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Screening of bioactive compound from soil mycoflora and its therapeutic effect on fish borne pathogens of grass carp, *Ctenopharyngodon idella* (Valenciennes)

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Abstract
Soil samples were collected from diverse agricultural fields of Erode Taluk. There were 7 fungal genera which include 11 fungal species observed in composite soil sample in which 9 were identified and 2 of the *Aspergillus* species could not be identified. Among the fungal genera the genus *Aspergillus* was predominant in the test soil sample with five of its species. Total bacterial population in the gills and intestine of test fish were counted and recorded. The high percentage of occurrence was shown by *E. coli* and *Aeromonas hydrophila* (17.2%). There were 9 bacterial genera isolated from *C. idella* and identified as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *B. cereus*, *E. faecalis*, *Salmonella*, *Vibrio*, *Aeromonas hydrophila* and *Proteus*. The bacterial pathogens were tested against selected antibiotics. Biofilm was produced as black colonies. *Trichoderma* species 1 was found to exhibit high antimicrobial activity against all selected pathogens.

Keywords: composite soil, bacterial pathogen, grass crap, therapeutic effect

1. Introduction
Nature is a rich source of bioactive metabolites with therapeutic potential. Soil are the main source of fungal genetic resource and recognized as a prolific secondary metabolite producers. Large quantities of decomposable organic matter added to agricultural soil every year have a significant outcome of soil microbial community.

Soil fungi plays a pivotal role in evaluation of soil conditions and in stimulating plant growth by biochemical transformation and mineralization activities in soils [1]. Type of cultivation and crop management practices found to have greater influence on the activity of soil fungi [2]. Continuous use of chemical fertilizers over a long period may cause imbalance in soil mycoflora and thereby indirectly affect biological properties of soil leading to soil degradation [3]. Fungi are an important component of the soil micro biota [4]. Micro fungi play a focal role in nutrient cycling by regulating soil biological activity [5]. There are many fungal species being exploited for the production of bioactive molecules such as antibiotics, enzymes and organic acids but still a lot remains untapped from diverse soil habitats and not yet assayed for their antibacterial activity [6].

Antibiotic resistance in bacteria is one of the emerging health related problems in the world. Extensive efforts have been made to identify bioactive compounds derived from natural resources, in particular from various fungi in order to develop safe, non-toxic and efficient antimicrobial agent. Fungi of soil have been the best choice among natural resources. Screening the bioactivity of fungal extract is essential in medical applications. This screening is tried with the bacterial pathogens of fish grass carp Ctenopharyngodon idella. Fishes are readily susceptible to microbial attack particularly bacteria [7]. Bacteria can enter the fish body through the gills &mouth. The gills are the primary target where the bacteria invades the fish's body and damages internal organs. By monitoring the bacterial contents of fish organs, the quality of fish can be measured because it will affect the storage life and quality of fish meat. Thus to predict the chances of disease out breaks and to design preventive measures detailed information of the bacterial load and types of bacteria associated with the organs in healthy fish is needed.

The bioactive compound from soil fungi have become an environment friendly, alternative compound to antibiotics. In the current health scenario, the emergence of new infectious diseases, the resurgence of several infections and increase in bacterial resistance has created the necessity for studies directed towards the development of new antimicrobials and discovery of eco-friendly novel antibiotics. Thus there is an urgent need for the production of effective and eco-favoured bioactive compounds (anti-microbials) from inexpensive bio-resources and this could be attained through this type of research. Hence the present study has been undertaken to investigate the bioactive compound from fungal diversity in composite soil samples of various agricultural fields in Erode Taluk.

2. Materials and Methods

2.1. Collection of soil sample and culture of fungi

Soil samples were collected from the sites where agricultural fields and cultivation are more in different regions of Erode Taluk. The selected sites were Anthiur, Bhavani, Thindal, Poondurai, and Modakurichi. The soils were collected from various crop fields such as paddy, sugarcane, maize, turmeric, banana, tapioca etc., and all the soils have been mixed well and composite soil was prepared to have a good collection of soil fungi. The samples (approximately 5g each) were collected using some clean, dry and sterile polythene bag along with sterile spatula. All the samples were transferred to the laboratory. It was processed to remove lumps and debris, sieved and air dried for 3-5 days at 18 ºC. The composite soil sample was prepared by mixing a minimum of 10 soil cores on a same area (10 m^2). The samples were kept at 100C until used.

