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THERAPEUTIC EFFECTS OF MANGO (*Mangifera indica*) AND CASHEW (*Anacardium occidentale*) LEAVES EXTRACTS AGAINST CERTAIN PATHOGENIC BACTERIAL STRAINS FROM *Clarias gariepinus*

[1]Olusola, S. E, [2]Olorunsola, R. A. and [3]Omileye, O. E.**

[1]Department of Biological Sciences (Fisheries and Aquaculture Programme), Ondo State University of Science and Technology, Okitipupa. [2]Department of Biological Sciences (Zoology Programme), Ondo State University of Science and Technology, Okitipupa. [3]Department of Biological Sciences (Microbiology Programme), Ondo State University of Science and Technology, Okitipupa. **Corresponding author's e-mail: se.olusola@osustech.edu.ng; belloolus@yahoo.com, Tel.: +2348034110139, +2348051026979

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ABSTRACT

The study was carried out to evaluate the potential therapeutic effects of mango (*Mangifera indica*) and cashew (*Anacardium occidentale*) leaves extracts against nine fish pathogens: *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus substillis, Samonella typhi, Staphylococcus epidermis, Streptococcus iniae, Aeromonas hydrophila* and *Aspergillus niger* using pour plate method. Phytochemical screening and minimum inhibitory concentration of ethanolic and methanolic extracts of mango and cashew leaves were determined using standard methods. Data were analyzed using ANOVA at P = 0.05. The results of antimicrobial properties revealed that both plants extracts showed antimicrobial properties. However, ethanolic and methanolic extracts of mango leaves exhibited the highest antimicrobial activities against all the pathogens investigated. No antimicrobial properties were recorded in the negative control (distilled water) and no antimicrobial activities were recorded for *Streptococcus iniae* and *Aspergillus niger* in erythromycin (50 mg/ml and 100 mg/ml) which serve as the positive control. The phytochemical screening for metabolites indicated the presence of saponins, alkaloids, tannins, glycosides, phenols and protein while flavonoids and steroids were not detected in both plants. The minimum inhibitory concentration of methanolic extracts of mango and cashew leaves is 1000 μg/ml and minimum inhibitory concentration of ethanolic extracts of mango and cashew leaves is 500 μg/ml. The results indicate the possibility of using mango and cashew leaves extracts in the treatment of microbial infections in fishes.

Keywords: Fish diseases, *Mangifera indica, Anacardium occidentale*, Antimicrobial, Phytochemical screening

INTRODUCTION

Fish are abundant in most bodies of water. They are found in nearly all aquatic environments, from high mountain streams to the abyssal and even hadal depths of the deepest oceans with 33,100 described species, fish exhibit greater diversity than any other group of vertebrates (Gupta *et al*., 2008). Bacterial disease is responsible for heavy mortalities in both culture and wild fishes throughout the world and most of the causative microorganisms are naturally occurring opportunist pathogens which invade the tissue of a fish and render them susceptible to infection (Rahman *et al*., 2009).

Continuous use of chemical antimicrobial has been the inducing factor for appearance of more and more microbial strains resistant to classic antimicrobial agents (Kiessling *et al*., 2002). Increasing use of chemical antimicrobial has created a situation leading to an ecological imbalance and enrichment of multiple multi-resistant pathogenic microorganisms. Current disease management tends to concentrate on environmental-friendly, preventive methods such as the use of natural products that have antimicrobial and immunodulatory properties.

Herbal medicine is a common element in Ayurvedic, Homeopathic and Naturopathic treatments. Herbs or herbal products also have a role in aquaculture at present time (Direkbusarakom, 2000). At the moment, nearly 30% or more of the modern pharmacological drugs are derived directly or indirectly from plants. The commercial value of various innumerable drugs and pharmaceuticals derived from tropical forest systems on worldwide basis is projected at 20 billion dollars a year (Sharif and Banik, 2006).

