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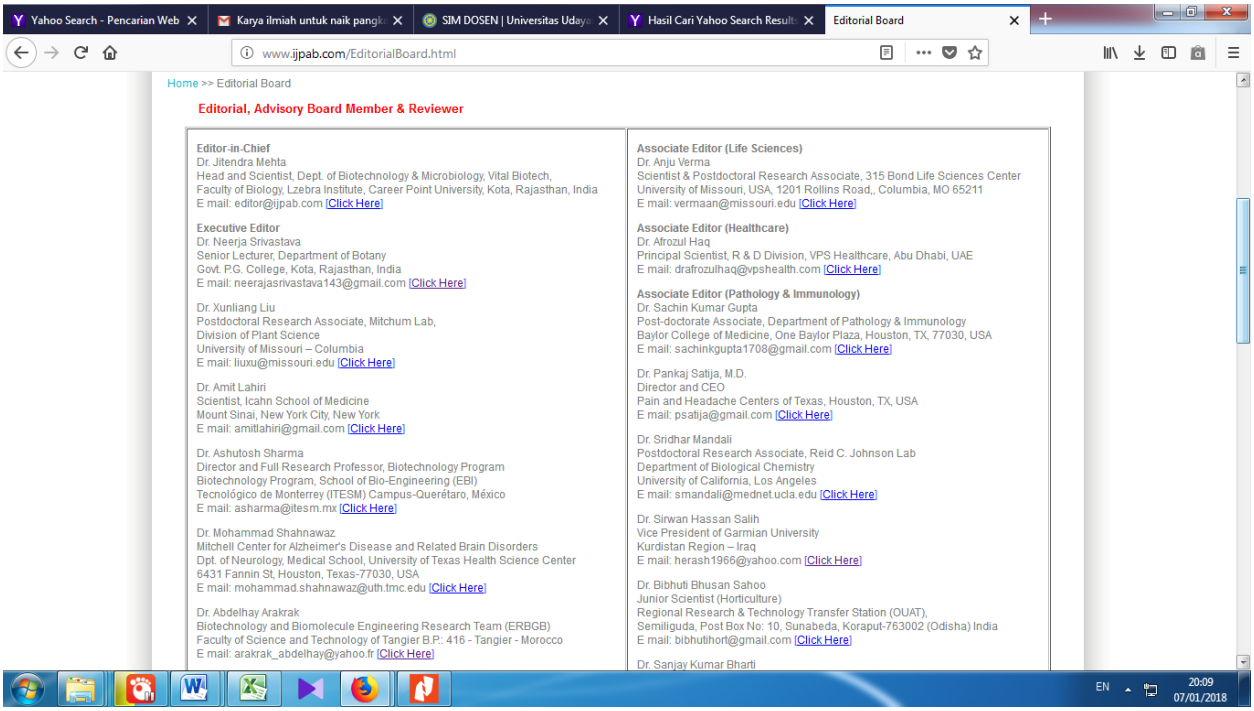

