

## ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS*, *L. PLANETARIUM*, *STREPTOMYCES* AND *SACCHAROMYCES CEREVISIAE* WITH EXTRACTS OF DATE PALM AND DRIED SHELL OF POMEGRANATE TO REDUCE AFLATOXIN M1 IN IRAQ

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### ABSTRACT

This study aimed to evaluate the efficiency of the bioproduct of the bacteria *Lactobacillus acidophilus*, *L. Planetarium*, *Streptomyces* and *Saccharomyces cerevisiae* with extracts of date palm and dried shell of pomegranate to reduce aflatoxin M1 produced by *Aspergillus flavus*. The antifungal activities of microbes and extracts were evaluated using the disk diffusion and agar well diffusion methods, the inhibitory zones were recorded in millimetres. The results showed that the population of fungi was higher in milk spoilt samples, three isolates diagnosed as *Aspergillus flavus* A1, A2 and A3. The results of this study showed that all treatment used against *Aspergillus flavus* A2 were showed antifungal, inhibition zone reached of treatment 5 -19.3 mm. The results of this study were encouraging, despite the need for clinical studies to determine of the real effectiveness and potential toxic effects *in vivo*. These results were revealed the importance of some microbial and plant extracts in control of aflatoxin M1. Using thin layer chromatography (TLC) method, show results generally reveal that all starters were characterized by their ability to gradually degrade AFM1.

**KEYWORDS:** *Lactobacillus*, *Streptomyces*, *Saccharomyces cerevisiae*, aflatoxin M1.

### INTRODUCTION

Mycotoxins are toxic fungal secondary metabolites produced by fungi (molds). Mycotoxins are known to grow on nuts, grains, corn, fruits and milk. (Robens and Cardwell, 2003; Risan, 2016a). They are also known to be the most toxic / carcinogenic compounds of all the mycotoxins (Durakovic *et al.*, 2012; Risan, 2016 b). Aflatoxins are a potent carcinogen and can contaminate a wide range of agricultural products regularly consumed by humans and ingestion of aflatoxin-contaminated foods increases the risk of developing hepatocellular carcinoma (Yu and Yuan 2004; Varga *et al.*, 2011). Aflatoxins are produced by toxigenic strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and are found in feed as aflatoxin B1, B2, G1, and G2 and found in milk as aflatoxin metabolite M1 and M2. (Talebi *et al.*, 2011; Arab *et al.*, 2012 ). Probably most concerning for humans are its indirect effect on children through milk, as children are more vulnerable to toxins and are known to ingest more milk when compared to adults. (Unnevehr and Grace 2013). Based on chromatography and fluorescence characteristics, all aflatoxins known to date can be classified into 18 different types. The major

ones are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), as well as M1 (AFM1) and M2 (AFM2) (Lerda, 2010). AFM1 and AFM2 are hydroxylated forms of AFB1 and AFB2 (Dors, 2011). When AFB1 in contaminated feed or foodstuffs is ingested by domestic animals, such as dairy cows, the toxin undergoes liver biotransformation and is converted into aflatoxin M1 (AFM1), becoming the hydroxylated form of AFB1. AFM1 is excreted in milk, tissues and biological fluids of these animals (Oatley *et al.*, 2000; Peltonen *et al.*, 2001; Murphy *et al.*, 2006) and in this form can be taken up by consumers. A linear relationship between the concentration of AFM1 in milk and the concentration of AFB1 in contaminated feeds consumed by the animals has been reported. It was found that about 0.3% to 6.2% of AFB1 ingested with feed is transformed into AFM1 in milk (Bakirci, 2001; Creppy, 2002). Currently the limits of AFM1 in milk are highly variable, depending on the degree of development and economic status of the countries. European Communities and Codex Alimentarius have fixed the limit to a maximum of 0.05ppb (Mohammadi, 2011). Aflatoxin M1 also exhibits a high level of genotoxic activity and certainly represents

a health risk because of its possible accumulation and linkage to DNA (Makun *et al.*, 2012; Shundo and Sabino, 2006). Aflatoxin M1 is categorized as a group 2B carcinogen (probable human carcinogen) (Darsanaki and Miri 2013). Pasteurization, a heating process that milk undergoes to kill bacteria, and sterilization have little effect on removing aflatoxin from milk (Flores-Flores *et al.*, 2015). Biocontrol to counteract aflatoxin contamination during storage has been tested with some success with probiotic yeast and bacterial strains. *Saccharomyces cerevisiae* resulted to be one of the most effective microorganisms for binding AFB1 (Shetty and Jespersen, 2006). Probiotics, such as Lacto Acid Bacteria and *Saccharomyces* sp., have been frequently employed as binding agents, due to their Generally Recognized as Safe (GRAS) status, high binding abilities, and wide distribution in nature. Commercial *Lactobacillus* and *Streptococcus* strains have been shown to reduce to varying degrees AFM1 concentration in milk and yoghurt ((Sarimehmetoglu and Kuplulu, 2004; Ayoub *et al.*, 2011; El Khoury *et al.*, 2011). At least 7000 different secondary metabolites have been discovered in *Streptomyces* isolates (Berdy, 2005; Amin *et al.*, 2016; Qasim and Risan 2017). These are small molecules, usually between 100 – 3000 Daltons, that are biologically active outside the producer cell, many being antibiotics that inhibit enzymes and cellular processes (Chater *et al.*, 2010). Therefore, this study aimed to use bioproduct of the bacteria *Lactobacillus acidophilus*, *L. plantarum*, *Streptomyces* and *Saccharomyces cerevisiae* with extracts of dates palm and dried shell of pomegranate to reduce aflatoxin M1 produced by *A. flavus*.

