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Antifungal Activity of Kebar Grass Leaf Extracts on the Growth of Aflatoxigenic *Aspergillus flavus* in Food Model Media

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Abstract

Some fungal species are toxigenic, for example Aspergillus flavus which can produce aflatoxin. Various methods have been conducted to reduce aflatoxin contamination in foods, among others using antimicrobial compounds derived from natural plant extract. Kebar grass (Biophytum petersianum Klotszch) is one of herbs that has potency as antimicrobe.

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The objective of this study was to investigate the effect of three types of model media i.e. carbohydrate-enriched media, fat-enriched media, and protein- enriched media containing kebar grass extract on the growth of aflatoxigenic A. flavus. Kebar grass extract at concentrations of 5, 10, and 20 mg mL^{-1} was extracted using various solvents, i.e. hexane, ethyl acetate, methanol, hexane-ethyl acetate, hexane-ethyl acetate-methanol, and aquadest extracts was tested on the growth of A. flavus.

The result showed that kebar grass extract of hexane-ethyl acetate-methanol at concentration of 20 mg ml^{-1} in carbohydrate- enriched medium, fat-enriched medium, and protein- enriched medium caused higher growth inhibition of A. flavus than other extracts, i.e. 96.2, 100, and 96.1%, respectevely. The other extracts had growth inhibition less than 90%. The results obtained in the present study suggest that kebar grass extract can be used to inhibit the growth of A. flavus toxigenic.

Keywords: Antifungal activity; aflatoxigenic Aspergillus flavus; Biophytum petersianum Klotzsch; food model media

1. Introduction

Foods are easily spoiled by either bacteria or fungi. During pre and post-harvest handling foodstuffs can be infected by fungi. Some fungal species among others *Aspergillus flavus* can produce toxins (aflatoxins) which can cause liver cancer on human and animal [1]. Many efforts have been done to extend the shelf life of foods and the safety, for example using thermal and non-thermal processing techniques or the use of preservatives such as synthetic or natural antioxidants and antimicrobes. Plant metabolites have been known to have antimicrobial and antioxidant activities and some of them are classified as Generally Recognized as Safe Substances (GRAS) [2,3].

One of the biological material that could be used as antimicrobe is kebar grass (*Biophytum petersianum* Klotzsch), a plant belong to family Oxalidaceae. In Indonesia, the plant is found in Kebar District, West Papua. The plant grows naturally and spread almost all over Kebar District [4]. Besides in Kebar District, this plant is also found in Southeast Asia and Africa, especially in Mali region. Mali community use this plant for the treatment of wounds, inflammation, ulcers, malaria, and fever [5]. Papuan community, especially in Kebar District often use this plant as a fertility drug.

It was reported that kebar grass contain bioactive compounds such as alkaloids, saponins, tannins, phenolic, flavonoids, triterpenoids, steroids and glycosides compounds [4,6]. As kebar grass contains fairly complete bioactive groups, therefore this plant is potential could be used as antimicrobe. [7] reported that the leaf extract of kebar grass has antibacterial activity against Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumoniae)* and Gram-negative bacteria (*Klebsiella pneumonia, Salmonella Typhii, Proteus vulgaris, Escherichia coli),* but there are no reports concerning the effect of kebar grass against toxigenic mold.

Foodstuff can consist of carbohydrate, fat and protein. Kind of substrates is a factor which will determine the growth and aflatoxin content produced by aflatoxigenic *A. flavus.* In order to be applied as *A. flavus* growth

inhibitor on food, it is necessary to study the effects of kebar grass leaf extract on the growth of aflatoxigenic *A. flavus* in food model. The objective of this study was to study the effect of three types of model media i.e. fat-enriched media, carbohydrate-enriched media, and protein-enriched media containing kebar grass extract on the growth of aflatoxigenic *A. flavus.*

2. Materials and Methods

2.1. Extraction and Determination of Phytochemicals Content of Kebar Grass Extracts

Kebar grass leaves and aflatoxigenic A. flavus

Kebar grass were collected from Kebar district, Manokwari county, West Papua province, in Februari, 2013. Kebar grass was identified by Indonesian Institute of Science (LIPI). Kebar grass were then air-dried for about 2 weeks until their colour turned into golden brown and they are easily broken. The leaves were then crushed into 40 mesh coarse powder using a blender. One strain of aflatoxigenic *A. flavus* BCCF0219 obtained from Indonesian Research Center for Veterinary Science culture collection was used in this study.

