Phytochemical and pharmacological analysis of C*roton macrostachys* roots

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The 80% aqueous ethanol extract of *Croton macrostachys* A. Rich (Euphorbiaceae) roots and the six isolated compounds were tested for oral glucose tolerance test, antibacterial activity and brine shrimp lethality test. The 80% aqueous ethanol extract significantly escalated postprandial blood glucose levels ($P \leq 0.05$) in mice. The extract also exhibited a weak antibacterial activity against some Gram-positive and Gram-negative bacteria and a weak antifungal activity against *Candida albicans*. Among the six isolated compounds, one compound significantly elevated postprandial blood glucose ($P \leq 0.05$), while two compounds exhibited a weak antibacterial activity, but none exhibited antifungal activity. The crude extract and compounds were both cytotoxic to brine shrimps.

Key words: *Croton macrostachys*; hyperglycaemia; antimicrobial; cytotoxicity

INTRODUCTION

Croton macrostachys A. Rich (Euphorbiaceae) is an evergreen medium-size shrub which is widely encountered in south-east Africa, and particularly common in Tanzania. It is among plants listed by Zigua traditional healers in Handeni District (Tanga region), for the treatment of symptoms within the expanded diabetes diagnostic criteria [1]. The plant is used in different parts of East Africa for the treatment of malaria [2, 3, 4] stomachache and dysentery [3, 5] and as an ascaricide and taenicide [2, 5, 6]

It is also used to stop bleeding in child birth [5], for treatment of cough [3], rheumatism [7], stomachache and as a purgative in cases of ascariasis [3, 7, 8]. Other uses include treatment of gonorrhoea, ringworm infestation, haemorrhoids [8] and venereal diseases [3]. The seeds are eaten to induce abortion [6], while the seed oil is used as a purgative [3]. An extract of the stem bark showed mitogenic activity on human lymphocytes and mice spleen lymphocytes [9]. Both methanol and dichloromethane extracts of the leaves and stem gave positive antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi [10, 11]. Similarly extracts of the plant possess molluscicidal activity [12, 13, 14]. Crotepoxide, a cyclohexane diepoxide, with antitumor activity against the rat models of Lewis lung carcinoma and sarcoma was isolated from a 95% ethanol extract of the seeds [15, 24]. Lupeol and betulin, were also reported from the stem bark [16].

This study is a continuation of a previous study [17] in which the authors reported the isolation of the compounds 3β-acetoxy taraxer-14-en-28-oic acid (**1**), trachyloban-19-oic acid (**2**), trachyloban-18-oic acid (**3**), neoclerodan-5,10-en-19,6β;20,12-diolide (**4**), 3α,19-dihydroxytrachylobane (**5**), and 3α,18,19-trihydroxytrachylobane (**6**) (Figure 1). This paper is a report of some biological activities of an 80% ethanol extract of the root bark and of six compounds isolated from the root bark.

