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EFFICIENCY OF PLANT EXTRACTS ON *ASPERGILLUS* GROWTH AND AFLATOXIN B1 PRODUCTION IN *ZEA MAYS*

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Abstract

The detection of fungi contaminating maize grain and the effect of four plant extracts *Azadirachta indica, Eucalyptus globulus Glycyrrhiza glabra* and *Zingiber officinale* on the growth of *A. flavus* and its ability to produce AflatoxinB1. The results showed that the incidence of *Aspergillus* spp., was 52.75% of the isolated fungi, of which 29.50% was due to *Aspergillus flavus*, followed by *Penicillium* spp., with an incidence of 21.06%, and then *Fusarium* spp., with a rate of 18.13%. The percentage of toxin-producing *A. flavus* isolates reached 70.8% out of 24 isolates. The results showed the effect of alcoholic plant extracts at a concentration of 10 mg/ml on the fungal growth activity of *A. flavus*, the alcoholic extract of neem leaves was superior to the alcoholic extract with an inhibition rate of 92.79% than that of the control treatment, followed by ginger extract with an inhibition of 60.14%, then eucalyptus extract with a medium inhibition rate of 53.88%. While the licorice extract showed a weak inhibition rate of 17.77 %. The lowest inhibitory concentration for the growth of the fungus for neem extract was 24 mg/ml. While the lowest inhibitory concentration of ginger extract was 48 mg/ml, while eucalyptus and licorice extract did not achieve complete inhibition of fungal growth despite using a concentration higher than 48 mg/ml for both types. The results indicated that the neem plant extract inhibited the production of AFB1 toxin in YES media by 100% at a concentration of 12 mg/ml, followed by ginger extract at a concentration of 24 mg/ml, while the eucalyptus extract achieved a complete inhibition of AFB1 production at the last concentration (48 mg/ml). The extract of licorice plant did not show a complete inhibition of toxin production, as the highest percentage of inhibition was 39.98% at a concentration of 48 mg/ml.

Key words: Plant extracts, Inhibition, *Aspergillus*, Aflatoxin, Fungi, Growth.

Introduction

Many agricultural crops, especially grain crops are infected with pathogenic and saprophytic fungus, whether in the field or during harvesting and storage. Fungal injuries cause a lot of direct and indirect losses of crop, lack of yield and quality. Fungi also caused public health problems and cattle not only through their contamination for consumer food or animals but in production of mycotoxins. It is unfortunate that the contamination of agricultural crops and their products with various fungi, including fungi that produce mycotoxins, is difficult to avoid. International reports indicate that 25% of global agricultural production is contaminated with mycotoxins (Eskola *et al*., 2020); this is a high percentage that represents the degree of danger to human health and the health of his livestock. The number of fungal toxins has more than 400 compounds now; possess a dangerous biological activity to human health, livestock and pets (Mina *et al*., 2022). Among this large number of toxins, aflatoxins represent the most harmful and dangerous group, being highly effective even at low concentration levels estimated at parts per billion. There are attempts all over the world to treat contamination of agricultural products especially grain crops with aflatoxins (AFB1). *Aspergillus parasiticus*, *Aspergillus flavus* are the main fungal species responsible for the secretion of AFB1 and the most common microorganisms that contaminate feed and other agricultural products (Rajarajan *et al*., 2021). Despite the efficiency of some chemical fungicides in controlling pathogenic and toxigenic fungi, the presence of traces or residues of these compounds on agricultural commodities are no less dangerous than the presence of mycotoxins, especially if the treated agricultural commodity is the last stage in production and is intended for consumption such as grains and nuts. Treatment of Seed is the most effective and cost-effective method of controlling seed borne fungal infections, producing mycotoxins, and preventing biodeterioration of cereal grains. Currently, the use of aqueous and alcohol plant extracts in the treatment of stored grains or commodities an effective and appropriate way to control the problems of pathogenic and toxic fungi and to limit their production of toxins, because they are mostly safe for the health of and animals, found naturally or can be cultivated, easy access and the important thing is their humans effectiveness in controlling the spread of toxic fungi and reducing their production of mycotoxins(Jihad *et al*., 2021). In current study four kinds of aqueous extracts plant were tested included *Azadirachta indica, Eucalyptus globulus Glycyrrhiza glubra* and *Zingiber officinale* on growth of *A. flavus* and AFB1production.

