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Potential Useful Application of Indigenous Extracts in Ornamental Fishes Affecting *Aeromonas hydrophila* Infection

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Abstract

Bacterial lesions, which include septicemia or bacterial toxins present in organs of fish and ulcers of fish skins, result in huge economic loss to aquaculture farmers. The causative organism for these types of bacterial diseases is a group of A. hydrophila type bacterium. Although there are commercially available vaccines and antibiotics to control bacterial diseases, they are often proven to be inefficient. Moreover, antibiotics lead to acquired resistance in bacteria, and the drug residual in fish, resulting in tight regulations for their usage. This calls for new alternative solutions that can be safe and cost-effective to prevent and treat fish diseases. Plant extracts are well known for their anti-microbial properties and they could be potential alternative candidates. Usage of plant extracts as therapeutics is largely used for human pathologies but there is limited knowledge for the same to treat piscine disease. The anti-microbial properties of extracts from Almond leafs, Turmeric powder and Rock salt was compared to their antimicrobial effect with commercially available antibiotic Chloramphenicol. The concentration of the extracts for each compound in mg/ml was studied as 30, 50 and 75 respectively. The bactericidal property was found effective in the mixture compound (Almond leaf+Rock salt+Turmeric powder) with an average inhibition of 18 mm at 75 mg/ml concentration. While, the almond leaf and turmeric extract at the same concentration found an average inhibition of 10 mm, each. It was speculate that among three extracts other than rock salt could serve as potential alternative therapeutics to treat bacterial septicemia associated with *A. hydrophila* in fish that would have fewer or no regulations and be cost-effective.

Keywords: Chloramphenicol; Aquaculture; Hemorrhagic septicemia; Turmeric; Bacterium

Introduction

Aquaculture is a growing industry having extending hands in ornamental fishes. The rearing and breeding of ornamental fishes is a care taking process which required skilled potentials and commitment. Even though, proper management implemented there are some failures occurred in this industry in the form of invaded pathogens. Parasitic as well as bacterial outbreaks are a serious limiting factor in aquaculture farming. It may be due to contaminated water, feed or transfer of species and once occurs it leads to death of fishes causing loss of heavy economy ultimately. Different studies were conducted to minimize fish feed costs by using medicinal plants "back to nature" as feed additives instead of using synthetic drugs of serious side effects [1]. The disease challenge is an in vitro technique which gives the chance to determine the performance and immunity of fish when exposed to xenobiotic bacteria present in their natural habitats [2].

Aeromonas hydrophila causes disease in fish known as “Motile Aeromonas Septicemia” (MAS), “Hemorrhagic Septicemia,” “Ulcer Disease,” or “Red-Sore Disease.” *Aeromonas hydrophila* is a ubiquitous gram-negative rod-shaped bacterium which is commonly isolated from fresh water ponds and which is a normal inhabitant of the gastrointestinal tract. Experimental demonstration shows that fish which are in poor environments due to unsatisfactory water quality such as high nitrite levels, low levels of Dissolved Oxygen (DO), or high levels of carbon dioxide (CO_2) are more susceptible to infection by *Aeromonas hydrophila*. Additionally, a seasonal incidence of a higher number of rep-ted fish deaths in the spring is associated with decreased water temperatures.

The chemicals and antibiotics cause bacteria to develop resistance [3], retention of the chemical compound in the tissues of fish leading to bio magnification [4]. Thus, there is huge environmental and human health risks associated with antibiotic and other chemical treatments. The vaccines are proven to be of low efficacy and there are strict regulations for the use of antibiotics in aquaculture. Therefore, there is a need to find new alternative solutions to prevent as well as treat fish diseases.

Indian almond leaves contain several different flavonoids, including Kaempferol and Quercetin [5]. These leaves are used widely in aquarium as a soft, acidic and tannin rich waters which mimic natural condition for species, mainly Betta fish. It has been reported that adding almond leaves trigger spawning since compounds released by the leaves will alter the chemistry of water, making the aquarium more similar to the habitat of fishes [6].

Turmeric contains bioactive compounds with powerful medicinal properties. Curcumin is the main active ingredient in turmeric. It has powerful anti-inflammatory effects and is a very strong antioxidant [7,8]. It is a natural anti-inflammatory compound where it matches effectiveness of some anti-inflammatory drugs [9]. It is an antioxidant that can neutralize free radicals due to its chemical structure. It is reported that this compound boosts the activity of body’s own

antioxidant enzymes. There are few reports which showed that fish has significant higher specific activities of digestive enzymes due to turmeric supplementation [10,11]. Also, Daneshyar, Alizadeh, et al. concluded that supplementation of turmeric powder in broiler chickens diets can decrease the concentrations of saturated fatty acids and triglycerides in thigh meat and improve the meat quality as a result [12].

