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## Antifungal and antimycotoxigenic properties of chemically characterised essential oil of *Boswellia serrata* Roxb. ex Colebr.

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### ABSTRACT

In the present investigation, the antifungal and antimycotoxigenic activities of *Boswellia serrata* essential oil were evaluated *in vitro* and in viable maize. The GC-MS analysis of *B. serrata* essential oil showed a total of 29 constituents, among which, 3-carene (34.74%),  $\beta$ -ocimene (13.78%), D-limonene (8.25%),  $\beta$ -caryophyllene (6.65%) and terpinolene (5.39%) recorded the highest percentage. The *B. serrata* essential oil showed promising antifungal activity against 15 different field and storage fungi with percentage of mycelial inhibition ranged between 15.9–56.3% at  $1\ \mu\text{L mL}^{-1}$ . Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were ranged between  $0.039\text{--}0.625\ \mu\text{L mL}^{-1}$  and  $2.5\text{--}10.0\ \mu\text{L mL}^{-1}$ , respectively, against the same fungi tested. The production of aflatoxin B<sub>1</sub> and fumonisin B<sub>1</sub> were completely inhibited by *B. serrata* essential oil at  $6\ \mu\text{L mL}^{-1}$  *in vitro*. The ergosterol content was drastically decreased with the increasing concentration of *B. serrata* essential oil *in vitro*. Viable maize model confirmed that aflatoxin B<sub>1</sub> and fumonisin B<sub>1</sub> contents were significantly inhibited with increasing seedling vigour of maize. The results revealed that *B. serrata* essential oil could be explored for management of mould and mycotoxin contaminations in food grains and feedstuffs.

### ARTICLE HISTORY

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## Introduction

Fungi are significant destroyers of foodstuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and production of mycotoxins.<sup>[1]</sup> Mycotoxins are toxic, structurally diverse, non-volatile and relatively low-molecular weight (MW <700) secondary metabolic products produced by a wide range of filamentous fungi such as species of *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium*, and they contaminate food, feed and various agricultural commodities either before harvest or under post-harvest conditions.<sup>[2,3]</sup> According to Food and Agricultural Organization (FAO) of the United Nations, it is estimated that around 25% of the world's food grains are significantly contaminated with mycotoxins.<sup>[4]</sup> Aflatoxins and fumonisins are the most common and wide spread contaminants in food and feedstuffs, which are mainly produced by species of *Aspergillus* and *Fusarium*, respectively.<sup>[5,6]</sup> *Aspergillus flavus* and *Fusarium verticillioides* are the predominant contaminants in food grains, maize in particular, by producing aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and fumonisin B<sub>1</sub> (FB<sub>1</sub>), respectively. The hepatotoxicity, teratogenicity, mutagenicity, immunosuppression, hemorrhage properties of AFB<sub>1</sub> and FB<sub>1</sub> to human has been well reported.<sup>[7,8]</sup> Because of their toxic properties, AFB<sub>1</sub> and FB<sub>1</sub> have been listed as Group 1 and Group 2B human carcinogens by the International Agency for Research on Cancer (IARC),<sup>[9]</sup> respectively. The management of mould infestations and mycotoxins accumulation in food grains was generally

achieved using synthetic chemicals, but residues of these chemicals cause damage to animal, plants and human.<sup>[10,11]</sup> The use of essential oils (EOs) for management of mould and mycotoxin contaminations in foodstuffs would be of great importance due to hazardous effects of synthetic fungicides.<sup>[12,13]</sup>

*Boswellia serrata* Roxb. ex Colebr. belongs to the family Burseraceae commonly found in India, northern Africa and Middle East.<sup>[14]</sup> *B. serrata* has been investigated for various bioactive properties such as anti-arthritic, antiproliferative, antioxidant, analgesic, anti-inflammatory, renal and cardio protective activities.<sup>[15–19]</sup> Kasali et al.<sup>[20]</sup> reported the various volatile constituents predominantly comprised monoterpenoids, of which  $\alpha$ -pinene was major constituents from *B. serrata* bark. Antimicrobial activities of methanolic extract and resin oils of *B. serrata* against different human pathogenic bacteria and yeast have been previously reported.<sup>[21–25]</sup> Mishra and Dubey<sup>[26]</sup> have reported the antifungal activity of *Boswellia serrata* essential oil (BSEO) against *A. flavus*. Although the antimicrobial activity has been previously reported, there are no reports on antimycotoxicogenic property of BSEO and antifungal activities against wide range of field and storage fungi. In this article, we have reported the antifungal activity of BSEO against fifteen different field and storage fungi, and its inhibitory efficacy on AFB<sub>1</sub> and FB<sub>1</sub> production from toxigenic strains of *A. flavus* and *F. verticillioides*, respectively.