Chemicals

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

Single extraction using organic solvents

A method of [8] was used to obtain the extract of kebar grass leaves. Powder of kebar grass leaves was extracted using maceration method using various organic solvents, i.e. a non-polar (n-hexane), semi-polar (ethyl acetate), and polar organic solvent (methanol). The kebar grass powder (200 g) was placed in a glass jar (volume 1000 mL), then 800 mL n-hexane was added. Its mixture was macerated for one hour at room temperature (28 – 30 °C), stirred in a shaker that combined with sonification for 30 minutes, followed by further maceration for one hour then filtered using Whatman No.1 filter paper. The extraction was conducted three times. The extract was collected and dried using a rotary evaporator to form a thick concentrate. The remaining solvent was removed using nitrogen gas. The yield of extract was calculated as the percentage of extract (mg extract/100 g kebar grass powder). Extraction with ethyl acetate and methanol was done with the same way. The extraction process was performed in triplicate. The extracts stored at 4°C until used.

Extraction using distilled water

Kebar grass leaves was extracted in distilled water on 60 ^{0}C for one hour. The extract was centrifuged at 5000 xg for 10 minutes, the supernatant was collected and dried using freeze drier to form a powder and the dry weight of extract was determined. The yield of extract was calculated as the percentage of extract (mg extract/100 g kebar grass powder). The extraction process was performed in triplicate. The extract was stored at 4°C until used [9].

Successively extraction by using n-hexane-ethyl acetate-methanol

Powdered kebar grass (200 g) was extracted by maceration (1 hr) method combined with sonication (30 min) at room temperature (28 – 30 °C) using three types of organic solvent, i.e. n-hexane, ethyl acetate, and methanol solvents, followed by Whatman No.1 paper filtration. Maceration was repeated for 2 times and filtrate was collected. The remaining residue of the plant material was dried for 24 hours and extracted using ethyl acetate and methanol solvent sequentially in a similar manner. The filtrate was collected and dried with a rotary evaporator to form a thick concentrate. The hexane-ethyl acetate-methanol filtrate was called as HEM extract. Residual solvent in HEM extract was removed by nitrogen gas [8]. The extract stored at 4 °C until used.

Phytochemical test

Phytochemical tests were performed on crude organic extract and aqueous extract of kebar grass leaves. The tests were conducted to determine the content of flavonoids, tannins, alkaloids, phenylhydroquinon, triterpenoids, and saponins [10].

2.2. Antifungal Activity Screening of Kebar Grass Extract in Food Model Media

Preparation of agar medium enriched with carbohydrate, fat, and protein

Carbohydrate-enriched medium was prepared by the addition of 30% (w/v) glucose on Bacto Agar. The medium was sterilized in an autoclave at 121 °C for 15 minutes [11].

Medium enriched with fat was prepared based on [12]. One hundred grams of grated coconut flesh was homogenized using 300 mL of hot distilled water for five minutes. The mixture was filtered adjusted its pH into 7.0 using NaOH 2M. Agar powder was added (20 g L^{-1}), and sterilized in an autoclave at 121°C for 15 minutes.

Protein-enriched medium was prepared using the method of [13] with a slight modification on type of protein source. 30% (w/v) skim milk obtained from Difco Laboratory was added to bacto agar medium and sterilized in an autoclave at 121°C for 10 minutes.

Preparation of A. flavus conidia

The conidia of *A. flavus* were prepared using the method of [14] with a slight modification. The fungus was cultured in PDA slant and incubated at 28°C for 5 days. The conidia were harvested by adding 10 mL of sterile distilled water and collected aseptically. Conidial suspension was dissolved in Tween 80 (0.5%) and centrifuged (2000xg) at 28 – 30 °C for five minutes. Conidial concentration was calculated using a haemacytometer and adjusted to 10^6 CFU mL^{-1}.

