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Antibacterial Activity of Bioactive Compounds from Endophytic Fungi against *P. aeruginosa* isolated from Freshwater Fishes

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ABSTRACT

With the increasing occurrence of bacterial resistance against available antibiotics, it has now become necessary to investigate newer sources for antimicrobials. Endophytic fungi have the aptitude to co-exist with their host plants without affecting any harm and are useful to both the plant and the fungi. The current study aimed to evaluate the antimicrobial activity of the bioactive compounds of the some endophytic fungi isolated from five medicinal plants against *P. aeruginosa*. A total of 30 distinct colonies of the endophytic fungi were isolated from five selected medicinal plants. Most of the isolates were obtained from *H. sabdariffa* L., *O. europaea* L., *M. piperita* L., *M. oleifera* Lam. and *A. indica* A. Juss. Most of the fungal isolates belonged to *Alternaria* sp., *Cladosporium* sp., *Ulocladium* sp., *Chaetomium* sp., *A. niger*, *E. nidulans* and *Fusarium* sp. A total of 34 intra- and extra-cellular metabolites from 17 endophytic fungal isolates were evaluated for their antibacterial activities against *P. aeruginosa*, some endophytic fungal metabolites showed antibacterial activities *as A. niger* extract isolated from neem which exhibited the most potent antibacterial activity 16.5± 0.71 mm against *P. aeruginosa*. In addition, *Cladosporium*, sp. isolated from neem, *Chaetomium* sp. extracts and *Alternaria* sp. (isolated from mint), showed antibacterial activity with a zone of inhibition, 13± 1.41, 12.5± 0.71and 12.5± 0 mm, respectively. In contrast, none of the *Ulocladium* sp., *Alternaria* sp. extracts (isolated from olive), *Cladosporium* sp., and *E. nidulans* extracts (isolated from mint) showed any antibacterial activities against *P. aeruginosa.*

INTRODUCTION

The efficiency of modern medicines is pretentiously reducing especially due to the ever-increasing bacteria resistance that is currently an issue of great public health concern (**Alpert, 2017**). Antibiotic resistance signifies a great problem for fish health managers

who are responsible for choosing effective antibiotics that can be used for the treatment and prevention of disease (**El-Bahar *et al.*, 2019 and Sherif *et al.*, 2021**). Commercial use of antibiotics in aquaculture needs to be reduced and replaced by other equally effective and non-resistance causing natural and bioactive products. Medicinal plants and their extracts have been employed for treating fish diseases (**Thanigaivel *et al.*, 2015**). Endophytes are the group of microorganisms, which live in plants inside their intercellular spaces without causing harmful to their host plant. Endophytes are extremely diverse fungi (**Gomes *et al.*, 2018**), actinomycetes or bacteria (**Zhang *et al.*, 2019**). Endophytic strains have possible to secret secondary metabolites, which defend plants against pests, insects and other pathogenic organisms, thus endophytes signify a promising source of novel bioactive compounds for pharmacological suggestions (**Abdalla *et al.*, 2020).** Endophytic fungi have the aptitude to co-exist with their host plants but without affecting any harm to its and are beneficial to both the plant and the fungi (**Manganyi *et al.*, 2019**). The symbiotic association may be documented to the production of bioactive compounds that function in competitiveness, growth promotion and protection of the host against herbivores and pathogens. These features authority them to be ideal applicants for bio-control processes and thus are applicable in a gricultural and medical industries (**Gouda *et al.*, 2016**). There are several researches on the antimicrobial activity of the ethnoveterinary plants of the present study against fish pathogens (**Bariyyah *et al*., 2019 and Hoseinifar *et al*., 2020)** but according to our knowledge, none of them studied the antibacterial activity of the endophytic fungi extracts of these plants against fish pathogens.

The current study aimed to evaluate the antimicrobial activity of the bioactive compounds of the endophytic fungi isolated from five medicinal plants against *P. aeruginosa*, that are implicated in aquaculture problems.

MATERIALS AND METHODS

2.1. Fish sampling and sites of fish farms.

A total of 217 clinically diseased fishes, 165 *O. niloticus* (60-300 g) and 52 *C. gariepinus* (350-800 g) were collected haphazardly from different privet farms in Ismailia Governorate, Egypt, during the period from August, 2018 to September, 2019. Fishes were transferred to the Animal Health Research Institute Lab, Ismailia governorate.

2.2. Clinical and postmortem examination of fishes

2.2.1. Clinical examination:

Fishes were examined clinically for any abnormal lesions according to **Noga (2010).**

2.2.2. Postmortem examination:

Fish samples were washed thoroughly with sterile distilled water prior to bacteriological examination. The collected fishes were dissected under sterile conditions according to the methods described by **Austin and Austin (2016).**

2.3. Isolation and identification of bacteria:

According to **Austin and Austin (2016**), the bacteria were isolated from different organs of each fish (liver, kidney, spleen, gills and external lesions if present), were inoculated in Tryptone Soy Broth and incubated at 28°C for 24 hrs. A loopful from all tubes was streaked on Tryptone Soy Agar and then was incubated aerobically at 28°C for 24 hrs. Pure culture colonies were selected and subcultured on TSA and incubated aerobically at 28°C for 24– 48 hrs for further specific isolation.

2.4 Identification of bacteria using API 20E Kit (API Bio Merieux, France)

Bacterial isolates were pre-grown on TSA and evaluated using the API 20E test kit following the manufacturer's protocol (**https://apiweb.biomerieux.com**) for biochemical characterization.

2.5 Antimicrobial susceptibility test

Antimicrobial susceptibility tests was performed according to **CLSI (2016**) using the Kirby-Bauer modified disc diffusion technique for *P. aeruginosa* on a total of 60 bacterial isolates (30 for each) against 22 antimicrobial discs (Table 8).

2.6 Molecular characterization using PCR

Selected isolates identified *P. aeruginosa* by conventional biochemical tests were tested by PCR to confirm the identification and to detect some virulent and resistant gene. Nine pairs of primers were supplied from Metabion (Germany) or Biobasic (Canada). They have specific sequence and amplify specific products **Table (1).**

Table (1): Oligonucleotide primers sequences.

Tested bacteria	Target gene	Primers sequences	Amplified segment (bp)	Reference
P. aeruginosa	*16S rDNA*	F: GGGGGATCTTCGGACCTCA	956 bp	**(Spilker *et al*., 2004)**
		R: TCCTTAGAGTGCCCACCCG		
	***opr*L**	F: ATG GAA ATG CTG AAA TTC GGC	504 bp	**(Xu *et al*., 2004)**
		R: CTT CTT CAG CTC GAC GCG ACG		
	***mex*R**	F: GCGCCATGGCCCATATTCAG	637 bp	**(Sánchez *et al*., 2002)**
		R: GGCATTCGCCAGTAAGCGG		

2.7 Collection of plant samples: Five medicinal plants, viz hibiscus (H. *sabdariffa* L.), mint (*M. piperita* L.) moringa (*M. oleifera* Lam.), neem (*A. indica* A. Juss.) and olive (*O. europaea* L.) were collected through July to December, 2018. The used parts of each plant and site of collection are listed in the **Table (2).**The plants were designated according to their ethno veterinary usages in the aquaculture field. The collected plant materials were kept in separate polythene bags up to transported to laboratory for further

isolation of endophytic fungi. The plant samples were authenticated by a taxonomist in the Department of Botany, Faculty of Science, Ismailia.

