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Identification, characterization of fungus which infects domestic fishes and its prevention using plant extracts

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Abstract
Fish fungus is one of the main concerns of people who culture fishes in hatcheries and aquariums as they adversely affect fishes leading to its death. Chemical formulations are used to treat fungus growing on fishes but not all fishes can be cured with the chemical formulations and the effects of these chemicals maybe shown when human consumes those fishes. Hence, it is always better to go for medicine from natural sources to treat these kinds of diseases. In this study, we have isolated fish fungus by culturing infected part of fishes *Carassius auratus* and *Cyprinus carpio* on Potato dextrose agar. The fungus was allowed to grow for 5–7 days at 28-30 ˚C, white spongy and grey colonies were observed. Genomic DNA was isolated from fungus and ITS gene was PCR amplified. They were further characterized using Sanger sequencing method to characterize its taxonomic nature of fungus and its growth properties. Further few common plants having antimicrobial properties were used to inhibit the growth of these funguses using agar well diffusion method. The results showed that the isolated funguses are *Aspergillus fumigatus* and *Saprolegnia parasitica* when sequences were subjected to NCBI BLAST. The results of antimicrobial studies highlighted the effect of ethanolic leaf extracts of *Cardiospermum helicacabum*, *Kirganelia reticulata*, *Urena lobata*, *Vitex negundo*, *Scoparia dulcis* and *Pongamia pinnata* plants against growth of above mentioned pathogenic fungus.

Keywords: antifungal activity, fish fungus, plant extracts, DNA bar coding

1. Introduction
Fishes play a major role in maintaining the ecosystems as they store nutrients in their tissues, transport it to other aquatic animals and also when they excrete it out from their body the necessary nutrients gets settled down in aquatic bodies. The products obtained from fishes have always been useful to man and its meat contains cod liver oil which is rich in Vitamin A and B. Fishes getting infected to fungus is the most common criteria responsible for mortality and economic losses in fish aquarium industries (Eakaphun *et al*., 2003) [1]. The most common infection that affects fishes is superficial, cotton like growth on the skin or gills. These lesions usually start as small infections which later spread rapidly over the surface of the body. Newly formed lesions will be white and over time becomes red, brown or green. George, *et al*. (1998) [2] reported that typical saprolegniasis grow on surface of skin and usually do not penetrate deeply into muscle. More than 80 fungal species are isolated from different kinds of aquatic fungus belonging to Saprolegnia, Pythium, Thraustotheca, Achlya, Aphanomyces, Dictyocha and Protachlya, which are considered as special parasites of temperate fish in India. Among these Saprolegnia and Achlya are most virulent parasite when compared to others (Sati and Khulbe, 1991) [3]. Aspergillus, Penicillium, Absidia and Pseudallscheria species are reported contaminated trout pellet feed (Diaz *et al*., 2009; Cutuli *et al*., 1991; Alinezhad *et al*., 2011) [4, 5, 6]. *Aspergillus fumigatus* is a human pathogen and environmental contaminant which leads to internal and external infection in fish (Alinezhad *et al.*, 2011; Saleemi *et al.*, 2012; Firoozbhakhsh *et al*., 2005; Ebrahimzadeh *et al*., 2007) [6, 7, 8, 9, 17]. Saprolegnia and Aphanomyces species are devastating infections on fish in aquaculture, fish farms and aquarium fish tanks. As a result *Saprolegnia parasitica* is now economically important pathogen, especially on edible fishes which demands investigations on developing treatment strategies (Pieter van West, 2006) [10]. So growing fish for consumption as well as its ornamental value plays vital roles in the ecosystem.

Culturing fishes with quality and preventing them from having infection is especially necessary with edible fishes. So we end up using chemicals which prevent fungus and that can still remain in the body of fishes which transfers to the human body causing ill effects. These chemicals also might cause mutations in human beings. The strategies used so far, for the prevention of fungus is to isolate the infected fishes and treat them with chemicals and transferring to clean water. The chemicals used are malachite green, chloromycetin, phenoxethol etc. Malachite green is a toxic material which is used as a stain at low concentration as anti-parasite, antifungal and antibacterial agent against fungal infection in fishes (Due *et al*., 1998) [11]. As it is considered as carcinogenic and mutagenic matter, it is no longer used by FDA (Food and Drug Administration) for edible fishes (Moghaddam *et al*., 2004) [12]. Many countries have refused to use this in aquatic environments, and an alternative number of studies are supported to find safety treatments which does not harm the environment. One good safety approach is using plant extracts and their oils that are traditionally used for treatment of human diseases and as antimicrobials (Al-Mayah, 2013) [13].