Mycelial Growth Assay

The effect of kebar grass extract on mycelial growth of *A. flavus* was tested using [15] and [16] methods. Each kebar grass extract dissolved in their solvent to reach a concentration of 50 mg mL^{-1}, pipetted aseptically

onto the food model media to produce concentration 5, 10, 20, 50 mg mL^{-1} in food model media. Ten mililitres model medium containing extract was poured into each Petri dish (9 cm in diameter) and left it until solid. Five microlitres of conidial suspension of *A. flavus* was inoculated on the surface of each test media, and then incubated at room temperature (28 –30 °C) for 10 days. The growth of mycelia was determined by measuring the diameter of colony (in mm) every 24 hours. As control, the fungus was cultured in food model media without kebar grass extract. Two replicates were used in this experiment. The percentage of inhibitory growth was determined using the following formula:

$$\text{Growth Inhibition} = \frac{(dc - dt)}{dc} \times 100\% \quad \ldots\ldots(1)$$

where *dc* = diameter of fungal colony in medium without kebar grass extract (mm)

dt = diameter of fungal colony in medium containing the extract of kebar grass (mm)

2.3. Statistical Analyses

The results were analyzed using Microsoft Excel 2007. All the values are expressed as means ± standard deviation (n=2).

3. Results and Discussion

3.1 Yield and Physical Properties of Kebar Grass Extracts

Yield and physical properties of kebar grass extracted with single solvent and the combination of various solvents is presented in Table 1.

Table 1 Yield and physical properties of kebar grass extract

Physical properties	Type of extracts					
	n-Hexane extract	Ethyl acetate extract	Methanol extract	n-Hexane-ethyl acetate extract	n-Hexane-ethyl acetate-methanol extract	Aquadest extract
Colour	Greenish brown	Brown	Dark brown	Greenish brown	Dark brown	Murky brown
Appearance	Viscous, oily	Viscous	Fluid, somewhat viscous	Viscous	Fluid, somewhat viscous	Brown powder
Yield (% w/w)	2.94	6.15	14.17	1.55	12.05	3.28

Extraction using hexane-ethyl acetate produced the lowest extract yield (1.55%), while a single extraction of methanol produced the highest yield (14.17%). Extract yield of hexane-ethyl acetate was low, because some components were soluble in nonpolar and semipolar solvents, such as steroids were extracted by nonpolar solvent, therefore the less that can be extracted by a semipolar solvent. The yield of a polar extract, either extracted using a single solvent (methanol) or graded solvent (hexane-ethyl acetate-methanol) was high, because of the more polar components were able to be extracted well using polar solvents.

The difference of solvent polarity resulted different phytochemical components of extract. Phytochemical components produced by various organic solvents is presented in Table 2.

Table 2 Phytochemicals of kebar grass extracts

Group Compound	Type of extracts						
	Kebar grass powder	Hexane extract	Ethyl acetate extract	Methanol extract	Hexane-ethyl acetate extract	Hexane-ethyl acetate-methanol extract	Aquadest extract
Alkaloids	+	-	+	+	-	-	+3
Flavonoids	+2	+	+2	+3	+2	+3	+2
Phenolhydroquinon	+2	-	+2	+3	+	+3	+2
Steroids	+	+2	+3	+	+3	+2	+
Triterpenoids	+2	+	+	+	+	+	+
Tannins	+3	-	-	+2	-	+	+3
Saponins	+3	-	-	+2	-	+2	+

Note : + = weak positive; +2 = positive; +3 = strong positive ; - = not detected

Hexane can extract nonpolar components such as waxes, fats and oils, including essential oils. However, extraction using hexane also resulted in less flavonoids. Other components that could be extracted using hexane solvent are steroids and triterpenoids. Extraction using ethyl acetate can extract alkaloids, flavonoids, phenylhydroquinon, steroids and triterpenoids, whereas extraction using methanol can extract components such as alkaloids, flavonoids, phenolhydroquinon, steroids, triterpenoids, tannins and saponins. According to [10], alkaloids are components of semipolar alkaline containing one or more nitrogen atoms and are widely used in medicine. Alkaloids are also often used as an antibacterial ingredient that is bactericidal [17,8]. Another class of compounds extracted, either using nonpolar, semipolar or polar solvents are flavonoids and phenolhydroquinon, but more strongly extracted in polar solvents, due to these compounds are polar [18]. As an antioxidant [19], flavonoids and phenolhydroquinon also have antimicrobial properties [18,20]. Steroids strongly extracted by hexane and ethyl acetate solvents. This is because most of the steroid is nonpolar and semipolar, so it can be extracted using nonpolar and semipolar solvents. Triterpenoids extracted