Table (2): Ethnoveterinary uses of the medicinal plants

Common and Scientific name	Used part	Collection site	Ethnoveterinary uses in the aquaculture field	References
Hibiscus ***H. sabdariffa*** **L.**	Leaves Stem Root	Experimental farm of Faculty of Agriculture, SCU, (Egypt).	Antibacterial against *Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp. and *staphylococcus* sp.	**(Awad and EL-Makhzangy, 2015)**
			Antibacterial against *A. hydrophila*	**(Bariyyah *et al.*, 2019)**
Mint ***M. piperita*** **L.**	Leaves Stem Root	Experimental farm of the Botany Department, Faculty of science, SCU.	Antibacterial against fish pathogens	**(Klūga *et al.*, 2017)**
Moringa ***M. oleifera*** **Lam.**	Leavs Twigs	Experimental farm of the Botany Department, Faculty of science, SCU	Antibacterial against *A. hydrophila*	**(El-Gawad *et al.*, 2020)**
			Antibacterial activity against *Micrococci* sp., *B. subtilis, E. coli* and *P. fluorescens*	**(Ubiogoro *et al.*, 2019)**
Olive ***O. europaea*** **L.**	Leaves Twigs	Experimental farm of the Botany Department, Faculty of science.	Antibacterial activity against *E. tarda*	**(Zemheri-Navruz *et al.*, 2019)**
			Immunostimulant and Antioxidant	**(Hoseinifar *et al.*, 2020)**
Neem ***A. indica*** **A. Juss**	Leaves Twigs	Experimental farm of the Botany Department. Faculty of science.	Antibacterial activity against *Micrococci* sp., *E. coli* and *P. fluorescens*	**(Ubiogoro *et al.*, 2019)**

2.8 Surface sterilization and isolation of fungal endophytes

Samples were wash away in running tap water for 10 min to eliminate any debris, and finally washed with distilled water for 1.0 min and this process was repetitive three times. The samples were air dehydrated, and then cut aseptically using a sterile blade into 1 × 1 cm^2 segments, which were surface sterilized with 70 % ethyl alcohol for 1.0 min, soaking in 4 % sodium hypochlorite solution for 3 min. and then washed with 70 % alcohol for 1.0 min, and finally washed with sterile distilled water for three times (**Liang *et al.*, 2012).** The samples be located blotted dry by sterile filter paper. Segments were inoculated onto Potato Dextrose Agar supplemented by chloramphenicol (50 mg/L) to destroy bacterial growth, and incubated for 4–6 days at 28°C. A control of the water

washes from the surface sterilized samples were streaked onto antibiotic-free PDA and hatched under the similar conditions. After 3 days of incubation, the first fungal hyphae were visible from the edge of the samples. Different fungal strains developed from each sample and individual strains were isolated by transferring hyphal tips onto antibiotic-free PDA medium. All isolates were maintained in PDA slants and kept at 4°C. They be located check every day for three weeks and individual fungal colonies were transported to fresh PDA plates for purification and identification purposes.

2.9 Identification of endophytic fungal isolates

For tentative identification, microscopic slides for each fungal mycelium were prepared and examined by a light microscope. Taxonomic identification of the fungi was founded on their morphological characters and the mechanism of spore creation. Isolates that unsuccessful to produce reproductive structures after 3-4 months of incubation be present referred to as sterile mycelium, and separated into morphospecies; this group of fungi is prevalent in endophyte studies **(Lacap *et al.*, 2003)**.

2.10 Fermentation and extraction of fungal secondary metabolites

Each fungal strain was separately inoculated into PDB via placing mycelial agar plugs of actively growing pure culture (6 mm in diameter) in 250 mL Erlenmeyer flask having 100 mL of the medium. Each flask was hatched at 25 ± 2°C for four weeks with periodical shaky at 150 rpm and inspected periodically for possible contamination. After incubation, the culture filtrate was take out and filtered through Whatman®No.1 filter paper to separate mycelia. For extracellular metabolites, the fermentation broth (filtrates) were poured in 1000 mL separating funnels, then were extracted thrice with an equal volume of ethyl acetate (1:1, v/v). The organic phase was collected, and the solvent were allowable to evaporate by air drying to additional concentrate the residue. For intracellular metabolites, the mycelial biomasses were harvested, thoroughly washed, and macerated in ethyl acetate (1:1, v/v). Then, the mycelia were homogenized thoroughly. The supernatants (organic phase) were further treated as explained above for the extracellular metabolites (**Astuti and Nababan, 2014**).

2.11. Evaluation of antibacterial activity of fungal endophytes.

The modified Kirby–Bauer disc diffusion method was used to estimate the antibacterial activity of the endophytic extracts (**Abdallah *et al.*, 2020**). The antibacterial activities of the endophytic extracts were tested against *P. aeruginosa*.

2.12 Preparation of bacterial inoculum

Prior to the experiment, pure cultures of *P. aeruginosa* strains were subcultured in nutrient agar and incubated for 18 h. at 37°C in order to extent the exponential phase, then adjusted by addition normal saline to be equivalent to McFarland standard 0.5.

About 500 µL of each culture was spread over plates containing Mueller–Hinton agar in 90 mm sterile Petri dishes.

2.13 Determination of minimum inhibitory concentration (MIC)

All bioactive extracts viewing potent antimicrobial activity was advance determined for their MIC by a microliter broth dilution technique **Balouiri *et al.*, (2016)**. A sterile 96 micro-titer plates were arranged by addition of 100 µL of sterile TSB into each of the wells of the plate by aseptic procedure. The endophyte extracts (50 mg/mL) were supplementary to the first row of the micro-titer plate at a volume of 50 µL. A volume of 100 µL of positive control (amikacin 50 mg/mL) was used for the tested bacteria, which were adjusted to McFarland scale 0.5 and then 50 µL was added to all wells. The test bacteria (*P. aeruginosa*) were chosen as they represent the most resistant isolates. The micro-titer plates were incubated at 28°C for 24 hrs for bacteria. After incubation, a volume of 40 µL nitro blue tetrazolium chloride (NBT, 0.02 mg/mL) was added to all well of the micro-titer plate to show viability. The MIC value of the extract was reserved as the lowest concentration that appearances no microbial growth (the wells with bacterial growth displayed deep blue color with precipitation) in relative to the culture control as a reference.

RESULTS

3.1. Examination of the fish samples

3.1.1. The clinical examination of *O. niloticus* and *C. gariepinus* revealed that some fishes showed irregular hemorrhages wholly over the fish body mainly at the base of fins, tail, anal opening, mouth and fins rot; with unilateral or bilateral exophthalmia, darkness of skin, skin ulceration, detachment of scales, abdominal distention, and increased in mucous secretion are the common clinical signs. **Photo (1, 2, 3, 4, 5, 6)**.

