

The Potential of Different Extraction Methods of Soursop (*Annona muricata* Linn) Leaves as Antimicrobial Agents for Aquatic Animals

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Abstract A cup plate diffusion method was employed to determine the antibacterial and antifungal activities of aqueous, ethanolic and methanolic extracts of soursop (*Annona muricata*) leaves against four clinical strains of bacteria isolates from *Clarias gariepinus* and *Oreochromis niloticus*. They were *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus iniae*, *Aeromonas hydrophila* and *Aspergillus niger*. Phytochemical screening and Minimum Inhibitory Concentration (MIC) of soursop leaves were established through standard methods. Data obtained were subjected to ANOVA at P = 0.05. The diameter of zone of inhibition varies depending on pathogens and method of extraction. An average diameter of zone of inhibition ranges from without inhibition zone in control to 20 ± 0.01 mm, without inhibition zone in control to 21 ± 0.02 mm and without inhibition zone in control 25 ± 0.01 mm for aqueous, ethanolic and methanolic extracts respectively. The extracts displayed higher activities to the Gram positive organisms. Chloramphenicol and distilled water were used as control. Phytochemical screening indicated the presence of tannins, glucosinolates, phenols, amino acids and polysterols and absence of saponins. The result of MIC of soursop leaves extracts on the pathogens investigated was 500 µg/ ml, 500 µg/ ml and 1000 µg/ml for aqueous, ethanolic and methanolic extracts respectively. The results indicated that different methods of extraction of soursop leaves had antimicrobial activity on the pathogens (*B. subtilis*, *S. aureus*, *S. iniae*, *A. hydrophila* and *A. niger*) and suggested that soursop extracts can be used in fish farming to inhibit bacterial growth and improved fish health.

Keywords Soursop leaves; Fish pathogen; Phytochemical; Antimicrobial

1 Background

The use of antibiotics and chemotherapeutics for prophylaxis and treatment in fish farming has been widely criticized for its negative impact (FAO, 2002). Issues associated with the uses of antibiotics in disease management should therefore focus on the uses of natural plant products that possess immuno-modulatory and antimicrobial activities. Immunostimulants are chemical compounds that stimulate the non specific immune system when given alone or the specific immune mechanism when given with an antigen, thereby making the animal more resistant to microbial and parasitic infections (Cuesta et al., 2005).

Therefore, using immunostimulants may act as attractive alternative to prevent infections in fish and improve growth (Secombes, 1994; Raa, 1996). Scientific studies revealed that the inclusion of medicinal plants in the diet of aquatic animals improved the immune system of fish and increased disease resistant properties (Chakrabarti et al., 2012). Some plant extracts were shown to have antimicrobial and immunomodulatory activities such plants include *A. muricata* leaves which appear to have anti-viral activity, anticarcinogenic and genotoxic effects and wound healing activity (Gajalakshmi et al., 2012).

Annona muricata Linn were group to the family of Annonaceae with an extensive pan-tropical distribution and it is known as corosol. It is a prevalent small tree and originated from Central America (Alassane et al., 2004). The seeds and leaves of these species were found to contain more than 50 mono-THF acetogenins which possess much of the diverse biological activities (Galalakshmi et al., 2012). However, there is scanty information of its use in aquaculture or its use in fish farming has not been investigated. The present study was investigated to examine the

possible use of *A. muricata* as potential antimicrobial in the farming of *C. gariepinus*.

2 Results

2.1 Determination of phytochemical in soursop leaves

Phytochemical screening of soursop leaves showed the presence of tannins, flavonoids, glucosinolates, phenol, amino acid and polysterols while saponins was not detected. The metabolites were in moderate quality (++) and small quality (+) as shown in Table 1.

Table 1 Determination of some important phytochemical of soursop leaves

Parameters	Soursop leaves
Saponins	-
Tannins	+
Flavonoids	+
Glucosinolates	+
Phenol	+
Proteins	++
Polysterols	+

Note: ++ = Moderate quantity; + = Small quantity; - = Absent

2.2 Microbial load of *Clarias gariepinus*

The microbial load of fish tissues (skin, live, intestine and gills) were determined and the results showed that highest enterobacteriaceae and total viable counts were recorded in gills and the least in liver while no enterobacteriaceae and total viable counts were recorded in the control as shown in Table 2.

