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EVALUATION OF THE EFFICIENCY OF SOME CHEMICAL (FYLEX), NANOPARTICLES (MGO) AND BIOLOGICAL FACTORS IN INHIBITING OF *ASPERGILLUS FLAVUS*

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Abstract

The Fylex extract exert a high inhibition effect against *A . flavus* growth on PDA medium, as the fungus growth was completely inhibited by 100% at a concentration of 0.2 and 0.3% of studied extract, while the lowest inhibition percentage (71%) was found at a concentration of 0.1%. Whereas magnesium oxide nanoparticles showed the highest inhibition ratio of *A. flavus* (100%) was detected at 0.2% and the lowest inhibition ratio (81.66%) was at concentration 0.5%. Moreover, the addition of *G. lucidum* powder to PDA medium with a concentration of 2.5 mg increased the inhibition rate of *A. flavus* growth which was 54.4%, while the lowest inhibition ration (18.22%) was found at a concentration of 1000 mg. The milky liquid (brocade milk) of *Calotropis porcera* plant showed the highest rate of inhibition against *A. flavus* growth at a concentration of 0.5%, which was (64.88%), while the lowest rate of inhibition (53.33%) was seen at a concentration of 0.1%. The results of the current study indicated that the studied treatments have a high inhibitory efficiency against the growth of *A. flavus*.

Key words: *Zea mays*, Aflatoxin, *A. flavus*, Fylex, MgO NPs, *G. lucidum*, *Calotropis porcera* Latex.

Introduction

The yellow corn cereals (*Zea mays*) occupies the third position after wheat and rice in terms of production and cultivated areas in the world; it is one of the most important food crops for humans and animals (FAO, 2012). The yellow corn cereals are expose to many fungi and their toxins during field harvesting, transportation, and storage (Chulze, 2010). The most prominent of these mycotoxins are Aflatoxins, which are secondary metabolites produced naturally by some *Aspergillus* species including *A. flavus* and *A. parasiticus* (Levin, 2012). Many foods and feed are contaminate with these mycotoxins which has carcinogenic effects on the liver (VanEgmond and Jonker, 2004). There are four main types of aflatoxin: B1, B2, G1 and G2 (Essa, 1996). The US Food and Drug Administration (FDA) has set the AFB1 tolerance ratio for all human foods to a maximum of 20 ppb, and for milk (0.5ppb) (Turcksess and Wood, 1997; Payne *et al*., 2010). Due to the harmful effects of aflatoxins, most research efforts have focused on methods and strategies that inhibit the growth of fungi and prevent the formation or production of aflatoxins and decontamination of aflatoxins (Holmes *et al*., 2008; Ahmadzadeh *et al*., 2015). Among them is Fylex, a liquid commercial product consisting of a group of organic acids: Formic acid, Lactic acid, Propoinic acid, Arthrophosphoric acid, Sorbic acid, Citric acid, water and ammonia produced by Selko company (Netherland). Nanoparticles have an inhibitory effect on the growth of microorganisms (Rico *et al*., 2011). These nanoparticles have a large role in managing of plant infection with fungi in comparison withusing of industrial fungicides. Nanoparticles are safe compared to agricultural chemicals (Mohendra *et al*., 2012). Many fungi have a medical importance as a result of possessing biologically active compounds; *G. Lucidum* is one of them with medicinal efficiency, which is characterized by a group of biological activities including antimicrobials, antioxidants, anti-tumors, anti-inflammatory, and immune enhancement (Sevindik, 2018). *Calotropis porcera* plant belongs to the family of Aclepidaceae and its Arabic name is milk weed. It is medicinal plant and its various parts have been used as anti-oxidants and anti-inflammatory agents (Choedon *et al*., 2006). The plant tissues contain lactic liguid (Latex), which is a skin-inflaming substance if it comes in contact

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with it. Latex contains trypsin and toxic substances including Calotroxin, Calotropin and Uscharin. It is used in India mixed with other plants as a topical treatment for some cases of rheumatoid disease as well as a strong laxative, toothache and as a vomit for guinea fever (Khan *et al*., 2007; Saratha and Subramanian, 2010).

