

Full Length Research

Influence of bioactive metabolites extracted from three species of nematode-trapping fungi on the growth of *Escherichia coli* and *Staphylococcus aureus*

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The effect of different concentrations of cultures filtrate, crude filtrate extracts and fungal mycelia crude extracted from three species of nematode-trapping fungi (*Arthrobotrys dactyloides*, *Arthrobotrys oligospora* and *Dactylella brochopaga*) on the growth of *Escherichia coli* and *Staphylococcus aureus* by using a disc diffusion technique was examined. All fungal extracts exhibited an inhibitory action against both bacterial isolates. It was noted that the crude filtrate extract is more efficient in inhibitory action than cultures filtrate and fungal mycelia crude extract. Moreover, cultures filtrate and crude filtrate extracts possessed higher inhibitory effect against *E. coli* when compared with *S. aureus*. It was also noted that the crude filtrate extracts inhibited the growth of bacteria more efficiently than that of cultures filtrate. Extracts of *A. oligospora* showed the best inhibition effect, followed by *D. brochopaga* then *A. dactyloides*. The cultures filtrate of *A. oligospora* showed the highest inhibitory ability against *E. coli* and *S. aureus* which amounted to 13.17 and 11 mm, respectively, followed by *D. brochopaga* which amounted to 9.67 and 8.58 mm, respectively. The crude filtrate extracts of *A. oligospora* exhibited an inhibitory action reaching 16.42 and 14.50 mm, respectively, followed by *D. brochopaga* (13.17 and 11.42 mm, respectively). A comparison between the inhibitory action of three commercial drugs and the fungal extracts showed that the cultures filtrate and crude filtrate extracts rendered a greater bioactivity than the examined drugs.

Key words: Nematode-trapping fungi, *Arthrobotrys oligospora*, antibacterial, antibiotics, bioactive metabolites, growth inhibition zones.

INTRODUCTION

In order to antagonize or kill their competitors, many microorganisms produce toxic metabolites, such as antibiotics (Herrera-Estrella et al., 2016). Toxins are particularly important for parasitic microorganisms, because they facilitate infection by debilitating the host (Morton et al., 2004). Nikapitiya (2012) reported that production of secondary metabolites is one of the characteristic features of microorganisms. More than 50,000 bioactive compounds have been isolated from the extracts of micro-organisms with a diversified arrangement of chemical structures, which showed antimicrobial, anti-tumor and agrochemical activity. Devi et al. (2012) stated that in a microbial world, the top bioactive compounds

producers are Actinomycetes (45%), fungi (38%) and bacteria (17%).

In the production of secondary metabolism, the fungi of most important microorganisms are compounds resulting from the culture media, which is not necessary for the growth of the fungi and its producing extracellular ability (Suay et al., 2000; Zhang et al., 2009). Secondary metabolism includes several compounds such as mycotoxins, antibiotics, enzyme inhibitors, pharmaceutical importance, pesticides and hormones that induce the growth of plants and animals (Demain and Fang, 2000). However, less information regarding the antimicrobial bioactive metabolites produced by the nematophagous

fungi is available. Several studies indicate that some nematophagous fungi have the ability to produce materials against microorganisms when grown in liquid culture, such as *Arthrobotrys*, *Nematoctonus* and *Hohenbuehelia* (Jansson et al., 1997). It was observed that the amount of secretion of these materials increases with an increase in the number of trapping devices. The nematode-trapping fungi (Orbiliomycetes, order Orbiliales and family Orbiliaceae) are a group belonging to the nematophagous fungi (Herrera-Estrella et al., 2016). These fungi are predatory (or nematode-trapping) which produce special mycelia nematode-trapping structures that may be either adhesive or non-adhesive, and by which nematodes are efficiently captured. The filaments of this fungus that penetrate nematodes and grow within it can be excreted toxic materials against microorganisms (Swe et al., 2011). Several antibiotics diagnosed to be produced by the nematode-trapping fungus *Arthrobotrys* include: oligosporon, oligosporol, oligosporol B, 40, 50-dihydro-oligosporon and linoleic acid (Li et al., 2007), and these antibiotics showed antimicrobial and toxic ability toward bacteria (Degenkolb and Vilcinskas, 2016). In this report, an attempt was made to investigate some fungal isolates of the nematode-trapping fungi that are isolated from the Iraqi soils for their bioactive metabolite production in culture media as a preliminary step for further investigation of the specific antimicrobial compounds to be tested against two types of bacteria.

