Chemical Constituents and Antibacterial Activities of the Stem Bark Extracts of *Ricinodendron heudelotii* (Euphorbiaceae).

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Studies were carried out on the stem bark of *Ricinodendron heudelotii* (Baill) Pierre ex Pax (Euphorbiaceae). The phytochemical examination of the n-hexane and methanol/methylene chloride extracts by silica gel chromatography afforded aleuritolic acid 1 and labda-8(17),13-dien-3β,15-diol 2, which were isolated for the first time along with E-ferulic acid octacosylate 3. The structures were elucidated using spectroscopic methods. The antimicrobial activity of the n-hexane and MeOH/CH_2Cl_2 extracts evaluated using disc diffusion method showed that these extracts were more active against some of the microorganisms tested than some commercially available antibiotics used. For the eight microorganisms tested, the minimal inhibitory concentration (MIC) obtained varied from 4.6±1.2 to 22±4 mg/ml for the n-hexane extract and from 5.1±1.2 to 20±1.5 mg/ml for the MeOH/CH_2Cl_2 extract. *Streptococcus faecalis* was found being the most susceptible organism with a MIC of 4.6 mg/ml for the n-hexane extract. These antimicrobial properties probably explain partly, the use of this plant in traditional medicine.

Ricinodendron heudelotii (Baill.) Pierre ex Pax belongs to Euphorbiaceae family. It is a large tree, which reaches more than 30 m in height and grows in the equatorial forest of Madagascar and on the West Coast of Africa[1]. In Cameroon, this wild tree grows throughout the humid lowland rainforest. The seeds are important ingredients in culinary usage[2]. The bark extract of this plant is use against cough, as poison antidote[3] and for the treatment of intestinal diseases[4]. The leaves are used to treat dysentery[3]. The stem bark contains two dinorditerpenoids, heudelotinone and 1,2-dihydroheudelotinol, E-ferulic acid octacosylate and 3-methyl orsellinate[3] and some natural chemopreventive agents[5]. A survey of literature revealed no report of antimicrobial activities study on this plant extract. The aim of this present investigation was to isolate the chemical constituents of *R. heudelotii* extracts and to determine if its use in traditional medicine for treatment of microbial infections can be substantiated through *in vitro* experiments.

The stem bark was collected from Buea, South West province, Cameroon in August 1997. The identification was authenticated with the Voucher specimen (N° 50811/NHC) that has been deposited in the national herbarium Yaounde, Cameroon. Air-dried powdered stem bark 7.5 kg was extracted successively with n-hexane and MeOH/CH_2Cl_2 (1/1) at room temperature. After removal of the solvent by concentration under reduced pressure, 26 g and 54 g of the n-hexane and MeOH/CH_2Cl_2 residue were obtained. These extracts were qualitatively very similar on TLC analysis and were combined. The combined extract was subjected to flash chromatography on silica gel using a mixture of n-hexane/ethyl acetate/methanol of increasing polarity to furnish five fractions; A (7 g), B (5 g), C (15 g), D (12 g), E (30 g). Fraction B (5 g) was subjected to column chromatography using n-hexane/ethyl acetate as eluents to yield compound 3 (E-ferulic acid octacosylate) 120 mg, compound 1 (aleuritolic acid) 60 mg. The same operation on the fraction D using a mixture of n-hexane-ethyl acetate-methanol afforded compound 2 (labda-8(17),13-dien-3β, 15-diol) 45 mg for the first time from a natural source. The structures have been elucidated using NMR techniques. Compound 1 (fig. 1) (aleuritolic acid): amorphous white solid 60 mg; MS: m/z 456 (M^+), 287, 248, 234, 207, 191, 189; IR (KBr, cm^{-1}): 3432, 1688, 1451,

