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Effect of kebar grass (*Biophytum petersianum* Klotzsch) leaf extract on the growth and morphological structure of aflatoxigenic *Aspergillus flavus*

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Abstract

The objective of this study was to investigate the antifungal activity of kebar grass (*Biophytum petersianum* Klotzsch) extract (KGE) on the mycelial growth, conidiation and morphological structure of two types of aflatoxigenic fungus, which are *Aspergillus flavus* BCCF 0219 and *A. flavus* BIO 2236. They were isolated in the three types of model media, namely carbohydrate-enriched medium, fat-enriched medium and protein-enriched medium with five concentrations of KGE (12, 14, 16, 18, and 20 mg/mL) on each media. The best extract concentration of that inhibits the growth of *A. flavus* BCCF 0219 was found in the carbohydrate-enriched medium (95.7%), which was 12 mg/mL, whereas at *A. flavus* BIO 2236 was found in the fat-enriched medium (100%), which was 16 mg/mL. Based on SEM observation results, it was found that the mechanisms involved in fungal growth inhibitory by the KGE were by morphological alterations of the hyphal development, and the collapse of the entire hyphae. These findings indicated that KGE as a potential natural antifungal agent, particularly against aflatoxigenic fungi.

1. Introduction

Kebar grass (*Biophytum petersianum* Klotzsch), belonging to the family Oxalidaceae, is a native plant of Kebar District, West Papua, Indonesia. The plant grows naturally and widespread all over the Kebar District (Santoso *et al*., 2007). This plant is also found in Southeast Asia and Africa, especially in Mali region. Mali community uses this plant for the treatment of wounds, inflammation, ulcers, malaria, and fever (Inngjerdingen *et al*., 2008). Papuan community, especially in Kebar District, often uses this plant as a fertility drug.

Several studies reported that kebar grass has potential as antibacterial, hypoglycemic agent, immunomodulatory agent, chemoprotective agent, hypocholesterolemic agent, apoptosis, anti-inflammatory, antitumor and prostaglandin biosynthesis (Natarajan *et al*., 2010). Furthermore, the use of methanol extract of kebar grass can affect the expression of COX-2 (Guruvayoorappan and Kuttan, 2008) and stimulate immune system cells in mice (Inngjerdingen *et al*., 2008). Some phenolic components that affect the system COX-1/COX-2 also have been isolated by Bucar *et al*. (1998). Kebar grass extract also shows the effect of apoptosis on B16F-10 cells and is able to regulate NO production and cytokine-associated macrophages to tumor formation (Guruvayoorappan and Kuttan, 2007).

Although Natarajan *et al.* (2010) have reported the antibacterial activity of kebar grass, there have been no reports of the antifungal activity of grass kebar. During the storage period, fungal growth on foodstuffs can lead to an increase in moisture content and humidity of the storage material. In this condition, the damage occurs so fast that nitrogen source materials needed to grow will be reduced and fungi need to maintain his life by utilizing the carbon source in the material as a source of energy. This situation will take place of fungi by doing secondary metabolism that produces mycotoxins (Syarief *et al*., 2012).

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Fungal contamination and their mycotoxin production lead to 25 – 40% or even more post-harvest economic losses in developing countries. About 4.5 billion people in underdeveloped countries are exposed to mycotoxins (Murugan *et al.,* 2013). Aflatoxins are mycotoxins produced by *A. flavus* and *A. parasiticus* that are hepatotoxic, mutagenic, teratogenic, and carcinogenic to humans and animals (Alberts *et al*., 2006). Besides harmful to human and animal health, aflatoxin contamination in food and feed also resulted in the loss of significant economic ranged from $85-100 million in the USA (Alberts *et al*., 2009), so that contamination of aflatoxigenic fungi received worldwide attention (Sohaibani *et al*., 2011).

Type of substrates is a factor which determines aflatoxin content produced by aflatoxigenic *A. flavus.* Since it is difficult to completely avoid aflatoxin contamination, the technology needed to reduce aflatoxin contamination without affecting the flavor and nutritional value of food and safety. As foodstuff can consist of carbohydrate, fat and protein, therefore as a preliminary screening, it is necessary to investigate the effects of kebar grass in inhibiting the growth of aflatoxigenic *A. flavus* in food system model and the mechanism of the growth inhibition itself.

