Antibacterial Assays of the Solvents Partitioned Portions of Methanol Stem Bark Extract of *Bauhinia rufescens* Lam [Leguminosae-Caesalpinioideae].

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ABSTRACT

The *in-vitro* antibacterial activities of the organic solvents partitioned portions of the methanol stem bark extract of *Bauhinia rufescens* Lam were evaluated in some Gram positive and Gram negative bacteria using the hole-in-plate disc diffusion technique. Test microorganisms were *Corynaebacterium spp.* and *Staphylococcus aureus* (Gram positive); *Proteus vulgaris, Pseudomonas aeruginosa* and *Shigella dysenteriae* (Gram negative) bacteria.

The diameters of inhibition zone exhibited by all the portions on the Gram positive bacteria ranged from 11.17 ± 0.33 mm to 23.67 ± 0.33 mm while on Gram negative organisms the values were from 10.67 ± 0.17 mm to 22.00 ± 0.00 mm. The results revealed that most of the portions were highly sensitive against *S. aureus* and *P. aeruginosa* which had highest MIC/MBC values of 1.56/1.56 mg/ml (ethylacetate and n-butanol portions) respectively. All the portions studied recorded comparatively higher activity index against *S. aureus* and *P. aeruginosa* when computed with ciprofloxacin than when computed with erythromycin or gentamicin; while activities of *S. dysenteriae* and *P. vulgaris* relates closer to gentamicin than ciprofloxacin. Similarly, *Corynaebacterium spp*. relates averagely closer to erythromycin than the broad-spectrum ciprofloxacin throughout the portions.

Overall, this extract portions were found to be more sensitive to Gram positive (17.90) than Gram negative (17.15) species studied. Therefore, this study further confirms the traditional use of the stem bark of *B. rufescens* in some parts of Northern Nigeria as a remedy against diarrhea, dysentery, and other related diseases which are caused by most of the organisms studied.

(Keywords: *Bauhinia rufescens*, organic portions, antibacterial, stem bark, *in-vitro*, Leguminosae)

INTRODUCTION

The continuous search for antimicrobials from natural sources, particularly plants, is on the increase notably due to incessant resistance posed by synthetic antibiotics to many infectious diseases. The availability, cheapness, and accessibility of these plants in Tropical and Sub-tropical Africa coupled with the global crisis of drug-resistances makes it convenient for in-depth survey of medicinal plants from this part of the world (Usman et al., 2009a). These plants are cheaper and more accessible to most of the population in the World whose medicinal value lies in some chemical substances that produce a definite physiological action on the human body (Edeoga et al., 2005).

In recent years, multiple drug resistance in both human and plant pathogenic micro organisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Loper et al., 1991; Davis 1994; Service 1995; Güllüce et al., 2004). Very little information on the antimicrobial effects of this plant has been documented (Maillarde et al., 1991). It is therefore, pertinent to scientifically screen medicinal plants from this part of the country which are used locally by the traditional healers for the treatment of various infectious diseases; with a view to validating their traditional usage and also serve as a starting material for the development of clinically useful chemotherapeutic compounds through the bio-assay procedure.

Briefly, the plant *Bauhinia rufescens* Lam is a scandent shrub or small tree belonging to the giant family Leguminosae, subfamily Leguminosae-caesalpiniodeae; usually 1-3 m high, sometimes reaching 8 m; often scraggy, stunted and multi-stemmed. Bark ash-grey, smooth, very fibrous and scaly when old, slash pink, twigs arranged in 1 plane like a fishbone, with thornlike, lignified, lateral shoots, 10 cm long. The leaves are very small, bilobate almost to base, with semi-circular lobes, glaborous, with long petioles, greyish-green, less than 3 cm long. Flowers are greenish-yellow to white and pale pink, petals 5, spathulate, 15-20 mm long; stamens 10, filaments hairy at the base. Fruits aggregated, long, narrow pods, twisted, up to 10 cm long, glaborous, obliquely constricted, shining dark red-brown, with 4-10 seeds each (FAO-UNEP, 1983; Burkill, 1995). The plant is deciduous in the drier area and evergreen in the wetter area, often found in the dry Savannah region, especially near streams or river banks; occuring throughout West Africa and extends across Africa up to Sudan. It has wide array of medicinal and socio-cultural uses.

