Phytochemical Screening and Antimicrobial Properties of the Leaf and Stem Bark Extracts of *Strychnos Spinosa*

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Abstract: The study was carried out to ascertain the antimicrobial properties inherent in the aqueous, ethanol and methanol leaf extracts of *Strychnos spinosa* on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*, as well as the phytochemical screening of the extracts. Agar well diffusion method and Macro Broth dilution method were used in determining the antimicrobial activity and minimum inhibitory concentration (MIC) respectively. The extracts showed good inhibitory activities against *E. coli* and *P. aeruginosa*, but minimally against *S. aureus* and *C. albicans*. The inhibitions of the test organisms were measured by the diameter of zone of inhibition. The methanol extracts of the leaves and stem barks were the most potent against *E. coli* while the ethanol extracts, especially the leaf gave the highest potency against *P. aeruginosa* with a minimum inhibitory concentration (MIC) of 125 mg/ml . The extracts gave different MICs against the test organisms using the double-fold dilution method, with concentrations ranging between 31.25 to 500 mg/ml. The minimum lethal concentrations (MLC) of the extracts were 250 mg/ml and 500 mg/ml. The study revealed that the extracts were more bacteriostatic than bactericidal. The results obtained were tested at $P \leq 0.05$ level of significance using the one-way analysis of variance (ANOVA). The phytochemical screening revealed the presence of alkaloids, tannins, saponins and glycosides in the plant parts used.
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Key words: Phytochemicals, Zone diameter of inhibition, Minimum inhibitory concentration, Minimum lethal concentration.

Introduction

Plants are used medicinally in different countries and are a source of many potent and effective drugs (Srivastava *et al*., 1996). According to Ghani, 1990 and Dobelis, 1993, the active principles of many drugs that are found in plants which are responsible for their therapeutic values are secondary metabolites. Some of these active compounds include alkaloids, glycosides, flavonoids and tannins (Chhetri *et al*., 2008). The phytochemical research of plants is considered an effective approach in the discovery of new anti-infective agents from higher plants (Duraipandiyan *et al*., 2006).

The genus *Strychnos* belongs to the family *Loganiaceae*. *Strychnos* is known by its very toxic substances like strychnine and curare. Originally, these substances have been prepared by cooking the plant bark with water and thickening to a paste. The residue, a brown resinous paste with a bitter taste is used by indigenous people for arrow poisons (Hoet *et al*., 2007).

Strychnos spinosa has been used in different places as remedy for many ailments and disease conditions. The antimicrobial activity of *Strychnos spinosa* against *Candida albicans* and *Aspergillus niger* among other microorganisms was reported by Nwozo *et al*., 2010. Other researchers have also reported the antiplasmodial (Frederich *et al*., 2002, Bero *et al*., 2009)as well as the antitrypanosomal properties of *S. spinosa (*Nwozo *et al*., 2010)

Materials and Methods

Processing of the Plant Materials

This was done according to the methods described by Jigna and Chanda,(2006) and Mann *et al*., 2011. The leaves and stem bark of *S. spinosa* were shade-dried at the temperature of $25^0C \pm 2^0C$ to a constant weight to aid pulverization. The dried samples were then pulverized using a blender. This was done to enhance the maximum penetration of extracting solvents.

Extraction of the Plant Materials

This was done according to the method described by Kubmarawa *et al*., (2007). Exactly 50g of the blended leaves and stem bark were weighed and introduced into 250 ml of distilled water, ethanol and methanol respectively. The mixtures were agitated at 30 minutes interval for 3 hours and then soaked for 72 hours (3 days). Subsequently, the soaked materials were filtered into containers using Whatman's No. 1 filter paper. The filtrates were evaporated to dryness using a vacuum evaporator and

rotary shaker. The extracts were covered and stored in a refrigerator at 4^0C until needed.