Photo (1): Naturally infected *O. niloticus* showing hemorrhages all over the body.
Photo (2): Naturally infected *O. niloticus* showing darkness of the skin.
Photo (3): Naturally infected *O. niloticus* showing detachment of scales.
Photo(4): Naturally infected *O. niloticus* showing hemorrhages and abdominal distention

.
Photos (5) and (6): Naturally infected *C. gariepinus* showing mouth and fins rot**.**

Photo (**7): ** Naturally infected *O. niloticus* showing pale anemic gills.

Photo (8): Naturally infected *O. niloticus* showing congestion of liver.

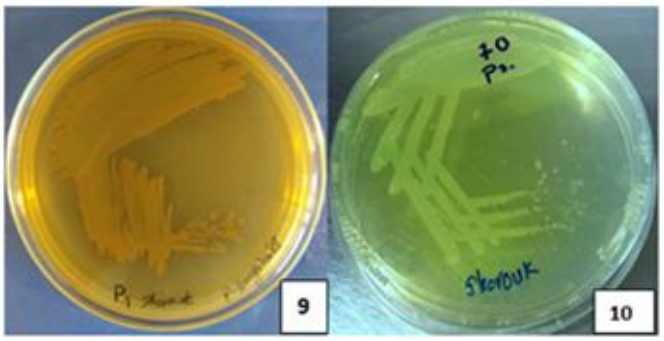
3.1.2. Post-mortem examination of the fish samples: The observed postmortem pictures included abdominal dropsy with reddish ascetic exudates, the gills of infected fishes were severely congested, covered with mucous in some cases and pale anemic in other as shown in **photo (7, 8)**. The liver of examined fishes varied from enlarged pale anemic or yellow in some cases to deep brown with focal hemorrhages (mottled), in other cases congestion of kidney, spleen, liver with distended gall bladder, and also few amount of yellowish sanguineous fluid was found in the abdominal cavity. The intestinal tract was usually hemorrhagic and inflamed with accumulation of some reddish fluid.

3.2.Isolation and identification of bacterial fish pathogens. The initial colonial characteristics of *P. aeruginosa* isolates were listed in the **Table (3)** and **photo (9,10).**

Table (3): Initial colonial characteristics of *P. aeruginosa*.

	Sheep blood agar	**MacConkey agar**	**TSA agar**	**Pseudomonas agar base**
P. aeruginosa	Large, flat colonies (3–4 mm), with a serrated edge, usually hemolytic, with the green-blue pigment, pyocyanin. The colonies have a characteristic fruity odor.	Pale Non-lactose. fermenter colonies, but the green-blue pigment is often superimposed on what would otherwise be pale colonies.	Mucoid large flat clonies with the green-blue pigment, pyocyanin and a characteristic fruity odor.	Small colonies, mucoid and smooth with different blue – green pigment,

Photo (9): *P. aeruginosa* on TSA: Mucoid, large, flat, irregular-edged colonies.
Photo (10): *P. aeruginosa* on pseudomonas agar base: Mucoid, large, flat, spreading and greenish colonies with green pigment.



3.3 Biochemical identification of the isolates by using API 20E: Presumptive *P. aeruginosa* were confirmed by using the numerical profile supplied in the API 20E system as shown in **Table (4)**.

Table (4): Reading the API20E. BioMérieux (2009)

TESTS	*P. aeruginosa*		TESTS	*P. aeruginosa*	
ONPG	Colorless	-	**GLU**	Yellow	+
ADH	Red/orange	+	**MAN**	Blue	+
LDC	Yellow		**INO**	Blue	-
ODC	Yellow	-	**SOR**	Blue	-
CIT	Blue-green/blue	+	**RHA**	Blue	-
H2S	Colorless/gray	-	**SAC**	Blue	-
URE	Red/orange	+	**MEL**	Blue-green	-
TDA	Yellow	-	**AMY**	Blue	-
IND	Yellow	-	**ARA**	Blue-green	-
VP	Colorless	-	**OX**	Violet	+
GEL	Black diffuse	+			

3.4.Prevalence of positive samples for *P. aeruginosa* among the examined fishes were listed in **Table (5).**

Table (5): Prevalence of positive samples for *P. aeruginosa*.

Fish type	No. of fish samples	No. of positive fish samples for *P. aeruginosa*	
		No.	%
O. niloticus	165	23	13.9
C. gariepinus	52	14	26.9
Total	217	37	17.1

3.5. Prevalence of *P. aeruginosa* isolated from fishes were listed in **Table (6)**.

Table (6) Prevalence of *P. aeruginosa* from examined fishes

Fish type	No. of fish samples	No. of isolates	No. of positive isolates for *P.aeruginosa*		Other bacterial species	
			No.	%	No.	%
O. niloticus	165	183	25	13.7	85	46.4
C.gariepinus	52	79	37	46.8	25	31.6
Total	217	262	62	23.7	110	41.9

3.6.Frequency distribution of *P. aeruginosa* isolates in different organs of examined fish samples were listed in **Table (7).**

Table (7) Prevalence of *P. aeruginosa* from examined fishes

Type of fishes	No. of isolates	Organs									
		Skin		Liver		Gills		Kidney		Spleen	
		No.	%	No.	%	No.	%	No.	%	No.	%
O. niloticus	25	6	24	6	24	9	36	4	16	0	0
C. gariepinus	37	4	10.8	11	29.7	13	35.1	8	21.6	1	2.7
Total isolates	62	10	16.1	17	27.4	22	35.5	١٢	19.4	1	1.6

3.8. Results of antimicrobial susceptibility test for the bacterial isolates: Results of antimicrobial susceptibility of 30 *P. aeruginosa* isolates recovered from fishes (*O. niloticus* and *C. gariepinus*) to different antimicrobial agents were summarized in **Table (8).**

3.9. MAR index of *P. aeruginosa* isolates:

The MAR index values were higher than 0.2. They was 0.57 for *P. aeruginosa*.

3.10. Molecular identification of *P. aeruginosa* isolates:

3.10.1 Five representatives, biochemically identified *P. aeruginosa* isolates were confirmed at the species level based on their 16S rDNA gene sequences**.** All the tested isolates were positive for 16S rDNA at 956 bp fragment as shown in **Photo (11)** .

Table (8): Results of susceptibility test for 30 isolates of *P. aeruginosa.*

Antibiotic	Code	Interpretive standards (mm)					
		Sensitive		Intermediate		Resistant	
		No	%	No	%	No.	%
Amikacin	AK	23	76.7	4	13.3	3	10
Gentamicin	CN	4	13.3	22	73.3	4	13.3
Neomycin	N	0	0	24	80	6	20
Streptomycin	S	9	30	3	10	18	60
Imipenem	IPM	30	100	0	0	0	0
Ceftriaxone	CRO	9	30	0	0	21	70
Ciprofloxacin	CIP	26	86.7	2	6.7	2	6.7
Flumequine	UB	2	6.7	0	0	28	93.3
Norfloxacin	NOR	26	86.7	1	3.3	3	10
Amoxi/ clav. a.	AMC	4	13.3	5	16.7	21	70
Trimethoprim/ sulphamethoxazole	SXT	9	30	1	3.3	20	66.7
Lincomycin	MY	0	0	0	0	30	100
Colistin	CT	28	93.3	0	0	2	6.7
Erythromycin	E	0	0	0	0	30	100
Aztreonam	ATM	3	10	2	6.7	25	83.3
Nitrofurantoin	F	1	3.3	1	3.3	28	93.3
Ampicillin	AMP	8	26.7	3	10	19	63.3
Penicillin	P	0	0	0	0	30	100
Chloramphenicol	C	6	20	0	0	24	80
Nalidixic acid	NA	2	6.67	8	26.7	20	66.7
Oxolinic acid	OA	10	33.3	0	0	20	66.7
OxyTetracyclin	T	5	16.7	0	0	25	83.3

