Journal of Microbiology and Pathology

Research Article Open Access

Screening of Herbs and Spices for Antimicrobial Properties against *Flavobacterium columnare*

Grace Ramena[1]*, Yathish Ramena[2], William Duke[1] and Nitin Challa[1]

[1]*Department of Aquaculture and Fisheries, University of Arkansas, Arkansas, USA*
[2]*Great Salt Lake Brine Shrimp Cooperative, Inc., Mountain Green, Utah*

Abstract

Bacterial diseases like columnaris caused by *Flavobacterium columnare*, result in huge economic loss to aquaculture farmers. Although there are commercially available vaccines and antibiotics to control bacterial diseases, they are 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. We have evaluated the anti-microbial properties of extracts from eleven herbs and spices and compared their antimicrobial effect with commercially available antibiotic Oxytetracycline (Terramycin® 200), that is used to treat columnaris. We found that all the extracts (50 mg/ml) we have tested, had bactericidal effects against *Flavobacterium columnare* (3×10^8 CFU/ml). Interestingly, *Syzygium aromaticum, Cuminum cyminum* and *Thymus vulgaris* showed highest antimicrobial activity against Flavobacterium columnare with an average inhibition of 20 mm and *Cinnamomum verum* showed 18 mm, which is similar to Oxytetracycline (30 µg). We speculate that the extracts we have identified could serve as potential alternative therapeutics to treat columnaris in fish that would have fewer or no regulations and be cost-effective.

Keywords: Bacterial diseases; Fish diseases; Antimicrobial

Introduction

Aquaculture is the main source of high quality protein for the growing human population. In the United States, Arkansas is a one of the leading producers for food, bait, sport and ornamental fish. The growing demand for world's seafood supply forces the farmers to raise fish in high densities. Therefore fish are prone to stress conditions like overcrowding, imbalance in water quality parameters, immune suppression that lead to disease outbreak [1-3]. Bacterial diseases like columnaris cause morbidity and sudden mass mortalities in wide variety of fish in aquaculture ponds. These sudden mortalities result in huge economic loss to aquaculture farmers. The disease outbreaks call for chemical treatment to control the economic loss. Although there are commercially available vaccines and antibiotics to control bacterial diseases, they are proven not to be efficient in combating the economic loss.

Flavobacterium columnare is a causative agent for columnaris There are various antibiotics like Oxytetracycline (Terramycin® 200), Sulfadimethoxine (Romet-30), and Florfenicol (Aquaflor®) and various chemicals like Hydrogen peroxide (H_2O_2), potassium permanganate ($KMnO_4$), Sodium chloride (NaCl), formalin, copper sulphate (CuSo4) to treatment these bacterial diseases. These treatments are associated antibiotic resistance, fish tissue residuals, kill plankton in ponds causing water quality imbalance, may cause irritation to fish and even lead to mortalities when used at wrong dose or at poor water quality management.

The chemicals and antibiotics cause bacteria to develop resistance [4], retention of the chemical compound in the tissues of fish leading to bio magnification [5]. 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.

Plant extracts having bioactive compounds could be potential candidates of replacement for antibiotics as shown by many studies. The anti-bacterial effects associated with plant extracts can be credited to the presence of components like alkaloids, flavonoids, steroids, and phenolic compounds [6-11]. Plant extracts have bactericidal and bacteriostatic effects and also may cause weight gain; act as appetite stimulant, and immune stimulants etc. In the current study, we evaluate the antibiotic property of crude methanol extracts of eleven herbs and spices like: cinnamon (*Cinnamomum verum*), clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*), sage (*Salvia officinalis*), turmeric (*Curcuma longa*), ginger (*Zingiber officinale*), garlic (*Allium sativum*), onion (*Allium cepa*), coriander (*Coriandrum sativum*), dillweed (*Anethum graveolens*) against *Flavobacterium columnare* and compare their activity with Oxytetracycline [12].

Materials and Methods

Plant extracts preparation

Dry ground Turmeric (*Curcuma longa*), Garlic (*Allium sativum*), ginger (*Zingiber officinale*), Onion (*Allium cepa*), Cinnamon (*Cinnamomum verum*), Cuminb (*Cuminum cyminum*), Clove (*Syzygium aromaticum*), Thyme (*Thymus vulgaris*), Coriander (*Coriandrum sativum*), and Dill weed (*Anethum graveolens*) all are

***Corresponding author:** Grace Ramena, Department of Aquaculture and Fisheries, University of Arkansas, Pine Bluff 1200 N, University Drive, Mail, Slot 4912, Room #133, Pine Bluff-71601, Arkansas, USA, Tel: 870-575-8137; E-mail: ramenagrace@gmail.com

Received December 27, 2017; **Accepted** March 27, 2018; **Published** April 05, 2018

Citation: Ramena G, Ramena Y, Duke W, Challa N (2018) Screening of Herbs and Spices for Antimicrobial Properties against *Flavobacterium columnare*. J Microbiol Pathol 2: 107.

