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Identification and Determination of Minimum Inhibitory Concentrations of Plant Extracts having Antimicrobial Activity as Potential Alternative Therapeutics to Treat *Aeromonas hydrophila* Infections

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Abstract

Aeromonas hydrophila causes infections in wide range of organisms including humans. It causes serious and life threatening lethal infections in humans. Motile aeromonas septicemia causes huge economic loss to fish farmers. Antibiotic use and abuse is a huge problem that is environmentally lethal, leading to antibiotic resistance and difficulty in treating bacterial infections. There is always a need to find new and safe alternatives to antibiotics to deal with bacterial infections. In this study we have evaluated 11 herb and spice extracts for their antimicrobial activity against *Aeromonas hydrophila* and compared their activity with commercially available antibiotic to treat this disease. We found that clove and cinnamon methanol extracts had significantly higher antibacterial effect against *A. hydrophila* when compared to oxytetracycline and other extracts. Both the extracts have higher activity at the lowest concentration tested (5 mg) and can potentially serve as alternative therapeutics to *A. hydrophila* infections in humans as well as fish.

Introduction

A. hydrophila is a gram-negative rod-shaped bacilli bacterium belonging to the family Aeromonadaceae. It has a single polar flagellum and is highly motile and it is found in sewage, and brackish water. *A. hydrophila* virulence factors include ability to make adhesions, release cytotoxins, lipases, biofilm formation etc. It is known to be associated with motile aeromonad septicaemia (MAS) in many fresh water fishes and is thought to be spread through accidental abrasions [1]. Cases of this bacterial infection are reported in many countries across the globe from south East Asia to the United States of America. This bacterium affects a variety of cultured food fish like hybrid striped bass, channel cat fish, American eel (*Anguilla rostrata*), Goldfish (*Carassius auratus*), Snakehead fish (*Ophiocephalus striatus*), Tilapia (*Tilapia nilotica*), Carp (*Cyprinus carpio*), Chinook salmon (*Oncorhynchus tshawytscha*), Rainbow trout (*Oncorhynchus mykiss*), Golden shiner (*Natemigonus crysoleucas*) etc. and it contributes to a substantial economic loss to the aquaculture industry, which is worth more than $ 423 million. MAS is also known as Hemorrhagic Septicemia, Ulcer Disease, or Red-Sore Disease, where the bacteria and its toxins (cytotoxins, enterotoxins, hemolysins etc.) can be found in various organs of the infected fish [2,3]. These bacterial toxins and enzymes form pores in the host epithelial cells. The *A. hydrophila* pili adhere to the host epithelial lining and colonize in the intestinal tract [4]. It also infects the skin and gill epithelium causing hemorrhagic septicemia [5]. In case of a hemorrhagic septicemia the fish may show focal hemorrhages in the gills and the opercula, abscesses, ulcers, exophthalmia. Symptoms include lack of appetite, pale gills, swimming abnormalities, skin ulcerations, and bloated appearance. In case of an acute infection the fish may undergo sudden mortality [6].

Dunnet's Test-Comparision of Cinnamomum verum with other extracts (50 mg) and Terramycin® 200 (50 µg)				
Degrees of Freedom = 51.1				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**
Cinnamomum verum	*Cinnamomum verum*	10.36	N/A	N/A
Cinnamomum verum	*Syzygium aromaticum*	9.76	0.9013	<0.0001
Cinnamomum verum	*Allium Sativum*	3.2	0.9013	<0.0001
Cinnamomum verum	*Salvia officinalis*	2.36	0.9013	<0.0001
Cinnamomum verum	*Thymus vulgaris*	2.2	0.9013	<0.0001
Cinnamomum verum	*Coriandrum sativum*	2.1	0.9013	<0.0001

Cinnamomum verum	*Cuminum cyminum*	2.02	0.9013	<0.0001
Cinnamomum verum	*Anethum graveolens*	0.8	0.9013	<0.0001
Cinnamomum verum	*Allium cepa*	0	0.9013	<0.0001
Cinnamomum verum	*Zingiber officinale*	0	0.9013	<0.0001
Cinnamomum verum	*Curcuma longa*	0	0.9013	<0.0001
Cinnamomum verum	Terramycin® 200	3.64	0.9013	<0.0001
Cinnamomum verum	Methanol	0	0.9013	<0.0001

Table 1: Dunnet's test comparing the significance of antimicrobial activity of *Cinnamon verum* to all extracts (50 mg) and Oxytetracycline (Terramycin® 200).