using hexane, ethyl acetate, and methanol solvent, can be divided into four groups, namely true triterpene, steroids, saponins and cardiac glycosides [10]. Triterpenoids are non-volatile terpenoid compounds. Terpenoids are the building blocks of essential oils. Besides having biological activity, triterpenoids also have antimicrobial properties [21]. Tannins and saponins are highly polar compounds, they can be extracted only using polar solvents. Both classes of compounds have antimicrobial activity [18]. Saponins are surface active compounds and it is like soap, which can be detected by their ability to form foam and hemolyzed blood cells. Saponin has expectorant action, which is very useful in the management of upper respiratory tract inflammation. Saponins was also reported to have antidiabetic properties [22].

3.2. Antifungal Activity of Kebar Grass Single Extract Extracted Using Single Solvent

Antifungal activity of kebar grass extracts at single extraction solvents (hexane, ethyl acetate, methanol, and distilled water) and at single concentrations (5, 10, 20, and 50 mg mL^{-1}) in three types of media (fat-enriched, carbohydrates-enriched and protein- enriched medium) is presented in Figure 1.

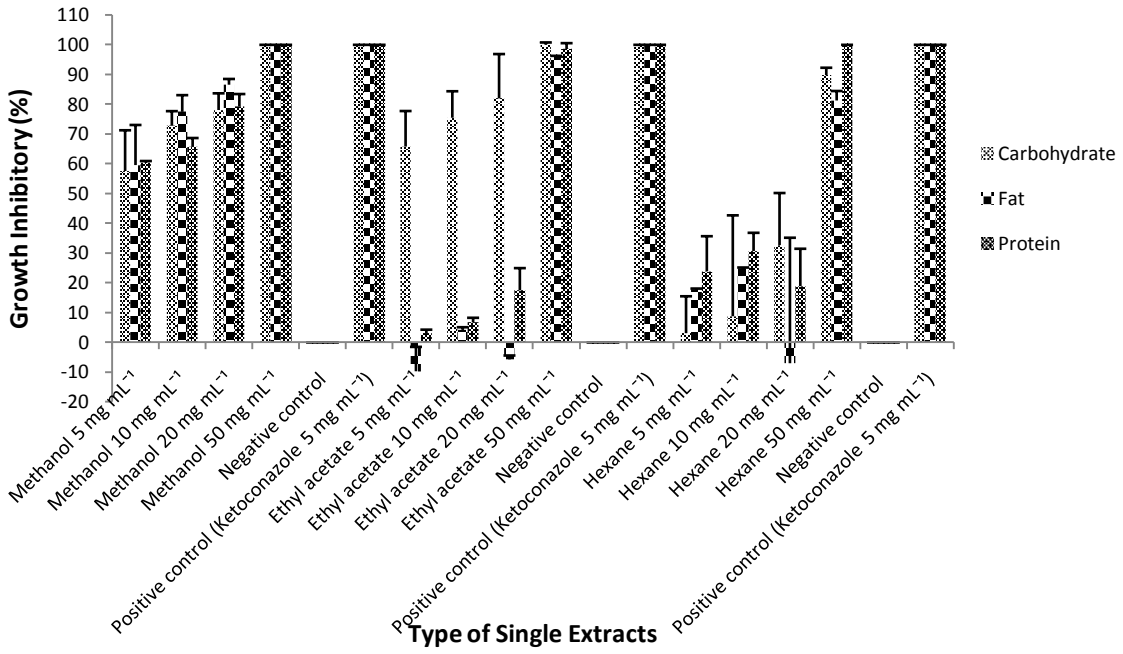
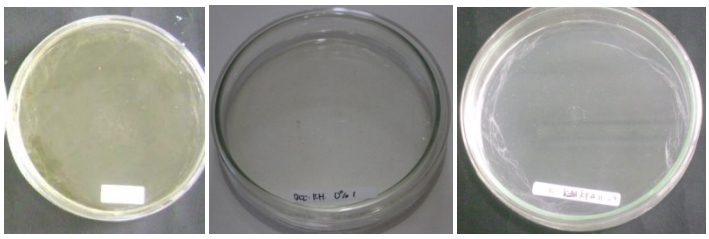