Organic, Great value products and Sage (*Salvia officinalis*) (Organic, McCormick) were purchased from Wal-Mart. Each of the plant materials were incubated in methanol (99.9% pure, Fisher chemicals) for 24 hours on a shaker at 140 rpm at room temperature. Later, the incubated plant material was filtered (Whatman, Sigma, Germany) to remove the large plant debris material. Next, the filtrate was passed through a vacuum filter (CORNING 250 ml filter system (430771) 0.2 um NY (Nylon) sterilizing, low extractable membrane Non-pyrogenic polystyrene) to ensure only the methanol soluble materials were obtained. The filtrate was concentrated in the vacuum at 25°C on a rotavapour (Buchi Rotavapor R-200, BUCHI, and Switzerland). The concentrated plant extract for antimicrobial activity was dissolved in methanol at a concentration of 1 g/ml for further usage

Bacterial culture and plate preparation

The *Flavobacterium columnare* (LV359 01) strain was a gift from Dr. Miles Lange at the USDA in Stuttgart, AR. The bacterial stock was thawed on ice and was inoculated in *Flavobacterium columnare* Growth Media (FCGM) broth prepared according to Farmer et al. 2004. After overnight incubation on shaker at 30°C the bacterial culture was centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in Normal saline (0.85% NaCl) and adjusted to a McFarland Turbidity standard - 1 (3×10^8 CFU). Then a volume of 333 µl of bacterial suspension was spread on 10 cm FCGM agar plate using a sterile cotton swab. The plant extracts (50 µl, a final concentration of 50 mg) were loaded onto sterile whatmann filter discs (9 mm) (Fisher Scientific). The discs were placed at the center of the FCGM agar plate. The zones of inhibition were recorded after an overnight incubation at 30°C temperature to the nearest mm. The experiments were done in five replicates.

Statistical Analysis

Dunnet's test was employed to find the difference between the antimicrobial activity between extracts and oxytetracycline (50 µg). The statistical analysis was performed by using SAS version 9.4.

Results

We have prepared methanol extracts for eleven herbs and spices and tested them against *Flavobacterium columnare*. We have used 50 mg plant extracts to screen for any antimicrobial activity and compared the activity with Oxytetracycline which was also our positive control and methanol served as negative control. We found that all of our methanol extracts we tested showed antimicrobial activity against *Flavobacterium columnare* (Figure 1). Cumin, clove and thyme showed highest activity as shown in red bars with an average inhibition zone of 19 to 20 mm and cinnamon had an antimicrobial activity with 18 mm growth inhibition zone. Garlic, coriander and sage had an average growth inhibition zone of 16 mm, whereas onion and ginger showed an activity of 13 mm. Dill weed and turmeric had showed lowest inhibition with a growth inhibition zone of 10 to 11 mm. Our results suggest that cumin, clove, thyme and cinnamon have significant efficacy as shown in Figure 2, in inhibiting the growth of *Flavobacterium columnare*.

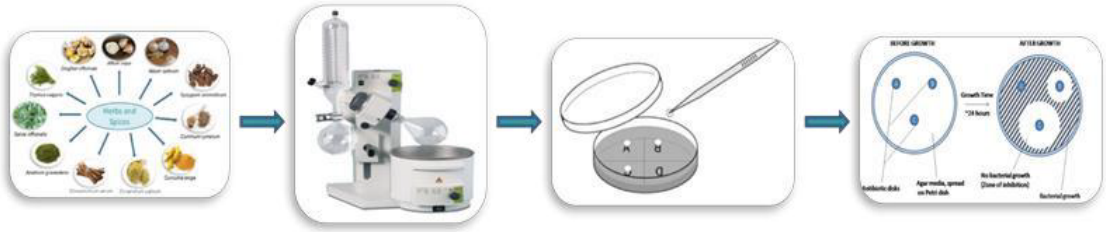

Figure 1: Experimental design for plant extracts preparation and antimicrobial tests against *Flavobacterium columnare*.