Mangifera indica is a large evergreen tree, 10-45 m high with a strong trunk and heavy crown. Native from tropical Asia, it is sufficiently warm and damp and is now completely naturalized in many parts of tropics and sub-tropics (Ross, 1999). *Mangifera indica* leaves were reported to possess antibacterial activity (Doughari and Manzara, 2008), anti-ulcerogenic action (Severi *et al*., 2009), hypoglycaemic activity (Aderibigbe *et al*., 2001) and atherogenicity (Muruganandan *et al*., 2005). Seed kernels possess anti-diarrhoeal activity (Sairam, 2003), effectiveness for dyslipidemia (Anila and Vijayalakshmi, 2002). Bark and stem possess immunomodulatory (Makare *et al*., 2001), anti-inflammatory and neuroprotective activity (Lemus–Molina *et al*., 2009).

Also, the cashew tree (*A. occidentale*) is a tropical evergreen tree that produces the cashew seed. The cashew tree is large and evergreen, growing to 10–12 m (33–39 ft) tall, with a short, often irregularly shaped trunk. The leaves of the tree possess medicinal benefits and have been used as remedy for diarrhea, reduce high blood sugar and blood pressure levels. It also possesses antibacterial and anti-inflammatory properties (Agedah *et al*., 2010).

The *A. occidentale* and *M. indica* leaves are phytotherapic plants used in folk medicine that is believed to have active components that help to treat and manage various diseases. The many parts of *A. occidentale* and *M. indica* have been used in traditional medicine to manage various diseases but there is a dearth of information about its uses in fish farming. The aim of this study was to evaluate the effectiveness of various extracts of *A. occidentale* and *M. indica* leaves, as antimicrobial agents against some fish pathogens.

MATERIALS AND METHODS

Plant Collection and Identification

Mangifera indica and *A. occidentale* leaves were collected from Igbodigo-Igbokoda, Okitipupa Local Government, Ondo State. These plants were identified at the herbarium unit of Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa, where a voucher specimen was kept for future reference.

Preparation and Extraction of Plant Materials

The *M. indica* and *A. occidentale* leaves samples were washed in tap water, air dried, and ground into powder. The extraction of *M. indica* and *A. occidentale* leaves were done as described by Olusola *et al*., (2017). The air–dried extracts of *M. indica* and *A. occidentale* leaves were kept in a separate container and stored at 25 °C until required.

Media Preparation

Media such as nutrient broth (Oxoid), nutrient agar (Biolife), potato dextrose agar (PDA) (Oxoid) and Mueller-Hinton agar (HiMedia) used were prepared according to manufacturer's instruction. All these media were allowed to cool after sterilization to about 45 °C before pouring into petri dishes.

Microorganism Isolation and Counts

Escherichia coli, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus substillis*, *Streptococcus iniae*, *Aeromonas hydrophila* and *Salmonella typhi* were isolated from *Clarias gariepinus*. Characterization and biochemical test were done in the Department of Biological Sciences (Microbiology Laboratory), Ondo State University of Science and Technology, Okitipupa. *Staphylococcus epidermis* and *Aspergillus niger* were collected from the laboratory stock of the Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa. The pure cultures were sub-cultured into nutrient broth and slants were preserved in the refrigerator at 4 °C until required for the study.

The gills, skin, intestine and liver samples of *Clarias gariepinus* obtained from Teaching and Research Farm, Ondo State University of Science and Technology, Okitipupa were separately homogenized using a sterilized mortar and pestle and were placed into each sterile clapped test tube containing 9 ml of sterilized distilled water and homogenized (Shalaby *et al*., 2006). Serial dilution was carried out and 1 ml each from 10^{-4} to 10^{-5} dilutions were dispersed into Petri dishes that were appropriately labeled and molten sterilized medium (allowed to cool after sterilization to

about 45 °C) was poured aseptically into them. The plates were swirled gently for even distribution of inoculum 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 enterobacteriacea counts were determined and the results were expressed in $\log_{10}$CFU/g.

