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Full Length Research Paper

Comparison of the chemical constituents and *in-vitro* antibacterial efficacies of the extracts of *Croton zambesicus* Muell ARG. stem-bark

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The stem bark of *Croton zambesicus* was extracted using soxlet apparatus with petroleum ether, ethyl acetate, methanol and distilled water respectively. The extracts were screened for chemical components. The results revealed the presence of carbohydrates, saponins, cardiac glycosides, flavonoides, terpenes/steroids and alkaloids. *In vitro* antimicrobial study of the extracts revealed strong broad spectrum activity, especially for methanol extract. This is evident from the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) test results in which the methanol extract had MIC and MBC of 1.560 mg on *Staphylococcus aureus* and *Escherichia coli*, followed by ethyl acetate extract with MIC value of 3.125 mg and 6.25 mg for MBC on *Pseudomonas aeruginosa*. Petroleum ether extract showed the highest activity on *Shigella dysenteriae, S. aureus* and *E. coli* with MIC value 25 mg and 50 mg for MBC while the aqueous extract did not show activity at all. The result of this study provide more basis and credence for the use of this plant in the treatment of ailments whose causative agents are some of the pathogenic microbes used in this study, and thus suggest the possible usefulness of *C. zambesicus* in the treatment of bacterial and fungal infections.

Key words: *Croton zambesicus*, chemical constituents, antimicrobial, efficacies, extracts.

INTRODUCTION

The history of the use of herbal medicine may be as old as the history of man kind. Thus the use of plants as medicine is an ancient practice common to all societies especially the African society (Usman and Osuji, 2007). Despite the level of world's development, the traditional medicinal methods, especially the use of medicinal plants still play a major role in the developing countries of Africa south of the Sahara and more so, the use of herbal remedy have risen in developed countries in the last decades (Kianbakht and Jahaniani, 2003; Usman et al., 2007).

Medicinal plants have been used for traditional treatment of numerous diseases in many parts of the world and in rural areas; they continue to be used as the primary source of medicine (Chitme et al., 2003; Enzo, 2006). Over 80% of the world's population still use plants as their primary source of medicine (Codell, 2000; Kim, 2005) and in Africa particularly in Nigeria, herbal medicine has become part of the people's culture with about 70% depending mainly on traditional medication (Akiniyi and Tella, 1991; Osuinde and Isibor, 1998; Ajoku et al., 2001; Afawodi, 2001; Bauso and Olutimayin, 2001; Geidam et al., 2007). This over dependence is due to high cost of conventional primary health service (Geidam, 2007).

Croton zambesicus muell Arg. is of the *Croton linn* and a member of Euphorbiaceae family (Arbonnier, 2004). It is known as *Koriba* or *Icen maser* in Hausa, *Ajekofola* in Yoruba, *Mfam* in Ekoi (Agishi and Shehu, 2004; Arbonnier, 2004) and *Moramora* in Kilba language (Reuben et al., 2008; 2009a; 2009b; 2009c). Watt and Breyer-Brandwikj, (1962), Adjanohoun et al. (1989) and Okokon et al. (2005) have reported that the decoction

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from the leaves and stem-bark of *C. zambesicus* have been used in folk medicine as anti-hypertensive, anti-microbial (in urinary infection) and treatment of malaria. Other medicinal uses as reported by Abbiw (1990) and Arbonnier, (2004) include smoke of burnt leaf for convulsions due to fits and epilepsy, leaf decoction for diarrhea, dysentery, headaches, measles, high blood pressure, intestinal worms and roots as laxatives.

The aim of the present study is to identify the chemical constituents of the various extracts of *C. zambesicus* and to compare the *in vitro* anti-bacterial efficacies of the extracts against some bacterial organisms with the view of providing validity for its reported folkloric medicinal uses.

MATERIALS AND METHODS

Sample collection and identification

The stem-bark of *C. zambesicus* Muell Arg. was collected in Mubi, Adamawa State, Nigeria. The stem-bark of *C. zambesicus* was authenticated by Prof. S. S. Sanusi and voucher specimen with No. 19052001/2 was deposited at the Department of Biological Sciences, University of Maiduguri, Maiduguri, Nigeria.

