Plant Archives Vol. 20 Supplement 1, 2020 pp. 1051-1057 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ASSESSING THE EFFICACY OF CERTAIN NANO, NATURAL AND CHEMICAL MATERIALS IN FUNGAL INHIBITION AND AFB1 TOXIN REDUCTION OF ASPERGILLUS *FLAVUS* ISOLATED FROM PEANUT ON PDA MEDIA

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Abstract

This study was conducted in the efficiency of some nanomaterials (magnesium oxide), natural products (fish oil) and chemical (Phylex). Seed testing results revealed presence of different fungal genera, including *Aspergillus*, *Penicillium*, *Rhizopus*, *Alternaria*, *Fusarium*, *Mucor* and *Cladosporium*. In addition, *A. flavus* scored the highest sovereignty followed by *A. niger* and *A. ochraceus* in peanut samples at frequency rates 40, 12.28 and 10% respectively. The emergence rates before and after surface sterilization were 78.125-80.89, respectively. Results confirmed that 9 out of 15 isolates *A. flavus* isolates were capable to produce AFB1 when grown in rice medium. It was observed when adding MgO nanoparticles at concentrations of 1%, 2% and 3% concentrations resulted in fungal growth inhibition by 95% and 100%, respectively. Besides Phylex treatment could inhibit *A. flavus* growth by 100%, 100% and 74.8% when 0.03 and 0.02 and 0.01 concentrations were used, respectively. While fish oil treatment at concentrations of 2%, 4% and 6% inhibited fungal growth up to 80.4%, 100% and 100% respectively.

Key word: Nanomaterials, peanut field, PDA, *Aspergillus flavus*.

Introduction

Peanut (*Arachis hypogaea* L.) is one of the oily leguminous crops. It is grown worldwide due to the nutritional value of the crop as it contains high protein and oil contents. The oil and protein percentages in | peanut seeds range 42-52% and 25-35%, respectively (Al-Sayuki, 1995). Whereas, Carbohydrate content is 5%. Peanutcontains important nutrients such as Zn, Fe, Mg, Ca, K, vitamins B, A, K and high proportions of the unsaturated fatty acids (oleic acid 54% and linoleic acid 34%). Thus, it is a preferred nutrition as it minimizes blood cholesterol (Hinds, 1995). In Arabian region, peanut is one of the most 13 important crops. It comes the second after olive (Al-Sayuki, 1994). In Iraq, the peanut crop has been introduced into commercialization recently, but the expansion of its cultivation has not archived the level of the economic importance. The area cultivated with this crop was 24196 yards until the year 2009 (Ministry of Agriculture, Statistic Department). The peanut is infected with many fungal pathogens such as *Aspergillus*, *Penicillium* at throughout pod collection and storage as well as transport. These fungi are known for their high ability to produce secondary metabolism materials of highly toxic and carcinogenic effects on human and animal health known as mycotoxins. Most studies indicate that these fungi are able to produce more than one type of toxin during their growth (Smith *et al*., 1994).

Many methods are used inhibit mycotoxins in crops, vegetables and fruit generally including physical methods. Among others, these methods have extensively been used to reduce and resist fungal toxins intreated agriculture and food products because of their high efficiency to decrease levels or remove aflatoxin. In addition, these methods keep the food value treated and is considered as safe methods for public health (Samara sseewa *et al*., 1994). The nanomaterials production is a new technology that derived from nanometers. This technology was first introduced by the Japanese scientist Norio Taniguchi in 1974. It is defined as a group of separation, composition

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and integration of materials at level of atoms and molecules (Cortez and others, 2411). Nanotechnology enables to control of the material or their dimensions smaller than 100 nm by madding, observing and measuring of the properties (Lie *et al.*, 2006, Al-Hoshan *et al.*, 2007). The modern studies confirmed that the halogenated MgO nps nanomaterials particles have a strong effect on the microorganism as they affect cellular membranes prop and their food transported properties (Jin, 2011). A number of studies and applications on the MgO nps nanomaterials particles as an adsorption agent and the destruction of toxic chemicals were performed (Lange and Obendoef, 2012). Al-Qaysi (2015) proved the high efficiency of the importer and local MgO nps nanomaterials particles when inhibited *A. flavus* growth by 100% in stored corn seeds, as well as the highest efficiency to reduce Aflatoxin B1 compared to the a microbic magnesium oxide.

