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Determination of Minimum Inhibitory Concentrations of Antimicrobial Herb and Spice Extracts against *Flavobacterium columnare*

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Abstract

Bacterial diseases like columnaris caused by *Flavobacterium columnare*, has huge economic impact on aquaculture industry. Proven the low efficacy of vaccines and tight regulations with antibiotic usage, there is a need for alternative therapeutics. We have previously found that methanolic extracts (50 mg/ml) from eleven herbs and spices had bactericidal effects against *Flavobacterium columnare* (3×10^8 CFU/ml). In the present study we determined the minimum inhibitory concentrations of all the eleven extracts and found that clove had the best significant growth inhibitory effect at the lowest concentration tested. Our results suggest that clove, cinnamon, thyme and cumin could serve as potential alternative therapeutics to deal with columnaris in fish.

Keywords: Bacterial; Clove; Cinnamon; Fish

Introduction

One of the leading causes of fish mortality for ornamental, cultured and wild fish is bacterial infection. In the United States, *Flavobacterium columnare* is one of the most common freshwater pathogens. This bacterium causes columnaris disease, second only to enteric septicemia in catfish aquaculture [1]. This species went through multiple changes in its name before *F. columnare* was assigned in 1996 [2]. Today there have been more than 36 species of fish infected by *F. columnare*, including commercially raised species such as channel catfish, tilapia, koi, rainbow trout, chinook salmon, and zebrafish [3,4]. Thus, this disease corresponds to major economic losses [5]. The catfish industry in the United States alone loses millions of dollars every year due to columnaris disease.

Flavobacterium columnare is a gram-negative bacillus bacterium with yellow pigmentation and rhizoid-forming colonies. These colonies are capable of attaching to the epidermis of fish, such as on their gills and tails. Different morphologies of the bacteria (e.g. rhizoid forming and non-rhizoid forming) affect adherence to the fish [6]. After attaching to the fish, *F. columnare* causes skin lesions that deteriorate fins and eat away at the flesh [7]. This gives the disease some of its common names, such as "fin rot", "saddleback disease" and "cottonmouth disease". This disease is a worldwide phenomenon, occurring in both tropical and temperate freshwater fish, some strains becoming more virulent at higher temperatures than others, and vice versa [8]. Though previously thought to only be transmitted by fish to fish contact, it has been shown that *F. columnare* can travel horizontally through water. In fact, this bacterium can survive for 10 days without a host in fresh water [9]. This means that removing infected fish from water inhabited by healthy fish does not guarantee preventing the spread of infection. Due to this, antibiotics and chemicals are used in an attempt to inhibit the growth of *F. columnare*.

There are various antibiotics capable of treating bacterial infections. However, they do not all treat the same species of bacteria. In addition, many can cause adverse side effects. Due to this, the use of antibiotics is heavily regulated. For aquaculture purposes, the FDA has approved only 3 drugs to be used: oxytetracycline, florfenicol, and sulfadimethoxine/ormetoprim [10]. As oxytetracycline is the most commonly used of these three, it is often used as a positive control in studies. While some studies have shown the effectiveness of florfenicol and oxytetracycline in the inhibition of *F. columnare*, it has also been shown that oxytetracycline is not a guaranteed inhibitor. In addition, bacteria are capable of developing resistances to antibiotics. Not only does this remove a solution to fish disease outbreaks, it can also cause resistances in bacteria that infect humans, resulting in the inability of antibiotics to alleviate human diseases [11]. The culmination of all of this calls for other means to control bacterial disease outbreaks.

Chemicals present a potential solution, as *F. columnare* is susceptible to the use of potassium permanganate, salt, hydrogen peroxide, chloramines [12], copper sulfate, and diquat. Despite the effectiveness of these chemicals, this usage could adversely affect the environment and the fish themselves, by polluting the water and deteriorating the external microbiome of the fish that provides protection from disease [12,13]. Since antibiotics and chemicals are not reliable for controlling bacterial outbreaks for a long period of time, alternatives are needed to safely and effectively prevent outbreaks. This leads to the investigation into natural compounds that inhibit bacterial growth, a field that has seen more interest in recent years.