Preparation and extraction of plant material

The collected stem-bark of *C. zambesicus* was pulverized using wooden pestle and mortar. The sample was air-dried at room temperature and four hundred grams (400 g) of the pulverized and air-dried plant part was extracted by hot continuous, successive and exhaustive extraction using sohxlet apparatus. Petroleum ether, ethyl acetate, methanol and distilled water respectively were used (gradient extraction). The extracts were concentrated *in vacuo*, weighed and labeled petroleum ether (PEE), ethyl acetate (EAE), methanol (MTE) and aqueous fractions (AQE) respectively, and stored aseptically in the refrigerator at 4°C until use (Trease and Evans, 2002). All work was carried out in accordance with the general guidelines for methodologies on research and evaluation of traditional medicine (WHO, 2000; Geidam et al., 2007).

Phytochemical analysis

The various extracts of *C. zambesicus* were subjected to qualitative chemical screening for identification of the various classes of active chemical constituents (carbohydrates, tannins, phlobatannins, saponins, glycosides, terpenes/steroids, flavonoids, anthraquinones and alkaloids). The phytochemical analysis was done according to standard methods (Harbon, 1993; Sofowora, 1993; Trease and Evans, 2002).

Preparation of microbial cultures

Gram positive, gram negative and fungal laboratory isolates of *Staphylococcus aureus, Streptococcus pyogenes, Shigella dysenteriae, Escherichia coli, Pseudomonas aerugenosa, Proteus vulgaris* and *Candida albicans* were all obtained from human clinical cases at the University of Maiduguri Teaching Hospital (UMTH), Maiduguri, Nigeria. The isolates were propagated and stored on nutrient agar plate. The nutrient agar medium was obtained in dehydrated form (Oxoid Ltd. England) and was prepared according to the manufacturer's specification. All stock cultures were maintained on nutrient agar plate at 4°C and sub-cultured in nutrient broths (Oxoid Ltd. England) at 37°C for 8 h prior to antimicrobial testing. One milliliter of the cultures was then used to flood the agar plate.

Preparation of stock solution of extracts

Stock solutions of the extracts at 500 mg/ml each were prepared by dissolving 1 g of extracts into 2 ml of diluents.

Preparation of nutrient agar plate

Nutrient agar was prepared according to the manufacturer's specification and 25 ml of the agar was poured into sterile Petri dish. This was allowed to solidify and dry. Using a sterile cock-borer of 9 mm diameter, three equidistant holes (wells) per plate were made.

Antimicrobial sensitivity testing

The plate-hole diffusion method as described by Kudi et al. (1999), Ogundepe et al. (2000) and adopted by Reuben et al. (2008, 2009a, 2009b, 2009c) was used to determine the antibacterial activity of the extracts. One milliliter of the broth cultures were used to flood the agar plates. The three equidistant holes (wells) per plate were then filled with different extract solutions at concentrations of 500, 400 and 300 mg/ml respectively. This was done in triplicates and the plates were then incubated at 37°C for 18 h. The antibacterial activities were observed and the zone of growth inhibition measured using a transparent meter rule. Zones of inhibition ≥ 10 mm were recorded (Vlietink et al., 1995; Kudi et al., 1999; Geidam et al., 2007; Reuben et al., 2008, 2009a, 2009b, 2009c).

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the extract fractions of *C. zambesicus* stem-bark were determined using the method adopted by Greenwood (1989), Vollekova et al. (2001), Dahiru et al. (2004), Usman et al. (2005, 2007), Geidam et al. (2007) and Reuben et al. (2008, 2009a, 2009b, 2009c). Ten sterile test tubes were arranged in six rows in a test tube rack, each row for one of the six microorganisms used for the test and 4 ml of sterile nutrient broth was poured into all the tubes using pipette. In addition, 1 ml of the stock extract solution at concentration of 500 mg/ml was diluted to 5 ml to give concentration of 500 mg/ml and 4 ml of these was dispensed into the first test tube containing 4 ml of nutrient broth. This made concentration of 400 mg/ml. Thereafter, there was a serial dilution of the extracts in each row to obtain concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 mg/ml, respectively. The test organisms (0.2 ml) were put into each of the test tubes using a pipette and incubated at 37°C for 24 h. Then MIC was determined and recorded as the lowest concentration of the extracts that completely inhibited the growth of the test organisms by showing no turbidity. The content of the tubes were further sub-cultured for 24 hrs to determine minimum bactericidal concentration (MBC). This effect was demonstrated when no growth occurred on the sub-cultured medium after MIC determination.

RESULTS

Table 1 present the result of the form and color of the extracts of *C. zambesicus*. The result revealed higher yield of the methanol soluble fraction of the extracts

Table 1. The yields of the various extracts of *C. zambesicus* Muell Arg. stem bark.