MATERIALS AND METHODS

Collection and Identification of Plant: The stem bark of *Bauhinia rufescens* was collected in June 2008 from Gathla village, Gwoza, Borno State, Nigeria (Long. 13° 31.369'E, Lat. 11° 00.562'N). The plant specimen was identified and authenticated by a Plant Taxonomist, Prof. S.S. Sanusi, with the Department of Biological Sciences, University of Maiduguri. The herbarium specimen was deposited at the Post Graduate Research Laboratory, Department of Chemistry with voucher number - #003/2008 provided. The stem bark of the plant was cleaned, chopped into pieces, air-dried under shade for seven days and pulverised into fine powder and then coded "*Plant material".*

Extraction of Plant Materials: The air-dried powdered plant material (2000 g) was extracted exhaustively with 85 % methanol in distillled water using soxhlet apparatus as described by Lin et al. (1999). The choice of methanol as the solvent was in line with Ahmad et al. (1998) and Lin et al. (1999), that methanol was a better solvent for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents such as water, ethanol and hexane. The combined methanolic extracts were concentrated to dryness at reduced pressure using rotary evaporator and the extract coded "BRME" – *Bauhinia rufescens methanol extract*. About 200 g of the BRME was then successively dried-partitioned with n-hexane, chloroform, ethylacetate, n-butanol and residual aqueous portions to afford BRNH, BRCF, BREA, BRNB and BRRA respectively. The portions were then subjected to *in vitro* antimicrobial susceptiblity test while the MIC and MBC of the susceptible organisms determined accordingly.

Antimicrobial Studies:

Test Microorganisms: The Gram positive organisms were: *Corynaebacterium spp., Staphylococcus aureus*, while Gram negative organisms were: *Proteus vulgaris, Pseudomonas aeruginosa, Shigella dysenteriae*. Standard susceptibility antibiotic discs used were: Ciprofloxacin (5 μg/disc); Erythromycin (5 μg/disc), Gentamicin (10 μg/disc), produced by Oxoid Ltd., Hampshire, England. These organisms were clinical isolates obtained from the Department of Medical Microbiology and Department of Veterinary Medicine, University of Maiduguri, Maiduguri – Nigeria.

Antimicrobial Susceptibility Studies: The portioned portions of the methanol extract of *B. rufescens* was subjected to preliminary antimicrobial evaluation on two Gram-positive and three Gram-negative strains using the hole-in-plate disc diffusion technique as described by Forbes et al. (1990); Vlietinck et al. (1995); Usman et al. (2007a).

The extracts were made in five different stock concentrations of 12.5 mg/ml, 25 mg/ml, 50 mg/ml, 100 mg/ml and 200 mg/ml; prepared by dissolving 0.125 g, 0.25 g, 0.50 g, 1.00 g, and 2.00 g respectively into 10 ml each of 85 % methanol in distilled water (v/v) – as vehicle. The micro organisms were maintained on agar slants until use. The inocula was then prepared by subjecting the test organisms in nutrient broth and incubated for 24 hours at 37°C. After incubation, the broth cultures were diluted to 1:1000 for Gram positive bacteria and 1:5000 for the Gram negative bacteria. One millilitre of the diluted cultures was inoculated into 19 ml sterile molten nutrient agar (48°C) and sabaround dextrose agar prepared according to manufacturer's

specification was poured into sterile Petri dishes. These were gently swirled and allowed to solidify. Afterwards, holes of 9 mm diameter were bored onto the solidified and inoculated nutrient agar plates using sterilized number VI cork borer.

All of the holes were filled with equal volumes of 0.1 ml of each portioned portion equivalent to 1.25, 2.5, 5.0, 10, 20 mg/hole. Standard discs were placed on bacterial – inoculated nutrient agar plate; the extracts were allowed to diffuse into the agar for an hour. Thereafter, plates were then incubated overnight at 35 ^{o}C and 37 ^{o}C for fungi and bacterial strains respectively. At the end of the incubation period, inhibition zones were recorded in millimeters as the diameter of growth-free zones around the bored holes using a transparent meter rule. The extract was independently tested in triplicate. Diameters of zones of inhibition $\geq$ 10 mm exhibited by plant extracts were be considered active (Zwadyk 1972; Usman et al., 2007a).

Activity Index (AI): This was estimated as 100 x diameters of inhibition zone of extract ÷ diameters of inhibition zone of the standard antibiotic (expressed as %) (Shahidi, 2004).

Percent Actvitiy (PA): This was calculated as 100 x number of susceptible strains to a specific extract ÷ total number of tested bacterial strains. This will be expressed as % Gram positive, % Gram negative and %T as total activity against both Gram positive and Gram negative (Shahidi, 2004).

Spectral Intensity Index (SII): This was determined as mean diameters of inhibition zones (mm) of all sensitive bacterial strains to a specific sample x %T ÷ 100 (Shahidi, 2004).

Determination of Minimum Inhibitory Concentration (MIC):

The MIC was determined using the nutrient broth dilution technique as described by Vollekovà *et al.* (2001). The minimal inhibitory concentration value was determined for the microorganisms that were sensitive to the extracts under study. Each extract was first diluted to the highest concentration (50 mg/ml) in 85 % methanol in distilled water (v/v), and then two-fold serial dilution of each extracts were then be made to a concentration ranging from 0.039 to 25 mg/ml using nutrient broth (13 g/l). To the suspension, 5ml of each extract concentration was added into nutrient broth and then 1.0 ml of standardized broth cultures containing 1.0 x 10^{7} CFU/ml were seeded into each test tube and then incubated at 35^{o} c for 18-24 hours. MIC was defined as the lowest concentration where no turbidity was observed in the test tubes.

