# The antagonist effect of 2-phynel ethyl acetate and ethyl acetate against growth of *Aspergillus flavus* in wheat

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# Committee decision

This thesis (The antagonist effect of 2-phynel ethyl acetate and ethyl acetate against growth of *Aspergillus flavus* in wheat)

Was successfully defended and approved on……..

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# Dedication

**To**

***My Mom & Dad***

***My Only Sister and Brothers***

***My Best Friends***

***From the bottom of my heart I would like to say I'm extremely thankful for giving me support, love & pushing me farther than I thought I could go.***

## Acknowledgment

*Above all, I'm thankful to Almighty Allah for giving me strength, patience, and ability to write and complete this thesis.*

*I would like to express the deepest appreciation to my supervisor Dr. Wafa Masoud, for her understanding, wisdom, patience, enthusiasm and encouragement. Her support and guidance saved me several times from frustration and despair.*

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*I would like to thank technical laboratory staff for their help.*

*Finally, I would like to thank my Father for encouraging me to register for this program.*

**الإقرار**

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

**The antagonist effect of 2-phynel ethyl acetate and ethyl acetate against growth of *Aspergillus flavus* B1 in wheat**

أقر بأن ما اشتملت عليه هذه الرسالة إنما هي نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيثما ورد، وأن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أية درجة علمية أو بحث علمي لدى أي مؤسسة تعليمية أو بحثية أخرى.

**Declaration**

The work provided in this thesis, unless otherwise referenced, is the researcher own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: Abeer Khaled "Sayed Ahmad" اسم الطالبة: عبير خالد "سيداحمد"

Signature: التوقيع:

Date: التاريخ:

# List of Contents

# List of Tables

## List of Figures

# List of abbreviation

| Abbreviation | Details |
|---|---|
| FAO | Food and Agriculture Organization |
| *A. flavus* | *Aspergillus flavus* |
| AFs | Aflatoxins |
| IARC | International Agency for Research on Cancer |
| AFB1 | Aflatoxin B1 |
| AFB2 | Aflatoxin B2 |
| HCC | hepatocellular carcinoma |
| ARIJ | The Applied Research Institute – Jerusalem |
| $a_w$ | water activity |
| EST | expressed sequence tags |
| HBV | hepatitis B virus |
| PDA | potato dextrose agar media |
| CAM | Coconut agar medium |
| PCR | polymerase chain reaction |
| aflR | Aflatoxin regulatory gene |

# The antagonist effect of 2-phynel ethyl acetate and ethyl acetate against growth of *Aspergillus flavus* in wheat.

**By**

**Abeer Khaled "Sayed Ahmad"**

**Supervisor**

**Dr. Wafa Masoud**

## Abstract

Wheat is an important agricultural products in the world. Wheat is the main ingredient in many feedstuffs and is used as a feed stock to produce ethanol. *Aspergillus flavus* is a pathogenic and saprotrophic fungus that produce aflatoxin B1 (AFB1). Aflatoxin B1 is known as a carcinogenic compound. This toxin has been reported to contaminate crops, mainly corn, cotton, peanuts, wheat and tree nuts.

It was the main goal of the present study to evaluate the antifungal activity of 2-phenyl ethyl acetate and ethyl acetate against growth of *Aspergillus flavus*. Eleven samples of dry wheat were collected from markets and stores in Tulkarem city in Palestine and were tested for their contamination with *A. flavus.* Two isolates of *A. flavus* were detected in wheat samples, culture technique (coconut agar media CAM) were used. One of the fungal isolates was found to produce aflatoxin B1, while the other did not produce aflatoxin B1. Different concentrations of 2-phenyl ethyl acetate and ethyl acetate were examined against growth of *A. flavus*. It was found that 2-phynel ethyl acetate was effective in reducing *A. flavus* growth. Growth of *A. flavus* was reduced by 2-phynel ethyl acetate at concentrations of 62.9, 78.4 and 93.9 μg/L. At a lower concentration of 2-phynel ethyl acetate, growth of *A. flavus* was reduced and the color of fungal colonies changed from green to white indicating a change in its metabolic activities. Ethyl acetate was found to have very weak effect on fungal growth. The findings of the present study demonstrated the potential use of 2-phenyl ethyl acetate as a preservative in wheat against fungal growth and production of aflatoxin B1, As well as the possibility of using CAM culture technique as a cheap, good and available method in the Developing countries.

# 1. Introduction

Wheat is known to be very important nutritious food because more than 20% of the calories obtained from the daily diet are provided by wheat (Wiese, 1987). Moreover, wheat has a caloric content and protein more than that of any other cereal crops. Wheat is cultivated in almost all over the world. Compared to corn, barley, and sorghum, wheat is very essential crop. In addition, wheat is economically very important because the amount of wheat that is traded between countries exceeds the amount of other grains. Wheat is a main ingredient in many food stuffs as well as it is used in starch production, and it is used to produce ethanol according to Food and Agriculture Organization (FAO, 2009).

Cereal grains are much suspected to be contaminated by fungi. Many studies documented fungal contamination of grains (Cleveland *et al.*, 2003; Roige *et al.*, 2009; Riba *et al.*, 2010; Sardiñas *et al.*, 2011).

*Aspergillus flavus* is a pathogenic and saprotrophic fungus (Machida & Gomi, 2010). This fungus has been reported to be found in soils causing disease to very important agriculture crops such as cereal grains, legumes, and tree nuts (Ramírez-Camejo *et al.*, 2012). *Aspergillus flavus* infection causes serious problems in some crops, such as yellow mold in peanuts and ear rot in corn either before or after harvest (Amaike, 2011). Although fungal contamination can occur before crops harvest, *A. flavus* infections don't show symptoms before harvesting. Symptoms appear during storage and / or transport. Several strains

of *A. flavus* have been found to form high amounts of aflatoxin, which, when consumed in certain quantities, are toxic to human beings (Agrios, 2005). Furthermore, *A. flavus* is a pathogenic fungus that causes Aspergillosis in persons with low immunity (Amaike, 2011).

*Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. toxicarius*, *A. minisclerotigenes* and *A. parvisclerotigenus* are reported to secrete Aflatoxins (AFs), which are toxic secondary metabolites. (Pildain *et al.*, 2008). During storage with favorable conditions for fungal growth, mainly temperature and high humidity, AFs can contaminate crops.

According to International Agency for Research on Cancer (IARC, 1982) Aflatoxin B1 has been included as a Group 1 carcinogen because it's proved to cause cancer in human beings (Castegnaro & Wild, 1995). Thus, aflatoxin B1 is considered as the most common of the four naturally occurring AFs (B1, B2, G1 and G2). Pittet (1998) reported that those toxins contaminated about 25-40% of cereals in the world.

Unfortunately, toxin contamination in foods remain during manufacturing, even under high temperature and high pressure. Aflatoxin B1 has chronic toxic effects when exposed to certain proportions, as it increase the occurrence of hepatocellular carcinoma (HCC). (Kucukcakan & Hayrulai-Musliu, 2015). This type of Aflatoxin is produce by *Aspergillus flavus* fungus, which lives in soil and

then contaminates the crops when spreads by the airborne conidia (Hedayati *et al*., 2007).

Food and Agriculture Organization (FAO, 2003), reported that the permitted amount of aflatoxin B1 range from 1–20 µg/kg in different types of products, and about 5–50 µg/kg in cattle feed. Wheat was one of the crops that has been found to be contaminated with aflatoxin.

To control aflatoxin contamination from *A. flavus* and to protect food and feedstuffs, many possible control strategies can be used in cereals such as treatment of cereals by fumigants, fungicides, or biocontrol agents (Kong *et al*., 2010). However, the use of fungicides against *A. flavus* has negative effects. Those negative effects include the generation of resistant strains of *A. flavus* to fungicides as well as the environmental pollution caused by fungicide residues, which affect food safety (Droby, 2005). Therefore, effective biological controls against aflatoxin producing fungi is a priority, where there will be fewer risks to human and environment.

Recently, using of microorganisms to produce new chemical compounds with high commercial value have gained considerable attention (Amador *et al.*, 2003).

Volatile compounds 2-phenyl ethyl acetate and ethyl acetate produced by *Pichia anomala* and *Pichia kluyveri* have proved to be effective in reducing *Aspergillus ochraceus* growth and ochratoxin A formation (Masoud *et al.*, 2006).

The main goal of this study was to:

Evaluate the effect of 2-phenyl ethyl acetate and ethyl acetate against growth of *Aspergillus flavus.*

The objectives of this study were to:

1- Determine the presence of aflatoxigenc *Aspergillus flavus* in eleven different samples of wheat, collected from Tulkarem city in Palestine by microscopic and macroscopic taxonomic keys.

2- Identify Aflatoxin B1 producing *Aspergillus flavus* by a special medium (coconut agar medium) and by Polymerase chain reaction (PCR).

3- Determine the antifungal activity of 2-phenyl ethyl acetate and ethyl acetate against growth of *Aspergillus flavus* by exposing the fungus to these volatiles.

# 2 Literature review

## 2.1 Wheat in Palestine

The Mediterranean climate- Palestine is one of the countries that has this climate- is characterized by a rainy winter and dry summer, which give rise to a distinctive type of agriculture.