Figure 1 Effect of type and concentration of a single extract of kebar grass on growth inhibitory of *A.flavus* in three types media models

Figure 1 showed that the growth inhibition of *A. flavus* at concentration 50 mg mL^{-1} of methanol and ethyl acetate extract in carbohydrate-enriched medium was 100%, while hexane extract at concentration of 50 mg mL^{-1} caused 89.8% of growth inhibition. At a lower extract concentration of 5, 10, and 20 mg mL^{-1}, all types of extracts caused growth inhibition of less than 50%. At concentration of 20 mg mL^{-1} ethyl acetate extract had growth inhibitory more higher than n-hexane and methanol extracts. Observation on mycelia

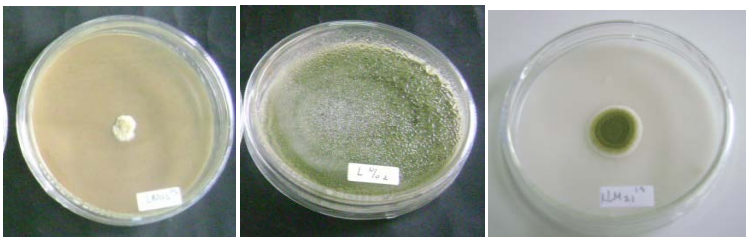
growth of *A. flavus* on carbohydrate- enriched medium on 10x24-hour incubation period after being treated with extract is presented in Figure 2(a-c).



(a) (b) (c)

Figure 2 The growth of *A. flavus* on carbohydrate- enriched medium treated using 20 mg mL^{-1} of ethyl acetate extract (a), negative control (b), and solvent control (c) after 10x24 hours of incubation

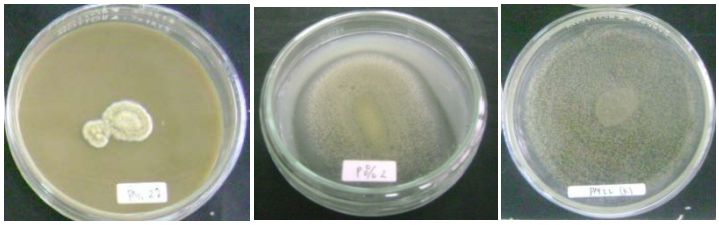
Figure 1 showed that on fat- enriched medium, methanol and ethyl acetate extracts at a concentration of 50 mg mL^{-1} shows inhibitory of *A. flavus* growth more than 90%, that is 100 and 96.3% respectively, while the hexane extracts only caused 81.3% and the water distilled extracts caused only 45.4% growth inhibition. At lower concentrations of 5, 10, and 20 mg mL^{-1}, all types of extracts caused growth inhibition less than 90%. At concentration of 20 mg mL^{-1} methanol extract had growth inhibitory more higher than n-hexane and ethyl acetate extracts. Mycelial growth of *A. flavus* on fat-enriched medium treated with methanol extract after 10x24-hours of incubation period are presented in Figure 3(a-c).



(a) (b) (c)

Figure 3 The growth of *A. flavus* on fat- enriched medium treated using 20 mg mL^{-1} of methanol extract (a), negative control (b), and solvent control (c) after 10x24 hours of incubation

Inhibitions of kebar grass extract using methanol, ethyl acetate and hexane at concentration of 50 mg mL^{-1} each in protein-enriched medium were 100, 98.6, and 100%, respectively, while at a lower concentration of 5, 10, and 20 mg mL^{-1}, all kinds of extracts produced growth inhibition less than 90%. At concentration of 20 mg mL^{-1} methanol extract had growth inhibitory more higher than n-hexane and ethyl acetate extracts.Mycelial growth of *A. flavus* after 10x24-hours of incubation period and after being treated using extract is presented in Figure 4(a-d).



