The influence of some plant extracts in control of fungi contaminating tissue culture media of date palm (*Phoenix dactylifera* L.)

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Abstract

The study aimed to isolate and diagnose the fungi associated with tissue palm growing media and their resistance to the method of poisoning the growing media of medicinal plants (cloves , ginger , currants , black seed) , compared with the effect of the chemical pesticide Beltanol . The results of isolation and diagnosis showed the dominance of the *Aspergillus* fungus in most of the samples under study and in a percentage appearance of 80% , while the rates of appearance of the rest of the species ranged between 40-60% . The outcomes of the test for pathogenicity in infecting the seeds of the four fungi that were chosen in this study showed that the seed germination rate was 0.0% and the seed inhibition rate was 100%, compared to the comparison treatment, which had a germination rate of 100% and an inhibition rate of 0.0% . The PCR analysis proved that the isolates belong to the fungi. *Aspergillus niger , Penicillium chrysogenum, , Aspergillus flavus and Meyerozyma guilliermondii*, as the isolates were recorded Fungi in the National Center for Biotechnology Information (NCBI) and under the special symbols (PQ895505.1 , PQ895508.1 , PQ895505.1 and PQ895494.1) respectively. The effectiveness of the pesticide Beltanol against the tested fungal isolates was studied, and showed that it was most effective on the two Fungi *P. chrysogenum* and *A. flavus* by 100% and 88% % respectively . the alcoholic clove extract at a concentration of 20% was superior in inhibiting fungi by 98.15% over the fungi *A. niger, A. flavus, and P. chrysogenum*, and 96.2% against the fungi. *M. guilliermondii*, and the aqueous extract of cloves at a concentration of 20% gave the highest antioxidant activity against the fungus *A. flavus,* at a rate of 88.88%, compared to the comparison treatment, which had an inhibition rate of 0.00%. The alcoholic extract inhibited the growth of the fungus at higher rates than the aqueous extract. The inhibition rates of the pathogens increase with increasing concentration of aqueous and alcoholic extracts.

Keywords : tissue culture , *Phoenix dactylifera* L. , Fungi .

Introduction

The date palm (*Phoenix dactylifera* L.) is grown in many regions of the world, and Iraq occupies a distinctive role in its cultivation, widely distributed in most Iraqi governorates [1]. The date palm reproduces in two ways: either through seeds, which takes a long time and produces varieties that differ from the mother genetically, or through Seedling: Due to the small number of seedlings produced by one palm tree [2] , attention has been drawn to this To tissue culture to compensate for the decrease in the number of date palms, and despite the efficiency of tissue culture technology in terms of the abundance of plants that can be produced from a single plant-Ttissue culture faces major challenges, the most important of which is contamination with microorganisms, as they cause rot and browning of the planted plant part and the destruction of its tissues. The reason is due to toxic and growth-inhibiting substances and enzymes secreted by contaminated microorganisms [3,4] Fungi are among the most important pollutants in date palm tissue farms in Iraq, their broad ability to grow anywhere and the availability of a suitable food source in the culture media for tissue cultivation. especially sugars, which stimulate their growth and development, means significant losses in the numbers of plant tissue cultures [5] Many studies have used fungicides to control fungal contamination in tissue culture laboratories [6].

Despite the high efficiency of fungicides, their use may lead to an impact on the structural formula of carbohydrates and also affect the biological metabolism of nitrogen in the treated plant, and toxicity. High levels of plant tissue [7,8] , so modern research has turned to use highly effective friendly materials as an alternative to chemical pesticides A study to evaluate the effectiveness of aqueous extract and ginger powder against fungi isolated from potato tuber slices indicated that ginger powder was highly effective against the fungi *Penicillium sp. Aspergillus niger and Colletotrichum* sp. The cause of rot in the “Ghana” cultivar had a complete inhibition rate (100%). The aqueous extract tested at a concentration of 30% was more effective with an inhibition rate of 80%. This encourages the use of extracts. Aqueous extract and *Zingiber officinale* powder were used as a fungicide to improve the shelf life of potato tubers [9] In another study, the results showed that the aqueous extract of cloves inhibited the radial growth of the fungi *Aspergillus flavus, Aspergillus niger,* and crassa Neurosporo, and its effectiveness increased as the concentration increased and decreased as well. The severity of infection with contaminated fungi compared to the control indicated Results from qualitative chemical examination on the active compounds in the plant extract , The extract contains effective antifungal compounds [10] , study on essential oils and their main compounds reported their antifungal activity against *Colletorichum gloeosporoides and Penicillium digitatum*, which are present as main compounds in Nigella sativa seeds and clove bud essential oils as environmentally friendly and antifungal agents[11]Therefore, the study aimed to isolate and diagnose fungi associated with palm tissue culture media and combat them using some plant extracts.

