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Antimicrobial Activity of Leaf Extracts of Neem (*Azadrichta indica*) and Tumeric (*Curcuma longa*) Rhizome Against Some Pathogens Isolated from *Clarias gariepinus*

S.E. Olusola [1]*, S. Fakoya [2], and I.B. Omage [2]

[1] Department of Biological Sciences (Fisheries and Aquaculture Programme), Ondo State University of Science and Technology, Okitipupa, Nigeria
[2] Department of Biological Sciences (Microbiology Programme), Ondo State University of Science and Technology, Okitipupa, Nigeria

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Abstract: The antimicrobial activities and diameter of inhibition of aqueous, ethanolic and methanolic extracts of Neem leaves (NL) and Turmeric rhizome (TR) were evaluated against four strains of bacteria isolates from *Clarias gariepinus* using agar well diffusion method. Minimum Inhibitory Concentration (MIC) and phytochemical screenings of NL and TR were determined using standard methods. The NL and TR of aqueous, ethanolic and methanolic extracts had inhibition zones of 20±0.02 mm 10±0.01 mm, 25±0.02 mm 20±0.01 mm and 19±0.02 mm 20±0.02 mm diameter respectively against *Bacillus subtilis*; 25±0.03 mm 25±0.01 mm, 22±0.02 mm 25±0.03 mm and 25±0.03 mm 23±0.01 mm against *Staphylococcus aureus*; 25±0.03 mm 25±0.02 mm, 15±0.01 mm 25±0.02 mm and 15±0.03 mm 20±0.01 mm against *Streptococcus iniae*; 26±0.02 mm 25±0.01 mm, 17±0.01 mm 15±0.02 mm and 10±0.02 mm 10±0.02 mm diameter against *Aeromonas hydrophila*; 04±0.01 mm 0.3±0.02 mm, 05±0.02 mm 0.5±0.02 mm and 08±0.02 mm 02±0.03 mm diameter against *Aspergillus niger*. The MIC of NL and TR of these extracts on the bacteria tested were 1000 µg/ml. The phytochemical screening of these plants indicated the presence of saponins, tannins, flavonoids and polysterols. The plants may be used as promising source of pharmaceutical agents against fish pathogens in the organic aquaculture.

Key words: Antibacterial; phytochemical; neem leaves; turmeric; fish pathogens.

Introduction

The main goals of aquaculture industry are to optimize growth and to produce high - quality fish [6]. The outbreak of diseases in fish farming is a major obstacle worldwide and this brought economic loss to the industry [6]. The large -scale settings of aquatic animal husbandry have resulted in an increased antibiotics resistance in bacteria potentially pathogenic to fish and related environment. Continuous use of antibiotics in aquaculture need to be reduced and replaced with alternative processes for treating fish diseases to avoid the emergence of antibiotic resistance in pathogenic and environmental bacteria [10]. Disease management should therefore concentrate on environmental - friendly, preventative methods such as the use of medicinal plants / immunostimulants. Medicinal plants are rich source of antimicrobial agents [17]. An antimicrobial is a compound that kills or inhibits the growth of microbes such as bacterial, fungal, viral etc such a compound is said to have antibacterial activity [15]. Medicinal plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids which

*Corresponding author (S.E. Olusola)
E-mail: < belloolus@yahoo.com, se.olusola@osustech.edu.ng >

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have antimicrobial properties [22]. As antibiotics, plant extracts could control and limit the growth and colonization of numerous pathogenic and non- pathogenic species of bacteria in the fish [8].

Studies concerning antimicrobial properties of herbal extracts against bacteria with fish such as *Clarias gariepinus* which is an economically important cultured fish species in Nigeria with culture importance *in vitro* and *in vivo* are still limited. Hence, this study was attempted to evaluate the antimicrobial activity of neem leaves and turmeric rhizome against the fish pathogens.

Materials and methods

Plant collection

The neem leaves and turmeric rhizome were used in the study and these plants were obtained in Igodan Lisa, Okitipupa, Ondo State and were identified by a botanist, Dr D. O. Aworinde in the Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa.

