ANTIBACTERIAL ACTIVITIES OF THE EXTRACTS FROM LEAVES OF *ACANTHUS MONTANUS* (Nees) T. Anders. (ACANTHACEAE)

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Summary

The present study was designated to evaluate the antibacterial activities of methanol crude extract (M-L), hexane, ethyl acetate and acetone fractions from the leaves of *Acanthus montanus* (*A. montanus*). This plant is used as traditional folk medicine in Cameroon for the treatment of infectious diseases. The antibacterial activities of the extracts against *B. cereus*, *B. subtilis*, *B. megaterium* and *B. stearothermophilus* were tested using disc diffusion method. The inhibition parameters were determined using macrodilution method. Phytochemical analysis of these extracts was also conducted. The acute toxicity of M-L was also studied. The results showed that hexane and ethyl acetate fractions exhibited a significant antibacterial effect against all the strains studied. M-L presented a moderate activity where as acetone fraction was inactive. These activities may be the results of the presence of either of tannins, glycosides, polyphenols, triterpenes, leucoanthocyans, cardiac glycosides, phlobatannins, flavonoids, lipids and sugars found in the leaves of this plant. The ratio of the minimal bactericidal concentration (MBC) over the minimal inhibitory concentration (MIC) indicates the bactericidal effect of the plant. M-L was found to be fairly non-toxic to the rats treated *per os.* These results support common use of leaves of *A. montanus* in the treatment of some infectious diseases.

Keywords: *Acanthus montanus*, Antibacterial activities, *Bacillus*, toxicity

Acanthus montanus a plant belonging to the *Acanthaceae* (1), is widely distributed in the centre, west and south provinces of Cameroon. Its leaves are frequently used as folk remedies to treat various ailments such as constipation, diarrhoea, indigestion and nausea (decoction of leaves) (2). Despite all these potential beneficial effects, experimental studies of the biological properties of *A. montanus* extracts are lacking. In recent years, multiple drug/chemical resistance in both human and plant pathogenic microorganisms have been developed due to the indiscriminate use of commercial antibacterial drugs/chemical commonly used in the treatment of infectious diseases (3, 4, 5). This situation forced the scientists to the searching of new antibacterial substances from various sources like medicinal plants (6, 7). It is essential to investigate *A. montanus* for antibacterial activity and for phytochemical substances that may be a medical and economic value. Furthermore, it is necessary to evaluate the toxicity of *A. montanus*.

The present study was conducted to investigate antibacterial properties of methanol crude extract, hexane, ethyl acetate and acetone fractions from the leaves of *A. montanus* against four strains of *Bacillus*, and to evaluate the oral acute toxicity of the methanol extract in rat with the hope that the results would provide information on the safety of this extract prior to the evaluation of its therapeutic efficacy in humans.

Materials and methods

Plant material

The leaves of *A. montanus* were collected in Mbouda in the west province of Cameroon in January 2003 and identified by the National Herbarium in Yaoundé, where the voucher specimen was deposited under the reference number, 22730/SFR/CAM.

Experimental animals

Male and female Albino Wistar rats, weighing 100 - 120 g were obtained from the animal house of the Biochemistry Department (University of Yaoundé I). They were housed under uniform husbandry conditions of 12 h - light: 12 h - dark cycle and temperature (26 ± 2°C) and allow free access to drinking water and standard laboratory diet. Rats were deprived of food 16 – 18 h prior the experiment.

Extraction procedure

Fresh leaves of the plant were ground and macerated with methanol in the ratio 1:5 (w/v) at room temperature for 24 h. The concentrated methanol extract was decanted, filtered with Wathman number 1 filter paper and concentrated in vacuo bellow 40°C using a rotary evaporator to give the crude extract (3.14% w/w) used for the investigations (8). The methanol crude extract (M-L) was fractionated by successive extraction using hexane, ethyl acetate and acetone. The hexane (H-L), ethyl acetate (E-L), acetone (A-L) fractions and residue (R-L) were obtained respectively at: 7.33% w/w; 3.8% w/w; 1.37% w/w and 68.6% w/w.

Phytochemical tests

Phytochemical tests for alkaloid, tannins, glycosides, polyphenols, triterpenes, leucoanthocyans, cardiac glycosides, phlobatannins, flavonoids, lipids, saponines, anthraquinones, sterols and sugars were carried out as described by (9). Each of the test was qualitatively expressed as positive (+) or negative (-).

Microorganisms

The test microorganisms *Bacillus cereus* F3748, *Bacillus subtilis* NCTC 3610, *Bacillus megaterium* 8174 were obtained from the Microbiology Laboratory, Institute of Food Research Reading, UK and *Bacillus stearothermophilus* CNCH 5781 from the Institute Appert, France.