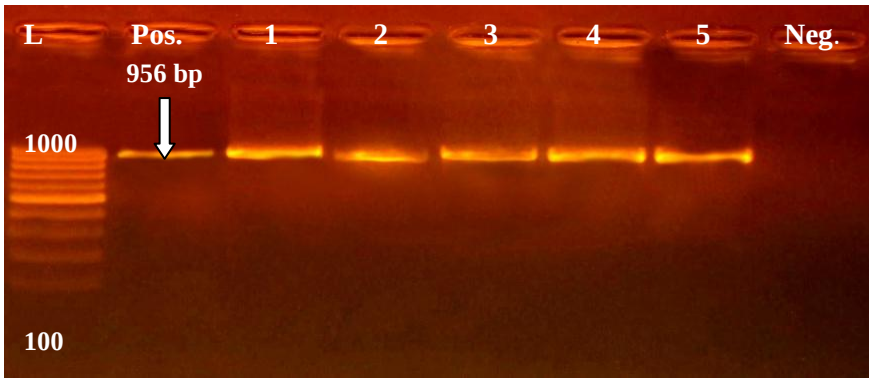


Photo (11): Agarose gel electrophoresis showing the result of PCR for detection of 16S rDNA of *P.aeruginosa*. Lanes 1 - 5: positive amplification of 956 bp of 16S rDNA gene in the 5 tested samples. L: Lane [Gelpilot 100 bp ladder (Qiagen, 100-1000 bp)]. Neg.: Negative control. Pos.: Positive control.

3.10.2. Molecular detection of *opr* L gene responsible for virulence and *mex* R resistance genes in *P. aeruginosa* isolates: The PCR results for *P. aeruginosa* showed that *opr* L virulence gene and *mex* R resistance were detected in all five studied isolates at 504 and 637 pb, respectively.

3.11. Biodiversity of the endophytic fungi connected with the selected medicinal plants. A total of 30 distinct colonies of the endophytic fungi were isolated from healthy parts of the five selected medicinal plants **(Table 9).**

Table (9): Fungal endophytic colonies isolated from medicinal plants.

Plant	Plant part	Isolate No.	Endophytic fungi isolates	Plant	Plant part	Isolate No.	Endophytic fungi isolates
Hibiscus (*Hibiscus sabdariffa* L.)	Leaves	1	*Alternaria* sp.	**Neem (Azadirachta indica A.Juss.)**	Twig	2	*Cladosporium* sp.
	Leaves	2	*Alternaria* sp.		Leaves	4	*Asprigillus niger*
	Leaves	3	*Alternaria* sp.	**Olive (*Olea europaea* L.)**	Twig	1	*Unidentified* sp.
	Leaves	4(1)	Sterile mycelium		Twig	2	*Cladosprium* sp.
	Leaves	4(2)	*Alternaria* sp.		Leaves	3	*Penicillium* sp.
	Leaves	4(3)	*Alternaria* sp.+ *Ulocladium* sp.		Leaves	4	*Fusarium* sp.
	Leaves	5	*Alternaria* sp.		Twig	5	*Cladosporium* sp.
	Leaves	6	*Drechslera* sp.		Twig	6	*Cladosporium* sp.
	Leaves	7	*Alternaria* sp.		Leaves	7	*Cladosporium* sp.
	Leaves	8	*Alternaria* sp.		Twig	8	*Emerisella nidulans*
Mint (*Mentha piperita* L.)	Root	1	*Alternaria* sp.		Twig	9	Sterile Mycelium
	Root	2	*Chaetomium* sp.	**Moringa (*Moringa oleifera* Lam.)**	Twig	1	*Alternaria* sp.
	Root	3	*Alternaria* sp.		Twig	2	*Alternaria* sp.+ *Ulocladium* sp.
	Root	4	*Chaetomium* sp.		Twig	3	*Alternaria* sp.
	Stem	5	*Ulocladium* sp.		Twig	4	*Alternaria* sp.

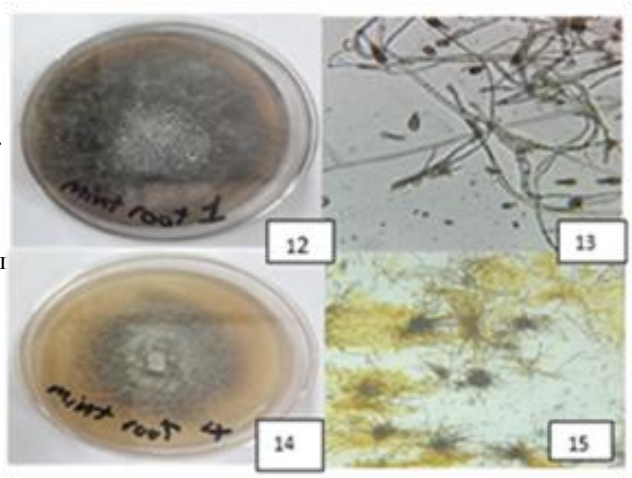


Photo (12): *Alternaria sp*. culture isolated from mint.

Photo (13): *Alternaria sp*. microscopically.

Photo (14): *Chaetomium sp.* culture isolated from mi

Photo (15): *Chaetomium sp*. microscopically

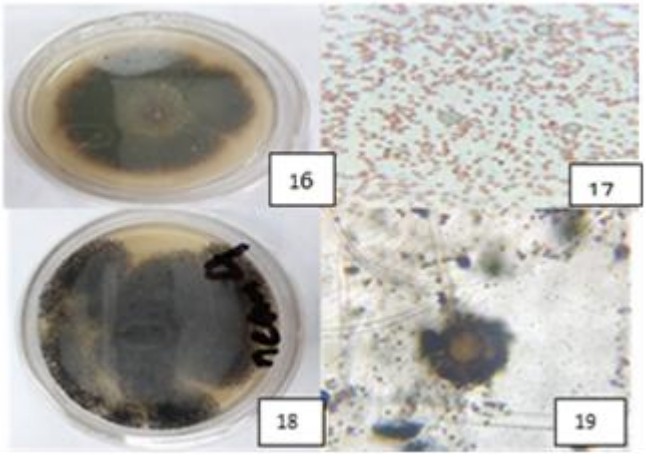


Photo (16): *E. nidulans* culture isolated from olive

Photo (17): *E. nidulans* microscopically.
Photo (18): *A. niger* culture isolated from neem.
Photo (19): *A. niger* microscopically.