Determination of Minimum Bactericidal Concentration (MBC):

The MBC was determined using the broth dilution technique previously described by Vollekovà et al. (2001) as adopted by Usman et al. (2007a,b) by assaying the test tubes resulting from MIC determinations. A 1oopful of the content of each test tube was then inoculated by streaking on a solidified nutrient agar plate and then incubated at 35^{o}C for 24 hours for possible bacterial growth. The lowest concentration of the sub-culture that shows no bacterial growth was considered the minimum bactericidal concentration.

Statistics:

The statistical analysis involved the determination of mean differences among the zone of inhibition exhibited by the extracts against each organism and the standard antibiotics analyzed using One-way ANOVA with Student-Newman-Keuls Multiple comparisons test performed using GraphPad InStat® (GraphPad Software, 1998).

RESULTS AND DISCUSSION

The extractive relative percentage values of the solvents partitioned portions were found to be 2.34 % w/w (yellowish-brown viscous oil), 6.64 % (yellowish-brown mass), 2.99 % (brown mass), 12.31 % (light-brown mass), 36.71 % (reddish-brown mass) for n-hexane (NH), chloroform (CF), ethylacetate (EA), n-butanol (NB) and residual aqueous (RA) portions respectively (Usman et al., 2009b).

Our earlier report on preliminary phytochemical studies of the partitioned portions showed the presence of aloes, anthraquinones derivatives, cardenolides and cardiac glycosides, flavonoids, resins, saponins and tannins (Usman et al.,

2009b). These are compounds that are known to have various sort of curative effects against most pathogenic organisms as reported by many researchers (Dangoggo et al., 2005; Geidam et al., 2007; Usman et al., 2007a).

Figures 1-5 shows susceptibility pattern of portioned portions of the methanol stem bark extract of *Bauhinia rufescens* and standard antibiotic discs against some Gram positive and Gram negative organisms studied.

Figure 1 shows the effects of 20 mg/hole level of the portioned portions, the results shows that the activities against Gram positive and Gram negative organisms ranges from 11.17 ± 0.33 to 23.67 ± 0.33 mm and 10.83 ± 0.17 to 22.00 ± 0.00 mm respectively. The diameters of inhibition zones exhibited by *Corynaebacterium spp.* was found to be significantly different (P<0.01, 0.001) between most portions with Ciprofloxacin (CIP) and Erythromycin (ERY) but insignificant (P>0.05) different was noted between n-hexane (NH) and all the portions except residual aqueous (RA) and likewise insignificant activities was observed between all portions with that of Gentamicin (GEN). The broad spectrum antibiotic CIP has showed indifferent activity (P>0.05) between the activities expressed by the portions against *S. aureus* except to that of RA which was significantly different (P<0.001). The activities against the Gram-negative organisms studied showed that at 20mg/hole level, the extract portions exhibited a non significantly activities (P>0.05) between portions and Gentamicin but were significantly (P<0.001) higher compared to Erythromycin and Ciprofloxacin against *P. vulgaris*. The portions seem to be insignificantly different (P>0.05) compared to the individual portion and also when RA was compared with ERY on *P. aeruginosa*. No significant difference (P>0.05) was noted between ERY and EA, CF; similarly, n-butanol portion was insignificant (P>0.05) with n-hexane in terms of their activities at this dose. Other tests were significant (P<0.05, 0.01) compared with each other.