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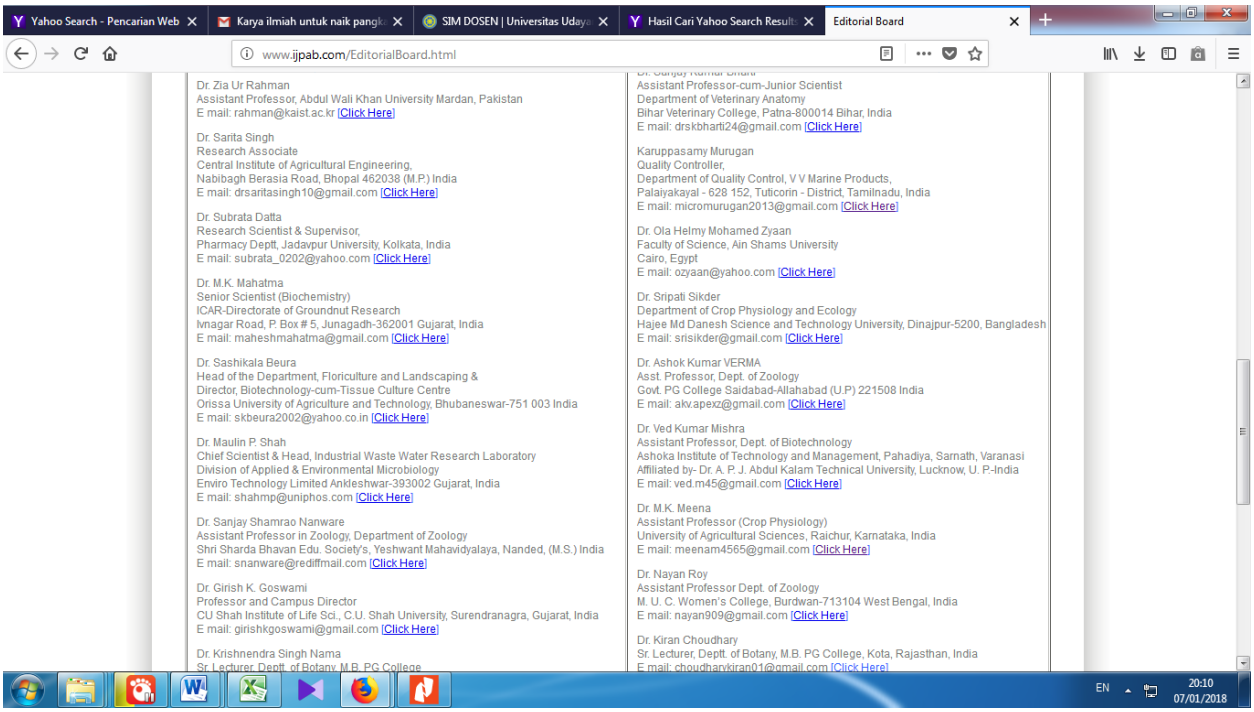