Wheat is a vital crop that respond well to the environmental conditions of the Mediterranean region. According to the Palestine Ministry of Agriculture, wheat is considered as an important crop with 143,326 dunum of agricultural land (which constitutes 9.6% of the agricultural land) were cultivated with wheat in the West Bank during the year 2012/2013. Wheat is estimated to be 25,926 tons of the total annual production of in the West Bank (The Applied Research Institute – Jerusalem (ARIJ), 2015).

Wheat is considered very important crop as it is the main component of bread, which is a main and an important food item in the Palestinian meals. Palestinian families spend 31.6 JD per month in the West Bank, and 28.5 JD in Gaza Strip, to buy wheat (whether in the form of bread or other things), according to the Palestinian Central Bureau of Statistics (Palestinian Central Bureau of Statistics, 2006). Therefore, it is very important to obtain wheat, with good quality and free contaminations such as aflatoxin B1.

## 2. 2 *Aspergillus flavus*

Link 1809 described *Aspergillus flavus* as a fungal species, which only form asexual spores, conidia, and form mycelium or sclerotia (resistant structures*)* during winter time. When conditions are suitable, sclerotia germinate to form conidia (asexual spores) or they produce additional hyphae, which can spread to air and soil (Bhatnagar *et al.*, 2000). The sexual stage of *A. flavus* is classified as *Petromyces flavus* (Horn *et al.*, 2009).

Preharvest or postharvest agricultural crops, mainly corn (ear rot), peanuts (yellow rot), and cotton seeds can be infected by *Petromyces flavus* (Klich, 2007; Michailides & Thomidis, 2007).

*Aspergillus flavus* is distributed widely in the environment, due to its ability to produce many airborne conidia, which can easily be transformed by insects as well as by air movements. The most important factor in the atmosphere that seems to have a major effect on fungal growth is moisture (Gibson *et al*., 1994). It has been reported that the optimum growth of *A. flavus* was at a water activity ($a_w$) of a range of 0.86 and 0.96 (Vujanovic *et al*., 2001). The optimum temperature for growth of *A. flavus* was reported to be around 37 ° C, but fungal growth can be observed at temperatures ranging from 12 to 48 °C (Vujanovic *et al*., 2001).

*Aspergillus flavus* is reported as a velvet fungus, which has yellow to green or brown with a golden to red-brown invert (Figure 1). The length of its conidiophores are not the same. The conidiophores are rough, pittted and spiny

that can be uniseriate or biseriate. The conidiophore covers the entire vesicle, and indicates the phialides in all directions (Figure 2). Conidia is globose to subglobose, clearly manifested, and diameter ranges from 3.5 to 4.5µm. *A. flavus* is classified into two groups according to its sclerotia size. The S strain, which is known to form many small sclerotia. The L strain, which was observed to form larger sclerosis (Cotty, 1989). From the S strain, there are some isolates that has been known as SB, which have been reported to produce only aflatoxin B, while other isolates from the same strain, which are called SBG, form both aflatoxin B and G (Cotty, 1989).

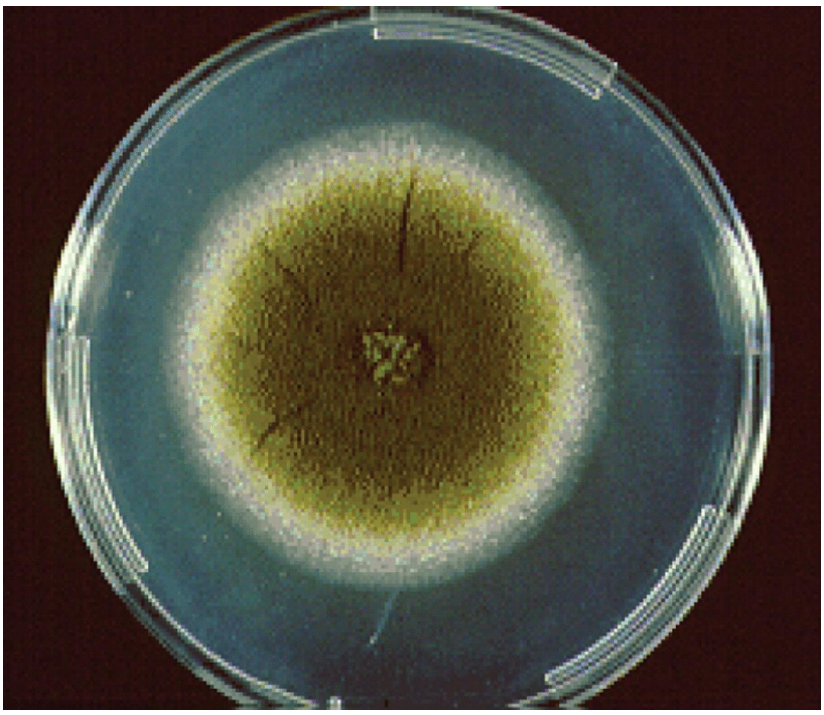

**Figure 1.Macroscopic features of *A. flavus* on Czapek's agar (Hedayati et al., 2007).**

Link (1809) explained microscopic features of *A. flavus* as shows in figure 2. Conidiophores are heavy-walled, uncoloured, and rough, with less than 1 mm

long. The vesicles are elongated at the beginning of growth, and after that become beneath globose or subglobose, with a diameter of 10 to 65 μm. Phialides are uniseriate or biseriate. The length of primary branches reaches 10 μm, while the length of the secondary branches reaches 5 μm. Conidia is usually a globose to subglobose, and echinulate clearly, with a diameter of 3.5 to 4.5 μm.

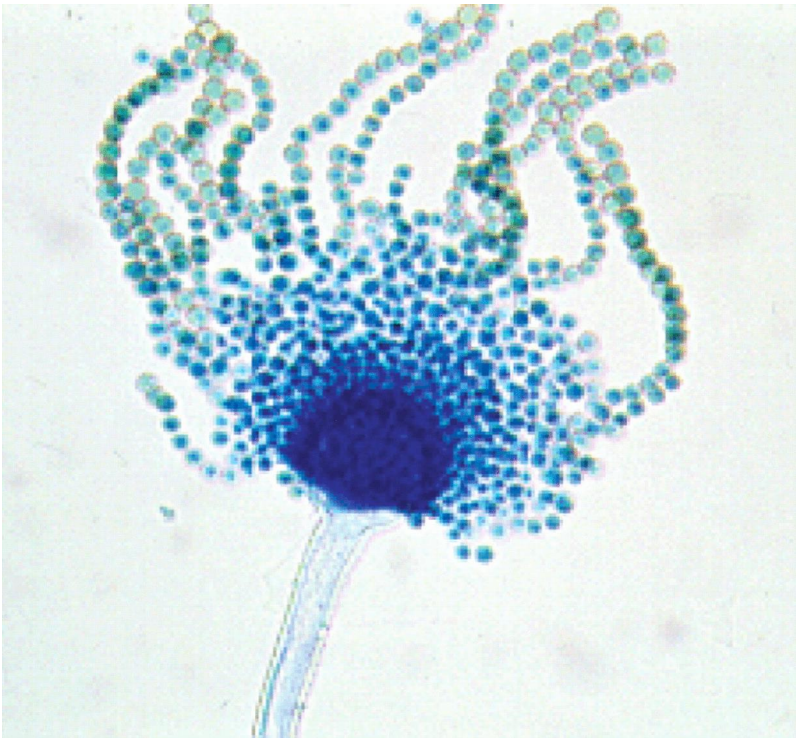

**Figure 2. Microscopic features of *A. flavus* (Hedayati et al., 2007).**

A 5X sequence of the whole genome of *A. flavus* has been released in 2005 by the J. Craig Venter Institute (Cleveland *et al.*, 2009; Payne *et al.*, 2010; http://www.aspergillusflavus.org/ genomics/, NCBI or Broad Institute1).

The US Department of Agriculture's Southern Regional Research Center in New Orleans, Louisiana have generated 7,218 unique expressed sequence tags (ESTs)

from *A. flavus* from 19,618 ESTs and identify it before sequencing of the fungal genome (Cleveland *et al.*, 2009). It has been reported that the length of *A. flavus* genome is about 37 mb on eight chromosomes that encode more than 12,000 functional genes (Chang & Ehrlich, 2010).

*Aspergillus flavus* can exist in extreme conditions, which make it easy for this mold to compete with other organisms for the nutritional ingredients that present in both soil and plants (Bhatnagar *et al.*, 2000).

Latest studies have shown that some strains of *A. flavus* produce aflatoxin while other strains do not produce aflatoxins (Orum *et al*., 1997). There are 13 known types of aflatoxins with aflatoxin B1 being the most toxic. Contamination of crops with *A. flavus* does not always mean the occurrence of aflatoxins in high levels (Patel *et al.*, 2015). The occurrence of aflatoxins varies with the climatic conditions prevailing at the time of harvest and transport and during subsequent conditions (Smith & Moss, 1985; Cotty & Jaime-Garcia, 2007). Moisture level of grains at harvest is an important determining factor to subsequent mold colonization (Smith & Moss, 1985). High moisture levels (20 - 25% wet weight) are required for the growth of fungi in living plant or in decaying organic material, whereas fungi capable of growing in stored products can usually exist at moisture content of (12-18%) (Smith & Moss, 1985). In many cases, development of the fungus usually stops when the temperature is below -1.5 ° C and the grain moisture is 12 % or below (Smith & Moss, 1985).