A preliminary study in our laboratory was conducted to select the type of extract that gave the best growth inhibition of *A. flavus.* In our preliminary study, we have examined the fungal growth inhibitory activity of six Kebar grass extracts (KGE): four extracts from single solvent extraction by using n-hexane, ethyl acetate, methanol, and water. The other two extracts were obtained from successively extracted from n-hexane-EtOAc-MeOH and n-hexane-EtOAc. The result showed that n-hexane-EtOAc-MeOH of KGE had the highest inhibition of *A. flavus* BCCF 0219 and *A. flavus* BIO 2236 growth. In this study, we further determined a *minimum inhibitory concentration* (MIC) of KGE against the growth of aflatoxigenic *A. flavus* and the mechanism involved. The chemical profile of KGE was also analyzed by using pyrolysis GC-MS.

2. Materials and methods

2.1 Plant materials

Kebar grass was collected from Kebar district, Manokwari county, West Papua province. Kebar grass was identified by the Indonesian Institute of Science (LIPI).

2.2 Chemicals

All solvents were of analytical grade purchased from JT. Baker, USA.

2.3 Plant extracts preparation

Kebar grass leaves were air-dried for about 14 days or until the colour turned into golden brown and they were easily broken. The leaves were then crushed into coarse powder using a blender. As much as 200 g powdered kebar grass was extracted by maceration (1 hr) method combined with sonification (30 mins) at room temperature using three kinds of organic solvent, i.e. n-hexane, ethyl acetate, and methanol solvents, followed by Whatman No.1 paper filtration. Maceration was repeated for two times and the filtrate was collected. The remaining residue of the plant material was dried for 24 hrs and was extracted using ethyl acetate and methanol solvent sequentially in a similar manner. The filtrate was collected and dried with a rotary evaporator. The hexane-ethyl acetate-methanol filtrate was called as HEM extract. Residual solvent in HEM extract was removed by nitrogen gas (Deng *et al*., 2011). The extract was then stored at 4°C before used for the analysis of chemical composition, antifungal activity and morphological changes of fungal structure.

2.4 Fungal isolate

Isolates of *A. flavus* BCCF 0219 and *A. flavus* BIO 2236 were obtained from the Research Institute for Veterinary Science (BBLITVET) and SEAMEO BIOTROP Culture Collections, respectively.

2.5 Preparation of fungal conidia

Fungal conidia were prepared using a method by Joseph *et al*. (2005) with a slight modification. Each fungal isolate was cultured in PDA (PDA; Oxoid, England) slant and incubated at 28°C for 5 days. The conidia were harvested by adding 10 mL of sterile distilled water and collected aseptically using a sterile inoculation loop. The conidial suspension was dissolved in Tween 80 (0.5%) and centrifuged (2000 x *g*) at 28°C for 5 mins. Conidial concentration was calculated using a haemocytometer and adjusted to 10^6 CFU/mL. The suspension was used to test the activity of kebar grass extract in inhibiting the growth of *A. flavus* isolates.

2.6 Preparation of food media model

Food model media is a medium for growing fungus that is made to mimic the conditions of real food. In this study food model media was made by adding carbohydrates, proteins, and fats to the agar media in order to mimic carbohydrate-rich foods, protein-rich foods, and fat-rich foods. Carbohydrate-enriched medium was prepared by the addition of 30% (w/v) glucose (E Merck, Germany) on Bacto Agar (Difco, USA) (Shih and Marth, 1974).

Fat-enriched medium was prepared using a method of Riba *et al.* (2010). As much as 100 g of grated coconut flesh was homogenized using 300 mL of heat distilled water for 5 mins. The mixture was filtered using four layers of cheesecloth. The filter obtained was adjusted its pH into 7.0 using 2N NaOH. Agar powder (Difco, USA) was added (20 g/L). Both media were sterilized in an autoclave at 121°C for 15 mins.

Protein-enriched medium was prepared using a method of Mellon and Cotty (1998) with a slight modification. As much as 30% (w/v) skim milk (Difco, USA) was added with bacto agar medium and sterilized in an autoclave at 121°C for 10 mins.

2.7 Py-GC/MS analyses of kebar grass extract

Samples of kebar grass polar extract were pyrolyzed by using a CDS Pyroprobe 2020iS connected on line to a gas chromatograph with a Shimadzu GC–MS mass detector (GCMS-QP2010). Samples were introduced into a quartz capillary tube in each experiment. The capillary was automatically introduced into the centre of a platinum resistor which heats up in an inert atmosphere. The products generated in pyrolysis were rapidly removed from the reaction zone (quartz capillary surrounded by the resistor) using a flow of helium, through a transfer line at the temperature of 280°C until introduced into the gas chromatograph provided with a mass spectrometry detector for analysis. Pyrolysis temperature was 400°C. GC analysis was carried out using a Rtx-5MS fused silica capillary column (60 m×0.25 mm×0.25 μm). The column temperature was 50 – 280°C. Helium was used as a carrier gas at a constant pressure of 100 kPa. Identification of different compounds obtained in this analysis was performed by using the Wiley 7 mass spectral library (Valdés *et al.*, 2013).