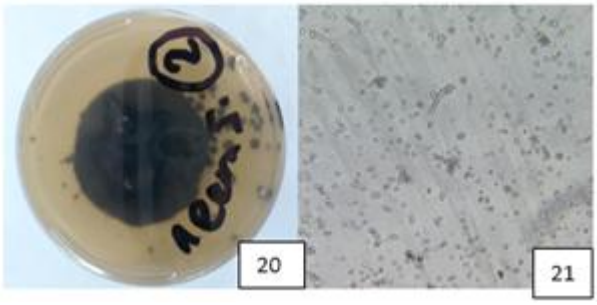


Photo (20): *Cladosporium* sp. culture isolated from neem.
Photo (21): *Cladosporium* sp. microscopically.

3.12. Evaluation of antibacterial activity of fungal endophytes. A total of 34 extra- and intracellular metabolites from 17 endophytic fungal pure isolates were evaluated for their antibacterial activities against the previously isolated fish pathogenic bacteria *P. aeruginosa*. Most of the endophytic fungal metabolites showed antibacterial activities against *P. aeruginosa*. The diameter of inhibition zones ranged from 7± 0 - 18± 1.41mm. Data has been listed in **Table (10)** as mean ± SD.

Table (10): The antibacterial activities of extra- and intra-cellular metabolites from endophytic fungal species

Plant	Extract type	Extract No.	Isolate No.	Endophytic fungi isolates	Mean zone of inhibition (mm)(±SD)
					P. aeruginosa
Hibiscus	**Filtrate**	6	1	*Alternaria* sp.	10 ± 1.41
		8	5	*Drechslera* sp.	10.5±0.71
	Mycelium	15	1	*Alternaria* sp.	8.5±0.71
		17	5	*Drechslera* sp.	11.5± 0.71
Mint	**Filtrate**	64	1	*Alternaria* sp.	11.5± 0.71
		65	2	*Chaetomium* sp.	11± 1.41
		70	4	*Chaetomium* sp.	12± 0
		72	5	*Ulocladium* sp.	-
	Mycelium	73	1	*Alternaria* sp.	-
		75	2	*Chaetomium* sp.	7.5± 0.71
		79	4	*Chaetomium* sp.	11.5± 0.71
		81	5	*Ulocladium* sp.	8± 1.41
Moringa	**Filtrate**	10	1	*Alternaria* sp.	10± 1.41
	Mycelium	19	1	*Alternaria* sp.	9± 1.41
Neem	**Filtrate**	2	2	*Cladosporium* sp.	13± 1.41
		4	4	*A. niger*	16.5± 0.71
	Mycelium	12	2	*Cladosporium* sp.	11± 1.41
		13	4	*A. niger*	10.5± 0.71
Olive	**Filtrate**	24	2	*Cladosporium* sp.	10± 0.71
		25	3	*Penicillium* sp.	8.5± 0.71
		30	5	*Cladosporium* sp.	11.5± 0.71
		32	6	*Cladosporium* sp.	10.5± 0.71
		46	4	*Fusarium* sp.	8.5± 0.71
		50	7	*Cladosporium* sp.	9± 0
		51	8	*Emerciella nidulans*	10.5± 0.71
		54	9	*Mycelia sterilia*	9.5± 0.71
	Mycelium	35	2	*Cladosporium* sp.	9.5± 0.71
		37	3	*Penicillium* spp.	8.5± 0.71
		41	5	*Cladosporium* sp.	7± 0
		44	6	*Cladosporium* sp.	8± 0
		55	4	*Fusarium* sp.	9.5± 0.71
		57	7	*Cladosporium* sp.	-
		59	8	*Emerciella nidulans*	-
		61	9	Sterile mycelium	7.5± 0.71

3.13. Minimum inhibitory concentration (MIC): All active extracts that revealed the most potent antimicrobial activity were further determined for their MIC by a microtiter broth dilution procedure. The *A. niger* extracellular fungal extracts showed MIC of 1.56 mg/mL *P. aeruginosa* **(Table 11).**

Table (11): Minimum inhibitory concentration

Plant	Extract type	Extract No.	Isolate No.	Endophytic fungi isolates	MIC (mg/ml)
					P. aeruginosa
Mint	Filtrate	64	1	*Alternaria* sp.	-
		70	4	*Chaetomium* sp.	12.5
Neem	Filtrate	2	2	*Cladosporium* sp.	25
		4	4	*A. niger*	1.56
Olive	Filtrate	51	8	*E. nidulans*	-
Positive control				Amikacin (50 mg/mL)	6.25

DISCUSSION

The secondary metabolites from the cultures of endophytic fungi have been originate to have cytotoxic, antiviral, antimicrobial and anticancer activities (**Nisa *et al.*, 2015**). The outcomes of the present study shown that out of 217 fish samples examined, 13.3% (23) were found to harbor *P. aeruginosa* in *O. niloticus*. These results nearly agree with the results obtained by **El-Bahar *et al.* (2019)** who identified 11/80 *P. aeruginosa*, representing 13.75% in *O. niloticus*. Furthermore**, Algammal *et al.* (2020)** isolated *P. aeruginosa* from 52 *C. gariepinus* with prevalence 26.9% (14). The current results of *P. aeruginosa* similar to the results obtained by **Magdy *et al.* (2014)** who detected *P. aeruginosa* in 27.5% (11/40) of the collected *C. gariepinus*.

The present results showed that *P. aeruginosa* identified with total prevalence of 21.5%, out of 183 bacterial isolates from *O. niloticus*. While, the results of *P. aeruginosa* agree with the results obtained by **El-Gamal *et al.* (2018)** who isolated *P. aeruginosa* with a prevalence of 15, 13.3 and 12% in *O. niloticus*. Higher results obtained by **Magdy *et al.* (2014)** who reported 34.4%. Thus, consistent with the present research results *P. aeruginosa* are the most common Gram-negative bacterial pathogens isolated from diseased freshwater fish **(El-Bahar *et al.*, 2019 and Sherif 2020).**

The whole distribution of *P. aeruginosa* in different organs of *O. niloticus* was 6 (24%), 9 (36%), 6 (24%), 4 (16%) and 0 (0%) in skin, gill, liver, kidney and spleen, respectively. **Begum *et al.* (2019)** found the highest prevalence of *Pseudomonas* sp. isolated from *O. niloticus* retrieved from the skin (20.7%), while **Matter *et al.* (2018)** isolated *P. aeruginosa* from the liver of cat fish only.

The extensive use of antimicrobials in husbandry and aquaculture promotes the emergence of antimicrobial-resistant zoonotic pathogens (**Sherif *et al.*, 2021**). Twenty two antibiotics belonged to 14 different antibiotic groups were tested against 30 isolates of *P. aeruginosa*. Concerning to *P. aeruginosa* isolates, the majority of *P. aeruginosa* strains were found to be highly resistant against most of the used antibiotics, the isolates showed 100% resistance against lincomycin, erythromycin, and penicillin as in Table (9). The present antibiogram results agree with **Magdy *et al*. (2014)** who reported that *P. aeroginosa* isolates showed sensitivity to colistin sulphate, and resistance against erythromycin, lincomycine, nitrofurantoin and (sulphamethoxazole + trimethoprim). Also, intermediate sensitivity to gentamycin has been reported. In contrast, the sensitivity to oxytetracyclin, nalidixic acid and oxolonic acid disagree with the present results which showed resistance against tested bacteria.Concerning this point, **Rajpakshe *et al*. (2012)** tested the sensitivity of *P. aeruginosa*, where high resistance was detected against, aztreonam, cefoxitin, nitrofurantoin and colistin methane sulphonate. Higher sensitivity was performed bynorfloxacin, ciprofloxacin and tetracyclin. The variation in antibiotic sensitivity test of isolated *P. aeroginosa* was designated by **Du *et al*. (2018)** who described that *P. aeruginosa* is intrinsically resistant to numerous antibiotics because of the low permeability of its outer-membrane, the constitutive expression of several efflux pumps, and the making of antibiotic-inactivating enzymes (e.g.,cephalosporinases).