Preparation of Standard Drug and Extract concentrations

Three concentrations (100, 200 and 400 mg/ml) of the six extracts: aqueous leaf, aqueous stem bark, ethanol leaf, ethanol stem bark, methanol leaf and methanol stem bark were prepared using methods described by Nascimento *et al*.(2000) and Mallikharjuna *et al*. (2010). In addition, 250 mg/ml of chloramphenicol and 200 mg/ml of ketoconazole were prepared using the same procedures and they served as antibiotic and antifungal controls respectively. The extracts were sterilized using a membrane filter.

Phytochemical Screening

The extracts were analysed for the presence of alkaloids, glycosides, steroids, tannins, reducing sugars, anthraquinones, phlobatannins and saponins as described by Trease and Evans, (1989), Siddiqui and Ali, (1997) and Sofowora (2006).

(i) Alkaloids

To 1 ml of each extract filtrate, 3 drops of Mayer's reagent were added. The mixture was then treated with few drops of 2% hydrochloric acid on a boiling water bath. The presence of turbidity in the observed tube indicates the presence of alkaloids.

(ii) Glycosides

To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulfuric acid were added and observed for a reddish brown coloration at the junction of two layers and a bluish green colour at the upper layer.

(iii) Steroids and Terpenoids

1 ml of the extract filtrates was treated with 3 drops of acetic anhydride. Then concentrated sulfuric acid was carefully added to the side of the test tube. The presence of a brown ring at the boundary of the mixture indicates a positive result.

(iv) Tannins

To 0.5 ml of extract solution, 1 ml of distilled water and about 2 drops of 10% ferric chloride solution were added. The mixture was observed for a blue or green-black coloration.

(v) Reducing sugars

To 0.5 ml of extract solution, 1 ml of distilled water was added and about 6 drops of Fehling's solution and warmed. The Fehling's solution was prepared by mixing equal volumes of equimolar concentration of Fehling's solutions A and B. The formation of brick red precipitates is an indication of the presence of reducing sugars.

(vi) Anthraquinones

2 ml of benzene was added to 1 ml of extract solution. Then, 2 ml of ammonia solution was added. The occurrence of turbidity is an indication of positive result.

(vii) Phlobatannins

1 ml of the extract solution was boiled with 1 ml of 1% aqueous hydrochloric acid and was observed for the deposition of a red precipitate to indicate phlobatannins presence.

(viii) Saponins

1 ml of the extract solution was boiled with 5 ml of water for 5 minutes and decanted while still hot. The following tests were performed to detect the presence of saponins.

(a) Frothing test: 1 ml of the mixture was further diluted with 4 ml of distilled water and shaken vigorously. It was then observed on standing for a stable froth.

(b) Emulsion test: This test was performed by adding 2 drops of olive oil to the frothing solution and shaking the mixture vigorously. The mixture was then observed for emulsion.

Preparation of the Test Organisms

Stock cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* were obtained from the University of Abuja Teaching Hospital (UATH) and authenticated using cultural and morphological identification, microscopy after Gram's staining as well as biochemical characterization of test organism using protocols described by Cheesbrough, (2002) and maintained in appropriate media in a refrigerator for future use. Suspensions of the test organisms were made in comparison with 0.5 MacFarland standards to give a cell density of 1.0×10^8 cells/ml ascertained using the Standard Curve according to Isu and Onyeagba, (1998).

Preparation and sterilization of media

All the media used in this study were obtained in powdered form and constituted in distilled water according to the manufacturers' instructions. The various quantities and volumes of water depended on the particular medium. A weighed quantity of each medium was dissolved in specific volume of de-ionized water in a chemical flask, which was stoppered properly. It was sterilized by autoclaving at 121^oC and 15 p.s.i for 15 minutes and cooled to 45-50^oC before dispensing into pre-sterilized dishes. These were left to gel on the work benches. Glass materials used in this work were also sterilized by autoclaving at 121^oC and 15 p.s.i. 15 minutes. They were then brought out and allowed to cool down properly before use.