Herbs and spices have been used for thousands of years to create medicines for various illnesses. Studies have shown that it is not these plants that are useful for illnesses, but rather the bioactive compounds that the plants produce as secondary metabolites and the antifungal and antibacterial qualities they possess. Thus, plant extracts can become complements to antibiotics due to proven synergism, or potentially even substitutes for antibiotics [14]. Also, because the plant extracts are produced naturally, their environmental impact would be substantially lower than that of the currently used synthetic substances. In this study we have determined the minimum inhibitory concentrations of plant extracts that we have previously identified to be having antimicrobial properties against *Flavobacterium columanare*.

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Materials and Methods

Plant materials and methanolic extract preparation

Eleven organic herb and spice powders were purchased from Walmart to test their antibacterial properties. They were turmeric (*Curcuma longa*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), onion (*Allium cepa*), cinnamon (*Cinnamomum verum*), cumin (*Cuminum cyminum*), clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*), coriander (*Coriandrum sativum*), dill weed (*Anethum graveolens*) and sage (*Salvia officinalis*). To prepare the Methanolic extracts, 50 g of plant powder was dissolved in 400 ml of 99% and incubated for 24 hours on a shaker at room temperature. The mixture was filtered using Whatmann Number 4 filter paper and a vacuum filter to remove the solid particles, allowing the bioactive compounds dissolved in methanol. These solutions were processed in a Buchi Rotavapor R-200 to evaporate the methanol. Then evaporated extracts were resuspended to final concentration of 1 $g{\cdot}mL^{-1}$ methanol.

Bacterial strain and growth medium

The LV-35901 strain of *Flavobacterium columnare* used in this study was generously provided by Dr. Miles Lange, USDA Stuttgart, AR. The growth medium used was FCGM, that has 8.0 g Triptone, 1.0 Magnesium Sulfate, 0.74 g Calcium Chloride, 1.5 g Sodium Citrate, 0.8 g Bacto™ Yeast Extract, 1.75 g Sodium Chloride, and made up to one liter with sterile Millipore distilled water. To make plates, 9.0 g Agar was added to this mixture. Neomycin and polymyxin B were added for selectivity.

McFarland turbidity standards

To consistently maintain the same number of bacteria on the petri dishes, the bacterial solution was compared to a McFarland Turbidity Standard No. 1. The standard was created by preparing 1% solutions of Sulfuric acid and Barium chloride. 0.1 mL of the 1% Barium chloride solution was added to 9.9 mL of the 1% Sulfuric acid solution. This solution had the same turbidity of a bacterial solution with 3×10^8 Colony Forming Units (CFU)/mL. In order to compare the bacterial solution to the standard, the growth media was removed from the solution so its color would not impact viewing the turbidity. We centrifuged the bacterial culture at 1000 rpm for 5 minutes and discarded the supernatant. This bacterium was then re-suspended in saline up to 10 mL. This bacterial solution was then held next to the standard in front of a light to compare turbidities. If the bacterial solution was more turbid than the standard, saline was added to the solution to dilute it. If the bacterial solution was not turbid enough, more bacterium was added. When the solution and the standard were similar, the bacterial solution was used to inoculate the petri dishes.

Antimicrobial tests for minimum inhibitory concentrations (MIC)

We used 10 cm petri dishes and maintained equal volume of growth medium in all the dishes. Sterile cotton swabs were used to spread out the bacteria to cover the entire plate. Then, 5 replicates of Whatman 9 mm discs were loaded with 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µl of each extract. The concentration of each methanolic extract was 1 mg extract/µl. five replicates of Oxytetracycline served as positive and methanol as negative controls. For Tetracycline MIC and dose response experiments, the experiment was conducted just like the plant extracts, but with a concentration of 1 µg/µl to achieve final concentrations. The zones of inhibition were recorded after an overnight incubation at 30°C temperature to the nearest mm.

