

## Invitro Antimicrobial Activities of *Mitracarpus scaber* Against Some Common Bacteria of Aquatic Origin

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### ABSTRACT

Some plants have been reported to be of medicinal values and reserve some antimicrobial properties. One of such plants is *Mitracarpus scaber* and its effect on bacterial growth is evaluated. The study aimed at evaluating the phytochemical analyses and antimicrobial potentials of *Mitracarpus scaber* against aquatic bacteria. Leaves of *Mitracarpus scaber* and phytochemical analysis and antimicrobial investigation of ethanolic and aqueous extracts of the leaves were carried out against bacteria isolated from diseased Catfish from various farms. The phytoconstituents detected include saponins, tannins, flavonoids, terpenoids, steroids, anthraquinones and alkaloids in both aqueous and ethanolic extracts. The ethanolic extract had zones of inhibition similar to that of standard antibiotics (enrofloxacin) across all tested microbes. The lowest minimum inhibitory concentration of ethanolic extract of *M. scaber* was against *Bacillus* sp with 10mg/ml while the highest was 85mg/ml against *Staphylococcus* species. The results of the assays showed promising evidences that *M. scaber* is a potential antibacterial agent against aquatic microbes. However, further studies are recommended to fractionate its constituents and determine the *in vitro* and *in vivo* anti-microbial activities and the exact mechanism of action of the constituents.

**Keywords:** *Mitracarpus scaber*; aquatic bacteria; aqueous extract; ethanolic extract; antimicrobial

### INTRODUCTION

One of the clogs in the wheel of advancement of global aquaculture is disease outbreak, especially of Bacterial origin (Ajadi *et al.*, 2018). Many of these bacteria are normal flora, but under stress or favourable conditions, they multiply, invade

and spread to various tissues and organs (Marian M. Cahill, 1990).

*Aeromonas*, *Vibrio*, *Staphylococcus* and *Bacillus* species are among the common bacteria of aquatic origin. Others include *Pseudomonas*, *Streptococcus*, *Escherichia*, *Edwardsiella* and *Renebacterium* (Austin and Austin, 2012). *Aeromonas hydrophila* is an

opportunistic bacterium and is reportedly ubiquitous in the aquatic environment with high tendency to cause diseases in fresh water species such as African cat fish (Anyanwu *et al.*, 2015). Various species of *Vibrio* that cause vibriosis in fish are among the commonest fish bacterial pathogens that cause severe clinical signs and high mortality rate (Ina-Salwany *et al.*, 2019).

More often than not, the immediate measure taken to stem the situation is the use of antibiotics. This approach, however, when indiscriminately employed, results in antibiotic resistance and drug residue (Chuah *et al.*, 2016). Apart from decreased production, as a result of decreased efficacy of these agents both chemotherapeutically and prophylactically, the widespread administration of these drugs may lead to the development of resistant human pathogens (De La Pena *et al.*, 2009).

The use of phyto-genic agents has been explored as a better and safer alternative to the menace of antimicrobial resistance. Medicinal plants have been incorporated into preventive and control measures against disease outbreaks and profound successes have been reported (Menanteau-Ledouble *et al.*, 2015; Karaskova *et al.*, 2015; Yang *et al.*, 2015). These plants contain chemicals that are categorised as primary or secondary metabolites. The primary metabolites are the common proteins, sugars, amino acids, purines and pyrimidines of nucleic acids and chlorophyll. Secondary metabolites include the remaining /chemicals which are produced from the primary metabolites. These include alkaloids, Phenolics, terpenoids, steroids tannins, and volatile oils (Okwu *et al.*, 2010).

*Mitracarpus scaber* is one of the tropical plants that have high medicinal values and their use in traditional medicine have been widely explored on terrestrial animals (Alli-Emmanuel *et al.*, 2003) as well as in humans (Ekpendu *et al.*, 1994). *Mitracarpus scaber* is a perennial plant, abundant in African soil and is used for the treatment of bacterial, fungal and parasitic infections in terrestrial animals (Sanogo *et al.*, 1996; Cimanga *et al.*, 2004).