It is also known to cause disease in amphibians, mammals, reptiles and birds. Notably, *A. hydrophila* is a known to be associated with human pathogenesis in immunocompromised individuals [7]. It causes gastrointestinal infections, soft-tissue infection and skin infections, and bacteraemia [8,9]. It also causes meningitis septic arthritis, osteomyelitis, myositis, urinary tract infections and haemolytic uremic syndrome [8,10,11] have reported that *A. hydrophila* was associated with a life-threatening infection called strongyloidiasis.

Antibiotics like Oxytetracycline, Sulfadimethoxine/ormetoprim and Florfenicol are shown to be effective against *A. hydrophila.* Other effective chemicals are H_2O_2 (250 mg/l-500 mg/l), NaCl and $KMnO_4$ and among them, H_2O_2 is considered to be the most effective against *A.hydrophila*, because H_2O_2 is considered to be environment friendly and readily degrades in water [12]. However, H_2O_2 may reduce algal and zooplankton in the pond water when treated for long. On the other hand, the microbes get acclimatized to multiple exposures of H_2O_2 and bacteria amplify back after the treatment is ceased. $KMnO_4$ at concentrations less than 100 mg/l are reported to be less effective in controlling strains of *A. hydrophila,* but $KMnO_4$ is known to oxidize the organic load in pond.

Antibiotics inhibit bacterial growth by multiple mechanisms. The most general ones are bactericidal and bacteriostatic effects. a) Bactericidal effect: The antibiotic interferes with bacterial cell wall formation that leads to cell death. E.g. penicillin, fluoroquinolones, and metronidazole. b) Bacteriostatic effect: The antibiotic interferes with bacterial DNA replication or protein translation etc. and therefore results in growth inhibition. E.g. tetracycline, sulfonamides, chloramphenicol and macrolides. However, bacteria develop resistance to antibiotics via multiple mechanisms. Bacteria take up genetic elements like plasmids and transposable elements that are responsible for transfer of genetic material from one bacterium to the other. This can render the bacteria with resistance against antibiotics and the two types of antibiotic resistance are inherent and acquired. In inherent resistance, bacteria are generally not susceptible to a particular class of antibiotics, which can be due to either the drug cannot enter the bacterial cell wall, or the affinity of the drug and bacteria is low or none. On the other hand, acquired resistance is the bacterium gains ability to resist a particular drug to which it was previously susceptible to. This can be due to lateral DNA transfer during transformation, transduction, or conjugation. There are several studies showing bacteria gaining resistance against previously used antibiotics, E.g. Oxytetracycline [13-15]. According to FAO, OIE and WHO in December 2003 antibiotic resistant microbes in food are more dangerous than the presence of antibiotics in food. Duran and Marshall, have isolated 162 bacterial species that had resistance to 10 antibiotics from ready to eat shrimp obtained from grocery stores. This is hazardous to human health and is a major concern. This rises an urgent need to find alternatives to treat and prevent bacterial infections and in our case *A. hydrophila.*

Dunnet's Test-Comparision of Terramycin® 200 (50 μg) with plant extracts (50 mg)				
Degrees of Freedom = 51.1				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**
Terramycin® 200	*Cinnamomum verum*	10.36	0.9013	<0.0001
Terramycin® 200	*Syzygium aromaticum*	9.76	0.9013	<0.0001
Terramycin® 200	*Allium Sativum*	3.2	0.9013	<0.0001
Terramycin® 200	*Salvia officinalis*	2.36	0.9013	<0.0001
Terramycin® 200	*Thymus vulgaris*	2.2	0.9013	<0.0001
Terramycin® 200	*Coriandrum sativum*	2.1	0.9013	<0.0001
Terramycin® 200	*Cuminum cyminum*	2.02	0.9013	<0.0001
Terramycin® 200	*Anethum graveolens*	0.8	0.9013	<0.0001

Terramycin® 200	*Allium cepa*	0	0.9013	<0.0001
Terramycin® 200	*Zingiber officinale*	0	0.9013	<0.0001
Terramycin® 200	*Curcuma longa*	0	0.9013	<0.0001
Terramycin® 200	Terramycin® 200	3.64	N/A	N/A
Terramycin® 200	Methanol	0	0.9013	<0.0001

Table 2: Dunnet's test comparing antimicrobial activity of all extracts (50 mg) against *Aeromonas hydrophila* to Oxytetracycline (Terramycin® 200).