Here a study was conducted to find out the effective use of turmeric, rock salt and almond leaf with special reference to the timing, dosage, method of administration, and suggested mode of action in fish. Antibiotics are frequently used to control disease caused by bacterium, but there is an increasing risk of developing antibiotic resistant. Herbal drugs in disease management are gaining success, because they are cost effective, eco-friendly and have minimal side effects. A large portion of the world population, especially in developing countries depends on the traditional system of medicine for a variety of diseases. Several hundred genera are used medicinally and plants are vital sources for potent and powerful drugs.

Materials and Methods

Sample preparation

Red leaf of Almond (100 mg), turmeric powder and rock salt (100 mg each) were grounded and dried in oven at 55-60 °C for 24 hours. Boiled in 100 ml distilled water and allowed to settlement. The upper aqueous layers were used for bactericidal studies. A concentration of 30 mg, 50 mg and 75 mg were used for the study.

Bactericidal studies

Diseased fish samples were collected from the nearby center and brought to lab for isolation of bacteria. The samples were macerated in normal saline and using sterile loop made streak culture in blood agar medium. These plates were incubated for 24 hours and identification of bacteria was done using micro-plating technique. A swab was used for lawn culture in Mullein Hinton Agar (MHA) medium. For sensitive studies disc was prepared using Whatman no.1 filter paper equally sized (6 mm) which was sterilized in hot air oven at 110 °C for 24 hours. Concentrations of 30 mg, 50 mg and 75 mg of the respective extract were loaded into it. These were placed in the bacterium containing MHA medium and kept it for 24 hours incubation. The zone of incubation was recorded in mm for determining the bactericidal activity.

Preparation of normal saline

A known volume of 0.9 g NaCl is dissolved in 100 ml distilled water. Autoclaved and used the medium for isolation of bacteria.

Preparation of Blood Agar medium

Suspend 40 g in 1 litre of distilled water. Bring to a boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 minutes. Pour it into the petri dish in molted condition. Allow to settle till the media gets solidify. Add 0.5 ml of the bacteria suspended in normal saline into the petri dish and spread using a sterile glass rod. Incubate the plate under 37 °C for 24 hours.

Mullein Hinton Agar (MHA) medium preparation

Suspend 38.0 g in 1000 ml distilled water. Heat it to dissolve medium completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Cool to 45-50 °C. Mix well and pour into sterile petri plates.

Statistical analysis

Dunnet's test was employed to find the difference between the antimicrobial activity between extracts and Chloramphenicol at 75 mg/ml concentration. The statistical analysis was performed by using SPSS analytical software. ANNOVA was performed to compare the difference between compounds and treatments.

Results

Tested extracts of three components Almond leaf, Turmeric powder, Rock salt and mixing of these three in 1:1:1 ratio against bacterium. During the initial concentration of 30 mg/ml and 50 mg/ml the results of extracts was presumably very low in the sensitivity studies. Very low observable zone of inhibition was found in *A. hydrophila* inoculated medium in these concentrations. A low value of 0-6 mm was found for turmeric, almond leaf extract, and rock salt but 10 mm was observed in the mixed extract. While, increasing the concentration to 75 mg/ml the zone of inhibition (ZOI) was predominantly high getting an average of 18 mm in the mixed compound. Therefore, the mixing compound is found very effective in this concentration for treating *A. hydrophila* infected fishes. Rock salt extract was found no effect in all the concentration for treatment of *A. hydrophila* bacteria. It has been reported that turmeric had bactericidal property which has a sensitive zone of 10 mm which is similar to the present study [13]. The sensitive test is found effective result in the 75 mg/ml concentration for almond leaf extract, turmeric extract and mixed extract which is shown in Figure 1. Almond leaf is rich in various tannins; astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins, which may be the reason for bactericidal property of the almond leaf extract [14]. The minimum inhibitory concentration has been determined to be 75 mg/ml of extracts for the treatment of *A. hydrophila* infected Ornamental fishes. ANNOVA results showed in comparison with N=7 samples there is significant variation between compound and treatments ($P<0.001$) shown in Table 1. Within compound and treatment also showed significant variation ($P<0.001$) shown in Table 2. These results showed that as the dosage increases there is variation in the inhibition ratio i.e. zone of inhibition increases when dosage increases. While performing Dunnet's test the comparison with control chloramphenicol showed significant variation as the results was not comparable. But there is inhibitory effect in mixer compound which has the inhibition effect most closely to the antibiotic (Table 3).