## 2.3 Aflatoxin

Aflatoxins were first known in 1960, when a more than 100000 people and many farm animals died due to toxic outbreak of Turkey "X" disease in England (Klich *et al.*, 2000; Devero, 1999). The word aflatoxin came from "a" for *Aspergillus* genus, "fla" for the species flavus and "toxin" (Bakırdere *et al.*, 2012).

Aflatoxins are formed by fungal species through a pathway that is composed of series steps catalyzed by specific enzymes from simple intermediates of primary metabolism, like acetates, malonate, mevalonate and certain amino acids (Steyn, 1998). The main biosynthetic reactions include condensation, oxidation / reduction, alkylation and halogination steps which create a remarkable range of secondary compounds (Smith & Moss 1983; Steyn, 1998). There are four kinds of aflatoxins, they include B1, B2, G1, G2, which are known to directly contaminate food and feeds.

Aflatoxins are crystals that are soluble in mild polar solvents such as chloroform, methanol, and dimethyl sulfoxide. AFs were reported to dissolve in water to a value of 10-20 mg / L (Feddern *et al.*, 2013). Table 1 represented the physical and chemical features of aflatoxins (European Mycotoxins Awareness Network, 2012).

**Table 1. Physical and chemical properties of aflatoxins.**

| Aflatoxin | Molecular formula | Molecular weight | Melting point | UV absorption (ε (L $mol^{-1}$ $cm^{-1}$)) 260-365 nm |
|---|---|---|---|---|
| **B1** | $C_{17}H_{12}O_6$ | 312 | 268-269 | 12,400-21,800 |
| **B2** | $C_{17}H_{12}O_7$ | 314 | 286-289 | 12,100-24,000 |
| **G1** | $C_{17}H_{14}O_6$ | 328 | 244-246 | 99,60-17,700 |
| **G2** | $C_{17}H_{14}O_7$ | 330 | 237-240 | 8,200-16,100 |

Aflatoxin B1, (AFB1) is reported as the strongest liver carcinogens of all myctoxins (Kuilman *et al.*, 1998). AFB1 and AFB2 are only two toxins that are produced by *A. flavus*. On the other hand, *A. parasiticus* was reported to produce four types of aflatoxins, which include AFB1, AFB2, G1 and G2 (Mclean & Dutton, 1995). Table 2 indicates the averages and ranges of the maximum permissible levels (μg / kg) for some aflatoxin (groups) (Shephard, 2003).

**Table 2: Medians and ranges of maximum tolerated levels (µg/kg) for some (groups of) aflatoxins in 1995 and 2003 and numbers of countries known to have relevant regulations.**

| | 1995 | | | 2003 | | |
|---|---|---|---|---|---|---|
| **Aflatoxin/matrix combination** | **Median (µg/kg)** | **Range (µg/kg)** | **Countries** | **Median (µg/kg)** | **Range (µg/kg)** | **Countries** |
| **Afla B1 in foodstuffs** | 4 | 0-30 | 33 | 5 | 1-20 | 61 |
| **Afla B1+B2+G1+G2 in foodstuffs** | 8 | 0-50 | 48 | 10 | 0-35 | 76 |
| **Afla M1 in milk** | 0.05 | 0-1 | 17 | 0.05 | 0.05-15 | 60 |
| **Afla B1 in feedstuffs** | 5 | 5-50 | 25 | 5 | 5-50 | 39 |
| **Afla B1+B2+G1+G2 in feedstuffs** | 20 | 0-1000 | 17 | 20 | 0-50 | 21 |

These toxins have closely similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds, the molecular formulas and chemical structures are in Figure 1 as established from elemental analysis and mass spectrometric determinations (Martins, 2014).

**B1: $C_{17}H_{12}O_6$ B2: $C_{17}H_{14}O_6$**

**G1: $C_{17}H_{12}O_7$ G2: $C_{17}H_{14}O_7$**

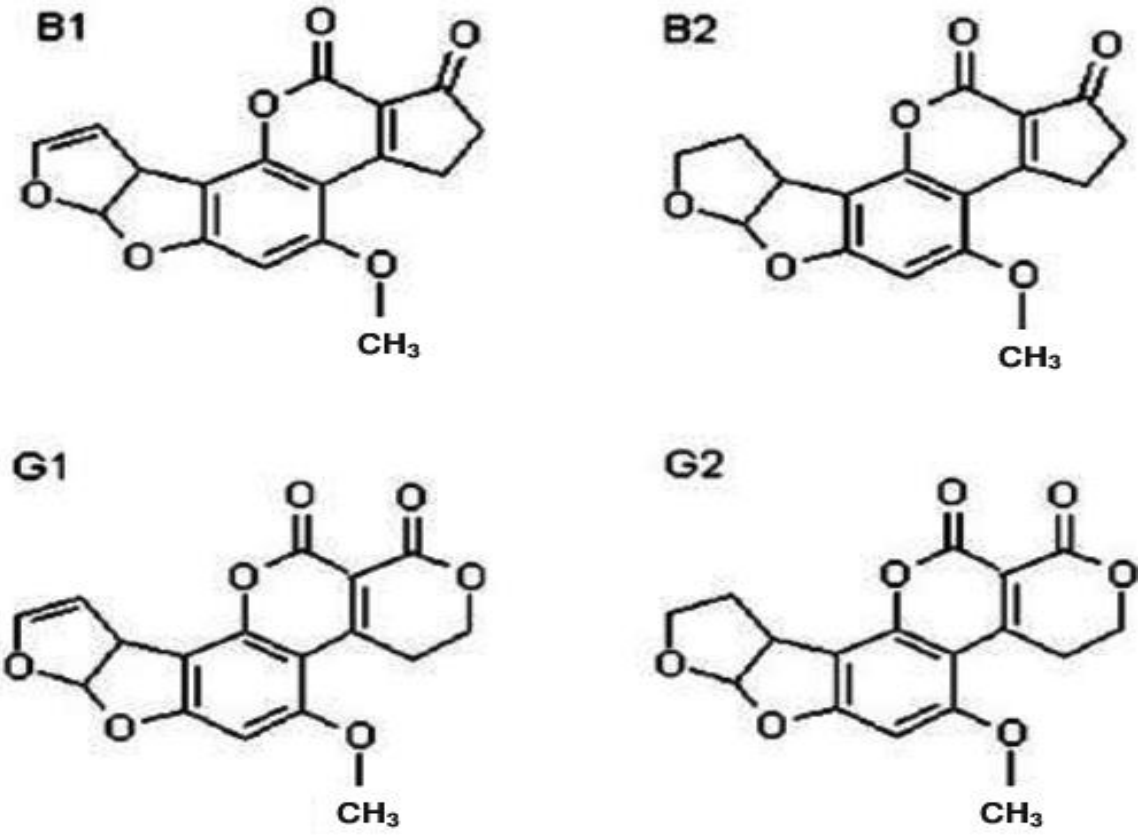


**Figure 3. Chemical structure of aflatoxins B1, B2, G1 and G2.**

Aflatoxin B2 and G2 were classified as dihydro derivatives of B1 and G1, respectively (Patel *et al.*, 2015).

## 2.3.1 Occurrence of aflatoxins in food and animal feed

Aflatoxins can contaminate crops in the field before harvest. After harvest, the toxin will contaminate with crops delayed drying as well as during inadequate storage conditions where moisture and temperature are suitable for mold growth.

In addition, insects or rodent infestations facilitate mold contaminations of stored crops (Hedayati *et al.*, 2007).

Aflatoxins were detected in several varieties of foods and feeds like: corn, cotton seed, cereals nut, rice, figs, wheat, almonds, spices and others (Sarma *et al.*, 2017). It is worrisome in Asia since cereal and nut are the main ingredients in the diet. Animal consumption of animal feed contaminated with aflatoxin may sometimes cause contamination of milk, egg and meat products. However, the commodities most susceptible to be contaminated with aflatoxins are peanuts, corn and cottonseed (Pittet, 1998; Patel *et al.*, 2015).

Corn is the most crop that is susceptible to be contaminated with aflatoxins worldwide, because it is cultivate in climates suitable for aflatoxins formation. Food processing stages usually do not affect the stability of aflatoxins. However, the procedures used in corn processing can diminish the level of aflatoxins (Patel *et al.*, 2015). For example, in processes employed to make tortillas in which alkaline condition or oxidizing steps are used, aflatoxins are reduced (Razzaghi-Abyaneh, 2013). Contamination of cornmeal and cottonseed was found in dairy rations with aflatoxin resulting in contamination of dairy products and milk with aflatoxin M1 (Patel *et al.*, 2015).

