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EVALUATION OF THE EFFICACIES OF *SARCOCEPHALUS LATIFOLIUS* DRIED LEAF EXTRACTS ON COMMON BACTERIAL FISH PATHOGENS

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ABSTRACT
Sarcocephalous latifolius is a known medicinal plant among local fish farmers in rural areas. It has been used for the traditional treatment of some fish diseases in southern Nigeria**.** Dried leaf extracts of *Sarcocephalous latifolius* (ethyl acetate, chloroform, and aqueous) were investigated for fish antibacterial activity. They were found to be effective against all tested bacterial strains with varied degree of sensitivities. Ethyl acetate fraction was found to be more effective than others. Ethyl acetate fraction showed maximum activity against the tested strains and minimum inhibition concentration (mic) of 6.46mg/ml for *Staphylococcus aureus*, 6.76mg/ml for *Serretia mercenses* and 8.13mg/ml for *Bacillus subtilis*. It was noted that the chloroform extract fraction inhibited the bacteria more than the aqueous extract. The use of the plant for bacterial infection treatment by local farmers is supported by this research.

KEYWORDS: *Sarcocephalous latifolius*, antibacterial, pathogens, fish.

INTRODUCTION
Fish is not only significant as a wellspring of animal protein for man but have also been used as major model organisms for diverse biomedical research activities. Fish can also suffer from various types of diseases like other animals. Diseases affecting fish are threat to the economic viability and sustainability of aquaculture farming not only in Nigeria but also in many other parts of the world. Pathogenicity of bacterial becomes an issue when fish are not nutritionally and physiologically balanced, or when other important environmental factors such as water quality and stocking density are compromised. Outbreak of disease is one of the primary reasons for economic setback in aquaculture (Plumb, 1997). Many microorganisms such as bacteria are capable of causing diseases in fish. They cause large scale mortalities, reduction in production capability and lower the quality of aquatic organisms. Bacteria can take different routes such as the gills, skin and other parts into fish body (Douglas, 2007). Growth and development of fish are adversely affected by these infections. The prevention of fish disease is therefore necessary for the benefit of the fisheries industry, the improvement of fish farming production capability, and the increase in fish resources. Chemotherapeutic control of fish disease is expensive therefore, fish disease infections invariably lead to lower harvest, higher production cost and huge economic losses to fish farmers. The use of a wide range of synthetic antibiotics in large quantity, including the non-biodegradable antibiotics that are used in human medicines as prophylactic and chemotherapeutic in aquaculture practice has led to a series of problems for human and animal health as well as aquatic environment (Daniel, 2002). The uncontrolled use of these antibiotics has also resulted in the emergence of antibiotics resistant bacteria in aquaculture environments. Studies have also shown that many synthetic antibiotics remain in the bottom sediment and in the aquatic environment for a long period after administration (Miranda and Zemelman, 2002). The sedimentary microbial community may also be affected by the residues of these antibiotics. Many of the antibiotics used in aquaculture practices have also been implicated for sensitization reaction and other unwanted side effects. Therefore, there is need to gradually transit to natural antibiotics that are abundantly available in plants and herbs. Plants and herbs are considered as powerful sources of medicine that have been for a period of time. The medicinal properties of plants may be due to the presence of different phytochemicals, which can cause a definite physiological action in animal body. Plant parts such as roots, leaves and fruits contain bioactive compounds like alkaloids, essential oils, peptides and unsaturated long chain aldehydes, that make them a rich source of medicine. The objective of this research is to carry out

antibacterial activities on the extracts of *Sarcocephalous latifolius* using some common fish pathogens.

MATERIALS AND METHODS

Materials

Reagents/ Chemicals. All chemicals used were of analytical grade and purchased from reputable chemical outlets in Benin, Benin City.

METHOD

Plant Sample Collection, Identification and Preparation

Sarcocephalus latifolius leaves were collected from around Ekiadolor, Ovia Northeast Local Government Area, Edo State. The plant was taxonomically identified in the department of Plant Biology and Biotechnology, faculty of life sciences University of Benin, Benin City. Fresh leaves of the plants were collected, cleaned and washed and shade dried at room temperature for two weeks and then ground into coarse powder using a mechanical grinding machine.

Fish Sample Collection, Identification and Preparation

A total number of ten (10) live *catfish* (*Clarias gariepinus*) of average weight of between (450-600g) were collected from the Nigerian Institute for Oceanography and Marine Research farm situated at Degele community in Sapele local government area of Delta state. The fish were then transported in a clean plastic container to the Pharmaceutical Microbiology Laboratory, University of Benin. Benin City, for isolation and identification of needed microorganisms.

