In-Vitro Antimicrobial Efficacy Study of *Borreria Verticillata* Stem Bark Extracts Against Some Dermatophytes and Drug Resistant Pathogens

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Abstract- The search for more effective antimicrobial agents among materials of plant origin with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs is the goal of this research. Extracts from the stem barks of Borerria verticillata were screened for their antimicrobial activities. Solvents used included hexane, chloroform, ethyl acetate, methanol and aqueous solvents. The BVR plant parts were air dried and powdered before being soaked in solvents for 3 days. The extracts were tested for the presence of different phytochemicals qualitatively, and were also tested against some drug resistant organisms Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi) and some fungi implicated in dermatophytic infections (Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis, Epidermophyton flocossum). Agar well diffusion and broth dilution methods were used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) at concentrations of 512mg/ml to 4mg/ml. Randomised complete block design was used to determine whether there exist any significant differences among the treatment means of the antimicrobial activity of the leaves of Borreria verticillata. The findings of this study, could therefore justify the use of this plant in traditional medicine in the treatment of bacterial infections and fungal infections most especially dermatophytes.

Index Terms- Borreria verticillata, crude extracts, anti-drug resistant pathogens, anti-dermatophytic properties

I. INTRODUCTION

Plants have always provided a good source of anti-infective agent and have remained effective in the fight against microbial infections (Olukoya *et al*., 1993). The known success of traditional medicine has guided the search for new chemotherapeutic alternatives to eliminate the infections caused by drug-resistant microbes and to reduce the harm caused by antibiotic (Giamarellou, 2006; Bocanegra-Garcia *et al*., 2009).
Many studies all over the world have showed that these medicinal plants and their extract have multi-antimicrobial properties (Bocanegra-Garcia, 2009; Boklari, 2009 Al-Juraifani, 2011; Bakht, 2011). While 25 to 50 % of current pharmaceuticals are derived from plants, none is used as antimicrobials (Cowan, 1999). Biological effects of these plants on prokaryotic and eukaryotic organisms have been discussed by few studies (Bakkali, 2008; Al-Zubaydi *et al.*, 2009). However, plants have an almost infinite ability to synthesize compounds that have diverse bioactive properties that we cannot synthesize.

In most developing countries, the use of indigenous, natural drugs is a common practice because life-saving synthetic drugs are beyond the reach of the poor people. In countries such as China, India, West African countries and other developing countries, it is not only the unavailability or inaccessibility of modern pharmaceuticals that drives people to traditional remedies, but, more importantly, the existence of a medical system enshrined within their customs (Ndamba *et al., 1994*).

Now, there has been an upsurge in the interest in herbal remedies in several parts of the world. Many of these herbal remedies have found their ways into orthodox medical practice (Cowan,1999).

For instance, In Nigeria, some of the plant materials used in the treatment of ailments include, the Ogbolo roots *(Cissus popularea)* used by the Yoruba of Western Nigeria to improve low sperm count (oligospermia) and lack of sperm (azospermia) seeds of *Ricinus communis* used as contraceptives (Sofowora, 1984), while *Grewia mollis* is used for the treatment of typhoid and paratyphoid fevers by natives of central (middlebelt) Nigeria while *B. verticillata* a perennial shrubby false-button weedy herb found in most parts of the world. The leaves are used in some parts of Nigeria for curative purpose as one of the traditional medicines. They have been commonly used effectively to cure Eczema-*Tinea versicolor*, ring worm-*Tinea capitis*, scabies and other skin lesions (e.g., infectious dermatitis) toothache, headache, and dyspepsia. The juice obtained from the aerial part is applied topically for the treatment of skin diseases.

The aim of the study is to screen for the antimicrobial activity of the *stem bark Borreria verticillata stem bark* against some drug resistant pathogenic bacteria and some dermatophytes while the specific objectives include the identification of the phytochemical constituents of the various parts of *Borreria verticillata* plant in various solvents, To validate or disvalidate the

efficacy of the various plant part extracts by evaluating their anti-dermatophytic and anti-drug resistant microbial activities.