Several studies [16-19] show that the bioactive compounds produced by plants have antifungal and antibacterial properties [20] found that 31 brazilian plant methanolic extracts had anti-bacterial activity against *F. columnare* and *A. hydrophila* [21] found that aqueous garlic extracts had antimicrobial against 18 isolates of *Edwardsiella tarda, Citrobacter freundii, Escherichia coli, Vibrio parahaemolyticus* and *Vibrio vulnificus, Staphylococcus aureus* and *Streptococcus agalactia*. Other spices like Clove, [22] Cumin, Coriander, Onion, Dill weed also showed promising antibacterial properties [23,24]. Therefore, we propose to find environmental and health safe plant extracts that could serve as alternative therapeutics to treat *A. hydrophila* infections. In this study, we have screened eleven herb and spice methanolic extracts that have antimicrobial activity against *A. hydrophila*; determined their minimum inhibitory concentrations and compared their activity to oxytetracycline.

Dunnet's Test-Comparision of Syzygium aromaticum with other extracts (5 mg) and Terramycin® 200 (5 μg)				
Degrees of Freedom = 51.1				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**
Syzygium aromaticum	*Cinnamomum verum*	3.8	0.04867	0.0004
Syzygium aromaticum	*Syzygium aromaticum*	4.02	N/A	N/A
Syzygium aromaticum	*Allium Sativum*	0	0.04867	<0.0001
Syzygium aromaticum	*Salvia officinalis*	0	0.04867	<0.0001
Syzygium aromaticum	*Thymus vulgaris*	0	0.04867	<0.0001
Syzygium aromaticum	*Coriandrum sativum*	0	0.04867	<0.0001
Syzygium aromaticum	*Cuminum cyminum*	0	0.04867	<0.0001
Syzygium aromaticum	*Anethum graveolens*	0	0.04867	<0.0001
Syzygium aromaticum	*Allium cepa*	0	0.04867	<0.0001
Syzygium aromaticum	*Zingiber officinale*	0	0.04867	<0.0001
Syzygium aromaticum	*Curcuma longa*	0	0.04867	<0.0001
Syzygium aromaticum	Terramycin® 200	0	0.04867	<0.0001
Syzygium aromaticum	Methanol	0	0.04867	<0.0001

Table 3: Dunnet's test comparing the significance of antimicrobial activity of *Syzygium aromaticum* against *Aeromonas hydrophila* to all extracts (5 mg) and Oxytetracycline (Terramycin® 200).

Materials and Methods

Plant extract preparation

Plant materials were purchased in powdered form, from Walmart the eleven methanol extracts tested are dill weed (*Anethum graveolens*), turmeric (*Curcuma longa*), garlic (*Allium sativum*), onion (*Allium cepa*), thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum verum*), cumin (*Cuminum cyminum*), ginger (*Zingiber officinale*), clove (*Syzygium aromaticum*), coriander (*Coriandrum sativum*), and sage (*Salvia officinalis*). 50 gm of herb or spice was dissolved in 400 ml of methanol and incubated on a shaker for 24 hrs, then filtered using Whatman filter paper and processed in a Buchi Rotavapor R-200. After complete evaporation the extracts were dissolved in 1 gm/ml methanol prior to use.

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Bacterial culture and antimicrobial tests

Aeromonas hydrophila (AH-2100) strain was a generous gift from Dr. Kidon Sung: National Center for Toxicology Research, AR. Frozen stocks of bacteria was inoculated into LB broth and cultured at temperature 32°C for 3 hours on a shaker at 140 rpm. The culture was then centrifuged at 2000 rpm for 2 minutes. The supernatant was discarded, and the pellet was resuspended in 0.85% saline and adjusted to 0.5 McFarland turbidity standard. Using a sterile cotton swab, 1.5 × 108 CFU/ml bacteria was spread onto Tryptic Soy Agar (TSA) plate for antimicrobial tests. Each extract was tested using disc diffusion method at 50mg to identify the extracts with antimicrobial activity against *A. hydrophila*. To determine the Minimum Inhibitory Concentrations (MIC), sterile 9 mm Whatmann discs were loaded with 5 μl, 10 μl, 15 μl, 20 μl, 25 μl, 30 μl, 35 μl, 40 μl, 45 μl and 50 μl of each extract (1 mg/μl). Five replicates of Oxytetracycline served as positive and methanol as negative controls. MIC was also performed for Oxytetracycline (Terramycin® 200) (1 μg/μl). The plates were incubated overnight at 32°C and zones of inhibition were recorded to the nearest mm for MIC. The plates were left in the incubator for over 72 hrs to confirm the bactericidal effect of the extracts.