## MATERIALS AND METHODS

### Sample preparation

A total of six samples from three types of imported milk powder, were collected from Baghdad city markets. The samples were transported to Mycology laboratory at College of Biotechnology, Al-Nahrain University. Twenty grams of milk powder were taken in a flask and filled up to 200ml with distilled water (dissolved by stirring for 5 minutes). Milk samples were centrifuged for 10 minutes / 3500g/ 10 °C. The upper creamy layer was removed and the lower phase was used for the tests.

### AFM1 standard solution

Stock solution for AFM1 was obtained from Immunolab GmbH (Kassel, Germany). Ten milliliter of chloroform was added to 10 µg AFM1 standard bottle that was obtained from Immunolab GmbH. Concentration in the bottle became 1 µg/ml in chloroform. The solution bottle was sealed and wrapped with aluminium foil, and stored in a cool (4 °C) dry place.

### Preparation of Medium of fungi

Solid medium potato dextrose agar (PDA) was used for the isolation. The medium was used in accordance with the manufacturers' instructions, 39g/1000ml, with 250 mg of the antibiotic Chloramphenicol per 1000 ml.

### Isolation and identification of fungus *A. flavus*

Five fold serial dilutions of each sample were prepared. Serial dilution was carried out, where 1m (**from sample preparation**) of each sample was transferred into a test tube containing 9 ml of sterile distilled water and the test tube was shaken and labelled as  $10^{-1}$ , from this tube 1 ml was also transferred into another tube containing 9 ml of the sterile distilled water and labeled as  $10^{-2}$ . The procedure was repeated up to  $10^{-5}$ . The test tube  $10^{-3}$  was used. One ml from the dilution factors of each  $10^{-3}$  test tube was transferred into sterile petri-dishes, containing prepared potato dextrose agar (PDA) added 250 mg of the antibiotic Chloramphenicol per 1000 ml. The diluted samples were used to inoculate the prepared medium using pour plate method. The agar plates were allowed to solidify and placed in an inverted position at  $25 \pm 2^\circ \text{C}$  for 5 days. After incubation, colonies of different shape and colours were observed on the plates. Pure culture of each colony type on each plate was obtained. This was done by sub-culturing each of the different colonies onto PDA plates and incubated at  $25 \pm 2^\circ \text{C}$  for 5 days (Jiha, 1995). The identification of fungi was based on macroscopic and microscopic examination. Macroscopic examination was based on color and nature of the hyphae. In microscopic examination, In microscopic examination, the technique was adopted for identification of unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide, where a small portion of the mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with  $\times 10$  and  $\times 40$  objective lenses. The species encountered was identified in accordance (Rocha *et al.*, 2012).

### Bacteria *Lactobacillus plantarum*, *Lactobacillus acidophilus*

Use bacteria *Lactobacillus plantarum* and *Lactobacillus acidophilus* were obtained from the Biotechnology Research Center, Al-Nahrain University, Baghdad-Iraq. Bacteria were grown routinely on MRS agar medium. Suspend 67 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure ( $121^\circ \text{C}$ ) for 15 minutes. Bacteria incubated at  $30^\circ \text{C}$  for 48 h.

### Preparation of *Saccharomyces cerevisiae* strain extract

The strain of *Saccharomyces cerevisiae* used in this study include strain from Hangzhou Bioactive Yeast Company, Ltd, Istanbul, Turkey, which was obtained from local markets of Baghdad city in Iraq. It was grow on yeast extract peptone dextrose medium (YPDA) agar composed of 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract and 20 g/L agar and kept at  $4^\circ \text{C}$  before use.

### Bacteria *Streptomyces* spp

Pure culture of *Streptomyces* sp was obtained from Mycology laboratory at College of Biotechnology, Al-Nahrain University. (Risan, 2017). *Streptomyces* strain was cultured on 90-mm diameter Petri dishes containing International *Streptomyces* project Medium slants (ISP-2) (4g Yeast extract, 10g Malt extract, 4g Dextrose, 20 agar and 1 L water) supplemented with tetracycline 50 µg/ml and 50 µg/ml cycloheximide, then incubated at 28°C for 7 days, Purified *Streptomyces* isolate was stored at 4 °C. Until microbial assays were performed on them (Qasim and Risan, 2017; Risan *et al.*, 2017).

#### **Preparation of Pomegranate peel extract**

Pomegranate fruit were purchased from local markets in Baghdad city. Collected peels and arils were then rinsed with tap water. Ethanol extraction of Pomegranate Peels was carried by removing Pomegranate arils and the fruit peels separately. The peels were cut into 0.5–0.75 cm<sup>2</sup>. The peels were air-dried in a low light at room temperature for 1 week. The material was thereafter ground in an electric blender to produce a powder separately. Forty grams of blended peels were placed in 250 ml flasks, followed by adding 100 ml of solvents having an increasing polarity: acetone, 95% ethanol. The flasks were then shaken at room temperature for 18 h prior to filtration. The filtrates were concentrated under reduced pressure with a rotary evaporator at 40 °C. These crude extracts were kept at 4 °C until use.

#### **Preparation of date extract**

Fresh fruit samples consisted of Ajwa varietie of date palm (*Phoenix dactylifera* L.), collected during the 2016. Immediately after harvesting, date fruits were selected (for colour and size), were obtained and surfaced-sterilized with 1% sodium hypochlorite for 30 seconds and rinsed in three (3) changes of sterile distilled water according to the method of Chukwuka *et al.*, (2010).