2.2. Isolation of fungi from the soil samples

The soil micro fungi were enumerated by two methods, namely Soil Dilution and soil plate method on different media such as Potato Dextrose Agar and Czapek, Dox Agar.

2.3. Soil dilution plate method

1 gram of soil sample was suspended in 10ml of double distilled water to make microbial suspensions (10-1 to 10-5). Dilution of 10-3, 10-4 and 10-5 were used to isolate fungi. 1 ml of microbial suspension of each concentration were added to sterile Petri dishes (triplicate of each dilution) containing 15 ml of sterile Potato Dextrose Agar and Czapek, s Dox Agar [8]. One percent streptomycin solution was added to the medium before pouring into petriplates for preventing bacterial growth. The Petri dishes were then incubated at 28± 2 ^{0}C in dark. The plates were observed everyday up to three days.

2.4. Soil plate method

About 0.005g of soil was scattered on the bottom of a sterile petri dish and molten cooled (40-45 °C) agar medium (PDA) & (CZA) was added, which was then rotated gently to disperse the soil particles in the medium. The Petri dishes were then incubated at 28 ± 20 C in dark for three days [9].

2.5. Identification of the soil fungi

Fungal morphology were studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto phenol cotton blue and observe under compound microscope for the conidia, conidiophores and arrangement of spores. The fungi were identified with the help of literature [10, 11]. Physico-chemical analysis of soil: The collected soil was characterized for its physico-chemical properties. The physico-chemical parameters were measured by standard methods.

2.6. Collection of fish

The grass carp *Ctenopharyngodon idella* were procured from the fisheries named Seraguvae, near Nerunchipettai, Mettur, Erode. The collected fish samples were transferred directly to the laboratory in an ice box with a minimum of delay, where they were prepared for acclimatization.

2.7. Maintenance of test fishes

The fishes with uniform weight (10-12 gms) and length (9-11 cm) were acclimatized in the laboratory under constant conditions. Fishes were reared in 20 L capacity plastic containers filled with de-chlorinated tap water (27 ± 1 °C). Fishes were fed twice daily with the standard commercially available fish feed pellets at 2% of their body mass. Medium has been changed every day and fishes were kept without stress for about 10 days.

2.8. Microbial analysis

2.8.1. Pre-enrichment

Fishes were dissected and tissue samples were taken under complete aseptic conditions from intestine and gills. Five grams of the test samples were weighed aseptically into sterile blender container and thoroughly homogenized with 100 ml of sterile peptone broth. The homogenate was incubated at 37 °C for 24 h. After incubation serial dilutions were made.

2.8.2. Total bacterial population

Serial diluted sample were plated on the nutrient agar medium and incubated at 37 °C for 24 h, after incubation the samples were observed, the colonies were counted and recorded.

2.8.3. Antibiotic sensitivity assay

All isolates were subjected into antibiotic stability test [12-14]. The susceptibility of isolates of bacteria to antimicrobial agents was examined by a disc diffusion assay. The bacterial isolates were tested against the following antibiotic discs (HiMedia): Gentamycin,10 µg; Ceftizomine, 30µg; Ceftriaxone 30µg; Erythromycin 15µg; Kanamycin 30µg; Penicillin 5µg; Bacitracin 10 units; Amikacin 30 µg; Tetracycline 30µg and Ampicillin 30µg. After 30 min of pre-diffusion time, the plates were incubated at 37 ºC for 18 - 24 h. After the incubation period, the diameter of the inhibition zone was measured and compared with the interpretive chart of Performance Standards for Antimicrobial Disk Susceptibility Tests, Dec. 1993 (Hi-Media) and classified as resistant, intermediate and sensitive.

2.8.4. Biofilm formation and isolation of biofilm producing isolates

Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved at 121 °C for 15 minutes. The isolates were inoculated and incubated the plates aerobically for 24 to 48 hours. Biofilm production was indicated by black colonies with a dry crystalline consistency whereas biofilm non-producers remain pink, though occasional darkening at the center of the colony was observed [15].

2.9. Characterization of pure fungal isolates
Characterization of pure fungal isolates was carried out based on macroscopic and microscopic examinations. Pure fungal isolates showing great antibacterial activities during secondary screening were cultivated on PDA. The different growth morphologies, including top colour, mycelium mat, mycelium end reverse colour, medium colour and perimeter were observed and recorded. Intact structures of the fungi, including structure of hyphae, conidia, spores and conidiophores were observed under light compound microscope.

