

Sensitivity of Fish Pathogenic Bacteria to Almond (*Terminalia Catappa*) Leaves and Bitter (*Vernonia Amygdalina*) Leaves Extracts

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Abstract

The use of antibiotics in aquaculture to treat infection has resulted into the development of resistant strains which have rendered antibiotic treatment ineffective. Therefore, alternative ways of treating diseases must be found, this study aimed to evaluate the antimicrobial activities of almond (*Terminalia catappa*) leaves and bitter (*Vernonia amygdalina*) leaves extracts on some fish pathogens such as *Staphylococcus aureus*, *Staphylococcus albus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Aeromonas hydrophila*, *Escherichia coli*, *Salmonella typhi*, *Streptococcus iniae* and *Aspergillus niger* using well-diffusion method. Phytochemical screening and minimum inhibitory concentration of methanolic and ethanolic extracts of *T. catappa* and *V. amygdalina* leaves were determined using standard methods. Data were analyzed using descriptive statistics. The result of the study revealed the diameter of inhibition zone in methanolic and ethanolic extracts of almond leaves while no diameter of inhibition of zone were recorded for *S. albus*, *B. subtilis*, *A. hydrophila*, *S. typhi*, *S. iniae* and *A. niger* in methanolic and ethanolic extracts of bitter leaves. The result of the phytochemical screening shows the presence of saponins, phenols, flavonoids, amino acids but triterpenes and steroids were not detected in the methanolic and ethanolic extracts of almond leaves. Minimum inhibitory concentrations of methanolic and ethanolic extracts of almond and bitter leaves on the bacteria tested were 1000µg/ml respectively. This investigation provides information on using almond and bitter leaves extracts to control bacterial diseases in aquaculture and it could be concluded that the methanolic and ethanolic extracts of almond and bitter leaves shows promise as an alternative antimicrobial source for use in veterinary medicine.

Keywords: Antimicrobial; Almond Leaves; Bitter Leaves; Phytochemical; Fish Pathogen

Abbreviations: PDA: Potato Dextrose Agar; ANOVA: Analysis of Variance; SPSS: Statistical Package for Social Science.

Introduction

Aquaculture has been a growing activity for the last 20 years worldwide and this impressive development has been attended by some practices potentially damaging to human and animal health [1]. The large-scale settings of aquatic animal husbandry have resulted in an increased antibiotic resistance in bacteria potentially pathogenic to fish and related environment [2-4]. Continuous use of synthetic antibiotics in aquaculture reveals the threats to consumers and non-target organism in the environment [5,6]. Treatments of bacterial diseases with various herbs have been safely and widely used in organic agriculture, veterinary and human medicine [7]. Since ancient times, medicinal plants have been used for the treatment of common infectious diseases [8] and treatments with plants having antibacterial activity are a potentially beneficial alternative in aquaculture [5]. Medicinal plants as the alternative agents are effective to treat the infectious diseases and mitigate many of side effects that are associated with synthetic antimicrobials [9]. In addition, plant-derived phyto-medicines provide a cheaper source for treatment and greater accuracy than chemotherapeutic agents [9]. Among the common fish pathogens are *P. aeruginosa*, *A. hydrophila* etc. that cause infectious diseases. *Aeromonas hydrophila* is one of the most common bacterial pathogens in freshwater fish and has been recognized to be the aetiological agent of several distinct pathological conditions including tail/fin rot and hemorrhagic septicemia especially in freshwater and ornamental fish [10]. The ability of some herbs and seaweeds to inhibit activity of bacteria having potential interest as fish pathogens has been documented [1,6,7,11-13]. Most diseases in fishes are due to poor water quality, stress and over-crowding which lowered the immune system because the presence of microbes [14]. *Terminalia catappa* and *Vernonia amygdalina* exhibited a wide range of pharmacological activities including antibacterial, antifungal, antiprotozoal, antidiabetic, anti-cancer, anti-inflammatory and antioxidant [15,16]. However, there is little or no information on their uses in fish farming. Hence, this study was assessed to evaluate the effectiveness of *T. catappa* and *V. amygdalina* as antimicrobial agents in treating infectious fish diseases.