2.8 Antifungal activity of Kebar grass extract in food model media

2.8.1 Mycelial growth assay

The effect of kebar grass extract on mycelial growth of *A. flavus* tested was conducted using Tian *et al.* (2011) methods. The concentrations of kebar grass extract added to food model media were 12, 14, 16, 18, and 20 mg/mL. A total of 10 mL of medium containing extract was poured into the medium in Petri dish (9 cm in diameter) and left until it was solid. Five microliters of conidial suspension of *A. flavus* isolate was inoculated on the surface of each test media and incubated at 28°C for 10 days. The diameter of the colony (in mL) was observed every 24 hrs. Fungal cultured in food model media without kebar grass extract was used as the negative control. The inhibitory percentage was calculated using the following formula:

$$(dc - dt)/dc \text{ x } 100\%,$$

Where dc = diameter of control colony and dt = diameter of fungal colony in treatment set

In this study, the concentration of extract giving >90% of growth inhibitory activity was expressed as *Minimum Inhibitory Concentration* (MIC) and it was used in the subsequent test (Manso *et al.*, 2013). Two replicates were used for each treatment combination, including the control.

2.8.2 Sample preparation for scanning electron microscopy analysis

A mycelial disk (2-mm diameter) was taken at the periphery of the colony grown on the cellophane disk placed on the model media with 0, 1, 1.5, 2 MIC with and without kebar grass polar extract after ten days of incubation. It was then dried at 60°C on a small glass slide (1 cm × 1 cm). Samples were fixed in 2.5% glutaraldehyde for 30 mins at room temperature. They were rinsed in distilled water (three times, 15 mins each). The specimens were then dehydrated in a graded ethanol series (30, 50, 70%, 10 mins for each ethanol dilution and 30 mins in 100% ethanol). The samples were then coated with gold-palladium using an ion sputter-cutter (JEOL JFC 1100). The preparations were observed using a SEM Zeiss EVO 50 (Germany) (de Billerbeck *et al.*, 2001).

3. Results

3.1 Chemical composition of methanol extracts

The chemical composition of kebar grass methanol extracts that extracted by graded extraction using hexane -ethyl acetate-methanol solvents is presented in Table 1. A total of 64 components were identified using pyrolysis/gas chromatography/mass spectrometry (Py/GC/MS), consist of a class of carboxylic acids (7.81%), amines (1.56%), phenolics (26.56%), terpenes (15.60%), alcohols (10.94%), hydrocarbons compounds (20.3%), benzene derivatives (6.25%), carbohydrate derivatives (4.69%), steroidal saponin (1.56%), haloalkane (1.56%), and sterols (3.13%), 10 components were left unidentified.

3.2 Fungal growth inhibition

MIC values of kebar grass polar extract against isolates of *A. flavus* BCCF 0219 and *A. flavus* BIO 2236 are presented in Table 2. Inhibition of fungal growth was influenced by the concentration of extract given. The concentration of kebar grass polar extracts of three food model media affected on the delay of fungal growth. The

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Table 1. Chemical composition of kebar grass polar extract