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1387; ^{1}H NMR ($CDCl_3$, 400 MHz): 0.67 (3H, s, Me), 0.70 (3H, s, Me), 0.83 (3H, s, Me), 0.84 (3H, s, Me), 0.85 (3H, s, Me), 3.09 (1H, m), 5.4 (1H, dd, J = 3.3 and 7.9 Hz, CH=). A positive response to Liebermann-Burchard reaction and spectra data indicated it to be aleuritolic acid[6]. Compound 2 (fig. 2) (labda-8(17),13-dien-3β,15-diol): white solid, 45 mg, mp: 149.9-150.5; MS (m/z): 306 (M^+), 291, 273, 255, 175, 147; IR (KBr, cm^{-1}): 3300, 3030, 1670, 1380, 1360; ^{1}H NMR ($CDCl_3$, 400 MHz): 0.69, 0.77, 0.99, 1.17 [Me-20, Me-19, Me-18, Me-16; s], 3.25 (1H, dd, J=11.6 and 6.0 Hz, H-3), 4.14 (2H, d, J=6.9 Hz, H-15), 4.53 and 4.85 (2H, H-17), 5.39 (1H, dd, J=1.2 and 6.9 Hz, H-14). ^{13}C NMR ($CDCl_3$, 100 MHz): 14.5 (q, C10), 15.4 (q, C19), 16.3 (q, C16), 21.8 (t, C11), 24.0 (t, C6), 27.9 (t, C2), 28.3 (q, C18), 37.1 (t, C1), 39.1 (s, C4), 39.3 (s, C10), 54.6 (d, C5), 56.0 (d, C9), 59.4 (t, C15), 78.8 (d, C3), 106.7 (t, C17), 123.1 (d, C14), 140.4 (s, C13), 147.9 (s, C8). Compound 3 (fig. 3) (E-ferulic acid octacosylate): white solid 80 mg, mp: 74-75; MS (m/z):

