaining the cell function and integrity (Tian *et al.* 2012). Hence, ergosterol is a potential target for antifungal drug. Different classes of antifungal agents *viz.*, polyene antibiotics, azole derivatives and allylamines/thiocarbamates, they target ergosterol, the major sterol in the fungal plasma membrane (Georgopapadakou 1998). The primary mechanism of action by which azole antifungal drugs inhibit fungal growth is disruption of normal sterol biosynthetic pathways, leading to a reduction in ergosterol biosynthesis (Kelly *et al.* 1995). The depletion of ergosterol disrupts the structure of the plasma membrane, making it more vulnerable to further damage, and alters the activity of several membrane-bound enzymes, such as those associated with nutrient transport and chitin synthesis. Severe ergosterol depletion may additionally interfere with the hormone-like functions of ergosterol, affecting cell growth and proliferation (Georgopapadakou 1998). Keeping this point in view, the antifungal mode of action of TK was assessed by measuring the total intracellular ergosterol contents in fungal cells with increasing concentrations of TK. Our observations revealed that there was an adverse effect on ergosterol content, therefore, it confirms the considerable impairment of the biosynthesis of ergosterol in plasma membrane of *A. flavus* and *F. verticillioides.* However, further experiments are required to understand the exact mode of action of the TK on plasma membrane and its correlation with the inhibition of aflatoxin biosynthesis.

Plant products are expected to be more advantageous over synthetic fungicides because of their biodegradable nature, and the abundance of raw materials because of luxuriant growth of the plants and their renewable nature makes use of plant products economical for practical application (Marin *et al.* 2011). The identification of antifungal compounds from plants is one of the promising and alternative strategies for preventing fungal-deterioration and mycotoxin contaminations. Results presented in this study confirmed that the TK isolated from *S. torvum* leaves was effective for the inhibition of growth of important mycotoxigenic fungi commonly associated with deterioration of food and feedstuffs. TK inhibited the growth of toxigenic strains of *A. flavus* and *F. verticillioides*, and their toxin productions at even high water activity levels, and was also found effective against a wide range of field and storage fungi. *Solanum torvum*, being the plant used in traditional medicine, possesses broad spectrum antifungal activity against important field and storage moulds that would probably be a good source of antifungal agents for prevention of fungal-deterioration and mycotoxin contaminations. This study indicates that the compound TK has considerable antifungal and antimycotoxigenic activity, deserving further toxicological investigations for future application.

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Conflict of Interest

No conflict of interest declared.

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