Isolation and Identification of Bacteria Used.

The bacteria used for this work were isolated from the body, gills and intestine of the fresh fish. It was carried out aseptically on Nutrient Agar (NA) media. The agar plates were incubated at 25^0c for 36-48hours. Indivdual colonies were separated from the plates on the basis of colour, shape and size and sub-cultured on nutrient agar slant to obtain pure culture.

A series of morphological, physiological and biochemical tests were carried out to characterize the suspected bacteria. A presumptive identification tests such as Gram-staining, motility, oxidase activity and catalase production were performed on the bacteria isolate.

Soxhlet Extraction of Plant Leaves

1000g of ground dried leaves of *Sarcocephalus latifolius* was weighed and placed in a thimble-holder in a soxhlet extractor. A 5-litre capacity round bottom flask, which was at the base of the soxhlet extractor, was placed on a heating mantle. The leaves powder was extracted with 3.5L methanol. The methanol crude extract was dried and the weight noted. This was fractionated into three fractions [ethyl acetate, chloroform and aqueous] using vacuum liquid chromatography method. The fractions were dried and used for this study.

Phytochemical Screening of *Sarcocephalus latifolius* Leaves Extracts

Qualitative investigation of the presence of secondary metabolites were carried out on Ethyl acetate, chloroform and aqueous fraction of *Sarcocephalus latifolius leaves* using the standard procedures described by Trease and Evans (1989) and Harborne (1991).

Alkaloids Test

The extract was dissolved in 1.0 ml of 1 % hydrochloric acid, HCl and treated with three drops of Dragendorff's reagent. Orange-red precipitation indicated the presence of the alkaloid compounds.

Phenols (Ferric chloride Test)

The extract was added with 1.0 ml of 5 % ferric chloride, $FeCl_3$ solution. The blackish green colours indicated the presence of flavonoids.

Steroids Test

The extract was dissolved in 1.0 ml of chloroform. 2.0 ml of concentrated tetraoxosulphate (vi) acid (H_2SO_4) was slowly added to form a lower layer which is yellow in colour with green fluorescence. A reddish brown colour on upper layer was interpreted as a steroid ring.

Tannins Test

2.0 ml of distilled water was added to about 1g of the extract in a test tube and boiled for few seconds, three drops of 0.1 % of $FeCl_3$ was added. The brownish green colouration indicates the presence of tannins.

Siphoning Test

Each of the crude extract was added with 1.0 ml of distilled water and boiled in test tube for 15 min. After cooling, the mixture was shaken and a persistent froth was formed indicating the presence of tannin.

Preparation of the Culture Media

Nutrient Agar (NA)

28g of nutrient agar powder was weighed and transferred into a clean sterile 1Liter conical flask. Sterile distilled water was added, the flask was properly sealed and shaken and then boiled until completely dissolved. The medium was thereafter autoclaved at 121^0C for 15mins. It was allowed to cool at 45^oC and mixed well before dispensing aseptically into 20ml volume Petri dishes. The medium was allowed to set on these plates and were used thereafter.

Preparation of test microorganisms

The test organisms are gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa and Serrretia marcenses)* isolated from the fish body, gills and guts. All these test organisms were

obtained and maintained as pure isolates on nutrient agar slants at 4°C.

Agar-well Diffusion Method

This was carried out using the agar diffusion technique as described by Osadebe and Ukwueze (2004). 0.1ml of 24 -hour broth culture of the test organisms was aseptically inoculated on the surface of solidify sterile dried Nutrient Agar (NA) plates by spread method using sterile glass rod. Five wells were made in the plates using a sterile cork borer (8mm diameter). The wells were sealed at the bottom with one drop of sterile nutrient agar to prevent diffusion of the extracts under the agar.

Preparation of Different Extract Concentrations

The extract was double diluted to different concentrations (500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml) which were used for the antibacterial assay.

Five tubes were arranged in a row in a test tube rack. 2ml of sterile distilled water was added to the first tube, while 1ml was added to the other four tubes. 1000mg of the extract was weighed. This 1000mg was transferred and dissolved in the first test tube containing 2ml of sterile distilled water. 1ml was transferred from the first tube into the second tube containing 1ml of sterile distilled water to get 500mg/ml.1mlof the suspension was transferred from the second tube into the third tube to get 250mg/ml. The same process was repeated to get 125mg/ml, 62.5mg/ml and 31.25mg/ml. 0.1ml of the prepared concentrations were then introduced into the corresponding wells using a sterile Pasteur pipette. The negative control well was filled with 0.1ml of tetracycline. The positive control well contained sterile distilled water.