Table 2 Microbial load of *C. gariepinus* (liver, skin, intestine and gills)

Organ	Organism	Microbial load (log ₁₀ CFU/g)
Control	Enterobacteriaceae counts	-
	Total viable counts	-
Liver	Enterobacteriaceae counts	1.60 ± 0.01
	Total viable counts	2.10 ± 0.02
Skin	Enterobacteriaceae counts	2.15 ± 0.02
	Total viable counts	2.54 ± 0.01
Intestine	Enterobacteriaceae counts	2.12 ± 0.02
	Total viable counts	2.36 ± 0.03
Gills	Enterobacteriaceae counts	2.25 ± 0.04
	Total viable counts	2.43 ± 0.06

2.3 Detection of antimicrobial activities of soursop leaves

Aqueous, methanolic and ethanolic extracts of soursop leaves shows antibacterial and antifungal properties in the study against all tested pathogens with methanolic extracts had higher inhibition zone of diameter compared to ethanolic and aqueous extracts while no antimicrobial activity were recorded in the negative control (distilled water). But the positive control (chloramphenicol) at 10 mg/ml and 20 mg/ml recorded varies inhibition of zone of diameters among the pathogens tested except *A. hydrophila* that did not show diameter of zone of inhibition in aqueous, ethanolic and methanolic extracts of soursop leaves (Table 3).

2.4 Determination of minimum inhibitory concentration (mic) of aqueous, ethanolic and methanolic extracts of soursop leaves

The MIC of aqueous, ethanolic and methanolic extracts of soursop leaves against four pathogenic bacteria isolated from *C. gariepinus* and *O. niloticus* were assessed and the results revealed that 500 µg/ml prevented growth against the pathogens tested except *A. hydrophila* and *S. iniae* that had 1000 µg/ml respectively (Table 4).

Table 3 Detection of antimicrobial activities (diameter of inhibition zone, mm) of aqueous, methanolic and ethanolic extracts of soursop leaves

Pathogen	Soursop leaves			Control		
	Aqueous	Methanol	Ethanol	Chloramphenicol (10 mg/ml)	Chloramphenicol (20 mg/ml)	Distilled water
<i>Bacillus subtilis</i>	18±0.03	17±0.03	12±0.03	15±0.01	18±0.04	-
<i>Staphylococcus aureus</i>	15±0.01	25±0.01	21±0.02	13±0.01	20±0.01	-
<i>Streptococcus iniae</i>	09±0.02	17±0.02	15±0.03	21±0.02	25±0.02	-
<i>Aeromonas hydrophila</i>	20±0.03	25±0.03	11±0.03	-	-	-
<i>Aspergillus flavus</i>	03±0.01	06±0.01	02±0.01	ND	ND	-

Note: - = No inhibition; ND = Not determined

Table 4 Minimum inhibitory concentration of aqueous, methanolic and ethanolic extracts of soursop leaves

Method of extraction	Isolates	Minimum inhibitory concentration in µg/ml							
		2000	1000	500	250	125	62.50	31.25	15.63
Aqueous extract	<i>Bacillus subtilis</i>	-	-	-	+	+	+	+	+
	<i>Staphylococcus aureus</i>	-	-	-	+	+	+	+	+
	<i>Streptococcus iniae</i>	-	-	-	+	+	+	+	+
	<i>Aeromonas hydrophila</i>	-	+	+	+	+	+	+	+
	Control	-	-	-	-	-	-	-	-
Ethanolic extract	<i>Bacillus subtilis</i>	-	-	-	+	+	+	+	+
	<i>Staphylococcus aureus</i>	-	-	-	-	+	+	+	+
	<i>Streptococcus iniae</i>	-	-	-	+	+	+	+	+
	<i>Aeromonas hydrophila</i>	-	+	+	+	+	+	+	+
	Control	-	-	-	-	-	-	-	-
Methanolic extract	<i>Bacillus subtilis</i>	-	-	+	+	+	+	+	+
	<i>Staphylococcus aureus</i>	-	-	+	+	+	+	+	+
	<i>Streptococcus iniae</i>	-	+	+	+	+	+	+	+
	<i>Aeromonas hydrophila</i>	-	-	+	+	+	+	+	+
	Control	-	-	-	-	-	-	-	-

Note: + = indicating growth showed by turbidity of the broth; - = no growth

3 Discussion

The phytochemical screening of the present study revealed the presence of tannins, flavonoids, glucosinolates, phenol, amino acid and polysterols while saponins was not detected which agreed with the report of Wisdom et al. (2014) and Anganna and Nursyam (2016) who observed the presence of these metabolites.

The result of this study revealed that microbial loads in the liver, intestine, skin and gills of *C. gariepinus* vary, with the gills having the highest values of enterobacteriaceae and total viable counts. This study were in agreement with the report of Shalaby et al. (2006) that bacterial loads is greater in the skin and gills than in any part of the fish, as these parts are the ones constantly exposed to infections.