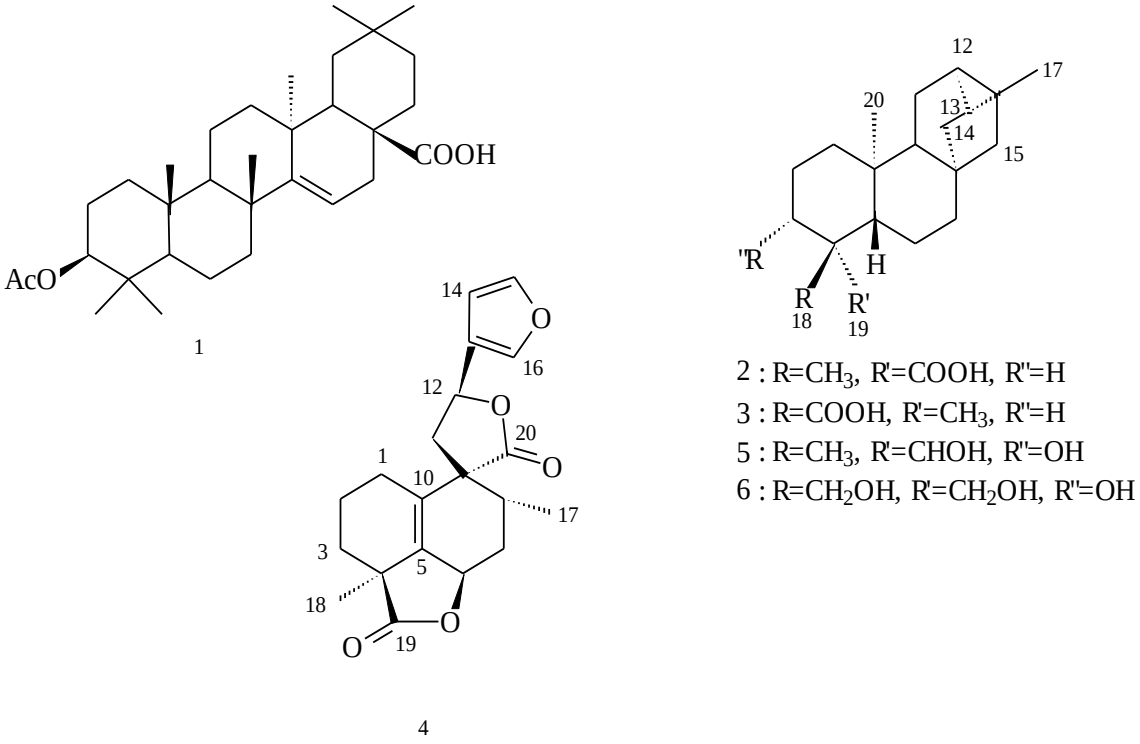

Figure 1: Structures of compounds isolated from *C. macrostachys*

MATERIALS AND METHODS

Plant material

The roots of *C. macrostachys* were collected from Msaje village, Handeni District, Tanga region, Tanzania by Dr M.J. Moshi and identified by the botanist, E.B. Mhoro. Voucher specimen No. MJ-53 was deposited at the Herbarium of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania.

Reagents and solvents

Petroleum ether, ethyl acetate, and ethanol were purchased from Fisher Scientific UK Ltd (Bishop Meadow Road, Loughborough, Leicestershire, LE 11 5RG, UK). Saboraud's dextrose agar (SDA) and Mueller Hinton agar were purchased from Oxoid Ltd (Basingstoke, Hampshire, England), while dimethylsulfoxide (DMSO) was from Sigma (Poole, Dorset, England). Brine shrimp eggs were bought from O.S.I. Marine Lab. Inc., 3550 Arden Road, Hayward, CA 94545, USA. Sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast, Tanzania.

Extraction and isolation

Air-dried roots (500 g) were ground into small particles and extracted with 80% aqueous ethanol. The filtrate was concentrated *in vacuo*, and freeze-dried to afford 140 g of residue, from which 5 g were chromatographed on silica gel using petroleum ether and increasing proportion of ethyl acetate. Six compounds, 3β-acetoxy taraxer-14-en-28-oic acid (**1**), trachyloban-19-oic acid (**2**), trachyloban-18-oic acid (**3**), neoclerodan-5,10-en-19,6β;20,12-diolide (**4**), 3α,19-dihydroxytrachylobane (**5**) and 3α,18,19-trihydroxytrachylobane(**6**) were

isolated from the crude 80% ethanol extract as reported previously [17]. Pure compounds were identified using both physical and spectroscopic techniques together with comparing to previously reported data [17].

Assays

The brine shrimp larvae were used to test extracts and isolated compounds for cytotoxic activity. Testing was performed as previously described [18]**,** whereby cyclophosphamide was used as a standard test drug.

Antibacterial and antifungal activities of extract and isolated compounds were done using the disc-diffusion method [19]. Six standard bacteria, *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (NCTC 10662*), Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhi* (NCTC 8385) and *Bacillus anthracis* (NCTC 10073) were used while the fungi, *Candida albicans* (Strain HG 392) and two local strains of *Aspergillus niger* and *Aspergillus fumigatus* were included. The standard drugs, ampicillin and gentamicin (for bacteria) and clotrimazole (for fungi) were used for comparison.

The *in vivo* assay (oral glucose tolerance test) was performed as described in the literature [20, 21].

Data analysis

The mean results of brine shrimp mortality against the logarithms of concentrations were plotted using the Fig P computer program (Biosoft Inc, USA), which also gives the regression equations. The regression equations were used to calculate LC_{16}, LC_{50} and LC_{84} values. The confidence intervals (95% CI) were calculated according to the method of Litchfield and Wilcoxon [22].