Incubation of the Plates

The plates were left on the bench for about 40 minutes for pre-diffusion of the extracts and then incubated at 37^0C for 24hours.

Antibacterial Activity of the Extract Fractions

The antibacterial activity of each extract was determined by measuring the zone diameters of inhibition (mm) against each test bacterium using a metric ruler. The experiment was carried out in duplicates and the average values of the result were taken as antibacterial activity. The above method was repeated for each extract fraction.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibition concentration of each extract fraction was quantitatively determined by using the graph method described by Chinedu *et al*. (2012) with little modifications. The mean zones of inhibition were recorded. A graph of the square of the corresponding mean zones of inhibition was then plotted against the log concentration of the extracts. A straight line of best fit was drawn and extrapolated to the log concentration axis(.X-axis).

The resultant intercept was recorded as the log minimum inhibitory concentration (MIC) against that organism. The antilog of which gave the minimum inhibitory concentration (MIC) of the extract that can inhibit the growth of the test microorganism.

RESULTS AND DISCUSSIONS

Phytochemical Screening

Table 1: Phytochemical Screening results of Ethyl acetate, Chloroform and Aqueous fraction of *Sarcocephalus latifolius.*

S/N	Phytochemical	Ethyl acetate fraction	Chloroform fraction	Aqueous fraction
1	**Alkaloids**	+	+	+
2	**Phenolics**	+	+	+
3	**Steroids**	+	+	-
4	**Tannins**	+	+	+
5	**Saponnins**	+	+	++

Table 2: Inhibition zone diameter (mm) of the ethyl acetate fraction of the SL.

Zone of inhibition diameter(mm) Microorganisms					
concentration (mg/ml)	***E. coli***	***P. aeruginosa***	***S. marcescens***	***B. subtilis***	***S. aureus***
500	22.5	19.5	12.5	18.0	20.0
250	19.0	17.5	11.5	16.5	15.5
125	17.0	16.0	10.0	13.5	15.0
62.5	11.0	14.5	9.0	12.0	14.0
31.25	7.0	8.0	5.0	9.5	10.5

Table 3: Inhibition zone diameter (mm) of the chloroform fraction of the SL.

Zone of inhibition diameter(mm)					
Microorganisms					
concentration (mg/ml)	*E. coli*	*P. aeruginosa*	*S. marcescens*	*B.subtilis*	*S. aureus*
500	18.5	16.5	9.0	14.5	17.0
250	16.0	14.0	7.5	13.0	12.0
125	12.0	12.5	6.0	11.0	10.5
62.5	8.0	10.0	5.5	9.0	9.5
31.25	4.0	5.0	2.0	6.0	4.5

Table 4: Inhibition zone diameter (mm) of aqueous fraction of the SL.

Zone of inhibition diameter(mm)					
Microorganisms					
Concentration (mg/ml)	*E. coli*	*P. aeruginosa*	*S.marcescens*	*B.subtilis*	*S.aureus*
500	17.0	13.5	6.5	12.0	14.0
250	13.0	11.5	5.0	10.5	9.5
125	9.0	10.0	4.0	7.5	8.5
62.5	0.0	0.0	0.0	0.0	0.0
31.25	0.0	0.0	0.0	0.0	0.0

Determination of the Minimum Inhibitory Concentration

Table 5: Inhibition zone diameter square (mm^2) and log concentration of the ethyl acetate fraction of the SL.

Zone of inhibition squared (mm^2)					
Microorganisms					
log concentration (500mg/ml- 31.25mg/ml)	*E. coli*	*P. aeruginosa*	*S. marcescens*	*B.subtilis*	*S.aureus*
2.699	506.25	380.25	156.25	324.00	400.00
2.3979	361.00	306.25	132.25	272.25	240.25
2.0969	289.00	132.25	100.00	182.25	225.00
1.7959	121.00	272.25	81.00	144.00	196.00
1.4949	49.00	240.25	25.00	90.25	110.25