### 2.3.2 The favorite factors for aflatoxin production

Interactions between fungus, the host and the environment are considered as the main causes of fungal growth and aflatoxin contamination (Smith & Moss, 1985). When these factors combined in an appropriate way, they will determine the extent of infection with the fungus, the amount and type of aflatoxin formed. However, fungal growth and the toxin produced are affected by the appropriate substrate, although the precursor factors that initiate the formation of toxins are not well known (Patel *et al.,* 2015). There are many factors for mold infection and toxin production in the host plant. The most important are water stress, stress caused by high temperatures, and insect damage in the host plant (Atkin, 1997). Aflatoxin formation is affected by growth associated with mold or other microbes (Smith & Moss, 1985; Patel *et al.,* 2015). Aflatoxins are formed by *A. flavus* in many starchy cereal grains mainly, wheat, com, rice and millet, which have a moisture content of about 18% *i.e.* in equilibrium with relative humidity 85% and at a temperature (12 ° C to 42 ° C) with an optimum temperature at 27 ° C to 30 ° C (Razzaghi-Abyaneh, 2013). The maximum moisture content of aflatoxin production is about 30% (Jacobson *et al,.* 1993). *A. flavus* grows slowly below 12°C and most rapidly at 36°C, but production of aflatoxin will stop at temperature below 12°C or above 42°C (Klich, 2007). Under optimal growth conditions, *A. flavus* can produce few quantities of aflatoxin within 24 hours but more quantities will be formed after few days (Jacobson *et al,.* 1993). Although *A. flavus* contaminate many crops, it does not produce the same quantities of

aflatoxin on all of them. Aflatoxins are also much more likely to be formed in worm to hot humid regions on drought - stressed plants (Jacobson *et al*,. 1993). The optimum temperature for the formation of aflatoxin B1 and G1 on rice was found at 28 ° C. However, the yield of aflatoxin B1 was not lowered at this temperature (Betina, 1984).

### 2.3.3 Effects of aflatoxin on human health.

Aflatoxins enters the human body by ingesting foods contaminated with fungal growth products or by eating animals and their products that have previously been exposed to aflatoxin in feed. (Rustom; 1997; Devero; 1999). Aflatoxin's toxicity to humans can be affected by environmental conditions, exposure level, exposure time period, health, age, gender, and the nutrients in the diet. Ingesting a large amount of toxin in a short time will result in acute intoxication that can cause immediate death. On the other hand, small ingested toxin will lead to a chronic disease, for an extended period of time (Sibadna *et al.,* 1997).

Humans are infected by aflatoxin from eating foods contaminated with aflatoxicosis products. In many countries, acute aflatoxicosis has been reported, mainly developing countries. The increased risk of acute aflatoxicosis in humans is caused by many factors. Those factors include inadequate availability of food, suitable environmental conditions for fungal growth in crops, as well as the absence of regulatory systems that can monitor and control production of aflatoxin. Expression of diseases caused by aflatoxins in persons seems to be

affected by age, gender, nutritional status. In addition, infections like viral hepatitis B (HBV) or parasite infection can lead to expression of diseases caused by aflatoxins (Berek *et al.,* 2001; Benet & Klich., 2003). Benet & Klich, 2003 found that aflatoxin B1 can cause human hepatocellular carcinoma. Consumption of aflatoxins can also cause hepatic failure, encephalopathy and Reye syndrome. Furthermore, exposure to aflatoxins is dangerous to the health and well-being of the fetus and newborn (Hayes., 1980; Benet & Klich., 2003).

## 2.4. *Pichia anomala* and *Pichia kluyveri*

*Pichia anomala* is ascomycetous yeast, which reproduces sexually by forming hat-shaped ascospores and asexually by budding (Kurtzman, 1998). Candida pelliculosa is the asexual state of *P. anomala* (Kurtzman, 1998). *Pichia anomala* can grow under high osmotic pressure, low water activity, and high and low pH it also can grow in the presence or absence of oxygen (Fredlund *et al.,* 2002). Furthermore, high-sugar food products can be spoiled by *P. anomala* (Tokuoka & Ishitani,. 1991) as well as spoilage of silage (when a silo is opened for feed-out, silage is exposed to ambient air, and undesirable microbes including bacteria, yeasts and molds grow by consuming residual water and soluble carbohydrates and organic acids. The importance of this process, referred to as 'aerobic deterioration' or 'spoilage', depends upon its extent) (Jonsson & Pahlow, 1984). *Pichia anomala* was reported to present in foods and clinical isolates (Hazen, 1995). However, *P. anomala* was not particularly tolerant against ethanol and

acetate (Kalathenos *et al.,* 1995; Fredlund, 2002), inspite of its ability to grow over a broad pH range and at high osmotic pressure. This yeast has been reported as spoilage yeast in yoghurt (Kosse *et al.,* 1997) cheese (Westall & Filtenborg, 1998), the animal feed corn silage (Kitamoto *et al.*, 1999). *Pichia anomala* was reported also as wine yeast (Mingorance-Cazorola *et al.*, 2003; Rojas *et al.*, 2003).

*Pichia anomala* has been reported as a biocontrol yeast against a number of filamentous fungi. *Botrytis cinereal,* which cause grey rot disease in grape-vine was inhibited by *P. anomala* (Masih *et al.,* 2000). *P. anomala* was found to have an antagonist effect against *Rhizoctonia solani*, *Fusarium equiseti*, *Botrytis fabae*, *Phytophthora infestans* (Walker *et al.*, 1995), *Penicillium roqueforti*, *Aspergillus candidus* (Björnberg & Schnürer, 1993), *Penicillium verrucosum* (Petersson *et al*., 1998) and *Aspergillus ochraceus* (Masoud *et al.,* 2006).

*Pichia kluyveri* is mainly heterothallic yeast but few strains appear homothallic. This yeast reproduces asexually by budding and sexually by producing two to four hat-shaped spores in each ascus (Kurtzman, 1998). The asexual state *of P. kluyveri* is *Candida eremophila*. *Pichia kluyveri* has been found in vegetables, milk and dairy products (Tudor & Board, 1993), fruits (Spenser *et al*., 1992; Abranches *et al*, 2000), cocoa (Jespersen *et al*., 2005; Nielsen *et al*., 2005) and coffee (Masoud *et al.,* 2006). It was found that these yeast species can produce killer toxins that suppress the growth of other yeasts (Radler *et al*, 1985; Zorg *et al*., 1988; Stamer *et al*., 1992; Abranches *et al*., 2000).

## 2.5 Volatile organic compounds as biocontrol

Hydrocarbons, alcohols, esters, fatty acids, aldehydes, ketones, cyclohexanes, thioesters, phenols, benzene derivatives, heterocyclic compounds and sulfur containing compounds are volatile compounds produced by yeasts (Dufour *et al.,* 2003). Volatile organic compounds are small molecules usually 300 Da in molecular mass with high vapor pressure and low water solubility (Morath *et al.,* 2012). Fredlund *et al* (2004) stated that ethyl acetate, a major ester produced by *P. anomala*, was able to minimize growth of *Penicillium roqueforti*. *Sporidiobolus pararoseus* was reported to inhibit growth of mycelia and germination of spores in *Botrytis cinerea* by its ability to produce 2-ethyl-1-hexanol (Huang *et al.,* 2012). On the other hand, *Candida intermedia* was reported to produce 1, 3, 5, 7-cyclooctatetraene, 3-methyl-1-butanol, 2-nanon, and phenyl ethyl alcohol during its interaction with *Botrytis cinerea* (Huang *et al.,* 2011). Furthermore, *P. anomala*, *P. kluyveri* and *Hanseniaspora uvarum* inhibited growth of *Aspergillus ochraceus* and its ability to produce ochratoxin A during coffee processing by their volatile compounds (Masoud *et al.* 2005). It was found that the main volatiles produced by these yeasts, which inhibit fungal growth and its ability to produce ochratoxin A, were 2-phenyl ethyl acetate and ethyl acetate (Masoud *et al.,* 2005).

## 2.6 Biocontrol growth of *Aspergillus flavus* and production of aflatoxin B1

Many studies have focused on developing aflatoxin control strategies, including genetic engineering for crop resistance, biological control with competitive, nonaflatoxigenic strains of the fungus *A. flavus* (Cotty, 1994), and regulation of aflatoxin biosynthesis by fungicides, pesticides, inhibitory substances originating from plants, and microbial substances (Ono *et al.,* 1997; Hua. *et al.,* 1999). However, the effectiveness of those control strategies was limited. The ability of various microorganisms to control production of aflatoxin have been investigated. Bacteria, yeast, actinomycetes, radial fungi, and algae was reported to reduce the amounts of aflatoxin in food and feed (Sweeney & Dobson 1998). It was reported that *Streptomyces* sp. produced Aflastatin A, inhibited the production of aflatoxin (Ono *et al.*, 1997). The saprophytic yeasts, like *Candida krusei* and *P. anomala* have also been found to inhibit production of aflatoxin (Hua. *et al.,* 1999). However, effective methods have not been developed to prevent aflatoxin contamination. It was found that the volatile substances produced by *P. anomal*a and *P. kluyveri* initiated growth of *Aspergillus ochraceous* and prevent it to produce ochratoxin A (Masoud, 2006).

# 3. Materials and Methods

## 3.1 Isolation of *Aspergillus flavus* from wheat samples

Eleven wheat samples (from feed and food) were collected from villages in Tulkarem governorate, in the period of April to June 2018. One hundreds g of each sample were ground. All samples were kept in plastic bags at room temperature.

Ten g of each sample were mixed with 90 mL of sterile 0.85% NaCl (w / v) in 250 mL flasks and incubated for 1 hour at 25 ° C while stirring at 100 rpm. Ten µl of each suspension were inoculated into potato dextrose agar media (PDA) plates and incubated at 27 ° C, for 5 days. Each sample was prepared in triplicate.