II. MATERIALS AND METHODS

Sample Collection and Preparation

Plant Material

Borreria verticillata is a dicotyledonous plant, which has a wide distribution in Nigeria. It consists mainly of trees and shrubs. The leaves are opposite, whole and entire (Benjamin, 1979). The plant is known by different names in various parts of Nigeria. Among the Hausas, it is called Nyenyere. The Yorubas call it Irawo ile, the people of Edo State call it, Akhevemosibe. The TIVs in Benue Sate call it Wantiyo kporou, while the Ibibios call it Abia-ikana.

Borreria verticillata stem barks was collected from Ucha village, a village adjacent to the University of Agriculture, Makurdi Local Government of Benue State, Nigeria. A quality evaluation of the plant material was carried out in the Department of Biological Sciences, University of Agriculture, Makurdi

Sample Preparation

The *Borreria verticillata* plant which was readily available in the rainy season was uprooted from the soil. The *Borreria verticillata* leaves was washed with running tap water to remove dirty prior to drying process. The sample was cut into small pieces and air died for 21 days to reduce moisture content and grinded into power with the aid of a pistol and mortar.

Extraction of Plants Material

Aqueous solutions of the milled plant parts was prepared by soaking 100 g of each in 250 ml hexane for four days. The resulting mixture was subjected to gravity filtration and the filtrates were concentrated by evaporation in a water bath, dried and weighed.

The procedure was repeated on the residue using the following solvents: hexane, ethyl acetate, chloroform and methanol sequentially in order of polarity. The extracts were stored in desiccators (Ushie and Adamu 2010).

Phytochemical Assay

Preliminary phytochemical screenings were carried out on the crude extracts as described by Brain and Turner (1975), Sofowora (1993), Edeoga *et al.* (2005), Harborne (1973), Okoli *et al.* (2010) and Ushie *et al*. (2010) to identify the presence of the classes of secondary metabolites (Alkaloids, flavonoids, tannins, saponins, glycosides, cardiac glycosides, terpenes, steroids, phenol).

Test for Alkaloids

0.5 g of the extract was stirred with 2 M aqueous hydrochloric acid (5.0 ml) on a steam bath. 1.0 ml of the filtrate were separately treated with a few drops of Mayer's reagent, Drangendoffs' reagent, Wagner's reagent. The resulting solution was observed for colour changes.

Test for Tannins

0.5 g of each of the plant extracts was boiled with distilled water (100 ml) for 5 minutes. To 2.0 ml of the cooled solution (filtrate) a few drops of ferric chloride was added. The colour change was recorded.

Test for Glycosides

A small portion of each of the plant extracts was placed in two separate test tubes of 0.1 M H2SO4 was added to one and distilled water (5.0 ml) added to the other. The test tubes were heated for 45 minutes in a water bath. The cooled solutions were made alkaline with a solution of 2 M NaOH.

Fehling solutions (5.0 ml) A and B (ratio1:1) was added to the two test tubes and were allowed to stand for 3 minutes. The solution of the extracts in distilled water serves as control. The changes in reaction were observed and recorded.

Test for Saponins

The froth test and emulsion test as described by Harborne (1975) were used to determine the presence of saponins. A small portion of each of the plant extracts was added to distilled water (20 ml) in a 100 ml beaker, boiled and filtered and the filtrate used for the test.

(a) Froth test: 5 ml of the filtrate was diluted with water (20 ml) and shaken vigorously and allowed to stand for 30 minutes. The result was recorded.

(b) Emulsion test: 2 drops of olive was added to the frothing solution and shaken vigorously. The result was recorded.

In order to remove 'false-positive", the blood haemolysis test was performed on the extract that frothed water.

Test for Anthraquinones

0.5 g of each of the plant extracts was shaken with benzene (2.0 ml) and filter where necessary. 10 % ammonia solution (4.0 ml) was added to the filtrate. The resultant mixture was shaken and the reaction observed and recorded.

Test for Flavonoids

(a) Lead Acetate Test: 0.5g of the extract dissolved in 5 ml of distilled water. 10 % of lead acetate solution (1.0 ml) was added. The colour formation was recorded.

(b) Iron (III) chloride. To a solution of 0.5 g of the extract in water, two drops of iron (III) chloride was added. A colour change noted and recorded.