#### **Preparation of date extract for antifungal activity**

Ajwa date, *Phoenix dactylifera* L, was used in its ripe stage. 25, 50 and 100 g date was suspended in 500 ml sterile distilled water for 24h, and then homogenized in a Waring blender at a maximum speed. The homogenized extract was filtered through a double layer of cheesecloth. All media were sterilized at 121°C for 10 min. Freshly prepared extract was used throughout. (Belmir *et al.*, 2016).

#### **Efficiency bacteria *Lactobacillus plantarum* and *Lactobacillus acidophilus* in growth inhibition of *A. flavus* fungus in culture medium**

Disc diffusion method described by Cassandra *et al.*, (2004) was used to determine the antifungal activity of two Lactic acid bacteria (LAB) *in vitro*. Three sterile Whatmann No. 1 filter disks placed on a Potato Dextrose Agar (PDA), plates were inoculated by disc diameter 0.5 cm of *A. flavus* at a rate of one disc in the center of each dish. A potential antifungal substance (10µl) is then applied on these filter disks. Plates were incubated

aerobically at 25°C and examined for inhibition zones around the filter disks during 7 days.

#### **Efficiency of *Saccharomyces cerevisiae* extract in growth inhibition of *A. flavus* A2 fungus in culture medium**

Petri dishes contained 20 ml of PDA have been used for well-diffusion assay. Wells have been prepared in the PDA plates. In agar well diffusion 10 µl of concentrations 1 g/L, 1.5 g/L and 2 g /L of *Saccharomyces cerevisiae* extract plates were inoculated by disc diameter 0.5 cm of *A. flavus* at a rate of one disc in the center of each dish. Diameters (in mm) of growth inhibition zones were measured after incubation at 25°C for 5 days.

#### **Efficiency bacteria *Streptomyces* spp in growth inhibition of *A. flavus* A2 fungus in culture medium**

Antimicrobial activity of *Streptomyces* isolate was determined to carry out by Agar- Well Diffusion method (Murray *et al.*, 1995). Screening for the antagonistic activity of *Streptomyces* sp. against *A. flavus* A2. The strain of *Streptomyces* sp. was screened for their *in vitro* antagonism against *A. flavus* A2, Briefly, 20, 30 and 40 ul of the cell free supernatant was applied of *Streptomyces* strain on potato dextrose agar (PDA) plate. (Yuan and Crawford, 1995). Diameters (in mm) of growth inhibition zones were measured after incubation at 28°C for 5 days. (Risan, 2017).

#### **Efficiency of Pomegranate peel extract in growth inhibition of *A. flavus* fungus in culture medium**

Petri dishes contained 20 ml of PDA have been used for well-diffusion assay. Wells have been prepared in the PDA plates. In agar well diffusion 15 µl of concentrations 150, 200 and 400 µl of Pomegranate peel extract plates were inoculated by disc diameter 0.5 cm of *A. flavus* at a rate of one disc in the center of each dish. Diameters (in mm) of growth inhibition zones were measured after incubation at 25°C for 5 days.

#### **Efficiency of date extract in growth inhibition of *A. flavus* A2 fungus in culture medium**

Petri dishes contained 20 ml of PDA have been used for well-diffusion assay. Wells have been prepared in the PDA plates. In agar well diffusion 15 µl of concentrations 100, 150 and 200 µl of date extract plates were inoculated by disc diameter 0.5 cm of *A. flavus* at a rate of one disc in the center of each dish. Diameters (in mm) of growth inhibition zones were measured after incubation at 25°C for 5 days.

#### **Determining fungi producing of aflatoxin**

Use *Aspergillus Flavus Parasiticus* Agar medium (AFPA) (consist of agar (20 g), Sucrose (30 g), potassium monohydrogen phosphate (10 g), magnesium sulfate water (0.5 g), iron sulfate water (0.01 g), mercuric chloride (0.0005 mg), Corn steep liquor (0.5 g), distilled water (1000 ml). This medium is Considered from



differential media for the detection of isolates producing to aflatoxin from *A. flavus* (Pitt and Hocking, 2009).

Cultured Isolates on AFPA medium to test their ability to production aflatoxin and characterized isolates that aflatoxin ability to produce Aspergillic acid, which reacts with ferric ammonium citrate and gives bright orange - yellow colour in the background medium of developing colony within 48 hours, and at (28 C<sup>0</sup>) as a characterized sign, while isolates non - producing to aflatoxin does not have the ability to produce Aspergillic acid.

### The detection about capability of *A.flavus* on producing aflatoxin M<sub>1</sub> by using Thin layer chromatography

#### Extraction of aflatoxin M<sub>1</sub>

Followed the method to (Aryantha and Lunggani, 2007), a quantity of 5g of milk was taken and put in a blender (each treatment as in paragraph (Above) with the amount 25 ml of chloroform then mixed for three minutes and filtrated by filter paper and the filtrate was concentrated in an oven at 60 °C until drying.

#### Diagnose aflatoxin M<sub>1</sub>

To detect for aflatoxin used technique (Thin layer chromatography) (TLC) Type (Glass sheets silica gel) measuring 20 × 20 cm equipped Company (SIGMA Chemical Co.) and use the aflatoxin standard M<sub>1</sub> equipped company (Promega - USA), According to the method used by (Bokhari, 2002; Stroka *et al.*, 1999), platelets activated for one hour before the use and use a separate system and component of chloroform and acetone (97: 3), attended the standard M<sub>1</sub> dissolving 1 mg of it in 1 ml of chloroform. Put the standard toxin M<sub>1</sub>, on format spots on a plate of silica gel a distance 1.5 cm from the bottom edge by lattice tube at a rate of (10) microliters, then put spots fungus sample beside standard toxin with left distance (1.5 cm) between spots, then placed in a tank, lifted the plate and left to dry and check under ultraviolet (360 nm) to observe the starred with compared spots starred resulting from fungus extract and color of standard toxin (Shotwell *et al.*, 1981; Risan and Muhsin 2015).

