Phytochemical Screening and Antimicrobial Studies of Ethyl Acetate Extract of *Croton zambesicus* Muell Arg. Stem Bark.

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ABSTRACT

The pulverized stem bark of *Croton zambesicus* was subjected to gradient extraction with soxlet and the ethyl acetate extract was phytochemically screened for chemical composition. The result revealed the presence of terpenes and steroids in moderate concentrations, cardiac glycoside and flavonoids in slight concentration. *In vitro* antimicrobial assay of the extract using agar plate hole diffusion and nutrient broth dilution techniques revealed the extract to have broad spectrum activity on gram positive and gram negative organisms and a fungal strain. The highest activity was shown on *P. aerugenosa* with MIC of 3.125 mg/ml and MBC of 6.25mg/ml and the lowest activity were shown on *S. aureus* and *E. coli* with MIC value of 50 mg/ml and MBC of 100 mg/ml. This study provides some scientific base for the folkloric medicinal use of this plant as remedy for ailments whose causative agents are the pathogens studied. The activities observed could also be attributed to the phytochemicals detected, some of which have been associated with antibacterial activity.

(Keywords: antimicrobial studies, *Croton zambesicus*, ethyl acetate, phytochemical screening)

INTRODUCTION

Natural substances of botanical origin have been used throughout the world for human and animal health care (Sexana, 2003 and Enzo, 2006), especially in Africa. Thus plants are a source of medicine in Africa and almost all developing countries of the world. Herbs and herbal usage has become a global issue in recent times, especially due to the high poverty level and the cheapness of herbal medicines and the belief that it has minimum or no side effects, (Igoli, *et al.,* 2005).

The lack of apparent resistance development by pathogens to the phytochemicals present in herbal preparations as well as their good absorbance and distribution to the area of infection [perhaps due to the fact that apart from alkaloids, they are mostly compounds associated to sugar (glycon) moiety which is absorbed and distributed in the system without problem] thus carrying the active phytochemicals (aglycone) in the infected areas, make these compounds even more attractive (Odama *et al.* 1997and Olutimeyin *et al*.2001). Recent studies have confirmed the efficacy of some of these herbal remedies (Lal *et al*. 1994 and Singh, 1994).

Croton zambesicus muell Arg.(Keay, 1989 and Arbonnier, 2004) commonly known as *Koriba* or *Icen maser* in Hausa; *Ajekofole* in Yoruba; *Mfam* in Ekoi (Agishi and Shehu, 2004); and *Moramora* in Kilba belongs to the *Euphorbiaceae* family. It is a shrub of 10-16m high, often branching low down with a spreading crown and characteristic hanging leaves, silvery beneath. The bark is whitish to pale gray, slash thin and yellowish with a strong pharmaceutical smell. Flowering usually occurs at the beginning of the dry season. It inhabits the Sudan and Guinea Savanna zones and is distributed from Cameroon to tropical Africa (Arbonnier, 2004). It is one of the plants that has wide application in African folkloric medicine. Some of the uses include leaf decoction as antimicrobial (to treat infection), antihypertensive, and in treating fever associated with malaria. The leaf alkaloidal fraction has been

reported to possess a wide spectrum of antibacterial property (Arbonnier,2004 and Okokon *et al. (* 2004, 2005).

C. zambesicus which has wide application in African folkloric medicinal usage has some of the sugar attachments (aglycons) to be therapeutically effective on several microbes. This study therefore is aimed at investigating the phytochemical constituents and ascertaining the *in vitro* effects of the ethyl acetate extract on some microbes with a view to getting a better natural therapeutic agent that could be a solution to cases of resistance by pathogens to some of the chemotherapeutic agents as has been reported by Shalit *et al*., (1989) and Usman *et al*. (2007) that prevalence of *S. aureus*-resistant strains to conventional antibiotics have increased in recent times in some hospitals.