Compound	Group	%*
Ammonium carbamate	Carboxylic acid	1.42
Trimethylamine	Amine	0.29
Izal	Phenol	0.68
1- Menthene	Terpene	0.2
Acetic acid	Carboxylic acid	0.77
l-Limonene	Terpene	2.35
1,3-Cyclopentadien, 5,5-Dimethyl-2-Propyl-	Terpene	0.11
2,6-Dimethylstyrene	Benzene derivatives	0.22
Guaiacol	Phenol	0.81
Beta-Ocimene-X	Terpene	0.14
3,5,5-Trimethyl-2-cyclohexenone	Benzene derivatives	0.1
1,5,8-p-menthatriene	Terpene	0.07
trans-p-Mentha-1(7),8-dien-2-ol	Terpene	0.07
p-Ethylphenol	Phenol	0.15
2-Methoxy-4-methylphenol	Phenol	0.13
Pyrocatechol	Phenol	2.21
2,3-Dihydro-benzofuran	Benzene derivatives	0.39
4-Cyclopentene-1,3-dione, 4-propyl-	Alicyclic hydrocarbons	0.05
3-Methoxy-pyrocatechol	Phenol	0.14
p-Ethylguaiacol	Phenol	0.3
Hydroquinone	Phenol	0.33
4-methyl catechol	Phenol	0.74
4-ethenyl-2-methoxy-phenol	Phenol	0.17
2,6-Dimethoxyphenol	Phenol	0.41
p-Methoxyacetophenone	Phenol	0.06
1-N-Pentadecyl-Decahydronaphthalene	Alicyclic hydrocarbons	0.12
Tetradecane	Aliphatic hydrocarbons	0.1
3,7,11-Tridecatrienenitrile, 4,8,12-trimethyl-	Terpene	0.3
p-Hydroxyacetophenone	Phenol	0.79
Pentadecane	Aliphatic hydrocarbons	0.2
ar-Curcumene	Terpene	0.07
trans-3-Oxabicyclo[5.3.0]decan-2-on, 9-Isopropyliden	Alicyclic hydrocarbons	0.08
1,6-Anhydro-beta-D-glucopyranose (Levoglucosan)	Carbohydrate derivatives	1.74
Lauric acid	Carboxylic acid	0.15
2,3,5,8-Tetramethyl-decane	Aliphatic hydrocarbons	0.4
1,2,4-Cyclopentanetrione, 3-methyl-5-(1-oxopropyl)-	Alicyclic hydrocarbons	0.14
1,6-Anhydro-beta-D-glucofuranose	Carbohydrate derivatives	0.19
Cyclopentaneethanol, 2-(hydroxymethyl)-beta,3-dimethyl-	Alcohol	0.13
alpha-Sinensal	Terpene	0.55
Hexahydrofarnesol	Alcohol	0.27
4,8-Dimethyl-1-nonanol	Alcohol	0.31
Myristic acid	Carboxylic acid	0.55
p-Benzylphenol	Phenol	0.65
Neophytadiene	Terpene	1.59
Cholestan-3-one, cyclic 1,2-ethanediyl acetal, (5. alpha)-	Steroidal saponin	1.06
1-Bromo-tetracosane	Haloalkanes	0.53
Vitamin E	Phenol	9.83
Alpha-Tocopherol-acetat	methylated phenol	9.13
6,10,14-hexadecatrien-1-ol,3,7,11,15, tetramethyl	Alcohol	2.64
Palmitic acid	Carboxylic acid	4.58
n-Nonacosane	Aliphatic hydrocarbons	2.38
n-Triacontane	Aliphatic hydrocarbons	2.11
beta-Tocopherol	Phenol	3.38
n-Tetratetracontane	Aliphatic hydrocarbons	6.42
Cholestan-7-one, cyclic 1,2-ethanediyl acetal, (5. alpha)-	Benzene derivatives	3.65
Stigmasta-5,22-dien-3-ol, acetate, (3.beta.22Z)-	Sterol	0.98
Alpha-Tocopheryl-beta-D-mannosid	Carbohydrate derivatives	7.55
Oleat-stigmast-5-en-3-ol	Sterol	6.31
2,10-Dodecadien-1-ol, 3,7,11-trimethyl-, (Z)-	Alcohol	0.46
1,30-Triacontanediol	Alcohol	2.59
Stearaldehyde	Aliphatic hydrocarbons	0.81
Solanesol	Alcohol	3.77
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-	Aliphatic hydrocarbons	2.25
2-Hexadecanone	Aliphatic hydrocarbons	0.36
Unknown (10 compounds)		8.57
Total		100

*percentage of the content of each constituent in total extract

higher concentration of the extract the longer fungal growth delayed. The longer lag phase of *A. flavus* BCCF 0219 isolate, i.e. up to 2 x 24 hrs, was observed in carbohydrate-enriched media after being exposed by kebar grass extract, at concentrations 14 and 16 mg/mL; lag phase was longer by the increase of concentration of the extract. The lag phase of isolate BIO 2236 in carbohydrate-enriched media after being exposed with kebar grass extract at a concentration of 12 mg/mL reached 2 x 24 hrs and the lag phase was extended by the increase of concentration of the extract. Kebar grass polar extract with concentration of 14 mg/mL in fat-enriched media extended the lag phase of isolates of *A. flavus* BCCF 0219 and *A. flavus* BIO 2236 up to 3x24 hours and 1x24 hours, respectively. Slow growth of *A. flavus* BIO 2236 isolate was observed after 2 x 24 hrs; and when the concentration of kebar grass extract was increased the lag phase of *A. flavus* isolate was getting longer. The similar pattern was also shown by isolates of *A. flavus* BCCF 0219 and *A. flavus* BIO 2236 in protein-enriched media with the addition of kebar grass extract. The extract at concentrations of 12 and 14 mg/mL extended the lag phase of isolate *A. flavus* BCCF 0219 up to 1 x 24 hrs, while the extract concentration of 12 mg/mL extended the lag phase of *A. flavus* BIO 2236 isolate up to 1 x 24 hrs. Again, by increasing the extract concentration, the longer the lag phase of both isolates.