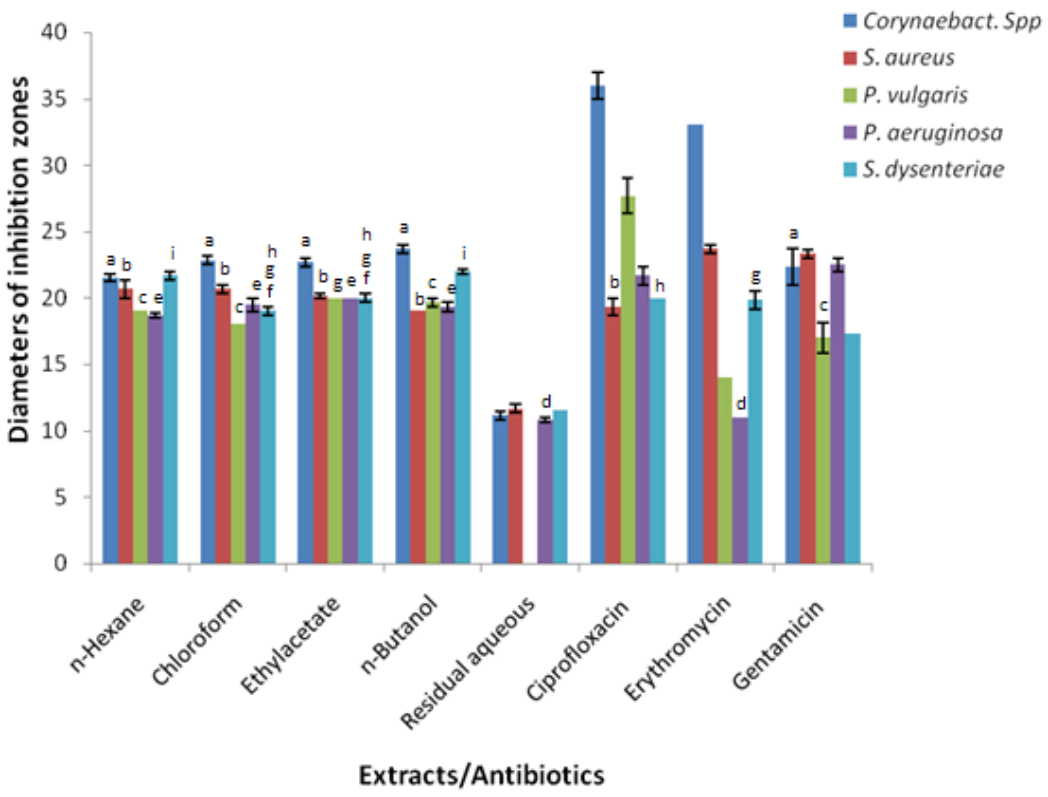

Figure 1: Pattern of Diameters of Inhibition Zone of the Partioned Portions of *B. rufescens* Stem Bark Extract against some Pathogenic Bacteria at 20 mg/hole; Same Letters on Same Organism's Activity are Insignifcantly (P>0.05) Different.

The zones of inhibition reported in Figure 2 showed that the activities against Gram positive and Gram negative organisms ranges from 11.50 ± 0.00 to 23.00 ± 0.00 mm and 11.33 ± 0.33 to 18.67 ± 0.17 mm, respectively. Thus, activities against *Corynaebacterium spp.* at 10 mg/hole was found to be insignificant (P>0.05) between portions and also between GEN; significant variation of the data was noted among other comparisons.

The activities due on *S. aureus* indicated no difference (P>0.05) between the data exhibited by the all the portions and that of CIP; similar layout was observed for the activities on *P. vulgaris* between the portions and like wise GEN whose activities differ insignificantly (P>0.05); but this trend was different on *P. aeruginosa* where only data expressed by CF and NH, and NH and NB showed insignicancy (P>0.05) while other comparison were (P<0.01, 0.001) as the case may be. The inhibition expressed by the portions were insignificantly (P>0.05) different to those of GEN. The activities of GEN relative to NB, CF were insignificant (P>0.05) compared against *S. dysenteriae*. Other tests of means were significant (P<0.01, 0.001) compared to each other.

Figure 3 showed the results the activities at 5 mg/hole dose of the portions on the organisms, results against Gram positive and Gram negative organisms ranges from 11.50 ± 0.00 to 19.00 ± 0.00 mm and 10.67 ± 0.17 to 18.33 ± 0.33 mm, respectively. The DIZ of inhibition against *Corynaebacterium spp.* were highly significant (P<0.001) not for NH and CF and also NB and EA where there was no statistical difference (P>0.05) noted. The results against *S. aureus* was found to be insignificantly different (P>0.05) when EA, NH and NB were compared. There was no significant (P>0.05) difference observed on the data generated when EA, NH, and NB were compared with GEN and likewise between ERY and CF, other comparison the inhibition zones on *P. vulgaris* were found to be significantly (P<0.001) different. These extract portions NH, CF, EA, NB showed some degrees of insignificance (P>0.05) and also between ERY and RA against *P. aeruginosa.* The inhibition against *S. dysenteriae* was insignificantly (P>0.05) different when NH, CF, EA, NB were compared with each other.