Materials and Methods

Isolation of contaminated fungi

Samples were collected from tissue culture laboratories of palm plants from the governorates of Iraq (Baghdad, Najaf, Basra) from the date of (7/1/2023 to 10/15/2023) and they were in the multiplication stage for the Barhi variety, they were transferred to the pathology laboratory in the Plant Protection Department - College of Agriculture - University of Kerbala. The samples were kept in the refrigerator until they were processed. The contaminated fungi were isolated after symptoms of contamination appeared. Fungi on tissues after preparing the sterilized PDA culture medium by autoclaving At a temperature of 121°C and a pressure of 15 pounds/ang for 20 minutes, with the addition of the antibiotic Chloramphenicol at a rate of 250 mg per ature, then the dishes were incubated in the incubator at a temperature of 25±2°C for seven days, then they were re-purified by taking a disk with a diameter of 0.5 cm from the edge. The mushroom colony was placed in a petri dish containing the PDA culture medium, and the dishes were incubated again in the incubator at a temperature of 25±2 for seven days, The fungal samples were then purified in a sterile atmosphere, and then the samples were preserved for the purpose of conducting tests on them. The appearance rates of the fungi were taken according to the following equation.

$$\%\ \text{fungal isolates appearing} = \frac{\text{the number of fungi that appeared in the samples}}{\text{the total number of samples}} \text{x}100$$

Testing the pathogenicity of fungal isolates

The pathogenicity test was conducted using susceptible radish seeds on sterilized MS medium in the incubator at a temperature of 121 and a pressure of 15 pounds per and for 20 minutes at a rate of (4.43 grams per liter) for the fungal isolates that were previously isolated and purified, which are about 32 fungal isolates, in order to reduce their number and select them. The most virulent ones and used in subsequent tests and experiments. The medium was prepared and then inoculated with fungal isolates from pure 5-day-old cultures with three replicates for each, using a 0.5 cm diameter cork auger and placed in the dishes. The dishes were then planted with sensitive red radish seeds after sterilizing the seeds with sodium hypochlorite at a concentration of 2% for two minutes, then washed well with water. Sterile was distilled, dried by placing it on sterile filter paper, and then transferred by Sterile forceps, which were placed on the plates with 10 seeds in each plate in a circular manner. As for the control treatment, three plates were left containing MS culture medium only, and the seeds were planted on them. Then they were placed in the incubator and incubated at a temperature of 25°± 2ºC. After 5 days, the percentage of germination was calculated. Moreover rotten seeds and the percentage of inhibition using the following equations:

$$\%\ \text{germination} = \frac{\text{treatment number of sprouted seeds}}{\text{compared to sprouted seeds number}} \text{x}100$$

$$\% \text{ inhibition} = \frac{\text{by treatment sprouted seeds number} - \text{comparison in sprouted seeds number}}{\text{by comparison sprouted seeds number}} \text{x100}$$

Molecular diagnosis of selected fungal isolates

To study the genetic and hereditary structure of the fungal isolates and compare it with the genome of global isolates, they were diagnosed by analyzing the sequence of the DNA bases. The PCR products were sent to the Macrogen company in South Korea determine the nucleotide sequence of the genetic region Internal Transcribed Spacer (ITS) and after receiving the sequences nucleotides of fungal isolates sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program to compare them with the data available at the National Center for Biotechnology Information (NCBI) within the electronic gene bank that belong to the fungal isolates that have been diagnosed globally[12].