Dunnet's Test-Comparision of Cinnamomum verum with other extracts (5 mg) and Terramycin® 200 (5 μg)				
Degrees of Freedom = 51.1				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**
Cinnamomum verum	*Cinnamomum verum*	3.8	N/A	N/A
Cinnamomum verum	*Syzygium aromaticum*	4.02	0.04867	0.0004
Cinnamomum verum	*Allium Sativum*	0	0.04867	<0.0001
Cinnamomum verum	*Salvia officinalis*	0	0.04867	<0.0001
Cinnamomum verum	*Thymus vulgaris*	0	0.04867	<0.0001
Cinnamomum verum	*Coriandrum sativum*	0	0.04867	<0.0001
Cinnamomum verum	*Cuminum cyminum*	0	0.04867	<0.0001
Cinnamomum verum	*Anethum graveolens*	0	0.04867	<0.0001
Cinnamomum verum	*Allium cepa*	0	0.04867	<0.0001
Cinnamomum verum	*Zingiber officinale*	0	0.04867	<0.0001
Cinnamomum verum	*Curcuma longa*	0	0.04867	<0.0001
Cinnamomum verum	Terramycin® 200	0	0.04867	<0.0001
Cinnamomum verum	Methanol	0	0.04867	<0.0001

Table 4: Dunnet's test comparing the significance of antimicrobial activity of *Cinnamomum verum* against *Aeromonas hydrophila* to all extracts (5 mg) and Oxytetracycline (Terramycin® 200).

Statistical analysis

To determine the statistical significance between the antimicrobial effects of plant extracts and comparison with antibiotic, the experiments were done in replicates of five and Dunnet's test was performed using Generalized Linear Model (GLM), by SAS version 9.4.

Results

To identify the plant extracts that has antimicrobial effect against *Aeromonas hydrophila*, we have initially screened eleven herb and spice methanol extracts at concentration of 50mg. Only 8 out of eleven extracts showed antimicrobial activity against *Aeromonas hydrophila*. Interestingly, cinnamon and clove showed highest antimicrobial activity with 10.36 mm and 9.76 mm against *Aeromonas hydrophila* when compared to other extracts and oxytetracycline 3.64 mm (Figure 1). Although the concentrations of our extracts are in milligrams and the antibiotic we compared to is in micrograms, we think it is comparable for two reasons.

Dunnet's Test-Comparision of Cinnamomum verum with other extracts (5 mg) and Terramycin® 200 (5 μg)				
Degrees of Freedom = 51.1				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard Error**	**P-Value**

Cinnamomum verum	*Cinnamomum verum*	3.8	N/A	N/A
Cinnamomum verum	*Syzygium aromaticum*	4.02	0.04867	0.0004
Cinnamomum verum	*Allium Sativum*	0	0.04867	<0.0001
Cinnamomum verum	*Salvia officinalis*	0	0.04867	<0.0001
Cinnamomum verum	*Thymus vulgaris*	0	0.04867	<0.0001
Cinnamomum verum	*Coriandrum sativum*	0	0.04867	<0.0001
Cinnamomum verum	*Cuminum cyminum*	0	0.04867	<0.0001
Cinnamomum verum	*Anethum graveolens*	0	0.04867	<0.0001
Cinnamomum verum	*Allium cepa*	0	0.04867	<0.0001
Cinnamomum verum	*Zingiber officinale*	0	0.04867	<0.0001
Cinnamomum verum	*Curcuma longa*	0	0.04867	<0.0001
Cinnamomum verum	Terramycin® 200	0	0.04867	<0.0001
Cinnamomum verum	Methanol	0	0.04867	<0.0001

Table 5: Dunnet's test comparing antimicrobial activity of all extracts against *Aeromonas hydrophila* (5 mg) to Oxytetracycline (Terramycin® 200).