Also, the result of the present study showed that aqueous, ethanolic and methanolic extracts of soursop leaves had antimicrobial properties and inhibited the growth of organisms tested. This observation was in accord with the report of Anganna and Nursyam (2016), Oyedeji et al. (2015) and Haro et al. (2014). The methanolic extracts of soursop leaves had significantly higher ($P < 0.05$) inhibition zone of diameter compared to aqueous and ethanolic extracts. Utilization of different types of solvents (aqueous, ethanol and methanol) gives effects to the inhibition of microbial growth resulting in *B. subtilis*, *S. aureus*, *S. iniae*, *A. hydrophila* and *A. flavus*. The inhibitory effects of aqueous, ethanolic and methanolic extracts of soursop leaves can be as a result of the active components of soursop leaves able to enter the cell wall, cell membranes and inhibits cell metabolism. Soursop leaves as antimicrobial agents for fish culture can be an essentially important in reducing the economic losses for fish farmer as a result of the world threatens of infectious diseases.

However, the MIC of the present study revealed that 500 µg/ml was the least concentration that prevented the growth of bacteria after 24 hours incubation except *A. hydrophila* and *S. iniae* who had 1000 µg/ml for aqueous and ethanolic extracts respectively while *S. aureus* recorded 250 µg/ml in ethanolic extracts of soursop leaves. The results were in agreement with the report of Oyedeji et al. (2015), Haro et al. (2014) and Bello et al. (2013).

4 Materials and Methods

4.1 Plant collection

The soursop (*A. muricata*) leaves used in the study was obtained in Teaching and Research Farm, Ondo State University of Science and Technology, Okitipupa and was identified by Dr D.O. Aworinde in the Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa.

4.2 Preparation and extraction of soursop leaves

Soursop leaves was extracted as described by Ajaiyeoba and Fadare (2006). The fresh leaves soursop were air-dried for 2 weeks and grounded with a hammer mill, 100 g of the powder of the plant leaves were immersed in 300 ml of water, ethanol and methanol for 72 hours and 48 hours respectively. The plant leaves were rightly blended with water, ethanol and methanol, filtered using a sterile muslin cloth after which the extracts was acquired, air-dried and store at 25°C until further study.

4.3 Source of test organisms

Microorganisms isolated from *Clarias gariepinus* juveniles were *Aeromonas hydrophila*, *Streptococcus sp.*, *Bacillus subtilis* and *Staphylococcus aureus*. 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 laboratory 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), kept in the refrigerator at 4°C until further study.

4.4 Counting of microorganisms

The tissues of *C. gariepinus* were individually softened and put into sterile bottles containing sterilized distilled water and homogenized (Shalaby et al., 2006). Serial dilution was adopted and 1ml each from 10⁻¹ to 10⁻⁶ dilution factors were dispersed into Petri dishes that were correctly labeled and molten sterilized medium was poured aseptically into Petri dish. The plates were twisted kindly 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 enterobacteriaceae counts were ascertained, the result were expressed in log₁₀CFU/g.

4.5 Antimicrobial assay

A cup plate diffusion assay as described by (Bello et al., 2013) 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 the plant extracts was placed into each well (Bello et al., 2013). Distilled water was used as negative control while antibiotics, chloramphenicol (10 mg/mL and 20 mg/mL) were used as positive control. The Petri dish was assessed for diameter of zones of inhibition which was scored positive, if the width of the clear zone was 10 mm or longer. The diameter of zones of the inhibition was taken to be proportional to the logarithm of the antimicrobial compounds in soursop leaves (Maria et al., 1994).

4.6 Minimum inhibitory concentration

Double dilution of 2000 µg/ml of soursop leaves was 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 (Stokes and Ridgeway, 1980). 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 (Osoba, 1979).

4.7 Phytochemical screening

4.7.1 Detection of saponins

a) Froth test: Extracts of 5 g 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.

4.7.2 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.

4.7.3 Tannins

Extract of 0.1 g was taken up in 10 ml distilled water and filtered. Then a few drops of ferric chloride (FeCl_3) 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.

4.7.4 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.

4.7.5 Glucosinolates

Extract of 0.1 g was dissolved in 5 ml of chloroform followed by filtration as described by Adeoye and Oyedapo (2004) method. Concentrated tetraoxosulphate (vi) 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.

4.7.6 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 (vi) acid (H_2SO_4) and shaken. The red colour produced within a few minutes indicated a positive reaction.

4.7.7 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.

4.8 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 soursop 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).

5 Conclusion

The result of the present study showed that *A. muricata* leaves had antimicrobial properties and it is therefore suggested that aqueous, methanolic and ethanolic extracts of soursop leaves can be used in fish culture as alternative to conventional vaccines, synthetic antibiotics and chemotherapeutic agents. The use of *A. muricata* leaves extracts as natural antimicrobial agents in fish culture system may be environmentally friendly, fully biodegradable, safe and non-toxic (toxin binders), effective against a number of opportunistic pathogens and no resistant problems.

Authors' contributions

This work was carried out by three authors. Authors OSE, FS and OIB designed the study and wrote the protocol, authors OSE and FS supervised the study. Author OIB performed the experiment and the statistical analysis as well as managed the literature searches.

Also, author OSE wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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