As for the chemical methods, a number of these methods has been tested for their ability to destroy and remove the mycotoxins especially the aflatoxins. Chemical substances tested include acids, alkaline, gases, aldehyde and others (Phillips *et al*., 1994, Scott, 1998). The Phylex material, which is a commercial liquid product, produced by the Selko company (Netherlands), contains a group of acids: propionic acid, formic acid, lactic acid, orthophosphoric acid, citric acid, sorbic acid and other materials such as evaporation preventive materials, publisher materials, water and ammonia, were used and their ability to decompose mycotoxin as well as application of biological methods such as species of bacteria *Flavobacterium aurantiacum* has been proven (D'souza and Brachett, 2001).

The use of bread yeast *Saccharomyces cerevisiae* could reduce more than 15% from aflatoxin type B1 when added to poultry feeds (Abdolamir-Allameh *et al.*, 2011). The essential oils mode of action against fungi can be in two way either a fungi static, which stops the growth of fungus or a fungicidal (Sharma *et al*., 2009) or use the fish oil (Eureka) which is a commercial product.

Therefore, the study aimed to test the efficiency of three concentrations of materials (Phylex, fish oil and MgO nanomaterials) to inhibit the isolation growth that production of the aflatoxinB1 *in vitro*.

Materials and Methods

The peanut samples were collected from local markets in Baghdad province (Al-Shurjeh, New Baghdad, Al-Kadhimiya, Al-Baya'a, Al-Saydiya, and Palestine Street). The fungi complex contaminating peanut was isolated and diagnosed by diluted method and dilution plate count to identify growing fungi (Andres, 1992).

Abbot 94 ml of sterile saline solution with 85% concentration 85% was added to 10g of each peanut sample and well-agitated for two minutes to obtain the first dilution at 10^{-1}. The dilution serial (10^{-1} to 10^{-3}) was prepared into 9 ml of sterile saline solution and well-agitated for each dilution. One ml of each dilution was transferred into Petri dishes under sterile laboratory condition. After that 20 ml from prepared PDA was poured into plates then incubated at temperature 28°C for 5 to 7 days.

The rate frequency of accompanied fungi of laboratory samples was calculated according to the following equation:

$$Frequency\% = \frac{The\ isolated\ fungi\ number}{Total\ isolated\ numebr} \times 100$$

*Using rice media for growing *A. flavus* isolates.

The rice medium was used to develop *A. flavus* isolates and production of the aflatoxin B1 according to method West *et al.,* (1973).

Extract aflatoxin B1 from rice medium

The weight of 25 grams of dried peanut samples was ground and placed a 250 ml capacity flask then 25 ml of methanol and chloroform was added. After that the mixture was shacked for 60 minutes for homogenizing. The mixture was filtered through Whatman 2 filter paper, then 25 ml of methanol 90% was added and separated by separator funnel. Transfer the supernatant was transferred into separator funnel and 25 ml hexane and 25 ml methanol 90% was added and separated with separator funnel. The lower layer containing methanol was dried by water bath and collected. The sample was mixed with chloroform and distilled water 25:25 ml in separator funnel and washed twice with distilled water. After agitating, the mixture in the funnel was left until the two layers separated (The upper layer was discarded). The lower layer (chloroform) was passed through the filter paper which contains 10 gm of Na_2SO_3. The supernatant was taken and dried in a water bath then 3 ml of Asto natural as an appropriate solvent (AOAC, 2005) was added.