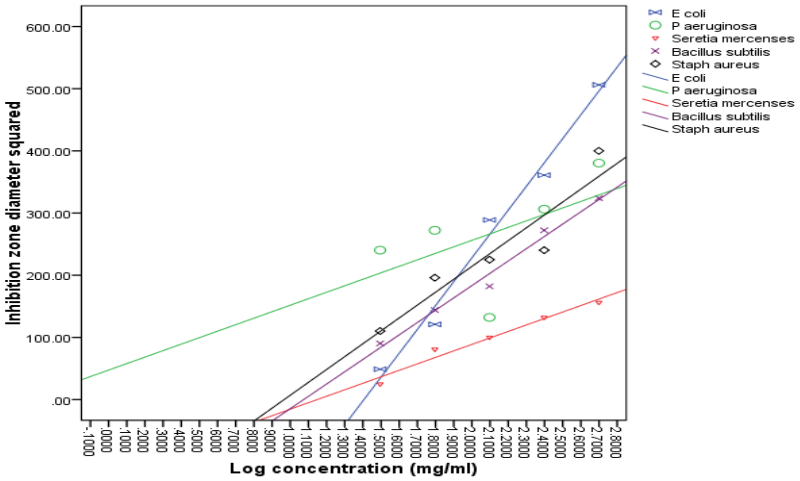

Figure 1: ethyl acetate fraction of SL.

Table 6: Inhibition zone diameter square (mm^2) and log concentration of the chloroform fraction of the SL.

Zone of inhibition squared (mm^2)					
Microorganisms					
log concentration (500mg/ml- 31.25mg/ml)	*E. coli*	*P.aeruginosa*	*S.marcescens*	*B.subtilis*	*S.aureus*
2.699	342.25	272.25	81.00	210.25	289.00
2.3979	256.00	196.00	56.25	169.00	144.00
2.0969	144.00	156.25	36.00	121.00	110.25

1.7959	64.00	100.00	30.25	81.00	90.25
1.4949	16.00	25.00	4.00	36.00	20.25

Table 7: Inhibition zone diameter square (mm^2) and log concentration of aqueous fraction of the SL.

Zone of inhibition squared (mm^2)					
Microorganisms					
log concentration (500mg/ml- 31.25mg/ml)	***E. coli***	***P. aeruginosa***	***S.marcescens***	***B. subtilis***	***S.aureus***
2.699	289.00	342.25	42.25	144.00	196.00
2.3979	169.00	132.25	25.00	110.25	90.25
2.0969	81.00	100.00	16.00	56.25	72.25
1.7959	0.00	0.00	0.00	0.00	0.00
1.4949	0.00	0.00	0.00	0.00	0.00

Table 8: Log of concentrations of extracts of SL.

Test organism	Gram	Ethyl acetate fraction	Chloroform fraction	Aqueous fraction
E. coli	_	1.320	1.440	1.580
P. aeruginosa	_	∞∞	1.220	1.595
S. marcescens	_	0.830	1.050	1.030
B. subtilis	+	0.910	1.100	1.470
S. aureus	+	0.810	1.330	1..520

These values were extrapolated from the x-axis of the graph and the antilogarithm gave the minimum inhibitory concentration (MIC) of the extract fractions in mg/ml for SL.

Table 9: The minimum inhibitory concentration (MIC) for SL.

Extracts MIC values(mg/ml)	*Escherichia. coli* (Gram –ve)	*Pseudomonas aeruginosa* (Gram-ve)	*Serretia marcenses* (Gram -ve)	*Bacillus subtilis* (Gram +ve)	*Staphylococcus aureus* (Gram +ve)
Ethyl acetate fraction	20.89	Not defined	6.76	8.13	6.46
Chloroform fraction	27.54	16.60	11.22	12.59	21.38
Aqueous fraction	38.02	39.36	10.72	29.50	33.11