Table 2. MIC and MFC value of kebar grass polar extract of isolates *A. flavus* BCCF 0219 and *A. flavus* BIO 2236

Type of media model	Isolate	MIC (mg/mL)
Carbohydrate	*A. flavus* BCCF 0219	12
	A. flavus BIO 2236	12
Fat	*A. flavus* BCCF 0219	14
	A. flavus BIO 2236	16
Protein	*A. flavus* BCCF 0219	14
	A. flavus BIO 2236	16
Positive control (ketoconazole)	*A. flavus* BCCF 0219	5
	A. flavus BIO 2236	5

3.3 Conidial production and SEM Observation of fungal structure

MIC values determined in the previous stage were used as the basis for assessing the influence of kebar grass methanol extracts against conidial production and fungal structure. Concentrations of kebar grass extract used for this test were 0, 1, 1.5, 2 MIC. Kebar grass methanol extract at concentration of 1 MIC inhibited the production of conidia during 10 days of incubation. On carbohydrate-enriched media mycelium growth of *A. flavus* BCCF 0219 isolate was very thin and the conidiation occurred within 7 x 24 hrs incubation with a few conidial productions; while on the carbohydrate-enriched media with kebar grass extract, growth of *A. flavus* BCCF 0219 isolate did not occur. The growth of

A. flavus BCCF 0219 isolate on fat-enriched media without kebar grass extract was fast, had compact colony and its conidiation occurred at 1 x 24 hrs incubation with abundant conidial production. The growth of *A. flavus* isolates on media with kebar grass extract was slow and failed to form conidia. The growth of *A. flavus* BCCF 0219 isolate on protein-enriched media without kebar grass extract was fast and showed a uniform colony, and conidiation occurred after 1 x 24 hrs incubation with abundant conidial production. Moreover, growth of *A. flavus* BCCF 0219 isolate on protein-enriched media with kebar grass extract showed slow growth and conidiation occurred at 10 x 24 hrs incubation with very few conidial productions.

Kebar grass methanol extract with a concentration based on MIC values obtained in the previous stage not only inhibited the growth of *A. flavus* isolates but also inhibited conidiation. On carbohydrate-enriched media, the growth of *A. flavus* BIO 2236 isolate was fast but with a very thin mycelium and the conidiation occurred within 8 x 24 hrs incubation with abundant conidial production and sclerotium was not formed. On carbohydrate-enriched media with kebar grass extract, growth of BIO 2236 isolate was slow and conidiation did not occur. Isolate of BIO 2236 inoculated on fat-enriched media without kebar grass extract showed very rapid growth and the conidiation occurred after 1 x 24 hrs incubation with abundant conidial production and its sclerotium was formed at 3 x 24 hrs incubation. Meanwhile, on fat-enriched media was added with extract of kebar grass the fungal growth was slowly and conidiation did not occur. Isolate of BIO 2236 grown on protein-enriched media without kebar grass extract showed rapid growth and the conidiation occurred at 2 x 24 hrs incubation and sclerotium formation began at 4 x 24 hrs incubation, whereas in protein-enriched media with kebar grass extract, the isolate growth was slow and failed to form conidia.

Observation on fungal morphological structure using SEM is shown in Figures 1-5. Kebar grass extract with concentration of 1 MIC on carbohydrate-enriched media resulting growth inhibitory of BCCF 0219 isolate up to 100%. Hyphal structure of BCCF 0219 isolate on fat-enriched media with kebar grass extract was thin and diameter size became smaller than the control (Figure 1b), whereas the conidia were not formed (Figure 1d). On protein-enriched media, fungal hyphae structure became thinner than control, hyphae was wound (Figure 2b) and the size of the conidia was also smaller than the control (Figure 2d).