	Extracts			
	Petroleum ether	**Ethylacetate**	**Methanol**	**Distilled water**
Color	Yellowish	Dark brown	Brown	Brown
Form	Oily paste	Mass	Mass	Mass
Extract weight (g)	17.197	50.56	52.023	26.4
Yield w/w (%)	4.30	12.64	13.01	6.6

52.023 g (13.01% w/w) followed by ethyl acetate fraction (50.563 g, 12.64% w/w), the aqueous soluble fraction (26.40 g, 6.60% w/w) and petroleum ether fraction (17.197 g, 4.30% w/w). The result of the phytochemical screening of the various extract fractions of *C. zambesicus* is as presented in Table 2. The result revealed the presence of carbohydrates, cardiac glycosides, saponins, flavonoids, alkaloids, terpenoids and steroids.

The result of the *in vitro* antimicrobial sensitivity test is as presented in Table 3. The result shows that *S. aureus, S. pyogenes, E. coli* and *P. aeruginosa* are susceptible to various concentrations of petroleum ether; ethyl acetate and methanol extract fractions. *S. dysenteriae* showed susceptibility to the petroleum ether and methanol fractions but resistance to the ethyl acetate extract. *P. vulgaris* exhibited resistance to all the extracts while *C. albican* showed resistance to the petroleum ether fraction, but susceptibility to ethyl acetate and methanol fractions. All the test organisms exhibited resistance to the aqueous extract.

The result of the MIC test is as presented in Table 4. The least MIC value for petroleum ether extract was observed against *S. dysenteriae*, *S. aureus* and *E. coli* followed by *P. aeruginosa* and then *S. pyogenes*. The ethyl acetate extract showed least MIC for *P. aeruginosa* followed by *C. albicans, S. pyogenes* and *S. aureus* and finally *E. coli*. The methanolic extract exhibited the least MIC for *S. aureus* and *E. coli* followed by *P. aeruginosa,* the *S. dysenterea* and *C. albicans,* while *S. pyogenes* showed the least MIC value.

The result of the MBC test is presented in Table 5. The result revealed least MBC value in petroleum ether extract against *S. dysenteriae, S. aureus* and *E. coli*, followed by *S. pyogenes* and *P. aeruginosa*. The ethyl acetate fraction showed least MBC against *P. aeruginosa* followed by *C. albicans*, then *S. pyogenes* and finally *S. aureus* and *E. coli*. The methanol extract fraction showed least MBC against *S. aureus* and *E. coli* followed by *P. aeruginosa,* then *S. dysenterea* and *C. albicans* and finally *S. pyogenes.*

DISCUSSION

The result of the phytochemical screening of the various extracts of *C. zambesicus* revealed the presence of carbohydrates, alkaloids, flavonoids, saponins, cardiac glycosides, terpenes and steroids. Phytochemicals are generally said to be non -nutritive plant chemicals that have protective or disease preventive properties. They are reported to protect the plants, but recent research demonstrates that they can protect humans against diseases. Some of the compounds that have been revealed in the plant under study, especially saponins, flavonoids, terpenes and steroids have been reported to have antibacterial/curative properties against several ailments whose causative agents are some of the organisms studied (Nweze et al., 2004; Hassan et al., 2004; Sartorato et al., 2004; Nwaogu et al., 2007; Usman et al., 2007) and so the activities observed in the present study is not unconnected with the presence of these phytochemicals. The *in vitro* antimicrobial assay of the extracts, which revealed various zones of inhibition of bacterial and fungal organisms indicate a broad spectrum antimicrobial property of the extracts.

The strength of the antibacterial efficacy of the extracts is expressed by the MIC and MBC respectively. Thus, the strongest activity for petroleum ether extract fraction was shown on *S. dysenteriae, S. aureus* and *E. coli* respectively with MIC of 25 mg/ml and MBC of 50 mg/ml while ethyl acetate extract had its own on *P. aeruginosa* with MIC of 3.125 mg and MBC of 6.25 mg. The methanol extract fraction contains more strong bioactive constituents like the saponins, flavonoids, terpenes/ steroids all in moderate concentration. It therefore has its strongest activity on two bacterial species; one gram positive *S. aureus* and one gram negative *E. coli* with MIC and MBC value of 1.560 mg. All the others have MIC's and MBC' s above 12.5 mg except for *P. aeruginosa* which have MIC and MBC value of 12.5 mg. The high activity observed of the methanol extract with as low MIC and MBC value of 1.56 mg is not unconnected with the moderate presence of saponins, terpenes/steroids and flavonoids which have been associated with antimicrobial activity and in addition, the synergistic effect of these phytochemicals might also have contributed immensely to make the extract more active than the others, more so that saponin is absent in petroleum ether and ethyl acetate fractions (Erich, 1977; Hammer, 1999). It was also observed that the methanol extract have stronger antimicrobial activity, followed by

Table 2. The results of phytochemical screening of extracts of *Croton zambesicus* Muell Arg. stem bark.