MATERIALS AND METHODS

Plant Collection and Identification: The plant parts of *C. zambesicus* Muell Arg. were collected in Mubi (Long. 13^{o} $15^{/}$ E, Ltd.10^{o} $15^{/}$ E) located in Adamawa State, Nigeria and was authenticated by Prof. S.S. Sanusi of the Biological Sciences Department, University of Maiduguri, Maiduguri, Nigeria.

Preparation and Extraction of Plant Material: The stem bark of *C. zambesicus* was gabled for removal of adultrants and pulverized. It was air-dried at room temperature and four hundred grams (400 g) of the pulverized stem bark was exhaustively defatted and successively soxlet-extracted with petroleum ether (60-80^{O}C), ethyl acetate, methanol, and distilled water, respectively. The extracts were concentrated *in vacuo* and a dark brown curd 12.64% (w/w), coded EAE (ethyl acetate extract), was kept until use.

Phytochemical Screening: The EAE was screened phytochemically for the presence of its chemical constituents using standard procedures of analysis (Harborne, 1993; Sofowora, 1993; and Trease and Evans, 2002).

Test Organisms: The bacterial and fungal microbes used include *Staphylococcus aureus (gram +ve), Streptococcus pyogenes (gram +ve), Shigella dystenteriae (gram -ve), Escheriua coli (gram –ve), Pseudomonas aeruginosa (gram -ve), Proteus vulgaris (gram –ve),* and *Candida albicans* fungus. All species were isolates obtained from human clinical cases at the University of Maiduguri Teaching Hospital (UMTH) Maiduguri, Nigeria. Bacteria were maintained at 4^{o}C on nutrient agar plates before use. The fungus was maintained on inhibitory mould agar at room temperature.

Antibacterial and Antifungal Activity: The plate-in-hole diffusion assay as described by Onoruvwe and Olorunfemi, 1998; Hugo and Russell, 1998; and Ogundipe *et al*. 2000 was used to determine the growth inhibition of bacteria by the plant extract (EAE). The tests were carried out by using a stock concentration of 500 mg/ml prepared by dissolving 1.0 g of the extract in 2 ml of distilled water.

Nutrient agar was prepared and 25 ml each was pored into sterile petri dishes. This was allowed to solidify and dry. Using a sterile cork-borer of 9 mm diameter, three holes (cups) per plate were made in the agar and they were inoculated with 0.5ml over night suspension of the bacteria/fungus. Thereafter, the wells (holes) were filled with the extract solution at varying concentrations of 500 mg/ml, 400 mg/ml, and 300 mg/ml, respectively. This was done in triplicate and the plates were inoculated at 37^{o}C for 18hrs. The antibacterial/antifungal activities were observed and measured using a transparent ruler and recorded if the zone of inhibition was 10 mm (Vlietinck *et al*., 1995).

Minimum Inhibitory Concentration (MIC): MIC is defined as the lowest concentration where no visible turbidity is observed in the test tube. Vollekova *et al*. (2001) method with modification by Usman *et al*. (2007) was employed. In this method, the broth dilution technique was utilized where the plant extract was prepared to the highest concentration of 500 mg/ml (stock concentration) in sterile water and serially diluted ranging from 0.780 mg/ml to 200 mg/ml using nutrient broth and later inoculation with 0.2 ml suspension of the test organisms.
After 18 hrs of incubation at 37^{o}C, the test tubes were observed for the presence of turbidity. The least concentration where no turbidity was observed was determined and noted as the minimum inhibitory concentration.

Minimum Bactericidal Concentration (MBC) Minimum Fungicidal Concentration (MFC): The MBC/MFC is defined as the lowest concentration where no bacterial/fungal growth is

observed. This was determined from the broth dilution resulting from the MIC tubes by sub-culturing to antimicrobial free agar as described by Vollekova *et al*. (2001) and Usman *et al*. (2007). The contents of the test tubes from MIC were streaked using a sterile wire loop on antimicrobial free agar plate and incubated at 37^{o}C for 18 hrs. The lowest concentration of the extract which showed no bacterial/fungal growth was noted and recorded as the MBC or MFC value.