The multi antibiotic resistance profile was noted with an MAR index for the total isolates of *P. aeruginosa* which described by **Vivekanandhan *et al*. (2002)** as 0.57. MAR index is a good tool for risk assessment; generally, the acceptable value is 0.2, with high values indicating the existence of multipleantibiotic resistance as in the present study. Similar or higher values of MAR index were found in other reports as **Matyar *et al.* (2010),** who obtained the MAR index values ranged from 0.2 to 0.73 for the *Pseudomonas* strains. **Nguyen *et al.* (2014),** also estimated the MAR index mean values of 0.457 of *Pseudomonas* isolates, indicated that these isolates were exposed to high risk sources of contamination where antibiotics were commonly used. lower MAR index for *P. aeruginosa* 0.3 obtained by **Rajpakshe *et al.* (2012**). Thus, alternative measures should be implemented to limit the abuse of antibiotics in aquacultural production.

In the present study, the results were recognized by bands at 956 bp that are specific for the *P. aeruginosa*, which is consistent with the study of **El-Bahar *et al.* (2019)** who detected 16S rDNA in 11 isolates of *P. aeruginosa* isolated from *O. niloticus* with bands observed at 956 bp.

The PCR results detected the *opr*L gene as an indication for *P. aeruginosa* virulence for the all tested isolates at 504 bp which agree with the results of many other studies where**, Abd El Tawab *et al.* (2016)** detected the *opr*L gene that was amplified in all 6 and 12 studied strains (100%), respectively giving product of 504 bp.

The *mex*R gene is one of the regulatory genes of the MexAB-OprM efflux system of *P. aeruginosa* **(Suresh *et al.*, 2018)**, which contributes to the natural resistance of *P. aeruginosa* to a wide range of antibiotics including fluoroquinolones, β-lactams and β-lactamase inhibitors, whereas MexXY-OprM contributes to aminoglycoside resistance. The detection of *mex*R gene in all samples of the present study could explaine the existence of MDR among *P. aeruginosa* isolates.

Few information are accessible on the antimicrobial activities of the endophytic fungi isolated from different medicinal plants against fish pathogens (**Septiana *et al.*, 2017**). In the current study, endophytic fungal extracellular metabolites which were extracted from *Alternaria* sp. and *Drechslera* sp. isolated from medicinal plant (*H. sabdariffa* L.) showed antibacterial activities against *P. aeruginosa* with inhibition zone diameter of 9± 0 mm and 10.5± 0.71mm, respectively. Moreover, the endophytic fungal intracellular metabolites exhibited antibacterial activities with inhibition zone diameter of 9± 1.41, 8.5 ± 0.71, 8.5± 0.71 and 11.5± 0.71mm, respectively. Several researches evaluated the antibacterial activities of different parts of the whole plant *H. sabdariffa* L. against fish pathogens **(Bariyyah *et al.*, 2019)**, but according to our knowledge no reports are available on antimicrobial activities of *H. sabdariffa* L. endophytic fungi metabolic extracts against fish pathogens.

In the current study most of the *M. piperita* L. isolated endophytic fungi from the root and only one isolate (*Ulocladium* sp.) has been obtained from the stem. **Martins *et al.* (2016)** explained that the higher diversity and abundance of the fungal endophytes in the roots than in the aboveground organs (leaves and twigs). The extracellular metabolites of *Alternaria* sp., *Chaetomium* sp. and *Ulocladium* sp. showed antibacterial activities against *P. aeruginosa* with inhibition zone diameter ranging from 12.5± 0.71mm and 7.5± 0.71mm. However, *Ulocladium* sp. did not show any activity against *P. aeruginosa.* In addition, all the intracellular metabolites of the same endophytic fungi possessed antibacterial activities with inhibition zone diameter ranging from 11.5± 0.71 to 7.5± 0.71 mm against *P. aeruginosa* except *Alternaria* sp., which did not show activity against *P. aeruginosa.* Many researchers investigated the antibacterial and immunostimulant activities of *M. piperita* L. in different forms (powder, extract and oil) against different fish bacterial pathogens **(Klūga *et al.*, 2017)**.

The current findings revealed that secondary metabolic extract of endophytes isolated from *M. oleifera* Lam. exhibited *in vitro* antibacterial activity, where the mean growth inhibition zones of extracellular metabolites of the *Alternaria* sp. two isolates against *P. aeruginosa* were 10± 1.41 and 10± 1.41mm, respectively. Other studies evaluated the antibacterial and the antifungal activities of the secondary metabolites of their endophytes **(Arora and Kaur, 2019; Mwanga *et al.*, 2019)**. However, none of them discussed the antimicrobial activities of these endophytes against fish pathogens.

In the present study *Cladosporium* sp. and *A. niger* were isolated from *A. indica* A. Juss. Similar results obtained **Chutulo and Chalannavar (2018)**, where *Cladosporium*

sp. and *Aspergillus* sp. were isolated in addition to other endophytic fungal species. *A. indica* A. Juss. is widely used as feed additive in fish farms for its immunostimulant and antimicrobial activity which has been discussed in several past researches **(Thanigaivel *et al*., 2015; Ubiogoro *et al*., 2019)**.

In the present study higher colonization of fungal endophytes have been detected in the twigs than in the leaves of *O. europaea* L. (3 isolates from leaves and 6 isolates from stem), these findings are in accordance with those found by some other investigations **(Martins *et al*., 2016; Gomes *et al*., 2018).** The present study revealed that the extracellular metabolites which were extracted from *Cladosporium* sp. (no 2), *Penicillium* sp., *Cladosporium* sp. (no 5), *Cladosporium* sp. (no 6), *Fusarium* sp. and *Cladosporium* sp. (no 7) possessed antibacterial activity against *A. hydrophila***.** Overall the results stressed for the first time the antibacterial potential of endophytic fungi from *O. europaea* L. against the fish pathogens and the possibility to be exploited for their antimicrobial agents.

The MIC was evaluated for the most potent extracts that could inhibit the fish bacterial pathogens which included the extracellular extracts of *Alternaria* sp., *Chaetomium* sp. (no 4), *Cladosporium* sp., *A. niger* and *E. nidulans*. The antimicrobial activities of these fungi were fully discussed in former reports (**Yadav *et al*., 2014; Alburae *et al*., 2020)**. The MIC range of the endophytic fungal extracts varied from 1.56 - 25 mg/ml. *A. niger* fungal extracts have the least MIC range (1.56 mg/ml) against *P. aeruginosa* which means this possessed the most significant antimicrobial activity. The same results obtained by **Yadav *et al.* (2014)** they reported that the endophytic extract of *A. niger* exhibited the least MIC range (1.87 mg/ml) against *K. pneumoniae*, *P. aeruginosa* and *S. flexneri.*

CONCLUSION

The present study provides further scope for isolating characteristics of each compound recognized to be present in the extracts of endophytic fungi and understanding their pharmacological properties to aid further drug progress. Further studies need to be carried out to isolate and characterize the active compound in neem. The endophytic fungi isolated from neem exhibited the highest antibacterial activity against *P. aeruginosa* and also to determine antibacterial activity against other fish bacterial pathogens. Assessment of the safety and toxicity of antimicrobial fungal endophytes extracts is important before implementing the uses of these compounds. So, further research is needed to prove the antibacterial effect from the fungal endophytes extracts against a wide range of MDR bacterial fish pathogens, as well as the antiviral and antifungal activities.