## 3.2 Isolation of the *Aspergillus flavus* strains

After fungal colony formation. Smears of fungal growth were stained with lactophenol cotton blue®. For the taxonomic fungal identification, microscopic portion (vesicle, conidiophore; (conidia format and size), and phialide characteristics) and macroscopic colony surface and reverse characteristics (coloration, texture, and format) were observed.

Taxonomic identification was based on microscopic and macroscopic features proposed by Klich (2002).

## 3.3 Analysis of aflatoxin production by culture techniques

The potential to produce aflatoxin by the isolates, which were identified as *A. flavus* was evaluated through the production of fluorescence under ultraviolet (UV) light on Coconut agar medium (CAM) (Davis *et al.,* 1987). Two handred ml of coconut milk, 600 ml of distilled water, pH 6.9, and 16 g of Agar were mixed for medium preparation (Lin & Dianese, 1976). The spores of *A. flavus* were transferred to plates containing 20 mL of CAM. The inoculated plates were incubated at 27 °C for 7 days, and a UV transilluminator with emission at 365 nm was used to observe the fluorescence. This experiment was done in triplicates. Samples containing fluorescent isolates (were considered AFB production) were kept on potato dextrose agar (PDA) medium.

## 3.4 DNA extraction

The genomic DNA from *A. flavus* was extracted from 5 to 7 days old cultures. Fine spatula was used to scrap out the fungal mass from the culture plate (Aamir *et al.,* 2015). Then, this fungal mass was placed with 500 µl buffer solution (400 mM Tris HCl [pH 8.0], 60mM EDTA [pH 8.0], 1% sodium dodecyl sulfate) in a 1.5 ml Eppendorf tube. Homogenization of fungal mass was done twice in a Pellet Pestle Motor® (Kontes, Sigma-Aldrich, Germany). One hundred and fifty µL of potassium acetate (pH 4.8; which was made of 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water) was added to the

tube, then it was kept at room temperature for 15 min, then for the 1 min tube was vortexed and centrifuged at 10000 X g. The supernatant was placed in another 1.5 mL Eppendorf tube and centrifuged again. This step was repeated one more time. The supernatant was mixed with an equal volume of isopropyl alcohol. The tube was centrifuged at 10000 X g for 3 min, then the supernatant was discarded. The pellet was washed in 300 µL of 70% ethanol. After centrifugation of sample at 10000 X g for 1 min, the supernatant was discarded. Finally, the DNA pellet was air dried and dissolved in 50 µL of TE buffer, 5 µL of the product were analyzed in a 1.7 % agarose gel containing ethidium bromide (0.25 µg/mL) run at 100 V for 45 min to verify DNA existence.

The product was visualized and photographed under UV light (UVITEC serial NO. 12 102304, France). One µL of pelleted DNA was used as a template for PCR reaction mixture (Khare *et al.,* 2018).

## 3.5 Molecular identification of aflatoxin producing *Aspergillus flavus* by polymerase chain reaction (PCR)

The molecular identification of the fungal isolates was performed via the amplification of the aflatoxin regulatory gene (aflR) fragments of aflatoxigenic fungal genomic DNA (Hashim *et al.,* 2013). The sequences of the forward and reverse primers of the aflR gene use were (5'-AACCGCATCCACAATCTCAT-3') & (5'-AGTGCAGTTCGCTCAGAACA-3') (Integrated DNA Technologies, Belgium). The amplification reactions were performed using a Vetri™ 96 well

thermal cycler (Applied Biosystems company, California, USA) in PCR tubes containing 25 µL of the following reaction mixture: 10x red buffer (2.5µL); 50 mM MgCl2 (0.95 µL); 1.5 U of Taq red DNA polymerase (1.25 µL); 10 mM dNTPs (0.5 µL); 100 µM of each primer (0.125 µL); and 50 to 400 ng of the DNA (1 µL) sample, free DNase water (18.55 µL). Samples were placed in the PCR machine where they were heated for 5 min at 94 °C. Then the PCR reaction was performed for 25 cycles of 1 min and 30 seconds at 94 °C, 1 min and 30 seconds at 56 °C, and 2 mins at 72 °C. PCR samples were incubated for 10 min at 72 °C and then were frozen at –20 °C until further use. Ten µL of each of the PCR produces were analyzed in a 1.5 % agarose gel containing ethidium bromide (0.25 µg/mL) run at 100 V for 60 min.

The PCR products were visualized and photographed under UV light (UVITEC serial NO. 12 102304, France). Negative control (no DNA template) was used in to test whether there is contamination of reagents and reaction mixtures with DNA. A 100 bp DNA Marker (100 bp DNA ladder RTU, CAT. DM001- R500, Gene DireX) was used to estimate the size of the DNA bands.

## 3.6 Effect of exposure of *Aspergillus flavus* to volatiles (2-phynel ethyl acetate and ethyl acetate) on fungal growth

Fungal spores were harvested from (PDA plates that were inoculated with *A. flavus* and incubated for 7 days at 30 ◦C.

Suspensions of $10^5$ spores/ml were prepared in 0.85% (w/v) NaCl solution and by counting under light compound microscope using a hemocytometer.

Six different concentrations from 2-phynel ethyl acetate and ethyl acetate were obtained by dilution with distilled water. Concentrations of 2-phynel ethyl acetate and ethyl acetate prepared were C1 (15.4µg/L), C2 (32µg/L), C3 (47.5µg/L), C4 (63µg/L), C5 (78.4µg/L) and C6 (93.9µg/L) these concentrations have been selected based on the experiment, where it was re-experiment several times. Ten ml of each concentration of 2-phynel ethyl acetate and ethyl acetate were placed in petri dishes. The lids were removed and the PDA plate which were inoculated with spores ($10^5$/ml) of *A. flavus* was inverted over other plate that contained 10 ml of one of the volatile compounds. The two plates were combined together with parafilm, after plates were incubated at 30 ◦C for one week (Masoud *et al.*, 2005). Triplicates concentration were done.

# 4. Results

## 4.1 Identification of *Aspergillus flavus* isolates

Two isolates of *A. flavus* were detected from the fungal cultures in wheat samples. They were identified by examining their morphological features as described by Klich (2002).

### 4.1.1. Macroscopic characterization of the isolates on Potato Dextrose Agar (PDA)

After the first 3 days of incubation white color of the mycelium was observed. After 3 days, an olive and dark green conidia were observed (Figure 4). They were plain and flat at the edges but were raised in the center and wrinkled in an almost cerebriform pattern.

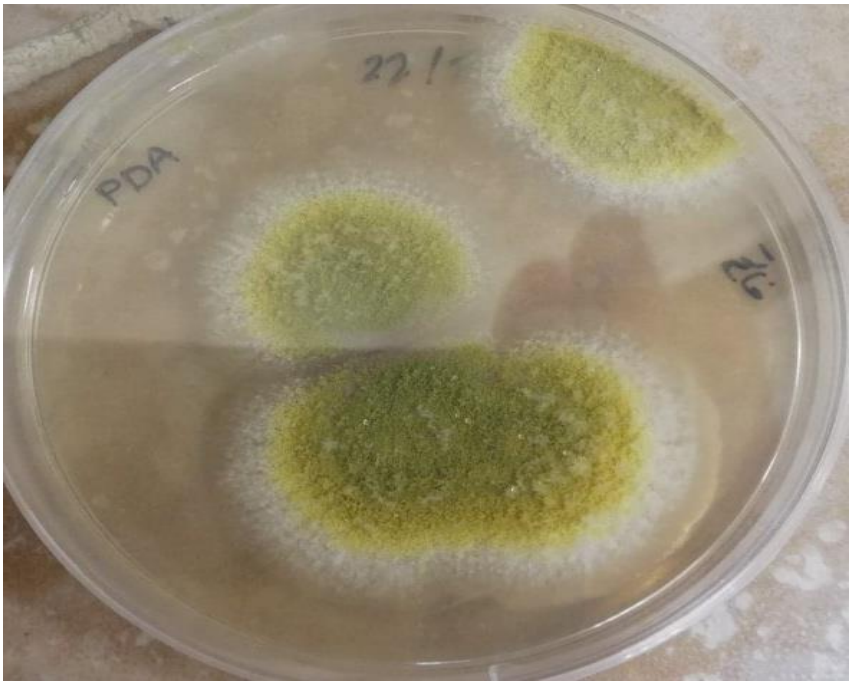


**Figure 4. Colony morphology of *Aspergillus flavus* isolate on Potatoe dextrose agar (PDA)**

All isolates produced secretions (droplets of liquid) that were either uncolored or brown in color. The colonies were surrounded by a white border.

## 4.2 Microscopic characteristics of the *Aspergillus flavus* isolates

To ensure their accurate identification, the microscopic properties (conidiophores, vesicles, conidia) of these isolates were examined under a light microscope. (Figure 5).