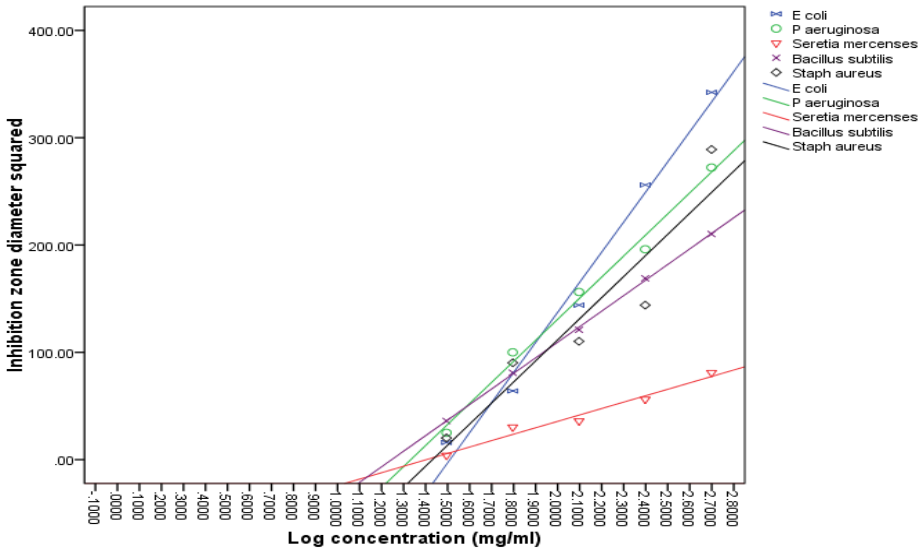

Figure 4: Antibacterial efficacy of SL extracts against 5 different bacteria from Catfish.

The phytochemical screening (Table 1) showed that all the fractions are loaded with phytochemicals that have been reported to show anti- inflammatory and antimicrobial activities.

The antibacterial sensitivity of the extract fractions and their potency were assessed quantitatively by determining the zones inhibition diameter (ZID) and minimum inhibitory concentration (MIC). The results

illustrated in tables 1 to 8 indicated that the fractions from the leaves of *Sarcocephalus latifolius* showed antibacterial activity against both the Gram-positive bacteria (*Bacillus subtilis and Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Serretia marcenses* and *Pseudomonas aeruginosa*). However, the ethyl acetate fraction of SL was more potent antibacterial agents against the Gram-positive bacteria than the gram-negative bacteria.

In this study, it was noted that the growth of *Staphylococcus aureus* was remarkably inhibited by the ethyl acetate fraction giving a MIC value of 6.46mg/ml. This observation was in agreement with reported work of (Mohammed *et al.,* 2015). *Staphylococcus aureus* is one of the most dangerous species of all the many common *Staphylococcal* bacteria. These Gram-positive, sphere-shaped bacteria can cause a wide range of illnesses, from minor skin infections to life threatening diseases. This was followed by *Serretia marcenses,* which has a MIC value of 6.67mg/ml. The growth of *Bacillus subtilis* was also inhibited but to a lesser extent by ethyl acetate fraction with MIC of 8.13mg/ml.

Again, the ethyl acetate fraction of *Sarcocephalus latifolius* had antibacterial activity against *Escherichia coli*, which is known to be multi-drugs resistant. The ability of this extract to inhibit *Escherichia coli*, a Gram negative bacterium, is of a great importance even though it was at the highest concentrations of the extract (20.89 mg/ml). Boussadaada *et al.* (2008) reported that Gram-negative bacteria are resistant to antibiotics. These bacteria are generally less sensitive to the activity of plant extracts. The difference in bacterial sensitivity to the fractions may be due to the nature of the bacterial species. Gram positive bacteria are more susceptible because they have only outer peptidoglycan layer which is not strong enough to serve as effective permeable barrier to drug constituents. The gram-negative bacteria on the other hand, have an outer phospholipidic membrane which contains the structural lipopolysaccharide compound. This makes the cell wall to serve as a strong barrier to drug constituents. Despite these barriers, phytochemical constituents from this plant leaves extract was able to inhibit the growth of this pathogenic strain. The aqueous fraction showed higher MIC values when compared to the ethyl acetate and chloroform fractions. This indicates that the bioactive compounds in the aqueous fraction are less active as antibacterial agent as compared to compounds fractionated into ethyl acetate and chloroform solvents.

The low sensitivity of the water extract of *Sarcocephalus latifolius* leaves extract observed in this study against the tested bacteria is in agreement with the earlier works done by (Aiyegoro *et al.,* 2008). He reported that aqueous extracts of plants generally exhibit little or no anti-microbial activities. The little or no activity of the aqueous fraction of this plant may not be unconnected to the fact that the active principles from plant materials are not readily extractible in water. Again, water being a universal solvent might have extracted different categories of compounds that may interact antagonistically in their overall activities (Martin and Eloff, 1998).

CONCLUSION

The antibacterial activity of leaves extract and fractions of *Sarcocephalus latifolius* against Gram-negative and Gram-positive bacteria appears to be as a result of the synergistic effect of the bioactive compounds present in the extracts.

Conflict of interest

The authors declare no conflict of interest.

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