(a) (b) (c)

Figure 4 The growth of *A. flavus* on protein- enriched medium treated using 20 mg mL^{-1} of methanol extract (a), negative control (b), and solvent control (c) after 10x24 hours of incubation

Apart from the type of extract, it is suspected that the difference of the pattern of growth inhibition is caused by the different content of active compounds in each kind of extract. In the methanol extract, all of antimicrobial active components can be extracted, while the non-polar and semi-polar extracts only extracted the specific components. In the methanol extract, flavonoids and phenolhydroquinons were detected in larger amount than in other kinds of extracts, whereas tannins and saponins were only detected in the methanol extract. Tannins was reported to posses physiological astringent properties, which hasten wound healing and ameliorate inflamed mucus membrane, it also has hemostatic properties [22]. Saponin has the property of precipitating and coagulating red blood cells. It is known that saponins can change the membrane permeability leading to the rupture of the cell [23].

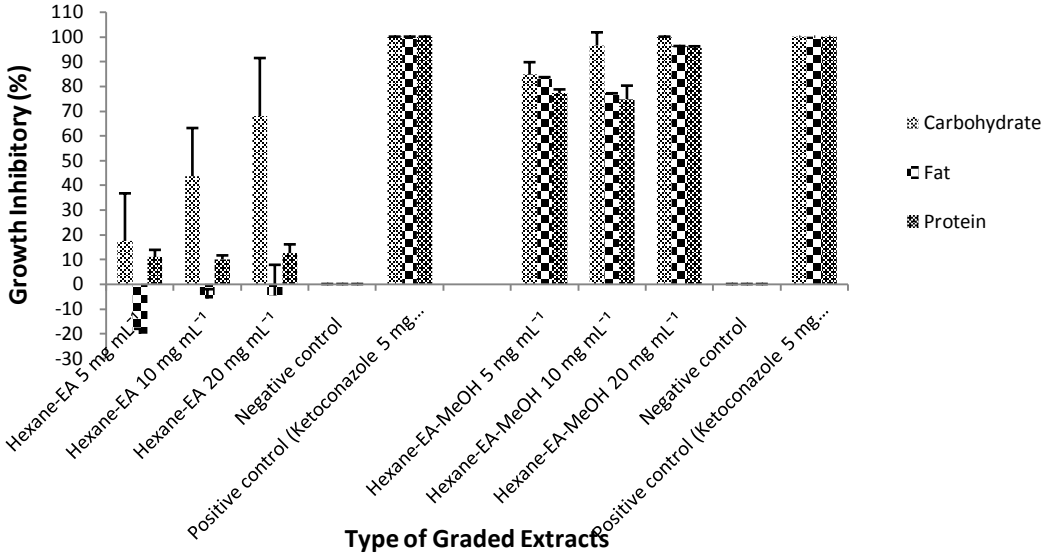
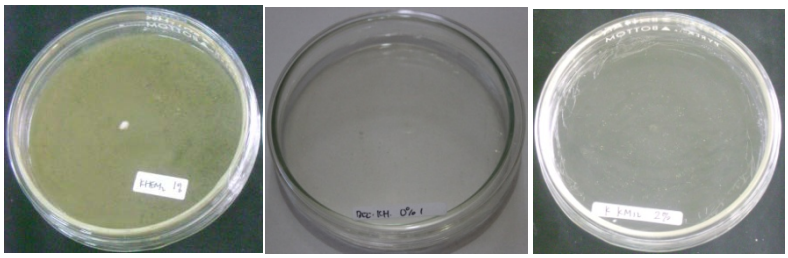


Figure 5 Effect of type and concentration of kebar grass extract on growth inhibitory of *A. flavus* in various media models

3.3. Antifungal Activity of Kebar Grass Graded Solvent Extract

In the **carbohydrate- enriched medium,** hexane-ethyl acetate extract led to growth inhibition of less than 90%, but the inhibitory percentage was higher than other medium models, both on the concentration of

extract 5, 10, or 20 mg mL^{-1} (Figure 5). Hexane-ethyl acetate-methanol extract caused growth inhibition 96.4 and 100%, respectively of the concentration extract of 10 (Figure 6) and 20 mg mL^{-1}.