3. Results and Discussion
3.1. Isolation of soil fungal isolates
In all the five selected locations and among the 7 identified genera, the genus Aspergillus was found to be dominant with four of its species (i.e.) *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus* and *Aspergillus fumigatus*. This was followed by the genus Mucor. Next to Mucor, Trichoderma species was found to be abundant in two sites (I and III). It was observed that Penicillium, Actinomycetes and Rhizopus were represented as a single representation of its genus. More number (4) of fugal species was noted in soil samples of site I and III (Table 1).

3.2. Colony characteristics of fungal species
Table 2 explained the colour of the colony and its growth pattern. *Rhizopus* was a fast growing cottony colony, mostly white in colour. *Fusarium* was pale colored white cottony culture which was fast growing. At maturity it became yellow. A clear black colored colony with moderate growth was noted as *Aspergillus niger*. *Aspergillus flavus* was differentiated as yellow - green colony with rapid growth. There was slow growth observed in *Aspergillus fumigates* with bluish grey colour. Two of the Aspergillus species could not be identified. Mucor was greyish in colour with rapid growth. Penicillium showed moderate growth and dirty white colour. Actinomycetes were white powdery colony with moderate growth. Trichoderma was notable with concentric rings. It was also a fast growing colony and compact in nature with greenish patches.

3.3. Cultural characteristics of fish pathogens on selective media
In each medium, the particular bacterial species has grown well. Based on the colour of the colony on a specific medium the identification of bacterial species was done. Metallic green colour colony on EMB, pink colour on MacConkey, yellow green on Cetramide agar, blue colour on Chromogenic agar, black colour on SS agar, yellow colour on TCBS agar, yellow colour on Starch ampicillin agar, light brown colour on chromogenic agar indicated the presence of *E. coli*, *Klebsiella pnemoniae*, *Pseudomonas areuginosa*, *Enterococcus faecalis*, *Salmonella*, *Vibrio*, *Aeromonas hydrophila* and *Proteus* respectively (Table 3). The species was further confirmed by the biochemical tests.

3.4. Bacterial pathogens isolated from the gills of grass carp, *C. idella*
Totally in the gills of the fish grass carp there were eight species of bacteria in which *Salmonella* and *Aeromonas* were represented thrice, *E. coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* were represented twice whereas *Proteus*, *Vibrio* and *Bacillus* where represented once (Table 4).

3.5. Bacterial pathogens isolated from the intestine of grass carp, *C. idella*
In the intestine of test species, *E. coli* and *Klebsiella* were equally dominated followed by *Vibrio*, *Salmonella*, *Aeromonas hydrophila*. All bacterial species observed were represented both in the gills and intestine except *Bacillus* in the gills and *Klebsiella* in the intestine (Table 5). Total bacterial population in the gills and intestine of test fish were counted and recorded. The high percentage of occurrence was shown by *E. coli* and *Aeromonas hydrophila* (17.2%) [16]. The bacterial population was more in the gills than in the intestine. The bacterial species were identified using colony morphology, selective media and biochemical tests. There were 9 bacterial genera isolated from *C. idella* and identified as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *B. cereus*, *E. faecalis*, *Salmonella*, *Vibrio*, *Aeromonas hydrophila* and *Proteus*.

3.6. Occurrence of the bacterial genera in grass carp, *C. idella*
In the selected fish samples on the whole, nine bacterial genera were represented. The high percentage of occurrence (17.2) was represented by *E. coli* and *Aeromonas hydrophila*. *Salmonella* species showed 14% of occurrence, this was followed by *Vibrio*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis* with 10.3% of occurrence. Less percentage of occurrences was shown by *Proteus* (7%) and the least (3.4%) occurrence was noted in *Bacillus* (Table 6).

3.7. Antibiotic susceptibility
The zone of inhibition (diameter) was measured and classified as resistant and sensitive. Out of 29 isolates of 9 bacterial genera, 23 of the isolates were resistant to Erythromycin and Amikacin followed by Penicillin. These drugs have no effect on the bacterial pathogens, where Tetracycline was found to be an effective antibiotic to kill the bacterial pathogen. Among the bacterial isolates *Aeromonas* showed highest percentage of resistance towards antibiotics (Table 7).