Our extracts are crude methanol extracts and not pure compounds, therefore the concentrations of the bioactive compounds in crude extracts are still low when compared to the antibiotic and on the other hand, antibiotic usage is tightly regulated in terms of concentration and time. We performed Dunnet's test to find if the antimicrobial effect of cinnamon is significant when compared to other extracts and the antibiotic. Table 1 show that cinnamon had significantly higher ($p<0.0001$) bactericidal effect against *A. hydrophila*. When we compared the activity of oxytetracycline with all the extracts (Table 2), we find that cinnamon and clove both had higher activity than all the other extracts and the antibiotic.

Since eight of our plant extracts showed promising antimicrobial activity, we set out to determine the minimum inhibitory concentrations for each of extracts as well oxytetracycline. Figure 1 shows the MIC after 24 hrs of incubation for cinnamon and clove, interestingly we found that at 5 mg cinnamon had 3.8 mm and clove had 4.02 mm growth inhibitory activity. The minimum bactericidal concentrations (MBC) were similar to MIC after 72 hrs of incubation. Tables 3 and 4, are Dunnet's test showing the significantly higher antimicrobial activity of cinnamon and clove compared to other extracts and antibiotics ($p<0.0001$), while $p=0.0004$ when compared clove and cinnamon. When we compared the oxytetracycline activity (Table 5) at the lowest concentration, there is no significant difference ($p=1$) in activity when compared extracts except for cinnamon and clove which had significantly higher activity ($P<0.0001$). Figures 2-6 show the MIC for extracts and Figure 6 shows the concentration dependent growth inhibition of *A. hydrophila* for all the extracts. While the Figures 7 and 8 shows the MIC, MBC and concentration dependent growth inhibitions by oxytetracycline.