MATERIALS AND METHODS

Growth of fungi on culture fermentation

The broth cultures of three species of nematode-trapping fungi (*A. dactyloides*, *A. oligospora*, and *D. brochopaga*) were prepared in inoculated flasks (500 ml) containing 300 ml of corn meal broth (CMB) with 5 discs (6 mm in diameter) cut from the axenic fungal culture of each isolate and incubated at $25 \pm 2^\circ\text{C}$ for 2 weeks on a rotary shaker. The cultures were prepared with an appropriate number of flasks for the following experiments (Kim et al., 1999). Fungal cultures were filtered on Whatman No. 1 filter paper and the pH was adjusted to 3 by a dilution of HCl. It was considered as a stock solution, and was used in the following experiments:

1. Using cultures filtrate: Different concentrations (25, 50, 75 and 100% of distilled water was used for dilution) of the standard solution were prepared, and their inhibition activity against bacterial growth was evaluated.
2. Crude extracts: Fungal cultures were filtered on Whatman No. 1 filter paper and the pH was adjusted to 3 by drops of HCl for each fungal filtrate. The filtrate was extracted in ethyl acetate (1:1 v:v) using a separating funnel. The organic layer was collected by dehydration of water using Na_2SO_4 . The filtrate was filtered again and placed in Petri dishes, then left to be dried at room

temperature. About 0.1 mg of the dried extract was dissolved in 1 ml ethanol as stock extract solution to be used for further experiments.

Antimicrobial bioactivity assay

After the filter paper discs (6 mm) were sterilized by autoclave, 20-25 discs were soaked in each concentration of cultures filtrate and fungal crude extract solution for 5 min. The soaked paper discs were placed on the surface of Muller-Hinton agar medium streaked with 0.2 ml of bacterial suspension of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923), that are provided by the microbiology laboratory at A-Sader hospital, Missan governorate. The initial suspension contained about 10^8 colony forming units (CFU)/ml. Additionally, 1:100 dilutions of the initial suspension were prepared in sterile 0.9% saline. Plates were incubated at 37°C for 24 h. The appearance of inhibition zones around the filter paper disc indicates the bioactivity of cultures filtrate and crude metabolites of the tested fungal isolates (Kim et al., 1999). The diameters of the clear zones were measured and compared with the control agar plates containing discs with solvent only, after which triplicates were made for each of the fungal extract.

Bioactivity of fungal mycelia crude extract

The mycelium of each fungal culture was harvested on filter paper, dried in an oven at 40°C and ground in a mortar with ethyl acetate solvent (1:1 v:v). The bioactivity of the mycelium extract of each fungal species was examined against the two selected bacterial isolates by using a disc diffusion technique as mentioned above.

Sensitivity of the bacteria isolates against commercial antibiotics

Three commercial antibiotics brought from pharmacies in Missan governorate, namely: Cefalotaxine, 500 mg; Penicillin, 500 g (Samarra Drugs Company) and Neomox (Amoxicillin), 500 mg (Neopharma company UAE) were selected and the sensitivity of the two bacterial isolates was tested against each of these antibiotics by using a disc diffusion method at a concentration of 30 mg antibiotics/100 ml distilled water.

Cytotoxicity test

Cytotoxicity of the fungal crude extracts was examined by using human RBC following a previously described method (Xian-guo and Ursula, 1994).

Statistical analysis

For data analysis, SPSS 11 statistical program for ANOVA was applied using completely randomized design

Table 1. Growth inhibition zones (mm diameter) exhibited by the different concentration of the fungal culture filtrate against *E. coli* and *S. aureus*.