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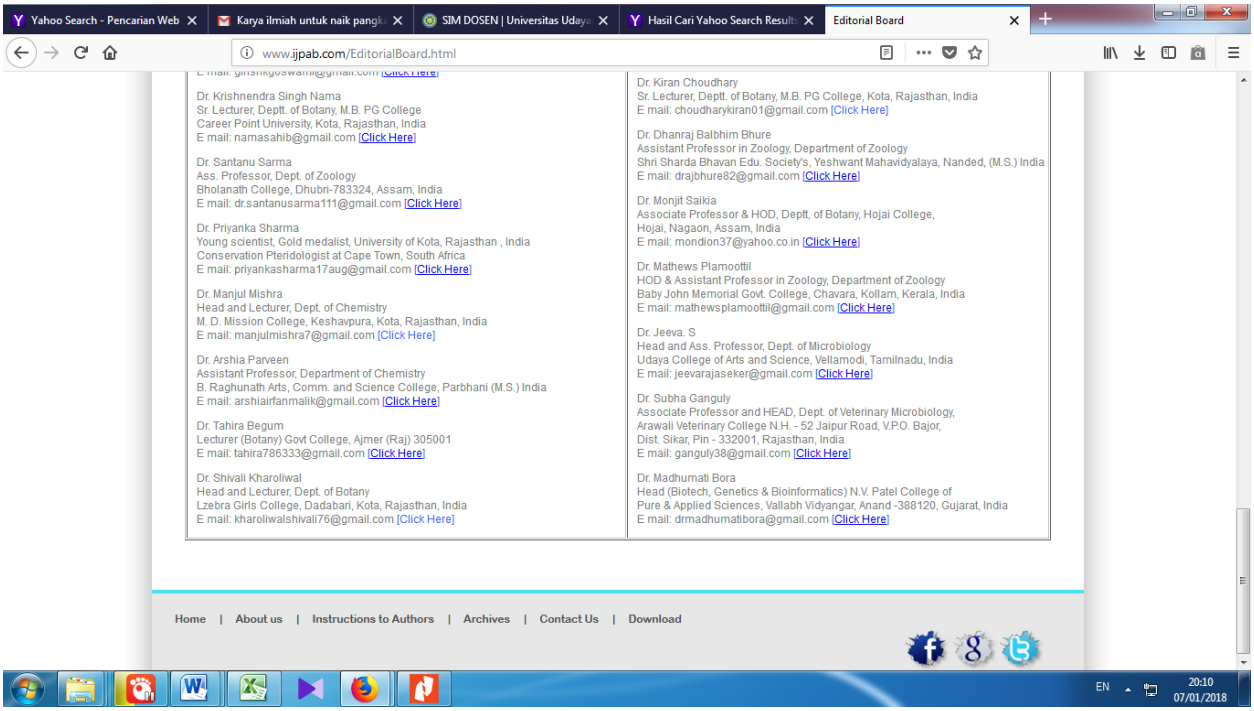
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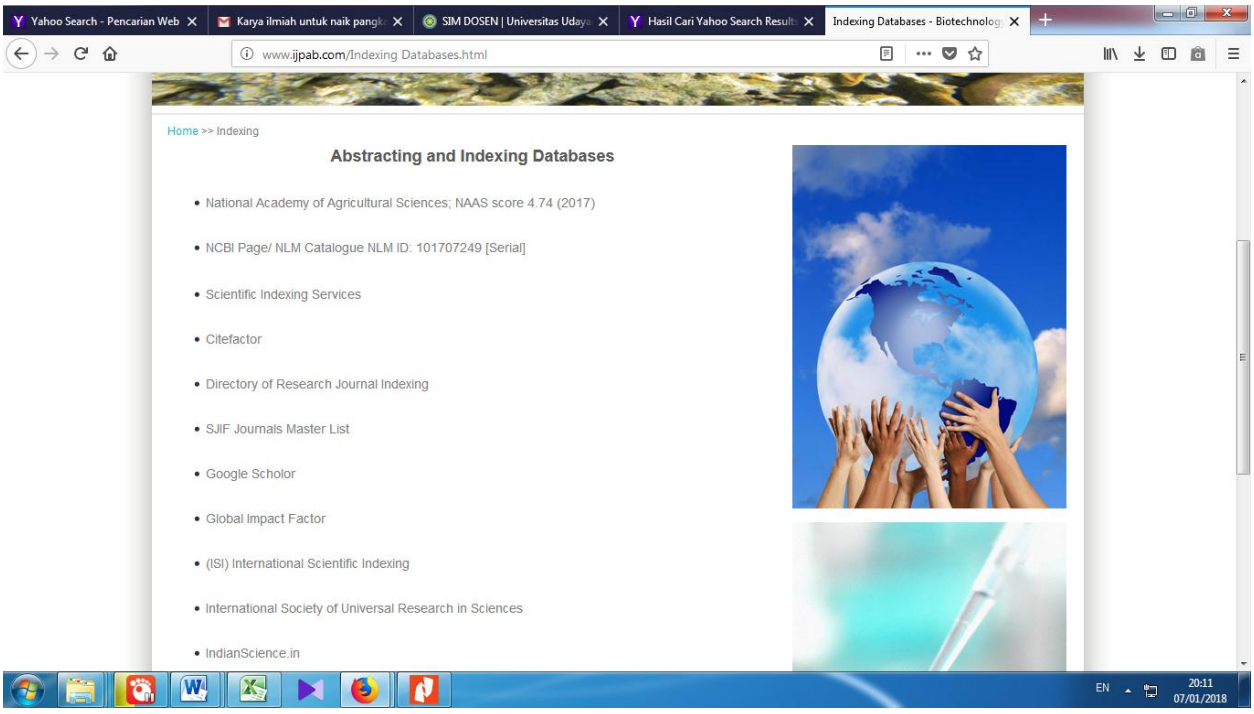
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Research Article