Testing the chemical pesticide Beltanol against fungal isolates

To test the effect of the pesticide on the fungi, it was mixed at 1 ml per liter with the PDA food medium sterilized in an autoclave at a temperature of 121 and a pressure of 15 lbs/ang for 20 minutes. Then the dishes were inoculated with the pathogenic fungus with 3 replicates for each concentration using a sterile cork perforator with a diameter of 0.5 mm with the presence of control treatment: Then the plates were incubated at a temperature of 25°C±2°C for a week After that, the result was taken by measuring the diameter of the inhibition zone, which represents the fungal growth zone, using a ruler, and the inhibition rate was measured according to the following equation:

$$\% \text{ Inhibition} = \frac{\text{treatment in fungi growth rate} - \text{comparison in fungi growth rate}}{\text{comparison in fungi growth rate}} \text{x100}$$

Preparation of extracts

Four medicinal plants were tested: cloves, ginger, Gujarat, and black seed. The selected medicinal plants were collected from local markets, then ground and preserved to make extracts of both aqueous and alcoholic types, according to the table below:

Table (1): Medicinal Plants used in the study

Seq	Plant name and scientific name	English name	The part used
1	*Syzygium aromaticum*	Indian clove	Flower buds
2	*Zingiber Officinalis*	Ginger	Roots
3	*Hibiscus sabdariffa*	Gujarat	Cup leaves
4	*Nigella sativa*	Black seed	Seeds

Aqueous extract

After the plants used in the experiment were obtained from local markets, the method of [13] was followed, by weighing 100 grams of dry plant powder and dissolving it in 1000 ml of boiled distilled water after cooling it to 60 degrees Celsius, then placing it

on the Magnetic Sterile Hotplate device. For 2 hours, then filter the mixture using medical gauze. Then it was filtered using Whatman No. 1, 2, 3 and 6. The filtrate was distributed in the tubes of the centrifuge at a speed of 3000 rpm for 10 minutes, after which the filtrate was subjected to evaporation utilizingof a rotary evaporator under low pressure and temperature (40-50)°C.

Dry the remainder using dishes with a large surface area in the oven at a temperature of 40°C until the water evaporates completely, thus obtaining a dry powder of the aqueous extract. It is placed in tightly sealed sterile glass tubes, after which it is marked and stored in the refrigerator at a temperature of 4°C until use. In subsequent tests, the extracts were kept in the refrigerator in sterile test tubes for further use.

Alcoholic extract

Following the method of [13], the procedure was proceed 100 grams of plant powder were weighed and dissolved in 1000 ml of ethyl alcohol at a concentration of 70%, and the mixture was placed in a shaking incubator for 24 hours at a temperature of 35 degrees Celsius After that, the mixture was filtered using medical gauze and then using papers . Whatman No.1, 2, 3 and 6

The filtrate was distributed in the tubes of the centrifuge at a speed of 3000 rpm for 10 minutes, after which the remaining filtrate was exposed to evaporation employing of a rotary evaporator under low pressure and temperature (40-50) C. Then the remainder was dried using dishes with a large surface area in the electric oven at a temperature 40°C, The process was repeated several times in order to obtain a sufficient quantity of the extract, then it was placed in tightly sealed sterile glass tubes. After that, it was labelled and stored in the refrigerator at a temperature of 4°C until use in subsequent tests.

Testing the antagonistic activity of extracts (aqueous and alcoholic) against fungal isolates

The sensitivity of the fungi to the extracts was tested by mixing the extract at concentrations (0, 15, 20) with the PDA nutrient medium, sterilized in an autoclaved for 20 minutes. Then the dishes were inoculated with the pathogenic fungus in 3 replicates for each concentration using a sterilized cork perforator with a diameter of 5. mm. Then the plates were incubated at a temperature of 25°C±2°C for a period of time A week later, the results were taken by measuring the diameter of the inhibition zone, which represents the area of fungal growth, using a ruler The inhibition rate was measured according to the following equation:

$$\% \text{ Inhibition} = \frac{\text{treatment in fungi growth rate} - \text{comparison in fungi growth rate}}{\text{comparison in fungi growth rate}} \text{x100}$$

Statistical analysis

All experiments were carried out according to complete randomization (C.R.D.) as one-factor or two-factor experiments as needed. The means were compared according to the L.S.D (Less Significant Differences) method and under the probability level 0.05 [14].