Leaves of *M. scaber* have been extensively used in traditional medicine in the tropics for the treatment of headaches, toothaches, skin infections, amenorrhea, dyspepsia, hepatic diseases, venereal diseases and leprosy (Bisignano *et al.*, 2000). The aerial part of *M. scaber* are used in the treatment of skin diseases such as superficial fungal diseases, produced as lotion and skin ointment (Sanogo *et al.*, 1996). The plant contains active compounds that are responsible for the antifungal, anti-inflammatory and anti-parasitic activities (Ekpendu *et al.*, 1994; Cimanga *et al.*, 2004). There is, however, dearth of information on the use of this plant against bacterial infections of aquatic origin.

In this study, the phytochemical analyses and antimicrobial potentials of *M. scaber* against aquatic bacteria including *Aeromonas*, *Bacillus*, *Vibrio* and *Staphylococcus* spp were evaluated.

## MATERIALS AND METHODS

### Plant collection

Leaves of *M. scaber* were collected from premises of Lagos State University staff quarters, Nigeria. They were authenticated at the Department of Plant Biology,

University of Ilorin. A voucher specimen was deposited and voucher number was issued as UILH/002/558. The leaves were washed with clean water, air dried for two weeks at room temperature and pulverized into powdery form for extraction.

### **Aqueous and Ethanoic Extraction**

280g of powdered *M. scaber* was extracted by maceration at room temperature in 140ml each of 98% ethanol and distilled water for 72 hours according to Mungole *et al.* (2010). The extracts were stored at 4°C until needed for use. The percentage yield of extract was calculated (table 2).

### **Phytochemical Screening**

Qualitative phytochemical analysis of each extract was done according to standard established procedures. 1 g of each ethanolic and aqueous extracts of *M. scaber* was dissolved in 99 ml ethanol and water respectively to obtain 1% (w/v) stock concentration. The resultant extracts were subjugated to phytochemical screening according to the previously described method (Krishnaiah *et al.*, 2009). Saponins, tannins, flavonoids, cardiac glycosides, terpenoids, steroids, anthraquinones and alkaloids were screened for.

### **Test for tannins**

0.5 g of powdered sample of *M. scaber* was boiled in 20 ml of distilled water in a test tube and then filtered. 0.1% FeCl<sub>3</sub> was added to the filtered samples and observed for brownish green or a blue black colouration, which showed that the plant contained presence of tannins.

### **Test for saponins**

2 g of powdered samples of *M. scaber* was boiled together with 20 ml of distilled water in a water bath and filtered. 10 ml of the filtered sample was mixed with 5 ml of distilled water in a test tube and shaken vigorously. The product was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which suggested the presence of saponins.

### **Test for flavonoids**

A few drops of 1% NH<sub>3</sub> solution was added to the extract of *M. scaber* in a test tube. A yellow colouration was observed indicating the presence of flavonoid.

### **Test for terpenoids**

5 ml of extract of *M. scaber* was mixed with 2 ml of CHCl<sub>3</sub> in a test tube. 3 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was gently added to the mixture to form a layer. An interface with a reddish brown coloration formed indicated the presence of terpenoids.

### **Test for cardiac glycosides**

5 ml of extract of *M. scaber* was mixed with 2 ml of glacial CH<sub>3</sub>CO<sub>2</sub>H containing a drop of FeCl<sub>3</sub>. The resultant mixture was gently added to the 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> so that the concentrated H<sub>2</sub>SO<sub>4</sub> was underneath the mixture. A brown ring colouration indicated the presence of the cardiac glycoside constituent.

### **Test for anthraquinones**

5 ml of *M. scaber* was mixed with organic solvent and filtered. 2ml of NaOH was added. A pink or violet colour in the base layer indicated the presence of anthraquinones.