Statistical analysis

Dunnet's test was employed to find the difference between the antimicrobial activity between extracts and oxytetracycline at all the ten concentrations testedand the minimum inhibitory concentration. The statistical analysis was performed by using SAS version 9.4.

Results

In our previous studies, we have screened eleven plant extracts for their antimicrobial activity against *Flavobacterium columnare*. We have tested one concentration 50 mg, of each extract against the bacterium. We found that all the eleven extracts had antimicrobial effect on *Flavobacterium columanre* and cumin showing highest growth inhibition, while cinnamon and clove showed similar activity as the antibiotic, Oxytetracycline.

Since our plant extracts showed promising results to deal with columnaris, we have set out to determine the Minimum Inhibitory Concentrations for all the eleven extracts in this study. We have tested 10 concentrations of each extract from 5 mg to 50 mg with increments of five. Consistent with our previous findings, we found that cumin, clove and thymus had highest growth inhibition of *Flavobacterium columnare* at the maximum concentration, 50 mg that we have tested. Cinnamon had shown similar activity as the antibiotic.

Figure 1 shows the growth inhibition of *Flavobacterium columnare* at increasing concentrations of cumin, clove, cinnamon and thyme. Figure 2 shows the effect of garlic coriander and sage. Figure 3 shows the effect of onion, and ginger; whereas the effect of dill weed and turmeric that had the least antimicrobial activity (Figure 4). The compiled effect of all the eleven extracts tested against *Flavobacterium columnare* is shown in Figure 5. From this compiled growth inhibitory curves among the extracts at various concentrations, we can see that clove had best effect at the minimum inhibitory concentration. Interestingly, when we ran the Dunnet's test to determine the significance of growth inhibitory effect of among the extracts and the antibiotic, we found that clove had significantly ($P<0.0001$) higher growth inhibitory effect at the lowest concentration 5 mg tested Table 1. Figure 6 shows the growth inhibition of *Flavobacterium columnare* in response to increasing concentrations of Oxytetracycline from 5 µg to 200 µg. notably, the effect of Oxytetracycline has plateaued at a concentration of 50 µg with an average of 19.5 mm and did not change with any further increase in concentration. We then ran a Dunnet's test (Table 2) to find if clove had significant growth inhibition when compared to Oxytetracycline and other extracts at all the 10 concentrations we tested. Interestingly, we find that clove similar effect like Oxytetracycline ($P=0.0676$), cumin ($P=0.7376$), thyme ($P=0.6995$), and cinnamon ($P=0.1492$). Clove had significantly higher effect than all the other extracts at all higher concentrations as shown in Table 2 ($P<0.001$).

Discussion

When compared to two other freshwater fish pathogens, *Streptococcus agalactiae* and *Aeromonas hydrophila*, in a study evaluating the antimicrobial effectiveness of 46 methanolic extracts, *F. columnare* was the most susceptible to herbal extracts. This was in terms of both minimum inhibition concentration and variety of extracts [15]. In another study, the seeds and oil of black cumin were found to be considerably inhibitorier towards *F. columnare* than oxytetracycline. This was found across 25 strains of the bacteria showing different responses to oxytetracycline. The major active compound in cumin is *p*-coumaric acid. This compound damages bacterial cell membranes