Antimicrobial Assay (Agar Well Diffusion Method)

This was done according to the methods described by Mallikharjuna *et al*., 2010. Twenty

milliliters (20 ml) of sterile molten Mueller Hinton agar was poured into a set of sterile Petri dishes under aseptic conditions and was allowed to solidify. Then, each plate was inoculated with 200 µl of pure cultures of the test organisms and was evenly spread with a sterile bent glass rod. After allowing the sensitivity agar surfaces to dry, 4 wells of 8 mm diameter each were made on the seeded agar plates at fairly equidistant positions using a sterile cork borer. Exactly 0.2 ml of the 100, 200 and 400 mg/ml of the extract were placed in corresponding wells for each microorganism. The fourth well contained the control. Chloramphenicol (250 mg/ml) served as the control for the bacteria- *S. aureus, E. coli and P. aeruginosa* while ketoconazole (200 mg/ml) served as control for the fungus- *C. albicans*. The procedure was repeated respectively for the aqueous, ethanolic, methanolic leaf and stem bark extracts of *S. spinosa*. They were allowed to stand for 30 minutes for proper diffusion and incubated at 37^0C for 24 hours. Plates were subsequently observed for zones of inhibition (if any) which were measured (in mm) using a transparent ruler.

Minimum Inhibitory Concentration (MIC)

The MICs of the extracts that showed activity against the organisms were determined according to the macro broth dilution technique as described by Trigg and Hill, (1996). Two drops of standardized suspensions of the test organisms were inoculated separately into a series of sterile test tubes containing 2 ml of nutrient broth each. Then, 3 drops of different dilutions of the extracts were separately added to the tubes. The dilutions were in two folds with these concentrations: 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5mg/ml and 31.25mg/ml. The tubes were then properly corked and incubated at 37^0C for 24 hours. The MIC was read as the least concentration that inhibited the growth of the test organisms (Isu and Onyeagba, 1998)

Minimum Lethal Concentration (MLC)

The MLC of potent extracts were determined by plating out the tubes that showed no growth (inhibited visible growth) during the MIC determination. Using a heat-sterilized wire loop, a loopful from each of such tubes was sub cultured onto extract-free agar plates and incubated for 24 hours at 37^0C. The MLC was recorded as the least concentration at which no growth was observed.

Statistical Analysis

The Univariate Analysis of Variance (ANOVA) at $P \leq 0.05$ was used to analyse the results obtained, $P \leq 0.05$ was considered to be significant and $P \leq 0.05$ was not significant.

RESULTS

Table 1: Phytochemical constituents of *S. spinosa*

Bioactive Component	Leaf Extracts			Stem bark Extracts		
	A	E	M	A	E	M
Alkaloids	+	+	+	+	+	+
Glycosides	+	+	+	-	+	-
Steroids & Terpenoids	+	+	+	+	+	+
Tannins	+	-	+	+	+	+
Reducing Sugars	+	-	+	+	+	+
Anthraquinones	-	+	+	-	-	+
Phlobatannins	-	-	-	-	-	-
Saponins	+	+	-	+	+	+

Key: += Present, - = absent (not detected), A= Aqueous, E= Ethanolic, M= Methanolic

Table 2: Zone Diameter (mm) of Inhibition of the Aqueous Leaf Extracts of *S. spinosa*.

Test Organisms	Concentration of Extracts (mg/ml)			Control
	100	200	400	
S. aureus	5.0 ± 0.7	7.0 ±0.3	9.0 ± 0.7	33.0 ± 0.3
E. coli	8.0 ± 0.3	9.0 ± 0.7	10.0 ± 0.3	39.0 ± 0.5
P. aeruginosa	6.0 ± 0.7	22.0 ± 0.5	24 ± 0.7	35.0 ± 0.3
C. albicans	8.0 ± 0.5	9.0 ± 0.7	9.0 ± 0.3	33.0± 0.5

Table 3: Zone Diameter (mm) of Inhibition of the Ethanol Leaf Extracts of *S. spinosa.*