Preparation and extraction of plant materials turmeric rhizome extraction

The turmeric rhizome were washed with clean sterile distilled water and allowed to air dry at ambient temperature (25°C) for 1hour. The air dry water coat/covering were neatly peeled off, washed and extracted as described by Azu *et al*.,[5]. One hundred gramme (100 g) of the fresh turmeric rhizomes were blended into paste and soaked in 150 ml of water for 72 hours, 150 ml ethanol and 150 ml methanol for 48 hours respectively. The pulp obtained was left in a clean, sterile glass container and shaken vigorously to allow for proper extraction and it was filtered using a sterile muslin cloth after which the extract was obtained, air-dried and store (4°C) until required.

Neem leaf extraction

The extraction of neem leaves was done as described by Ajaiyeoba *et al*.,[3]. The air-dried neem leaf was grounded with a hammer mill and 100 g of the fine powder of the plant leaf was soaked in 150 ml of water, 150 ml ethanol and 150 ml methanol for 72 hours and 48 hours respectively. The plant leaves were properly mixed with water, ethanol and methanol, filtered using a sterile muslin cloth after which the extraction was obtained, air-dried and store at 25°C until required.

Media preparation

All media 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 test organisms

The microorganisms isolated from *Clarias gariepinus* juveniles were *Aeromonas hydrophila* (-ve), *Streptococcus sp* (+ve), *Bacillus substilis* (+ve) and *Staphylococus aureus* (+ve). The isolation and characterization of bacteria using bio-chemical test was carried out at Microbiology Laboratory, Faculty of Science, University of Ibadan, Nigeria. *Aspergillus flavus* was collected from the stock of the Department of Microbiology, 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), pre-served in the refrigerator at 4°C until it is required for study.

Isolation of microoganism/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 [21]. Serial dilution was carried out and 1ml each from 10^{-1} to 10^{-6} 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. Total viable counts and entero-bacteriacea counts were determined, the result were expressed in Log_{10}CFU/g.

Antimicrobial assay

A well diffusion assay as described by Bello *et al*.,[7] 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 0.1 ml of these plants extract was placed into each well [7]. Distilled water was used as negative control while antibiotics, chloramphenicol (10 mg and 20 mg) 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 neem leaves and turmeric rhizome [16].

Minimum inhibitory concentration

Double dilution of 2000 µg/ml of neem leaves and turmeric rhizome extract were made in 2 ml volume of broth to 15.63 µg/ml. One row of the test was inoculated with 0.02 ml of 1 in 100 dilution of the overnight broth culture of the organism [25]. 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

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.

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 ($Fecl_2$) 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

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, 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 and Oyedapo method [2]. Concentrated 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

The Salkowski test: Extract of 1 g 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 (H_2SO_4) and shaken. The red colour produced within a few minutes indicated a positive reaction.

Detection of proteins and amino acids

Xanthoproteic test

The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Results

Determination of phytochemical in neem leaves and turmeric rhizome

The result of the phytochemical screening revealed the presence of saponins, tannins, glucosinolate, polysterol, flavonoids and protein. Protein in neem leaves and turmeric rhizome were present in abundant quantity (+++) while other showed moderate (++) and low quantity (+). Also, phenol were absent in both plants as shown in table 1.

Detection of antimicrobial activities of aqueous, ethanolic and methanolic extracts of neem leaves and turmeric rhizome

The results showed that aqueous, ethanolic and methanolic extracts of the neem leaves and turmeric rhizome exhibited antimicrobial pro-

Table 1. Determination of some important phytochemicals of neem leaves and turmeric rhizome

Parameters	Neem leaves	Turmeric rhizome
Saponins	++	+
Tannins	+	++
Flavonoids	+	++
Glucosinolates	+	+
Phenol	-	-
Proteins	+++	+++
Polysterols	+	+

perties against all pathogens tested. Although, the highest antimicrobial properties was observed in neem leaves extracts. Negative control (distilled water) did not show antibacterial activities against the pathogens while antibacterial properties were recorded in positive control (Chloramphenicol) at 10mg/ml and 20mg/ml except *A. hydrophila* (table 3).