Material and Methods

Isolation and diagnosis of fungi accompanying with yellow corn

A 400 of yellow corn seed from each sample (location) and sterilized with sodium hypochlorite solution at a concentration of (2%) for 3 minutes, then washed with distilled sterile water 3 times and dried using filter paper. The seeds were planted on the PDA culture medium, and by 5 seed in petridish. and sterilized using autoclave at 121°C and 1.5 kg/cm^2 pressure for 20 minutes; the antibiotic Tetracycline was added at a concentration of 10 mg/L to the culture medium. All dishes were incubated for 7 days at of 25±2°C. The end of fungal mycelium was transferred from all isolates of *A. flavus* for the purpose of purification by isolation needle and cultured on PDA culture medium in another dish, then incubated at 25±2°C for 7 days. The fungus was detected according to the morphological characteristics depending on the classification keys (Samson *et al*., 1984; Pitt and Ailsa, 2009).

Growing isolates of *A. flavus* in yeast extract sucrose (YES) to select the most aflatoxin B1 toxin productive isolate

The liquid Yeast Extract Sucrose (YES) medium was prepared according to Davis *et al*. (1966) method by dissolving 20g of yeast extract and 200g of sucrose in a liter of distilled water. The medium was prepared in conical flasks (250 ml) and then sterilized using autoclave at 121°C and 1.5 kg/cm^2 pressure for 20 minutes, and cooled to 45°C and Tetracycline was added at a concentration of 250 mg/L. The flasks containing YES medium were inoculated with two disk (5 cm) of *A. flavus* at 7 days of growth using cork borer, and then incubated at 25±2°C for 21 days.

Extraction of aflatoxin B1 toxin from yeast extract sucrose extract (YES) medium

After the incubation period, YES medium was filtered using a filter paper and similar volume of filtered medium of chloroform was added and placed on the shaker for an hour, then the suspension put in a funnel to separate the lower layer containing the chloroform and shake for 30 seconds with observing the expulsion of the gases formed for the purpose of separating the two layers to extract AFB1. The suspension was collected and passed on filter paper containing anhydrous sodium sulfate for removing the water from the sample, where 40g of ml of anhydrous sodium sulfate was added for each 100 ml of suspension. The filtrated suspension was collected in a glass bottle and kept in the freezer until using for subsequent tests (AOAC, 2005).

Detecting AFB1 toxin using Thin-Layer Chromatography (TLC)

The presence of aflatoxin B1 toxin was detected using Thin-Layer Chromatography device that coated with G60 silica gel with dimensions of 20 × 20 cm, and thickness of 0.20 mm. The plates were activated using electric oven at 110°C for an hour before use. The dried extract of collected samples was dissolved and 10 µl of sample using micro syringe was taken and placed on the plate at 2 cm from the bottom edge of the plate, and leaving a distance of 1.5 cm from the edge of the side plate. Similar distance was left between each sample and the standard toxin On the other hand, 5 µl was left to dry, then placed in the TLC- separation tank that containing the mobile phase (separation solutions) which consisting of chloroform - methanol in volumetric proportions (3-97). The TLC plate was monitored until the solution reached a distance of 2 cm from its upper edge and then the plate was raised and left to dry at laboratory temperature. After that, it was examined under ultraviolet radiation at a wavelength of 360 nm using UV viewing device and by matching the color brightness and intensity of the spots, RF factor of samples was detected in comparison with the standard solution of AFB1, AFB1 (Cocker *et al*., 1984). The rate of flow (Rf) of the studied samples and standard toxin was estimated according to the following formula:

Rf = distance from origin point to spot / distance from origin point to front

(Al-Adil and Abd, 1979)

Depending of the estimated results of RF, the most AFB1 toxin productive isolate was chosen for subsequent experiments, and called AFSC4.

Determination of the inhibition efficiency of Fylex, MgONPs, *Ganoderma lucidum* and milky liquid of *Calotropis porcera* (Latex) on *A.flavus* growth on PDA medium:

PDA medium was prepared by dissolving 41g of ready-made medium powder in a liter of distilled water, the medium was distributed in glass flasks with 50 ml/ flask of medium, then all flasks sterilizedusing autoclave at 121°C and 1.5 kg/cm^2 pressure for 20 minutes. Tetracycline was added at a concentration of 10 mg/l.

The studied treatments were added to the flasks which are including Fylex, MgO nanoparticles, *Ganoderma lucidum* powder and milk of *Calotropis porcera* (Latex) at concentrations (0.1, 0.15, 0.2, 0.3) ml, (0.5, 1, 1.5, 2) g, (2.5, 5, 10, 250, 1000) mg, (0.1, 0.2, 0.3, 0.5) ml respectively.

The treated medium was poured into Petri dishes with a diameter of 9 cm in three replicates for each concentration of treatment including control group (without treatment). Subsequently, The Petri dishes were inoculated with spores suspension of *A.flavus* that prepared by adding 10 ml of distilled water to dish containing *A. flavus* at 7 days of growth. The growing colony diameters were measured after 5 and 7 days of incubation and the inhibition ratio was calculated according to the following formula:

The percentage of Inhibition = (mean diameter of the comparison colony -mean diameter of the treatment colony) / mean diameter of the comparison colony x 100. (Karaman *et al*., 2001).