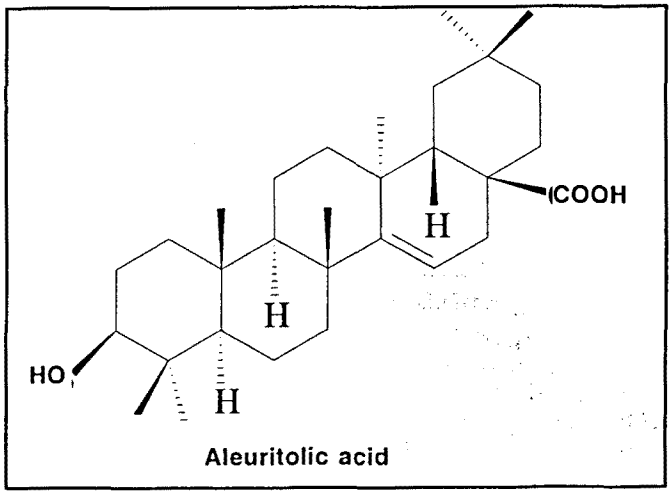

Fig. 1: Structure of compound 1

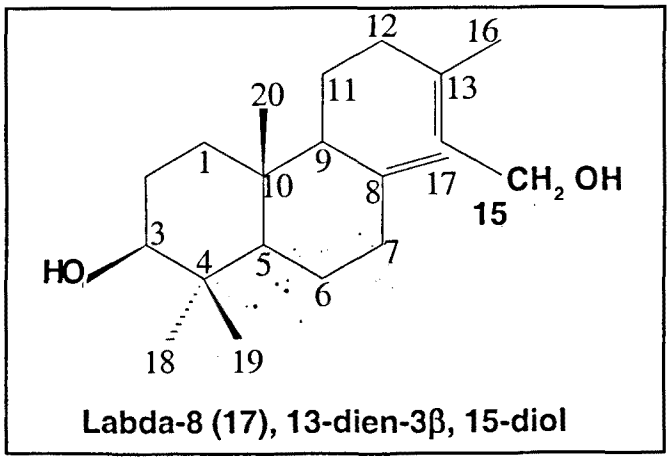

Fig. 2: Structure of compound 2

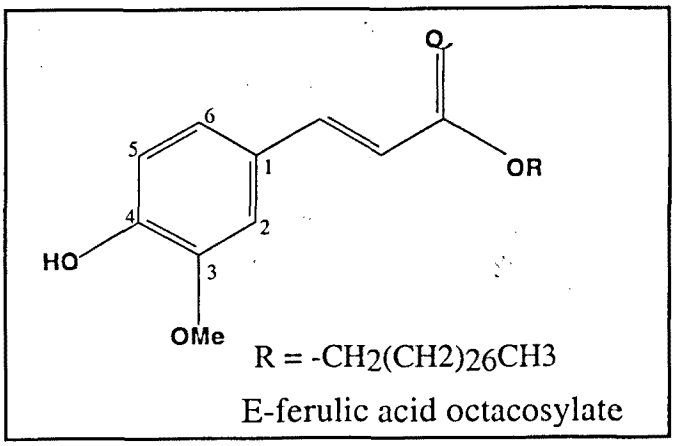

Fig. 3: Structure of compound 3

586 (M^+), 559, 425, 194, 177; IR (KBr, cm^{-1}): 3475, 1660, 1625, 1590, 1380, 840, 800; ^{1}H NMR ($CDCl_3$, 400 MHz): 0.92 (3H, t, Me), 1.25 (52H, s, $(CH_2)_{26}$), 3.91 (3H, s, OMe), 4.15 (2H, m, OCH_2CH_2), 6.28 (1H, d, J = 16 Hz, CH=), 6.93 (1H, dd, J = 1.1 and 8.1 Hz, H-5), 7.07 (1H dd, J = 1,1 and 8.1 Hz, H-6), 7.60 (1H, d, J = 16 Hz, CH=), ^{13}C NMR ($CDCl_3$, 100 MHz): 55.9 (t, OMe), 64.6 (t, OCH_2), 114.7 (d, CH=), 115.7 (d, CH=), 109.3 (d, CH), 123.0 (d, CH), 127.1 (d, CH), 144.6 (s), 146.8 (s), 147.9 (s), 167.4 (s). The data suggested it to be E-ferulic acid octacosylate[7].

Microorganisms used were, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Candida albicans* and *Aspergillus flavus*. These strains were isolated in the Laboratory of Microbiology of the National Advanced School Agro-Proces's Industries, University of Ngaoundere and *Actinomyces naeslundii*-ATCC 12104 was obtained from the Pasteur Centre of Garoua, Cameroon. Mueller-Hinton agar (20 ml) was poured into a Petri dish and inoculated with microorganisms. Sterilised filter paper disc (Whatman No. 1, diameter 6 mm) previously impregnated with 10 µl of each extract was dried and placed onto the surface of agar media. Dishes were then incubated at 37° for bacteria and 30° for fungi for 18 h and the diameter of inhibition zones (not including the diameter of the disc) were measured. Antibiotics (streptomycin, tetracycline, erythromycin, penicillin, chloramphenicol) and antifungal (Anaxeril®, myconazole, nystatin) obtained from Abtek Biologicals Ltd., Liverpool (Lot No 2903/P), were prepared as control for Gram+ and Gram- bacteria and fungi[8]. Extracts of 100 mg/ml were prepared by dilution of 1 g of crude extract in 10 ml of tween 80 at 50° in water bath. A series of test tubes containing each a suspension of 10^6 organisms/ml of trypticase soy broth (TSB) was prepared. Precise quantities of prepared extract (100

mg/ml) were added in order to obtain final concentration of extract ranged from 1 mg/ml to 100 mg/ml and all the test tubes were incubated at 37° for bacteria and 30° for fungi for 24 h. A blank assay with tween 80 was prepared as control. At the end of incubation, 1 ml of each test tube was inoculated in a trypto-casein-soy (TCS) agar poured in petri dishes and after incubation as mentionned above for 24 h, colonies were then counted. The MIC corresponds to the concentration of extract of first test tube in which there is not positive culture[9].