RESULTS

The preliminary phytochemical screening of the EAE is shown in Table 1. This reveals the presence of cardiac glycosides, flavonoids, terpene and steroids.

The *in vitro* antimicrobial screening result is as presented in Table 2. It shows the susceptibility of gram positive and gram negative organisms and a fungal strain.

The result of the minimum inhibitory concentration assay is as presented in Table 3. It reveals the concentrations of the extract which can inhibit the growth of the bacteria or fungal species under test. (bacteriostatic and fungistatic concentrations).

The result of the minimum bactericidal/fungicidal concentration assay is as shown in Table 4. It also reveals the concentrations of the extract which is bactericidal and fungicidal to the organisms.

Table 1: Phytochemical Screening of Ethyl Acetate Extract (EAE) of *Croton zambesicus* Muell Arg. Stem Bark.

S/No.	Constituents/Test	Ethyl Acetate 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(combined reducing sugar test)	_
3.	Cardiac glycosides	
	Killer-killani's test	+
4.	Flavonoids	
	i. Shinoda's test	+
	ii. $FeCl_3$ test	_
	iii. Pew's test	_
	iv. Sodium hydroxide test	+
5.	Saponins	
	Frothing test	_
6.	Steroidal nucleus(terpenes/steroids)	
	i. Salkowski test	+ +
	ii. Libarman-Burchard's	+ +
7.	Tannins	
	i. $FeCl_3$ test	_
	ii. Lead acetate test	_

Key: - = *Negative (absent)*
+ = *Positive (slightly present)*
++ = *Positive (moderately present)*

Table 2: *In vitro* Antimicrobial Susceptibility of Ethyl Acetate Extract of *Croton zambesicus* Muell Arg. Stem Bark.

Parameters	Extract Conc. mg/ml	Zone of Inhibition (mm)						
		S.d.	S.a.	S.p.	E.c.	P.a.	P.v.	C.a.
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
Extract (EAT)	400	R	18.0±0.00	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
Ethylacetate	1ml	R	R	R	R	R	R	R
TCN	25	10.0±0.00	25.0±0.00	28.0±0.00	12.0±0.00	10.0±0.00	N.T	13.0±0.00

Key:
S.d.=*S. dysenteriae*: S.a.=*S. aureus*: S.p.=*S. pyogenes*: E.c.=*E. coli*
P.a.=*P. aeruginosa*: P.v.=*P. vulgaris*: C.a.=*C. albicans*
N.T.=Not tested: R=Resistant (-ve); TCN (Tetracycline)=control
All data except TCN were mean of 3 values (x± SEM)

Table 3: Minimum Inhibitory Concentration (MIC) Values for Bacterial Isolates Against Ethyl Acetate Extract of *Croton zambesicus* Muell Arg. Stem Bark.

Bacteria	Extract concentrations(mg/ml)								
	0.780	1.560	3.125	6.25	12.5	25	50	100	200
S.d.	-	-	-	-	-	-	-	-	-
S.a.	-	-	-	-	-	-	β	+	+
S.p.	-	-	-	-	-	β	+	+	+
E.c.	-	-	-	-	-	-	β	+	+
P.a.	-	-	β	+	+	+	+	+	+
C.a.	-	-	-	β	+	+	+	+	+

Key:
S.d.=S. dysentarea: S.a.=S. aureus: S.p.=S. pyogenes: E.c.=E. coli
P.a.=P. aeruginosa: P.v.=P. vulgaris: C.a.=C. albicans
- =Resistance (growth of bacteria or turbidity)
+ =Concentrations showing no turbidity (inhibition of bacterial growth)
β = Least concentration showing no turbidity (MIC)

Table 4: Minimum Bactericidal Concentration (MBC) Values for Bacterial Isolates Against Ethyl Acetate of *Croton zambesicus* Muell Arg. Stem Bark.