Antibacterial activity

Disc diffusion method

One millilitre of inoculums (3.3×10^6 colony forming units) prepared from an overnight nutrient broth culture was used to seed each prepared and dried nutrient agar plate (10). The plates were allow to air dry for 5 - 10 min. Sterile paper discs (6 mm diameter) prepared from whatman number 1 filter paper were impregnated with (M-L) from a stock of 80 mg/ml; H-L,

E-L, A-L and R-L, from a stock of 20 mg/ml. Each disc contained 2.4 mg of M-L and 1.0 mg of each fraction. Negative control was prepared with methanol. Chloramphenicol (10 µg/disc) was used as positive reference for the susceptibility test. The discs were allowed to dry for 24 h, at the sterility condition. Each disc was then arranged and firmly presses on to agar surface of each plate. The inoculated plates were incubated at appropriate temperature for 24 h. The antibacterial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Each assay in this experiment was repeated twice (11).

Macrodilution assay

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of M-L, H-L, E-L and chloramphenicol were determined by macrodilution technique in nutrient broth for the microorganisms that were determined as sensitive in the disc diffusion method.

The bacterial strains were cultured overnight at appropriate temperature in nutrient agar. The test strains were suspended in nutrient broth to give a final density of 5 x 10^5 cfu/ml and these were confirmed by viable count. Extracts in water were first diluted to the highest concentrations (2400 µg/ml for M-L and 1200 µg/ml for H-L and E-L), and then serial two-fold dilutions were made with nutrient broth in the concentration range from 75 to 2400 µg/ml for M-L and 37.5 to 1200 µg/ml for H-L and E-L in 10 ml sterile test tubes containing 1 ml of the inocula. The final volume in each tube was 5 ml (12). The last tube containing 4 ml of nutrient broth without extract and 1 ml of the inocula was used as a negative control. Tubes containing the same concentration of extracts without microorganisms were also used as sterility control. Chloramphénicol at the concentration range of 5 to 80 µg/ml was prepared in nutrient broth and used as positive control. The contents of each tube were mixed on a plate shaker at 300 rpm for 20 s and then incubated at appropriate temperature for 18 h. Bacterial growth was determined by absorbance at 600 nm using the spectrophotometer UV-120-01. These values of absorbance were used to draw a concordance curve from which MIC, MBC and the dose that inhibits 50% (IC_{50}) growth after the incubation period were determined (11).

Acute toxicity

Eight rats were randomly divided into two groups of four animals per sex. The methanol extract of *A. montanus* was dissolved/suspended in distilled water and administrated by gavage at doses of 0, 4, 8, 12, 16, 20 and 24 g/kg. The general behaviour of rats was observed continuously for 1 h after the treatment and then intermittently for 4 h, and thereafter over a period of 24 h (13). The rats were further observed for up to 5 days following treatment for any signs of toxicity and deaths, and the latency of death. The LD_{100} and LD_{50} values were determined according to the method of (14). On the day 6, all rats were fasted for 16 – 18 h, and then sacrificed by decapitation. Blood was collected and centrifuged at 3000 g at 4°C for 15 min to obtain the serum, which was stored at -20°C until analysis for biochemical parameters.

Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M). Significant differences between control and experimental groups were assessed by Student's t-test. P-values less than 0.05 were considered to be significant.

Results

The results of phytochemical studies of the crude extract and fractions are shown in Table 1. M-L showed the presence of alkaloids, saponins, tannins, glycosides, polyphenols, cardiac glucosides, triterpenes, leucoanthocyans, phlobatannins, flavonoids, lipids and sugars; with the absence of anthraquinones and sterols. H-L fraction showed the presence of polyphenols, saponins, cardiac glucosides, leucoanthocyans, phlobatannins, flavonoids lipids, triterpenes, and sugars. E-L fraction showed the presence of tannins, glucosides, polyphenols, cardiac glucosides, triterpenes, leucoanthocyans, flavonoids, phlobatannins, lipids and sugars. A-L fraction showed the presence of alkaloids, tannins, triterpenes, polyphenols, leucoanthocyans, phlobatannins, flavonoids. R-L showed the presence of alkaloids, saponins, polyphenols, tannins, leucoanthocyans, phlobatannins, flavonoids and sugars.