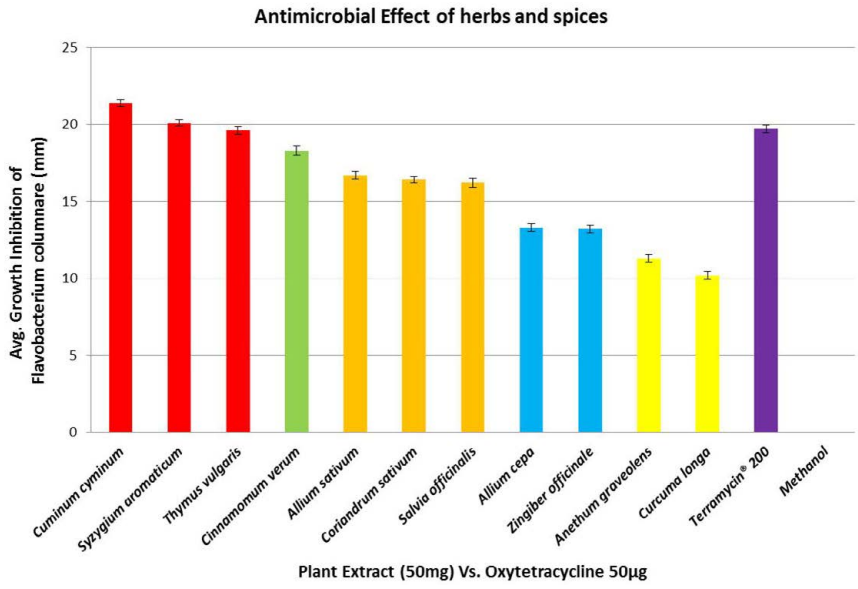

Figure 2: Antimicrobial activity of various herbs and spices against *Flavobacterium columnare*.

These results are well comparable to the commercially available antibiotics like Oxytetracycline. We have performed Dunnet's test and found that cumin showed significantly higher antimicrobial activity than Oxytetracycline or any other extract (Table 1) (P<0.001). When we compared the antimicrobial activity of Terramycin with all extracts (Table 2) cumin had higher activity than Terramycin. On the other hand, clove and cinnamon had similar activity as Terramycin.

Conclusion

Interestingly, more than four of our extracts had equal potency as Oxytetracycline which is a common antibiotic that is used to treat columnaris. This antibiotic has tight regulations for use, with a withdrawal period of 10 days. We hypothesize the herbs and spices would be safe to administer with fewer or no regulations when compared to antibiotics. The plant extracts have many benefits like antioxidant properties, increase immune response etc. Therefore, we speculate that cumin, clove, thyme and cinnamon can be used regularly because of their many benefits. This can even inhibit disease outbreak to begin with that can have great economic impact. However we need to identify the minimum inhibitory concentrations of our plant extracts to be able to use them on a regular basis and to have minimal side effects like cytotoxic and genotoxicity.

Dunnet's Test				
Degrees of Freedom=51				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**
Cuminum cyminum	N/A	21.4	N/A	N/A
Cuminum cyminum	*Syzygium aromaticum*	20.1	0.1556	<0.0001
Cuminum cyminum	*Thymus vulgaris*	19.6.	0.1556	<0.0001
Cuminum cyminum	*Cinnamomum verum*	18.3	0.1556	<0.0001
Cuminum cyminum	*Allium sativum*	16.7	0.1556	<0.0001
Cuminum cyminum	*Coriandrum sativum*	16.4	0.1556	<0.0001
Cuminum cyminum	*Salvia officinalis*	16.2	0.1556	<0.0001
Cuminum cyminum	*Allium cepa*	13.3	0.1556	<0001
Cuminum cyminum	*Zingiber officinale*	13.2	0.1556	<0.0001
Cuminum cyminum	*Anethum graveolens*	11.3	0.1556	<0.0001
Cuminum cyminum	*Curcuma longa*	10.2	0.1556	<0.0001
Cuminum cyminum	Terramycin® 200	19.7	0.1556	<0.0001
Cuminum cyminum	Methanol	0	0.1556	<0.0001

Table 1: Comparison of growth inhibition of *Flavobacterium columnare* by *Cuminum cyminum* vs. other extracts/antibiotic/methanol.

Dunnet's Tests				
Degrees of Freedom=51				
Antibiotic compared	**Extract**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**
Terramycin® 200	*Cuminum cyminum*	21.4	0.1651	<0.0001
Terramycin® 200	*Syzygium aromaticum*	20.1	0.1651	0.0503
Terramycin® 200	*Thymus vulgaris*	19.6	0.1651	1.0
Terramycin® 200	*Cinnamomun verum*	18.3	0.1651	<0.0001
Terramycin® 200	*Allium sativum*	16.7	0.1651	<-0001
Terramycin® 200	*Coriandrum sativum*	16.4	0.1651	<0.0001
Terramycin® 200	*Salvia officinalis*	16.2	0.1651	<0.0001
Terramycin® 200	*Allium cepa*	13.3	0.1651	<0.0001
Terramycin® 200	*Zingiber officinale*	13.2	0.1651	<0.0001
Terramycin® 200	*Anethum graveolens*	11.3	0.1651	<0.0001
Terramycin® 200	*Curcuma longa*	10.2	0.1651	<0.0001
Terramycin® 200	Terramycin® 200	19.7	N/A	N/A
Terramycin® 200	Methanol	0	0.1651	<0.0001

Table 2: Comparison of growth inhibition of *Flavobacterium columnare* by Terramycin® 200 vs. plant extracts/methanol.

Acknowledgements

We would like to thank Dr. Lin Xie for help with statistical analysis.

Conflict of Interest

No conflict of Interest.

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