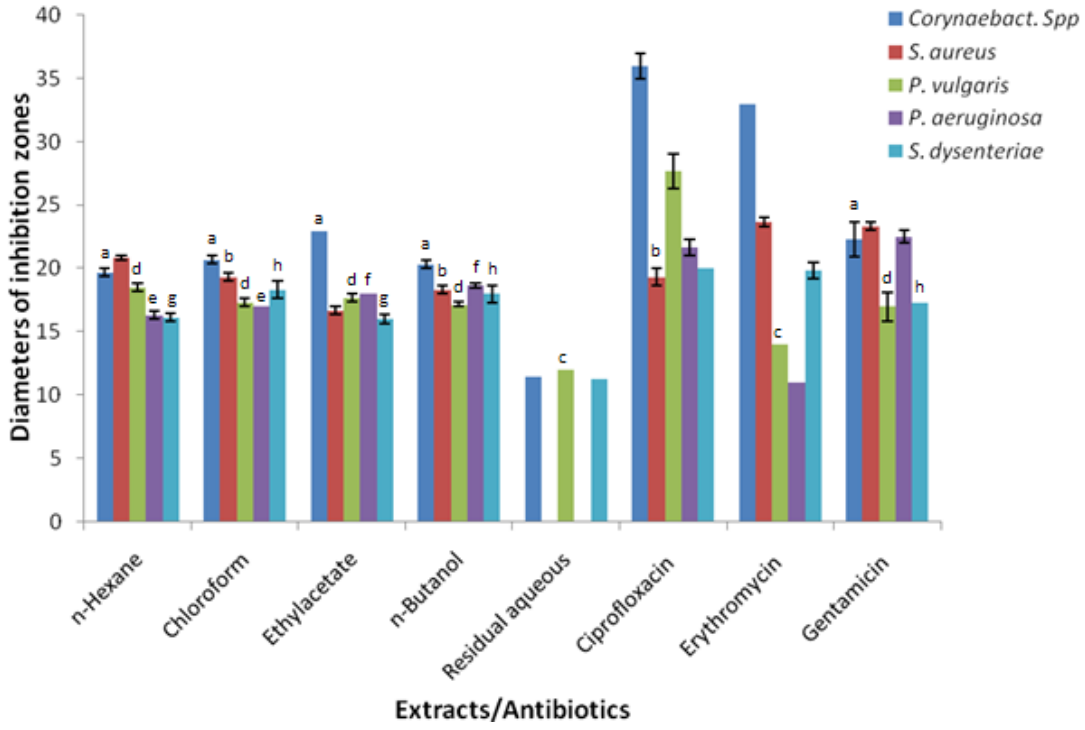

Figure 2: Pattern of Diameters of Inhibition Zone of the Partioned Portions of *B. rufescens* Stem Bark Extract Against some Pathogenic Bacteria at 10 mg/hole; Same Letters or Symbol on Same Organism's Activity are Insignifcantly (P>0.05) Different.

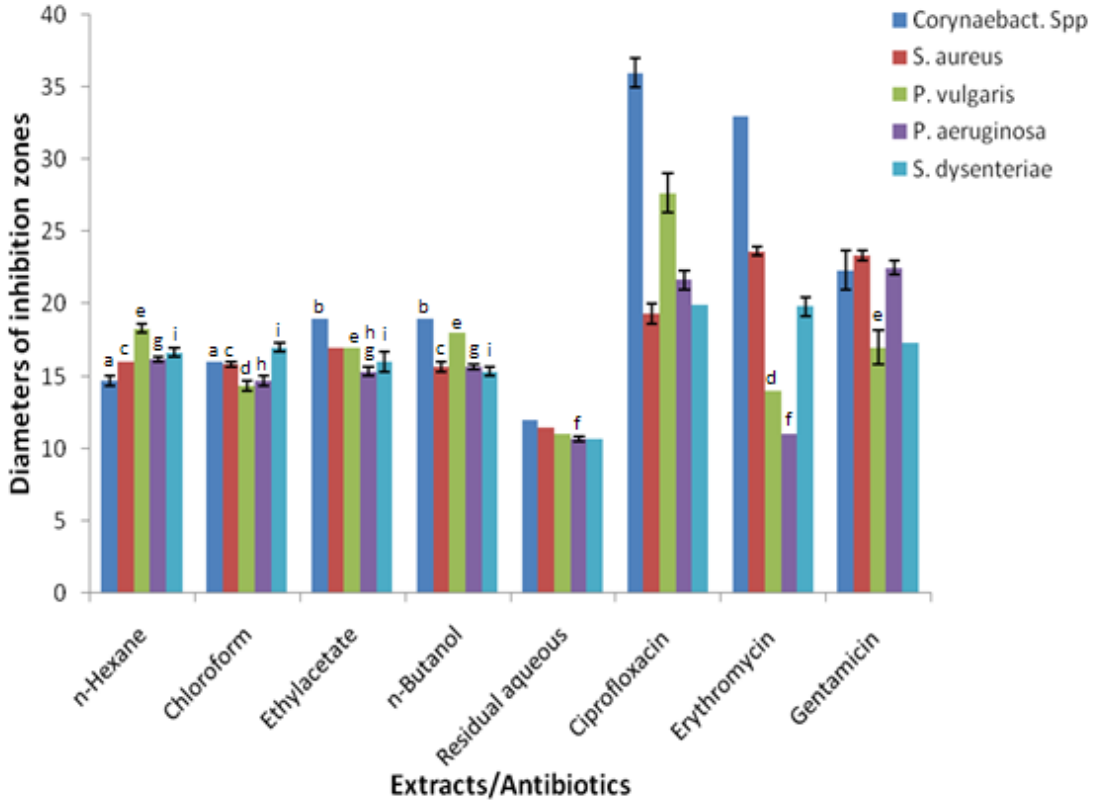

Figure 3: Pattern of Diameters of Inhibition Zone of the Partioned Portions of *B. rufescens* Stem Bark Extract against some Pathogenic Bacteria at 5 mg/hole; Same Letters on Same Organism's Activity are Insignifcantly (P>0.05) Different.