The results of antibacterial activity by disc diffusion assay showed in table 2 indicated that crude extract and ethyl acetate fraction possess an inhibition effect against all the microorganisms tested. The diameter of inhibition zone varied from 9 ± 0.87 to 14 ± 0.5 mm for M-L observed on *B. megaterium* and *B. stearothermophilus* respectively and 10.33 ± 0.57 to 14 ± 1 mm for E-L observed on *B. cereus* and *B. stearothermophilus* respectively. The residue and acetone fraction were inactive. Chloramphenicol used as standard antibacterial for the positive control gave diameters ranging from 22.33 ± 1.21 to 25.66 ± 1.52 mm observed on *B. megaterium* and *B. stearothermophilus* respectively. Antibacterial activities of hexane fraction were least observed with *B. subtilis, B. megaterium* and *B. stearothermophilus*, this fraction was inactive against *B. cereus.* The antibacterial effect of crude extract and ethyl acetate fraction was found to be half comparable to the antibacterial activity exhibited by chloramphenicol. The results of the macrodilution assay are shown in table 3

Table 1: Phytochemical screening of the crude methanol extract and fractions from *A. montanus*

Crude extract and fractions	Chemical Groups													
	Alk	Sap	Tan	Glu	Pph	Cgl	Tri	Leu	Su	Phl	Fla	Lip	Anth	Ster
M-L	+	+	+	+	+	+	+	+	+	+	+	+	-	-
H-L	-	+	-	-	+	+	+	+	+	+	+	+	-	-
E-L	-	-	+	+	+	+	+	+	+	+	+	+	-	-
A-L	+	-	+	-	+	-	+	+	-	+	+	-	-	-
R-L	+	+	+	-	+	-	-	+	+	+	+	-	-	-

Alk: Alkaloids **Pph:** Polyphenols **Su:** Sugars **Anth**: Anthraquinones
Sap: Saponins **Cgl**: Cardiac glucosides **Phl**: Phlobatannins **Ster:** Setrols
Tan: Tannins **Tri:** Triterpenes **Fla**: Flavonoids **Glu:** Glucosides
Leu: Leucoanthocyans **Lip**: Lipids

Table 2: Antibacterial activities of *A. montanus* extracts from the disc diffusion method

Extract and fractions tested	Inhibition zone diameters (mm)			
	Bacterial stains			
	B. cereus	***B. subtilis***	***B. megaterium***	***B. stearothermophilus***
Chloramphenicol (10 µg)	23 ± 0.68	25.3 ± 0.57	22.33 ± 1.21	25.66 ± 1.52
M-L (2400 µg)	11.5 ± 1	13 ± 2	9 ± 0.87	14 ± 0.5
H-L (1000 µg)	-	10 ± 0	8.8 ± 0.28	10.3 ± 0.57
E-L (1000 µg)	10.33 ± 0.57	12.16 ± 1.25	12.83 ± 0.28	14 ± 1
A-L (1000 µg)	-	-	-	-
R-L (1000 µg)	-	-	-	-
Negative control	-	-	-	-

(-): absence of inhibition zones

Table 3: MIC, MBC and IC_{50} values (µg/ml) of *Acanthus montanus* extracts in the macrodilution assay comparable to chloramphenicol

	Crude extract				Hexane fraction				Ethyl acetate fraction				Chloramphenicol			
Inhibition parameters / Bacterial strains	MIC	MBC	IC_{50}	$\frac{MBC}{MIC}$	MIC	MBC	IC_{50}	$\frac{MBC}{MIC}$	MIC	MBC	IC_{50}	$\frac{MBC}{MIC}$	MIC	MBC	IC_{50}	$\frac{MBC}{MIC}$
B. cereus	550	1050	250	1.9	Nd	Nd	Nd	Nd	270	500	135	1.8	18.5	33	9.5	1.8
B. subtilis	575	1000	300	1.73	160	280	80	1.75	115	215	60	1.8	9	16	4.5	1.8
B. megaterium	900	1600	450	1.77	300	560	150	1.8	240	450	120	1.8	36	70.5	18	1.9
B. stearothermophilus	550	950	300	1.72	150	270	75	1.8	120	220	55	1.8	8.5	14.5	4.5	1.7

(Nd): Not determined

The biochemical profiles of the treated and control rats are presented in table 4 and 5. The acute toxicity indicated the total lethal dose (LD_{100}) at 20 and 24 g/kg body weight for males and females respectively. The medium lethal dose (LD_{50}) values for male and female rats were 16 and 19 g/kg body weight respectively. Acute oral administration of *A. montanus* crude extract in the male (up to a dose of 16 g/kg body weight) did not cause any significant changes in alanine aminotransferase (ALT) total and direct bilirubin. However, urea, proteins, aspartate aminotransferase level were significantly increased ($p<0.05$) whereas creatinine level was significantly decreased ($p<0.05$). Alkaline phosphatase (ALP) level was significantly increased ($p<0.01$). In the female group (up to dose of 20 g/kg body weight), there is not any significant change in creatinine, ALP, total and direct bilirubin. However, urea, ALT level were significantly increased ($p<0.01$), AST level increased ($P<0.05$) whereas proteins level was decreased ($p<0.01$) as compared to the control group.