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The *Trichoderma asperillum* TKD Filtrate Potency in Reducing Contaminants of Aflatoxins B_1 Produced by *Aspergillus flavus* FNCC 6109 on Concentrate Feed

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ABSTRACT

Aspergillus flavus is a fungus that often contaminates concentrate feed. This fungus is able to produce aflatoxin B1, which is harmful to livestock and humans. The aim of this study is to determine the potential of the filtrate of Trichoderma asperillum TKD in reducing aflatoxin B1 produced by Aspergillus flavus FNCC 6109. The test is done by formulating concentrate feed with filtrate concentrations of 20 %; 40 % and 60 %. The level of aflatoxin B1 is determined by ELISA technique (Enzyme Linked Immuno Sorbent Assay). The characterization of secondary metabolites in the filtrate is analyzed by using GCMS. The results showed that 60 % of filtrate concentration was able to reduce aflatoxin B1 by 47.84 % which is significantly different (P≤0,05) to other treatments. The secondary metabolites contained in T. asperillum TKD filtrate are Butane, 1,1 - oxybis- (CAS) n - Butyl ether; Butane, 1,1 - dibutoxy - ; Pentansaeure, 2,24,4 - Tetramethyl; 1 - Tetradecene; Phenol, 2,4 - bis (1,1 - dimethylethyl- (CAS) 2,4; 1- Hexadecane (CAS) Cetene; 1 - Octadecene (CAS) alpha - Octadecene ; hexadecanoic acid , methyl ester (CAS) Methyl, n - Tetracosano - 1 , 4 - Heptanone (CAS) GBL; 1 - Dodecanol (CAS) n - Dodecanol and Behenic alcohol.

Keywords: *aflatoxin B_1, filtrate, T. asperillum TKD, concentrate.*

INTRODUCTION

A type of fungus that often contaminate concentrate feed is *Asperillus flavus*. This fungus is able to produce aflatoxins which are harmful to human and livestock. Under conditions of 85 % relative humidity and temperature of 25^0C to 35^0C, *A. flavus* growa excellently and produces aflatoxin[6]. Many aflatoxin has been known, but this fungus only produces aflatoxin B_1 and B_2 [15]. More than 80 % of commercial poultry feed are contaminated by aflatoxin B_1 with various levels of contamination[1].

Aflatoxin B1 is the most poisonous aflatoxin[6,23]. In high concentration, aflatoxin B_1 may cause death. Whereas at low concentration and in a long term it can cause necrosis of the liver and kidneys[20,8,21]. In addition, aflatoxin B_1 can cause a decrease in livestock reproductive performance, immune function and decreased milk production[11].

A. flavus growth in the concentrate during storage, will provide increased opportunities of aflatoxin content, it causes a decline in the quality of the feed. Therefore, the fungus needs to be controlled. Current control method that is being used are by using sodium calcium aluminosilicate, the combination of propionic acid and nisin [13, 14]. Such chemical control is expensive and can interfere with human health.

Based on this, it is necessary to utilize biological control *Trichoderma asperillum* TKD. This fungus was isolated from maize plants rhizosphere. It has been proven that it has the ability to inhibit *A. flavus* FNCC 6109 on a laboratory scale. As an antagonist, *T. asperillum* TKD is certainly has a mechanism to inhibit the growth of *A. flavus* FNCC 6109. Therefore, this study aims to see the *T. asperillum* TKD filtrate potency in inhibiting *A. flavus* FNCC 6109 so that the contamination of aflatoxin B_1 can be minimized.

MATERIALS AND METHODS

Well diffusion assay

The filtrate of *T. asperillum* TKD was obtained by inoculating this fungus into 100 mL of medium Yeast Extract Sucrose (axtract yeast 20g / L ; Sucrose 150g / L and 1 liter of distilled water) and then incubated at room temperature for 21 days. Liquid medium that has been grown by T. *asperillum* TKD was further filtered and evaporated by using *a speed vacuum device* (dry max plus). The ability of this Concentrated filtrate to its antifungi activity was then tested using the well diffusion method [12].