Morphological changes in the hyphal of BIO 2236 isolate grown on carbohydrate-enriched media with
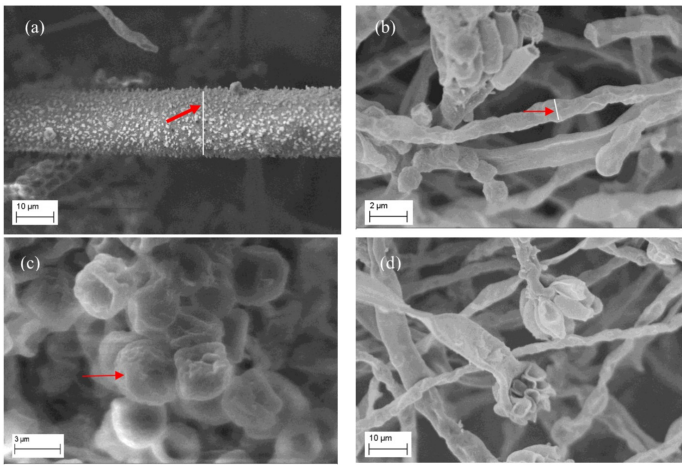
Figure 1. Structure of *A. flavus* BCCF 0219 on fat-enriched media (a) Hyphal structure of control, mag. 1000x, d=22.11 µm, (b) Hyphal structure treated using 1 MIC (14 mg mL^{-1}), mag. 2000x, d=3.23 µm, (c) Conidial structure of control, mag. 5000x, d=4.3 µm, (d) Conidial structure treated using 1 MIC (14 mg mL^{-1}), mag. 1500x

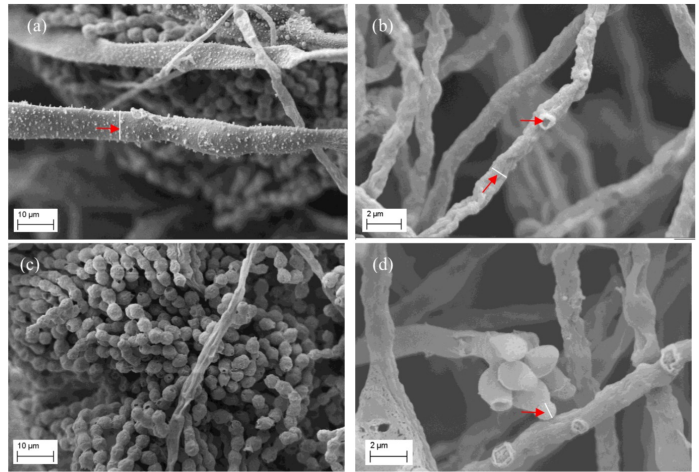Figure 2. Structure of *A. flavus* BCCF 0219 on protein-enriched media (a) Hyphal structure of control, mag. 1000x, d=9.72 µm (b) Hyphal structure treated using 1 MIC (14 mg mL-1), mag. 2000x, d=3.21µm, (c) Conidial structure of control, mag. 1000x, d=5.2 µm (d) Conidial structure treated using 1 MIC (14 mg mL-1), mag. 2500x, d= 2.5 µm

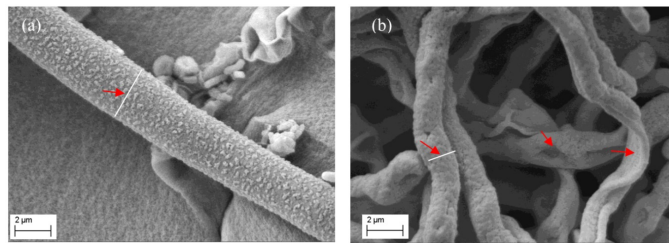Figure 3. Hyphal structure of A. flavus BIO 2236 on carbohydrate-enriched media (a) Hyphal structure of control, mag. 2000x, d= 8.85 µm and (b) Hyphal structure treated using 1 MIC (12 mg mL-1), mag. 3000x, d=2.64 µm
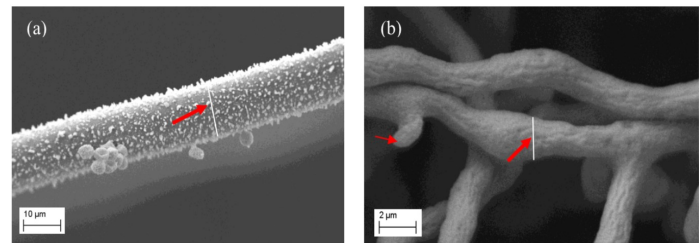

Figure 4. Hyphal structure of A. flavus BIO 2236 on fat-enriched media (a) control, mag. 1000x, d=17.95 µm and (b) 1 MIC (16 mg mL-1), mag. 5000x, d=1.27 µm

the hyphal, such as a wound on the hyphal surfaces, and formed short branches of hyphae (Figure 4b), as well as did not form any conidia. Isolate of BIO 2236 inoculated on protein-enriched media with kebar grass extract also suffered damage on the hyphae; it had rough and wound on hyphal surfaces (Figure 5b), short branches of hyphae were formed and failed to form conidia, whereas vesicle, phialide and metulae also folded (Figure 5d).

