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Antibacterial Activity of the Ethyl Acetate Extract of *Hypericum roeperanum* Schimp. Ex A. Rich. (Guttifereae) and Their Secondary Metabolites

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Abtract: The present study was designated to evaluate the antibacterial activities of the ethyl acetate crude extract and their secondary metabolites isolated from the twigs of *Hypericum roeperanum*. This plant is used as traditional folk medicine in Cameroon for the treatment of infectious diseases and gastrointestinal disorders. The antibacterial activities of the extract and their secondary metabolites against ten pathogens causing gastrointestinal disorder were tested using disc diffusion method. The inhibition parameters were determined using microdilution method. The results showed that ethyl acetate crude extract exhibited a significant antibacterial effect against all the strains studied. This activity is the results of the presence of 1,3,5,6-tetrahydroxyxanthone (Norathyriol) and 2,5-dihydroxyxanthone isolated from the plant using repeated column chromatography of the ethyl acetate crude extract. These compounds exhibited a significant antibacterial effect against all the strains studied. However, betulenic acid, 5,7,3,4-tetrahydroxyflavanone, 1,7-dihydroxyxanthone, 5-hydroxy-2-methoxyxanthone, 1,3,6,7-tetrahydroxyxanthone, lupeol, friedelin and friedelinol eight other compounds isolated from this plant were inactive. The ratio of the minimal bactericidal concentration over the minimal inhibition concentration indicates the bactericidal effect of the plant.

Key words: *Hypericum roeperanum*, antibacterial activities, compounds, extract, bactericidal

INTRODUCTION

Hypericum roeperanum a plant belonging to the Guttifereae (Berhaut, 1971; Junior *et al*., 2008) is widely distributed in Batcha in the west region of Cameroon. Its twigs are frequently used as folk remedies to treat various ailments such as abdominal pains, constipation, diarrhoea, indigestion and nausea (Noumi and Yomi, 2001). Despite all these potential beneficial effects, experimental studies of the chemical and biological properties of *H. roeperanum* 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 (Loper *et al*., 1991; Davies, 1994; Service, 1995). This situation forced the scientists to the searching of new antibacterial substances from various sources like medicinal plants (Clark, 1996; Cordell, 2000). It is essential to investigate *H. roeperanum* for antibacterial activity that may be a medical and economic value. The present study was conducted to investigate antibacterial properties of the ethyl acetate crude extract and secondary metabolites isolated from the twigs of *H. roeperanum* against ten pathogens causing gastro-intestinal disorder.

MATERIALS AND METHODS

Plant material: The twigs of *H. roeperanum* were collected at Batcha in June 2008, in the west region of Cameroon. The plant identification was done by a botanist (Mr. Nana) at the National Herbarium, Yaounde where the voucher specimen was deposited under the reference number 13189/SRF/Cam.

Microorganisms: The test microorganisms *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*,

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Salmonella typhi, *Schigella flexneri*, *Citrobacter freundi*, *Morganella morganii*, *Enterobacter cloake*, *Pseudomonas aeruginosa* and *Proteus vulgaris* were provided by the Medical Bacteriology Laboratory of the Institute Pasteur of Yaounde Cameroon.

Extraction and purification procedure: The above plant was cut into small pieces, air dried and pulverized. 10 kg of powder were obtained and macerated in dichloromethane/methanol (1/1, v/v) for 48 h, then filtered and the filtrate was concentrated under vacuum to afford 330 g of crude extract. Part of this extract (325 g) was reextracted with ethyl acetate and methanol to obtained 130 and 100 g respectively which were stored in the fridge till further use.

Part of the ethyl acetate crude extract (125 g) was subjected to silica gel 60 (0.063-0.200 mm) flash chromatography using pure n-hexane, n-hexane-ethyl acetate gradient system and pure ethyl acetate as eluent. Several fractions of 500 mL each were collected and combined based on their TLC profile to give eight series (A-E). Series B (3.0 g) was rechromato graphed over a Si gel column with varying proportions of hexane and EtOAc (0-20%) as eluant to give compounds 7, 9 and 10. Further silica gel column chromatography of series C using n-hexane/ethylacetate as eluent yielded compounds 4 and 5. Series F, which was obtained with n-hexane/ethylacetate (70/30) presented a precipitate which after filtration gave compound 5. The filtrate was further chromatographed over a Si gel column to afford compounds 3 and 6. Series G was also chromatographed over silica gel with hexane-EtOAc (2%) as eluant to afford a mixture of 1 and 2 which was not possible to purify after repeated column chromatography and PTLC.

Acetylation of 2,5-dihydroxyxanthone and the mixture of compounds 1 and 2: Dry pyridine (0.5 mL) and acetic anhydride (1.0 mL) were added separately to 10 mg of the mixture of 1 and 2 and the mixture was stirred at room temperature for 12 h. Similar reaction was carried out with 10 mg of compound 3. After the usual workup, the acetylated compounds (mixture of 1a and 2a ($THRG6_{AC}$) and 3a ($THRG4_{AC}$)) were obtained

General: Melting points were measured on a Buchi SMP-20 melting point apparatus. Aluminum sheet pre-coated with silica gel 60 F254 nm (Mereck) was used for thin layer chromatography and the isolated spots were visualized using both ultra-violet light (254 and 366 nm) and 50% sulfuric acid spray reagent. Column chromatography was carried out on silica gel (70-270 mesh, Merck grade) and flash silica gel (230-400 mesh, Merck). The chemical structure of each of isolated compound was determined on the basis of spectral data produced by one and two-dimensional Nuclear Magnetic Resonance (NMR), recorded on Brucker-Avance-500 MHz instrument. This spectrometer was equipped with 5 mm, ^{2}H and ^{2}C NMR probes operating at 500 and 125 MHz, with tetrametylsilane as internal standard. Mass spectra were recorded on a Finnigan MAT double focusing spectrometer Model 8230. The structures of the compounds were confirmed by comparing with reference data from available literature.