### Bacterial Isolation

The bacteria used for the antimicrobial analyses were obtained from samples (gills, kidneys and skin) collected from diseased African cat fish from various farms in Kwara state, Nigeria. The samples were analyzed at the Veterinary Microbiology laboratory, University of Ilorin, Nigeria according to method of Fagbemi *et al.* (2009). Briefly, 1 g of each sample was pre-enriched in 9 ml of buffered peptone water (Oxoid, Hampshire, UK) incubated at 37 °C for 24 hours. Pre-enriched sample (1ml) was then inoculated into 9 ml of nutrient broth (Oxoid, Hampshire, UK) and incubated overnight at 37 °C. The turbid nutrient broth was subsequently inoculated unto 5 % sheep blood agar (Oxoid, Hampshire, UK) incubated aerobically at 37 °C for another 24 hours. The resultant colonies were purified on nutrient agar (Oxoid, Hampshire, UK) and then subjected to Gram staining and biochemical characterization including oxidase, catalase and coagulase tests (Cowan and Steel, 2002). The isolates were kept in 20 % glycerol broth at -20 °C for future use.



**Figure 1.** one of the diseased fish sampled.

### Antimicrobial Screening

The bacteria (*Aeromonas*, *Vibrio*, *Staphylococcus* and *Bacillus*) were sub-cultured from the stock into freshly prepared nutrient agar (Oxoid, Hampshire, UK). 1.0 g of each of aqueous and ethanolic extract of *M. scaber* was dissolved in 10 ml of respective solvent. A concentration of 100mg/ml was obtained to investigate the antimicrobial activities of *M. scaber* extracts (Joshi *et al.*, 2009). Mueller-Hinton agar (Oxoid, Hampshire, UK) was used for the antimicrobial screening according to Hudzicki (2009). The agar was seeded with the test microbes to evenly cover the surface of the prepared plates with the inoculum (0.5 Mac Farland dilution equivalence of approximately  $1.5 \times 10^8$  CFU mL<sup>-1</sup>). The plates were allowed to dry and standard cork borer of 6 mm in diameter was used to bore holes (Fagbohun *et al.*, 2013). Each hole was labelled according to the extract to be dispensed in it. 0.1 mL of each extract was introduced into the respective well with sterile syringe. Enrofloxacin and ethanol were used as controls. The plates were aerobically incubated for 24 hours at 37 °C after which the zones of inhibition of growth were observed and measured using a transparent ruler. The zones of inhibition of the extracts were measured and compared with that of standard antimicrobials CLSI (2012a).

Minimum inhibitory concentration (MIC) of the ethanolic extract of *M. scaber* was determined using broth macrodilution method as previously described (Balouiri *et al.*, 2016). Two-fold dilutions (10, 20, 40, 80 and 160 mg/mL) of the extract was prepared in nutrient broth (Hi-media, New Delhi, India) for each of the isolates. 2 mL of each dilution was dispensed in different test

tubes. Well isolated colonies of each isolate were inoculated into each tube and the turbidity of each tube was adjusted to 0.5 % Mac Farland standard against a nephelometer (Oxoid, Hampshire, UK). The tubes were incubated with minimum agitation at 37 °C for 24 hours. The MIC of each isolate was taken as lowest concentration of the extract that completely inhibits growth of the isolates in tubes as indicated by clear broth when viewed by unaided eye (CLSI 2012b).

Because of the economic importance of *Aeromonas* species in fish production, and its high occurrence and distribution in this study, the need to further characterize them molecularly targeting 16S rDNA sequence for their identification became necessary. However, considering the economic challenge during the study, only two of the isolates were randomly selected and shipped to International Institute for tropical agriculture (IITA) Ibadan, Nigeria, for molecular identification.

DNA extraction was carried out according to method described by Ahmed *et al.* (2017) and PCR assay using 16S rDNA primers (16SR:

GTGCCAGCAGCCGCGCTAA, 16SR: AGACCCGGGAACGTATTAC) was done according to protocol described by Wagner *et al.* (2008). Partial genome sequencing of the amplified product was done at Bioscience laboratory of IITA, Ibadan. The obtained 16S rDNA sequences was compared with sequences available in GenBank database of National Center for Biotechnology Information (NCBI) using the algorithm BLAST program (Wagner *et al.*, 2008; Thomas *et al.*, 2014). MEGA version X was used to conduct phylogenetic analyses of the isolate and those obtained from NCBI (Wagner *et al.* 2008). Consensus sequence from the test isolate was generated using Bioedit and sequences of eight *Aeromonas* and seven *Pseudomonas* (outgroup) strains used in the phylogenetic tree were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>) and a phylogenetic tree was constructed. The tree was constructed using the maximum likelihood program with the Tamura-Nei model and Boot strapping 1000 times (Wang *et al.*, 2014).