The statistical analysis System -SAS (2012) program.

Results and discussion

The ability of different isolates to produce AFB1 toxin on the yeast medium (YES) using Thin Layer Chrotography (TLC):

The results of detection of AFB1 toxin using TLC technique of *A.flavus* isolates accompanying the maize cereals grown on the liquid yeast medium revealed that the isolates have the ability to produce AFB1 toxin, and the most toxin productive isolate was determined based on the size of the spot, its brilliance and the rate of flow compared with spot of standard AFB1 toxin (Wyllie and Morehouse, 1977).

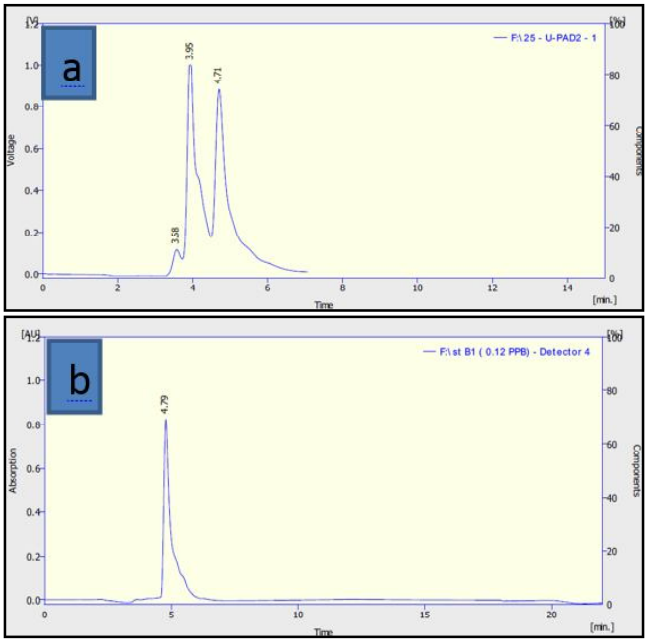

Fig. 1: The concentration curve of AFB1 toxin. The retention time is 4.79. a: the concentration of AFB1 toxin in AFSC4 isolate. b: the standard AFB1 toxin.

Determination of the most toxin productive isolate:

The results of the quantitative evaluation of AFB1 toxin produced by the most efficient isolates of *A. flavus*, which is A.f.25C4 grown on YES medium, showed the highest brilliance on the TLC plate (+++++) in comparison to the standard AFB1 toxin Fig. 1b. The concentration of toxin was 904 ppb/Kg Fig. 1a. Thus, this isolate was selected for subsequent tests that included the effect of some treatments on the growth of *A. flavus*.

Determination the efficiency of the effective concentrations of Fylex in inhibiting of *Aspergillus flavus* growth on PDA medium:

As shown in table 1, the results observed that Fylex extract inhibited the growth of *A. flavus* on PDA medium with high inhibitory efficiency. The inhibitory percentages were 71, 85, 100 and 100% for concentrations 0.1, 0.15, 0.2 and 0.3% respectively as compared to control treatment.

These findings were consistent previous studies including with Hasan and Husssein (2016), Husssein *et al*., (2018). Al-Jubouri (2016) reported that Fylex inhibited the growth of *A.flavus* by 74.8%, 100% and 100% using concentrations of 0.1, 0.2 and 0.3% respectively. Previous studies have been shown that brosil, which containing 99% propionic acid has a significant effect in inhibiting the growth of *Aspergillus flavus* growth to 100% (Alhiti, 1977). The high effectiveness of Fylex in inhibiting the growth of *A.flavus* due to it contains strong acids (Hussein *et al*., 2017) and this is in agreement with the findings of Al-Nazal *et al*., (2004) Al-Qassi (2010) which observed that some acids inhibit *A. flavus* growth which affects cereals in warehouses and animal feed.

Table 1: Determination the inhibitory effect of different concentration of Fylex extract on *A. flavus* growth on PDA medium.

Concent-ration (%)	Mean of fungal colony diameter (cm)	The inhibition percentage (%)
0.1	2.61	71%
0.15	1.35	%85
0.2	0	100%
0.3	0	100%
Compared	0.0	0.0
LSD value* P≤0.05	0.507 *	8.447 *

Determination the efficiency of the effective concentrations of MgONPs in inhibiting of *Aspergillus flavus* growth on PDA medium:

As shown in table 2, the results showed that magnesium oxide nanoparticles (MgONPs) has the ability to inhibit *A.flavus* growth after treatment with different concentrations at 0.5, 1, 1.5 and 2g/100 ml; the inhibition percentages were 81.66, 89.33, 98 and 100% respectively.