Materials and Methods

Plant Collection

The following plants were used in the study; almond leaves and bitter leaves. These plants were obtained in Ala quarters, Akure, Ondo State and were identified by Dr D. O. Aworinde in the Department of Biological Sciences (Botany Programme), Ondo State University of Science and Technology, Okitipupa.

Preparation and Extraction of Plant Materials

Leaves Extraction: The extractions of almond and bitter leaves were done as described by Ajaiyeoba and Fadare [17]. The air-dried almond leaves and bitter leaves were grounded with a hammer mill and 200g of the fine powder of these plant leaves were soaked in 1000 ml of ethanol and methanol for 48 hours. These plant leaves were proper mixed with ethanol and methanol at regular interval for homogeneity, filtered, using a sterile muslin cloth after which the extracts was obtained, air-dried and store at 25°C until required.

Media Preparation: Media such as Nutrient agar (Oxoid, Germany), Potato Dextrose agar (Oxoid, Germany), MacConkey agar (Oxoid, Germany), and Nutrient broth (Oxoid, Germany) used were prepared according to manufacturer's instruction. All these media are allowed to cool after sterilization to about 45°C before pouring into petri dishes.

Source of Micro Organisms

The microorganisms isolated from *C. gariepinus* were *A. hydrophila*, *P. aeruginosa*, *S. iniae* and *S. aureus*. The isolation and characterization of bacteria using biochemical test was carried out at Microbiology Laboratory, Faculty of Science and University of Ibadan. *Aspergillus Niger* and *S. albus* were collected from the laboratory stock of the Department of Biological sciences, Ondo State University of Science and Technology, Okitipupa. The pure cultures were labeled, sub-cultured on nutrient agar slants and nutrient broth(s) and potato dextrose agar (PDA), preserved in the refrigerator at 4°C until it is require for study.

Antimicrobial Assay

A well diffusion assay as described by Bello OS, et al. [18] was used. Pre-poured indicator pathogen (4 mm depth) was overlaid with a 10 ml soft agar (0.7%) lawn of indicator culture (thus generating a potential mat for the indicating of bacteria). Wells of 10 mm diameter were cut into these agar plates using cork borer and 0.1ml of these

plants extract was placed into each well [18]. Distilled water was used as negative control while antibiotics, chloramphenicol (0.1 µg and 0.3 µg) were used as positive control. The plates were examined for zones of inhibition which was scored positive, if the width of the clear zone was 10 mm or longer. The diameter of the inhibition zones were taken to be proportional to the logarithm of the antimicrobial compounds in almond and bitter leaves and expressed as the log₁₀ CFU/ml.

Isolation of Microorganism / Counts

The gills, skin, intestine and liver sample of *C. gariepinus* were separately macerated and put into sterile clapped test tube containing sterilized distilled water and homogenized [19]. Serial dilution was carried out and 1ml each from 10⁻³ to 10⁻⁵ dilution factors were dispersed into petri dishes that were appropriately labeled and molten sterilized medium was poured aseptically into petri dish. The plates were swirled gently for even distribution of inocula and allowed to set / gel and then incubated at 37°C for 24 hours. The organisms grew into visible different colonies after 24 hours. Enterobacteriaceae and Total viable counts were determined, the result were expressed in log₁₀ CFU/g.

Minimum Inhibitory Concentration

Minimum inhibitory concentration of almond and bitter leaves was carried out using Double dilution of 2000 µg / ml of these plants extract were made in 2 ml volume of broth to 3.96 µg / ml. One row of the test was inoculated with 0.02 ml of 1 in 10 dilution of the overnight broth culture of the organism [18]. The test was incubated at 37°C for 24 hour aerobically. The minimum inhibitory concentration was the lowest concentration that prevented the growth of bacterial after 24 hour incubation [20].