## Materials and methods

### Chemicals and culture media

All analytical grade solvents and culture media were purchased from Hi-Media (Mumbai, India). The synthetic fungicide copper oxychloride 50% WP (Fungicop-50) was purchased from Karnatak Agrochemicals Pvt Ltd (India) and zinc ethylene bithiocarbamate 75% WP (Indofil Z-75) was purchased from Indofil Chemicals Company (India). Microtiter plates (96-well) were purchased from Axiva (New Delhi, India). AFB<sub>1</sub> and FB<sub>1</sub> standards were obtained from Sigma (Germany). Silica gel 60 F<sub>254</sub> coated preparative thin layer chromatography (TLC) plates were procured from Merck (Germany).

### Extraction of essential oil from *B. serrata* leaves

Fresh leaves of *B. serrata* were collected from the southern part of Karnataka (India) during 2014–16. The plant samples were authenticated by Dr. Seetharam, Professor, Department of Biological Sciences, Bangalore University, Bengaluru (India) and the authenticated voucher specimens were deposited in the Department of Microbiology and Biotechnology, Bangalore University (Bengaluru) along with a proper voucher number (Voucher numbers: BUB/MB-BT/DCM/DST-SERB/24/2014–15). BSEO was extracted by hydro-distillation method using Clevenger-type apparatus.<sup>[27]</sup> The collected BSEO was stored in dark clean glass vial after removing water traces passing through anhydrous sodium sulphate and stored at 4°C for further analysis.

### Chemical profile analysis of BSEO by GC-MS

The chemical compounds present in the BSEO were identified by gas chromatography coupled to mass spectrometry (GC-MS, Thermo Electron Corporation Focus gas chromatograph, DSQ II model, Thermo Scientific, CA, USA). The GC conditions were as follows: Column, DB 5 ms with integrated guard column (Dimensions: 30mL x 0.25 mm ID x 0.25  $\mu$ m film thickness); initial column temperature was 40°C for 2 min and ramp temperature was 280°C for 5 min; helium served as carrier gas; sample flow rate was 1.0 mL min<sup>-1</sup>; injection volume was 1 $\mu$ L and scan mass ranged between 30 m/z–600 m/z with +ve polarity. The identification of individual component was done on the basis of retention times compared to those of authentic samples and matching spectral peaks

available with National Institute of Standards and Technology (NIST, U.S Department of Commerce, Gaithersburg) spectral libraries.<sup>[28]</sup>

## Antifungal activity of BSEO

### Fungal strains

The fungal species viz., *Aspergillus flavus*, *A. fumigatus*, *A. ochraceus*, *A. tamarii*, *A. terreus*, *Fusarium equiseti*, *F. udum*, *F. verticillioides* and *Penicillium citrinum*, which are associated predominantly with maize at a higher percentage, *Alternaria geophila*, *Curvularia tetramera*, *F. lateritium*, *F. oxysporum* and *Penicillium expansum*, which are frequently associated with sorghum at higher percentage, and *Alternaria brassicicola* (sunflower isolate) was collected from Department of Microbiology, University of Mysore, Mysore (India), were selected as test fungi for antifungal activity assay. The fungal species were previously isolated and reported by Thippeswamy et al.<sup>[29]</sup> Mycotoxigenic strains of *A. flavus* and *F. verticillioides* were isolated from maize seeds in our previous study.<sup>[5,30]</sup> The isolated fungi were maintained on Sabouraud dextrose agar (SDA) and the 7-days-old cultures were used for the assays.

### Determination of percent mycelial inhibition

The antifungal activity of BSEO was determined against fifteen different field and storage fungi by poisoned food technique following the procedure of Prakash et al.<sup>[31]</sup> with minor modifications. The requisite amount of BSEO was dissolved in 0.5 mL of acetone and added to molten SDA to achieve final concentrations ranged from 1 to 10  $\mu\text{L mL}^{-1}$ . A 5 mm disc of test fungi was placed at the centre of the plate and incubated at  $28 \pm 2^\circ\text{C}$  for 7 days. The Petri dishes containing media with 0.5 mL of acetone served as control. The fungi-toxicity of the extract in terms of percentage inhibition of mycelial growth was calculated using the formula:

$$\text{Percent mycelial inhibition (\%MI)} = \frac{dc - dT}{dc} 100$$

Where, dC = average mycelial growth in control and dT = average mycelial growth in treatment.

### Determination of MICs and MFCs

The broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of BSEO following the standard procedures with some modifications.<sup>[32,33]</sup> Briefly, 200  $\mu\text{L}$  of two-fold serially diluted BSEO in Sabouraud dextrose broth (SDB) ( $0.019\text{--}10.0 \mu\text{L mL}^{-1}$ ) was added separately to the wells of a sterile 96-well microtiter plate and inoculated with 15  $\mu\text{L}$  of fungal spore suspension ( $10^4$  spores  $\text{mL}^{-1}$ ) and incubated at  $28 \pm 2^\circ\text{C}$  for 72 hours. SDB containing same concentration of copper oxychloride 50% WP and zinc ethylene bithiocarbamate 75% WP served as positive control and SDB containing only Tween-80 served as negative control. After incubation, a 10  $\mu\text{L}$  of treated broths were streaked radially onto the SDA plates and incubated at the same condition. After the incubation period, the MIC/MFC values were recorded. Parallely, the results were also confirmed by adding a 50  $\mu\text{L}$  of iodinitrotetrazolium chloride (INT; 2 mg  $\text{mL}^{-1}$ ) to microtiter plate and incubated at  $37^\circ\text{C}$  for 30 min. The pale yellow-coloured INT was reduced to a pink colour which indicated the presence of fungal viability, while the yellow colour remained the same where the fungal growth was inhibited.