These results are consistent with Hussein (2017), Hussein (2017), Hussein *et al*., (2018). Furthermore, it also in agreement with many previous studies that observed the high effectiveness of nanoparticles of metals and their oxides in inhibiting plants pathogens (Clement *et al*., 1994; Rico *et al*., 2011). Al-Qaisi (2015) study found that the use of MgO nanoparticles with a concentration of 1, 2, 3% inhibited *A.flavus* growth by 95.53, 100 and 100% respectively, while using silicon dioxide nanoparticles (SiO_2) at similar concentrations show Inhibition percentage at 32.41, 54.82 and 76.17% respectively. MgO nanoparticles are a white, odorless and nontoxic powder that has a high melting point and high hardness. Nanoparticles are widely used in industries due to biocompatibility, biological degradation, and relatively low cost; magnesium oxide is used as an anti-microbial and anti-tumor agent (Tang and Lv, 2014). Superoxide anion O_{2-} is formed on the surface of the MgO nanoparticles that are released and bound with the fungal cell membrane causing the cells die after losing their contents (Yamamoto *et al*., 2010; Emamifar *et al*., 2011). Imada *et al*., (2016) found that the treatment of tomato MgO NPs generated Superoxide anion O_{2-} in its roots as a result of the interaction of MgO NPs with phenols in those roots. Additionally, in a study to control root rot of sugar beet caused by *Rhizoctonia solani*, *F.oxysporum* f. sp. betae and *Sclerotium rolfsii*, the nanoparticles of MgO, titanium dioxide (TiO_2) and ZnO were used and the inhibition percentage of studied fungi growth with using MgO NPs was 62.22, 55.71 and 77% respectively (El- Argawy *et al*., 2017). One of the theories that explain the mechanism by which MgO nanoparticles can inhibit fungi growth is their high surface area and small size that enables nanoparticles to easily penetrate the cell wall causing increased cell size and then death of the fungus as a result of the cytoplasm flow outside the cell (Tan and Bin-Feng, 2014; Ahmad *et al*., 2016). Interestingly, a previous study evaluated the efficiency of MgO nanoparticle in inhibition of fungi growth, and revealed that the inhibition rates reached 40, 60 and 90% for the concentrations of 0.5, 1 and 2% respectively and this is due to the lysis of fungal cell wall (Parizi *et al*., 2014).

Table 2: Determination the inhibitory effect of different concentration of MgO nanoparticles on *A. flavus* growth on PDA medium.

Concentration (%)	Mean of fungal colony diameter (cm)	The inhibition percentage (%)
0.5	1.65	81.66%
1	0.96	89.33%
1.5	0.18	98%
2	0.0	100%
Compared	0.0	0.0
LSD value* P≤0.05	0.488 *	9.361 *

Determination the efficiency of the effective concentration *Ganoderma lucidum* powder in inhibiting of *Aspergillus flavus* growth on PDA medium:

As shown in table 3, the results showed the efficiency of the *G. lucidum* powder in inhibiting *A.flavus* growth at low concentrations, in contrast to the high concentrations. The inhibition percentages were 54.4, 51.66, 46.33, 39.11 and 18.22% for concentration of 2.5,5, 10, 250 and 1000 mg/100 ml respectively.

These results are consistent with previous studies Jonathan and Awotona (2010), Kumar *et al*., (2010), Al-Easawi and Al-Bahrani (2016) and Shahid *et al*., (2016). These studies indicated that the organic and aqueous extracts of *G. lucidium* have anti-fungal activity against fungi causing plant diseases and the highest inhibition ratio was 64% using adopting agar absorption method, while the lowest percentage was 38% after treatment with aqueous extract using Agar well diffusion method, and the concentrations were 5%, 10%, 15% and 20%.

In MTT test to evaluate the anti-cancer activity of *G. lucidum* extracts, the methanol extract at concentration 15.6 µg/ml showed inhibition ratio of 53.96%, the ethanol extract at concentration of 31.2 µg/ml with inhibition ratio

Table 3: Determination the inhibitory effect of different concentration of *G. lucidum* on *A. flavus* growth on PDA medium.