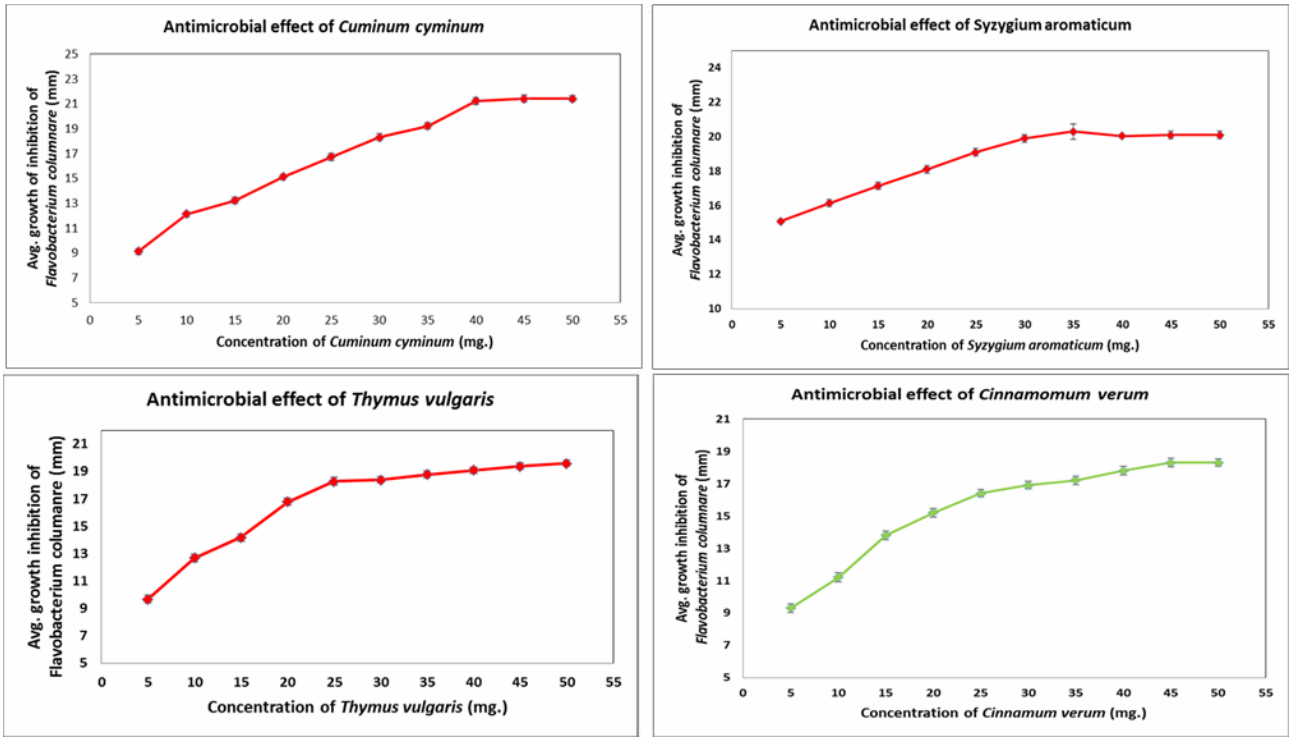

Figure 1: Antimicrobial effects of cumin, clove, thyme and cinnamon against *Flavobacterium columnare* in response to increasing concentrations from 5 mg to 50 mg.