### Effect of BSEO on AFB<sub>1</sub> and FB<sub>1</sub> production

The *in vitro* efficacy of BSEO on AFB<sub>1</sub> production by *A. flavus* was determined using Sucrose-magnesium sulphate-potassium nitrate-yeast extract broth (SMKYB) medium.<sup>[34]</sup> Briefly, 100  $\mu\text{L}$  of

spore suspension ( $10^4$  spores  $\text{mL}^{-1}$ ) of *A. flavus* were inoculated into SMKYB containing the requisite amount of BSEO ( $2.0$ – $10.0$   $\mu\text{L mL}^{-1}$ ) and incubated at  $28 \pm 2^\circ\text{C}$  for 10 days. The broth cultures were filtered through Whatman No. 1 filter article and the obtained culture filtrate was used for  $\text{AFB}_1$  extraction by adding an equal volume of chloroform. The chloroform layer was collected and evaporated *in vacuo*. The  $\text{AFB}_1$  content was determined qualitatively by TLC method and quantitatively by spectrophotometric method (UV-1800, Shimadzu, Japan). The amount of  $\text{AFB}_1$  content was calculated following formula:

$$\text{AFB}_1 \text{ content } (\mu\text{g L}^{-1}) = [D \times M/E \times L] 1000$$

where,  $D$  is absorbance,  $M$  is molecular weight of  $\text{AFB}_1$  (312),  $E$  is molar extinction coefficient of  $\text{AFB}_1$  (21,800) and  $L$  is path length (1 cm).

The *in vitro* efficacy of BSEO on  $\text{FB}_1$  production by *F. verticillioides* was determined following the procedure of Bailly et al.<sup>[35]</sup> with minor modifications. Briefly, 100  $\mu\text{L}$  of spore suspension ( $10^4$  spores  $\text{mL}^{-1}$ ) of *F. verticillioides* was inoculated into SDA containing the requisite amount of BSEO ( $2.0$   $\mu\text{L}$ – $10.0$   $\mu\text{L mL}^{-1}$ ) and incubated at  $28 \pm 2^\circ\text{C}$  for 10 days. To estimate  $\text{FB}_1$  production, the culture along with SDA was ground in acetonitrile-water (1:1, v/v) and filtered through 0.45  $\mu\text{m}$  porous membrane filter. The extracted  $\text{FB}_1$  was visualised on eluted TLC chromatograms by spraying with 0.5% *p*-anisaldehyde solution followed by heating at  $110^\circ\text{C}$  for 10 min. The amount of  $\text{FB}_1$  was estimated using spectrophotodensitometer (Biorad, Universal Hood II, 720BR/02170, USA) at 600 nm by comparing with known concentrations of standard  $\text{FB}_1$ .

### **Efficacy of BSEO on $\text{AFB}_1$ and $\text{FB}_1$ production in viable maize**

The efficacy of BSEO on  $\text{AFB}_1$  and  $\text{FB}_1$  production in maize corn was determined following the procedure of Garcia et al.<sup>[10]</sup> with some modifications. Briefly, freshly harvested maize samples were collected and the water activity ( $a_w$ ) was adjusted to 0.95 by adding sterile distilled water, based on the following formula:

$$W = A(b - a)/(100 - b)$$

where,  $W$  = volume of water required (ml),  $A$  = initial weight of the sample (g),  $a$  = initial moisture content (%) and  $b$  = required moisture content (%),<sup>[36]</sup> and separated into two sets. One set of maize samples were treated with different concentrations of BSEO ( $2.0$ – $10.0$   $\mu\text{L g}^{-1}$ ) and inoculated with 100  $\mu\text{L}$  of spore suspension ( $10^4$  spores  $\text{mL}^{-1}$ ) of *A. flavus* and *F. verticillioides*, separately. All treatments were incubated at  $28 \pm 2^\circ\text{C}$  up to 10 days. After incubation, the *A. flavus* treated maize seeds (10 g) were milled and subjected for  $\text{AFB}_1$  extraction by adding an equal volume of  $\text{CHCl}_3$ . After evaporation of chloroform, the residue was again re-dissolved in 1 mL of chloroform and subjected to qualitative and quantitative estimation following the standard procedures as described above.<sup>[35,37]</sup> Similarly, *F. verticillioides* treated maize seeds (10 g) were powdered using a warring blender and subjected to extraction of  $\text{FB}_1$  by adding 100 mL of acetonitrile-water (1:1, v/v), then filtered through Whatman No. 1 filter article and evaporated the acetonitrile-water on water bath. After that, the residue was re-dissolved in 1 mL of methanol, then 10  $\mu\text{L}$  of sample was spotted on TLC plate along with desired different concentrations of standard  $\text{FB}_1$ . Eluted the TLC plate using butanol-acetic acid-water (20:10:10, v/v/v) as a solvent system and allowed to air dry. The air-dried TLC plate was sprayed with 0.5% *p*-anisaldehyde in methanol-acetic acid- $\text{H}_2\text{SO}_4$  (85:10:0.5, v/v/v) solution and subjected to heat treatment at  $110^\circ\text{C}$  for 10 min in hot air oven (Labline, India). The amount of  $\text{FB}_1$  was estimated qualitatively and quantitatively by measuring the band intensity using spectrophoto-densitometer at 600nm and calculated the amount of  $\text{FB}_1$  by comparing with different concentrations of standard  $\text{FB}_1$ . The other set was treated with 10.0  $\mu\text{L}$  of BSEO and subjected to evaluation of natural seed borne mycoflora and seedling vigour of maize.<sup>[37,38]</sup> The percent incidences (PI) of the seed-borne fungi associated with maize seed samples were determined following the formula:

$$PI = \frac{\text{Number of seeds on which the fungus was encountered in each sample}}{\text{Total number of seeds tested in each of the samples}} \times 100$$

Simultaneously, to monitor the phytotoxic effect on maize, the seedling vigour was measured by recording the length of radicle and plumule of maize seedlings. The seedling vigour index (SVI) was determined following the formula.<sup>[39]</sup>

$$SVI = (\text{Average root length in cm} + \text{Average shoot length in cm}) \times \text{Germination percentage}$$

### **Effect of BSEO on ergosterol content in fungal plasma membrane**

Effect of BSEO on ergosterol content in the plasma membrane of *A. flavus* and *F. verticillioides* was detected by a method described previously by Abhishek et al.<sup>[30]</sup> Fifty  $\mu\text{L}$  of spore suspension of *A. flavus* and *F. verticillioides* containing  $10^6$  spores  $\text{mL}^{-1}$  was inoculated into respective medium (SMKYB for *A. flavus* and SDB for *F. verticillioides*) containing different concentrations of BSEO ( $2\text{--}10 \mu\text{L mL}^{-1}$ ) and incubated at  $28 \pm 2^\circ\text{C}$  for 4 days. A control set, tween-80, was kept parallel to the treatment. After incubation, mycelia were harvested and subjected to extraction of ergosterol. The extracted n-heptane layer was analysed by scanning spectrophotometry (UV-1800, Shimadzu, Japan) between 230 and 300 nm. The ergosterol content was calculated as a percentage of the mycelia wet weight by the following formula:

$$\% \text{ ergosterol} + \%24(28) - \text{dehydroergosterol} = (A_{282}/290)/\text{pellet weight},$$

$$\% 24(28) - \text{dehydroergosterol} = (A_{230}/518)/\text{pellet weight},$$

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

where, 290 and 518 are the *E* values (in percentages per cm) of 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  $\pm$  standard error. Analysis of variance was conducted, and the differences between values were tested for deviation significance by ANOVA with the SPSS 19 (IBM, Armonk, NY, USA) programme. Differences at  $p \leq 0.05$  were considered statistically significant.