Materials and Methods

Detection of fungi associated with maize grains: To detect the fungi associated with maize grains, including *Aspergillus flavus*, maize grains were collected from different places in the local agricultural markets and stores in the city of Ramadi. The seeds were sterilized with 3% sodium hypochlorite solution for 2 minutes, then rinsed with sterile distilled water several times and dried between dry sterile filter papers. The seeds were sown in Petri dishes containing pre-sterilized PDA media by

autoclaving (half an hour at 121°C and pressure 1.5 joules) to which the antibiotic streptomycin was added 200 mg/L (6 seeds in each dish). The dishes were placed in the incubator for 7 days at a temperature of 25 ± 2°C. The fungal isolates obtained from the dishes were purified and preserved in slants contain sterile PDA media containing the antibiotic for the purpose of diagnosis. The fungi accompanying the yellow corn seeds were identified according to the morphological shape and some reproductive and color traits, and the important taxonomic keys were adopted (Klich, 2002; Varga *et al.*, 2011). Because AFB1 is the most important and dangerous among the more than 400 toxic compounds secreted by various fungi as secondary metabolites, and because AFB1 is secreted from specific types of *Aspergillus* fungi, focus was placed on the isolates of *Aspergillus flavus*, as it is one of the most important and most dangerous fungal species that produce toxins.

The ability of *Aspergillus flavus* isolates to produce AFB1: The fungal isolates of *Aspergillus flavus*, which were detected with the fungi associated with maize grains (24 isolates), were tested on the production of AFB1 toxin. Isolates were grown in liquid nutrient medium according to the method used by Salim (2006). Qualitative and quantitative detection of the most AFB1 producing isolates (the most luminous) was carried out using TLC technology, accompanied by the standard substance on a densometer and was used in the subsequent tests in the study.

Preparation of plant materials: Plant materials were prepared for four different types of plants belonging to four different families as shown in (Table 1). Samples were collected from the markets and fields of the College of Agriculture/University of Anbar, according to availability, and the species were diagnosed by specialist in the Field Crops Department. The plant parts of each species were washed with water to remove dust and then sterilized with 2% sodium hypochlorite solution for 5 minutes. The plant parts were cut into slices or small pieces and then placed in an oven at a temperature of 40 degrees Celsius until drying. The plant parts were ground with a mill into a very fine powder.

Table 1. Plant species, their families and the parts used for each type in the study.

Plant scientific name	Common name	Family	Used part
Azadirachta indica	Neem	Meliaceae	leaves
Eucalyptus globulus	Blue gum	Myrtaceae	Leaves
Glycyrrhiza glabra	Liquorice	Fabaceae	root
Zingiber officinale	ginger	Zingiberaceae	rhizome

Preparation of plant extracts: 100 grams of dry powder was taken separately from each plant type and 1000 ml of ethyl alcohol was added mixed well and then left to soak for two days. The extracts were filtered through filter papers in a Buchner funnel as a first stage, and then to obtain a clear filtrate, the filtrate was placed again in the centrifuge in batches at a speed of 5000 rpm for 15 minutes. The total filtrate of each type was placed in a glass beaker in the oven, and then dried at 40°C, and the dry extract was kept at 4°C until use.

Test the effectiveness of plant extracts in inhibiting the growth of the fungus *Aspergillus flavus*: To evaluate the effectiveness of alcoholic extracts of different plant parts in inhibiting the growth of the mycelium of *Aspergillus flavus*, the Czapek dox broth method was used.

One gram of each plant extract was dissolved in 10 ml of culture medium and sterilized by passing through sterile filter paper (Millex.GP) 0.22 µm size, then the volume was completed to 100m of sterile CDB culture medium in a 150 mL beaker to obtain a concentration of 10 mg/mL plant extract and in three replications for each type of extract. The beakers were inoculated with a disc size 0.5 cm from the edge of the isolate of *A. flavus*. 3 beakers were left containing the culture medium with the fungal isolate only without any plant extract as a control treatment. The flasks were placed in an incubator at 25 ± 2°C for 7 days. After incubation, the flasks were taken out and their contents were filtered using filter papers that had been previously weighed. The biomass for the treatments were placed in an oven at 40°C until completely dry and then weighed. The percentage of fungal growth inhibition was estimated according to the equation below:

$$\text{Percentage of inhibition} = \frac{x - m}{x} \times 100$$

where X = Dry weight of biomass of fungus in control treatment
M = Dry weight of biomass of fungus in sample treatment