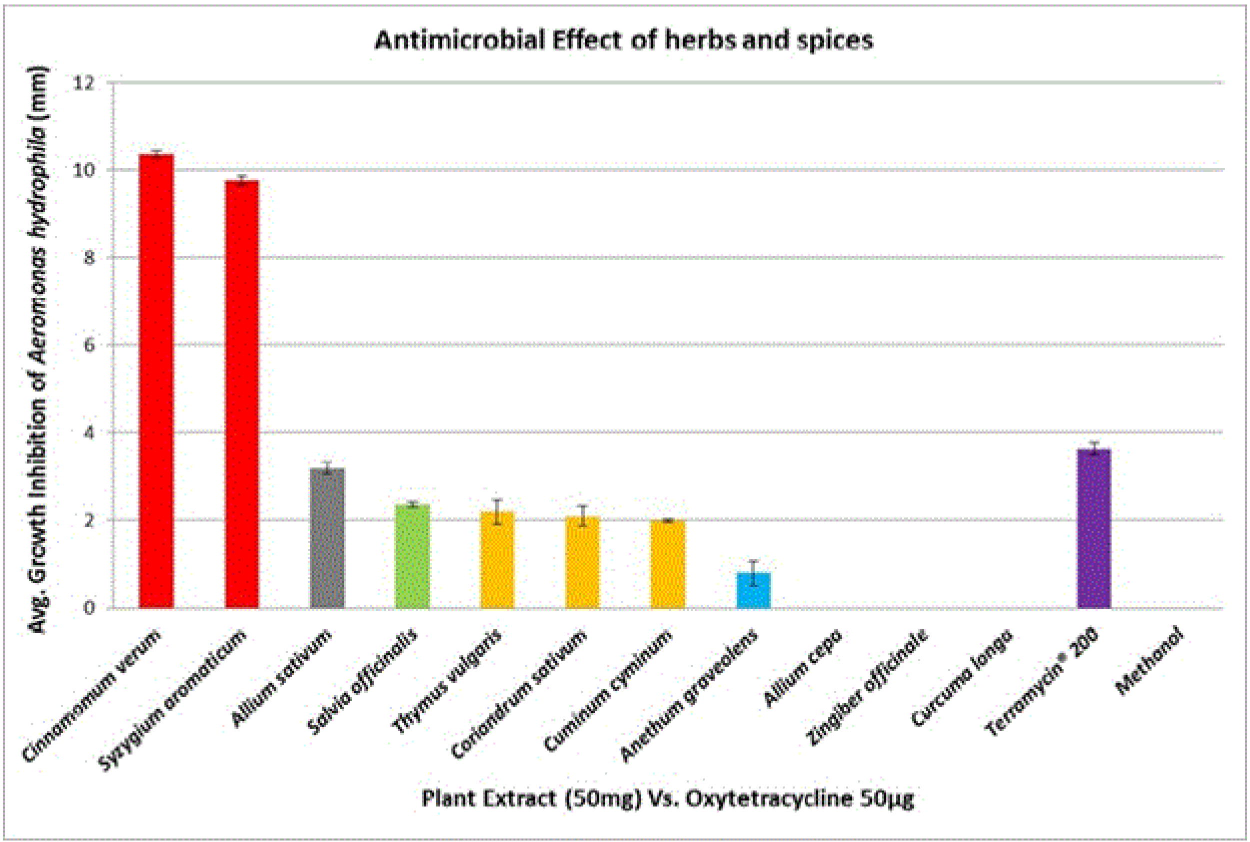

Figure 1: Screening of herb and spice extracts compared to Oxytetracycline for antimicrobial activity against *Aeromonas hydrophila.*

Overall, our results suggest that cinnamon and clove methanol extracts have better antimicrobial activity than other extracts and even the commercially used antibiotic, oxytetracycline (Figure 8).

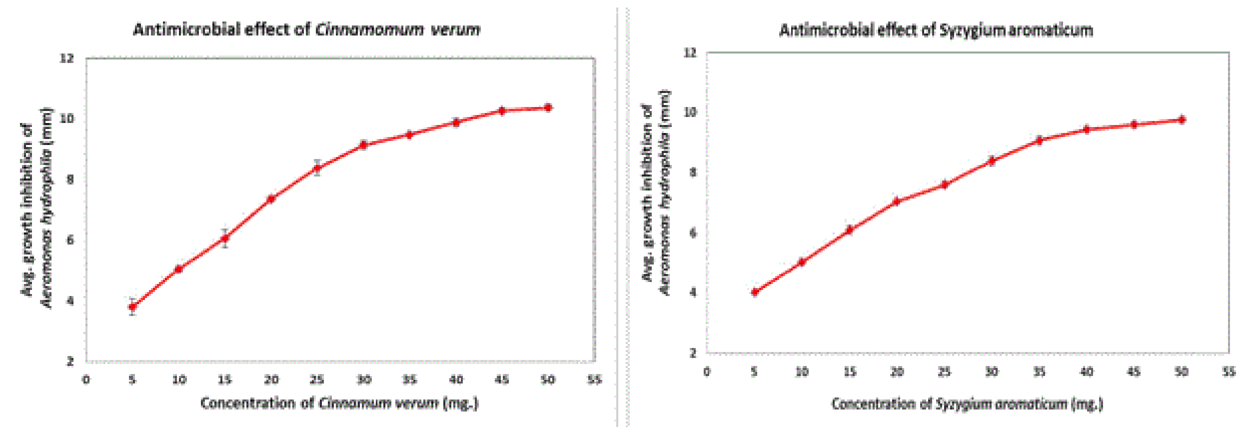

Figure 2: Curves showing minimum inhibitory concentrations against *Aeromonas hydrophila* for *Cinnamomum verum* and *Syzygium aromaticum.*

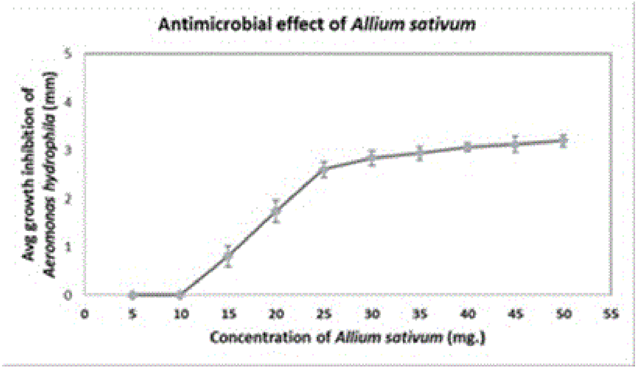

Figure 3: Minimum inhibitory concentration of *Allivum sativum* against *Aeromonas hydrophila.*