4. Discussion

Kebar grass polar extract inhibited the growth of *A. flavus* BCCF 0219 and BIO 2236 in a dosage response manner. In the present study, the polar extract of kebar grass leaf was reported as a potent inhibitor of *A. flavus* growth *in vitro*. The extract showed a fungistatic activity toward the fungus at all concentrations used which were significant for the concentrations ≥12 mg/mL compared to control of all food model media. Some other studies reported the activity of essential oil against conidiation; thus this result can be evaluated as the first report. The inhibitory effect of EOs against conidiation of different fungi has been previously reported (Gandomi *et al.,* 2009). Tataoui-Elaraki *et al*. (1993) studied the effect of three Moroccan EOs on fungal asexual reproduction stages including conidial production and reported that
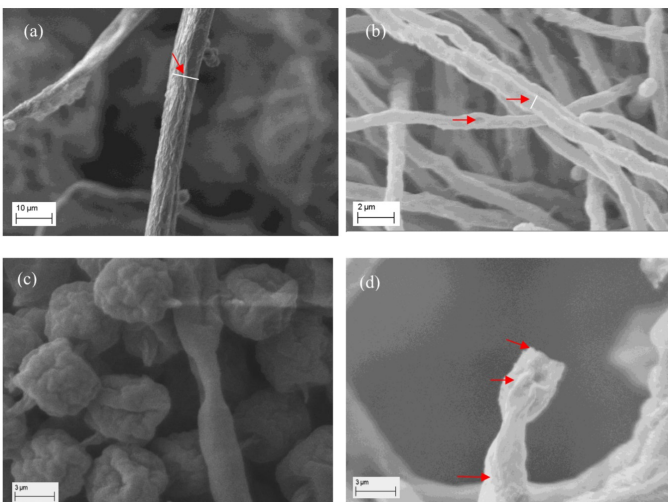

Figure 5. Structure of *A. flavus* BIO 2236 on protein-enriched media (a) Hyphal structure of control, mag. 1000x, d=8.96µm, (b) Hyphal structure treated using 1 MIC (16 mg mL-1), mag. 3000x, d=1.86 µm (c) Conidial structure of control, mag. 5000x, (d) Vesicle, phialide and metulae structure treated using 1 MIC (16 mg mL-1), mag. 5000x.

kebar grass extract also occurred; folding hyphae was formed, and minor injuries occurred on the surface of the hyphae (Figure 3b) while the conidia were not formed. Isolate of *A. flavus* BIO 2236 inoculated on fat-enriched media with kebar grass extract also suffered damage on

the partial inhibition of conidial production could be attributed to mycelial destruction or inhibition of fungal growth. Mahanta *et al.* (2007) suggested that the impact of *Cymbopogon citrates* L. essential oil on conidiation may reflect the effects of volatile compounds emitted by the oil on the surface of developing mycelia, and thus the stage to support conidial production involved in the switch from vegetative to reproductive development. Reduction of conidial production could limit the spread of pathogens by lowering the conidia load in the storage atmosphere and on surfaces (Tzortzakis and Economakis, 2007).

There are few reports on the growth inhibitory potential of kebar grass extract against bacteria and no report on the effect of plant extract on fungal especially against *A. flavus* growth and its effect on fungal morphological structure. Some researchers reported antibacterial activity of the methanolic extract of whole part of kebar grass against different pathogenic and medically important bacteria (Natarajan *et al.*, 2010).

We reported here for the first time the inhibition of fungal growth and conidial production of *A. flavus* which was exposed to different concentrations of kebar grass extract in relation to morphological alterations in fungal compartments. From the SEM observations, it was shown that hyphae of *A. flavus* treated with 1 MIC of the kebar grass extract was entirely collapsed and folded with the formation of short branches and undifferentiated tips. Morphological structure changes of fungal hyphae have been reported for *A. flavus* hyphae treated with *Cymbopogon citratus* oil (Helal *et al.*, 2007) and *Matricaria chamomilla* L. flower essential oil (Tolouee *et al.*, 2010).

An interesting observation in bioassays was the inhibition of conidial production in relation to fungal growth inhibition. Marked reduction in *A. flavus* conidial production by kebar grass extract reported in the present study might be a consequence of retardation in vegetative growth development through affecting the normal fungal branching pattern. In these results, with kebar grass extract, completely squashed and severely collapsed hyphae were recorded, which results in flattening and ultimately death of hyphae. The hyphae showed damage, loss of the cell wall integrity and rigidity, and the cell wall was folded. These observations indicate that the mode of antifungal activity of kebar grass extract, a result of the attack on the cell wall. Such effects induced by kebar grass extract may be related to the interference of extract bioactive components with enzymatic reactions of wall synthesis, which affects fungal morphogenesis and growth. Zambonelli *et al.* (1996) reported that fungal growth inhibition can be

associated with degeneration of fungal hyphae after treatment with *Thymus vulgaris* essential oil. Our observations are in agreement with the above reports.