As for the test procedure is, by preparing a sterile Petri dish filled with 200 mL of a suspension of spores of *A. flavus* FNCC 6109, then was poured with 15 mL MEA media and allowed to solidify. Right in the middle of the Petri dish a well was made by using a cork borer then 20 mL of the filtrate was deposited into it. Furthermore all Petri dishes were incubated at 28^0C for 3 days. The filtrate inhibitory ability can be determined by measuring the clear zone around the well.

Characterization of secondary metabolites of T. asperillum TKD through GC - MS analysis.

The secundary metabolites of *T. asperillum* TKD was obtained by mixing the concentrated filtrate (250 mL) with 500 mL of chloroform and methanol (2 : 1.v / v). This mixture was separated in a separatory funnel. The separation results which positively inhibit *A. flavus* FNCC 6109, were evaporated and fractionated using column chromatography (diameter 1.5 cm and length 50 cm) filled with silica gel. The column was passed with eluent chloroform and methanol (2 : 1 v / v) and then 25 mL were collected as fraction 1 and with the same way the next 25 mL were collected in order to obtain some fraction[17,24]. All fractions obtained were evaporated back by using vacuum rotary evaporator. Then a separation was performed using Thin Layer Chromatography (TLC) Keisel Del 60 F254 with the developer of N - Butanol : acetate acid : water (4 : 1 : 5) . Group of compounds which show the same signs of separation were combined in one fraction and their inhibitory activity against *A. flavus* p FNCC 6109 were re-tested. The compounds separation was continuously performed until pure compounds were obtain, then they were identified by using GCMS type QP2010, Shimadzu.

Effect of *Trichoderma asperillum* filtrate TKD against *A. flavus* FNCC 6109 and aflatoxin B1 FNCC 6109

Filtrate testing produced by *T. asperillum* TKD against *A. flavus* FNCC 6109 on concentrate feed was done by using a completely randomized design (CRD) with 5 types of treatment which are:

A: concentrate feed without *A. flavus* FNCC 6109 and without *T. asperillum* TKD filtrate.

B: concentrate feed with *A. flavus* FNCC 6109

C: concentrate feed with *A. flavus* FNCC 6109 and *T. asperillum* TKD filtrate with a concentration of 20 %

D: concentrate feed with *A. flavus* FNCC 6109 and *T. asperillum* TKD filtrate with a concentration of 40 %

E: concentrate feed with *A. flavus* FNCC 6109 and *T. asperillum* TKD filtrate with a concentration of 60 %

Concentrate feed used in this trial is concentrate feed with egg laying grower kind, which is formulated as follows: 400g corn ; 130g soy beans ; 210g rice bran ; 90g coconut cake ; 100g fish meal ; 55g turi leaves flour; 7g mineral salt and 8g B12 mineral. All the materials are mixed and then made in the form of pellets. Before formulating, concentrate feed was autoclaved to avoid contamination of other microbes in the test. 5 sterile bag were prepared and each were filled with 100 g of concentrate feed. The first bag is used as a control that contains only 100 g of concentrate with no treatment. 4 pockets were prepared, then filled with 100 g of concentrate that has been added with 5 ml of spore suspension of *A. flavus* FNCC 6109 to each of them, then mixed until uniform. The second bag was not given an additional filtrate. Then the third bag, was added with 15 mL of *T. asperillum* TKD filtrate with a concentration of 20 % , as well as the fourth and fifth bags each was added with 15 mL of filtrate with the concentration of 40 % and 60 % . All treatments were dried in an oven at 40 ° C for 24 hours. Then each of all of the ingredients with treatment were incorporated into sterile Erlenmeyer then sealed with cotton and kept at room temperature

for 30 days. After the storage period ended, an observation of *A. flavus* FNCC 6109 populations was performed by using a plating method and the aflatoxin B_1 content was determined by ELISA technique (Enzyme Linked Immuno Sorbent Assay).