The current study aims towards replacing malachite green with natural substances to avoid adverse effects on human health, finding out cost effective materials which are available throughout the year which will help to reduce economic losses. So use of plant extracts which are locally available serves as the best alternative. Our study was aimed to identify and characterize the fungal infections in two locally collected ornamental and edible fishes. Further 6 plants having antimicrobial activities such as *Cardiospermum helicacabum*, *Kirganelia reticulata*, *Urena lobata*, *Vitex negundo*, *Scoparia dulcis* and *Pongamia pinnata* were extracted using ethanol and checked its rate of inhibition towards inhibition of above isolated funguses.

2. Materials and Methods

2.1 Collection of fish and Culturing of fungus

The infected fishes were collected from aquarium shop and fish market near Peenya, Bangalore. The infected part from the surface of *Carassius auratus* and *Cyprinus carpio* fish skin was cultured on PDA at 28-30 ˚C for 5-7 days. The fungus was further used for molecular studies.

2.2 DNA Isolation

The DNA isolation was done using the C-TAB method. This DNA was run on Agarose gel, based on the ethidium bromide fluorescent dye staining. The DNA concentration and purity was checked by running the samples on 0.8% agarose gel depending on the intensities of the band when compared with 1Kb lambda DNA marker.

2.3 PCR

The purified DNA was then taken for polymerase chain reaction to amplify a single copy of desired part of DNA to multiple copies. The Primers used were for ITS. The amplified ITS gene was compared with 100bp ladder. The obtained PCR products were gel purified to get rid of salt impurities and further subjected for Sanger sequencing.

2.4 Sanger sequencing

Sangers sequencing was done to obtain the basic information of sequence with actual nucleotides. The results were obtained in the form of Electropherogram peaks which were converted to FASTA sequence. Sequencing files were obtained.AB1 format which was viewed using Finch TV software. Quality of the obtained sequence was observed through Electropherogram peaks. Further the sequencing data was analysed using NCBI BLAST server to identify the similar hit. Once we obtained the name of an unknown organism, further phylogenetic tree was constructed using Clustal Omega to understand its evolutionary relationship with other fungus.

2.5 Antifungal activity

2.6 Plant Collection and preparation of extracts

The plants were collected from GKVK medicinal garden, Bangalore. From the collected plant materials leaves were separated, cut into small pieces and shade dried. Separately dried materials of all plants were soaked separately in ethanol until the material became colourless. It was filtered and concentrated to get thick paste and then used for antifungal studies.

2.7 Agar well diffusion method

The fungal samples were spreaded on PDA agar plates and 5 wells were punched using cork boarer. Each well was filled with the extract possessing different concentrations (500µg, 250µg, 125µg), positive (Ampicillin) and negative controls (ethanol) were added in two wells. The plates were kept aside until the cultures grew and the zone of inhibition was measured after 4-5 days.

3. Results and Discussion

Two infected fish varieties including one ornamental and another edible fish were collected and analyzed. The infected part from the surface of *Carassius auratus* and *Cyprinus carpio* were isolated and cultured *in vitro*. Clinical symptoms of both fishes showed that caudal fins had acquired white edges where scales were eroded and tip of dorsal fin edges shredded and discoloured. Scales were eroded from abdomen region; haemorrhages and lesions were present all over the body surface with whitish edges which indicates fungal hyphae as shown in figure 1. These distinct fungal lesions were present on the surface of skin, were superficial and not penetrated deep into the muscles. The infected part was scrubbed and cultured on PDA plates which yielded growth of 2 kinds of fungus as shown in figure 2. Genomic DNA was isolated from both fungus, ITS region was amplified using primers and further subjected for Sanger sequencing as shown in figure 3. The resulting sequences which are shown below (F1 and F2) were subjected to NCBI BLAST which identified similar sequence based on similarity percentage and E value. The details of which are shown in table1. F1 was identified as *Aspergillus fumigatus* and F2 was identified as *Saprolegnia parasitica*. Figure 4 and figure 5 represents the phylogenetic relationship of F1 and F2 as analysed using Clustal Omega.