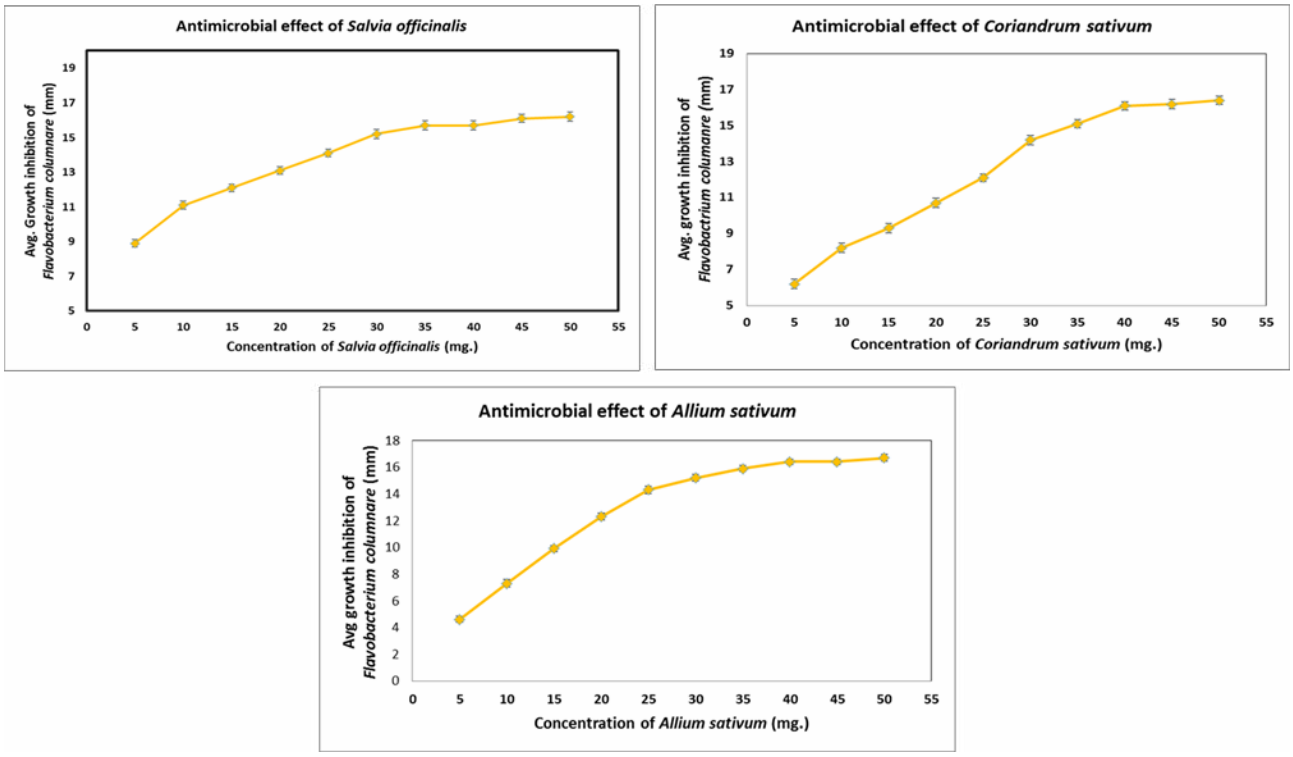

Figure 2: Antimicrobial effects of sage, coriander, and garlic against *Flavobacterium columnare* in response to increasing concentrations from 5 mg to 50 mg.

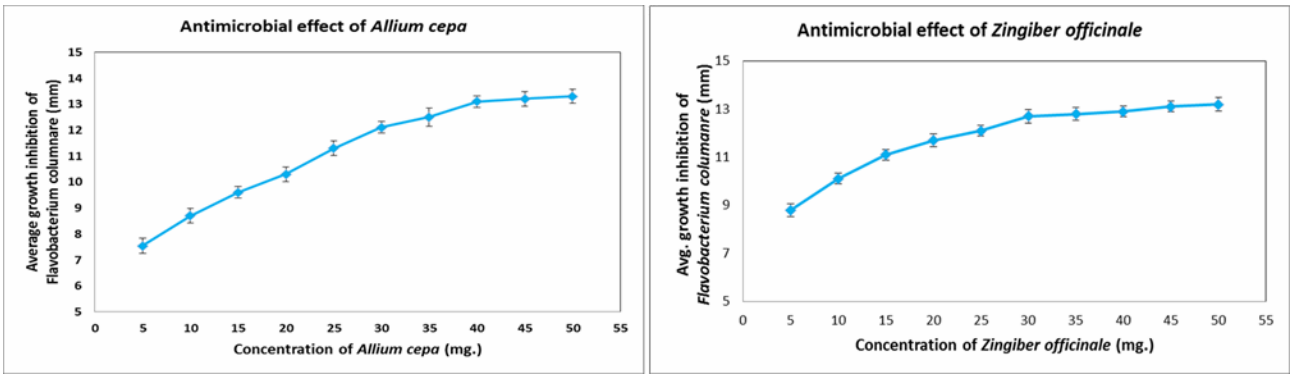

Figure 3: Antimicrobial effects of onion, and ginger against *Flavobacterium columnare* in response to increasing concentrations from 5 mg to 50 mg.