## RESULTS AND DISCUSSION

### Isolation of *Aspergillus flavus*

The species of fungi isolated and identified were ten isolates belonging to the genus *Aspergillus* spp and *Fusarium* species. Three isolates diagnosed as *A. flavus* (A1, A2 and A3). Table (1). The results showed that the population of fungi were higher in milk spoilt samples

**Table 1: Isolated species of the fungus which isolated from milk samples.**

	Isolate	Fungal species
1	A1	<i>Aspergillus flavus</i>
2	A2	<i>Aspergillus flavus</i>
3	A3	<i>Aspergillus flavus</i>
4	A4	<i>Aspergillus niger</i>

5	A5	<i>Aspergillus niger</i>
6	A6	<i>Fusarium</i> sp
7	A7	<i>Fusarium</i> sp
8	A8	<i>Fusarium</i> sp
9	A9	<i>Aspergillus</i> sp
10	A10	<i>Aspergillus</i> sp

Nura *et al.*, (2016) found *Aspergillus flavus* in five samples of Tigernut milk drink. *Aspergillus flavus* strain produces two most common aflatoxins (B1 and B2) (Amaike and Nancy 2011). *A. flavus* has a minimum growth temperature of 12 °C and a maximum growth temperature of 48C, the optimum growth temperature is 37 °C (Hedayati and Pasqualotto 2007). Nazir *et al.*, (2014) found that primarily identified as positive for *A. flavus* based on colony pigmentation and morphology of the conidial head. After a 7 day culture, colonies on PDA at 30°C were olive to lime green with a cream reverse.

The high prevalence of *A. flavus* is largely depends on long time storing in poor condition and unhygienic preparation, and its high adaptability to growth substrates in a wide range of environment and the production of spores (conidia) that remain viable even under extremely harsh conditions (Saleemullah *et al.*, 2006). Most prevalent fungi in pre- and post-storage were *Aspergillus* (mostly *A. flavus*), *Fusarium* and *Penicillium*. Our study showed that the rate of contamination with AFM1 in milk powder was 82.8% which is similar to other reults (Shipra *et al.*, 2004) which recorded that the rate of contamination with AFM1 in India was 87.3%. In Korea, 85% infant formula samples were found to be contaminated with AFM1 (Kim *et al.*, 2000). The reason for high contamination of milk powder samples, is probably the lack of information on the quality of the fodder given to lactating farm animals. The fodder may be contaminated with AFB1, due to the unpredictable climatic and environmental conditions. Earlier studies have shown that contamination of AFM1 in milk and dairy products is a result of exposure of AFB1 to dairy cattle through feedstuffs (Applebaum *et al.*, 1982).

### Morphological characterization of *A. flavus*

Colonies were identified by morphological characteristics, according to the qualities adopted in (Pitt and Hocking, 2009), colony characterized of *A. flavus* were Yellow - Green Color on PDA. Rocha *et al.*, 2012, show they characterized by the microscopic and morphological features as *A. flavus* with addition of Lactophenol cotton blue, presence of septate hyphae, colorless conidiophores, and conidiophores ends vesicle appeared in spherical shape with a series of one or two of sterigmata and conidia measured 3-6 µm and walls were rough (Gao *et al.*, 2007). *Aspergillus flavus* is distinguished by their bright yellow green (or less commonly yellow) conidial colour and rapid growth at both 25 and 37 °C. *A. flavus* produces conidia which are rather variable in shape and size, with relatively thin, smooth to moderately rough, walls, with most being finely roughened, vesicles of *A. flavus* are larger, up to

50 µm in diameter, and usually bear metulae. (Pitt and Hocking, 2009). An isolate of the fungus *A. flavus* A2 was chosen to be used in subsequent experiments.

#### Efficiency bacteria *Lactobacillus plantarum* and *Lactobacillus acidophilus* in growth inhibition of *A. flavus* fungus in culture medium

The antifungal activity of *Lactobacillus plantarum* showed in table (2). The Inhibition zone (mm) was observed against *A. flavus* A2. The original cell free supernatant (100%) of *L. plantarum* showed the highest Inhibition zone reached 16.6mm. *Lactobacillus plantarum* is highly effective in inhibiting the fungus *A. flavus* A2 in the culture medium compared with the control. While antifungal activity of *L. acidophilus* reached 19.1 mm.

**Table 2: Evaluation of of bacteria *Lactobacillus plantarum* and *L. acidophilus* activity against *A. flavus* A2.**

Treatments	Inhibition zone (mm)
<i>Lactobacillus plantarum</i> + <i>A. flavus</i> A2	16.6
<i>Lactobacillus acidophilus</i> + <i>A. flavus</i> A2	19.1
<i>A. flavus</i> A2	0.0