Detection of minimum inhibitory concentration (MIC) of neem leaves and turmeric rhizome

The MIC of aqueous, ethanolic and methanolic extracts of neem leaves and turmeric rhizome against 4 pathogenic bacterial isolated from *Clarias gariepinus* were investigated in the study and the result showed 1000 µg/ml, 2000 µg/ml of aqueous, ethanol and methanol extracts of neem leaves and turmeric rhizomes respectively against the tested pathogens except *S. iniae* in ethanol and aqueous extracts of neem leaves and turmeric rhizome respectively (table 2A-C)

Detection in microbial load in *Clarias gariepinus*

The microbial load of fish tissue (skin, gills, intestine and liver) were determined and the results show that the highest enterobacteriacea counts was recorded in skin and least in liver while no enterobacteriacea counts was recorded in control. Also, the highest total viable counts was recorded in skin and least in liver while no total viable counts was recorded in the control as shown in Table 4.

Table 2A. Minimum inhibitory concentration of methanolic extracts of neem leaves and turmeric rhizome

		Minimum inhibitory concentration in µg/ml							
Parameter	**Isolates**	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.63**
	Control (without isolates)	-	-	-	-	-	-	-	-
Neem	*Staphylococcus aureus*	-	-	+	+	+	+	+	+
leaves	*Streptoloccus iniae*	-	+	+	+	+	+	+	+
	Aeromonas hydrophila	-	-	+	+	+	+	+	+
	Bacillus subtilis	-	-	+	+	+	+	+	+
	Control (without isolates)	-	-	-	-	-	-	-	-
Turmeric	*Staphylococcus aureus*	-	+	+	+	+	+	+	+
rhizome	*Streptoloccus iniae*	-	-	+	+	+	+	+	+
	Aeromonas hydrophila	-	+	+	+	+	+	+	+
	Bacillus subtilis	-	+	+	+	+	+	+	+

Table 3. Detection of antimicrobial activities (diameter of inhibition zone, mm) of aqueous, methanolic and ethanolic extracts of neem leaves and turmeric rhizome

Pathogen	Diameter of inhibition zone, mm								
	Aqueous		Methanol		Ethanol		Chloramphenicol (10 mg/ml)	Chloramphenicol (20 mg/ml)	Control
	Neem leaves	Turmeric rhizome	Neem leaves	Turmeric rhizome	Neem leaves	Turmeric rhizome			
Bacillus subtilis	20±0.02	10±0.01	19±0.02	20±0.02	25±0.02	20±0.01	20±0.02	25±0.02	-
Staphylococcus aureus	25±0.03	25±0.01	25±0.03	23±0.03	22±0.02	25±0.03	18±0.02	23±0.01	-
Streptoloccus iniae	25±0.03	25±0.02	15±0.03	20±0.01	15±0.02	25±0.02	16±0.02	22±0.01	-
Aeromonas hydrophila	26±0.02	25±0.01	10±0.02	10±0.03	17±0.01	15±0.02	-	-	-
Aspergillus niger	04±0.01	03±0.02	08±0.02	02±0.00	05±0.02	05±0.02	ND	ND	-

- = No inhibition
ND = Not determined

Discussion

Medicinal plants having antimicrobial compounds in comparison with antibiotics, usually with no side effects, better patient tolerance, relative less expensive and acceptable due to long history of use and being renewable in nature [23]. The results of the study, revealed the presence of some phytochemical such as saponin, tannins, flavonoids, glucosides, polysterols and protein. This report was in agreement with Hariffa and Shanthi [14], Akpuaka *et al*.,[4], Natarajan *et al*., [18], Biswas *et al*.,[9], El-Manhmood *et al*.,[13] who reported the presence of flavonoids, Saponins, alkaloids ,tannins, steroids and phenolic compound which are associated with antimicrobial and curative property against pathogens.