S/N	Constituents/test	Pet. ether extract	Ethylacetete extract	Methanol extract	Aqueous extract
1.	**Alkaloids**				
	i. Dragendorff's test	-	-	+ +	+ +
	ii. Meyer's test	-	-	+ +	+ +
2.	**Carbohydrates**				
	i. Molisch' test	-	-	+	+ +
	ii. Barford's test	-	-	-	-
	iii. Fehling's test (reducing sugar test)	-	-	+ +	+ +
	iv. Fehling's test (comb red. sugar test)	-	-	+ +	+ +
3.	**Cardiac glycosides**				
	Killer-Killani's test	-	-	+	+ +
4.	**Flavonoids**				
	i. Shinoda's test	-	+	-	+ +
	ii. $FeCl_3$ test	-	-	+ +	+ +
	iii. Pew's test	-	-	+ +	+ +
5.	**Saponins**				
	Frothing test	-	-	+ +	+ +
6.	**Steroidal nucleus (terpenes, steroids)**				
	i. Salkwoski's test	+ +	+ +	+ +	+
	ii. Liberman-Burchard's test	+ +	+ +	+ +	+
7.	**Tannins**				
	I. $FeCl_3$ test	-	-	-	-
	ii. Lead acetate test	-	-	-	-

- = negative (absent), + = positive (slightly present),++ = positive (moderately present).

Table 3. The result of *in vitro* antimicrobial sensitivity testing of extracts of *C. zambesicus* Muell Arg. stem bark.

		Zones of inhibition (mm) (X ± SEM)						
Extracts	**Conc.(mg)**	**Sd**	**Sa**	**Sp**	**Ec**	**Pa**	**Pv**	**Ca**
Petroleum ether	500	29.3 ± 1.53	20.0 ± 1.00	33.3 ± 0.58	24.3 ± 1.15	21.3 ± 2.08	R	R
	400	25.3 ± 0.58	17.0 ± 0.05	30.0 ± 0.00	23.0 ± 2.16	19.0 ± 1.73	R	R
	300	22.6 ± 0.58	15.2 ± 0.29	27.0 ± 0.00	19.7 ± 0.58	14.7 ± 0.58	R	R
Ethyl acetate	500	R	20.3 ± 0.58	25.0 ± 0.50	31.0 ± 1.00	33.3 ± 0.58	R	18.2 ± 0.29
	400	R	18.0 ± 1.00	22.0 ± 0.00	23.0 ± 2.33	31.3 ± 1.15	R	17.3 ± 0.57
	300	R	15.0 ± 0.29	20.0 ± 0.00	20.0 ± 0.00	30.3 ± 1.15	R	15.5 ± 0.58
Methanol	500	30.0 ± 0.00	30.0 ± 0.00	31.5 ± 0.50	30.0 ± 0.00	31.7 ± 2.89	R	30.0 ± 0.00
	400	27.7 ± 0.58	30.0 ± 0.00	27.3 ± 0.50	28.3 ± 1.53	29.0 ± 1.00	R	29.7 ± 0.00
	300	25.0 ± 0.00	28.7 ± 0.58	23.0 ± 0.00	27.0 ± 0.00	26.7 ± 2.89	R	25.3 ± 1.53
Aqueous	500	R	R	R	R	R	R	R
	400	R	R	R	R	R	R	R
	300	R	R	R	R	R	R	R
Tetracycline	25	10	25	28	12	10	NT	13

Sd = *Shigella dysenterIae*; Sa = *Staphylococcus aureus*; Sp =*Streptococcus pyogenes*; Ec =*Escherichia coli*; Pa =*Pseudomonas aeruginosa*; Pv = *Proteus vulgaris*; Ca = *Candida albicans*, R = resistance (+ve); NT = not tested; Control = tetracycline 25 mg, n = 3.