Test for Terpenoids (Salkowski test)

A solution of each of the extract was made by dissolving 0.5 g of the extract in 2.0 ml of chloroform and concentrated H_2SO_4. The presence of terpenes in the sample was detected as the colour changes**.**

Test for Cardiac Glycosides

a) Steroidal Nucleaus (Salkowskii's Test): 0.5g of each extract was dissolved in 2cm^3 chloroform followed by the addition of concentrated sulphuric acid (H_2SO_4) to form a layer. A reddish brown ring colour at the interface signified the presence of steroidal nucleaus (i.e a glycone portion of the glycoside)

b) Keller – Killiani's Test: About 0.5g of each extract was dissolved in 2cm^3 of glacial acetic acid containing a drop of Iron III chloride ($FeCl_3$) solution. To the mixture, 1cm^3 of concentrated tetraoxosulphate (VI) acid (H_2SO_4) was

added down the side of the test tube. Reddish brown ring obtained at the interface indicated the presence of a digitoxose sugar component characteristic of cardelonides.

Test for Steroids

About 0.5g of each ewasxtract added with 2ml of chloroform. After which concentrated sulphuric acid (H_2S0_4) was added to the sides of the test tube.

Test for Phenols

Ferric chloride test: 0.5g of each extract is treated with 2ml of water and 10% aqueous ferric chloride solution. The blue or green coloration is observed.

Gelatin test: About 1% solution of gelatin containing 10% NaCl is added to the ethanolic extract. The white precipitate formation is observed

Source and Maintenance of Test Organisms

Gram-positive organisms (*Staphylococcus aureus, Bacillus subtilis)* and Gram-Negative organisms (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi)* were obtained and confirmed resistant to at least two of the convectional antibiotics such as chloramphenicol, ampicillin and cotromoxazole at the Medical Microbiology and Parasitology unit of the Clinical Laboratory department of the Federal Medical Centre, Keffi using the antibiogram susceptibility tests.

The fungi implicated in dermatophytic infections (*Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis, Epidermophyton flocossum*) were obtained at the Medical Microbiology and Parasitology department of the Ahmadu Bello University Teaching Hospital.

They were maintained on nutrient agar and Sarbaround's dextrose agar respectively (Oxoid, UK). Twenty-four hours old pure cultures were prepared for use each time.

Antimicrobial Bioassay

The bacterial assay procedures of Water Worth (1978) and Perez *et al*. (1990) was employed with small modification (Ushie and Adamu, 2010). The methods involved the preparation of the culture medium and inoculation. Aseptic technique was used to avoid contamination (Ushie and Adamu 2010)

The agar plates were inoculated by spreading a small volume (0.05 mL to 0.10 mL) of the liquid inoculums (sub-cultured nutrient broth) by means of an L-shaped glass rod in such a way that the surface of the agar in the plates was covered with microbes.

One microbe was inoculated to one plate making a total of ten plates for ten microbes.

Five wells for hexane, chloroform, ethyl acetate, acetone, and methanol extracts and two for the control were made through the aid of a sterile cork borer.

The plant extracts were diluted using dilution method and in each of the appropriately labelled well (hole) diluted plant extract was introduced. Ciprofloxacin and terbinafine were introduced in the other two wells (holes) as control.

The inoculated plate was left on the bench for about an hour to allow the extracts diffuse into the agar. The plates were aerobically incubated at 37ºC for 23 hours for the bacteria and 72 hours for the fungi.

The diameter of zones of inhibition was measured by means of linear instrument in millimeter (venier calliper) and recorded (Pedro *et al.*, 2002).

Determination of the minimum inhibitory concentration (MIC)

To measure the MIC values,

Suspension of micro-organisms were made in sterile normal saline and adjusted to 0.5 Macfarland standard (10^8 Cfu/ml) (NCCLS, 2000). From the stock solution, serial dilutions were made to 512, 256, 128, 64, 32, 16, 8, 4 mg/ml (NCCLS, 2000). The various concentrations of the stock were prepared in about different test tubes labelled 1-8 respectively,

These were assayed against the test bacteria. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth (Al Juraifini, 2011; Shahidi Bonjar, 2004).

Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC).

This was an offshoot of the previously determined MIC. Equal volume of the various concentrations of each extract and Sarbaround's dextrose agar (Oxoid, UK) were mixed in micro-tubes to make up 0.5ml of solution. 0.5ml of McFarland standard of the organism suspension was added to each tube (Shahidi Bonjar, 2004). The tubes were incubated aerobically at 37ºC for 24 hours for MDR-bacteria, and 72 hours for dermatophytes.

These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 hours.

The highest dilution that yielded no single bacterial/fungal colony was taken as the Minimum bactericidal/fungicidal Concentration (Al Juraifini *et al.*, 2011). This was carried out on some of the extracts with high antimicrobial activity and some of the highly sensitive organisms.

Statistical Analysis

Data obtained were subjected to analysis of variance and means separated according to Duncan's Multiple Range Test at $P = 0.05$.

Randomised complete block design was used to determine whether there existed any significant differences among the treatment means of the antimicrobial activity of *Borreria verticillata* plant leaves.

TABLE 1: Nature and yield of different solvents extract of the stem barks of *Borreria verticillata*

Solvents	**Colour of Extract**	**Texture of Extract**	**Yield of Extract(g)**	**Percentage Recovery (%)**
Hexane	Brownish	Hard solid	4.00g	4.1
Chloroform	Brown	Hard powder	3.60g	3.8
Ethyl actate	Dark brown	Sticky powder	2.00g	2.0
Aqueous	Light brown	Powder	3.30g	3.6
Methanol	Light brown	Powder	2.86g	3.4

TABLE 2: Result of the qualitative phytochemical screening of the crude extract of *Borreria verticillata* stem barks.

		Extracts				
Phytochemicals	**Reagents**	HE	CE	EAE	AE	ME
Alkaloids	a) Wagners	-	-	-	-	-
	b) Mayer	-	-	-	-	-
	c) Drangedroff	-	-	-	-	-
Tannins	Solutions of extracts plus Ammonia solution	-	-	-	-	-
Flavonoids	a) Lead acetate	+	+	-	-	+
	b) Ferric Chloride	-	-	-	-	+
Anthraquinone	Extract in benzene plus Ammonia solution	-	-	-	-	-
Terpenes	Extracts plus chloroform plus H_2SO_4	+	-	-	+	+

Saponins	a) Frothy test	+	+	+	+	+
	b) Emulsion test	+	+	+	+	+
Glycosides	Extracts plus dilute H_2SO_4 plus NaOH plus Fehiling solution	-	+	-	-	-
Cardiac glycosides	a) Kellner killani test	+	-	-	+	+
	b) Salwoski test	+	+	-	+	+
Steroids		-	-	-	-	-
Phenols	Extract solution plus Ferric Chloride	-	-	-	-	-

TABLE 3: Diameter of the zone of inhibition of the antimicrobial activity of crude extract in mm of *Borreria verticillata* stem barks on selected MDR bacteria strains and some dermatophytes.

Test organisms	**HE**	**CE**	**EAE**	**AE**	**ME**	**CPR**	**TER**
S. aureus	12.67b	13.67b	9.00b	0.00c	0.00c	25.33a	NA
Escherichia coli	10.00c	10.00c	15.33b	14.67b,c	10.00c	26.00a	NA
B. subtilis	6.00b	6.00b	0.00c	6.33b	10.67b	15.67a	NA
Salmonella typhi	2.33c,d	0.00c	6.67c	14.00b	13.67b	21.00a	NA
P. aeroginosa	10.00d	14.33b	23.00a	11.33b,c	14.33b	22.00a	NA
K. pneumoniae	3.00b	2.67b	0.00b	4.00b	4.67b	14.67a	NA
M. canis	8.67c	11.67b,c	12.33b	0.00d	0.00d	NA	22.00a
T. rubrum	0.00c	6.00b	0.00c	8.67b	2.00c	NA	23.33a

E. flocosum	1.67c	0.00c	3.33b	2.33b	0.00b	NA	24.33a
T. mentagrophytes	5.00b	0.00c	7.00a,b	4.67b	2.67b	NA	16.00a

Data are means of three replicates. Means followed by the same letter in each vertical column are not significantly different while means followed by different letter in each vertical column are significantly different according to Duncan's multiple range test ($P = 0.05$).