Antimicrobial Assay

The pour plate method (Perez *et al*., 1990; Bello *et al*., 2013) was employed for the determination of antimicrobial activity, in which the wells served as a reservoir of the sample dilutions and the standard dilutions. Pre- poured indicator [pathogen (4 mm depth)] was overlaid with a 10 ml soft agar (0.7%) lawn of indicator culture. Wells of 5 mm diameter were cut into these agar plates using cork borer and 0.1 ml of each of the leaf extracts was placed into each well (Bello *et al*., 2013).

Distilled water was used as negative control while antibiotics (Erythromycin) 50 mg/ml and 100 mg/ml were used as positive control. The plates were incubated aerobically at 37 °C for 24 hours. 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 was taken to be proportional to the logarithm of the antimicrobial compound in cashew and mango leaves (Maria *et al*., 1994).

Minimum Inhibitory Concentration (MIC)

The MIC values of extracts of *M. indica* and *A. occidentale* leaves were determined based on a micro broth dilution method in a test tube. 2000 µg/ml of *M. indica* and *A. occidentale* leaves extracts 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 (Bello *et al*., 2013). The test was incubated at 37 °C for 24 hours aerobically. The minimum inhibitory concentration was the lowest concentration that prevented the growth of bacterial after 24 hours incubation (Osoba, 1979; Bello *et al*., 2013).

Detection of Phytochemical in *M. indica* and *A. occidentale* Leaves Extracts

1. **Detection of Saponins**
   Froth Test: Extracts (0.5 ml) 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 (0.5 ml) was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.
2. **Detection of Phenols**
   Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenol.
3. **Tannin**
   Extracts (2 ml) were diluted with distilled water to 10 ml and filtered. Then few drops of ferric chloride reagent were added to 1 ml of the filtrate. The mixture was observed for the formation of blue, blue black, green or green black coloration or precipitate.
4. **Detection of Flavonoids**:
   Alkaline Reagent Test: Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color indicates the presence of flavonoids.
5. **Glucosinolates**:
   Extracts were treated with few drops of chloroform followed by filtration as described by Adeoye and Oyedapo (2004). Concentrated tetraoxosulphate (iv) 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.
6. **Test for Triterpenes and Steroids**:
   The Salkowski Test: Extract (2 ml) was warmed in 5 ml of chloroform solution, and then treated with a small volume of concentrated tetraoxosulphate (iv) acid and shaken. The red colour produced within few minutes indicates a positive reaction.
7. **Detection of Protein and Amino**

acids

Xanthoproteic Test: The residues were treated with few drops of concentrated nitric acid. Formation of yellow colour indicates the presence of protein.

8. **Test for Alkaloids:**
   Extracts (1 ml) were added to 1% of hydrochloric acid on steam bath and then filtered; about 1 ml filtrate was then added to 6 drops of Mayer's reagent. Appearance of cream white precipitate indicated the presence of alkaloids.

Statistical Analysis

The microbial load of fish tissue (skin, gills, intestine and liver) and antibacterial and antifungal activities (diameter of inhibition zone, mm) of *M. indica* and *A. occidentale* leaves extracts against nine tested pathogens resulting from the experiment were subjected to one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences Version 20.0).

RESULTS

Phytochemical Screening of *M. indica* and *A. occidentale* Leaves Extracts

The result of phytochemical screening of *M. indica* and *A. occidentale* leaves extracts using methanolic, ethanolic extracts is shown in table 1, revealing the presence of saponins, phenols, glycosides and tannin in both plant extracts. Flavonoids and steroids were absent in both plants. Alkaloid was present in mango leaves extracts but absent in cashew leaves extracts.

Table 1: Phytochemical Screening of Methanolic and Ethanolic Extracts of *M. indica* and *A. occidentale* Leaves

	Tests/Observation	Methanol Extracts	Ethanol Extracts
Mango Leaves	Alkaloids	+	+
	Flavonoids	-	-
	Saponins	+	++
	Protein	–	–
	Phenol	+	+
	Steroid	–	-
	Glycoside	+++	+++
	Tannin	+	+
Cashew Leaves	Alkaloids	–	–
	Flavonoids	–	–
	Saponins	+	+
	Protein	+	+
	Phenol	+	+
	Steroid	–	–
	Glycoside	+++	+++
	Tannin	++	+