Measurement of the lowest inhibitory concentration: The lowest inhibitory concentration of alcoholic extracts of the four plants was measured in the growth of the mycelium of the poisonous isolate *A. flavus* and its production of AFB1. Yeast Extract Sucrose liquid yeast extract medium was used, (by preparing 20 g of yeast extract with 200 g of sucrose / liter of sterile distilled water) with different concentrations of alcoholic extracts of plants (3, 6, 12, 24, 48 mg / ml) by dissolving the required amount of each extract in 10 ml of medium Yeast extract and then sterilize by passing it on a 0.22 micropure filter paper. Next, complete the volume by adding 90 ml of sterile yeast extract into 150 ml sterile glass flasks in three replications for each concentration. The flasks were inoculated with a 0.5 cm disc from the edge of the colony of the toxic fungal isolate *A. flavus* at the age of 7 days. Three flasks containing yeast extract medium were also inoculated without adding any alcoholic extract to the plants as a control treatment. The flasks were placed in the incubator at 25±2°C for 14 days. After the incubation period, the contents of each flask were filtered with a pre-weighed filter paper. Biomass weights were measured for all used concentrations of plant extracts, in addition to the control treatment and the lowest inhibitory concentration for each type of plant extract was estimated. The filtrate was kept for each beaker for the purpose of extracting AFB1 toxin and estimating its proportion.

Effect of plant alcoholic extracts on the production of AFB1 toxin: To measure the effect of the alcoholic extracts of the plants used on the production of AFB1 toxin, the method used by (El-Melegy *et al*., 2017) with some modification was used to extract the AFB1, 50 ml of growth medium was taken for each flask and 50 ml chloroform in a 150 ml separating funnel and shake gently while allowing the formed gases to escape more than once, then the lower layer of the mixture was passed over a layer of anhydrous sodium sulfate and the filtrate was received in a clean flask. The process was repeated again by adding another 50 ml of chloroform to the flask and collecting the filtrate. The filtrate was dried in a water bath at 50°C with vacuum pressure to accelerate drying. The precipitate and each replicate were re-dissolved with 1 ml of chloroform and a drip process (15 µl) of the extracts was carried out on Thin Layer Chromatography (TLC) sheets 20 x 20 cm. In addition to the standard aflatoxin (Sigma Chemical Co., St. Louis, USA), the plates were transported into transfer basin containing methanol-chloroform (3-97). After the migration was completed, the plates were lifted from the transfer basin and dried vertically at room temperature. The concentration of AFB1 was measured by ultraviolet radiation (365 nm) using (optical density meter).

Results

Detection of fungi associated with maize grains: The results of the detection of fungi associated with corn grains collected from different markets and warehouses in the city of Ramadi showed the appearance of six fungal species (Table 2). The most prevalent among all fungi was *Aspergillus* spp. (29.50%), and *A. flavus* was the most abundant species among the same genus, reaching 23.25%, followed by the genus *Penicillium* spp., where the percentage of its presence was 21.06%, while the presence of the genus *Fusarium* spp., 18.13%. Whereas, for the incidence of other fungi, it was less, it did not reach the percentages in which the first three fungi appeared. The appearance of each of the genus Rhizopus spp, Cladosporium spp and Mucor spp was 5.54%, 2.18% and 0.77%, respectively. This result shows the importance and danger of the presence of this common fungus on cereals and the potential danger it poses to human and animal health together, as it is one of the genera that excretes very dangerous and biologically influential mycotoxins at low concentration levels, especially the widespread type *A. flavus* that excretes AFB1 toxin, which is the most dangerous globally because of its growth in wide thermal ranges and its ability to withstand harsh environmental conditions.

Table 2. Proportions of the appearance of the fungi species associated with maize grains.

ID	Isolated fungi	Appearance %
1.	*Aspergillus* spp.	29.50
2.	*Aspegillus flavus*	23.25
3.	*Penicillium* spp.	21.06
4.	*Fusarium* spp.	18.13
5.	*Rhizopus* spp.	5.54
6.	*Cladosporium* spp.	2.18
7.	*Mucar* spp.	o.77

Isolates of A. flavus that produce AFB1 toxin: The test results of 24 fungal isolates of *A. flavus* obtained from laboratory detection on the fungi associated with maize grains showed that they produced AFB1 toxin after growing on medium and by transfer technique on TLC plates. 70.8% (17 isolates) of tested isolates produced AFB1 toxin at different levels (Fig. 1). The positive isolates showed clear fluorescence under ultraviolet rays that differed from one isolate to another according to the genetic potential of the isolate. The most fluorescent isolate (the most toxin-producing) was chosen for the subsequent tests.