REFERENCES

Abdalla, M.A.; Aro, A.O.; Gado, D.; Passari, A.K.; Mishra, V.K.; Singh, B.P. and McGaw, L.J. (2020). Isolation of endophytic fungi from South African plants, and screening for their antimicrobial and extracellular enzymatic activities and presence of type I polyketide synthases. S. Afr. J. Bot.,134**:**336-342. https://doi.org/10.1016/j.sajb.2020.03.021

Abd El Tawab, A.A.; Maarouf, A.A. and Ahmed, N.M. (2016). Detection of virulence factors of *Pseudomonas* species isolated from fresh water fish by PCR. BVMJ, 30(1):199-207.

Abdel-Wahhab, M.A.; El-Nekeety, A.A.; Hathout, A.S.; Salman, A.S.; Abdel-Aziem, S.H.; Sabry, B.A. and Jaswir, I. (2020). Bioactive compounds from *Aspergillus niger* extract enhance the antioxidant activity and prevent the genotoxicity in aflatoxin B1-treated rats. Toxicon, 181: 57-68. DOI**:** https://doi.org/10.1016/j.toxicon.2020.04.103

Algammal, A.M.; Mohamed, M.F.; Tawfiek, B.A. and Hozzein, W.N. (2020). Antibiogram and PCR-RFLP based detection of *Aeromonas hydrophila* complex isolated from *Oreochromis niloticus*. Pathogens, 9(3):238. **https://doi.org/10.3390/pathogens9030238**

Alpert, P.T. (2017). Superbugs: antibiotic resistance is becoming a major public health concern. Home Health Care Management & Practice, 29(2): 130-133.

Alburae, N. A., Mohammed, A. E., Alorfi, H. S., JamanTurki, A., Asfour, H. Z., Alarif, W. M., & Abdel-Lateff, A. (2020). Nidulantes of *Aspergillus* (Formerly Emericella): A treasure troveof chemical diversity and biological activities. Metabolites, 10(2):73. https://doi.org/10.3390/metabo10020073

Arora, D.S. and Kaur, N. (2019). Antimicrobial potential of fungal endophytes from Moringa oleifera. Appl. Biochem. Biotechnol., 187(2): 628-648. https://doi.org/ 10.1007/s12010-018-2770-y

Astuti, P. and Nababan, O.A. (2014). Antimicrobial and cytotoxic activities of endophytic fungi isolated from *Piper crocatum* Ruiz & Pav. Asian Pac. J. Trop. Biomed., 4: S592-S596. https://doi.org/10.12980/APJTB.4.2014APJTB-2014-0073

Austin, B. and Austin, D.A. (2016). Aeromonadaceae representative (*Aeromonas salmonicida*) Bacterial fish pathogens (p. 215-321): Springer.

Awad, M. and EL-Makhzangy, A. (2015). Efficacy of ciprofloxacin and clove extract on bacterial infection of *Clarias gariepinus*. Sciences, 5(01): 01-09.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. J. Pharm. Anal, 6 (2): 71-79. https://doi.org/10.1016/j.jpha.2015.11.005

Bariyyah, S.K.; Prajitno, A. and Yuniarti, A. (2019). Phytochemical screening and antimicrobial activity of roselle (*Hibiscus sabdariffa* l.) flower extract against *Aeromonas hydrophila*. J. Exp. Life Sci**.** 9 (2):65-69. https://doi.org/10.21776/ub.jels.2019.009.02.01

Begum, S.; Salauddin, M.; Hossain, M.K. and Begum, M.D. (2019). Antibiogram Study of Bacterial Pathogen from Tilapia Fish in Bangladesh. TURJAF, 7(4):658-664.

Chutulo, E.C. and Chalannavar, R.K. (2018). Endophytic mycoflora and their bioactive compounds from Azadirachta indica: A comprehensive review. J. Fungus, 4(2):42. DOI: 10.3390/jof4020042

CLSI, C. (2016). Performance standards for antimicrobial susceptibility testing. Clinical Lab Standards Institute.

Du, G.; Xiao, M.; Yu, S.; Wang, M.;Xie, Y. and Sang, S. (2018). *Phyllanthus urinaria*: a potential phytopharmacological source of natural medicine. Int J Clin Exp Med, 11(7): 6509-6520. www.ijcem.com /ISSN:1940-5901/IJCEM0070937

El-Bahar, H.M.; Ali, N.G.; Aboyadak, I.M.; Khalil, S.A. and Ibrahim, M.S. (2019). Virulence genes contributing to *Aeromonas hydrophila* pathogenicity in *Oreochromis niloticus*. Int. Microbiol, 22:479–490. https://doi.org/10.1007/s10123-019-00075-3

El-Gawad, E.A.; El Asely, A.M.;Soror, E.I.; Abbass, A.A. and Austin, B. (2020). Effect of dietary *Moringa oleifera* leaf on the immune response and control of *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*) fry. Aquac Int, 28(1):389-402 https://doi.org/10.1007/s10499-019-00469-0

El-Gamal, A.M., El-Gohary, M.S. and Gaafar, A.Y. (2018). Detection and Molecular Characterization of Some Bacteria Causing Skin Ulceration in Cultured Nile Tilapia (Oreochromis niloticus) in Kafr El-Sheikh Governorate. Int. J. Zool. Res, 14(1):14-20.

Furmanek-Blaszk, B. (2014). Phenotypic and molecular characteristics of an *Aeromonas hydrophila* strain isolated from the River Nile. Microbiol. Res., 169(7-8): 547-552.

Gomes, T.; Pereira, J.A.; Benhadi, J.; Lino-Neto, T. and Baptista, P. (2018). Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem. Microbial ecology, 76(3): 668-679.

Gordon, L.; Giraud, E.; Ganière, J.P.; Armand, F.; Bouju-Albert, A.; De la Cotte, N. and Le Bris, H. (2007). Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. J. Appl. Microbiol, 102(4):1167-1176.

Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. Front. Microbiol., 7:1538. https://doi.org/10.3389/fmicb.2016.01538

Hoseinifar, S.H.; Shakouri, M.; Yousefi, S.; Van Doan, H.; Shafiei, S.; Yousefi, M. and Faggio, C. (2020). Humoral and skin mucosal immune parameters, intestinal immune related genes and antioxidant defense in rainbow trout (*Oncorhynchus mykiss*) fed dietary olive (*Olea europea* L.) waste. Fish Shellfish Immunol., 100:171-178.