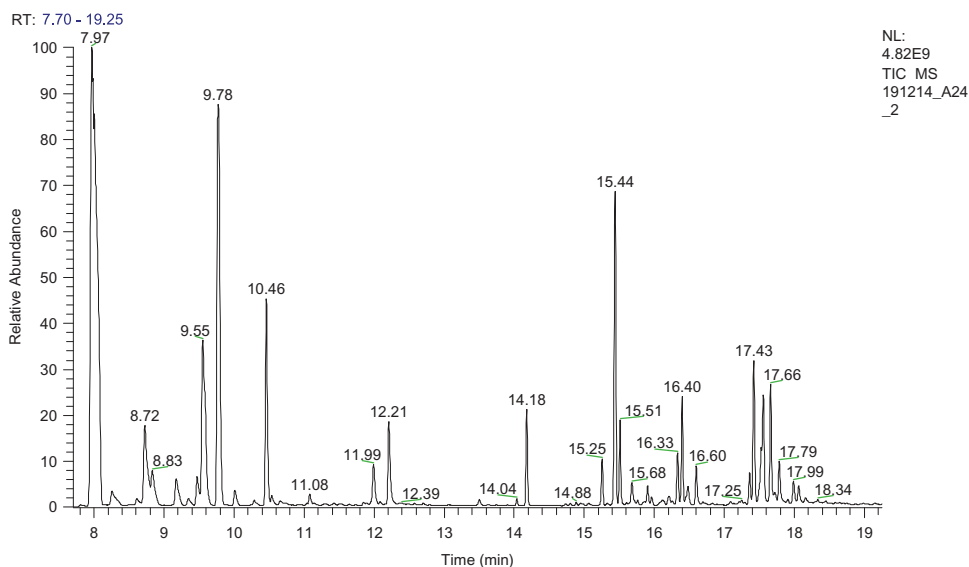
## **Results and discussion**

### **Phytochemical characterization of BSEO**

The extracted BSEO was clear to pale yellow in colour and its yield was 1.5% (v/w). The GC-MS analysis of BSEO revealed that a total of 29 phytoconstituents were present, among which 3-carene (34.74%),  $\beta$ -ocimene (13.78%), D-limonene (8.25%),  $\beta$ -caryophyllene (6.65%) and terpinolene (5.39%), were identified as major components with the highest percentage (Table 1; Figure 1). The geographical and seasonal variations have a significant effect on the yield and composition of essential oils.<sup>[40]</sup> Also the plant species, parts and extraction methods that may show chemotypic variations.<sup>[31,41]</sup> Although some of the components of BSEO reported by the previous workers were same,<sup>[42]</sup> there are variations in their percentage chemical composition in comparison with present study.

**Table 1.** Phytochemical constituents of BSEO.

S. no.	Compounds name	Percentage	Retention time
1	3-Carene	34.74	7.97
2	p-sec-Butylphenol	1.04	8.26
3	$\beta$ -Pinene	2.95	8.72
4	Vinyl crotonate	0.93	8.83
5	$\alpha$ -Phenylcyclopentaneacetic acid	1.24	9.17
6	D-Limonene	8.25	9.55
7	$\beta$ -Ocimene	13.78	9.78
8	Benzeneacetic acid	0.53	10.01
9	Terpinolene	5.39	10.46
10	(4E,6Z)-allo-Ocimene	0.36	11.08
11	Pinocarveol	1.36	11.99
12	$\alpha$ -Terpineol	2.25	12.21
13	Bornyl acetate	0.14	13.5
14	$\gamma$ -Elemene	1.77	14.18
15	$\beta$ -Caryophyllene	6.65	15.44
16	$\alpha$ -Bergamotene	1.64	15.51
17	Selina-3,7(11)-diene	0.54	15.68
18	$\alpha$ -Humulene	0.39	16.33
19	(+)-Ledene	1.0	16.60
20	Elixene	2.05	16.4
21	Borneol	0.41	17.43
22	Bornyl isovalerate	0.72	17.66
23	Palustral	0.58	17.36
24	Spathulenol	2.67	17.43
25	Globulol	3.58	17.56
26	Viridiflorol	2.39	17.66
27	2- Naphthalenemethanol	0.8	17.79
28	2,2-Dimethylpropionic acid	0.45	17.99
29	2,5-di-tert-Butylnitrobenzene	0.51	18.07

**Figure 1.** GC-MS Profile of BSEO.

### **Antifungal activity of BSEO**

The growth inhibitory effect of BSEO was screened against a panel of 15 fungal pathogens containing storage and field fungi and the obtained results are presented in [Table 2](#). The percentage of growth

**Table 2.** Antifungal activity of BSEO and synthetic fungicides against different field and storage fungi.

Test fungi	BSEO			CO		ZEB	
	%MI <sup>a</sup>	MIC	MFC	MIC	MFC	MIC	MFC
<i>A. brassicicola</i>	56.3 ± 0.5	0.039	2.5	39	625	39	78
<i>A. geophila</i>	48.8 ± 0.6	0.078	2.5	156	>1000	39	625
<i>A. flavus</i> <sup>b</sup>	15.9 ± 0.8	0.625	>10.0	625	>1000	156	1000
<i>A. fumigatus</i>	22.5 ± 0.8	0.156	5.0	312	>1000	312	1000
<i>A. ochraceous</i>	25.2 ± 0.3	0.156	5.0	312	625	78	625
<i>A. tamarii</i>	17.5 ± 0.6	0.625	>10.0	625	>1000	156	250
<i>A. terreus</i>	18.74 ± 1.1	0.312	5.0	312	>1000	156	250
<i>C. tetramera</i>	46.9 ± 1.7	0.156	2.5	312	>1000	39	625
<i>F. equiseti</i>	34.4 ± 1.2	0.078	5.0	312	>1000	39	125
<i>F. lateritium</i>	39.6 ± 1.5	0.156	2.5	625	>1000	39	125
<i>F. oxysporum</i>	22.38 ± 1.0	0.312	5.0	625	>1000	39	125
<i>F. udum</i>	35.56 ± 1.0	0.078	2.5	312	>1000	39	125
<i>F. verticillioides</i> <sup>c</sup>	18.21 ± 0.8	0.312	10.0	1000	>1000	39	125
<i>P. citrinum</i>	23.55 ± 0.5	0.625	10.0	1000	>1000	625	>1000
<i>P. expansum</i>	27.45 ± 0.7	0.312	10.0	625	>1000	625	>1000

CO: Copper oxychloride 50%; ZEB: Zinc ethylene bithiocarbamate 75%.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values are expressed in  $\mu\text{L mL}^{-1}$  in case of BSEO and  $\mu\text{g mL}^{-1}$  in case of synthetic fungicides.