For the antimicrobial activities, the inhibition zones were calculated as the difference between disc diameter (5 mm) and the diameters of inhibition[23] (Hewitt and Vincent, 1989). The mean inhibition zones were used to calculate the activity index. Activity index (AI) was calculated as the mean inhibition zone for test sample divided by the mean inhibition zone for the standard drug [19].

The data for glucose tolerance was analyzed using one-way analysis of variance (ANOVA) for repeated measurements. The Neuman-Keuls range test was used to determine differences at each point. Differences at each point were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Brine shrimp lethality test

The 80% aqueous ethanol extract of *C. macrostachys* was toxic to brine shrimps, giving an LC_{50} value of 13.4 (8.7-20.6) µg/ml. Compounds **2**, **3**, **4**, **5** and **6** exhibited toxicity to the brine shrimps, giving LC_{50} values of 9.5 (6.6-14.7), 4.4 (3.2-6.1), 9.6 (5.6-16.4), 27.6 (17.2-44.4) and 10.3 (6.6-16.1) µg/ml, respectively. Cyclophosphamide, which was tested in the same battery, gave an LC_{50} 16.3 (10.6-25.2) µg/ml.

Antimicrobial activity

Table 1 shows that the 80% aqueous ethanol extract of *C. macrostachys* roots exhibited a weak antibacterial activity against *S. aureus* and *S. typhi*. It also exhibited a weak antifungal activity against *C. albicans*. Among the six compounds which were isolated, compound **2** and **3** exhibited antibacterial activity against *S. typhi* and *B. anthracis*, while the rest were inactive.

Table 1: Antimicrobial activity of crude extract and compounds of *C. macrostachys* roots[a].

			Isolated compounds						Standard compounds		
Organisms tested	**IZ AI**	**AE**	**1**	**2**	**3**	**4**	**5**	**6**	**Clotrimazole (20 µg/disc)**	**Gentamicin (10 µg/disc)**	**Ampicillin (20µg/disc)**
S. aureus	IZ	5.0	-	-	-	-	-	-	ND	-	15.0
	AI	0.3									1.0
E. coli	IZ	5.0	-	-	-	-	-	-	ND	-	21.0
	AI	0.2									1.0
P. aeruginosa	IZ	-	-	-	-	-	-	-	ND	15.0	ND
	AI									1.0	
S. typhi	IZ	-	-	5.0	8.0	-	-	-	ND	ND	20.0
	AI			0.2	0.4						1.0
V. cholera	IZ	-	-	-	-	-	-	-	ND	ND	12.0
	AI										1.0
B. anthracis	IZ	-	-	-	-	-	-	-	ND	ND	10.0
	AI										1.0
C. albicans	IZ	5.0	-	-	-	-	-	-	30.0	ND	ND
	AI	0.2							1.0		
A. niger	IZ	-	-	-	-	-	-	-	35.0	ND	ND
	AI								1.0		
A. fumigatus	IZ	-	-	-	-	-	-	-	35.0	ND	ND
	AI								1.0		
A. flavus	IZ	-	-	-	-	-	-	-	20.0	ND	ND
	AI								1.0		
C. neoformans	IZ	-	-	-	-	-	-	-	20.0	ND	ND
	AI								1.0		
Penicillim spp	IZ	-	-	-	-	-	ND	ND	20.0	ND	ND
	AI								1.0		

[a]Each result is a mean of 3 readings.
AE = 80% aqueous ethanol extract; IZ = inhibition zone; AI = activity index; ND = not done;
- = negative

Effect on blood glucose

The 80% aqueous ethanol extract of *C. macrostachys* roots caused a dose-dependent delay in the clearance of postprandial blood glucose in mice, leading to hyperglycaemia in the treated groups (Figure 2). Hyperglycaemia was not significant at 100 mg/kg body weight dose, but at 400 mg/kg body weight there was a significant elevation of blood glucose levels as compared to both the solvent treated group and the group given 100 mg/kg body weight of extract ($P \leq 0.05$).