Similar types of fungus were isolated by Fauzia *et al* 2014, reporting that most prevalent genus was Aspergillus spp. which was isolated from C. auratus and X. maculatus. Though most of the fungus are regarded as opportunistic pathogen, few of them cause diseases such as Saprolegniasis, Aspergillosis, Scopulariopsis, Paecilomycosis and Penicillium infection (Refai *et al*., 2010) [15]. Environmental variables influence the intensity of aquatic fungal infections (Fadaeifard *et al*., 2011) [16]. These fungal species are presumably infectious and spread through the contamination of fish feed (Saleem *et al*., 2012) [17]. The isolated fungi from diseased fish *C. auratus* is *Aspergillus fumigatus*, though it is considered as

normal mycoflora but are responsible to produce diseases under particular environmental conditions. Hence, it demands more attention from the health point of view of ornamental fish. Another main type of fungal diseases is saprolegniasis which is caused by species *Saprolegnia parasitica* (Abdollah *et al*., 2009) [18]. All these fungi are considered as saprophytic organisms which are widely distributed in aquatic environment and they derive nutrients from organic source in water. They become pathogenic to fish only when fish are stressed, injured, poor nutritional condition, temperature shocked when incubation temperature is below the optimum (John, 1997) [20]. *Saprolegnia parasitica* is the main causative agent of disease in aquatic animals which holds responsible for maximum damage in developmental stages of edible fishes (Gieseker *et al*., 2006) [19].

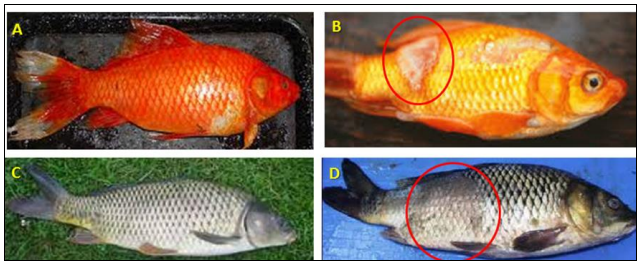

Fig 1: Showing healthy fishes and highlighting the infected part of fishes; Healthy and infected *Carassius auratus* - A, B and Healthy and infected *Cyprinus carpio* - C, D

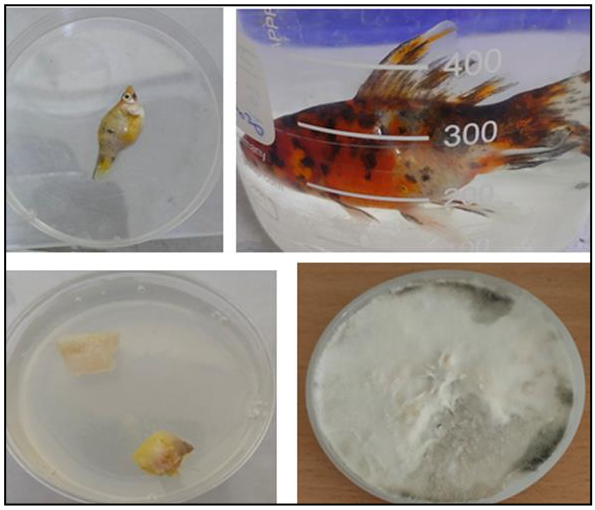

Fig 2: Showing culturing of infected parts from fishes and growth of isolated fungus**.**

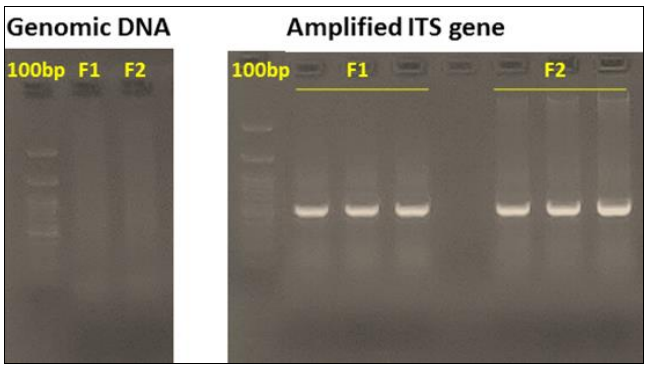

Fig 3: Genomic DNA isolated and PCR amplicons of ITS from fungus F1 and F2.