Fungal species	<i>E. coli</i>					<i>S. aureus</i>				
	Concentration					Concentration				
	25	50	75	100	Average	25	50	75	100	Average
<i>A. dactyloides</i>	0.00	2.33	6.33	11.33	5	0.00	1.67	5.33	10.0	4.25
<i>A. oligospora</i>	4.67	9.33	15.0	23.67	13.17	2.33	6.67	13.67	21.3	11
<i>D. brochopaga</i>	1.33	3.67	13.33	20.33	9.67	1.00	3.33	12.00	18.0	8.58
Average	2.00	5.11	11.56	18.44		1.1	3.9	10.3	16.4	
RLSD: Fungi = 0.88, concentration = 1.02						RLSD: Fungi = 0.80, concentration = 0.92				

Numbers represent average of three replicates ($P \leq 0.05$).

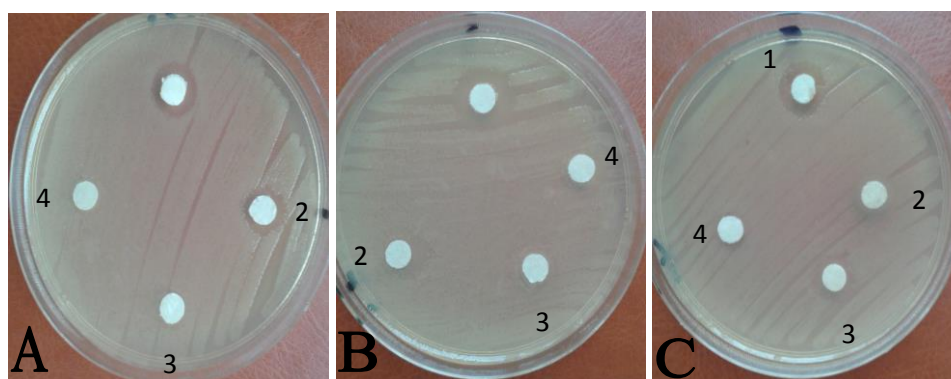


Figure 1. Inhibition zones exhibited by fungal cultures filtrate (A: *A. dactyloides*, B: *A. oligospora*, C: *D. brochopaga* against *S. aureus*). 1, 2, 3, and 4 represent concentrations of 100, 75, 50, and 25%, respectively.

(CRD) and revised least significant difference (RLSD) under the significant confidence level ($P < 0.05$).

RESULTS

The preliminary screening of three species of the nematode-trapping fungi for their bioactivity against *E. coli* and *S. aureus* revealed that the cultures filtrate and crude filtrate extracts of the examined species exhibited promising growth inhibitory action, and that *E. coli* was more susceptible than *S. aureus*. In general, crude extracts were more efficient than cultures filtrate, and those belonging to *A. oligospora* showed the highest inhibitory effect followed by those belonging to *D. brochopaga* and those belonging to *A. dactyloides*.

For the culture filtrate solution, *A. oligospora* has the highest inhibitory effect against the two bacteria species of *E. coli* and *S. aureus* with an inhibition zone equal to 13.17 and 11.00 mm respectively, followed by *D. brochopaga* with an inhibition zone of 9.67 and 8.58 mm, respectively, and then *A. dactyloides* with an inhibition zone of 5.00 and 4.25 mm, respectively. The results showed that high concentrations (100%) of the cultures

filtrate were mostly efficient in inhibiting the growth of *E. coli* and *S. aureus* with an inhibition zone equal to 18.44 and 16.40 mm, respectively, followed by a concentration of 75% resulting in an inhibition zone of 11.56 and 10.3 mm respectively, while a concentration of 25% showed less inhibitory ability against the two bacterial species with an inhibition zone of 2.00 and 1.10 mm, respectively (Table 1 and Figure 1).

On the other hand, crude filtrate extracts of *A. oligospora* also gave the highest inhibitory ability against both *E. coli* and *S. aureus* to reach 16.42 and 14.5 mm, respectively, followed by *D. brochopaga* with an inhibitory ability of 13.17 and 11.42 mm, respectively, and *A. dactyloides* with the least inhibitory ability of 8.58 and 7.50 mm, respectively. Moreover, when comparing the effect of concentrations, the concentration of 100% is the most influential on the growth of the tested bacteria (25.0 and 21.78 mm, respectively), followed by the concentration of 75% (14.67 and 13.44 mm, respectively), while the lowest inhibitory ability was observed in the concentration of 25% (Table 2 and Figure 2).