Detection of AFB1 using thin layer chromatography (TLC)

The dry extract of the samples of which the aflatoxin was extracted using 1 ml of chloroform and well-agitating, to ensure dissolving. Five µl of the sample was transferred by a micro-syringe spotted onto a TLC at 2 and 1.5 cm distances from the lower and lateral edges 1.5cm of plate, respectively. Similarly, the standard toxin was spotted. The spots were dried and TLC plate was placed into

TLC chamber containing separation solutions (consist of chloroform-methanol (97-3) and mixed well). When the separated solution was at 2cmdistance of the upper edge of TLC plate, the plate was removed and left to air dry. Spots were exanimate under UV light at 365 nm wavelength and observed color illumination and the RF value were identified and the illumination intensity was compared to the resulting spot of the standard the aflatoxin B1 (Cocker *et al*., 1984, Alrawi and Hussein, 2017).

Estimating of AFB1 using High Performance Liquid Chromatography (HPLC): The HPLC instrument model Sykam, (Germany) showed in scheme (1) was used to separate and quantify aflatoxin B1 (Holler, 2007).

Testing the efficiency of MgO nano materials, Phylex and Eureka to inhibit the growth of *Aspergillus flavus* on PDA medium *in vitro*:

1. MgO nanomaterials in the medium of PDA: Three MgO nanomaterial concentrations MgO (1, 2 and 3 g per 100 ml PDA medium after sterilization) were tested to find out the best concentration that would inhibit the growth of *A. flavus* isolate of the most aflatoxin B1 productive on. Each concentration was poured into 9 cm plates and cultivated with the highest aflatoxin B1 productive fungal isolate and incubated in at 27°C. Data was collected (3, 5, 7) days after incubation and compared to control by measuring the fungal growth of *A. flavus*.

2. Fish oil (eureka) with PDA medium: Three oil concentrations (2, 4 and 6 ml per 100 ml of PDA after sterilization) were tested to find out the best concentration that inhibits the mycelium growth of *A. flavus*. Fish oil-PDA mixtures were poured into 9 cm capacity plates and cultured with highest aflatoxin B productive isolate and incubated at 27°C. Seven days later, Data was collected and compared to control through measuring the fungal growth of *A. flavus*. The diameters of colony growth were measured every two days until the control plate was fully occupied. The inhibition rate was calculated according to the following equation:

$$Inhibition = \frac{Diameter\ mean\ of\ control - Diameter\ mean\ of\ treated\ colony}{Diameter\ mean\ of\ control\ colony} \times 100$$

Table 1: Percentages of fungi associated with peanut.

No.	Fungi	Incidence %
1	*Aspergillus*	78.125
2	*Penicillium*	46.875
3	*Alternaria*	6.25
4	*Rhizopus*	51.625
5	*Fusarium*	28.125
6	*Rhodotorula*	9.375
7	*Mucor*	3.125

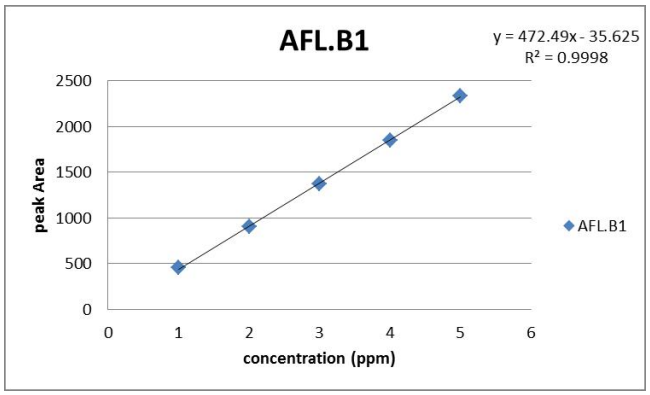


Fig. 1: Standard compound curve for aflatoxin type (B1).