## RESULTS

The phytochemical screening revealed the presence of high concentration of steroids and terpenoids in ethanolic extract while anthraquinones and saponins were much concentrated in aqueous extract (Table 1). The percentage yield of ethanolic and aqueous extracts were 3.6% and 2.3% respectively.

**Table 1.** Phytochemical screening (qualitative) for the ethanol aqueous extracts of *M. scaber*

Test	Ethanol	Aqueous
<b>Saponins</b>	+ve	++ve
<b>Tannins</b>	+ve	+ve
<b>Flavonoids</b>	+ve	+ve

<b>Cardiac glycosides</b>	-ve	-ve
<b>Terpenoids</b>	++ve	+ve
<b>Steroids</b>	++ve	+ve
<b>Anthraquinones</b>	+ve	++ve
<b>Alkaloids</b>	+ve	+ve

+ve: Present; -ve: Absent; ++ve: abundantly present

**Table 2.** Quantitative Phytochemical Determination

$$\text{Percentage(\%)} \text{ flavonoid content (w/w)} = \frac{\text{Weight of flavonoid extract gotten}}{\text{Weight of plant material used}} \times 100$$

**Table 2.1.** Quantitative *Flavonoid* Determination

Sample	Amount of sample used	Empty bottle weight	Bottle + Extract	Yield	Yield %
<i>Ethanol</i>	0.5g	62.995g	63.153g	0.158g	31.6%
<i>Aqueous</i>	0.5g	63.808g	64.009g	0.201g	40.2%

**Table 2.2.** Quantitative *Alkaloid* Determination

Sample	Amount of sample used	Empty bottle weight	Bottle + extract	Yield	Yield %
<i>Ethanol</i>	0.5g	0.594g	0.615g	0.021g	4.2%
<i>Aqueous</i>	0.5g	0.554g	0.588g	0.034g	6.8%

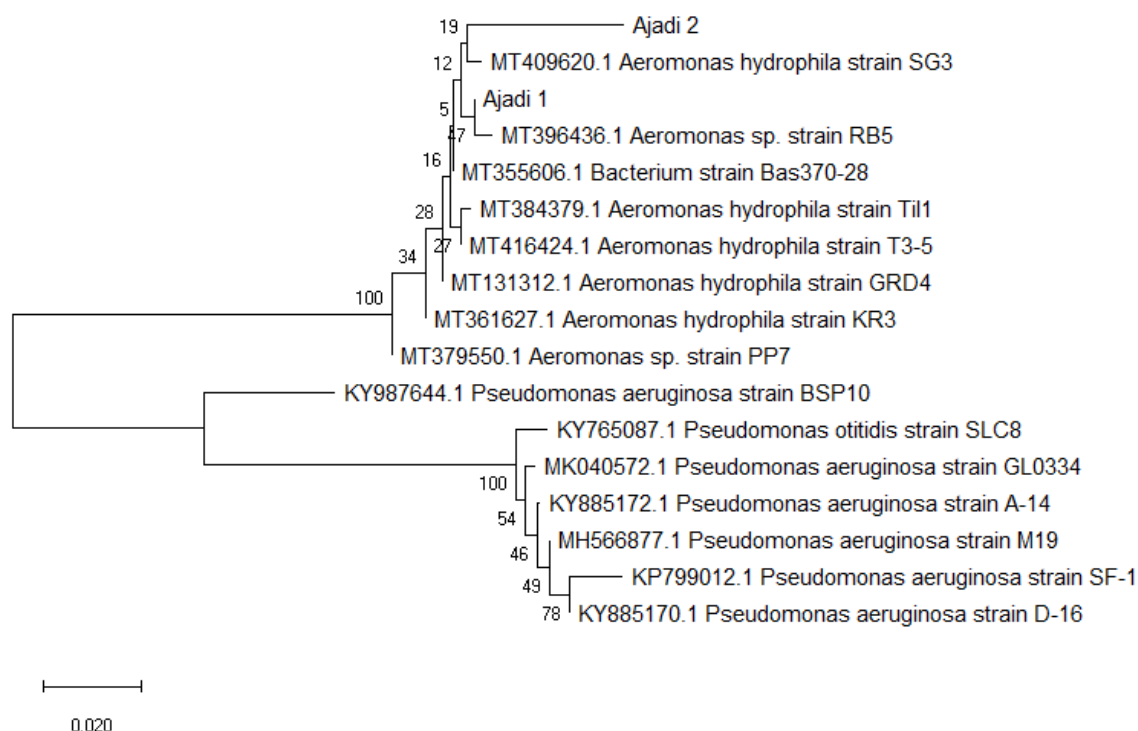
**Table 2.3.** Quantitative *Saponin* Determination