Results and Discussion

Isolation of contaminated fungi

The results (Table 2) showed the dominance of the *Aspergillus* fungus in most of the samples under study, with an occurrence rate of 80% for sample N3R3, which agreed with many studies [15,4 16], then it was followed by samples M2R1 and N2R2, with an occurrence rate of 60%. [17] mentioned that the fungus *Aspergillus* is one of the most important pollutants for tissue culture in banana plants, while [18,19] mentioned that the fungus *Penicillium spp* repeatedly appeared in the tissue culture of palm plants. While sample N7R2 was the least abundant, with a rate of 40%, it was characterized by high pathogenicity. The difference in occurrence rates is due to the fungi's differences in their ability to withstand environmental conditions and produce reproductive structures and spread in most parts of the biosphere [5].

Table (2): The percentage of appearance of fungal isolates contaminating tissue culture of palm plants

Seq	Name of fungi	% to appear
1	N3R3	% 80
2	N2R2	% 60
3	M2R1	% 60
4	N7R2	% 40

Testing the pathogenicity of laboratory-selected fungal isolates

The results (Table 3) showed that some fungi can infect seeds and cause their destruction by 100%. These are the four fungi that were chosen for this study, where the germination rate was 0.0% and the inhibition rate was 100%. Compared to the comparison treatment, which had a germination rate of 100% and an inhibition rate of 0.0%, this is due to the fungi's ability to parasitize and their possession of plant tissue-degrading enzymes such as cellulose, hemicellulose, and pectin, which are capable of decomposing and destroying plant tissues in addition to their production of toxins [20].

Table (3): Percentage of germination and inhibition of fungal isolates

Seq	Treat name	% germination	% inhibition
1	Comparison	100	0
2	N3R3	0	100
3	N2R2	0	100
4	M2R1	0	100
5	N7R2	0	100

Note: the numbers in the table are percentages of inhibition, and germination and each number is the result of three replicates.

Molecular diagnosis of fungal isolates

The results of nucleotide sequence analysis confirmed that the isolates belong to the *fungi Aspergillus niger , Penicillium chrysogenum , Aspergillus flavus , and*

Meyerozyma guilliermondii. The fungal isolates were registered at the National Center for Biotechnology Information (NCBI) under the special codes (PQ895505.1, PQ895508.1, and PQ895505). 1 PQ895494.1) respectively.

The molecular nucleotide sequences achieved the highest percentage of identity with the ITS genetic region when compared with the equivalent nucleotide sequences retrieved from GenBank at NCBI using the BLAST program. Nucleotide analyses were conducted using the MEGA program to analyze the isolates and draw a genetic tree between these isolates and similar isolates registered at the NCBI Center. It was built from the nucleotide molecular sequence of each isolates' ITS region the first record of *M. guilliermondii and P. chrysogenum* as contaminants of tissue culture.