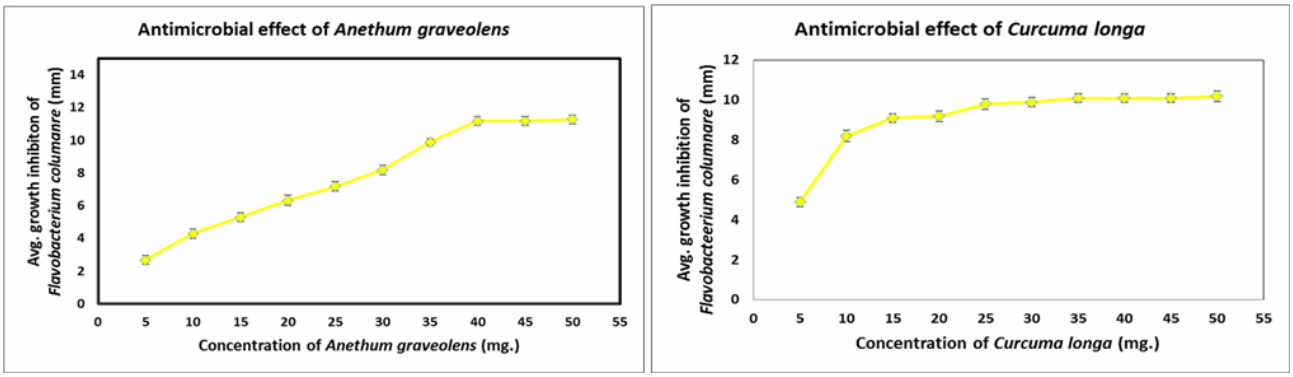

Figure 4: Antimicrobial effects of dill weed and turmeric against *Flavobacterium columnare* in response to increasing concentrations from 5 mg to 50 mg.