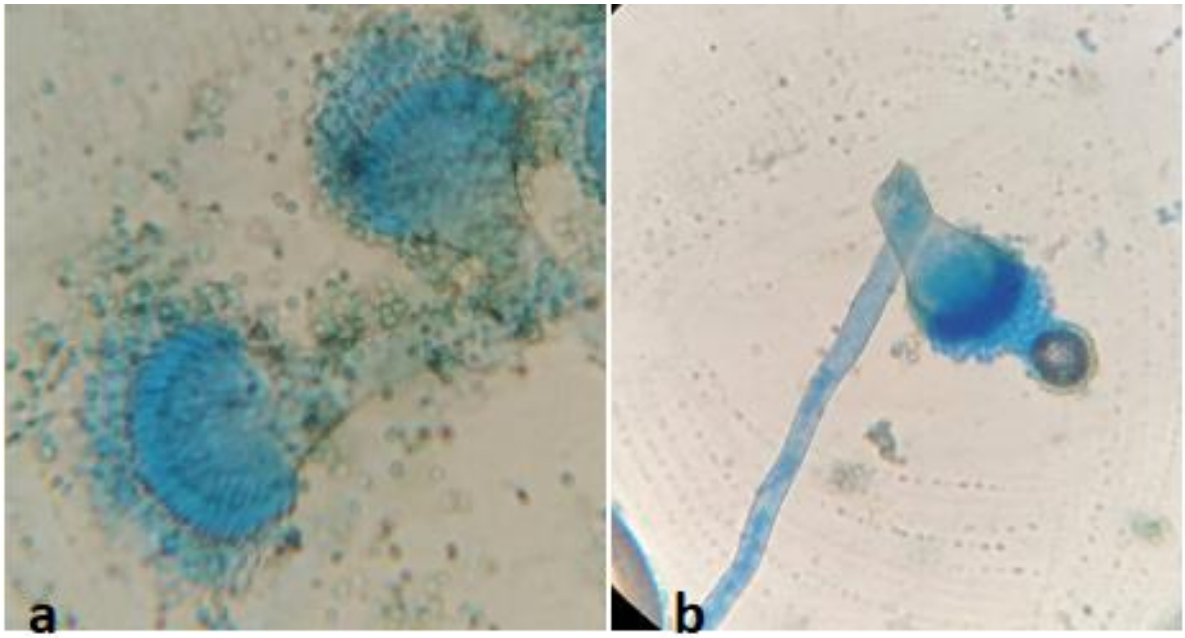


**Figure 5. Microscopic characteristics of *Aspergillus flavus* isolates under the 100 × objective of the light microscope. (a) *A. flavus* isolate under the 100 × objective of the light microscope, and (b) represent *A. flavus* isolate under the 40 × objective of the light microscope**

The conidiophores were non-colored, thick-walled, and rough or chiseled. There were vesicles that were subglobose in some isolates and globose in others. The cells were either uniseriate or biseriate or both. For biseriate cells, the phialides were borne on the metuale and in uniseriate cells. They were attached directly to the vesicles. The metulae covered nearly the entire surface of the vesicles and radiated from the vesicles in all directions. The conidia were spherical in shape with thin walls.

### 4.3 Fluorescence screening of aflatoxins on coconut agar medium CAM

The production of aflatoxins was investigated by observing the blue colour on the white medium around and in the background of the fungal colonies (Lin & Dianese, 1976).

Figure 6 shows the results obtained from the UV fluorescence screening of the *A. flavus*, showing both fluorescent and non-fluorescent isolates on CAM.

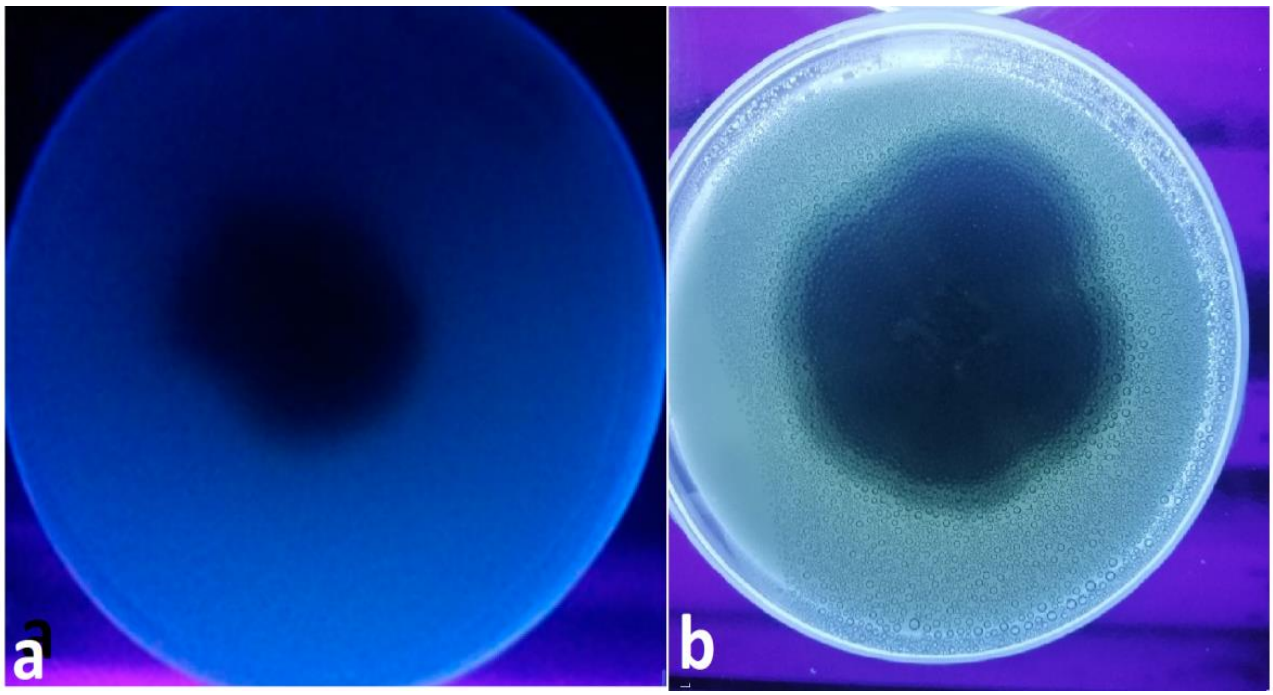


**Figure 6. Colonies of aflatoxigenic (fluorescent) (a) and non-aflatoxigenic (non-fluorescent) (b) strains of *A. flavus* observed under UV light (365 nm) after seven days of growth on Coconut Agar Medium (CAM).**

One of the isolates fluoresced blue on CAM when exposed to UV light, which indicates that it produces aflatoxin. On the other hand, no blue fluorescence was observed in the other isolates, which indicates that it is non-aflatoxigenic.

## 4.4 Molecular analysis

Figure 7. Shows gel electrophoresis of the fungal genomic DNA isolated from *A. flavus*.

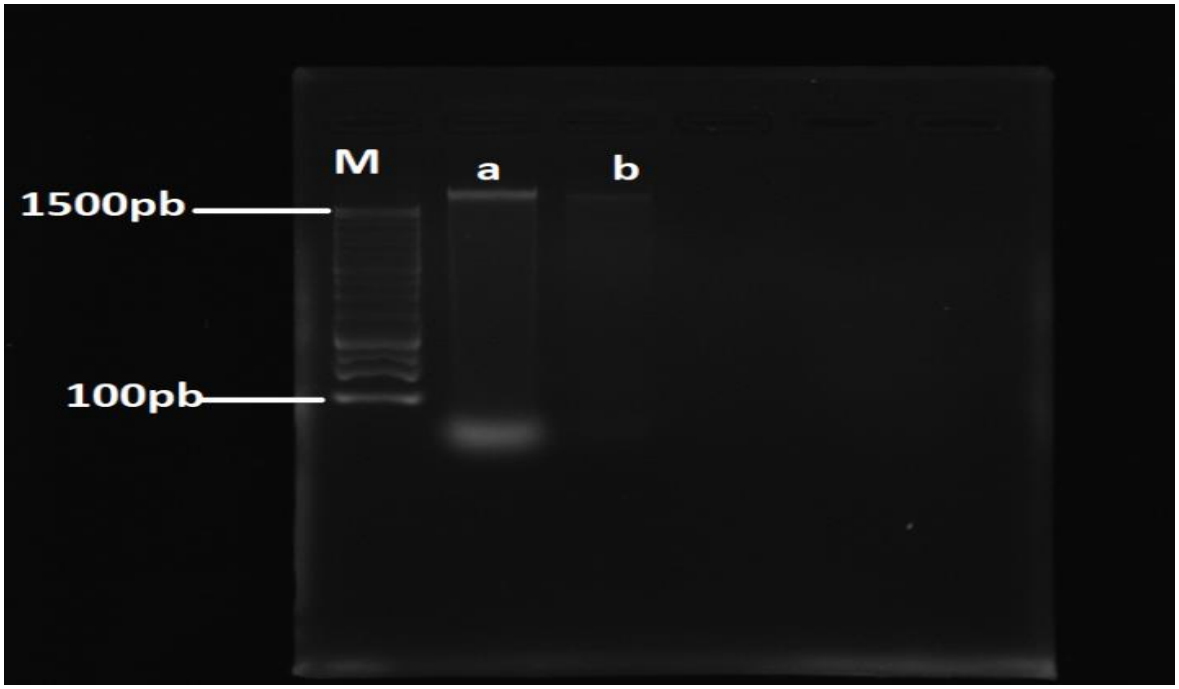


**Figure 7. Gel electrophoresis of the total genomic DNA isolated from *Aspergillus flavus*. M: 100 pb DNA RTU ladder (Cat. DM001-R500) as a molecular size marker. (a): fungal sample, (b): represent a negative control sample.**

The band shown in lane (a) confirms the presence of DNA, which means that it can be used to confirm the presence of the aflatoxin gene by relying on Khare's *et al.,* (2018) research.