Aflatoxins are a potent carcinogen and can contaminate a wide range of agricultural products regularly consumed by humans and ingestion of aflatoxin-contaminated foods increases the risk of developing hepatocellular carcinoma (Yu and Yuan 2004; Varga *et al.*, 2011). These results are similar to those reported by Batish *et al.*, (1990) who reported that *L. acidophilus* supernatant due to reduction the activity against tested fungi. The inhibition action of lactic acid strains may be due to reduced permitting sporulation (Onilude *et al.*, 2005). The antifungal activity of lactic acid may be due its ability to produce fungistatic bacteriocin-like substance, benzoic acid, methylhydantoin and mevalonolactone (Corsetti *et al.*, 1998 and Lavermicocca *et al.* 2003). Reduction of aflatoxins production by *A. flavus* by *L. acidophilus* may be due to the antifungal activity of strains against aflatoxins producing fungi. *L. acidophilus* inhibited the fungal growth and mycelial development as mentioned by Onilude *et al.*, (2005). Vanne *et al.*, (2000) showed that the growth of toxigenic storage fungi could be restricted by LAB *in vitro*, it could be safely concluded that the action of the lactic acid bacteria supernatant used in this work is being active against both of *A. flavus* and *A. parasiticus*. Sankar *et al.*, (2012) Isolated bacteriocin producing *Lactobacillus plantarum* strain from cow milk samples and it showed broad range of antibacterial activity against food borne pathogens. *L. fermentum* gave the strongest degradation of Aflatoxin BI followed by *L. delbruekii* and *L. plantarum* (Arina, 2002). Concerning the effect of lactic acid bacteria on reducing the concentration of aflatoxin in yoghurt, the obtained results

came in agreement with Mohamed (1998), who measured a reduction of aflatoxin M1 in yoghurt made by *L. acidophilus* and *Bifidobacterium bifidum* of 95.3 and 84.7% for AFM1 and B1, resp., after 5 days.

#### Efficiency of *Saccharomyces cerevisiae* extract in growth inhibition of *A. flavus* A 2 fungus in culture medium

All extracts from *Saccharomyces cerevisiae* inhibitory activity against *A. flavus* A2 (Table 3). Inhibition zone reached 12.0- 19.3 mm by used for *Saccharomyces cerevisiae* extract compared with the control.

**Table 3: Antifungal activity of *Saccharomyces cerevisiae* extract against *A. flavus* A2 fungus in culture medium.**

Treatments	Inhibition zone diameter (mm)	
<i>Saccharomyces cerevisiae</i> extract + <i>A. flavus</i> A2	1.0 g/L	12.0
	1.5 g/L	14.2
	2.0 g/L	19.3
<i>A. flavus</i> A2 (control)	0.0	

*Saccharomyces cerevisiae* resulted to be one of the most effective microorganisms for binding AFB1 (Shetty and Jespersen, 2006). Yeasts such as *Saccharomyces cerevisiae*, *Aureobasidium pullulans*, *Debaryomyces hansenii*, *Kluyveromyces* spp., *Pichia anomala* and *Pichia Guilliermondii* have been tested for their ability to suppress mycological growth and limit mycotoxin production on foods such as grapes, coffee beans, cereals, peanuts, and dairy products (Masoud and Jakobsen 2005; Bleve *et al.*, 2006; Masoud and Kaltoft 2006; Dimakopoulou *et al.*, 2008; Cubaiu *et al.*, 2009; Liu and Tsao 2009; Prado *et al.*, 2011; Somai and Belewa 2011). Studies focused on yeast antagonistic effects against *Aspergillus ochraceus* (Serna *et al.*, 2009; Velmourougane *et al.*, 2011), while fewer studies have examined its potential for biocontrol of aflatoxin-producing species such as *Aspergillus flavus* and *Aspergillus parasiticus* (Prado *et al.*, 2011; Somai and Belewa 2011). Recent work suggests that the yeasts, *S. cerevisiae* and *Tulbaghia violacea*, are antagonists of both *A. flavus* and *A. parasiticus* (Joannis-Cassan *et al.*, 2011; Prado *et al.*, 2011; Somai and Belewa 2011), but the antagonistic effects of these yeasts may be temperature dependent. Species of *Aspergillus* can flourish at temperatures ranging from 25–35°C, while optimal growth of *S. cerevisiae* occurs in a range of temperatures between 15–30°C (Sood, 2011). To further investigate both the biocontrol potential of yeast and the effect of temperature on its efficacy as a biocontrol tool, a pour-plate dilution method, using a commercially available baker's yeast incubated at three different temperatures, was employed with the expectation that a combination of higher levels of yeast and lower temperatures would lead to a decrease in the growth of

both *A. flavus* and *A. parasiticus*. More recently, a readily available commercial baker's yeast was reported to reduce the incidence of *A. ochraceus* and OTA in coffee (Velmourougane *et al.*, 2011). Similar results were obtained in this experiment using a commercially available baker's yeast to control the growth of *A. flavus* and *A. parasiticus*, but the inhibitory effects of the yeast were moderated by an interaction between the concentration of the yeast, temperature, and time. Some modicum of growth inhibition was achieved at all temperatures, but as expected, the combination of a high concentration of yeast and a lower temperature was most effective in limiting *Aspergillus* growth. Perhaps these toxins are effective in suppressing the growth of *A. flavus* and *A. parasiticus* when conditions are optimal for *S. cerevisiae*. The exact mechanism whereby *S. cerevisiae* limits the growth of *Aspergillus* is still poorly understood (Persons *et al.*, 2013)