Preparation of standard solutions

The amount of aflatoxin type B1 was calculated by using High Performance Liquid Chromatography (HPLC) according to the mentioned operational program mentioned below and through the application of straight line equations that have been obtained from standard compounds curves shown in fig. 1:

Mobile phase: H_2O: CH_3CN (60:40)

Flow rate: ml/min 1

Detector: UV 365 NM

Column: ODS

Size Injection: 100 µl

Results and Discussion

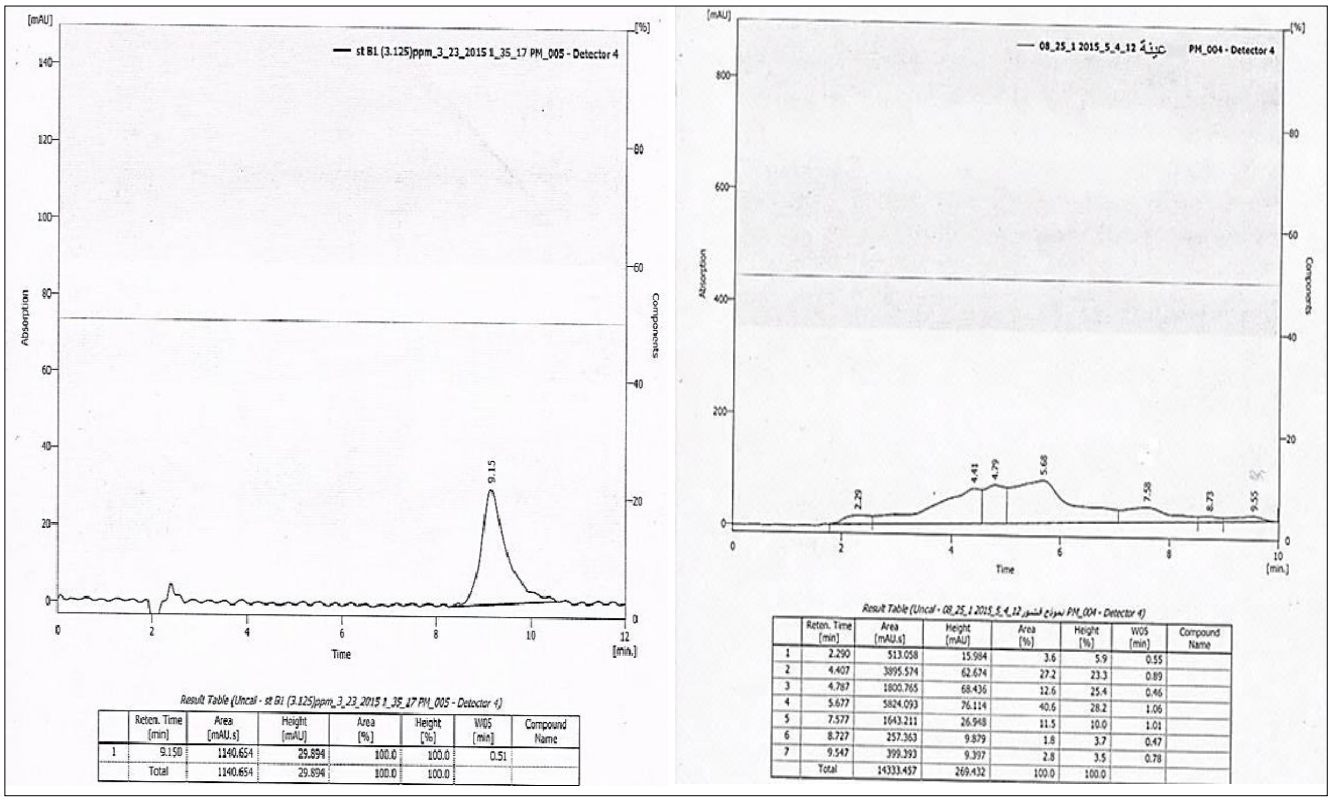
Isolation and diagnosis of fungi associated with peanut

The isolation and diagnosis results of peanut samples, randomly collected from different area in Baghdad, showed presence of the following fungi genera: *Aspergillus*, *Penicillium*, *Rhizopus*, *Alternaria*, *Fusarium* and *Mucor*. Similar to Bruand and Rowsell (2001) findings, species of the fungus *Aspergillus* namely, *A. flavus*, A. *niger* and *A. parasiticus* were the highest incidence percent in samples.