Sample	Amount of sample used	Empty bottle weight	Bottle + extract	Yield	Yield %
<i>Ethanol</i>	0.5g	70.021g	70.022g	0.001g	0.2%
<i>Aqueous</i>	0.5g	62.553g	62.569g	0.016g	3.2%

Table 2 shows percentage yield of the constituents, table 2.1 – 2.3 (flavonoid, alkaloid and saponin respectively). The bacteria isolated from diseased African catfish from various farms within Kwara state, Nigeria were *Aeromonas*, *Vibrio*, *Staphylococcus* and *Bacillus* (Table 3) *Aeromonas* spp was the most isolated bacteria present in all the farms. On phylogenetic analysis, the test isolates (presumptive *Aeromonas* species) associated genetically with different strains of *Aeromonas* especially, the strain SG3 with accession number MT409620.1 (Ajadi 1) and strain RB 5 with accession number 396436.1 (Ajadi 2). However, they did dissociate from the outgroup (*Pseudomonas* species) (fig. 2).

**Table 3.** Number of Bacterial isolates from diseased fish from different farms in Kwara State

Farm	Number of Samples	Number of Positive	Species Identified
Farm I	10	4	<i>Staphylococcus</i> spp (1), <i>Aeromonas</i> spp (3)
Farm II	6	3	<i>Aeromonas</i> spp (3)
Farm III	15	5	<i>Staphylococcus</i> spp (2), <i>Aeromonas</i> spp (2) <i>Vibrio</i> sp (1)
Farm IV	10	2	<i>Aeromonas</i> spp (2)
Farm V	8	4	<i>Aeromonas</i> spp (3), <i>Vibrio</i> sp (1)



**Figure 2.** Phylogenetic tree generated from *Aeromonas* and *Pseudomonas* (outgroup) type strains and consensus sequences from fish isolates. Bootstrap values are based on 1000 replications. The scale bar represents the nucleotide substitution per site

Both aqueous and ethanolic extracts of *M. scaber* showed varying degree of antibacterial activities at the concentration of 100mg/ml but ethanolic extract showed a better bacterial growth inhibition (Table 4). All the bacteria show similar rate of susceptibility to the ethanolic extract as to that of the control (enrofloxacin). Table 5 shows that the lowest MIC of ethanolic extract of *M. scaber* was against *Bacillus* sp with 10mg/ml and the highest was 80mg/ml against *Staphylococcus* sp.

**Table 4.** Zone of inhibition of ethanolic and aqueous extract of *M. scaber* (100mg/ml), Enrofloxacin and Ethanol

Test organisms	Ethanolic extract (mm)	Aqueous extract (mm)	Enrofloxacin (mm)	Ethanol (mm)
<i>Aeromonas</i>	19	16	6	8
<i>Vibrio</i>	27	24	26	6
<i>Staphylococcus</i>	31	16	33	6
<i>Bacillus</i>	31	16	32	6

The values of inhibitory zones included the 6mm of each of the agar well.