Concentration (mg)	Mean of fungal colony diameter (cm)	The inhibition percentage (%)
2.5	4.1	54.4%
5	4.35	51.66%
10	4.83	46.33%
250	5.48	39.11%
1000	7.36	18.22%
Compared	0.0	0.0
LSD value* P≤0.05	1.064 *	7.923 *

52.38%, while the aqueous extract at concentration of 62.5µg/ml the inhibition rate was 50.75% (Fathima and Reenaa, 2016). Furthermore, Shahid *et al*., (2016) found that methanol extract of *G. lucidum* at a concentration of 20% inhibited *Fusarium oxysporum* and *Alternaria alternate* growth by 57% and 52% respectively. The most of the biological activities of *Ganoderma* are belong to the presence of Triterpenoids, Steroids and Polysaccharides (Baby *et al*., 2015; Paterson, 2006). Wang and Ng (2006) isolated an antifungal protein called Ganoderm in from *G. lucidum* and this protein inhibited the fungal growth of plant pathogenic fungi including *B. cinerea*, *F. oxysporum* and *P. piricola*, which are pathogens infect cucumbers, cotton and apples respectively, where toxins are released causing risk to human and animal health.

As shown in table 3, it is found that the increase in the concentration of *G. lucidium* corresponds to a decrease in the rate of inhibition, which means that the high concentration of *G. lucidium* encouraged the growth of *A.flavus*. The explanation of this may be due to the various compounds which its presence increased with high concentration, which encouraged the growth of *A.flavus*. In addition, pH of *G. lucidium* powder is 5.9, and the optimum pH for conidia formation of *Aspergillus* is 6.5 and pH for *Aspergillus* growth is 7.5, thus increasing the concentration of *G. lucidium* in the medium containing *A.flavus* will change pH of the medium and makes it suitable to the growth of *Aspergillus* and production of conidia.

It is possible that one of the reasons that drive *G. lucidium* powder at high concentration is not able to inhibit *A.flavus* growth but rather stimulated its growth is due to its use in the form of a powder where the presence of various compounds such as proteins, carbohydrates, amino acids, sugars, mineral elements have stimulated the growth of *A .flavus*. This is consistent with study of Jonathan and Awotona (2010) which observed that some extracts of *G. lucidum* had inhibited microorganisms growt hat low concentrations. Danieli, (1957) indicated that the minimum inhibitory concentration (MIC) keeps the extracts strong because the secondary bioactive metabolic, which are vital products, remain available to cause damage to the studied microorganisms, thus the higher concentration may not be required which may be toxic to the host cells.

Table 4: Determination the inhibitory effect of different concentration of *Calotropis porcera*(Latex) on *A. flavus* growth on PDA medium.

Concentration (Ml)	Mean of fungal colony diameter (cm)	The inhibition percentage (%)
0.1	4.2	53.33%
0.2	3.8	57.77%
0.3	3.66	59%
0.5	3.16	64.88%
Compared	0.0	0.0
LSD value P≤0.05	0.574 *	8.440 *

Determination the efficiency of the effective concentration of milky liquid of *Calotropis porcera* (Latex) in inhibiting of *Aspergillus flavus* growth on PDA medium

The results of table 4 found that the milky liquid (brocade milk) (Latex) of *Calotropis porcera* plant has antifungal activity, as *A.flavus* is inhibited on PDA medium by 53.3%, 57.77%, 59% and 64.88% at concentrations 0.1, 0.2, 0.3 and 0.5% respectively in comparison to control group.

These results are consistent with findings of Mohan *et al*., (2007), Shobowale *et al*., (2013), Kori and Alawa, (2014) and Mohamed *et al*., (2019). Al-Baldawi (2012) indicated that the milky liquid (brocade milk) possesses antifungal activity against *A. ochraceus* and *F. graminearum*. The inhibition percentages were 55.50% and 74.50% respectively at a concentration of 0.1%, while it was 100% at concentration of 0.2 and 0.3% for both fungi. This activity os may due to the presence of bioactive components of this plant (Shobowale *et al*., 2013).

Furthermore, the antibacterial activity of Latex *C. procera* is may be due to the presence of Calactin, mudarin and calotropain, which are the active agent in latex (Parotta 2001; Kareem *et al*., 2008). Latex from *C.procera* extract is readily available and used in medicine to treat many diseases. It is used as a wound healing agent, anti-diarrhea, anti-inflammatory and anti-rheumatic. It is also used against malaria and skin infection (Saratha *et al.,* 2010 ; Upadhyay, R.K., 2014). Additionally, several Plants have been reported to contain anti-cancer, anti-microbial, anti-diabetic, anti-inflammatory and antioxidant properties (Kumar *et al*., 2010).

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