Similarly, Figure 4 (2.5 mg/hole dose) shows the effects of the portions against Gram positive and Gram negative organisms ranges from 11.67 ± 0.17 to 16.00 ± 0.00 mm and 11.17 ± 0.17 to 17.00 ± 0.00 mm, respectively. The activities of the portions at this dose against *Corynaebacterium spp.* revealed statistically insignificant (P>0.05) different between all the portions except when compared with the antibiotic disc where significant difference (P<0.001) was observed. Only 3 portions (NH, EA, NB) showed insignificant (P>0.05) variation on the activities expressed on *S. aureus* but other comparison exhibited various levels of significance (P<0.01; 0.001). At the same concentration, NH, CF, EA, NB, GEN, and ERY pair comparison between them showed a non significant (P>0.05) correlation when tested against *P. vulgaris;* on the other side, only NH,CF,EA comparison indicated insignificance level of differences (P>0.05) when the portions were tested on *P. aeruginosa* but other combinations were highly significant (P<0.001). There was no statistical difference (P>0.05) between Gen and CF, NB on *S. dysenteriae* and equally between NH and EA portions.

The data on Fig 5 shows the mean diameters of inhibition expressed by the portions at 1.25 mg/hole and the antibiotics discs. The results showed varying levels of inhibition against Gram positive and Gram negative organisms which ranges from 12.67 ± 0.33 to 18.67 ± 0.33 mm and 11.17 ± 0.17 to 19.67 ± 0.33 mm, respectively. The results showed significant difference on the majority of the portions when tested against *Corynaebacterium spp.,* except between CF, EA, and NB whose inhibition level did not show any significant difference (P>0.05). The results against *S. aureus* showed similar pattern to that of *Corynaebacterium spp.* Quite a different trend was observed against *P. vulgaris* where no significance (P>0.05) inhibition level was recorded between pair of NH, CF, EA, NB, RA, ERY, and GEN. Only two comparison (ERY and RA; NB and EA) were not significantly (P>0.05)

different when tested against *P. aeruginosa* but others were. The effects on *S. dysenteriae* was not significant (P>0.05) when activities of ERY was compared to NH, CIP; likewise CF and RA.

Table 1 shows the minimum inhibitory and minimum bactericidal concentrations of the susceptible portions. The n-butanol portion both had high MBC/MIC of 3.13/1.56 mg/ml against *P. vulgaris* and *S. dysenteriae*; equally higher MBC/MIC of 1.56 mg/ml was noted against *P. aeruginosa* and *S. aureus. Corynaebacterium spp*. had 3.13/1.56 exhibited by the ethylacetate and n-butanol soluble portions. On the overall, the dosages-dependent approach in this study is in line with similar work by Parekh et al. (2006); also the n-butanol portion was noted to be most susceptible and thus, contains bioactive phytochemicals against the tested organisms.

Table 2 shows the susceptibility pattern of the five portions, the spectral intensity index revealed that considering the organisms studied the portions were more susceptible to Gram positive (17.90) than Gram negative organisms (17.15) tested.

The AI (activity index) was designed to express the relation of the inhibition zone of the extract to that of standard antibiotics; it was found that both extracts showed varying level of correlation to either the broad spectrum antibiotic (ciprofloxacin), Gram negative susceptible antibiotic (Gentamicin) or Gram positive susceptible antibiotic (Erythromycin) with the extract's portions studied. The effects of the portions against *Corynaebacterium spp.* had relates well with erythromycin, while effects of most portions against *S. aureus* showed closer look to ciprofloxacin. Among the Gram negative organisms, *P. vulgaris* and *S. dysenteriae* had similar affects by most extract portions and Gentamicin but the effects on *P. aeruginosa* had a closer relationship to ciprofloxacin.