Data given are the mean values of three replicates  $\pm$  standard deviation ( $p \leq 0.05$ ).

<sup>a</sup>%MI of BSEO was determined at  $1 \mu\text{L mL}^{-1}$ .

<sup>b</sup>Aflatoxin B<sub>1</sub> producing strain.

<sup>c</sup>Fumonisin B<sub>1</sub> producing strain.

inhibition by the BSEO was estimated by measuring the growth diameter of colony grown on SDA with treatment and control. Most of the treated field fungi were susceptible at  $1.0 \mu\text{L mL}^{-1}$ . Among the fungi tested, *A. flavus* and *A. tamarii* were found to be most resistant organisms with percent mycelial inhibition 15.9 and 17.5%, respectively. The field fungi such as *A. brassicicola* (56.3%), *A. geophila* (48.8%) and *C. tetramera* (46.9%), were found to be most susceptible organisms at  $1.0 \mu\text{L mL}^{-1}$ . The MIC and MFC values of BSEO were ranged between  $0.039\text{--}0.625 \mu\text{L mL}^{-1}$  and  $2.5\text{--}>10.0 \mu\text{L mL}^{-1}$ , respectively. The BSEO showed concentration-dependent growth inhibitory activity against toxigenic strains of *A. flavus* and *F. verticillioides* with percent mycelia inhibition 62.6% and 75.5%, at  $10 \mu\text{L mL}^{-1}$ , respectively (Table 3). The MIC and MFC values of BSEO against toxigenic strains of *A. flavus* were 0.625 and  $>10 \mu\text{L mL}^{-1}$ , and against *F. verticillioides* were 0.312 and  $10 \mu\text{L mL}^{-1}$ , respectively (Table 2). The obtained results were compared with MIC and MFC values of synthetic fungicides viz., copper oxychloride 50% and zinc ethylene bithiocarbamate 75%. The MIC and MFC values of copper oxychloride 50% were ranged  $39\text{--}1000 \mu\text{g mL}^{-1}$  and  $625\text{--}>1000 \mu\text{g mL}^{-1}$ , respectively. Whereas the MIC and MFC values of zinc ethylene bithiocarbamate 75% were ranged from  $39\text{--}625 \mu\text{g mL}^{-1}$  and  $78\text{--}>1000 \mu\text{g mL}^{-1}$ , respectively. On comparative evaluation with synthetic fungicides, BSEO showed remarkable antifungal activities against different field and storage fungi tested. In antifungal evaluation, the storage fungi viz., species of *Aspergillus* and *Penicillium*, were more resistant when compared to field fungi viz., species of *Alternaria*, *Curvularia* and *Fusarium*. Among the methods used for antifungal activity evaluation, the better activity was observed in the broth microdilution method than poisoned food technique, because of more opportunity to test samples to come in close contact with test fungi as it has been earlier reported.<sup>[43]</sup>

Management of fungal diseases in crops is generally achieved using synthetic chemicals.<sup>[10,44]</sup> But, 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 microorganisms.<sup>[1,45]</sup> Essential oils, being the natural derivatives, are biodegradable and do not leave toxic residues or by-products to contaminate the environment.<sup>[44,46]</sup> The result of the present study confirms that BSEO showed significant antifungal activity against both field and storage fungi including mycotoxigenic strains of *A. flavus* and *F. verticillioides*.



**Table 3.** Antifungal and antimycotoxigenic activities of BSEO against toxigenic strains of *A. flavus* and *F. verticillioides*.

BSEO <sup>a</sup>	<i>A. flavus</i>			<i>F. verticillioides</i>		
	AFB <sub>1</sub> content <i>in vitro</i> ( $\mu\text{g L}^{-1}$ )	AFB <sub>1</sub> content <i>in vivo</i> ( $\mu\text{g kg}^{-1}$ )	% MI	FB <sub>1</sub> content <i>in vitro</i> ( $\text{mg L}^{-1}$ )	FB <sub>1</sub> content <i>in vivo</i> ( $\text{mg kg}^{-1}$ )	% MI
Control	1523.6 $\pm$ 10.1	1868.5 $\pm$ 15.6	0.0 $\pm$ 0.0	89.7 $\pm$ 3.1	48.5 $\pm$ 2.1	0.0 $\pm$ 0.0
2.0	497.4 $\pm$ 2.59	533.2 $\pm$ 6.3	31.83 $\pm$ 0.8	50.6 $\pm$ 1.8	29.6 $\pm$ 1.6	38.5 $\pm$ 2.0
4.0	182.6 $\pm$ 4.3	240.6 $\pm$ 4.2	45.43 $\pm$ 1.4	0.0 $\pm$ 0.0	19.6 $\pm$ 1.2	56.54 $\pm$ 1.2
6.0	0.0 $\pm$ 0.0	188.4 $\pm$ 1.8	52.22 $\pm$ 0.9	0.0 $\pm$ 0.0	15.2 $\pm$ 0.9	64.35 $\pm$ 0.6
8.0	0.0 $\pm$ 0.0	108.7 $\pm$ 2.5	58.20 $\pm$ 1.2	0.0 $\pm$ 0.0	11.1 $\pm$ 0.6	70.25 $\pm$ 0.5
10.0	0.0 $\pm$ 0.0	82.5 $\pm$ 1.7	62.65 $\pm$ 0.7	0.0 $\pm$ 0.0	2.6 $\pm$ 0.8	75.55 $\pm$ 0.8