ANNOVA				
Compound		**Mean**	**Std. Deviation**	**N**
Chloramphenicol	75	29.685714	0.7380799	7

	30	0.097143	0.0994509	7
	50	6.137143	0.1218899	7
Almond	75	10.004286	0.113116	7
	30	6.057143	0.1133893	7
	50	10	0.1732051	7
Mixer	75	18	0.2160247	7
	30	0	0	7
	50	0	0	7
Rock salt	75	0	0	7
	30	0.057143	0.0786796	7
	50	5.88	0.1064581	7
Turmeric	75	10.228571	0.4834745	7
	30	1.552857	2.6497182	28
	50	5.504286	3.639745	28
Total	75	13.583714	10.0243524	35
Dependent Variable: Effect				

Table 1: Statistical data showing variation between samples (ANNOVA).

Tests of Between-Subjects Effects					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F-value**	**Sig.**
Corrected Model	6354.556a	12	529.546	7457.591	0
Intercept	5936.977	1	5936.977	83610.34	0
Compound	3623.455	4	905.864	12757.26	0
Treatment level	897.249	2	448.625	6317.973	0
Compound Treatment Level	334.843	6	55.807	785.931	0
Dependent Variable: Effect; R Squared=0 .999, Adjusted R Squared=0.999					

Table 2: Mean significant variation of compared data.

Multiple Comparisons Dunnet's Test						
Compound	**Control**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	**Upper Bound**
Almond	Chloramphenicol	-24.272857*	0.1162982	0	-24.551683	-23.994031
Mixer	Chloramphenicol	-18.333333*	0.1162982	0	-18.612159	-18.054508
Rock salt	Chloramphenicol	-29.685714*	0.1162982	0	-29.96454	-29.406889
Turmeric	Chloramphenicol	-24.297143*	0.1162982	0	-24.575969	-24.018317

*The mean difference is significant at the 0.05 level.

Table 3: Dunnet's test for the comparison with the control Chloramphenicol.

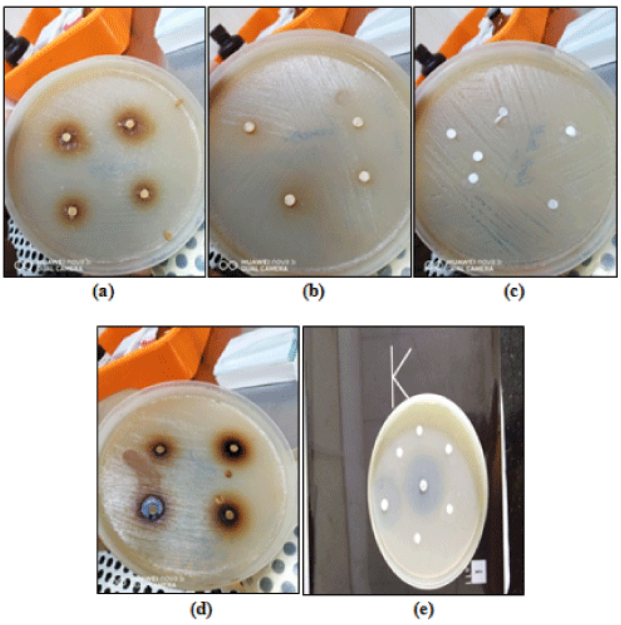

Figure 1: *A. hydrophila* sensitivity tests at a concentration of 75 mg/ml: (a) Almond leaf extract, (b) Turmeric extract, (c) Rock salt extract, (d) Mixed extract, (e) Chloramphenicol.

Conclusion

Diseases caused by *Aeromonas hydrophila* bacterium are some of the most widespread in freshwater fish culture. Nowadays, a large portion of the world population, especially in developing countries depends on the traditional system of medicine for a variety of diseases. Interestingly, the results gave satisfactory minimum concentration requirement for the treatment of *A. hydrophila* bacterium. The sensitivity studied in vitro, can be effective in culture system where there is risk associated with this bacterium. Septicemia associated infections can be cured with the application of almond leaf extracts, turmeric extracts and mixing extract solution. Additionally, phyto-medicines provide a cheaper source for treatment and greater accuracy than chemotherapeutic agents without causing toxicity.

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