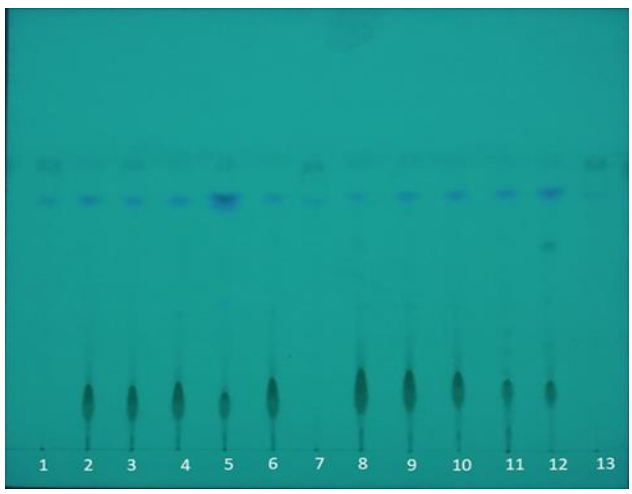

Fig. 1. Strains of *A. flavu* fungus isolated from maize grains that produce AFB1 toxin.
(1,7and 13 AFB1 stander solution. 2-6, 8-12 isolates of *A. flavus* associated with corn grains).

Efficiency of selected plant extracts in inhibiting the growth of A. *flavus*: The results of a study comparing the effect of alcoholic extracts at a concentration of 10 mg/ml for four plant species showed significant differences between them in their effect on the fungal growth activity of *A. flavus* (Table 3). The extract of neem leaves showed the highest inhibition value among the types of alcoholic extracts used, as the dry biomass weight was 0.0483 g, with an inhibition of 92.66% over the control treatment (0.654 g), followed by ginger extract, which showed a medium inhibition ability, with a biomass weight of 0.267 g, with an inhibition of mycelium 59.17%. Then eucalyptus extract with a biomass weight of 0.309 g and an inhibition rate of 52.75%. On the other hand, licorice extract showed a weak inhibition ability among other extracts, as the fungus biomass weight was (0.551 g) and the inhibition rate was 15.74%.

Table 3. Effect of extracts of plant species (10 mg/ml) on inhibiting the growth of mycelium.

Plant species	Dry weight of biomass (g)	Mycelium growth inhibition %
Azadirachta indica	0.048^{E}	92.66
Eucalyptus globulus	0.267^{D}	59.17
Zingiber officinale	0.309^{C}	52.75
Glycyrrhiza glabra	0.551^{B}	15.74
Control	0.654^{A}	0
L.S.D.	0.020	

The values in the column marked with similar letters do not differ significantly at the level of significance (p<0.05)

Estimation of lowest inhibitory concentration: This experiment was conducted using the food poisoning method to measure the lowest inhibitory concentration and the best plant extract in its effect on the growth of the mycelium. Liquid nutrient medium was used as it is suitable for fungus growth and production of AFB1 toxin. The results in this experiment were not different from the results of the previous experiment (10 mg/ml), and the results were expected in the superiority of the alcoholic extract of the neem plant in its ability to inhibit the growth of *A. flavus* than in the rest of the other types of extracts (Table 4). The results of fungal growth inhibition were higher and significant by using the alcoholic extract of neem plant in liquid medium than in the control treatment. The inhibition rate was 92.38% at a concentration of 12 mg/ml, and the lowest inhibitory concentration for the extract of the same plant (100%) was at a concentration of 24 mg/ml, and it was superior to the rest of the other types of plant extracts. These results are consistent with what was recorded by Mondall (2009), which indicated the efficiency of the alcoholic extract of neem leaves in resisting the fungus *A. flavus*. The ginger extract came in second, as the percentage of growth inhibition at a concentration of 12 mg/ml and a concentration of 24 mg/ml was 64.26% and 92.79%, respectively, while the lowest inhibitory concentration of ginger extract was in the growth of mycelium (100%) at a concentration of 48 mg/ ml as completely suppressing the growth of mycelium. Also, the use of the alcoholic extract of eucalyptus had an important and significant activity in reducing the growth of the mycelium. The rate of inhibition of the growth of the mycelium of the eucalyptus extract was 50.31% and 74.06% at the concentration 12 and 24 mg/ml, respectively.