Tableau 4: Biochemical parameters values of male rats in acute toxicity of the methanol extract from the leaves of *A. montanus*

Parameters tested	Extract dose (g/kg body weight)				
	0 (n = 4)	4 (n = 4)	8 (n = 4)	12 (n = 4)	16 (n = 2)
Urea (mg/l)	1,66 ± 0,26	1,70 ± 0,17	1,62 ± 0,71	1,90 ± 1,11	2,39 ± 0,2*
Créatinine (mg/l)	2,50 ± 1,13	2,38 ± 0,47	1,81 ± 0,22	1,46 ± 0,20*	1,46 ± 0,20*
Total protein (mg/ml)	4,96 ± 0,96	4,66 ± 0,75	5,49 ± 0,49	5,48 ± 0,84	6,53 ± 0,61*
ALP (UI/l)	14,25 ± 1,55	13,88 ± 1,37	15,75 ± 2,21	15,86 ± 1,93	17,85 ± 2,73**
AST (UI/l)	60,94 ± 8,34	61,25 ± 64,46	71,88 ± 5,52	86,25 ± 4,24*	86,88 ± 4,22*
ALT (UI/l)	39,5 ± 2,78	34 ± 3,36	36,5 ± 1,41	42,5 ± 3,10	44 ± 1,65
Total bilirubin (mg/l)	1,1 ± 0,02	1,09 ± 0,37	1,08 ± 0,62	1,33 ± 0,73	0,91 ± 0,68
Direct bilirubin (mg/l)	0,34 ± 0,05	0,27 ± 0,03	0,33 ± 0,06	0,33 ± 0,01	0,30 ± 0,75

* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)

Tableau 5: Biochemical parameters values of female rats in acute toxicity of the methanol extract from the leaves of *A. montanus*

Parameters tested	Extract dose (g/kg body weight)					
	0 (n = 4)	4 (n = 4)	8 (n = 4)	12 (n = 4)	16 (n = 3)	20 (n = 2)
Urea (mg/l)	1,78 ± 0,16	1,74 ± 0,02	1,66 ± 0,2	1,89 ± 0,22	2,00 ± 0,23	2,16 ± 0,23**
Créatinine (mg/l)	2,27 ± 0,98	2,03 ± 1,08	1,81 ± 0,09	2,15 ± 0,52	2,2 ± 0,36	1,53 ± 0,21
Total protéin (mg/ml)	6,49 ± 0,49	7 ± 0,65	6,62 ± 0,25	6,27 ± 0,60	5,31 ± 0,77**	5,4 ± 0,53**
ALP (UI/l)	14,62 ± 2,03	13,75 ± 0,64	12,75 ± 1,32	14,63 ± 1,17	13,87 ± 2,11	14,75 ± 1,70
AST (UI/l)	69,06 ± 3,64	75 ± 4,70	65 ± 4,74	88,88 ± 6,25*	99,25 ± 3,22*	101,38 ± 6,32*
ALT (UI/l)	32 ± 6,32	30 ± 9,59	41 ± 4,65	36,5 ± 5,20	43 ± 7,73	40,38 ± 7,39**
Total bilirubin (mg/l)	2 ± 0,97	1,11 ± 0,06	1,96 ± 0,74	1,36 ± 0,63	1,76 ± 0,54	1,62 ± 0,38
Direct bilirubin (mg/l)	0,22 ± 0,04	0,21 ± 0,01	0,20 ± 0,5	0,19 ± 0,04	0,2 ± 0,02	0,22 ± 0

* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)

Discussion and Conclusions

The presence of saponines, tannins and triterpenes may explain the antibacterial action of this plant. Since the antibacterial action of these phytochemical substances have been documented (8). Antibacterial effect observed vary according to different fractions. The highest antibacterial activity was obtained with ethyl acetate fraction. Hexane fraction exhibited a moderate activity. These differences observed can be attributed to distribution of active principles in different fractions due to their affinity with the solvent used during fractionation.