RESULTS AND DISCUSSION

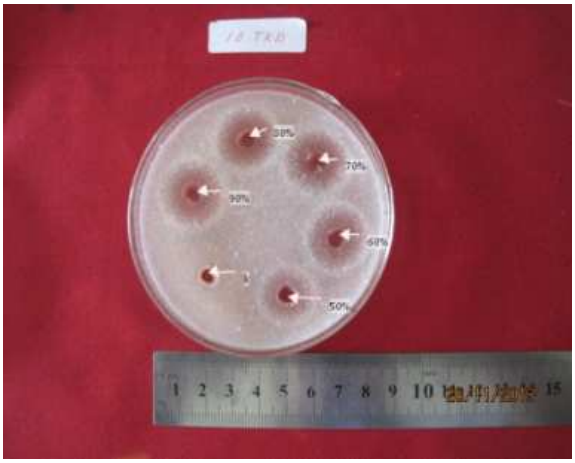
Trichoderma sp. is believed to have a better ability to live and has a fairly high power to compete in a variety of conditions[16]. In the competition, various mechanisms can occur such as antibiotics or enzymes production that can interfere with the physiological process of the competitors. In addition to having a high competition for nutrients and space, *Trichoderma* sp. can produce antibiotics that can inhibit or kill other microbes[4]. This was confirmed by a research which said that Trichoderma can produce secondary metabolites that may play a role in competition against other microorganisms[19].

The result of the inhibition of the filtrate of T. asperillum TKD against *A. flavus* FNCC 6109 showed that there is a clear zone around the well as shown in Figure 1. The clear zone indicates that *T. asperillum* TKD filtrate contains active compounds that could potentially inhibit fungi beeing tested. A similar study also stated that the filtrate of *T. harzianum* and *T. viride* able to inhibit the growth of *A. flavus* in the laboratory scale[3]. Furthermore a filtrate of *Trichoderma* sp. with a concentration of 75 ppm; 125 ppm; 250 ppm; and 500 ppm of *Trichoderma* sp . able to inhibit *Rhizoctonia bataticola*[9].

Table 1. Inhibition and Rf value of crude extract fraction of *T. asperillum* TKD filtrate against *A. flavus* FNCC 6109

Fraction	Rf	Inhibition
I	-	-
II	0,75	-
III	0,45 and 0,60	-
IV	0,48 and 0,64	+
V	0,48 and 0,59	-
VI	0,56	-
II	0,57	-

Fig. 1:



Inhibitory strength of some concentration of the *T. asperillum* TKD filtrate against *A. flavus* FNCC 6109 through the well diffusion method.

From crude extract of the bottom layer filtrate of *T. asperillum* TKD that has been columned with a mobile phase of 500 mL of eluent chloroform: methanol (2 : 1), 19 fractions were obtained. The same pattern of spots on TLC were combined into one fraction, so that at the end of the TLC, 7 fractions were obtained that have different patterns of spots. Based on the inhibition testing of the 7 fractions, only fraction IV inhibited the growth of *A. flavus* in FNCC 6109 in vitro (table 1 .). After analyzing the GCMC (*Gas Chromatography Mass Spectra*), spot with Rf value of 0.48 which is in fraction IV, eight

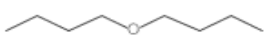
types of secondary metabolites were identified namely : Butane , 1,1 - oxybis- (CAS) n - Butyl ether ; Butane , 1,1 - dibutoxy- ; Pentansaeure , 2,24,4 - Tetramethyl ; 1 - Tetradecene ; Phenol , 2,4 - bis (1,1 - dimethylethyl- (CAS) 2,4 ; 1- Hexadecane (CAS) Cetene ; 1 - Octadecene (CAS) alpha - Octadecene and hexadecanoic acid , methyl ester (CAS) Methyl and n - Tetracosano - 1 . Spot with Rf value of 0.64 in fraction IV identified three types of compounds are: 4 - Heptanone (CAS) GBL ; 1 - Dodecanol (CAS) n - Dodecanol and Behenic alcohol .