3.8. Antimicrobial activity
Secondary screening and selection of potent isolates for antimicrobial activity was done by agar well diffusion assay. Trichoderma species 1 was found to exhibit high antimicrobial activity against all selected pathogens (Table 8). The bacterial pathogens were tested against selected antibiotics (i.e) Gentamycin, Ceftizomine, Ceftriaxone, Erythromycin, Kanamycin, Penicillin, Bacitracin, Amikacin, Tetracycline and Ampicillin. The zone of inhibition (diameter) was measured and classified as resistant and sensitive. Out of 29 isolates of 9 bacterial genera, 23 of the isolates were resistant to Erythromycin and Amikacin followed by Penicillin. These drugs have no effect on the bacterial pathogens, where Tetracycline was found to be an effective antibiotic to kill the bacterial pathogen. Among the bacterial isolates *Aeromonas* showed highest percentage of resistance towards antibiotics [17]. (Isoken *et al*., 2012). Biofilm was produced as black colonies. Out of 29 isolates, 17 isolates were found to be the biofilm producers. Except Bacillus, all bacteria were found to produce biofilm [18].

Table 1: Isolation of soil fungal isolates

S. No.	Fungal isolates	*E. coli*	Pseudo	*Vibrio*
1.	I	Anthiyur	Soil sample 1	*A. niger*, *Mucor*, *A. oryzae*, *Trichoderma* sp., *Fusarium* sp.
2.	II	Bhavani	Soil sample 2	*A. niger*, *A. flavus*, *Mucor*
3.	III	Perundurai	Soil sample 3	*A. niger*, *Mucor*, *A. fumigatus*, *Trichoderma* sp.
4.	IV	Aval Poondurai	Soil sample 4	*Penicilium* sp, Actinomycetes
5.	V	Modakurichi	Soil sample 5	*A. fumigatus*, *Rhizopus*, *Aspergillus* sp.

Table 2: Colony characteristics of fungal species

Fungal isolates	Colony colour on PDA	Growth pattern
Rhizopus sp.	White at maturity black	Fast growing; Cottony
Fusarium sp.	Pale colored; whitish to yellow	Fast growing; cottony aerial mycelium
Aspergillus niger	Black	Moderate growth with submerged mycelium
Aspergillus flavus	Yellow-green	Rapid growth
Aspergillus fumigatus	Blue-green to gray	Moderate to slow growth
Aspergillus spp (unknown 1)	Dark brown colour	Moderate to slow growth
Aspergillus spp (unknown 2)	Light brown with wriggle	Very slow growth
Mucor	White to beige or grey	Rapid growth
Penicillium	White with yellowish-green	Moderate to slow growth
Actinomycetes	White powdery	Moderate growth
Trichoderma sp.	White and scattered greenish patches become visible as the conidia are formed and may form concentric rings at times	Fast growing; wooly becoming compact in time

Table 3: Cultural characteristics of fish pathogens on selective media

Organisms	Character on selective media
Escherichia coli	EMB-Metalic green colour
Klebsiella pneumonia	Pink colour on Macconkey
Pseudomonas aeruginosa	Yellow green on cetramide agar
Bacillus cereus	White dull colour on nutrient agar
Enterococcus faecalis	Blue color on Chromogenic agar
Salmonella spp	Black colour on SS agar
Vibrio spp	Yellow colour on TCBS agar
Aeromonas hydrophila	Yellow colour on Starch ampicillin agar
Proteus	Light brown colour on chromogenic agar

Table 4: Bacterial pathogens isolated from the gills of grass carp *C. idella*

S. No.	Samples	Isolates
1.	Fish Sample 1	*E. coli*, *P. aeruginosa*
2.	Fish Sample 2	*A. hydrophila*, *Salmonella sp.*,
3.	Fish Sample 3	*E. coli*, *E. faecalis*, *A. hydrophila*, *Proteus*
4.	Fish Sample 4	*Salmonella* sp., *Vibrio* sp., *P. aeruginosa*, *E. faecalis*
5.	Fish Sample 5	*Bacillus* sp., *A. hydrophila*, *Salmonella* sp.

Table 5: Bacterial pathogens isolated from the intestine of grass carp *C. idella*

S. No.	Samples	Isolates
1.	Fish Sample 1	*Vibrio* sp., *E. coli*,
2.	Fish Sample 2	*E. coli*, *K. pneumoniae*, *Proteus*
3.	Fish Sample 3	*A. hydrophila*, *E. faecalis*
4.	Fish Sample 4	*Salmonella* sp., *K. pneumoniae*, *E. coli*
5.	Fish Sample 5	*K. pneumoniae*, *A. hydrophila*, *P. aeruginosa*, *Vibrio* sp.