The mycelia crude extract of the examined fungi revealed the lowest inhibition zones against *E. coli* and

Table 2. Growth inhibition zones (mm diameter) exhibited by the different concentrations of the crude filtrate extracts of fungal culture against *E. coli* and *S. aureus*.

Fungal species	<i>E. coli</i>					<i>S. aureus</i>				
	Concentration					Concentration				
	25	50	75	100	Average	25	50	75	100	Average
<i>A. dactyloides</i>	1.33	4.33	11.33	17.33	8.58	1.33	3.33	10.00	15.33	7.50
<i>A. oligospora</i>	6.00	11.0	16.67	32.00	16.42	5.00	10.0	15.67	27.33	14.50
<i>D. brochopaga</i>	2.67	8.33	16.00	25.67	13.17	2.33	6.00	14.67	22.67	11.42
Average	3.33	7.89	14.67	25.00		2.89	6.44	13.44	21.78	
RLSD: Fungi = 0.73, concentration = 1.05						RLSD: Fungi = 0.51, concentration = 0.73				

Numbers represent average of three replicates ($P \leq 0.05$).

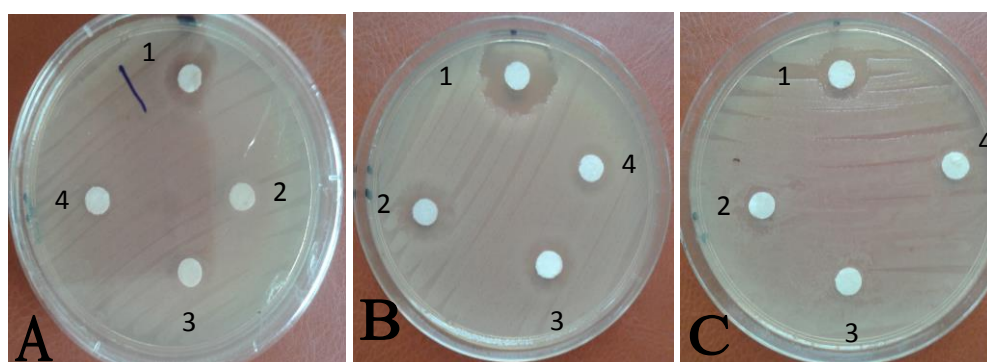


Figure 2. Inhibition zones exhibited by fungal crude extracts (A: *A. dactyloides*, B: *A. oligospora* against *E. coli*) (C: *A. dactyloides* against *S. aureus*). 1, 2, 3, and 4 represent concentrations of 100, 75, 50, and 25%, respectively.

S. aureus as compared with fungal filtrate extracts. However, *A. oligospora* mycelium showed relatively higher inhibition zones reaching 21 and 23 mm, respectively, followed by *D. brochopaga* and then *A. dactyloides* (Table 3).

The results showed that the effect of the commercial antibiotics varied on the growth sensitivity of the two bacteria isolates. *E. coli* and *S. aureus* isolates were more sensitive to Neomox with inhibition zones equal to 17.63 and 14 mm, respectively, followed by Cefalotaxine (14.21 and 3.30 mm, respectively). Comparatively, the crude fungal extracts exhibited a more inhibitory effect on the growth of both bacteria than that of the tested commercial drugs (Tables 1 and 4). However, cultures filtrate as well as crude extracts of the tested fungi did not show any hemolytic activity toward RBCs at all concentrations.

DISCUSSION

Many pathogenic bacteria are becoming resistant to synthetic drugs and hence an alternative strategy is very much needed. Fungi represent an enormous source for natural products with diverse chemical structures and

activities (Praveena and Padmini, 2011). A vast number of fungi have been utilized for the biotransformation process and many more are yet to be exploited for isolation of potential compounds. Nematophagous fungi have several strategies to survive and capture nematodes, such as, ability to secrete several substances including inhibitors of nematodes as toxins which have the antimicrobial capability (Liu et al., 2009). A total of 179 nematicidal compounds belonging to diverse chemical groups have been identified from nematophagous fungi (Li et al., 2007).