<sup>a</sup>BSEO treatment concentration: *In-vitro* evaluation ( $\mu\text{L mL}^{-1}$ ) and *in-vivo* evaluation ( $\mu\text{L g}^{-1}$ ). Data given are the mean values of four replicates  $\pm$  standard deviation ( $p \leq 0.05$ ).

### **Inhibitory efficacy of BSEO on AFB<sub>1</sub> and FB<sub>1</sub> production**

*In vitro* inhibitory effects of BSEO on AFB<sub>1</sub> production by *A. flavus* and FB<sub>1</sub> production by *F. verticillioides* were estimated using suitable culture medium. The mycelial growth and mycotoxin production were significantly inhibited by BSEO in a dose dependant manner (Table 3). The amounts of AFB<sub>1</sub> and FB<sub>1</sub> contents in control were found to be 1523.6 ± 10.1 µg L<sup>-1</sup> and 89.7 ± 3.1 mg L<sup>-1</sup>, respectively. AFB<sub>1</sub> production was completely inhibited by BSEO at concentration 6 µL mL<sup>-1</sup> of culture medium, while FB<sub>1</sub> was inhibited at 4 µL mL<sup>-1</sup>. It is interesting to note that no complete inhibition of fungal biomass was observed at these concentrations.

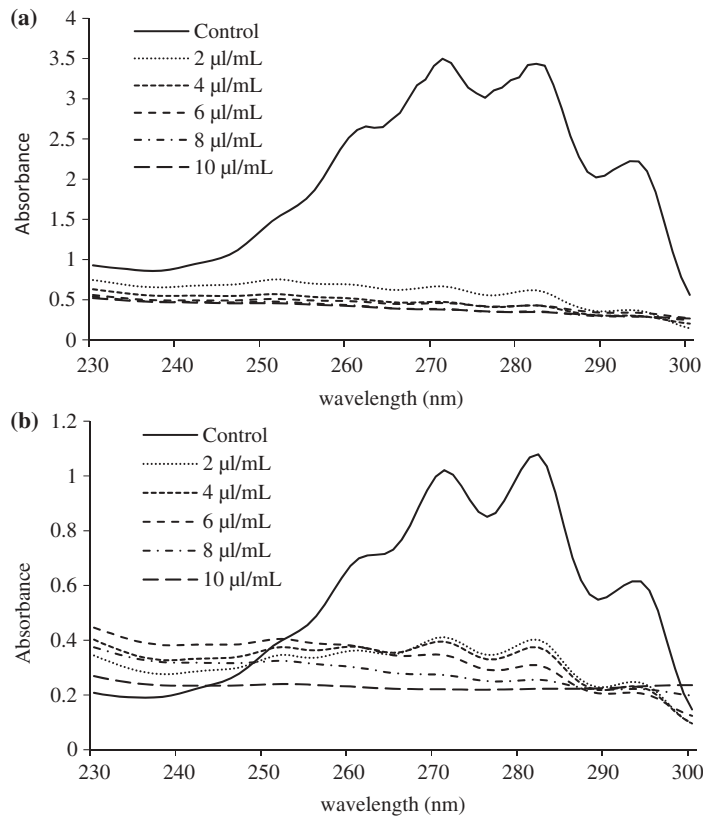
*In vivo* efficacy of BSEO on AFB<sub>1</sub> production by *A. flavus* and FB<sub>1</sub> production by *F. verticillioides* were evaluated using viable maize as model (Table 3). The amount of AFB<sub>1</sub> and FB<sub>1</sub> contents extracted from untreated maize (control) were found to be 1868.5 ± 15.6 µg kg<sup>-1</sup> and 48.5 ± 2.1 mg kg<sup>-1</sup>, respectively. In BSEO treated maize, AFB<sub>1</sub> and FB<sub>1</sub> contents were found to be 82.5 ± 1.7 µg kg<sup>-1</sup> and 2.6 ± 0.8 mg kg<sup>-1</sup>, respectively, at 10 µL mL<sup>-1</sup>. The effect of BSEO on inhibition of natural seed-borne mycoflora of maize was evaluated at 10 µL g<sup>-1</sup>. The results revealed that species of *Aspergillus* (PI 95.0%), *Fusarium* (PI 45.0%), *Penicillium* (PI 80.0%), *Alternaria* (PI 25.0%) and *Curvularia* (PI 15.0%) recorded as dominant fungi, but these fungi were inhibited significantly in maize sample treated with BSEO at 10 µL g<sup>-1</sup> of maize. When compared to control the percent incidences species of *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Curvularia* were drastically reduced in maize treated with BSEO (PI 35.0, 10.0, 25.0, 0.0, 0.0%), respectively. The increasing seedling vigour index was observed in BSEO treated maize samples (2260) than control (1654), which indicates that BSEO did not show adverse effect on germination of maize seeds. The results showed that BSEO was found to be effective in control of AFB<sub>1</sub> and FB<sub>1</sub> production in maize.