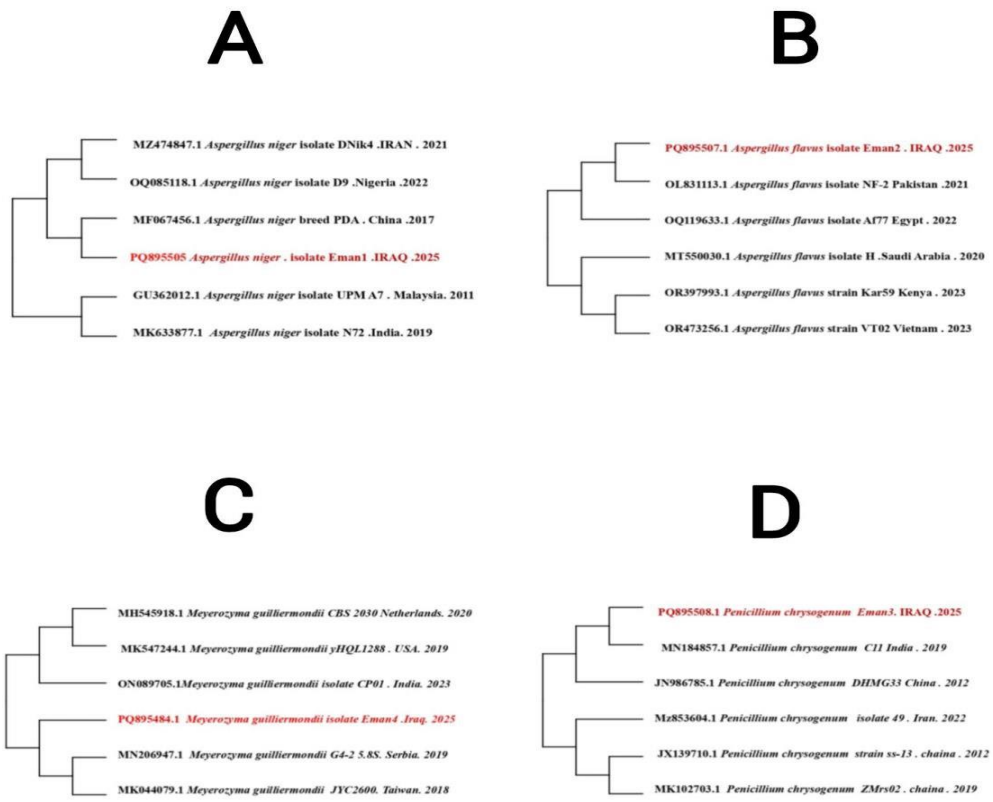

Figure (1): where A: genetic tree of the fungus *A. niger Isolat Eman1* B: Genetic tree of the fungus *A. flavus Isolat Eman2* C: Genetic tree of the fungus *M. guilliermondii Isolat Eman4* D: Genetic tree of the fungus *P. chrysogenum Isolat Eman3*

Evaluation of the efficiency of the chemical pesticide Beltanol in inhibiting the growth of laboratory pathogenic fungal isolates

The results of the effectiveness of the pesticide Beltanol against the fungal isolates tested (Table 5) showed that it had the most effect on the fungi *P. chrysogenum* and the fungus *A. flavus* by 100% and 88%, respectively, followed by the fungus *A. niger* by 79.62%. The results agreed with [17] , reported that the effectiveness of the pesticide was at an inhibition rate 100% of all tested fungi, including *Aspergillus sp*. And the

fungus *Penicillium sp.* It also showed that adding the pesticide butanol to the banana growing medium positively affected the fresh and dry weight of the tissues. While the fungus *M. guilliermondii* was the least affected by the chemical pesticide, at a rate of only 69.62%, The pesticide Beltanol had not been used to control it before. This study is considered the first of its kind to test the interaction between the pesticide Beltanol and the fungus *M. guilliermondii*.

Evaluating the efficiency of alcoholic plant extracts in inhibiting the growth of fungal isolates in vitro

The results (Table 4) showed that all alcoholic plant extracts had a significant effect in inhibiting the growth of fungal isolates, as the clove extract excelled with an inhibition rate of 98.15% at a concentration of 20% against *A. niger, P. chrysogenum, and A. flavus,* followed by ginger extract at a concentration of 20%. The inhibition rate was 98.15% for the two *fungi A. flavus M. guilliermondii,* while the inhibition rate for *A. niger* was 96.29%. This was compared with the comparison treatment, which amounted to 0.0%, the results agreed with [21], which showed that ginger extract is effective in combating the fungus *A. niger*, followed by other extracts, as the alcoholic extract of gujarat recorded a high inhibition rate against fungal growth , ranging from 68.51% to 88.88%, while black seed extract did not record good results, as It was Its highest percentage of inhibition was against the fungus *A. flavus*, which reached 35.18%.

Table (4): Effect of alcoholic extracts in inhibiting fungal isolates

Seq	Treatment	Percentage of inhibition				Treatment rate
		A. niger	*P. chrysogenum*	*A. flavus*	*M. guilliermondii*	
1	Control	0	0	0	0	0
2	Beltanol	79.62	100	88.88	69.62	84.53
3	Clove extract 20%	98.15	98.15	98.15	96.29	97.68
4	Clove extract 15%	96.29	96.29	96.29	94.44	95.83
5	Ginger extract 20%	96.29	79.62	98.15	98.15	93.05
6	Ginger extract 15%	94.44	68.51	88.88	94.44	86.57
7	Gujarat extract 20%	74.07	81.48	88.88	69.62	78.51
8	Gujarat extract 15%	70.37	75.92	83.33	68.51	74.53
9	Black seed extract 20%	18.15	23.89	20.18	35.18	24.35
10	Black Seed Extrat 15%	16.11	18.15	18.15	31.29	20.92
	Fungi rate					
	64.35		64.20		68.09	65.76
L.S.D for treatments = 2.189			L.S.D for fungi= 1.384			L.S.D for overlap= 4.377