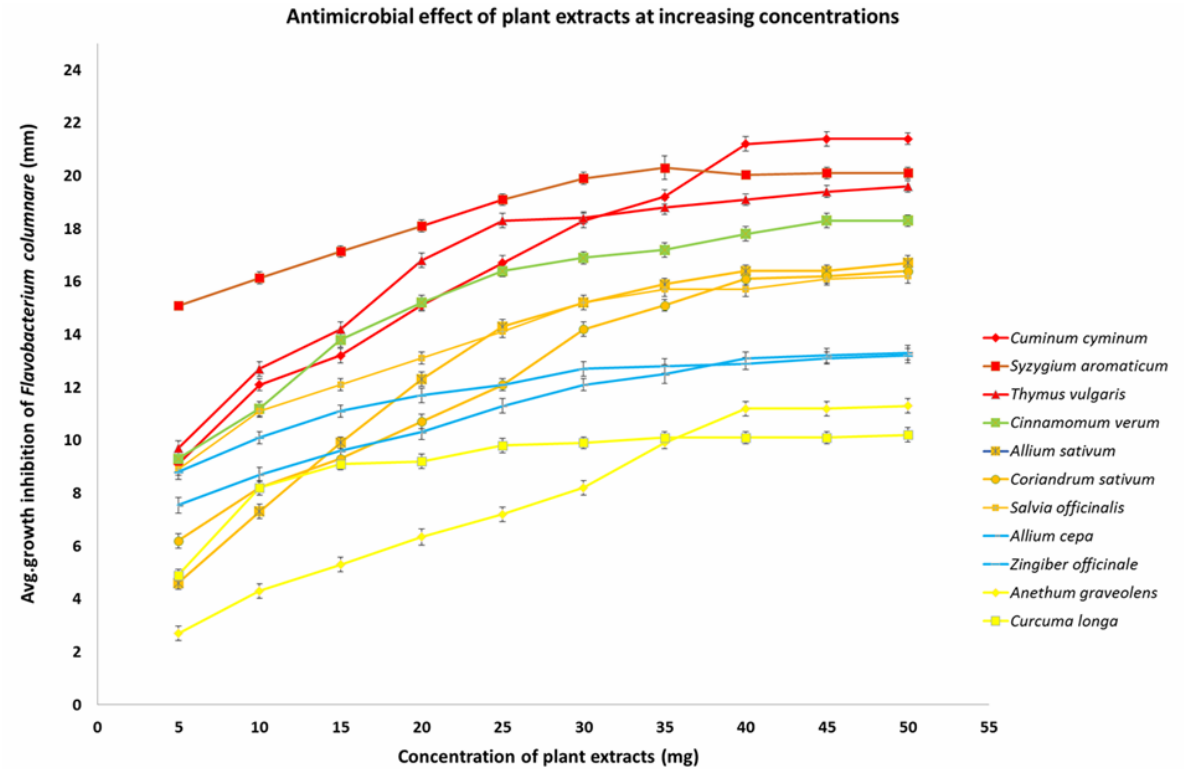

Figure 5: Antimicrobial effects of all eleven extracts against *Flavobacterium columnare* in response to increasing concentrations from 5 mg to 50 mg.

Dunnet's Test - Comparison of Syzygium aromaticum with other extracts (5mg) and Terramycin® 200 (5 µg)				
Degrees of Freedom= 47.1				
Extract compared	**Extract/Antibiotic**	**Mean Inhibition (mm)**	**Pooled Standard &Tor**	**P-Value**
Syzygium aromaticum	Syzygium aromaticum	15.08	N/A	N/A
Syzygium aromaticum	Cuminum cyminum	9.1	0.1581	<.0001
Syzygium aromaticum	Thymus vulgaris	9.7	0.1581	<.0001
Syzygium aromaticum	Cinnamomum verum	9.3	0.1581	<.0001
Syzygium aromaticum	Allium sativum	4.6	0.1581	<.0001
Syzygium aromaticum	Coriandrum sativum	6.2	0.1581	<.0001
Syzygium aromaticum	Salvia officinalis	8.9	0.1581	<.0001
Syzygium aromaticum	Allium cepa	7.54	0.1551	<.0001
Syzygium aromaticum	Zingiber officinale	8.8	0.1581	<.0001
Syzygium aromaticum	Anethum graveolens	2.7	0.1581	<.0001
Syzygium aromaticum	Curcuma longa	4.9	0.1581	<.0001
Syzygium aromaticum	Terramycin® 200	9.8	0.1581	<.0001
Syzygium aromaticum	Methanol	0	0.1581	<.0001

Table 1: Comparison of clove growth inhibition of *Flavobacterium columnare* with all the other extracts and antibiotic tested at the Minimum Inhibitory Concentration 5 mg, using Dunnet's test.

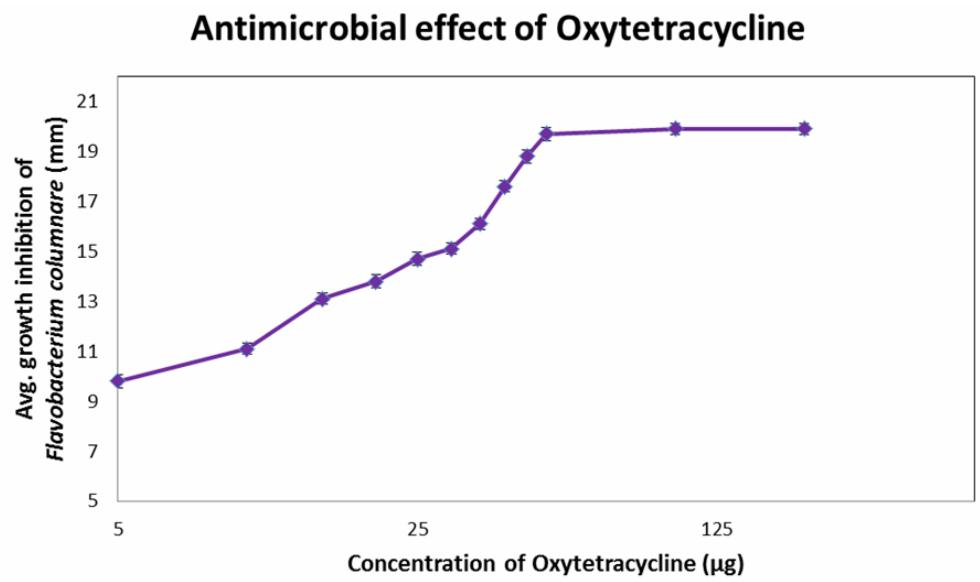

Figure 6: Antimicrobial effects of Terramycin® 200 (Oxytetracycline) against *Flavobacterium columnare* in response to increasing concentrations from 5 µg to 200 µg.