Selected compounds of kebar grass polar extract including phenolics (26.56%), terpenes (15.60%), alcohols (10.94%), steroidal saponin (1.56%) also reported has antifungal activity (Marei *et al.*, 2012). To the best of the author knowledge, this is the first study to establish the chemical composition of leaf extract and antifungal activity of kebar grass. Lin and Wang (2003) reported that kebar grass also have biflavon (cupressuflavone and amentoflavone), flavonoid (luteolin 7-methyl ether, isoorientin, 3'-methoxyluteolin 7-O-glucoside), 4-caffeoylquinic acid and 5-caffeoylquinic acid. Other study reported that there is a relationship between the chemical structures of the most abundant compounds in the tested oil and the antimicrobial activity. The extract rich in phenolic compounds are widely reported to possess high levels of antimicrobial activity (Skočibušić *et al.*, 2006). Since the active antimicrobial compounds of plant extract are phenolics and terpenes, it seems reasonable that their mode of action might be similar to that of other phenolic compounds. Most of the studies on the mechanism of phenolic compounds focused on their effects on cellular membranes, altering its function and in some instances structure, causing swelling and increasing its permeability. A consistent observation is an increase in K^+ and often cytoplasmic content effluxes from cells in response to the antimicrobial challenge. These effects may develop as a result of membrane depolarization by altered ion transport or through changes in membrane structure, inhibition of energy (ATP) generation by interference with glucose uptake or inhibition of enzymes involved in oxidative or substrate level phosphorylation. Increases in cytoplasmic membrane permeability appear to be a consequence of the loss of the cellular pH gradient, decreased ATP levels, and loss of the proton motive force, which lead to cell death. Other mechanisms for the antimicrobial activity of simple phenolic compounds have been proposed to include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with proteins. More complex natural phenolic compounds such as quinones, flavones, flavonoids and flavanols exert their antimicrobial activity by complexing irreversibly with nucleophilic amino acids in proteins leading to inactivation of the protein and loss of function. The most probable targets in microbial cells are surface exposed adhesions, cell wall polypeptides, and membrane-bound enzymes (Samapundo *et al.*, 2007). In addition, aflatoxin inhibitory effect of phenolic compounds through the mediation of oxidative stress levels in the fungi is also

reported by Kim *et al.* (2006). Kim *et al.* (2006) have reported that growth inhibition of *A. flavus* by the phenolics salicylic acid, thymol, vanillyl acetone, vanillin and cinnamic acid is via targeting the mitochondrial oxidative stress defence system. Since the mitochondria are responsible for providing acetyl-CoA, a main precursor for AF biosynthesis, disruption of mitochondrial respiration chain may account in part for the inhibitory effects of antifungal phenolics on aflatoxin production. Hence, these plant extract rich in phenolics compound could be used as markers for the elucidation of antioxidant-based inhibition of aflatoxin biosynthesis. The antimicrobial properties of alcohols were known to increase with molecular weight. It is also evident that terpene alcohols such as trans-p-Mentha-1(7),8-dien-2-ol, solanesol, 1-limonene exhibit strong antimicrobial activity, especially pronounced on whole cells, while hydrocarbon derivatives possess lower antifungal properties, as their low water solubility limits their diffusion through the medium (Prakash *et al.*, 2012). The extract rich in saponin compounds is reported to have antimicrobial activity too. It is known that saponins change the membrane permeability leading to the rupture of the cell (Brusotti *et al.*, 2013). These observations suggested that the antifungal effect of kebar grass leaf extract is probably due to the individual or synergistic effect of the secondary metabolites present in the extract.

5. Conclusion

A HEM extract of kebar grass possesses fungitoxic activities in inhibiting the growth of aflatoxigenic *A. flavus*, leading to irreversible deleterious morphological alterations and thus, it is worth exploiting for the bio management of aflatoxigenic *A. flavus*. The results of this study suggested the possibility to use HEM extract of kebar grass leaf as a natural preservative since it possesses strong antifungal activity. Further research regarding its application in the food system is needed to obtain information regarding the practical effectiveness of extract to prevent the growth of foodborne and spoiling microbes under the specific application conditions.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgements

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