Eucalyptus extract did not achieve 100% inhibition of mycelium growth even when it was used at the highest concentration (48 mg/ml). These results did not agree with what was mentioned by Al-Rahmah (2011) that eucalyptus leaves were effective in reducing the biomass of the fungus by 96.46% at a concentration of 20 mg/ml. The plant extract of licorice showed a weak inhibitory activity in different concentrations compared to the rest of the other extracts used. The highest inhibition was 40.08% when using 48 mg/ml of the plant extract. Also, none of the alcoholic extracts achieved 100% complete inhibition at a concentration of 12 mg/ml or less.

As for the efficiency of alcoholic extracts in inhibiting the production of AFB1 toxin, although the conditions for the production of mycotoxins are not necessarily the same that suit the growth and activity of the fungus that is secreting them (Safari *et al*., 2020; Hadi *et al.,* 2021), the results of this study showed that there is a direct relationship between the increase in the production of the toxin with the growth of the fungus and vice versa. All plant extracts showed different significant differences between them in the amount of AFB1 reduced toxin from the control treatment in which the alcoholic extract of neem plant was distinguished. The addition of the alcoholic extract of the plant with different concentrations caused a significant inhibition in reducing biomass in addition to reducing the production of AFB1 toxin, and for all concentrations (Table 4). The highest inhibition rate was at a concentration of 12 mg/ml upwards, as it achieved a 100% inhibition rate in the production of AFB1 toxin, although the fungus biomass weight was 0.048 and the percentage of mycelium growth inhibition was 92.38%. This result agrees with what was found by Christiane (2010) that the addition of neem leaf oil at a concentration of 4% w/w inhibited 95% of the concentration of AFB1 and AFB2, followed by ginger leaf extract, where the percentage of inhibition of AFB1 was (100%) at a concentration of 24 mg/ml for the extract. The eucalyptus extract showed 100% inhibition of AFB1 toxin production at the last concentration (48 mg/ml) only. On the other hand, the effect of licorice extract was the least among the used types. The inhibition rate at concentrations 12, 24 and 48 mg/ml was 31.55%, 36.14% and 39.98%, respectively.

Discussion

Reports indicate that AFB1 represents the most dangerous mycotoxin globally among more than 400 registered toxic compounds produced by various fungi (Eskola *et al*., 2020). In the current study, the results indicate a high contamination of maize grains with the most effective and most dangerous fungi in their production of toxins, and *A. flavus* is one of a very limited number of fungi that secrete AFB1 toxin. These results agreed with other studies that indicated the contamination of various types of cereals, including yellow corn, with toxin-secreting fungi. It is noted that the fungi Aspergillus and Penicillium in addition to the Fusarium compete in their spread in a common environment where temperature and humidity take their role in the supremacy of one of them over the other species (Mannaa & Kim, 2018).In addition, many studies indicated that many isolates of these species have the genetic ability to secrete toxins, as is the case in the results of this study, the percentage of *A. flavus* isolates that excrete AFB1 toxin was 70.8%. Herein lays the danger of these types. Since grain crops represent the final stage of agricultural production before consumption, it is very difficult to treat fungal contamination using chemical pesticides, regardless of the safety of the pesticides used. Natural plant extracts are a promising field in human search for alternatives to environmentally friendly chemical fungicides, with no or little toxicity to human and animal health, they are cheap and usually available in nature and biodegradable in the environment without negative effects. Alcoholic extracts of four plant species were tested as inhibitory compounds for the growth of mycelia and thus to reduce the production of AFB1 toxin. The results indicated that when using a uniform concentration of plant extracts (10 mg/ml) in their effect on the growth of the mycelium of *A. flavus*, all extracts made a significant difference in the dry weight of biomass as compared With the control treatment (Table 3). The neem extract was strongly distinguished in inhibiting the growth of the fungus with a degree of 92.66%, followed by the effect of ginger extract and then eucalyptus with a medium degree of inhibition almost and a weaker degree of licorice extract, the inhibition rates were 59.17%, 52.75% and 15.74%, respectively. In the