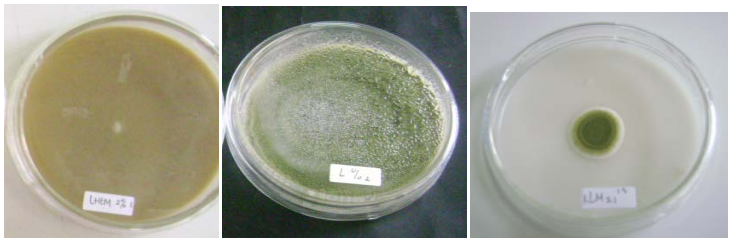


(a) (b) (c)

Figure 6 The growth of *A. flavus* on carbohydrate- enriched medium treated using 10 mg mL^{-1} of n-hexane-ethyl acetate-methanol extract (a), negative control (b), and solvent control (c) after 10x24 hours of incubation

In **fat-enriched medium,** hexane-ethyl acetate extract showed no inhibition on the growth of *A. flavus*, and tended to stimulate the growth of *A. flavus* (Figure 5). According to [10], steroid compounds belonging to the class of triterpenes, some of which include plant sterols that are only found in low level plants such of yeasts and molds. Ergosterol is a major component of the molds cell wall. The presence of high steroid in hexane-ethyl acetate extract did not inhibit the growth of *A. flavus*, otherwise a supporter of the *A. flavus* cell wall synthesis.

Hexane-ethyl acetate-methanol extract (polar extract) at concentration of 20 mg mL^{-1} caused 96.2% stunted growth of *A. flavus* which was higher than the single extract. It is thought that compounds extracted in graded extraction was more pure and has stronger activity. Mycelial growth of *A. flavus* on fat-enriched medium after 10x24-hours of incubation period and after being treated using extract is presented in Figure 7.

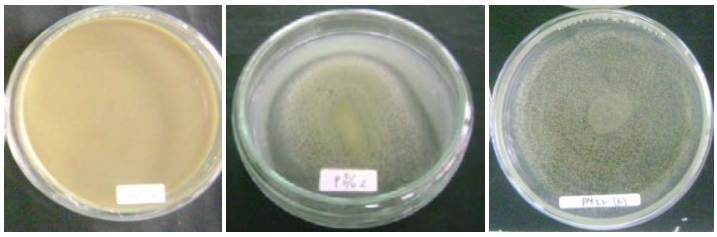


(a) (b) (c)

Figure 7 The growth of *A. flavus* on fat- enriched medium treated using 20 mg mL^{-1} of n-hexane-ethyl acetate-methanol extract (a), negative control (b), and solvent control (c) after 10x24 hours of incubation

Experiment on the **protein-enriched medium** showed that the kind of hexane-ethyl acetate extract with a concentration of 20 mg mL^{-1} caused growth inhibition less than 90%, while the kind of hexane-ethyl acetate-methanol extract led to growth inhibition of 96.1%. The lower concentration of ethyl acetate-hexane-

methanol extract produces less than 90% inhibition. Mycelial growth of *A. flavus* on protein-enriched medium after 10x24-hours of incubation period and after being treated using extract is presented in Figure 8.



(a) (b) (c)

Figure 8 The growth of *A. flavus* on protein- enriched medium treated using 20 mg mL^{-1} of n-hexane-ethyl acetate-methanol extract (a), negative control (b), and solvent control (c) after 10x24 hours of incubation

Extract HEM rich of flavonoids, phenolhydroquinon, steroids, triterpenoids, tannins, and saponins. The antifungal proprety in this study could be due flavonoids and tannins contents of the extract, both of which are known to posses appreciable antimicrobial activities [22]. Other while, triterpenoids can reduces the synthesis of ergosterol fungal cell membrane component and cause defective cell wall formation and leakage of cellular contents [24].

4. Conclusion

Kebar grass leaf extract was able to inhibit the growth of *A. flavus* in food model media. The most effective extract tested (20 mg mL^{-1}) with growth inhibitory 96.1 – 100% was extract of hexane-ethyl acetate-methanol (HEM). Therefore, the kebar grass leaf could be recommended as antifungal of aflatoxigenic *A. flavus*.

5. Acknowledgment

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