Test Organisms	Concentration of Extracts (mg/ml)			Control
	100	200	400	
S. aureus	7.0 ± 0.7	9.0 ±0.3	9.0 ± 0.7	32.0 ± 0.3
E. coli	8.0 ± 0.3	9.0 ± 0.7	10.0 ± 0.3	39.0 ± 0.5
P. aeruginosa	14.0 ± 0.7	24.0 ± 0.5	33.0 ± 0.7	37.0 ± 0.3
C. albicans	8.0 ± 0.5	10.0 ± 0.7	10.0 ± 0.3	33.0 ± 0.5

Table 4: Zone Diameter (mm) of Inhibition of the Methanol Leaf Extracts of *S. spinosa.*

Test Organisms	Concentration of Extracts (mg/ml)			Control
	100	200	400	
S. aureus	7.0 ± 0.7	9.0 ±0.3	10.0 ± 0.7	33.0 ± 0.3
E. coli	11.0 ± 0.3	19.0 ± 0.7	27.0 ± 0.3	40.0 ± 0.5
P. aeruginosa	12.0 ± 0.7	23.0 ± 0.5	31.0 ± 0.7	34.0 ± 0.3
C. albicans	9.0 ± 0.5	10.0 ± 0.7	10.0 ± 0.3	33.0 ± 0.5

Table 5: Zone Diameter (mm) of Inhibition of the Aqueous Stem bark Extracts of *S. spinosa.*

Test Organisms	Concentration of Extracts (mg/ml)			Control
	100	200	400	
S. aureus	5.0 ± 0.7	7.0 ±0.3	9.0 ± 0.7	33.0 ± 0.3
E. coli	8.0 ± 0.3	19.0 ± 0.7	25.0 ± 0.3	39.0 ± 0.5
P. aeruginosa	13.0 ± 0.7	25.0 ± 0.5	29 ± 0.7	35.0 ± 0.3
C. albicans	8.0 ± 0.5	9.0 ± 0.7	9.0 ± 0.3	32.0± 0.5

Table 6: Zone Diameter (mm) of Inhibition of the Ethanol Stem bark Extracts of *S. spinosa.*

Test Organisms	Concentration of Extracts (mg/ml)			Control
	100	200	400	
S. aureus	7.0 ± 0.7	9.0 ±0.3	9.0 ± 0.7	32.0 ± 0.3
E. coli	8.0 ± 0.3	13.0 ± 0.7	14.0 ± 0.3	39.0 ± 0.5
P. aeruginosa	13.0 ± 0.7	16.0 ± 0.5	18.0 ± 0.7	34.0 ± 0.3
C. albicans	8.0 ± 0.5	10.0 ± 0.7	10.0 ± 0.3	32.0 ± 0.5

Table 7: Zone Diameter (mm) of Inhibition of the Methanol Stem bark Extracts of *S. spinosa.*

Test Organisms	Concentration of Extracts (mg/ml)			Control
	100	200	400	
S. aureus	7.0 ± 0.7	9.0 ±0.3	10.0 ± 0.7	32.0 ± 0.3
E. coli	11.0 ± 0.3	26.0 ± 0.7	35.0 ± 0.3	39.0 ± 0.5
P. aeruginosa	13.0 ± 0.7	16.0 ± 0.5	18.0 ± 0.7	35.0 ± 0.3
C. albicans	9.0 ± 0.5	10.0 ± 0.7	10.0 ± 0.3	33.0 ± 0.5

The results obtained from the assay of the minimum inhibitory concentration (MIC) revealed the susceptibility of organisms, at different degrees, to extracts that showed antimicrobial activity.