Phytochemical Screening

Detection of Saponins:

- a) **Froth Test:** Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.
- b) **Foam Test:** Extract of 0.5 g was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of Phenols Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Tannins: Extract of 0.1 g was taken up in 10 ml distilled water, and filtered. Then a few drops of ferric chloride (FeCl₂) reagent were added to 1 ml of the filtrate. The mixture was observed for the formation of blue, blue-black, green or green-black colouration or precipitate.

Detection of Flavonoids

a) **Alkaline Reagent Test:** Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid (HCl), indicates the presence of flavonoids.

Glucosinolates: Extract of 0.1 g was dissolved in 5 ml of chloroform followed by filtration as described by Adeoye BA, et al. [21] method. Concentration tetraoxosulphate (iv) acid (Sulphuric acid) was carefully layered at the bottom of the tube without disturbing the solution. It was observed for the formation of a sharp brown ring at the chloroform/sulphuric acid interface.

Test for Triterpenes and Steroids:

a) **The Salkowski Test:** Extract of 3ml was warmed in 5 ml of chloroform for 30 minutes. The chloroform solution was then treated with a small volume of concentrated tetraoxosulphate (iv) acid (H₂SO₄) and shaken. The red colour produced within a few minutes indicated a positive reaction.

Detection of Proteins and Aminoacids

a) **Xanthoproteic Test:** The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Statistical Analysis

The microbial load of fish tissue (skin, gills, intestine and liver) and antimicrobial and antifungal activities (diameter of zone of inhibition, mm) of almond and bitter leaves against tested pathogens resulting from the experiment were subjected to one way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Science version 15.0)

Results

Determination of Phytochemical in Almond and Bitter Leaves

Phytochemical screening of almond and bitter leaves for metabolites showed the presence of tannins, saponins, flavonoids, phenol, protein and amino acids while glucosinolates, triterpenes and steroids were not detected in almond leaves but present in methanolic and ethanolic extract of bitter leaves. The values of these metabolites

were in abundant quantity (+++), moderate quantity (++) and low quantity (+) in both plants as shown in Table 1.

Table1: Phytochemical screening of methanolic and ethanolic extracts of almond leaves and bitter leaves.

	Phytochemical	Test Used	Ethanol	Methanol
Almond leaves	Saponins	Froth test	++	++
		Foam test	+	+
	Phenols	Ferric chloride test	+	+
	Flavonoids		+	+
	Tannins		+	+
	Glucosinolates		-	-
	Steroids and triterpenes		-	-
	Protein and amino acid	Xanthoproteic test	+	+
Bitter leaves	Saponins	Froth test	+++	+++
		Foam test	+++	+++
	Phenols	Ferric chloride test	+	+
	Flavonoids		+	+
	Tannins		+	+
	Glucosinolates		+	+
	Steroids and triterpenes		+	+
	Protein and amino acid	Xanthoproteic test	+	+

Keys: +++ = Present in high quantity, ++ = moderately present, + = present in low quantity, - = Negative or not present.

Antibacterial Activity of Almond and Bitter Leaves Extracts

The result revealed that the methanolic and ethanolic extracts of almond and bitter leaves showed antibacterial activities against the pathogens tested except *S. aureus*

and *S. albus* that did not show a diameter of inhibition zone in ethanolic extracts of almond leaves. Also, *S. iniae*, *S. albus*, *A. hydrophila*, *B. subtilis* and *S. typhii* were not present in the methanolic and ethanolic extracts of bitter leaves. This was represented in Table 2.

Table 2: Antibacterial activity (diameter of zone of inhibition, mm) of methanolic and ethanolic extract of almond and bitter leaves against isolates obtained from *C. gariepinus*, using distilled water as negative control and chloramphenicol as positive control.