Figures 3, 4 and 5 shows that, among the six isolated compounds, compound **5** caused a dose-dependent delay in the clearance of postprandial blood glucose at both 50 and 100 mg/kg body weight ($P \leq 0.05$). Compounds **2** and **6** had no effect on postprandial blood glucose as compared to controls ($P > 0.05$).

C. macrostachys is a herb commonly used by traditional healers for treatment of diabetes and bacterial infections. However, the scientific studies regarding to its anti-diabetic and anti-bacterial is limited. The results of the present study suggest that the plant crude extract together with compound **5**, does not lower blood glucose, but instead it causes hyperglycaemia. Hence, both the aqueous ethanolic extract and compound **5** delayed clearance of postprandial blood glucose, leading to hyperglycaemia. This would certainly worsen the diabetic state.

The other ethnomedical claim on the plant is treatment of bacterial infections such as dysentery and gonorrhoea. Two previous studies using dried stem bark ethanol and methanol extracts [11] reported antibacterial activity against *Bacillus albus* and no activity against *Escherichia coli*. In another study using acidified ethanol extract of the leaves [10], antibacterial activity was detected against both Gram-positive and Gram-negative bacteria. All the three studies reported negative antifungal activity against yeast and *Penicillium crustosum*.

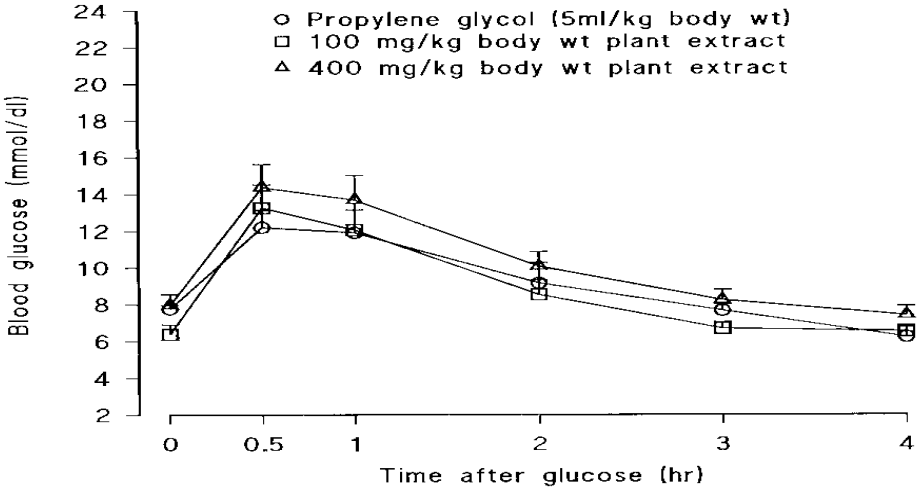

Figure 2: Effect of an 80% aqueous ethanol extract of *C. macrostachys* roots on blood glucose concentration in mice. Each point represents Mean ±SEM (n=10).

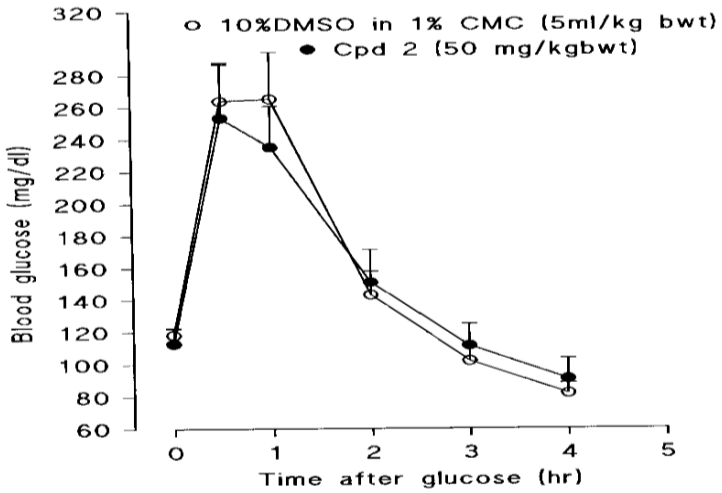

Figure 3: Effect of compound 2 on blood glucose concentration in mice. Each point represents Mean ±SEM (n=10).