Note: The numbers in the table are percentages of inhibition, and each number is the result of three replicates

The results agreed with [22] who only proved that clove extract is effective against *A. flavus* because it contains eugenol and caryophyllene (caryophyllene and eugenol). [23] found that clove extract is effective against the fungi *A. flavus and A. niger,* as

[24] showed that The effectiveness of clove oil against fungi *P. chrysogenum*, the reason for the superiority of cloves is due to the presence of eugenol as the main compound It also contains saponins, alkaloids, flavonoids, glycosides, tannins, and steroids. Cloves and their various extracts have various biological antifungal activities [25]. As for ginger, results of studies have shown that aqueous and ethanolic ginger extracts and raw juice contain important components such as flavonoids, alkaloids, phenols, terpenoids, and tannins, and a variety of active components, including anthocyanins, tannins, and pungent phenolic compounds known as gingerols, zingerone, shogaols, and sesquiterpenes [26,27] While the effect of the gujarat plant was due to phenolic compounds, which include flavonoids, which are the most abundant group of compounds in the plant. The study conducted by [28], showed that gujarat extracts have strong antimicrobial effects against many pathogens, which supports the potential of flavonoids as a therapeutic agent .It was found that the gujarat plant contains essential oils and important substances such as limonene and linalool. [29].

Shokri [30] discussed The antifungal potential of black seed seeds and their ability to combat many types of filamentous fungi and yeasts, and in another study conducted by Singh and others [31] they demonstrated the ability of ethanolic extract and black seed oil in combating the fungi *A.niger and A.niger and Penicillium viridicatum.*

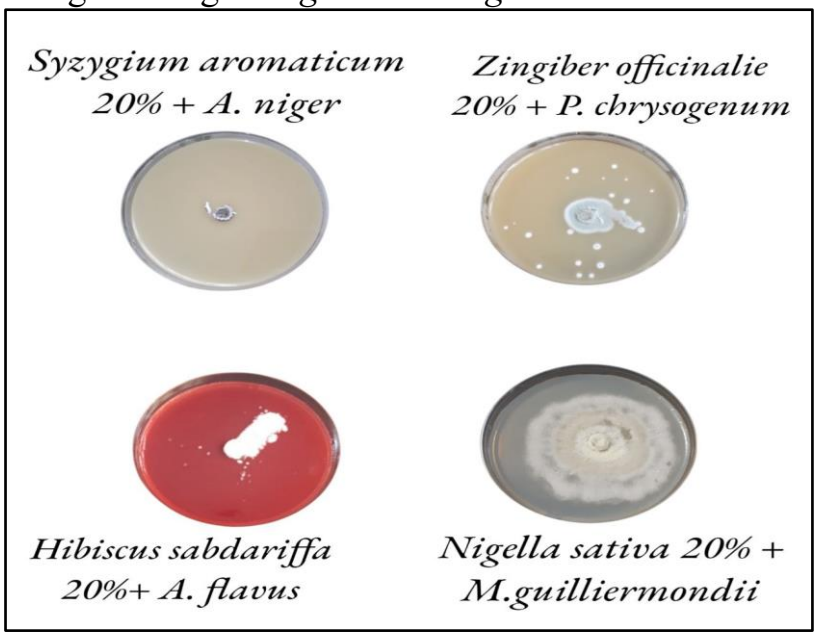

Figure (2): The effect of alcoholic extracts on fungal growth

Evaluation of the efficiency of aquatic plant extracts in inhibiting the growth of laboratory pathogenic fungal isolates

The results of an experiment on the effectiveness of aqueous plant extracts against the tested fungal isolates (Table 5) showed that they had lesser effect on the fungi compared to alcoholic extract Which is due to the fact that alcohol helps extract more active substances. The clove extract at a concentration of 20% gave an antagonistic activity against the fungus *A. flavus* with a rate of 88.88%. This is consistent with [32],

while the ginger extract was more effective against the fungi *A. niger* and the fungus *A. flavus*, by 72.22 and 79.62%, respectively, and the ginger extract had a greater effect on the fungi *A. niger* and the fungus *M. guilliermondii*. By 75.92 and 79.62, respectively, the effect was the same. The rest of the aqueous extracts was average, according to the table below.