Mycotoxin contamination of various foodstuffs and agricultural commodities is a major problem in the tropics and sub-tropics, where climatic conditions and agricultural and storage practices are favorable to fungal growth and toxin production.<sup>[47]</sup> Many species of *Aspergillus*, *Fusarium* and *Penicillium* are not only recognised as plant pathogens but are also sources of the important mycotoxins of concern in animal and human health.<sup>[48]</sup> Among the mycotoxins, aflatoxins and fumonisins are the most toxic secondary metabolites mainly produced by species of *Aspergillus* and *Fusarium*. *A. flavus* and *F. verticillioides* are two important mycotoxigenic moulds that colonise different kinds of food grains.<sup>[45,49]</sup> Aflatoxins are highly toxic polyketide secondary metabolites produced mainly by *A. flavus*. The human health impact of AFB<sub>1</sub> exposure is widespread in developing countries.<sup>[50,51]</sup> 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.<sup>[52]</sup> Both aflatoxins and fumonisins are relevant in food and feedstuffs due to their widespread occurrence and co-occurrence.<sup>[53]</sup> Maize, which is susceptible to common fungal infestation, was selected as model for this study. Conditions studied *in vivo* were closed to environment that may occur during pre- and post-harvest of maize. Results showed that the BSEO was effective in inhibition of fungal growth and toxin production in treated maize.

### **Effect of BSEO on ergosterol content in fungal plasma membrane**

The inhibition of ergosterol content in the plasma membrane of *A. flavus* and *F. verticillioides* by BSEO is shown in Figure 2. When compared to control, the percent inhibition of ergosterol content in the plasma membrane of *A. flavus* by BSEO was recorded to be 73.67, 79.77, 80.38, 82.21 and 83.94% at 2.0, 4.0, 6.0, 8.0 and 10.0 µL mL<sup>-1</sup> concentrations. Similarly, the percent inhibition of ergosterol content of *F. verticillioides* was found to be 55.87, 56.11, 59.47, 63.07 and 65.46% at 2.0, 4.0, 6.0, 8.0, and 10.0 µL mL<sup>-1</sup> concentrations, respectively.

Ergosterol is specific to fungi and is the major sterol component of the fungal cell membrane.<sup>[30]</sup> The major antifungal agents act by inhibition of ergosterol synthesis,<sup>[54,55]</sup> which leads to inhibition



**Figure 2.** Effect of different concentrations of BSEO on ergosterol content in the plasma membrane of *A. flavus* (a) and *F. verticillioides* (b).

of fungal cell growth and reduced ergosterol synthesis, hence the measurement of ergosterol content provides information pertinent to alteration and/or disruption of normal sterol biosynthetic pathways. The lower quantity of ergosterol observed with increasing concentrations of BSEO in comparison with the control revealed that BSEO considerably impaired the biosynthesis of ergosterol.

The development of natural antifungal agents would be helpful for decreasing the negative impact of synthetic chemical agents. The EOs of higher plants are well known for their antifungal and antioxidant activities. Recent reports revealed that EOs can be explored as food preservatives to improve the shelf life and quality of stored food products.<sup>[37]</sup> Due to their bioactivity in the vapour phase, this characteristic makes them attractive as botanical fumigants against moulds and mycotoxin contamination of food commodities.<sup>[56]</sup> Furthermore, some EOs have been generally exempted from the toxicity data requirements by Environment Protection Agency (EPA) in view of their favourable safety profile.<sup>[57]</sup> Some of the EO-based food preservatives are commercially available in European market.<sup>[31]</sup> In this article, we have reported the concentration-dependent antifungal, antiaflatoxigenic and antifumonisin activities of BSEO for the first time. Hence, the present findings indicate the possible use of BSEO as alternative agents for developing plant based preservatives against post-harvest fungal infestation and mycotoxins contamination of food commodities.

## Conclusion

Management of fungal contamination, biodeterioration and mycotoxin accumulation in foodstuffs is generally achieved using synthetic chemicals. 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 microorganisms. The identification of antifungal compounds or Eos from plants is one of the promising and alternative strategies for preventing fungal-deterioration and mycotoxin contaminations. The BSEO exhibited 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 mycotoxins contamination. Further, the significant antimycotoxigenic potency of BSEO offers possibilities for its application in preventing mycotoxins contamination in food and feed stuffs. The study suggests further investigations for large-scale practice in the food systems.

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