>F1_query
GACTTCGGGAGGATCATTTTTCCGTGAGGGCAGAAAGGGT
CCTTTCNCCCACCCGTGTCTATCGTACCTTGTTGCTTCGGC
GGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCTTGCGCC
CCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTG
TTCTGAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTT
AAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCC
CCTGATATTCCGGGGGGCATGCCTGGTCCGAGCGTCATTG
CTGCCCTCAAGCACGGCTTTGTGTTGATGGGCCCCCGATCC
CCCTCTCCCGGGGGACGGGGCCCCGAAAAGGCAGCGGCG
GCACCGCGTCCGGATCCTCGAACCCGTAAGGGCGGCTTTT
GTCACCAGCATCTGTAGGCCCCGGCGGGCGCCAGCCGAAC
ACCCAACTTTTTCTAAAGGTTGGA
>F2_query
TCCGTAGGTGAACCTGCGGAAGGATCATTACCACTTTATG
AGGCTTTGCGCTGCCCTTGTGGCAGCTAGCCGAAGGTTTCG
CAAGAAGCCGATGTCAATTTGAATCCTTTTTAAAATACGA
CTGATCAAAACTGCAGATAGAAATATCTGCATGCAATTGA
AATACAACTTTCAACAGTGGATGTCTAGGCTCGCACACCG
ATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGC
AGAATTCAGTGAGTCATCAAAATTTTGAACGCATATTGCA
CTTCCGGGTTAGTCCTGGGAGTATGTTTGTATCAGTGTCCG
TGAACACAAACTTGTTTCATTTCTTGATTGGGATGGAGCAG
ACTGTGAAGGTCTTGTAATTACAAGTCCTTTTAAACGACGG
TACCTATGCGTCCTAGTGAGATGTATTATTTAAAGGTATGC
CTGCGCTCCTTTCGAAAGTCTTGTGTGGCGGCACACAGCA
CTCAAAGAGAGAGCAAATCGCGGTAGTTTTGCTTGTACTT
CGGTACGAGTGGACACATATTGCTTTTTGTGATTTCTGCGA
GTCTGTTGTCAAAGTACAAGGCACGTAAGGAGAGTTGGTA
TGCTGGTGCATTTCTTGGCGTATGGAGGCAAATTGGGA

Table 1: Showing hits obtained and its other details from

Sl. No.	Hit obtained	Similarity Percentage	Query coverage	E value	Accession number
F1	*Aspergillus fumigatus* strain GAD14 internal transcribed spacer 1, partial sequence	99.8%	100%	0.0	MN638754.1
F2	*Saprolegnia parasitica* isolate 195_Dal.Ac-18_Striped_Bass-3 small subunit ribosomal RNA gene, partial sequence	99%	98%	0.0	MK850074.1