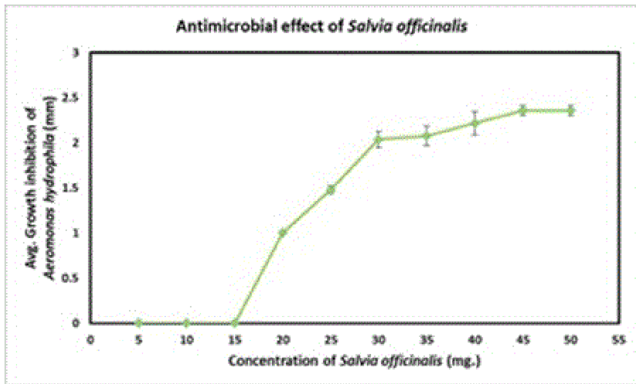

Figure 4: Curve showing minimum inhibitory concentration of *Salvia officinalis* against *Aeromonas hydrophila.*

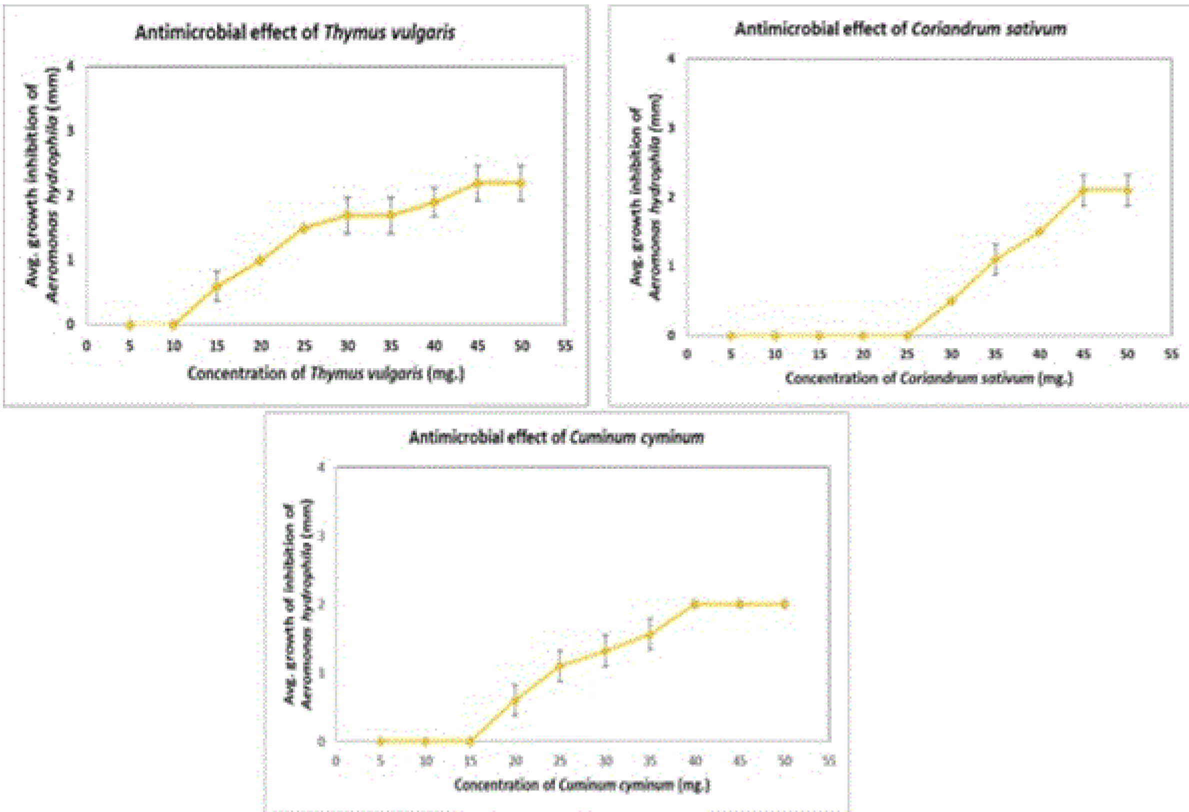

Figure 5: Curves showing minimum inhibitory concentrations of *Thymus vulgaris*; *Coriandrum sativum* and *Cuminum cyminum* against *Aeromonas hydrophila.*