The incidence percent of *Aspergillus* was 78.125% and the *A. flavus* was the most frequent species then *A. niger* and *A. ochraceus* when scored 44%, 28.6 and 8.3%, respectively (Table 1 and 2). Similar results were indicated by Al-Haiti (1977) and Hussein (2000) the high *A. flavus* dominancy in grain stores.

Table 2: Frequency percentages of *Aspergillus*species.

No.	Fungi	Incidence %
1	*A.flavus*	40
2	*A.niger*	27.6
3	*A. ochracuse*	8.3
4	*A.terveuse*	1.6



Result Table (Uncal - st B1 (3.125)ppm_3_23_2015 1_35_17 PM_005 - Detector 4)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	9.150	1140.654	29.894	100.0	100.0	0.51	
	Total	1140.654	29.894	100.0	100.0		

Result Table (Uncal - 08_25_1 2015_5_4_12 نموذج فشور PM_004 - Detector 4)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.290	513.058	15.984	3.6	5.9	0.55	
2	4.407	3895.574	62.674	27.2	23.3	0.89	
3	4.787	1800.765	68.436	12.6	25.4	0.46	
4	5.677	5824.093	76.114	40.6	28.2	1.06	
5	7.577	1643.211	26.948	11.5	10.0	1.01	
6	8.727	257.363	9.879	1.8	3.7	0.47	
7	9.547	399.393	9.397	2.8	3.5	0.78	
	Total	14333.457	269.432	100.0	100.0		

Fig. 2: HPLC of 12 samples and standard compound for aflatoxin B1 (St.).

The reason behind the high dominancy of *Aspergillus* was its ability to grow in the wide thermal range with allow moisture content during harvesting or in grain stores (Al-Saadi, 2000).

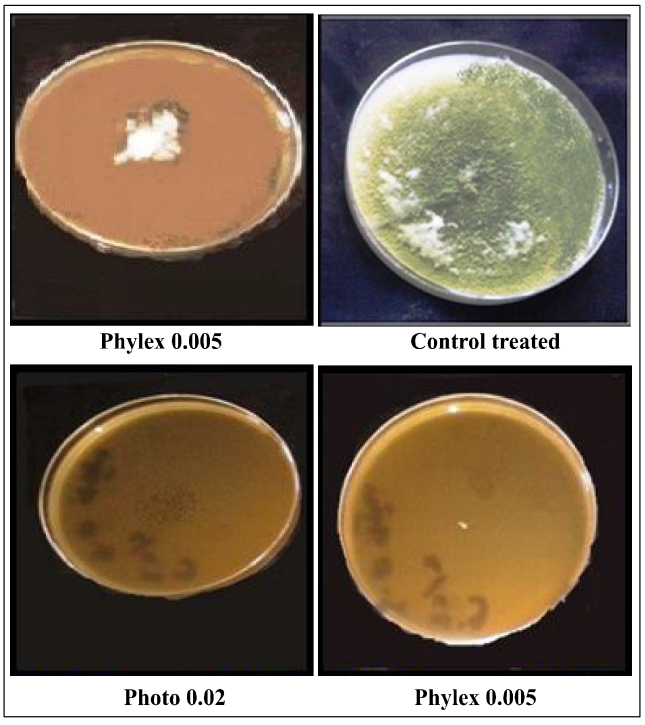


Fig. 3: Shows the Phylex activity in the inhibition of *A. flavus* on PDA medium.