NCBI BLAST

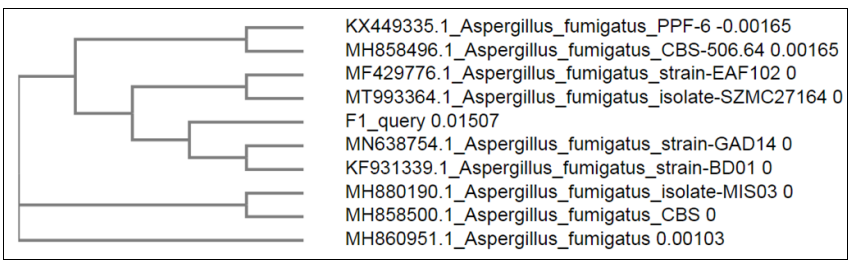

Fig 4: Phylogenetic analysis of F1 showing its closest relationship

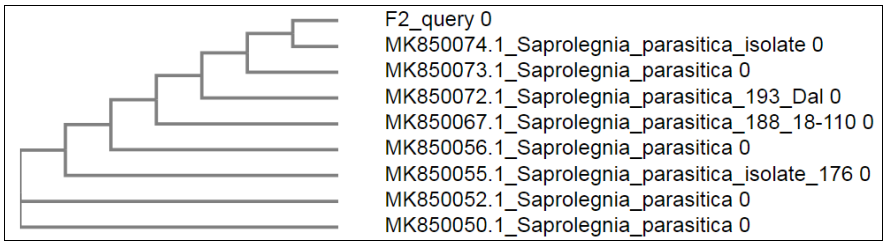

Fig 5: Phylogenetic analysis of F2 showing its closest relationship

The ethanolic leaf extracts of 6 plants such as *Cardiospermum helicacabum*, *Kirganelia reticulata*, *Urena lobata*, *Vitex negundo*, *Scoparia dulcis* and *Pongamia pinnata* (Figure 6) were prepared in different concentrations and checked for its inhibiting activity against *Aspergillus fumigatus* and *Saprolegnia parasitica*. The inhibition zones obtained were measured and tabulated as seen in table 2. All the plants are showing moderate inhibition against tested fungus when compared to standard. This might be due to the presence of effective inhibitory substances in these plants. Since all the plants have different phytochemicals the combination of extracts might give more excellent results. The active substances present in it cause membrane disruption of fungus, affecting their metabolic efficiency (Hili *et al*., 1997) [22]. They also interfere with fungal cellwall and DNA, inhibiting microbial enzymes rupturing cell membranes breaking their association with DNA (Draughon, 2004; Benkeblia, 2005; Unver *et al*., 2009) [23, 24, 25]. Further their attachment to cell membrane and lipophilic compounds gets ruptured and thereby inhibits the growth of fungi (Cowan, 1999) [26]

But in all the ways the results obtained exhibits potential in controlling and preventing infections in fish. So this recommends further investigations to develop alternative control strategies against ornamental and edible fishes. Because the use of malachite green began in 1933 and it was adopted as one of the milestone used in treatment of fish infections against all kinds of parasites (Meyer, F.P and Jorgenson 1983) [27]. Unfortunately, this chemical is still being used by fish farmers in some fisheries which highlight the connected problems and stimulate the research for new constituents of natural origin. In a more extensive study of the use of plant extracts against pathogenic fungi has been proved to be capable of inhibiting the growth or even causing the death of *S. parasitica* at comparatively low concentrations (Tampieri *et al*., 2001) [28]. Hence the results showed to have inhibitory action against *Aspergillus fumigatus* and *Saprolegnia parasitica* in different concentrations, which may lead to their future usage in aquaculture applications and department of fisheries.

Fig 6: Showing the plants taken for study: *Cardiospermum helicacabum* (A), *Kirganelia reticulata* (B), *Urena lobata* (C), *Vitex negundo* (D), *Scoparia dulcis* (E) and *Pongamia pinnata* (F).

Table 2: Showing Inhibition zones of plant extracts against *Aspergillus fumigatus* and *Saprolegnia parasitica*, Inhibition zones are shown in mm.

Extracts	*Aspergillus fumigatus*			*Saprolegnia parasitica*		
	500 µg/mL	250 µg/mL	125 µg/mL	500 µg/mL	250µg/mL	125 µg/mL
Cardiospermum helicacabum	12	10	8	10	9.5	8
Kirganelia reticulata	19	18.5	17	11.5	10	9.5
Urena lobata	17	16	15.5	10	8.5	8
Vitex negundo	13	11.5	10	9.5	8	7
Scoparia dulcis	14	13	11.5	10	9.5	8.5
Pongamia pinnata	16	16	14	11.5	10	9
Ciprofloxacin 3 µg/mL	24			23		

4. Conclusion

The ethanol extracts of the plants possess antimicrobial activity against fish fungus. Hence, it is recommended to use these herbal formulations to treat the fungal infections instead of using chemical formulations which may have adverse side effects. As we tested individual plants it showed moderate antifungal activity, but chances are that the activity will automatically get enhanced if two or more samples are clubbed together. In general, our study suggests that natural products derived from some medicinal plants have the potential to be used as medicine for fish infections. Further research should be planned to try using a combination of extracts so as to prevent fish from fungal infections and it is always good to use natural medicines over harmful chemicals.

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