Dunnet's Test - Comparison of Syzygium aromaticum with other extracts and Terramycin® 200 at all concentrations tested			
Degrees of Freedom=108			
Extract compared	**Extract/Antibiotic**	**Pooled Standard Error**	**P-Value**
Syzygium aromaticum	Syzygium aromaticum	N/A	N/A
Syzygium aromaticum	Cuminum cyminum	1.3544	0.7376
Syzygium aromaticum	Thymus vulgaris	1.3544	0.6995
Syzygium aromaticum	Cinnamomum verum	1.3544	0.1492
Syzygium aromaticum	Allium sativum	1.3544	0.0005
Syzygium aromaticum	Coriandrum sativum	1.3544	0.0002
Syzygium aromaticum	Salvia officinalis	1.3544	0.0058
Syzygium aromaticum	Allium cepa	1.3544	<.0001
Syzygium aromaticum	Zingiber officinale	1.3544	<.0001
Syzygium aromaticum	Anethum graveolens	1.3544	<.0001
Syzygium aromaticum	Curcuma longa	1.3544	<.0001
Syzygium aromaticum	Terramycin® 200	1.3544	0.0676
Syzygium aromaticum	Methanol	1.3544	<.0001

Table 2: Comparison of clove growth inhibition of *Flavobacterium columnare* with all the other extracts and antibiotic tested at all the ten concentrations tested using Dunnet's test.

and causes intracellular nucleotides and proteinaceous materials to leak out of the cell [16]. Clove and cinnamon have been found to have large quantities of eugenol, and cinnamon also has cinnamaldehyde [17,18]. Linalool is the major component in coriander, and when in contact with gram-negative bacteria it causes an increase in the permeability of the cell membrane [19]. Onion contains quercetin, a polyphenolic compound [20]. Allicin that is found in garlic completely inhibits RNA synthesis and partially inhibits DNA and protein synthesis, making it effective against bacteria [21]. The main source of antimicrobial activity in turmeric is due to the chemical curcumin [22]. The major components of dill are carvone and limonene [23]. Camphor, 1,8-cineole, α-thujone, and β-thujone accounts for the antimicrobial properties of sage [24]. The main compound responsible for antibacterial activity in ginger is sesquiterpenoids [25,26].

Since there is a need to find alternatives to antibiotics and chemicals for the treatment of bacterial diseases, we screened eleven plant extracts that naturally produce compounds with antimicrobial properties and determined their minimum inhibitory concentrations to control the growth of *F. columnare* [27]. Although we found Cumin to be more effective when used a higher dose of 50 mg, clove was very efficient when used at lowest dose of 5 mg. We conclude that clove has a significant effect on the growth of *Flavobacterium columnare* when compared to other plant extracts and the Oxytetracycline. We speculate that cumin and clove would effectively inhibit the growth of *Flavobacterium columnare* in vivo. However, there is still a need for further studies in determining the cytotoxic and genotoxic effects of our plant extracts with antimicrobial effects, on the host cell [28].

Conclusion

Consistent with our previous studies we saw growth inhibitions for all the eleven extracts we tested, against *Flavobacterium columnare*. We determined the MICs for all the eleven extracts. When we compared the growth inhibitory significance using Dunnet's test, we found that clove has best antimicrobial effect on *Flavobacterium columnare* at its minimum inhibitory concentration. Our data suggests that clove can be used as a therapeutic to prevent and treat columnaris disease.

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