Table 8: Minimum Inhibitory Concentration of Aqueous, Ethanolic and Methanolic leaf Extracts of *S. spinosa*

Test Organism	Solvent	MIC Value (mg/ml)
E. coli	A	500
	E	500
	M	125
P. aeruginosa	A	500
	E	125
	M	250

Key: A= Aqueous, E= Ethanol, M= Methanol

Table 9: Minimum Inhibitory Concentration of Aqueous, Ethanolic and Methanolic stem bark Extracts of *S. spinosa*

Test Organism	Solvent	MIC Value (mg/ml)
E. coli	A	250
	E	500
	M	125
P. aeruginosa	A	500
	E	125
	M	500

Key: A= Aqueous, E= Ethanol, M= Methanol

The minimum lethal concentrations of potent extracts were investigated and shown in Table 10. Since *S. aureus* and *C. albicans* both exhibited resistance to the extracts, the MLC investigation, which in this case, is the investigation of the minimum bactericidal concentration (MBC) was carried out on *E. coli* and *P. aeruginosa*.

Table 10: Minimum Bactericidal Concentration of Aqueous, Ethanolic and Methanolic leaf Extracts.

Test Organism	Solvent	MBC Value (mg/ml)
E. coli	A	NT
	E	NT
	M	250
P. aeruginosa	A	NT
	E	250
	M	NT

Key: A= Aqueous, E= Ethanol, M= Methanol, NT= Not Tested

Table 11: Minimum Bactericidal Concentration of Aqueous, Ethanolic and Methanolic stem bark Extracts

Test Organism	Solvent	MBC Value (mg/ml)
E. coli	A	500
	E	NT
	M	500
P. aeruginosa	A	NT
	E	250
	M	NT

Key: A= Aqueous, E= Ethanol, M= Methanol, NT= Not Tested

DISCUSSION

The qualitative analysis of phytochemicals in *S. spinosa* revealed the presence of alkaloids, steroids and terpenoids, tannins, reducing sugars and saponins in the experimented plant parts. This is in consonance with the findings of Kubmarawa *et al.,* 2007 and Nwozo *et al.,* 2010. However, the detection of glycosides (in leaf) and absence of phlobatannins in the plant parts used in this study is in contrast with the work of Kubmarawa *et al.,* 2007 and Nwozo *et al.,* 2010. These differences may be due to the difference in geographical locations and environmental conditions of the places where the plant was obtained or the use of different levels of extract concentrations Kubmarawa *et al.,* 2007.

The result showed that *S. spinosa* was mildly effective against *Staphylococcus aureus* and *Candida albicans* as both *S. aureus* and *C. albicans* were inhibited, to some extent, by the extracts. The activity of the plant against *C. albicans* is in consonance with the findings of Nwozo *et al.,* (2010) who reported that *S. spinosa* was active against *C. albicans* at concentrations between 20mg/ml and 50 mg/ml. However, the extracts showed marked activities against both *E. coli* and *P. aeruginosa.* The methanol stem bark extract and ethanol leaf extract appeared to be very effective against *E. coli* (Table 7) and *P. aeruginosa* respectively (Table 3). This is in agreement with the report of Verpoorte *et al.,* (1983) and McGaw *et al.,* (2000). However, the result is at variance with that of significant activity of the extracts of *S. spinosa* against *S. aureus*.

The MIC results revealed that methanol extracts of the plant were the most potent against *E. coli* of all the extracts as both extracts had an MIC of 125 mg/ml (Tables 8 and 9). Also, the ethanol extracts of both leaf and stem bark appeared to be the most effective against *P. aeruginosa,* having an MIC value of 125 mg/ml in both cases (Tables 8 and 9). The MBC assay revealed that most of the extracts

were rather more bacteriostatic than bactericidal at the tested concentrations. However, some extracts gave bactericidal actions against the test organisms. Statistically, it was revealed that for most of the plant extracts, there was generally no significant difference ($P \leq 0.05$) in their activities on the test organisms. Some extracts, however, showed significant differences ($P \leq 0.05$).

CONCLUSION

The results of this research have shown the antibacterial properties of *Strychnos spinosa* against certain microorganisms owing to the bioactive substances it possesses. This emphasizes the usefulness of the leaves and stem bark of the plant in the treatment of certain bacterial diseases in the traditional medicine practice and the need to harness this potential in the development of new antibiotics especially with the problem of development of resistance to known antibiotics by bacteria.

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