Table (5): Effect of aqueous extracts in inhibiting fungal isolates

seq	Treatments	Percentage of inhibition				Treatment rate
		A. niger	*P. chrysogenum*	*A. flavus*	*M. guilliermondii*	
1	Control	0	0	0	0	0
2	Beltanol	79.62	100	88.88	69.62	84.53
3	Clove extract 20%	51.85	48.15	88.88	48.15	59.26
4	Clove extract 15%	42.59	31.48	81.48	42.59	49.53
5	Ginger extract 20%	72.22	57.41	79.62	48.15	64.35
6	Ginger extract 15%	48.15	48.15	68.51	46.29	52.77
7	Gujarat extract 20%	75.92	38.88	48.15	79.62	60.64
8	Gujarat extract 15%	68.51	27.77	33.33	77.77	51.85
9	Black seed extract 20%	12.78	24.07	12.78	22.22	17.96
10	Black Seed Extrat 15%	9.26	18.15	12.78	12.78	13.24
	Fungi rate					
	46.09		39.40	51.44		44.72
L.S.D for treatments = 2.319 L.S.D for fungi= 1.467 L.S.D for overlap= 4.639						

Note that the numbers in the table are percentages of inhibition, and each number is the result of three replicate

Abdallah [33] mentioned that these black seed seeds are rich in biologically active compounds, containing alkaloids, steroids, saponins, terpenes, monoterpenes, and phenolic compounds. Despite the presence of these compounds, the results obtained were not good.

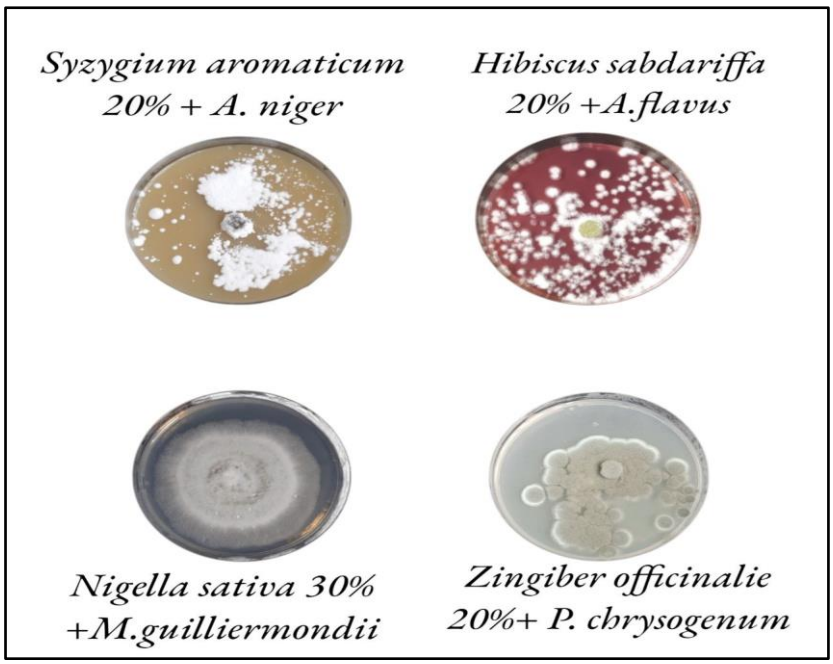

Figure (3): The effect of aqueous extracts on fungal growth

The percentages of inhibition also differed between the aqueous and alcoholic extracts, and this difference may be due to the difference in the polarity of the solvent used and the same extraction method for the studied plant. From the above, the importance of the type of solvent used in extracting active substances from the plant becomes apparent, which may have led to differences in the occurrence of the mechanism of toxic effect when The type of solvent varies, and this may be due to the presence of more than one active substance in the plant, However it does not dissolve in water, while alcohol is a suitable solvent for it. [34] stated that the chemical examination of clove extracts (aqueous and alcoholic), showed a difference in the percentages of active substances, as the ethanolic extract contains a higher percentage of flavonoids and contains saponins and quercetin, while the aqueous extract does not contain them.

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