Results of the antimicrobial activities with respect of the inhibition zones for bacteria tested obtained showed that the n-hexane extract was more effective against *S. faecalis* (12±0.5 mm) and less active against *P. fluorescens* (7±2 mm) (Table 1). For the fungi, the zone of inhibition was lower for *C. albicans* (3±0.7 mm) than those obtained for bacteria, demonstrating insignificant activities of this extract against these fungi. The n-hexane extract was found to be more effective against *P. fluorescens, B. subtilis, S. faecalis, E. coli* than most of the antibiotics. The MeOH/CH_2Cl_2 extract was more active against *B. subtilis* (10±1.5 mm) and less effective against *S. aureus* (5±0.6 mm). Compared to the n-hexane extract, it activities was also insignificant against the fungi (*C. albicans, A. flavus*) and was lower than those of the antifungal used. The n-hexane extract seemed to be more effective against all microorganisms tested than the MeOH/CH_2Cl_2 extract. So the non-polar compounds may having more antibacterial activity. This idea is confirmed by the MIC determination which showed that the n-hexane extract has the lower MIC (5.2±0.7 mg/ml) than the MeOH/CH_2Cl_2 extract (9±0.7 mg/ml) against *E. coli*. The n-hexane extract has the lowest MIC (4.6±1.2 mg/ml) of both extracts against *S. faecalis* (Table 2). E-ferulic acid octacosylate have shown antimicrobial activity against *E. coli, S. aureus, Mycobacterium ranae, P. aeruginosa, C. albicans, Klebsiella pneumonia, Proteus vulgaris* and *Trichomonas foetus*[10]. Isolated in good yield from these extracts, it is probably one of the compounds responsible of their antibacterial activity.

From the stem bark of *R. Heudelotii*, aleuritolic acid and labda-8 (17),13-dien-3β,15-diol were isolated for the first time along with E-ferulic acid octacosylate. The n-hexane and MeOH/CH_2Cl_2 extracts were found to inhibit the growth of five and four microorganisms respectively. On the basis of the determinate MICs, the effect of n-hexane extract against *S. faecalis* and *E. coli*, and of MeOH/CH_2Cl_2 extract against *B. subtilis* and *E. coli* were the most inter-

TABLE 1: ANTIMICROBIAL ACTIVITY OF METHANOL/METHYLENE CHLORIDE (1/1) AND N-HEXANE EXTRACTS OF THE STEM BARK OF *R. HEUDELOTII*.

Microorganisms	Zone of inhibition (mm)[a]									
	ME	HE	Str	Tet	Ery	Pen	Chl	Ana	Myc	Nys
P.fluorescens	-	7±2	2±1	4±1	-	4±0.5	-	2±0.5	5±2	-
B. subtilis	10±1.5	9±1.5	7±0.9	10±1	8±1	2±0.5	6±0.8	-	-	-
Staph. aureus	5±0.6	7.3±1	6±1.2	10±1	4±1.2	3±1.2	4.3±0.5	-	-	2.7±0.2
S. faecalis	-	12±0.5	8±1	6.5±1	5.5±1	7±0.7	1.5±0.2	7±0.6	-	1,5±0.6
E. coli	8±2	10±3	4.2±2	7±0.7	4±1	6.5±1	5±0.9	-	-	0,8±0.2
A. naeslundii-ATCC 12104	6±1.2	-	-	-	-	-	-	8±0.6	10±0.8	3±0.4
C. albicans	2±0.3	3±0.7	-	-	1.8±0.2	-	-	6±0.7	7±0.5	11±0.2
A. flavus	-	-	-	-	-	-	-	3±0.2	6±2	9±0.6

[a]Value are mean of four experiments±S.D, - denotes resistant strain(no zone of inhibition), ME is the methanol/methylene chloride (1/1) extract, HE is the n-hexane extract. Str is streptomycin (10 µg), Tet represents tetracyclin (10 µg), Ery stands for erythromycin (5 µg), Pen is penicillin (1 iu), Chl is chloramphenicol (10 µg), Ana denotes anaxeril® (10 µg), Myc denotes myconazole (5 µg) and Nys is nystatin (2 µg).