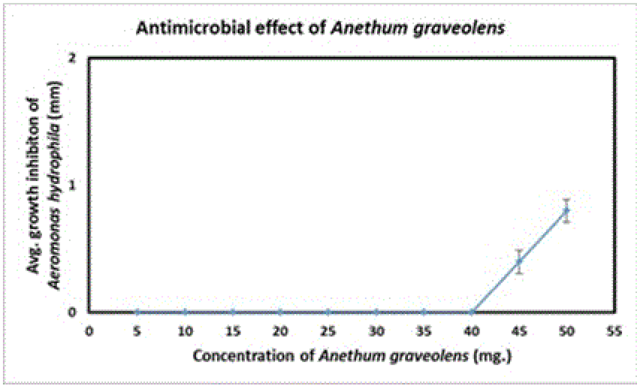

Figure 6: Curve showing Minimum Inhibitory concentration of *Anethum graveolens* against *Aeromonas hydrophila.*

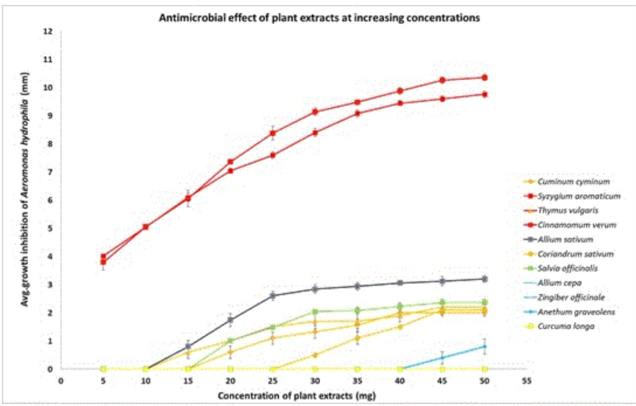

Figure 7: Curves showing Minimum Inhibitory concentrations of eleven methanol extracts tested against *Aeromonas hydrophila.*

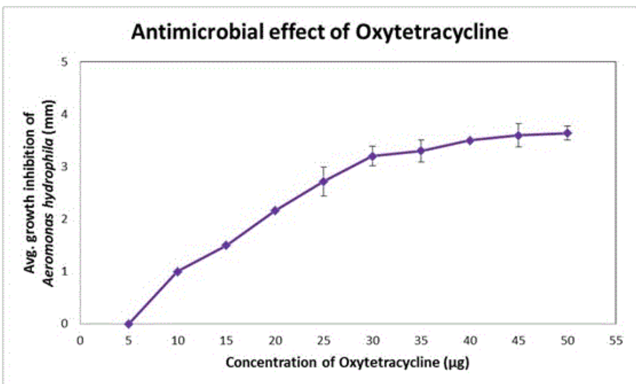

Figure 8: Curve showing minimum inhibitory concentration of Oxytetracycline tested against *Aeromonas hydrophila.*

Discussion and Conclusion

Aeromonas hydrophila infections cause huge economic loss to aquaculture industry. This bacterium is facultative pathogen causing pathogenic conditions in wide variety of organisms including humans. Since the commercially used antibiotics are tightly regulated and increased antibiotic resistance in bacterium, we set out to find new alternative therapeutics to treat this disease. Since the plant extracts contain bioactive compounds that are non-synthetic and safe to use with minimal or no regulations, we tested eleven methanol extracts against *A. hydrophila*. Some of the bioactive compounds in plant extracts: clove contains eugenol; cinnamon has eugenol and cinnamaldehyde [25,26]. We found that cinnamon and clove had highest antimicrobial activity when compared to other extracts. Linalool is the major component in coriander, and it can breach the cell membrane of gram-negative bacteria [27]. Garlic contains allicin, which inhibits RNA, DNA and protein synthesis of bacteria [28]. The major components of dill are carvone and limonene [29]. Camphor, 1, 8-cineole, α-thujone, and β-thujone account for the antimicrobial properties of sage [30]. Onion contains quercetin, a polyphenolic compound [31] ginger has sesquiterpenoid [32] and turmeric contains chemical curcumin [33]. However, in our studies against *A. hydrophila*, onion, turmeric and ginger did not show any activity [34,35]. Our results, suggest that clove and cinnamon can serve as potential and promising alternatives to antibiotics to deal *Aeromonas hydrophila* infections in fish as well as humans.

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