	Pathogens	Methanol	Ethanol	Control	Control 2 (chloramphenicol 10mg/ml)	Control 2 (chloramphenicol 30mg/ml)
Almond leaves	<i>S. aureus</i>	35.00 ± 0.03	-	-	13.00 ± 0.02	35.50 ± 0.01
	<i>S. albus</i>	20.00 ± 0.01	-	-	11.00 ± 0.01	35.00 ± 0.02
	<i>P. aeruginosa</i>	15.00 ± 0.05	15.00 ± 0.01	-	-	-
	<i>S. iniae</i>	15.00 ± 0.05	15.00 ± 0.05	-	16.00 ± 0.05	40.00 ± 0.01
	<i>A. hydrophila</i>	25.00 ± 0.10	20.00 ± 0.10	-	14.50 ± 0.03	43.00 ± 0.03
	<i>B. subtilis</i>	20.00 ± 0.06	8.00 ± 0.06	-	12.80 ± 0.07	38.00 ± 0.01
	<i>E. coli</i>	25.00 ± 0.02	10.00 ± 0.02	-	14.00 ± 0.01	43.00 ± 0.02

	<i>S. typhii</i>	25.00 ± 0.01	10.00 ± 0.01	-	15.00 ± 0.02	47.00 ± 0.01
Bitter leaves	<i>S. aureus</i>	7.00 ± 0.02	7.00 ± 0.01	-	13.00 ± 0.02	35.50 ± 0.01
	<i>S. albus</i>	-	-	-	11.00 ± 0.01	35.00 ± 0.02
	<i>P. aeruginosa</i>	11.00 ± 0.01	11.00 ± 0.03	-	-	-
	<i>S. iniae</i>	-	-	-	16.00 ± 0.05	43.00 ± 0.01
	<i>A. hydrophila</i>	-	-	-	14.50 ± 0.03	40.00 ± 0.03
	<i>B. subtilis</i>	-	-	-	12.80 ± 0.07	38.00 ± 0.01
	<i>E. coli</i>	7.00 ± 0.04	7.00 ± 0.02	-	14.00 ± 0.01	43.00 ± 0.02
	<i>S. typhii</i>	-	-	-	15.00 ± 0.02	47.00 ± 0.01

Keys: - = no diameter of zone of inhibition.

Microbial Load in *C. gariepinus*

The microbial load of organs in the fish varies distinctively. The result revealed that the total viable counts were generally higher than the enterobacteriaceae

counts. The highest value recorded was in the gills, followed by the skin, the least value recorded in the liver. This was shown in Table 3.

Table 3: Microbial load of *C. gariepinus* (liver, skin, intestine and gills).

Organ	Organism	Microbial load (log ₁₀ CFU/g)
Liver	Enterobacteriaceae counts	5.88 ± 0.50
	Total viable counts	6.26 ± 1.22
Skin	Enterobacteriaceae counts	6.67 ± 0.66
	Total viable counts	6.92 ± 0.84
Intestine	Enterobacteriaceae counts	6.33 ± 0.79
	Total viable counts	6.63 ± 0.66
Gills	Enterobacteriaceae counts	6.95 ± 0.54
	Total viable counts	7.19 ± 0.88
Control	Enterobacteriaceae counts	-
	Total viable counts	-

Determination of Minimum Inhibitory Concentration Assay of Methanolic and Ethanolic Extracts of Almond and Bitter Leaves on Isolated Fish Pathogens

The result of the experiment showed that minimum inhibitory concentration of 1000µg/ml of methanolic and ethanolic extract of almond leaves and bitter leaves was the minimum concentration that prevents the growth of bacteria after 24 hours of incubation. This is shown in Tables 4A & B.

Table 4A: The minimum inhibitory concentration assay of methanolic and ethanolic extract of almond leaves on isolated fish pathogen.

Pathogens	Minimum inhibitory concentration in µg/ml																			
	Methanol										Ethanol									
	2000.0	1000.0	500.0	250.0	125.0	62.5	31.25	15.63	7.81	3.96	2000.0	1000.0	500.0	250.0	125.0	62.5	31.25	15.63	7.81	3.96
<i>Staphylococcus aureus</i>	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
<i>Staphylococcus albus</i>	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+

<i>Streptococcus iniae</i>	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Aeromonas hydrophila</i>	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
<i>Bacillus subtilis</i>	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Escherichia coli</i>	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Salmonella typhi</i>	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Keys: + =Presence of growth or medium turbidity, - =No growth or turbidity observed.