Figure 8. Shows Agarose gel analysis of PCR products obtained from *A. flavus.* The primers used amplified the region from 540 to 1338 of Aflatoxin regulatory gene with a 798 base pairs size (Khare *et al.,* 2018).

The band of 798 bp was only observed for one of the two isolates of *A. flavus* (Fig 8). This means that the aflR gene is present in this isolate. This confirms the result obtained from CAM culture media for the two isolates of *A. flavus*.

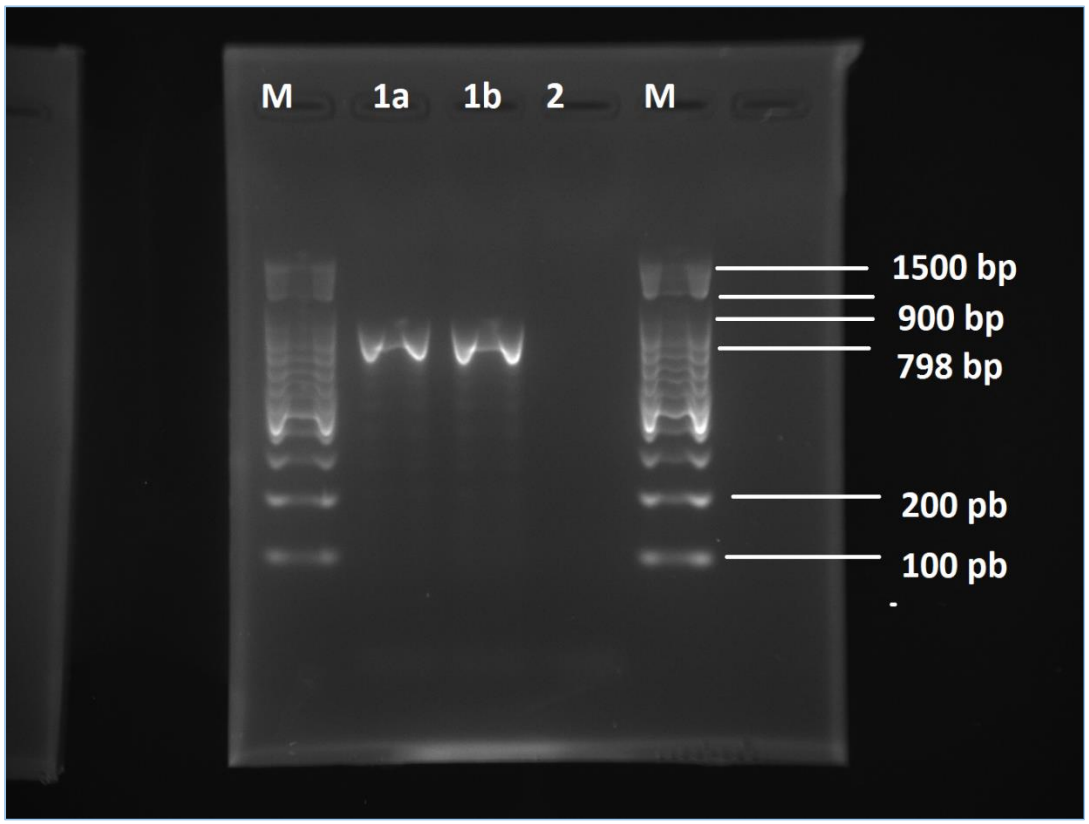


**Figure 8. Agarose gel electrophoresis of PCR products obtained from *Aspergillus flavus*. Lanes: (M) DNA marker (100 pb), (1a) first sample, (1b) replicate and (2) second sample.**

## 4.5 Effect of exposure of *A. flavus* to volatiles (2-phynel ethyl acetate and ethyl acetate).

The effects of six different concentrations C1 (15.4µg/L), C2 (32µg/L), C3 (47.5µg/L), C4 (63µg/L), C5 (78.4µg/L**),** C6 (93.9µg/L) of 2-phynel ethyl acetate on growth of *A. flavus* after 5 days of incubation at 30 $^{o}$C is shown in Table 3, growth of *A. flavus* was inhibited by 2-phynel ethyl acetate at concentrations of 62.9, 78.4 and 93.9µg/L. At a lower concentration of 47.5µg/L, growth of *A. flavus* was reduced. On the other hand, a little effect of 2-phynel ethyl acetate on the number of fungal colonies was observed at concentrations of 15.4 and 32µg/L.

The color of *A. flavus* colonies was observed to change from green to white (Figure 9) when exposed to low concentrations of 2-phynel ethyl acetate as shown in Figure 9.

**Table 3. The effect of exposure of six concentrations of 2-phynel ethyl acetate on growth of *A. flavus*.**

| | **Concentrations of 2-phynel ethyl acetate (µg/L)** | | | | | |
|---|---|---|---|---|---|---|
| | **C1** | **C2** | **C3** | **C4** | **C5** | C6 |
| | **15.4µg/L** | **32µg/L** | **47.5µg/L** | **62.9µg/L** | **78.4µg/L** | **93.9µg/L** |
| **Growth of *A. flavus*** | +++ | +++ | ++ | - | - | - |

**-** T No growth
+ Poor growth 1-5 colonies
++ Medium growth 10-20 colonies
+++ Strong growth 25 -30 colonies
++++ Sronger growth > 50 colonies

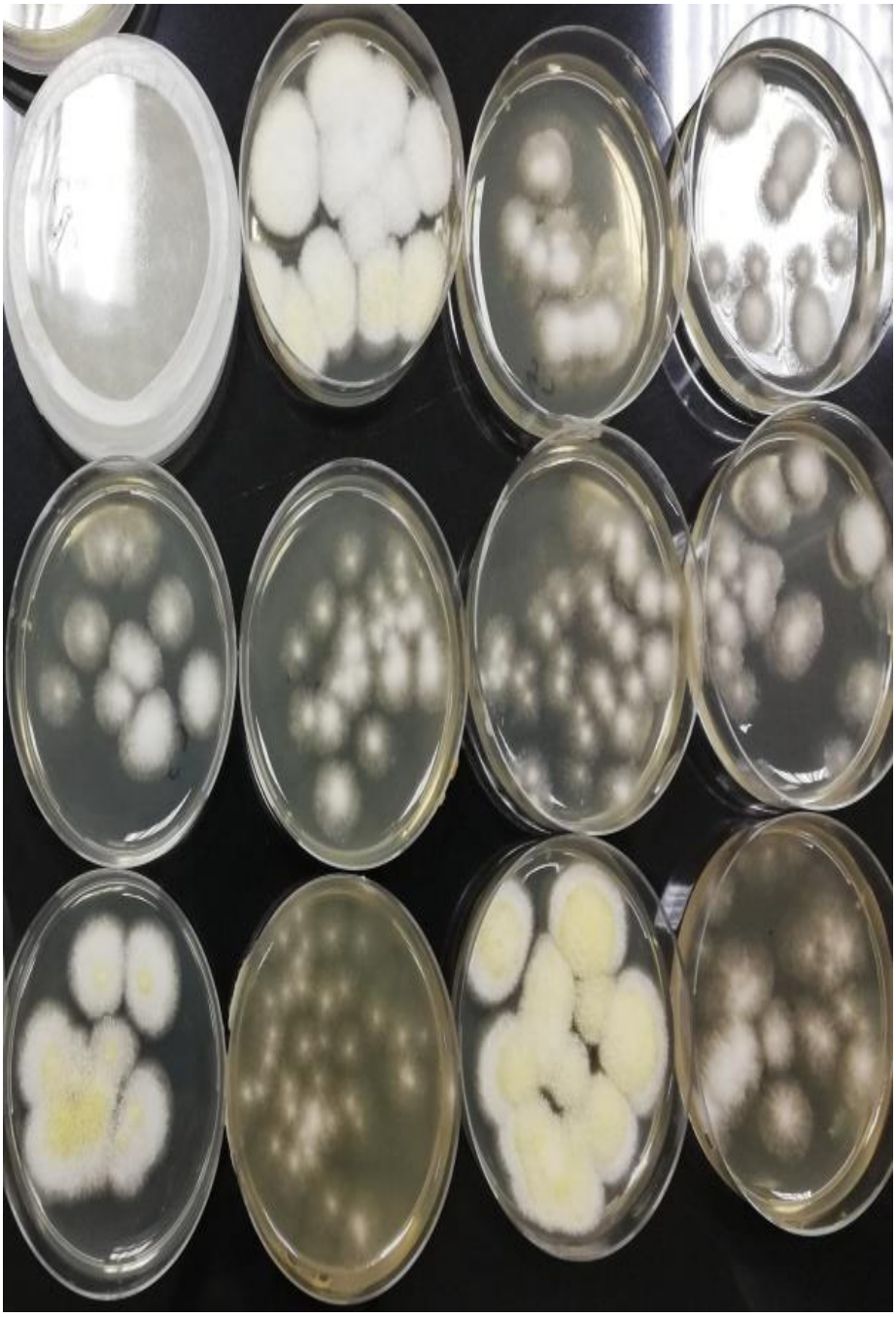

**Figure 9. The effect of 2-phynel ethyl acetate on the colour of *A. flavus*.**

Table 4. shows the effect of ethyl acetate at different concentrations on the growth of *A. flavus* after 5 days of incubation at 30 ºC. As shown in Table 4, ethyl acetate inhibited growth of *A. flavus* only at 93.9 µg/L, the highest concentration investigated. The fungal growth was reduced when exposed to 78.4 and 62.9 µg/L ethyl acetate. The three remaining lower concentrations of ethyl acetate showed no effect on *A. flavus* growth.