Literature describes HPLC[1], capillary electrophoresis and reversed phase HPLC[2] methods for the determination of enantiomers of carvedilol. Reported methods include sophisticated techniques, while the developed method having almost same sensitivity with simplicity. Carvedilol is chemically non-selective β receptor antagonists. In the present investigation, a new simple selective and sensitive difference spectroscopic method is reported for the determination of Carvedilol in bulk and formulations.

Standard stock solution was prepared by dissolving Carvedilol in methanol to make final concentration of 100 µg/ml as a stock solution. Different aliquots were taken from stock solution and diluted with 0.1 N NaOH and 0.1 N HCl separately to prepare the series of concentration from 1 to 14 µg/ml as a reference and test solutions respectively. Maxima and minima were obtained as 217 nm and 210 nm. The calibration curve was prepared by plotting amplitude versus concentration of carvedilol.

Tablets of two brands were used for the purpose of analysis. Twenty tablets were powdered and powder equivalent to the 5 mg was transferred in the volumetric flask and dissolved in methanol, then filter it and dilute the solution up to the mark by using methanol to prepare stock solution of 100 µg/ml. From stock solution take 1 ml of solution and dilute with 0.1 N NaOH to prepare working solution of 10 µg/ml. Similarly take 1 ml of stock solution and dilute it with 0.1 N HCl to prepare working solution of 10 µg/ml. The absorbance of the working solution was measured and the amount of Carvedilol was calculated from calibration curve. And the results are shown in Table 1.

TABLE 1: RESULTS OF THE MARKETED CARVEDILOL TABLETS

Formulation	Mg of drug taken from tablets	Amount found in (mg)	% Labeled claim *Mean±sd
T-1	5	4.95	99.3±0.38
T-2	5	4.95	99.3±0.38

*average of three determinations, T-1 Cardivas 3.125 mg manufactured by SUN Pharma Ltd. T-2 Carloc 3.125 mg manufactured by Cadila health cares Ltd.

All of the formulations contain excipients and binders, which are added alongwith the active drug constituents. These substances may cause some interference during the estimation of the active drug constituents. Interference from the excipients was confirmed by performing the recovery experiment for which standard addition method was employed. From the recovery result it is to be claimed that the method can be used for estimation of carvedilol in dosage form. The results obtained are shown in Table 2.

TABLE 2: OPTICAL AND STATISTICAL DATA

Parameters	Values
Maximum wavelength λmax	217 nm.
Mininimum wavelength λmin	210 nm
Calibration curve range	1-14 µg/ml
Molar extinction coefficient	3.455×10^4
Sandell's sensitivity	0.0117 µg/ml
Regression equation	Y=0.0706X+0.094
Slope	0.0706
Intercept	0.094
Correlation co-efficient (r)	0.995

Carvedilol shows maxima and minima at 217 nm and 210 nm, respectively by taking drug solution in 0.1 N HCl in reference cell and drug solution in 0.1 N NaOH in test cell. The calibration curve was found to be linear in the range of 1 to 14 µg/ml. From the result of recovery study shows in Table 1, it is concluded that the excipients present in the formulation do not interfere in the estimation of carvedilol. This method is also suitable for estimation of its dosage form. This method can be applied to marketed preparations. The slope, Intercept, co-relation co-efficient and optical characteristics are summarized in Table 2. The proposed method can be successfully used for the analysis of drug in marketed preparations with good precision, sensitivity and accuracy.

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