The least values of MIC and MBC were obtained with ethyl acetate fraction ranging from 115 to 270 µg/ml and 215 to 500 µg/ml respectively observed on *B. subtilis* and *B. cereus*. MIC and MBC obtained with chloramphenicol varied from 8.5 to 36 µg/ml and 14.5 to 70.5 µg/ml respectively, observed on *B. stearothermophilus* and *B. megaterium*. It was considered that if the extract display a MIC less than 100 µg/ml, the antimicrobial activity was good, from 100 to 500 µg/ml, the antimicrobial activity was moderate, from 500 to 1000 µg/ml, the antimicrobial activity was weak, over 1000 µg/ml the extract was considered inactive (15). These results showed that ethyl acetate and hexane fractions exhibited a moderate activity against all the strains tested. In all the cases, the ratio MBC/MIC suggested that *A. montanus* has a bactericidal effect. The antibacterial effect may be due to the pore formation in the cell wall and the leakage in cytoplasmic constituents by the active compounds present in this plant (16).

The LD_{50} for *A. montanus* extract for male and female rats were greater that 5000 mg/kg body weight thus, this extract was found to be fairly non-toxic (17). (AST and ALT) are good indicators of liver functions. The increase of these transaminases level observed indicates that *A. montanus* extract induces some damage to the liver. The difference of some biochemical parameters observed between female and male rats can be attributed to the hormonal difference.

Bacillus cereus, Bacillus subtilis, Bacillus megaterium and *Bacillus stearothermophilus* were found to be sensitive to *A. montanus* extracts. Crude extract was found to be fairly non-toxic. These results suggested that the leaves from *A. montanus* contain compounds with antibacterial properties which can be used as antibacterial agents in new drugs for therapy of infectious diseases in human. The active compounds were being purified and will be further identified to elucidate their mechanism.

References

1. Berhaut J. Flore illustrée du Sénégal Gouvernement du Sénégal Ministère du Développement Rural, Direction des Eaux et Forêts DAKAR, 1971: 385-386.

2. Noumi E, Yomi A. Medicinal plants used for intestinal diseases in Mbalmayo Region, Centre Province, Cameroon. *Fitoterapia* 2001; 72 (3): 246-254.

3. Davis J. Inactivation of antibiotics and the dissemination of resistance genes. *Science* 1994; 264: 375-382.

4. Loper JE, Henkels MD, Robert RG, Willet MJ, Smith TJ. Evaluation of streptomycin, oxytetracycline and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. *Plant Disease* 1991; 75: 287-290.

5. Service RF. Antibiotics that resist resistance. *Science* 1995; 270: 724-727.

6. Clark AM. Natural products as sources of news drugs. *Pharmaceutical research* 1996; 13: 1133-1141.

7. Cordell GA. Biodiversity and drug discorvery a symbiotic relationship. *Phytochemistry* 2000; 55: 463-480.

8. Deeni YY, Sadiq NM. Antibacterial properties and phytochemical constituents of the leaves of *African mistletoe* (*Tapinanthus dodeneifolius* (DC) Danser) (*Loranthaceae*): and ethnomedicinal plant of Hausaland, Northern Nigeria. *Journal of Ethnopharmacology* 2002; 83 (3): 235-240.

9. Odebiyi O, Sofowara EA. Phytochemical screening. Nigeria medical plants. *L. Loydia* 1978; 41: 231-234.

10. Deeni YY, Hussain HS. Screening for antimicrobial activity and alkaloids of *Nauclear latifolia*. *Journal of Ethnopharmacology* 1991; 35: 91-96.

11. Carbonnelle B, Denis F, Marmonier A, Pinon G, Vargue R. Bactériologie médicale : Techniques usuelles (ed). SIMED: Paris, 1987: 237-238.

12. Zgoda JR, Porter JR. A convenient microdilution method for screening natural products against bacteria and fungi. *Pharmaceutical Biology* 2001; 39: 221-225.

13. Twaij HA, Kery AA, Kharraji NK. Some pharmacological, toxicological and phytochemical investigation on *Centauroa phyllocophora. Journal of Ethnopharmacology* 1983; 9: 299-314.

14. Berhens B, Karber G. Mathematics for Naturalist and Agriculturalist. PWN, Warszawa, 1983: 212.

15. Fabiola BH, Greisiele LP, Neviton RS, et al. Screening of some plants used in the Brasilian folk medicine for the treatment of infectious deseases. *Mem Inst Oswaldo cruz, Rio de Janeiro* 2002; 97 (7): 1027-1031.

16. Gnanamani A, Shanmuga PK, Radhakrishnan N, Mary B. Antibacterial activity of two plants extracts on eight burn pathogens. *Journal of Ethnopharmacology* 2003; 86 (1):59-61.

17. Delongas JL, Burnel D, Netter P, et al. Toxicité et pharmacocinétique de l'oxychlorure de Zirconium chez la souris et le rat. *Journal of Pharmacology* 1983; 14(4): 437-447.