Quantifying aflatoxin B1 production of *A. flavus* isolates using thin layer chromatography (TLC) and High-Performance Liquid Chromatography (HPLC)

Table 3: Concentrations of Aflatoxin B1 in peanut samples.

No.	Samples	Aflatoxin B1 concentrations µg/gm (ppb)
1	Al-Saidia (Al-Alam district)	10
2	Al-Saidia (commercial street)	10
3	Al-Sinak	10
4	Al-Kadhimya	0
5	Al-Mashtal	20
6	Al-Otaeffia	0
7	Jamella	0
8	Al-Doora	104
9	Palestine street	10
10	New Baghdad	10
11	Al-Zaafaranya	100
12	Al-Shorja	110
13	Al-Mashtal	20
14	Al-Jameea district	10
15	Abu Graib	20
16	Al-Baeaa	10
17	Al-Shawaka	0
18	Al-Ameria	0
19	Abu Al-Jaber	10
20	Al-Karada	0

Table 4: The different concentrations of fish oil, Phylex and MgO nanoparticles inhibitory activities against *A. flavus* growth on the PDA medium.

No.	Treatment	Concentration %	Inhibition percentage %
1	Phylex	0.1	74.8
		0.2	100
		0.3	100
2	MgO nanoparticles	1	95
		2	100
		3	100
3	fish oil	2	84.4
		4	100
		6	100
4	LSD for comparison	-	0

TLC results of showed different aflatoxin B1 production of *A. flavus* fungus isolates isolated from peanut seeds based on illumination intensity. The reason for the difference in mycotoxin quantities produced by the isolates could be attributed to the experimental condition, the type of the separation system as well as the plates used for analyzing and genetic variation for the isolates produced mycotoxin (Reddy *et al.,* 2009). The presence of the mycotoxin was confirmed by HPLC when compared to the standard compound (Fig. 2) and the quantification of the mycotoxin concentration in the samples that contain aflatoxin B1. The results showed high variation in the concentrations (Table 3). Among others, Al-Shorja sample scored the highest concentration value (110 ppm).

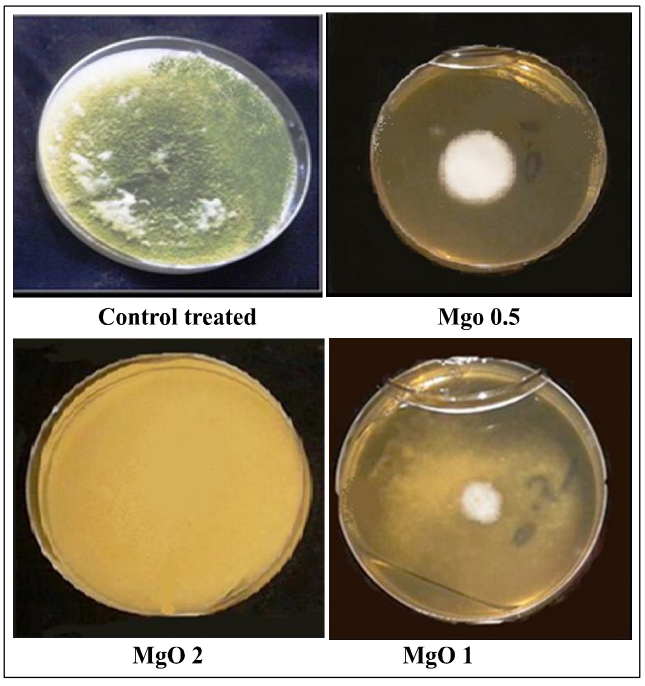


Fig. 4: Shows the MgO nanoparticles activity in the inhibition of *A. flavus* on PDA medium.