Antibiotic sensitivity test of each pathogenic species was performed under *in- vitro* to determine the diameter of zone of inhibition. The result of the study revealed that aqueous, ethanol and methanol extracts of neem leaves and turmeric (rhizome) showed antimicrobial activity and inhibited the growth of bacterial strains, *P. aeruginosa, B. subtilis, E. coli, S. iniae* which are in accord with the report of Bello *et al*.,[7], Yadah and Khan [27]; Chandrama *et al*.,[11] and Negi *et al*., [19]. Aqueous extracts of neem leaves and turmeric rhizome showed very high potential as antimicrobial agents. The result of the study revealed that the leaves extracts of neem showed an interesting inhibitory effects on all the tested pathogenic bacterial and it was proved that the antimicrobial properties of extracts from neem leaves either *in vitro* or *in vivo* react to the presence of several antimicrobial active ingredient in the leaves tree such desactylimbin, quercetin and sitosterol [24]. Also, the antimicrobial property of turmeric has been attributed to the presence of essential oil, an alkaloids, Curcumin and other curcuminoids, turmeric oil ,tumerol and veleric acid [12,26].

The minimum inhibiting concentration of neem leaves and turmeric rhizome revealed that 2000 µg/ml was the least concentration that revealed the growth of bacteria after 24 hours incubation. *Streptococcus iniae* had 250 µg/ml and 500 µg/ml for ethanolic extracts of neem leaves and aqueous extracts of turmeric rhizome respectively.

Table 2B. Minimum inhibitory concentration of ethanolic extracts of neem leaves and turmeric rhizome

Parameter	Isolates	Minimum inhibitory concentration in µg/ml 2000	1000	500	250	125	62.5	31.3	15.63
Neem leaves	Control (without isolates)	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	-	+	+	+	+	+	+
	Streptoloccus iniae	-	-	-	-	+	+	+	+
	Aeromonas hydrophila	-	+	+	+	+	+	+	+
	Bacillus subtilis	-	-	+	+	+	+	+	+
Turmeric	Control (without isolates)	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	+	+	+	+	+	+	+
	Streptoloccus iniae	-	+	+	+	+	+	+	+
	Aeromonas hydrophila	-	+	+	+	+	+	+	+
	Bacillus subtilis	-	+	+	+	+	+	+	+

Table 2C. Minimum inhibitory concentration of aqueous extracts of neem leaves and turmeric rhizome

Parameter	Isolates	Minimum inhibitory concentration in µg/ml 2000	1000	500	250	125	62.5	31.3	15.63
Neem leaves	Control (without isolates)	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	-	+	+	+	+	+	+
	Streptoloccus iniae	-	+	+	+	+	+	+	+
	Aeromonas hydrophila	-	-	+	+	+	+	+	+
	Bacillus subtilis	-	-	+	+	+	+	+	+
Turmeric	Control (without isolates)	-	-	-	-	-	-	-	-
	Staphylococcus aureus	-	-	+	+	+	+	+	+
	Streptoloccus iniae	-	-	+	+	+	+	+	+
	Aeromonas hydrophila	-	-	-	+	+	+	+	+
	Bacillus subtilis	-	-	+	+	+	+	+	+

Table 4. Microbial load of skin, gills, intestine and liver of *C. gariepinus*

Fish site	Organism	Microbial Load (log_{10}Cfu/g)
Liver	Enterobactericea counts	1.74±0.01
	Total viable counts	2.25±0.02
Intestine	Enterobactericea counts	2.33±0.02
	Total viable counts	2.48±0.03
Skin	Enterobactericea counts	2.82±0.01
	Total viable counts	2.96±0.06
Gills	Enterobactericea counts	2.45±0.04
	Total viable counts	2.56±0.01
Control	Enterobactericea counts	-
	Total viable counts	

Abalaka *et al*.,[1] and El-Mahmood *et al*.,[13] reported that the extracts obtained from *Azadirachta indica* added inhibitory effects on *B. subtilis, S. iniae, P. aeruginosa* and *E. coli* which were similar to our findings.

The result of this work revealed that microbial loads in the liver, intestine, skin and gill on *Clarias gariepinus* varies with the skin and gill have the highest values of enterobacteriacea and total viable counts. This results were similar to the observation made by Bello *et al*., [7] and Shalaby *et al*., [21] that bacterial load is greater on the skin and gill than any part of fish as these parts are ones constantly exposed to challenges.

Conclusion

The effectiveness of aqueous, ethanolic and methanolic extracts of neem leaves and turmeric rhizome were found to be varies from species to species. Neem leaves and turmeric rhizome effectively inhibited isolated fish pathogenic bacterial provides the aquaculturist with a promising management tool for treatment of fish pathogens.

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