Table 4B: Minimum inhibitory concentration assay of methanolic and ethanolic extracts of bitter leaves on isolated fish pathogen.

Minimum inhibitory concentration in µg/ml																				
Pathogens	Methanol										Ethanol									
	2000.0	1000.0	500.0	250.0	125.0	62.5	31.25	15.63	7.81	3.96	2000.0	1000.0	500.0	250.0	125.0	62.5	31.25	15.63	7.81	3.96
<i>Staphylococcus aureus</i>	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
<i>Staphylococcus albus</i>	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
<i>Streptococcus iniae</i>	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
<i>Aeromonas hydrophila</i>	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Bacillus subtilis</i>	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
<i>Escherichia coli</i>	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
<i>Salmonella typhi</i>	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Keys: + =Presence of growth or medium turbidity - =No growth or turbidity observed.

Discussion

Phytochemical screening of almond leaves and bitter leaves for metabolites showed the presence of tannins, saponins, flavonoids, phenol, protein and amino acids while glucosinolates, triterpenes and steroids were not detected in almond leaves but present in methanolic and ethanolic extract of bitter leaves. This result agrees with the report of Udochukwu U, et al., Okokon JE, et al. and Igile GO, et al. who reported the presence of these metabolites [22-24].

Methanolic and ethanolic extracts of almond and bitter leaves showed antibacterial activities against the pathogens tested except *S. aureus* and *S. albus* that did not show a diameter of inhibition zone in ethanolic extracts of almond leaves. Also, *S. iniae*, *S. albus*, *A. hydrophila*, *B. subtilis* and *S. typhi* were not present in the methanolic

and ethanolic extracts of bitter leaves. This correlates with Kigigah LT, et al., Akharaiyi FC, et al. and Nair R, et al. [25-27]. Almond leaves extracts had highest zone of inhibition compared to bitter leaves extracts. This revealed that almond leaves extract had better antimicrobial potential when compared with bitter leaves. The inhibitory effect on bacteria might be due to the active compound present in almond leaves. This result agrees with the report of Olusola SE, et al., Nargis A, et al. and Turker AU, et al. [28-30] who stated that plant extracts can be used as antimicrobials. The results were compared with the standard antimicrobics Chloramphenicol at 10mg/ml and 30mg/ml, the results revealed that Chloramphenicol at 30mg/ml had better diameter of zone inhibition when compared with ethanolic and methanolic extracts of almond and bitter leaves.

The organs in a fish have varying number of microorganisms present. Total viable counts of the organs were always higher than the enterobacteriaceae counts. Gills was presented to have the highest total viable counts of ($7.19 \pm 0.88 \log_{10}$ CFU/g) among all the organs followed by the skin total viable counts of ($6.92 \pm 0.84 \log_{10}$ CFU/g) due to their exposure to the aquatic environment, with the liver presenting the least value of total viable counts ($6.26 \pm 1.22 \log_{10}$ CFU/g). This result supports the report of Bello OS, et al. and Shalaby AM, et al. [18,19].

The result of the experiment showed that 1000 µg/ml of methanolic and ethanolic extracts of almond leaves and bitter leaves was the minimum concentration that prevents the growth of bacteria after 24 hours incubation. This result supports the report of Olusola SE, et al. and Owolabi MS, et al. [28,31].

Conclusion

This result revealed that almond leaves and bitter leaves had antimicrobial properties. However, the methanolic extracts of almond leaves had higher antimicrobial property when compared with the bitter leaves. Also, the minimum inhibitory concentration of 1000 µg/ml were recorded in methanolic and ethanolic extracts of almond and bitter leaves at 24 hours old incubation. This study justify the use of almond and bitter leaves as therapeutic and antimicrobial agents to treat fish pathogenic diseases that have developed resistance to existing synthetic antimicrobial agents.

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