It has been reported that the secondary metabolites produced by Trichoderma depends on the strain of Trichoderma (Ghisalberti and Sivasithamparam (1991). Furthermore, there are three groups of secondary metabolites produced by Trichoderma namely (i) an antibiotic that is volatile (6 - pentyl - a- pyrone (6pp) and derivatives of isocyanide); (ii) compounds that are readily soluble in water (heptelidic koningic acid or acid) , and (iii) peptaibols which is a class of linear or cyclic peptide, which is characterized by long chains consisting of 4-21 amino acids

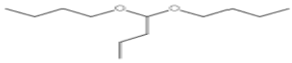
Vinale *et al*. (2006) isolate and characterize the secondary metabolites obtained from the filtrate of T. harzianum strain (T22 and T39) [22]. The secondary metabolites produced by *Trichoderma* spp. strain T22 is (1) azaphilone and strain T39 is 2 : butenolide ; 3 : harzianolide ; 4 : dehydro harzianolide ; 5 : harzianopyridone ; 6 : 6 - pentyl - a- pyrone ; 7 : 1 - hydroxy - 3 - methyl - anthraquinone ; 8 : 1,8 - dihydroxy - 3 - methyl - anthraquinone ; 9 : harziandione ; 10 : koninginin A ; 11 : heptelidic acid ; 12 : trichoviridin ; 13 : harzianic acid ; 14 : gliotoxin ; 15 : gliovirin ; 16 : viridin ; 17 : viridiol ; 18 : trichorzianines .

It has been also reported that a filtrate of Trichoderma sp. contains secondary metabolites such as : 6 - nonylene alcohol , massoilactone , methyl cyclopentane , methyl cyclohexane , methyl N- pyrollidine , dermadin , ketotriol , Koningin - A , 3 - methyl - heptadecanol , 2 - methyl heptadecacol , palmitic acid , 3- (2 ' -hydroxypropyl) -4 (hexa - 2' - 4 - dineyl) -2- (5H) -furanone and 3- (propenone) -4- (hexa - 2' - 4' - dineyl0-2- (5H) -furanone) detected by GCMS (Gas Cromathography Mass Spectra) [9].

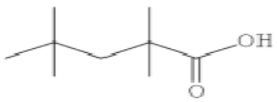
Figure 2. Molecular structure of secondary metabolites fraction IV on the spot with Rf values of 0.48 and 0.64 were identified by using GCMS



Butane, 1,1-oxybis-(CAS) n-Butyl ether

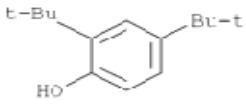


Butane, 1,1-dibutoxy-



Pentansaeure,2,24,4-Tetramethyl

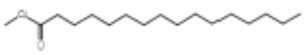
1-Tetradecene



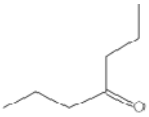
Phenol,2,4-bis(1,1-dimethylethyl-(CAS)2,4-