Keys: +++ Strong intensity reaction, ++ Medium intensity reaction, + Weak intensity reaction, – Not present

Antibacterial Activity of Methanolic and Ethanolic Extracts of *M. indica* and *A. occidentale* Leaves

The results of the present study revealed the antibacterial and antifungal activities of methanolic and ethanolic extracts of *M. indica* and *A. occidentale*. The *M. indica* leaves exhibited the highest activities against all the pathogens investigated, however, no antibacterial activity was recorded for *Streptococcus iniae* and *Aspergillus niger* in erythromycin (50 mg/ml and 100 mg/ml) as shown in table 2

Table 2: Antimicrobial Activity (diameter zone of inhibition, mm) of Methanolic and Ethanolic Extracts of *M. indica* and *A. occidentale* Leaves

Pathogens	Diameter Zone of Inhibition (mm)						
	Methanol		Ethanol		Control		
	Mango Leaves	Cashew Leaves	Mango Leaves	Cashew Leaves	Distilled Water	Erythromycin (50 mg/ml)	Erythromycin (100 mg/ml)
Staphlococcus aureus	26±0.02	16±0.01	36±0.03	26±0.04	-	16±0.04	20±0.06
Staphlococcus epidermis	16±0.01	12±0.03	26±0.04	16±0.02	-	18±0.05	22±0.04
Echerischa coli	22±0.03	16±0.05	32±0.03	28±0.04	-	18±0.02	20±0.03
Streptococcus iniae	26±0.03	12±0.07	18±0.02	18±0.05	-	-	-
Pseudomonas aeruginosa	25±0.04	12±0.02	30±0.04	18±0.03	-	28±0.01	32±0.05
Aeromonas hydrophila	28±0.06	26±0.04	32±0.05	26±0.05	-	22±0.02	24±0.04
Bacillus substillis	24±0.02	10±0.06	28±0.07	12±0.07	-	9±0.03	12±0.04
Samonella typhi	24±0.05	12±0.03	28±0.06	14±0.09	-	10±0.05	14±0.07
Aspergillus niger	18±0.04	14±0.02	20±0.05	16±0.03	-	-	-

Key: - No zone of inhibition

Determination of Microbial Load in *Clarias gariepinus*

The results of the present study showed that the skin has the highest total viable and enterobacteriacea counts followed by the gills and the least was observed in the liver while the enterobacteriacea and total viable counts were negative in the control (Table 3).

Table 3: Microbial Load of Skin, Gills, Intestine and Liver of *Clarias gariepinus*.

Fish Organs	Organism	Microbial Load (log_{10}CFU/g)
Skin	Enterobacteriacea counts	7.16 ± 0.46
	Total viable counts	7.21 ± 0.48
Liver	Enterobacteriacea counts	6.84 ± 0.45
	Total viable counts	6.94 ± 0.47
Intestine	Enterobacteriacea counts	6.98 ± 0.78
	Total viable counts	7.02 ± 0.48
Gills	Enterobacteriacea counts	7.13 ± 0.78
	Total viable counts	7.17 ± 0.48
Control	Enterobacteriacea counts	_
	Total viable counts	_

Determination of Minimum Inhibitory Concentration (MIC) of Methanolic and Ethanolic Extracts of *M. indica* and *A. occidentale* Leaves

The minimum inhibitory concentration of the ethanolic and methanolic extracts of *M. indica* and *A. occidentale* leaves against the pathogenic bacteria isolated from fish were examined in the present study and the results showed that minimum inhibitory concentration of methanolic extracts of *M. indica* and *A. occidentale* leaves is 1000 µg/ml and minimum inhibitory concentration of ethanolic extracts of *M. indica* and *A. occidentale* leaves is 500 µg/ml.