#### Efficiency bacteria *Streptomyces* sp in growth inhibition of *A. flavus* A2 fungus in culture medium

The antifungal activity of *Streptomyces* sp showed in table (4). The biological approaches to antifungal and mycotxins detoxification will be taken as a mean of bio-transformation or degradation of toxin by endogenous enzyme to a metabolites that is either nontoxic when ingested by animal or less toxic than that the original toxin and readily extracted from the body .In the present study, the antifungal effects of stationary or the exponential culture filtrate obtained from the strain of *Streptomyces* sp. Where, the filtrate of the stationary phase of *Streptomyces* sp. yielded a wide range of antifungal activity zones ranged from (12 -16.2 mm) in diameter. On the other hand, the antifungal activity zone (Table, 4). These findings imply that the antifungal potential of the exponential culture filtrate was probably related to the increased production of hydrolytic enzymes, particularly chitinase. It has been reported that chitinase from *Streptomyces* sp. was able to lysis the cell walls of fungus (El-Katatny *et al.*, 2001). There is a possibility that the increased antifungal activity against the fungi tested in these experiments by the stationary culture filtrate of *Streptomyces* sp. is a consequence of the production of extracellular secondary antifungal compounds. The production of secondary antifungal compounds has been already reported in many species of *Streptomyces* (Fguira *et al.*, 2005 and Taechowisan *et al.*, 2005; Risan *et al.*, 2017). Antifungal production by *S. hygrosopicus* can inhibit a broad range of fungal pathogens such as *Rhizoctonia solani*, *F. oxysporum* and *Sclerotinia homeocarpa* (Chamberlain and Crawford, 1999). The production of chitinase and  $\alpha$ -1,3-glucanase enzymes by *Streptomyces* was related to fungal growth inhibition and the biological control of fungal pathogens due to the ability of *Streptomyces* to degrade fungal cell walls (Mahadevan and Crawford , 1997).

**Table 4: Antifungal activity of bacteria *Streptomyces* sp against *A. flavus* A2 fungus in culture medium.**

Treatments	Inhibition zone
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		diameter (mm)
<i>Streptomyces</i> sp + <i>A. flavus</i> A2	10 $\mu$ l	12.0
	15 $\mu$ l	14.5
	25 $\mu$ l	16.2
<i>A. flavus</i> A2 (control)		0.0

#### Efficiency of Pomegranate peel extract in growth inhibition of *A. flavus* A 2 fungus in culture medium

The antifungal potency was initially determined by the agar well-diffusion method. Table 5 presents diameters of inhibition zones (clear zones around wells) exerted by the different extracts towards test fungus. On the other hand, all extracts from pomegranate fruit peels inhibitory activity against *A. flavus* A2 (Table 5), with the highest inhibition zones on ethanol extracts 9- 15.3 mm inhibition zones for Pomegranate peel extract compared with the control. (Foss *et al.*, 2014) show that pomegranate peel is rich in tannins, high-molecular weight plant polyphenols, which can be categorized into two chemically and biologically separate groups, condensed hydrolysable tannin and tannin, the latter composed of glycosyl esters and phenolic acids. Hydrolyzable tannins are parted into gallotannins containing gallic acid and ellagitannins, containing ellagic acid. Al-Zoreky (2009) reported that only water-methanol extract of peels have marked inhibition (12–20 mm inhibition zones) and the water extract was inactive against eleven microorganisms tested, such as *S. aureus* (2 strains), *B. subtilis*, *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Candida utilis*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Fungistatic activity of pomegranate peel varied with test organisms as it inhibited the growth of *Penicillium citrinum* for 8 days, *P. patulum* for 4 days and *P. roquefortii* and *Aspergillus ochraceus* for 3 days (Azzouz and Bullerman, 1982). Bharani and Namasivayam (2016), show that in the case of *P. aeruginosa*, maximum zone of inhibition was recorded at 100 $\mu$ L with 20mm followed by 50 $\mu$ L with 19mm; 10 $\mu$ L revealed 11mm of zone of inhibition whereas in the case of *S.aureus* and *S.typhii*, the zone of inhibition was around 21mm at the highest dosage level of 100 $\mu$ L. *E.coli* showed high sensitivity to high concentration of aqueous peel extract (100 $\mu$ L, 50 $\mu$ L, 10 $\mu$ L); 22, 21 and 7mm of zone of inhibition has been observed at the respective concentrations and in case of antifungal activity, Food poisoning technique was carried out to depict the growth inhibition of the *A. niger* and *A. flavus* against the aqueous peel extract. The maximum growth was seen in *A. niger* i.e. 14mm compared to *A. flavus* (13mm)

**Table 5: Antifungal activity of Pomegranate peel extract against *A. flavus* A2 fungus in culture medium.**

Treatments		Inhibition zone diameter (mm)
Pomegranate peel extract + <i>A. flavus</i> A2	150 $\mu$ l	9.0
	200 $\mu$ l	17.1



	400 µl	15.3
<i>A. flavus</i> A2 (control)		0.0

The extracts obtained from fruits of six popular pomegranate cultivars were found to be effective against *Bacillus megaterium* DSM 32, *Pseudomonas aeruginosa* DSM 9027, *Staphylococcus aureus* Cowan 1, *Corynebacterium xerosis* UC 9165, *Escherichia coli* DM, *Enterococcus faecalis* A10, *Micrococcus luteus* LA 2971, and three fungi, *Kluyveromyces marxianus* A230, *Rhodotorula rubra* MC12, *Candida albicans* ATCC 1023, inhibition zones ranging from 13-26 mm (Duman *et al.*, 2009). The antimicrobial effects of pomegranate were previously studied. Indeed, it is reported that the bark, leaves, flowers, and fruits of pomegranate are widely used as phytotherapeutic agents in Brazil (Mathabe *et al.*, 2005).