KEYS: HE= Hexane extracts; AE=Aqueous extracts, CE=Chloroform extracts, EAE= Ethyl acetate extracts, ME= Methanol extracts, NA= Not Applicable.

TABLE 4: Minimum inhibitory concentration (MIC) in mg/mL of the crude extract of the *Borreria verticillata* stem barks and control drugs.

Extracts	***S. aureus***	***E. coli***	***B. subtilis***	***S. typhi***	***K. pneumoniae***	***P. aeruginosa***	***T. mentagrophytes***	***T. rubrum***	***E. floccosu***	***M. canis***
HE	512	256	-	128	32	128	512	-	-	512
CE	128	256	512	-	8	-	512	-	-	512
EAE	-	-	-	512	256	64	-	512	-	-
AE	-	512	-	512	256	-	512	512	-	-
ME	512	512	256	128	64	8	-	512	-	512
CPR	256	128	-		-	64	NA	NA	NA	NA
TER	NA	NA	NA	NA	NA	NA	128	64	128	-

KEYS: HE= Hexane extracts; AE=Aqueous extracts, CE=Chloroform extracts, EAE= Ethyl acetate extracts, ME= Methanol extracts, NA= Not Applicable. CPR=Ciprofloxacin, TER= Terbinafine.

TABLE 5: Minimum Bactericidal/Fungicidal concentration (MBC/MFC) in mg/mL of the crude extracts of the *Borreria verticillata* stem barks and control drugs.

Extracts	*S. aureus*	*E. coli*	*B. subtilis*	*S. typhi*	*K. pneumoniae*	*P. aeruginosa*	*T. mentagrophytes*	*T. rubrum*	*E. floccosu*	*M. canis*
HE	-	-	-	512	-	-	512	-	-	-
CE	-	256	512	-	-	256	-	256	512	-
EAE	512	512	-	-	-	256	512	-	-	-
AE	-	-	-	-	-	-	-	-	-	-
ME	-	-	-	512	-	-	-	-	-	-
CPR	512	266	-		-	64	NA	NA	NA	NA
TER	NA	NA	NA	NA	NA	NA	128	128	256	-

KEYS: HE= Hexane extracts; AE=Aqueous extracts, CE=Chloroform extracts, EAE= Ethyl acetate extracts, ME= Methanol extracts, CPR=Ciprofloxacin, TER=Terbinafine
NA= Not Applicable.

III. DISCUSSION

The antimicrobial activity of *Borreria verticillata* against the growth of the selected isolates was observed visually and measured. This agreed with the research findings of Sofowora (1982); Benjamin (1975); which pointed out that *BVR possess* antimicrobial action at different concentration depending on the bacteria species.

It can be observed that the *BVR* plant possess relatively fair antimicrobial properties even in different solvents extracts. Cheesbrough (2000) pointed out that the active antimicrobial compound diffuses from the disc into the medium and the susceptible organisms are inhibited at a distance from the disc.
The MIC of the crude extracts from the leaves, stem barks and roots of the *BVR* plant proved that the plant has a relatively significant anti drug-resistant bacterial and anti dermatophytes activity (Benjamin, 1975).

Just like other species of the Spermacoce and Borreria genus have been found to demonstrate anti drug resistant bacterial and even antifungal and is even as effective as the antiomicrobial agents commonly used (Taylor, 2004).

The findings of this study, could therefore justify the use of this plant in traditional medicine in the treatment of bacterial infections and fungal infections most especially dermatophytes (Balde *et al.*, 1989; Conserva *et al.*, 2012; Abdullahi-gero *et al.*, 2014).

IV. CONCLUSION

This justifies and validates the claims by the herbal practitioners that the stem bark of *Borreria verticillata* are used to cure some illness and has confirmed that the stem bark of the aforementioned plant is of medicinal value due to its antimicrobial activity and could be exploited for use in pharmaceutical.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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