Table 6: Frequency of occurrence of the bacterial genera in grass carp *C. idella*

Bacterial genera	Number of isolates	% of occurrence
E. coli	5	17.2
Vibrio	3	10.3
P. aeruginosa	3	10.3
K. pneumonia	3	10.3
Salmonella spp	4	14
Bacillus	1	3.4
A. hydrophilia	5	17.2
E. faecalis	3	10.3
Proteus	2	7
Mean ± S.D	3.24 ± 1.30*#	11.11 ± 4.49*#

* 't' test significant at 5% (p<0.05) level, # Anova significant at 1% (p<0.01) level

Table 7: Antibiotic susceptibility of bacterial pathogens in grass carp *C. idella*

S. No.	Isolates	Antibiotics										% of susceptibility
		G	Ctx	Ctr	E	K	P	B	A	Te	Amp	
1.	*E. coli* 1	S	R	R	R	S	R	R	R	R	R	20
2.	*E. coli* 2	S	R	R	R	S	R	R	R	S	R	30
3.	*E. coli* 3	R	R	S	R	S	R	S	S	S	R	50
4.	*E. coli* 4	S	S	S	R	S	R	R	R	S	S	60
5.	*E. coli* 5	S	R	R	R	S	R	R	R	R	R	20
6.	*P. aeruginosa* 1	S	S	S	R	S	R	R	R	R	I	40
7.	*P. aeruginosa* 2	R	R	S	R	R	R	R	S	R	R	20
8.	*P. aeruginosa* 3	S	S	S	R	S	R	R	R	R	I	40
9.	*Vibrio* 1	S	S	S	R	R	R	S	S	S	R	60
10.	*Vibrio* 2	S	S	R	R	R	R	S	S	S	S	60
11.	*Vibrio* 3	S	S	R	R	R	R	S	S	S	S	60
12.	*Aeromonas* 1	R	R	R	R	R	R	S	R	S	R	20
13.	*Aeromonas* 2	S	S	S	R	R	S	R	R	R	R	40
14.	*Aeromonas* 3	R	R	R	R	R	R	S	R	S	R	20
15.	*Aeromonas* 4	S	R	R	R	R	S	S	R	S	R	40
16.	*Aeromonas* 5	R	R	R	R	R	R	S	R	S	R	20
17.	*Salmonella* 1	R	R	R	S	R	S	R	R	S	S	40
18.	*Salmonella* 2	R	R	R	S	S	R	R	R	S	S	40
19.	*Salmonella* 3	R	R	R	S	S	R	R	R	S	S	40
20.	*Salmonella* 4	R	S	S	R	R	R	S	R	R	R	30
21.	Kleb 1	S	S	S	R	S	R	R	R	R	S	50
22.	Kleb 2	R	R	R	R	S	R	S	R	R	S	30
23.	Kleb 3	S	S	S	R	S	R	R	R	R	R	40
24.	*Proteus* spp	S	S	S	S	R	S	S	R	R	S	70
25.	*Proteus* spp	R	S	S	R	R	S	S	S	R	S	60
26.	*Enterococcus*	S	S	S	R	S	R	R	R	S	R	50
27.	*Enterococcus*	R	S	R	S	R	S	R	R	R	S	40
28.	*Enterococcus*	R	R	R	R	R	S	R	R	S	R	20
29.	*Bacillus* spp	R	S	S	S	R	R	S	R	S	S	60

G – Gentamycin, Ctx – Ceftizomine, Ctr – Ceftriarone, E – Erythromycin, K – Kanamycin, P – Penicillin, B – Bacitracin, A – Amikacin, T – Tetracycline, Amp – Ampicillin

Table 8: Screening of anti-microbial activity producing fungi by seeded agar plate method

S. No.	Fungal isolates	*E. coli*	Pseudo	*Vibrio*	*Aeromonas*	*Salmonella*	Kleb	*Proteus*	Entero	*Bacillus*
1.	*Trichoderma* sp. 1	+	+	+	+	-	+	+	+	+
2.	*Trichoderma* sp. 2	-	-	-	-	-	+	+	+	-
3.	*Penicilium* sp,	-	-	-	-	-	-	-	-	+
4.	Actinomycetes	-	-	-	+	-	-	-	-	+
5.	*Rhizopus*	-	-	-	-	-	-	-	-	-

4. Conclusion

The present investigation strongly evidenced that the bioactive compound extracted from soil fungi was an effective bacteriocide. The fungal extract was a potent, effective, ecofriendly and a natural antibiotic against multi resistant pathogens. This fungal extract might help the fishery industry to reduce their medical expenses by reducing the cost of antibiotics and increasing resistance towards diseases.

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