Ahmad and Beg (2001), reported that alcohol extracts of pomegranate fruits showed antibacterial activity when tested against *S. aureus*, *E. coli* and *Shigella dysenteriae*. Prashanth *et al.*, (2001), also reported methanolic extracts of *Punica granatum* fruit rind to be active against all microorganisms tested in their study. Mathabe *et al.*, (2005), showed that methanol, ethanol, acetone, and water extracts obtained from pomegranate were active and effective against the tested microorganisms (*S. aureus*, *E. coli*, *Salmonella typhi*, *Vibrio cholera*, *S. dysenteriae*, *S. sonnei*, *S. flexneri*, *S. boydii*), showing an inhibition zone of 12-31 mm. Melendez and Capriles (2006), have also reported that extracts from pomegranate fruits possess strong in vitro antibacterial activity against many bacteria tested (*E. coli*, *Enterobacter cloacae*, *P. fluorescens*, *Proteus vulgaris*, *Alcaligenes faecalis*, *Serratia marcescens*, *E. aerogenes*, *S. aureus*, *Arthrobacter globiformis*, *M. luteus*, *B. cereus*, *B. subtilis*, *B. coagulans*, *Micrococcus roseus*, *M. phlei*, *M. rodochrus*, *M. smegmatis*; showing an inhibition zones of 11-31 mm). Food extracts may be more beneficial than isolated constituents, due to the other compounds present in the extracts can change the properties of bioactive individual component. (Oliveria *et al.*, 2008).

Eliana *et al.*, (2010), show that the maximum inhibition zones of peel Pomegranate fractions against *Candida albicans* ATCC 3153 were obtained in 200 µl concentrations by n- butanol fraction, water fraction and ethyl acetate fraction respectively, *C. albicans* ATCC 3153 only was sensitive to flower n- butanol fraction. In addition *C. albicans* ATCC 3153 was resistant to peel and flower Petroleumether fractions.

#### Efficiency of date extract in growth inhibition of *A. flavus* A 2 fungus in culture medium

The antifungal potency was initially determined by the agar well-diffusion method. Table 6 presents diameters of inhibition zones exerted by the different extracts towards test fungus. On the other hand, all extracts from date extract inhibitory activity against *A. flavus* A2

(Table 6), with the highest inhibition zones 5- 12.4 mm inhibition zones of date extract compared with the control. Shraideh *et al.*, (1998) reports The effect of Berhi date extract on the ultrastructure of *Candida albicans* was studied by scanning and transmission electron microscopy. Exposure of yeast to 5% (w/v) date extract showed evidence of weakening in the cell wall with indications of cell distortion and partial collapse in some cases as seen by scanning electron microscopy, increasing the concentration of date extract (20%, w/v) led to more drastic damage to the yeast with cell lysis and concurrent leakage of cytoplasmic material with eventual cell death, ultrastructural investigation showed irregular shapes of cells treated with date extract, with prominent effects on cell wall layers. Cell membranes lost their integrity, aggregation of the cytoplasmic contents and large detachment of plasmalemma from cell wall was observed in the treated cells, these results suggest that date extract may have multiple effects on *Candida albicans* with an increasing potential of using it for prophylaxis purposes.

**Table 6: Antifungal activity of date extract against *A. flavus* A2 fungus in culture medium.**

Treatments	Inhibition zone diameter (mm)	
Date extract + <i>A. flavus</i> A2	50 µl	5.0
	100 µl	9.0
	200 µl	12.4
<i>A. flavus</i> A2 (control)		0.0

#### Determining fungi producing of aflatoxin M1

*Aspergillus Flavus Parasiticus* Agar medium (AFPA) was used to test the ability of *A. flavus* for aflatoxin production, Table (7). Aflatoxins are the most intensively studied mycotoxins in dairy cows as the produce of AFM1 in dairy milk is of public health concern (Fink-Gremmels, 2008). After ingestion of aflatoxin-contaminated feeds, a part of the ingested aflatoxin B1 is hydroxylated to AFM1 in the liver (Kuilman *et al.*, 2000). Aflatoxins (AFs) are toxic, carcinogenic, immunosuppressive secondary metabolites produced by some *Aspergillus* species which colonize crops, including many dietary staple foods and feed components. AFB1 is the prevalent and most toxic among AFs. In the liver, it is biotransformed into AFM1, which is then excreted into the milk of lactating mammals, including dairy animals. AFM1 has been shown to be cause of both acute and chronic toxicoses. The presence of AFM1 in milk and dairy products has represented a worldwide concern since even small amounts of this metabolite may be of importance as long-term exposure is concerned. Contamination of milk may be mitigated either directly, decreasing the AFM1 content in contaminated milk, or indirectly, decreasing AFB1 contamination in the feed of dairy animals. (Giovati *et al.*, 2015). *A. flavus* varied in producing aflatoxin. Results revealed that the three isolates A1 and A2 are aflatoxin producers, but A3, can't produce

aflatoxin. This agrees with Pitt *et al.*, (1983), who found that (AFPA) medium differentia between isolates producing aflatoxin, and reported that colonies of *A. flavus* / *parasiticus* recovered on AFPA developed better reverse orange-yellow color than did colonies on *Aspergillus* Differential Medium (ADM). This observation was also made in the present study, particularly when comparing colonies on ADM and AFPA after 42- 44 h of incubation. Beuchat (1984), show that use of AFPA for detecting the *A. flavus* / *parasiticus* group may have some advantage over ADM for inexperienced workers, since color development of reverse colonies is quicker and more intense on AFPA. Gallo *et al.*, (2012), showed the of ability isolated *A. flavus* in producing the aflatoxin (55%). Out of 43 isolates, 9 (20.93%) produced Aflatoxins (AFs) including (AFB1, AFB2, AFG1, AFG2). Scherm *et al.*, (2005), found that differences between isolates in aflatoxin production, may due to the genes conferred by *A. flavus* responsible for the production of aflatoxin. Many countries have set maximum acceptable levels for AFM1 in milk and dairy products. US Food and Drug Administration (USFDA) set a maximum permissible level for aflatoxin M1 in milk of 0.5 µg/Kg while in Europe and some Africa and Asia countries, the maximum acceptable level of aflatoxin M1 in milk is 0.05 µg/kg (Van Egmond *et al.*, 2007).