experiment using the culture medium, the neem leaf extract showed a higher inhibition activity than the rest of the other extracts, and thus greater inhibition of AFB1 toxin reached 100% at concentration levels 12, 24 and 48 mg/ml (Table 4). And the results are close to the results of other studies that indicated the effectiveness of neem leaf extract in inhibiting the growth of the fungus and reducing the production of AFB1 toxin (Mondall *et al*., 2009; Christiane *et al*., 2010).The results were similar in the use of ginger extract and eucalyptus leaves with the results of (Al-Rahmah *et al*., 2011; Hadi & Mariod, 2022). The results of (El-Ghany *et al*., 2015), and the difference in the rates of inhibition of biomass or in the production of poison was according to the used concentration used. As for the use of licorice extract in inhibiting the mycelium and the production of AFB1 toxin, although it was significant in relation to the control treatment, it was not at the same levels as the effect of other types of extracts, and it may be necessary to use a high concentration to achieve similar results, as in the study of (Mohseni *et al*., 201; Mohammed *et al.,* 2022), which used the same extract at 500 mg / ml or more to inhibit the production of AFB1 toxin. The results of the study indicated a difference in the ability of the extracts to inhibit the mycelium and the production of mycotoxin with different concentrations, as the neem plant extract was the most efficient in inhibiting the growth of the mycelium and the production of the toxin by 100% at a concentration of 12 mg/ml. While it required the use of ginger extract at a concentration of 24 mg / ml and eucalyptus at a concentration of 48 mg / ml to achieve the same result. This may be due to the different concentrations of the same chemical compounds contained in the extracts of plant species, or the difference in the compounds affecting each extract and thus the difference in the type and degree of influence on the growth of the fungus and its production of toxin (Lorán *et al*., 2022). Some researchers (Nazzaro *et al*., 2013) attributed the effect of plant extracts to their effect on the walls of fungi or cell membranes, which hinders the ion exchange process in them or disrupts the osmosis process. Its effect may be dependent on its ability to penetrate through cell membranes and bind to some enzymes or wall proteins, it is possible that those active compounds in plant extracts participate directly or indirectly in the primary reactions of the manufacture of mycotoxins, which leads to obstruction or inhibition of their manufacture (Shuping & Eloff., 2017).

Table 4. The lowest inhibitory concentration of plant extracts in the growth of mycelium and AFB1 production.

Plant species	Concentration of extract(mg/ml)	Dry weight of biomass(g)	Concentration of AFB1 ppb	AFB1 inhibition %
Azadirachta indica	0.00	0.630^{F}	160.20	0
	3.00	0.272^{E}	52.90	66.97
	6.00	0.195^{D}	10.40	92.92
	12.00	0.048^{C}	0.00	100
	24.00	0.000^{B}	0.00	100
	48.00	0.000^{A}	0.00	100
L.S.D.		0.024		
Eucalyptus globulus	0.00	0.644^{F}	161.30	0
	3.00	0.558^{E}	102.40	36.51
	6.00	0.412^{D}	80.90	49.84
	12.00	0.320^{C}	67.10	58.40
	24.00	0.167^{B}	28.00	82.64
	48.00	0.020^{A}	0.00	100
L.S.D.		0.096		
Glycyrrhiza glabra	0.00	0.681^{F}	161.30	0
	3.00	0.659^{E}	149.90	7
	6.00	0.596^{D}	126.20	21.7
	12.00	0.543^{C}	110.40	31.5
	24.00	0.478^{B}	103.40	35.87
	48.00	0.408^{A}	96.80	39.98
L.S.D.		0.0354		
Zingiber officinale	0.00	0.680^{F}	161.20	0
	3.00	0.425^{E}	107.50	33.31
	6.00	0.306^{D}	40.80	74.68
	12.00	0.243^{C}	0.10	99.93
	24.00	0.049^{B}	0.00	100
	48.00	0.000^{A}	0.00	100
L.S.D.		0.036		

The values in the column marked with similar letters do not differ significantly at the level of significance (p<0.05)

Recommendations

The data of this research indicate that the alcoholic extracts of the types of plants studied, especially the extract of neem and ginger, in addition to eucalyptus, taking into account the results of another research, It can be adopted as fungicides or growth retardants for toxic fungi as an alternative to chemical pesticides whose residues may have harmful effects on human and animal health alike. And urging the continuation of the search for other plant extracts that may be more effective, while defining the mechanism of action and their effective active substances.

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