1- Hexadecane (CAS) Cetene

1-Octadecene (CAS)alpha-Octadecene



Hexadecanoic acid, methyl ester (CAS)Methyl



4-Heptanone(CAS)GBL

1-Dodecanol(CAS)n-Dodecanol

Behenic alcohol

Results of analysis of variance showed there are effect of the treatment which was given to the content of *A. flavus* FNCC 6109 on egg laying chicken concentrate feed grower for 30 days of storage. In Table 2 shows that the highest content of *A. flavus* 6109 FNCC happens in B_{30} treatment that is equal to 5.320 log10 CFU / g ± 0.563 and the lowest happens on the treatment of A_{30} that is 0.000 log10 CFU / g ± 0,00 . Statistically, these two treatments are significantly different (P≤0,05). The Absence of *A. flavus* FNCC 6109 on the A_{30} indicates that concentrate treatment for 30 days storage has no of *A. flavus* contamination. The average content of *A. flavus* FNCC 6109 on concentrate feed given 60 % filtrate of *T. asperillum* TKD at 3.054 log10 CFU / g ± 0.146 , when compared with treatment B_{30} (without giving filtrate) seems to be much lower and statistically significantly different (P≤0,05). This means giving a filtrate of *T. asperillum* of 60% is able to inhibit the growth of *A. flavus* FNCC 6109 to concentrate feed during 30 days of storage. From the data shown in Table 2 , there is a tendency that the more concentration of the filtrate of *T. asperillum* TKD given to the concentrate feed stored for 30 days the less the content of *A. flavus* FNCC 6109.

Table 2. Average content of *A. flavus* FNCC 6109 and aflatoxin B_1 on concentrate feed egg laying chicken grower after being stored for 30 days

Prameter	Treatment				
	A_{30}	**B_{30}**	**C_{30}**	**D_{30}**	E_{30}
A. flavus (Log_{10})	0,000±0,000^{d}	5,320±0,563^{a}	4,124±0,689^{b}	3,950±0,683^{b}	3,054±0,146^{c}
Aflatoksin B_1 (ppb)	51,640±6,11^{bc}	74,160±7,110^{a}	57,160±5,58^{b}	44,680±2,504^{cd}	38,680±6,395^{d}

Description: Values with different letter notation on the same line shows the average value significantly different (P≤0,05) , based on the Duncan test after analysis of variance (ANOVA) .

The content of aflatoxin B_1 on concentrate feed given with *T. asperillum* TKD filtrate looks decrease compared to concentrate feed which is only be given with a suspension of *A. flavus* FNCC 6109. The lowest content of aflatoxin B_1 in the concentrate feed found in the E_{30} treatment (given with *T. asperillum* TKD filtrate with concentration of 60 %) in the amount of 38.680 ± 6.395 ppb. This is significantly different (P≤0,05) with aflatoxin B_1 content in concentrate feed which is not be given with filtrate (B_{30}).

Storage of concentrate feed which is given a concentration of the filtrate of 60% for 30 days, is able to reduce aflatoxin B_1 up to 47.84 %. Decreased levels of aflatoxin in this study are consistent with research conducted by Choudhary (1992) who reported that *T. viridae* is able to reduce the level of aflatoxin B_1 to 73.5 % even against aflatoxin G_1, reduced to 100 % when the culture were given together. While Calistru *et al*. (1997) only proves filtrate of *T. harzianum* and *T. viride* able to inhibit the growth of *A. flavus* in the laboratory scale. In treatment A_{30} without suspense isolates of *A. flavus* FNCC 6109 and filtrate, turned out to contain aflatoxin B_1 of 51.640 ± 6.11 ppb [3].

This indicates that the raw materials used in preparing this concentrate was contaminated with aflatoxin B_1. The aflatoxins can be derived from corn, soybeans, rice bran, coconut meal, fish meal or flour leaves. It has been reported that most of the corn samples in Kupang , East Nusa Tenggara contain aflatoxin B_1 with different levels[18]. Likewise, another research discovered aflatoxin in some agricultural products such as corn , peanut , and soybean meal [7]. Some other research reported aflatoxin contamination in feed ingredients and feed in some areas of the province of Lampung and East Java [2]. The result also shows that in the samples (corn, peanuts, rice bran) a presence of aflatoxin with varying levels are detected.

CONCLUSION

The filtrate of *Trichoderma asperillum* TKD can inhibit *A. flavus* FNCC 6109 and reduce levels of aflatoxin B_1 in egg laying chicken grower concentrates feed. The filtrate contains 11 different secondary metabolites.

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