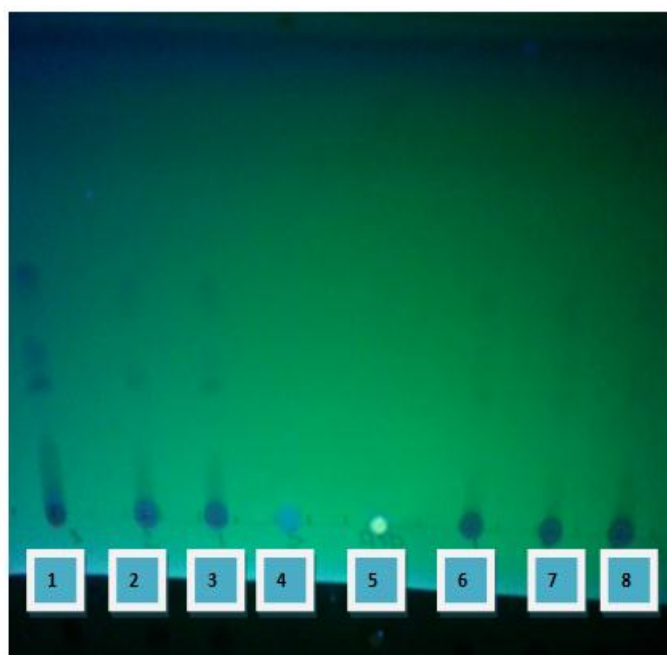
**Table 7: *Aspergillus flavus* isolates producing aflatoxin.**

Isolate	Fungal species	Producing of aflatoxin from <i>A. flavus</i>
A1	<i>Aspergillus flavus</i>	+
A2	<i>Aspergillus flavus</i>	+++
A3	<i>Aspergillus flavus</i>	-

(+): Light brilliance (+ + +): High brilliance (-): No brilliance.

#### Detoxification of AFM1 in milk by using Thin layer chromatography

One concentration 2g/L, 200µl and 400µl of *Saccharomyces cerevisiae*, Date extract and dried shell of pomegranate extract respectively with bacteria *Lactobacillus acidophilus*, *L. plantarum*, *Streptomyces* were examined using thin layer chromatographic (TLC) method comparison with standard aflatoxin M1. Results presented in Fig (1) generally reveal that all starters of bacteria *Lactobacillus acidophilus*, *L. plantarum*, *Streptomyces*, *Saccharomyces cerevisiae*, Date extract and dried shell of pomegranate extracts were characterized by their ability to gradually degrade AFM1.



**Fig. (1). Detoxification of AFM1 in milk by bacteria *Lactobacillus acidophilus*, *L. plantarum*, *Streptomyces*, *Saccharomyces cerevisiae*, Date extract and dried shell of pomegranate extracts by using Thin layer chromatography**

- (1) L. a. *Lactobacillus acidophilus*
- (2) L. p. *Lactobacillus plantarum*
- (3) St. *Streptomyces*
- (4) S. Standard aflatoxin M 1
- (5) ASP. Isolate produce aflatoxin M 1.
- (6) P.P. Pomegranate peel extract



(7) **D.** Date extract

(8) **S. c.** *Saccharomyces cerevisiae* extract.

Since the first analytical methods for aflatoxins were published in 1964. Thin Layer Chromatography (TLC) has been the only technique capable of detecting and quantitating aflatoxins at low levels. The aflatoxins are well suited for analysis by TLC since most of the compounds fluoresce strongly under long-wave UV light. Approximately 0.5 ng-spot can be routinely detected either visually or instrumentally. The TLC technique serves as both purification and quantitation step. Before TLC analysis, the aflatoxins are extracted from the sample, usually with an aqueous organic solvent, and the extract is initially purified by one or more techniques such as solvent partition, heavy metal precipitation, column filtration, or chromatography. These techniques affect the result of analysis published by the Association of Official Analytical Chemists (AOAC), the American Oil Chemist Society (AOCS), the European Economic Community (EEC), and the American Association of Cereal Chemists (AACC). Successful analyses of any commodity depend on the selection of appropriate methods for preparation of extract. Also crucial to successful analysis is thin layer chromatography itself. It is not sufficiently appreciated that a good quantitation requires efficient chromatography, i.e., separation of the analysis from each other and from other extractives. (Anon, 1980). Results agreed with those obtained by (El-Naggar *et al.*, 2006 ; Boudjelal *et al.* 2011). All such researchers obtained a single active spot in their isolated actinomycetes with different Rf values which were using different organic solvents in mobile phase, after applying the direct bioautography assay for determining their position, Maataoui *et al.*, (2014) obtained three spots on TLC plate and only one of them showed an antimicrobial activity.

These results agreed with those obtained by El-Tayeb *et al.* (2004) and Ababutain *et al.*, (2012) who stated that the bioactive antimicrobial metabolites were insoluble in n-Hexane while soluble in methanol, ethyl acetate, chloroform, n-butanol, acetone with different Rf values ranged from 0.92 cm to 0.3 cm. Emara *et al.*, (2000) and Maryamma *et al.*, (1990) also came to the same conclusion, when they used different Species of lactic acid bacteria, i.e., *L. casei* sp. *casei* (ATCC 15088), *Lactobacillus acidophilus* (ATCC 11975), *L. sp.* GG. ATCC 53103) and *L. rhamnosus* (ATCC 10863). The reduction level by these strains ranged from 26.2- 34.0%, depending upon the bacterial isolates.

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