Table 4: Minimum Inhibitory Concentration of Methanolic and Ethanolic Extracts of *M. indica* Leaves on Isolated Fish Pathogens

	Minimum Inhibitory Concentration (MIC) in µg/ml																			
	Methanol									**Ethanol**										
Pathogens	2000	1000	500	250	125	62.5	31.25	15.63	7.81	3.96	2000	1000	500	250	125	62.5	31.25	15.63	7.81	3.96
Salmonella typhi	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Streptococcus iniae	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Aeromonas hydrophila	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Staphylococcus aureus	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Staphylococcus epidermidis	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Escherichia coli	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Bacillus substillis	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Table 5: Minimum Inhibitory Concentration of Methanolic and Ethanolic Extracts of *A. occidentale* Leaves on Isolated Fish Pathogens

	Minimum Inhibitory Concentration (MIC) in μg/ml																			
	Methanol										**Ethanol**									
Pathogens	2000	1000	500	250	125	62.5	31.25	15.63	7.81	3.96	2000	1000	500	250	125	62.5	31.25	15.63	7.81	3.96
Salmonella typhi	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Streptococcus iniae	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Aeromonas hydrophilia	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Staphylococcus aureus	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
Staphylococcus epidermidis	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Escherichia coli	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Bacillus substillis	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

DISCUSSION

From this study, phytoconstituents such as saponins, tannins, alkaloids, phenols, steroids, glycosides and proteins were shown to be present in *M. indica* and *A. occidentale* leaves extracts. This result supports the report of Aiyelaagbe and Osamudiamen, (2009) and Cushnie and Lamb, (2011) who reported the presence of alkaloids, phenols, steroids, glycosides saponins and tannins in *M. indica* and *A. occidentale*. Phytoconstituents have been found to inhibit bacteria, fungi, viruses and pests (Marjorie, 1999).

The ethanolic and methanolic extracts of *M. indica* and *A. occidentale* leaves used in this study have antimicrobial activity against the tested strains with different diameters of inhibition zones from one strain to another. All the tested bacteria were sensitive to ethanolic and methanolic extracts of *M. indica* and *A. occidentale* leaves. This agrees with the report of Doughari and Manzara (2008) and Agedah *et al*. (2010) that mango and cashew plants used as spices have significant anti-bacterial activity. *Mangifera indica* leaves extracts showed the highest inhibition zone by well diffusion method compared to *A. occidentale* leaves extracts. It was also noted that ethanolic extracts have greater effect in the inhibition compared to methanolic extracts. The difference in antibacterial activity of plants extracts might be attributed to the age of the plants used, freshness of plant materials, physical factors (temperature, light water), time of harvesting of plant materials and drying method used before the extraction process.

The epithelial surfaces of fish such as skin, gill or gastrointestinal tract are the first contact areas for potential pathogens (Narvaez *et al*., 2010). The result of this work revealed that the microbial counts in the liver, intestine, skin and gill of *Clarias gariepinus* varies with the skin and gills having the highest values of enterobacteriacea and total viable counts. This agrees with report of Bello *et al*. (2013) that bacterial load is greater on the skin and gills than any part of fish as these parts are the ones constantly exposed to challenges.

Turbidity method was used to determine the lowest plant extract concentration that could inhibit the growth of the bacteria for effective evaluation of minimum inhibitory concentration. The results of minimum inhibitory concentration of the ethanolic and methanolic extracts of *M. indica* and *A. occidentale* leaves against nine pathogenic bacteria isolated from fish were examined in the present study and the results showed that minimum inhibitory concentration of methanolic extracts of *M. indica* and *A. occidentale* leaves is 1000 μg/ml and minimum inhibitory concentration of ethanolic extracts of *M. indica* and *A. occidentale* leaves is 500 μg/ml. The present study agrees with Doughari and Manzara, (2008).

CONCLUSION

The application of herbs to prevent and control microbial diseases is an alternative chemotherapeutic treatment. The present study revealed that *M. indica* and *A. occidentale* leaves have antimicrobial properties although the ethanolic extracts of *M. indica* leaves had higher zone of inhibition when compared with *A. occidentale* leaves extracts. The presence of more spectrums of phytochemical might be responsible for their therapeutic effect. The results of this study provide justification for the use of these plants in folk medicine to treat various infectious fish diseases.

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