**Table 4. The effect of exposure of six concentrations of ethyl acetate on growth of *A. flavus*.**

| | **Concentrations of ethyl acetate (µg/L)** | | | | | |
|---|---|---|---|---|---|---|
| | **15.4µg/L** | **32 µg/L** | **47.5µg/L** | **62.9µg/L** | **78.4µg/L** | **93.9µg/L** |
| **Growth of *A. flavus*** | ++++ | ++++ | ++++ | +++ | ++ | **-** |

**-** T No growth
+ Poor growth 1-5 colonies
++ Medium growth 10-20 colonies
+++ Strong growth 25 -30 colonies
++++ Stronger growth > 50 colonies

# 5. Discussion

Examining morphological traits and microscopic characteristics of fungal growth on PDA medium, is the most widely used method to identify fungi. In the present study, morphological characterization of fungal growth was carried out due to the lack of advanced facilities. With reference to the taxonomic key descriptions, a reliable identification of *A. flavus* was achieved. In general, isolates contained yellowish green, olive, or dark green colonies ringed by a white border, which eventually interfered with conidia. The production of sclerotia was announced to be a rare feature of *A. flavus*, despite it is one of its distinctive features. According to the taxonomic characterization which was written by Klich (2002) and Clayton (1977), the isolates morphological characterization in this study is similar to that of *A. flavus*, as shown in Figure 3.

Some species of *Aspergillus* possess the same characteristics, which makes it difficult to be distinguished from *A. flavus*. *A. oryzae* has the same color of *A. flavus* (Thathana *et al.,* 2017). A thorough examination using macroscopic and microscopic characteristics is required for a correct classification and identification. However, there is an agreement that some features are common between *Aspergillus* isolates even microscopically (Paulussen *et al.,* 2017). In this study, the characteristics of the conidia, conidiophores, and vesicles were evaluated. The uncommon characteristics of *A. flavus* were the globose vesicles with radiate sterigmata and rough conidiophore walls. The thin walls of the

globular conidia, which were light to medium roughness, were another characteristic of the *A. flavus*. Figure 4 showed these macroscopic characteristics, and they were consistent with those of the *A. flavus* characteristics previously described (Rodrigues *et al.,* 2007).

When the fungal colonies were examined under UV light, the aflatoxin-producing fungus showed blue fluorescence, while the non-producing isolates produced no fluorescence (Figure 5). In this study, we were able to distinguish between *A. flavus* isolates that can produce AFB1 and isolates that cannot produce AFB1 on CAM medium. Fluorescence was observed for one isolate of *A. flavus* of the two isolates investigated, indicating its ability to produce aflatoxin B1. Lin & Dianese (1976) showed the importance of coconuts to stimulate aflatoxin production by supporting growth, blue fluorescence, and aflatoxin production when it incorporated into CAM.

To distinguish the *A. flavus* which produced AFB1 from fungus-infected wheat grains, available sequence of PCR primers were used (Hashim & Abdulmalek, 2013). The sequences of the genes responsible for aflatoxin biosynthesis are highly conserved in *A. flavus* and *A. parasiticus* (Yu *et al.,* 2004). According to Khare *et al.,* (2018), the product of PCR in gel electrophoresis when use these primers will be 798 bp in size. In the result of this study, one strain was seen in gel electrophoresis with size was 798 pb. This means that the aflR gene is found in this strain. This confirms the result obtained from CAM culture media.

The volatile compound 2-phenyl ethyl acetate was the most effective compound against fungal growth and aflatoxin formation. Concentrations 93.9, 78.4 and 62.9 μg/L of 2-phenyl ethyl acetate were the most effective concentration since no growth of the fungus appeared, but the other concentrations showed effects but to varying degrees. Furthermore, the color of the fungal colonies was affected by 2-phenyl ethyl acetate, indicating that this volatile effected the characteristics of *A. flavus*. 2-phenylethanol was found to inhibit spore germination, gene expression and AFB1 formation by *A. flavus* (Hua *et al*, 2014). Many studies demonstrated the growth inhibition of fungi such as *A. flavus* volatiles present in plant or produced by microorganisms (Gueldner *et al.,* 1985). Roze *et al*. (2011) showed that, pentanoic acid methyl ester, 3, 5-octadien-2-one, hexanoic acid ethyl ester, and 2-pentenal from willow bark have the ability to prevent production of aflatoxin by *Aspergillus parasiticus*. Inhibition of aflatoxin production is a result of reduced expression of the *ver-1* gene, which is encodes the enzyme involved in the aflatoxin biosynthesis pathway. Volatile compounds of soybean, which include (E)-2-heptenal, (E)-2-hexenal, and aldehydes were reported to eleminate *A. flavus* growth and prevent it from producing aflatoxin (Cleveland *et al*., 2009). The biocontrol yeast, *P. anomala* was reported to produce 2-phenylethanol, which prevented the *A. flavus* to form aflatoxin due to down regulation of the aflatoxin biosynthesis genes expression (Hua *et al.*, 2014).

On the other hand, ethyl acetate was found to reduce fungal growth only at high concentration of 93.9 μg/L. Masoud (2006) demontrated that exposure of *A. ochraceus* to various concentrations of ethyl acetate did not inhibit ochratoxin A (OTA) formation.

# 6. Conclusions and Recommendations

The main conclusions obtained from the present study can be summarizes as follows:

- The coconut medium developed in this study seemed to be a competitive, reliable, good, quick and cheap tool to detect *A. flavus* producing AFB1.

- The volatile compound 2-phynel ethyl acetate was found to have antagonistic effect against growth of *A. flavus* and seemed to have an effect on its ability to produce aflatoxin B1.

Further studies:

- The mechanism of action of 2-phynel ethyl acetate against growth of *A. flavus* needs to be investigated.
- The effect of 2-phynel ethyl acetate on the colour change of *A. flavus* colonies required further investigation.
- The potential use of 2-phynel ethyl acetate as a preservative for wheat against *A. flavus* needs further studies.

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## Abstract (In the Second Language)

## التأثير المضاد لمركبات 2-فينيل إيثيل أسيتات والإيثيل أسيتات ضد نمو فطر الأسبرجيلوس فلافس في القمح

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## الملخص

يعتبر القمح محصول مهم ومن المنتجات الاستراتيجية في جميع أنحاء العالم ويستخدم أيضًا كمكون أساسي في العديد من المنتجات الغنية، والأعلاف المركبة، وإنتاج النشا، وكمخزون تغذية في إنتاج الإيثانول.

الاسبيرجلس فلافوس عبارة عن فطر ممرض منتشر عالميا وبشكل كبير، في بعض الظروف المعينة تكون له القدرة على انتاج أقوى مركب طبيعي سام ومسرطن معروف باسم الأفلاتوكسين بي 1 هذا السم له القدرة على تلويث العديد من المحاصيل مثل: الذرة والقطن والفول السوداني والقمح. يعتبر الأفلاتوكسين بي 1 من أكثر المسببات لسرطان الكبد من بين جميع السموم.

كان الهدف الرئيسي من هذه الدراسة هو معرفة النشاط المضاد لمركبات 2-فينيل ايثيل أسيتات وإيثيل أسيتات ضد نمو فطر الأسبرجيلوس فلافس. أحد عشرة عينة من القمح الجاف تم جمعها من مدينة طولكرم في فلسطين وتم فحصها لمعرفة إذا ما وجد فيها الأسبرجيلوس فلافس ام لا. تم العثور على عزلتين من الاسبرجيلوس فلافوس في عينات القمح تم التأكد أن واحدة منهما تنتج الافلاتوكسن بي 1والاخرى لم تنتجه. تم فحص تراكيز مختلفة من2-فينيل ايثيل أسيتات وإيثيل أسيتات ضد نمو الأسبرجيلوس فلافس. وجد أن 2-فينيل ايثيل أسيتات كانت فعالة ضد نمو الأسبرجيلوس فلافس. تم تثبيط نمو الأسبرجيلوس فلافس بواسطة 2-فينيل ايثيل أسيتات -بتراكيز 62.9 و78 و93 ميكروغرام / لتر. عند تركيز أقل من 2-فينيل إيثيل أسيتات، تم تقليل نمو الأسبرجيلوس فلافس وتغير لون المستعمرات الفطرية من الأخضر إلى الأبيض. وجد أن الإيثيل أسيتات لها تأثير ضعيف جدًا على نمو الفطريات. أوضحت نتائج الأطروحة الحالية إمكانية استخدام 2-فينيل ايثيل أسيتات كمادة حافظة في القمح ضد نمو الفطريات وإنتاج الأفلاتوكسين بي 1 وكذلك امكانية استخدام تقنية (وسط جوز الهند) كتقنية متوفرة ورخيصة وتعطي نتائج اولية جيدة للكشف عن وجود الافلاتوكسين بي 1.