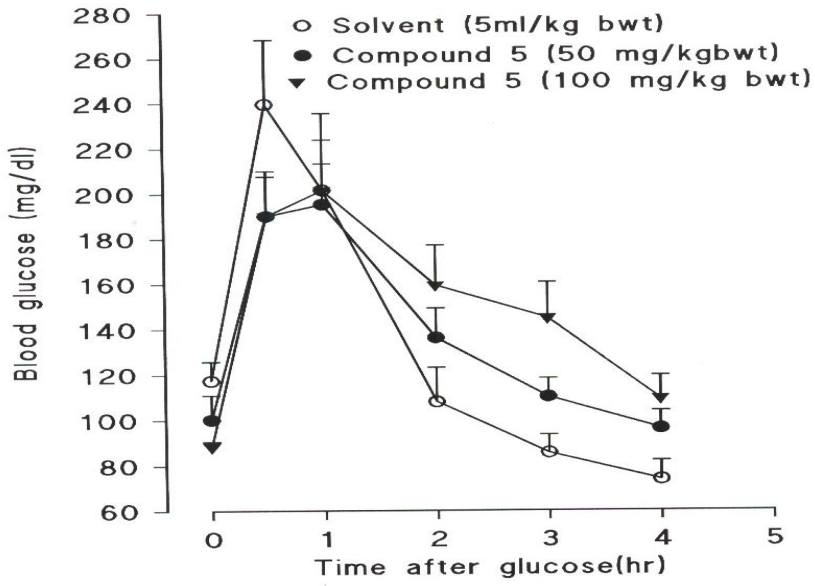

Figure 4: Effect of compound 5 on blood glucose concentration in mice. Each point represents Mean ±SEM (n=10).

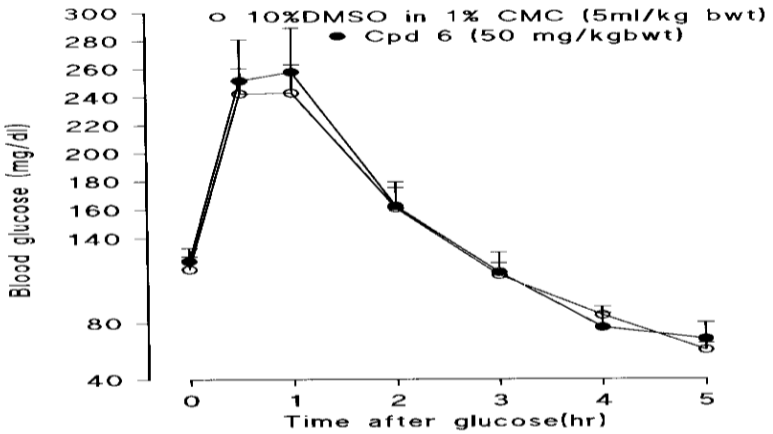

Figure 5: Effect of compound 6 on blood glucose concentration in mice. Each point represents Mean ±SEM (n=10).

The results obtained show that, the roots of *C. macrostachys* have a weak antibacterial activity against *S. aureus*, and a weak antifungal activity against *C. albicans*.

In a previous study, crotepoxide, a cyclohexane diepoxide with antitumor activity was isolated from an ethanol extract of *C. macrostachys* seeds [15, 24]. This study has shown that the aqueous ethanol extract of the roots also contains cytotoxic compounds. Five of the six compounds isolated from the root extract were toxic to brine shrimps at levels that are comparable with the standard anticancer drug cyclophosphamide. Compounds **2**, **3**, **4**, and **6** were more toxic at LC_{50} of 9.5, 4.4, 9.6 and 10.3 µg/ml respectively, while compound **5** (LC_{50} = 27.6 µg/ml) was slightly less toxic than cyclophosphamide. The detection of cytotoxic activity in this study is consistent with literature reports of other plants of the genus *Croton* [25, 26, 27, 28]. That suggests that the genus as a potential source of compounds with cytotoxic activities.

The present study provided scientific evidence that the plant cannot be used as an hypoglycaemic agent but rather as hyperglycaemic agent. On the other hand, the results of this study agree with traditional use of extracts of *C. macrostachys* for the treatment of bacterial and fungal infections. Further, the extracts may be toxic or have useful anticancer activity. Further work is required for the isolation and identification of other possible anticancer compounds from *C. macrostachys*.

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