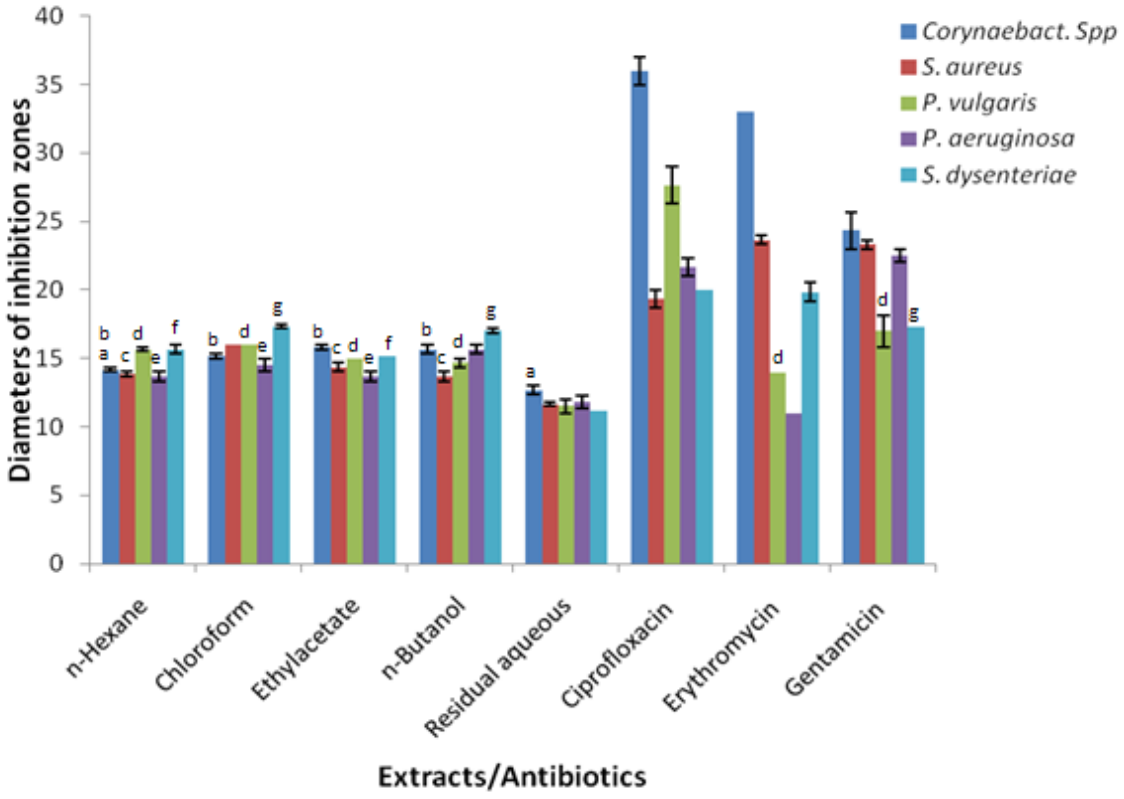

Figure 4: Pattern Diameters of Inhibition Zone of the Partioned Portions of *B. rufescens* Stem Bark Extract against some Pathogenic Bacteria at 2.5 mg/hole; Same Letters or Symbol on Same Organism's Activity are Insignifcantly (P>0.05) Different.