The Phylex inhibition activity against *Aspergillus* growth on PDA medium

The results shown in table 4, confirmed the high efficiency of Phylex added into the PDA medium on the inhibit *A. flavus* growth. The concentrations 0.2 and 0.3% caused 100% inhibition, whereas the percentage of inhibition was 74.8% at 0.1%, concentration. These results resembled to what Al-Heeti (1977) found that the bro-sil which contains 99% of the propionic acid inhibited fungi that infecting yellow-corn in up to 100%. In addition, acidic compounds were applied to inhibit fungal growth. Citric and lactic acids were used against *A. parasiticus* in wheat bread at 0.5% concentration 0.75%, respectively to prevent fungus growth and aflatoxin production. Abdul-Hamid (2000) found that the application of propionic acid at 0.2% concentration could inhibit fungal growth by 40-80% on daily basis. Jaber and Al-Salahy 2005 and IFST (2006) confirmed the effectiveness of a number of acids to inhibit many species of fungi that infecting grain in stores.

The MgO nanoparticles inhibitory activity against *Aspergillus* on PDA medium

The results in table 4, showed the inhibitory activity of MgO nanoparticles against *A. flavus* at 2% and 3% concentrations. The percentage of fungus growth inhibition on PDA was 100%, at 1% concentration of 1% with inhibition percentage up to 95% (Fig. 4). These results resembled number of studies pointed to the high inhibitory efficacy of MgO Nps against plant pathogens (Rico *et al*., 2011, Mohendra *et al.*, 2012, Abdul-Hasan

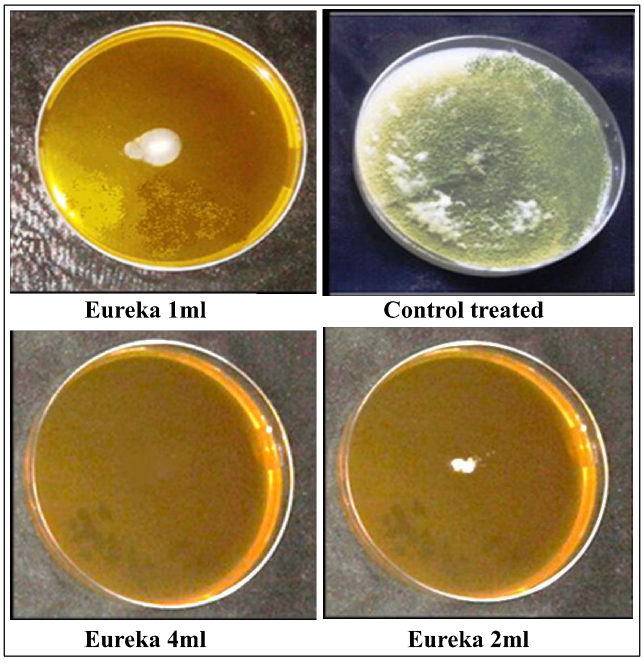


Fig. 5: Test of the efficacy of different concentrations from fish oil, Phylex and MgO nanoparticles on inhibition the growth of *A. flavus* on the PDA medium.

and Hussein, 2016). Whereas, Wani (2012) found that the use of MgO nanoparticles inhibited *Fusarium* spp. causing wilt diseases in the plant. Similarly, Al-Qaysi (2415) verified the high efficiency of MgO nanoparticles when scored inhibition percentage up to 100% at 2 and 3% concentration. While, 95.53% inhibition percentage was scored at 1% concentration.

Eureka (fish oil) inhibitory activity against *Aspergillus* on PDA medium

The addition of fish oil (Eureka) showed high inhibitory activity against the *A. flavus* growth. The percent of fungal growth inhibition on PDA medium was 100% at 4 and 6% concentrations. While it was 84.4% at 2% concentration of (Fig. 5). Fish oil action is to prevent the absorption of nutrients (*i.e.* phosphate and Sulphur) *Aspergillus* species because this material targets the fungal cell membranes.

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