**Table 5.** Minimum Inhibitory Concentration of Ethanolic Extract of *M. scaber*

Test organisms	Ethanol extract (MIC) (mg/ml)
<i>Aeromonas</i>	40
<i>Vibrio</i>	20
<i>Staphylococcus</i>	80
<i>Bacillus</i>	10

## DISCUSSION

Globally, the menace of drug resistance due to abrupt use of antibiotics is alarming and the need to stem this malady is expedient. Phytogetic agent is one of the panaceas to consider as a way of proactive measure. One of the many tropical plants that are traditionally used for medicinal purposes is *M. scaber*. It was revealed in this study, that plant extracts (aqueous and ethanol) contained secondary metabolites including saponins, tannins, flavonoids, terpenoids, steroids anthraquinones and alkaloids in varying degrees, this is in agreement with earlier study by Aberé *et al.* (2007).

These metabolites and several other aromatic compounds are responsible for the antimicrobial activities of the plant (Opoku *et al.* 2015). Previous studies have reported the antimicrobial activities exhibited by these phytoconstituents including tannins (Kolodziej *et al.*, 1999); flavonoids (Cottiglia *et al.*, 2001; Elsohly *et al.*, 2001), tarpenoids (Braca *et al.*, 2000) and anthraquinones (Houghton *et al.*, 2000). Saponins have been

reported to have antimicrobial activity and hence, it's an essential bioactive component of plant involved in plant disease resistance (Godswill *et al.*, 2010; Hossain *et al.*, 2013).

The activities of *M. scaber* reported in this study could probably be related to the presence of these phytoconstituents in the plant. In comparison, the ethanolic extract was seen to be more active against the tested bacteria than the aqueous extract and has similar activity with Enrofloxacin. This could be due to the insolubility of the phytoconstituents in water or denaturation of the active compound by hot water in the process of extraction (Opoku *et al.*, 2015). This is an indication that ethanol would be a better solvent for plant extraction for antibacterial analyses (Irobi *et al.*, 1994).

The use of enrofloxacin as a positive control is due to the fact that most isolated organisms from fish are susceptible to the antibiotic (Zhang *et al.*, 2010) and that Enrofloxacin is one of the antibiotics generally in use for the treatment of infection in fish production in Nigeria (Abalaka *et al.*, 2013). However, the incessant and indiscriminate use of chemotherapeutic



agents results in less efficient disease control and subsequent antibiotic resistance and eventual drug residue (Chuah *et al.*, 2016). Earlier studies have reported the antimicrobial, anti-inflammatory, antifungal and analgesic effects of *M. scaber* (Owolabi *et al.*, 2013; Bisignano *et al.*, 2000; Sanogo *et al.*, 1998; Ekpendu *et al.*, 1994; Irobi *et al.*, 1994). Ali-Emmanuel *et al.*, 2003 reported its effectiveness against bovine dermatophilosis.

Extracts of *M. scaber* possess active ingredients that are effective bactericidal (Adeshina *et al.*, 2019). This makes the plant to be essential in tradi-medicine for the treatment of various diseases such as infectious dermatitis, candidiasis, toothache and venereal diseases among others that are caused by several bacteria and fungi. The concentration of ethanolic extract of *M. scaber* to inhibit bacterial growth is lowest (10mg/ml) against *Bacillus* sp and highest (85mg/ml) against *Staphylococcus* sp. Therefore, in this study, the ethanolic extract of *M. scaber* is most potent against *Bacillus* sp and least against *Staphylococcus* sp. *Aeromonas* and *Vibrio* spp were moderately inhibited.

## CONCLUSION

Extracts of *M. scaber* showed moderate antimicrobial activities against test organisms including *Aeromonas*, *Bacillus*, *Vibrio* and *Staphylococcus* isolated from diseased African catfish. The results of the antimicrobial assay showed promising evidence that the plant (*M. scaber*) is a potential antibacterial agent against aquatic microbes. Further *in vivo* study is expedient to substantiate the antimicrobial potentials of this extract against these bacteria of aquatic origin.

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