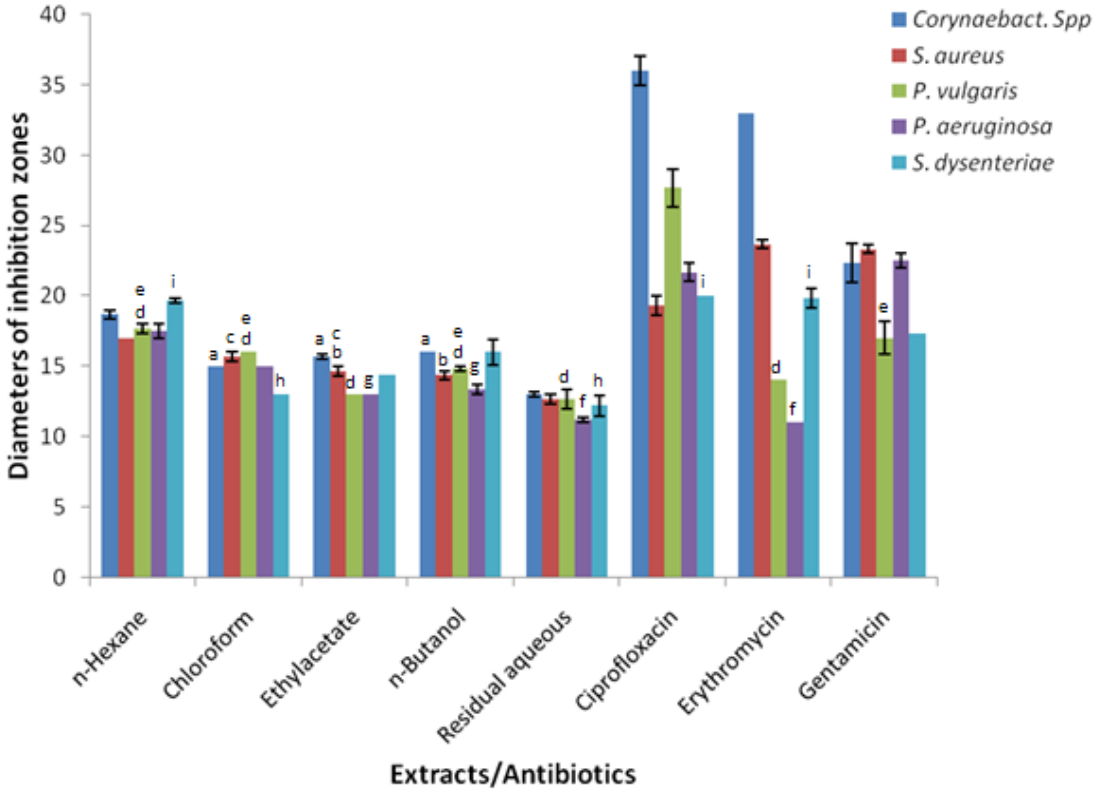

Figure 5: Pattern of Diameters of Inhibition zone of the Partioned Portions of *B. rufescens* Stem Bark Extract against some Pathogenic Bacteria at 1.25 mg/hole; Same Letters on Same Organism's Activity are Insignifcantly (P>0.05) Different.

Table 1: Minimum Inhibitory and Minimum Bactericidal Concentrations of Partitioned Portions of Methanol Stem Bark Extract of *B. rufescens*.

		Partitioned portions of *B. rufescens methanol extract*				
		n-Hexane	Chloroform	Ethylacetate	n-Butanol	Residual aqueous
S/N	Microorganisms	Concentrations (mg/ml)				
1	*Corynaebacterium spp.*	12.5** 6.25*	6.25** 6.25*	3.13** 1.56*	3.13** 1.56*	12.5** 6.25*
2	*Staphylococcus aureus*	6.25** 3.13*	3.13** 3.13*	1.56** 1.56*	6.25** 3.13*	12.5** 6.25*
3	*Proteus vulgaris*	12.5** 6.25*	3.13** 3.13*	6.25** 3.13*	3.13** 1.56*	12.5** 6.25*
4	*Pseudomonas aeruginosa*	12.5** 6.25*	12.5** 6.25*	3.13** 1.56*	1.56** 1.56*	12.5** 6.25*
5	*Shigella dysenteriae*	12.5** 6.25*	6.25** 6.25*	3.13** 3.13*	3.13** 1.56*	25.0** 12.5*

*Key: ** = MBC value* ** = MIC value*

Table 2: Sensitivity Pattern of Partitioned Portions of the Stem bark extract of *B. rufescens* against some Pathogenic Organisms.

Portion	Percent activity			Spectral intensity index			Activity index (%)									
	%G+	%G-	%T	G+	G-	µ	CR		SA		PV		PS		SG	
BRNH	100	100	100	17.71	17.15	17.43	[a]49.28	[b]53.75	[a]91.41	[b]74.65	[a]64.45	[c]104.88	[a]75.99	[c]73.20	[a]89.85	[c]103.69
BRCF	100	100	100	17.72	16.23	16.98	[a]49.82	[b]54.33	[a]90.53	[b]73.93	[a]59.02	[c]96.06	[a]74.45	[c]71.69	[a]84.66	[c]97.69
BREA	100	100	100	17.90	16.27	17.09	[a]53.43	[b]58.27	[a]85.71	[b]70.00	[a]59.75	[c]97.24	[a]73.83	[c]71.11	[a]81.50	[c]94.06
BRNB	100	100	100	17.57	16.70	17.14	[a]52.59	[b]57.36	[a]83.81	[b]68.44	[a]60.95	[c]99.24	[a]76.30	[c]73.47	[a]88.33	[c]101.96
BRRA	90	86.7	85.3	10.78	10.08	10.43	[a]33.53	[b]36.58	[a]49.15	[b]40.14	[a]34.09	[c]55.47	[a]41.07	[c]39.56	[a]56.82	[c]65.55

Key: CR, *Corynaebacterium* spp.; SA, *Staphylococcus aureus;* PV, *Proteus vulgaris;* PS, *Pseudomonas aeruginosa;* SG, *Shigella dysenteriae;* µ, mean; Computed with: a, Ciprofloxacin; b, Erythromycin; c, Gentamicin; BRNH, n-Hexane portion; BRCF, Chloroform portion; BREA, Ethylacetate portion; BRNB, n-Butanol portion; BRRA, Residual aqueous portion; G+, Gram positive; G-, Gram negative; T, total

CONCLUSION

In conclusion, these portioned portions were found to be effective, most especially n-butanol portions which had exhibited a broad-spectrum level of activities against the studied pathogenic microorganisms usually isolated from patients with the aforementioned diseases conditions.

Research is under way in our laboratory to isolate, purify, and characterize the bioactive compound(s) through the bioassay directed protocol. This study has therefore, laid credence to the traditional use of this plant parts in the cure of the diarrhea and related diseases in some parts of Northern Nigeria.

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