Nematode-trapping fungi are a group of nematophagous fungi that capture nematodes using adhesive or mechanical hyphal traps (Swe et al., 2011). The results of this study which showed that *A. dactyloides*, *A. oligospora* and *D. brochopaga* have the ability to secrete materials that inhibited growth of bacteria are in agreement with those of other studies (Pendse et al., 2013; Stadler et al., 1993a, b). Degenkolb and Vilcinskas (2016) reported that *A. oligospora* produce several metabolites, including three colorless oils namely: oligosporon, oligosporol A, and oligosporol B, and that the antimicrobial activity of the culture filtrate was detected. *A. conoides*, *D. brochopaga*, and *A. dactyloides*

Table 3. Growth inhibition zones (mm diameter) exhibited by fungal mycelia extract against *E. coli* and *S. aureus*.

Fungal species	<i>E. coli</i>	<i>S. aureus</i>
<i>A. dactyloides</i>	9	11
<i>A. oligospora</i>	21	23
<i>D. brochopaga</i>	13	14
RLSD	4.21	4.85

Numbers represent average of three replicates ($P \leq 0.05$).

Table 4. Growth inhibition zones (mm diameter) exhibited by different commercial antibiotics (30 mg/100 ml) and crude filtrate extracts against *E. coli* and *S. aureus*.

Commercial antibiotics	<i>E. coli</i>	<i>S. aureus</i>
Cefalotaxine	14.21	3.30
Penicillin	8.52	11.34
Neomox	17.63	14.00
<i>A. dactyloides</i>	17.33	15.33
<i>A. oligospora</i>	32.00	27.33
<i>D. brochopaga</i>	25.67	22.67
RLSD	3.41	4.23

Numbers represent average of three replicates ($P \leq 0.05$).

were also assigned as producers of these compounds (Anke et al., 1995). All three compounds also exhibited moderately cytotoxic and hemolytic effects, but no phytotoxic or mutagenic activity. Moreover, Silva et al. (2010) noted that *Monacrosporium thaumasium* has the ability to excrete toxic substances. Moosavi and Zare (2012) explained that *Paecilomyces lilacinus* secrete leucinostatin and lilacin.

The crude filtrate extract of the examined species of *A. dactyloides*, *A. oligospora* and *D. brochopaga* exhibited an inhibitory action against both bacterial strains of *E. coli* and *S. aureus*. However, a variation in the inhibitory action, based on the inhibition zones diameter, among the examined fungi was noticed. Extracts of *A. oligospora* and *D. brochopaga* revealed higher inhibition zones against *E. coli* and *S. aureus* (Table 2). Such variations may be attributed to the difference of the active constituents among fungal extracts. Fungi that grow in batch culture produce large amounts of secondary metabolites, including antibiotics, and vary in production, function and specificity to a particular fungus (Makut and Owolewa, 2011; Keller et al., 2005). The disparity in the inhibitory capacity of the tested fungi may be due to the different growth conditions. It was observed that some species produce a specific metabolic compound under certain liquid culture, but could not produce the same compound in another medium (Schulz et al., 1995). Dreyfuss and Chapela (1994) made an assumption that certain physical and biological change in natural

environment favors the production of a diverse range of secondary metabolites. Niu and Zhang (2001) found out that *A. oligospora* has a complement of secondary metabolites as numerous and diverse as those of other fungal taxa. Among the classes of compounds discovered in *A. oligospora* are polyketides, benzenoids and terpenoids. Additionally, other typical fungal secondary metabolites have also been observed in this species. Other *Arthrobotrys* species were also found to produce these or similar compounds. Noweer and Al-Shalaby (2014) found out that *D. brochopaga* and *A. dactyloides* have been developed and formulated in a compound named Dbx-1003 20% and Adx-1004 20%.

The results showed that the red blood cells were not lysed during the incubation period at temperatures of 37°C. According to this result, the compounds derived from the tested fungi are not toxic and therefore can be used as antibiotics. However, more experiments need to be conducted in order to ascertain the effectiveness of fungal extracts.

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