Table 4. Minimum inhibitory concentration (MIC) of extracts *C. zambesicus* Muell Arg. stem bark against some microbial isolates.

Extract	Organism	Extract conc. (mg/mL)								
		0.780	1.560	3.125	6.25	12.5	25	50	100	200
Petroleum ether	Sd	-	-	-	-	-	β	+	+	+
	Sa	-	-	-	-	-	β	+	+	+
	Sp	-	-	-	-	-	-	-	β	+
	Ec	-	-	-	-	-	β	+	+	+
	Pa	-	-	-	-	-	-	β	+	+
Ethyleacetate	Sa	-	-	-	-	-	-	β	+	+
	Sp	-	-	-	-	-	β	+	+	+
	Ec	-	-	-	-	-	-	β	+	+
	Pa	-	-	β	+	+	+	+	+	+
	Ca	-	-	-	β	+	+	+	+	+
Methanol	Sd	-	-	-	-	-	β	+	+	+
	Sa	-	β	+	+	+	+	+	+	+
	Sp	-	-	-	-	-	-	β	+	+
	Ec	-	β	+	+	+	+	+	+	+
	Pa	-	-	-	β	+	+	+	+	+
	Ca	-	-	-	-	-	β	+	+	+

Sd =*Shigella dysenterea*; Sa = *Staphilococcus aureus*; Sp =*Streptococcus pyogenes*; Ec =*Escherichia coli*; Pa =*Pseudomonas aerugenosa*; Ca =*Candida albicans*. - = Resistance (growth of microbes/turbidity), β = Minimum inhibitory concentration, that is, MIC (least extract concentrations that inhibit microbial growth/turbidity), + = Extract concentrations that inhibit microbial growth/turbidity.

Table 5. Minimum bactericidal/fungicidal concentration (MBC/MFC) of extract preparations of *Croton zambesicus* Muell Arg. Stem bark against some microbial isolates.

Extract	Organism	Extract conc. (mg/mL)								
		0.780	1.560	3.125	6.25	12.5	25	50	100	200
Petroleum ether	Sd	-	-	-	-	-	-	β	+	+
	Sa	-	-	-	-	-	-	β	+	+
	Sp	-	-	-	-	-	-	-	β	+
	Ec	-	-	-	-	-	-	β	+	+
	Pa	-	-	-	-	-	-	-	β	+
Ethylacetate	Sa	-	-	-	-	-	-	-	β	+
	Sp	-	-	-	-	-	-	β	+	+
	Ec	-	-	-	-	-	-	-	β	+
	Pa	-	-	-	β	+	+	+	+	+
	Ca	-	-	-	-	β	+	+	+	+
Methanol	Sd	-	-	-	-	-	β	+	+	+
	Sa	-	β	+	+	+	+	+	+	+
	Sp	-	-	-	-	-	-	β	+	+
	Ec	-	β	+	+	+	+	+	+	+
	Pa	-	-	-	β	+	+	+	+	+
	Ca	-	-	-	-	-	β	+	+	+

Sd =*Shigella dysenterea*; Sa = *Staphylococcus aureus*; Sp =*Streptococcus pyogenes*; Ec =*Escherichia coli*; Pa =*Pseudomonas aeruginosa*; Ca =*Candida albicans*, - = Resistance (growth of microbes/turbidity), β = Minimum bactericidal/fungicidal concentration, that is, MBC/MFC (least extract concentration that kill bacteria/fungi). + = Extract concentrations that kill bacteria/fungi.

ethyl acetate and petroleum ether fraction. The aqueous extract did not show any antimicrobial activity. The present study further substantiates earlier reports by Abbiw, (1990); Abo et al. (1999); Arbonnier et al. (2004) that the stem bark of *C. zambesicus* has antibacterial property and as well for its use in treatment of diarrhea and dysentery in folklore medicine.

Conclusion

The phytochemical analysis of *C. zambesicus* revealed the presence of pharmacologically active constituents which may explain the basis for the strong antimicrobial activity observed especially with the methanol fraction. The result of these studies has provided more basis and credence for the use of this plant in treatment of ailments whose causative agents are some of the pathogenic microbes used in this study, and thus suggest the possible usefulness of *C. zambesicus* in the treatment of bacterial and fungal infections. Finally, we suggest that further research work on the purification, characterization and structure elucidation of the constituents of the extracts be done with the view of getting the molecular and structural formula of the bioactive constituents as they may serve as novel compounds for the synthesis of new drugs with better therapeutic value.

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