Bacteria	Extract concentration(mg/ml)								
	0.780	1.560	3.125	6.25	12.5	25	50	100	200
S.d.	-	-	-	-	-	-	-	-	-
S.a.	-	-	-	-	-	-	-	β	+
S.p.	-	-	-	-	-	-	β	+	+
E.c.	-	-	-	-	-	-	-	β	+
P.a.	-	-	-	β	+	+	+	+	+
C.a.	-	-	-	-	β	+	+	+	+

Key:
S.d.=*S. dysenteriae*: S.a.= *S. aureus*: S.p.= *S. pyogenes*: E.c.=*E. coli*
P.a= *P. aeruginosa*: P.v.= *P. vulgaris*: C.a.=*C. albicans*
- =Resistance(growth of bacteria /fungi)
+ =Bactericidal/fungicidal concentrations
β= Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

DISCUSSION

The preliminary phytochemical screening of the ethyl acetate extract showed moderate presence of terpenes and steroids which are mainly present in essential oils whose antibacterial activities have been recognized for many years (Hammer *et al*., 1999). There was also slight presence of cardiac glycosides and flavonoids in the extract which might be responsible for the antibacterial property as flavonoids play a major role in bacterial inhibition (Wunwisa and Areeya, 2005).

The essential oils which contain the terpenes and steroids with other compounds have been known to have diverse therapeutic uses such as the management of upper respiratory tract, urinary tract, and digestive tract infections, Franstick,(1991). The combined effects (synergistic effect) of these phytochemicals are not unconnected to the broad spectrum activity exhibited by this extract. The *in vitro* antibacterial screening of the extract showed considerable inhibition against almost all the test organisms except for *S. dysenteriae* and *P. vulgaris* that showed resistance at all concentrations of the extract. It can also be observed that the extract exhibited greater zone of inhibition on *E. coli, P. aeruginosa* and *C. albicans* even at the lowest concentration as compared to the standard drug even though their concentrations are not comparable (Table 2).

The minimum inhibitory concentration assay also reveals that *P. aeruginosa* and *C. albicans* with MIC's of 3.125 mg/ml and 6.25 mg/ml are most strongly inhibited by the extract, buttressing the low MIC values as against *S. aureus* and *E. coli* with MIC value of 50 mg/ml each which are moderately inhibited (Table 3).

The highest bactericidal effect is shown on *P. aeruginosa* with lowest MBC value of 6.25 mg/ml followed by fungicidal concentration of 12.5 mg/ml for *C. albicans* and the least bactericidal effect had been shown on *S. aureus* and *E. coli* with high MBC value of 100 mg/ml, this concentration may even be toxic (Table 4).

On general consideration, the terpenes, steroids, flavonoids, and cardiac glycosides are all compounds known to have antimicrobial and

curative properties against several bacterial pathogens (Nweze *et al*., 2004; Hassan *et al*., 2004; Nwaogu *et al*., 2007; and Usman *et al*., 2007). From the MIC, MBC, and MFC results, it can be summarized that the extract has a broad spectrum of activity against both grams positive and negative organisms and a fungal strain. This result therefore gives a scientific base and credence to the claims of the therapeutic capabilities and folkloric usage of the plant for the treatment of various ailments. In addition, the study provides some validity for the use of the plant in African traditional medicine and probably as a source of chemotherapeutic agents.

CONCLUSION

The phytochemical analysis has revealed the presence of cardiac glycosides, flavonoids, terpenes, and steroids, some of which have been associated with antibacterial properties. The result of the *in vitro* antibacterial studies shows that the extract possesses antibacterial and antifungal properties. This is evident from the results of susceptibility test assay, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC). From the result of this study, there is validity for the folkloric usage of the plant in the treatment of ailments caused by these pathogenic micro organisms. This suggests the possible usefulness of *C. zambesicus* in the treatment of bacterial and fungal infections.

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