Klūga A, Terentjeva M, Kántor A, Kluz M, Puchalski C, Kačániová M. (2017). Antibacterial activity of *Melissa officinalis* L., *Mentha piperita* L., *Origanum vulgare* L. and *Malva mauritiana* against bacterial microflora isolated from fish. Adv Res Life Sci;1(1):75-80. DOI: https://doi.org/10. 1515/arls-2017-0013

Lacap, D.; Hyde, K. and Liew, E. (2003). An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. Fungal Divers., 12:53-66.

Liang, H.; Xing, Y.; Chen, J.; Zhang, D.; Guo, S. and Wang, C. (2012). Antimicrobial activities of endophytic fungi isolated from *Ophiopogon japonicus* (Liliaceae). BMC Complement Altern. Med., 12(1): 238.

Magdy, I.; El-Hady, M.; Ahmed, H.; Elmeadawy, S. and Kenwy, A. (2014). A contribution on *Pseudomonas aeruginosa* infection in African catfish (*Clarias gariepinus*). Res. J. Pharm., Biol. Chem., 5(5):575-58. https://www.researchgate.net/publication/266142025

Manganyi, M.C.; Regnier, T.; Tchatchouang, C.D.; Bezuidenhout, C.C. and Ateba, C.N. (2019). Antibacterial activity of endophytic fungi isolated from *Sceletium tortuosum* L.(Kougoed). Ann. Microbiol. 69(6) DOI: 10.1007/s13213-019-1444-5

Martins, F., Pereira, J.A., Bota, P., Bento, A. and Baptista, P. (2016). Fungal endophyte communities in above-and belowground olive tree organs and the effect of season and geographic location on their structures. Fungal Ecol., 20:193-201.

Matter, A.F.; El Asely, A.M.; Shaheen, A.A.; El-Gawad, E.A.; El-Abd, H. and Abbass, A.A. (2018). Phenotypic and molecular characterization of bacterial pathogens isolated from diseased freshwater fishes. Int J Fish Aquat Stud, 6(2):34-41.

Matyar, F.; Akkan, T.; Uçak, Y. and Eraslan, B. (2010). *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea). Environ. Monit. Assess**.**, 167(1-4):309-320.

Mwanga, Z.; Mvungi, E. and Tibuhwa, D. (2019). Antimicrobial Activities of Endophytic Fungi Secondary Metabolites from *Moringa oleifera*. Tanz. J. Sci.,45(3):463-476. DOI:10.4314/TJS.V45I3

Nguyen, H.N.; Van, T.T..; Nguyen, H.T.; Smooker, P.M.; Shimeta, J. and Coloe, P.J. (2014). Molecular characterization of antibiotic resistance in *Pseudomonas* and *Aeromonas* isolates from catfish of the Mekong Delta, Vietnam. Vet. Microbiol., 171(3-4):397-405. https://doi.org/10.1016/j.vetmic.2014.01.028

Nisa, H.; Kamili, A.N.; Nawchoo, I.A.; Shafi, S.; Shameem, N. and Bandh, S.A. (2015). Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: a review Microb. Pathog., 82:50-59.

Noga, E.J. (2010). Fish disease: diagnosis and treatment: John Wiley & Sons

Rajpakshe, A.; Prasad, K.P.; Mukherjee, S.C.; Kundan, K.; Brahmachari, R.K.; Meena, C.T. and Kumar, N. (2012)**.** In vitro sensitivity of three bacterial pathogens of koi carp (*Cyprinus carpio* L.) to certain antibiotics. J. Agric. Sci. Technol., 2: 93-98.

Sánchez, P.; Linares, J.F.; Ruiz-Díez, B.; Campanario, E.; Navas, A.; Baquero, F. and Martínez, J. L. (2002). Fitness of *in vitro* selected *Pseudomonas aeruginosa* nalB and nfxB multidrug resistant mutants. J. Antimicrob. Chemother., 50(5): 657-664.

Septiana, E., Sukarno, N. and Simanjuntak, P. (2017). Endophytic fungi associated with turmeric (*Curcuma longa* L.) can inhibit histamine-forming bacteria in fish. Hayati J. Biosci., 24(1); 46-52. https://doi.org/10.1016/j.hjb.2017.05.004

Sherif, A. H.; Gouda, M.Y.; Naena, N.A. and Ali, A.H. (2020). Alternate weekly exchanges of feeding regime affect the diversity of intestinal microbiota and immune status of Nile tilapia *Oreochromis niloticus*. Aquac. Res., 51(10): 4327-4339. **https://doi.org/10.1111/are.14778**

Sherif, A.H.; Gouda, M.; Darwish, S. and Abdelmohsin, A. (2021). Prevalence of antibiotic-resistant bacteria in freshwater fish farms. Aquac. Res., 52(5): 2036-2047. **https://doi.org/10.1111/are.15052**

Spilker, T.; Coenye, T.; Vandamme, P. and LiPuma, J.J. (2004). PCRbased assay for differentiation of P*seudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. **J**. Clin. Microbiol., 42(5):2074-2079.

Suresh, M.; Nithya, N.; Jayasree, P.; Vimal, K. and Kumar, P.M. (2018). Mutational analyses of regulatory genes, mexR, nalC, nalD and mexZ of mexAB-oprM and mexXY operons, in efflux pump hyperexpressing multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*. World J. Microbiol. Biotechnol., 34 (6):83. DOI: 10.1007/s11274-018-2465-0

Ubiogoro, O.E.; Alarape, S.A.; Saka, A.B. and Adeyemo, O.K. (2019). Growth performance and sensory parameters of African catfish (*Clarias gariepinus*) fed with a sublethal dose of neem leaf extract, and its antibacterial effects. Vet. Arh., 89(5): 709-721. DOI: 10.24099/vet.arhiv.0284

Vivekanandhan, G.; Savithamani, K.; Hatha, A. and Lakshmanaperumalsamy, P. (2002). Antibiotic resistance of *Aeromonas hydrophila* isolated from marketed fish and prawn of South India. Int. J. Food Microbiol., 76(1-2): 165-168.

Xu, J.; Moore, J.E.; Murphy, P.G.; Millar, B.C. and Elborn, J.S. (2004). Early detection of *Pseudomonas aeruginosa*–comparison of conventional versus molecular (PCR) detection directly from adult patients with cystic fibrosis (CF). Ann. clin. Microbial., 3(1):21.

Yadav, M.; Yadav, A.; Kumar, S.; Sharma, D. and Yadav, J.P. (2014). Evaluation of *in vitro* antimicrobial potential of endophytic fungi isolated from *Eugenia jambolana* Lam. Int. J. Pharm. Sci., 6(5): 208-211.

Zemheri-Navruz, F.; Acar, Ü. and Yılmaz, S. (2019). Dietary supplementation of olive leaf extract increases haematological, serum biochemical parameters and immune related genes expression level in common carp (*Cyprinus carpio*) juveniles. Fish Shellfish Immunol., 89: 672-676. https://doi.org/10.1016/j.fsi.2019.04.037

Zhang, C.; Tian, X. and Zhang, C.S. (2019). Diversity and probiotic activities of endophytic bacteria associated with the coastal halophyte *Messerschmidia sibirica*. Appl. Soil Ecol., 143:35-44. https://doi.org/10.1016/j.apsoil.2019.05.030