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 Mueller Hinton agar plate (Deeni and Hussain, 1991). 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 crude extract from a stock of 40 mg mL^{-1} and compound from a stock of 5 mg mL^{-1}. Each disc contained 1 mg of crude extract and 50 μg of each compound. Negative control was prepared with methanol used for dissolution of crude extract and compounds. Gentamycin (10 μg $disc^{-1}$) 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 37°C 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 (Carbonnelle *et al*., 1987).

Microdilution assays: The Minimal Inhibition Concentration (MIC) and the Minimal Bactericidal Concentration (MBC) of crude extract, active compounds and gentamicin were determined by microdilution technique in nutrient broth for the microorganisms that were determined as sensitive in the disc diffusion method. The bacterial strains were cultured overnight at 37°C in nutrient agar. The test strains were suspended in nutrient broth to give a final density of 5×10^{5} cfu mL^{-1} and these were confirmed by viable count. Geometric dilutions ranging from 75 to 2400 μg mL^{-1} of the crude extract and from 9.5 to 300 μg mL^{-1} of the active compounds were prepared in 96 well microtiter including one growth control (nutrient broth), one sterility control (nutrient broth+extract or compounds) and one negative control (nutrient broth+inocula). gentamicyn at the concentration range of 5 to 80 μg mL^{-1} was prepared in nutrient broth and used a s positive control. The contents of each tube

were mixed on a plate shaker at 300 rpm for 20 sec and then incubated at 37°C for 18 h. Bacterial growth was indicated by the presence of a white pellet on the well bottom (Carbonnelle *et al*., 1987).

Statistical analysis: The experimental results are expressed as the Mean±standard deviation (SD).

RESULTS

The ethylacetate crude extract (THR) of the twigs of *Hypericum roeperanum* upon repeated column chromatography, afforded ten known compounds, five xanthones: 1, 7-dihydroxyxanthone (THRG9) (Hu *et al*., 1999), 5-hydroxy-2-methoxyxanthone (THRG10) (Rath *et al*., 1996), 2, 5-dihydroxyxanthone (THRG4) (Tanaka and Takaishi, 2006), 1,3,5,6-tetrahydroxyxanthone or Norathyriol (THRG6) (Peres *et al*., 2000) and 1,3,6,7-tétrahydroxyxanthone (THRG5) (Peres *et al*., 2000); one flavonoid 5,7,3,4-tetrahydroxyflavanone (THRG7) and four triterpenes: betulenic acid (THRG2) (Kitajima *et al*., 2000), lupeol (THRG3) (Kouam *et al*., 2005), friedelin (THRG8) (Wabo *et al*., 2007) and friedelinol (THRG1) (Fig. 1). The structural identification of these compounds has been done using their NMR spectral data in conjunction with the available literature data in Fig. 1.

The results of antibacterial activity by disc diffusion assay showed in Table 1 indicated that crude extract, 1,3,5,6-tetrahydroxyxanthone and 2,5-dihydroxyxanthone compounds possess an inhibition effect against all the microorganisms tested. The diameter of inhibition zone varied from 10.66±0.4 to 15±0 mm for crude extract and 2,5-dihydroxyxanthone compound observed on *P. vulgaris* and *S. flexneri*, respectively. Gentamycin used as standard antibacterial for the positive control gave diameters ranging from 22±0.8 to 32.33±0.4 mm observed on *S. flexneri* and *K. Pneumonia,* respectively. The results of the microdilution assays are shown in Table 2. The least values of MIC and MBC were obtained with 2,5-dihydroxyxanthone and 1,3,5,6-tetrahydroxyxanthone

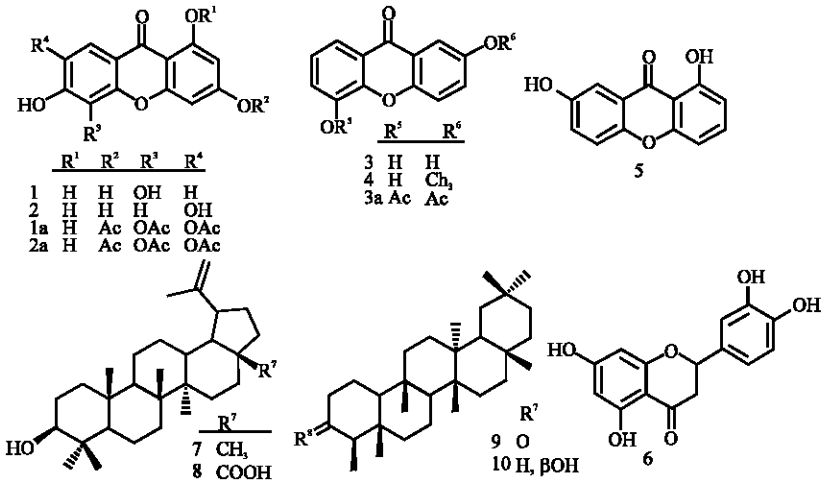

Fig. 1: Chemical structure of isolated compounds (1-10)

Table 1: Antibacterial activities of *H. roeperanum* crude extract and compounds from the disc diffusion method

	Inhibition zone diameters (mm)													
Bacterial stains	THRG	THRG1	THRG2	THRG3	THRG4	THRG5	THRG6	THRG7	THRG8	THRG9	THRG10	$THRG4_{AC}$	$THRG6_{AC}$	GM
E. coli														
K. pneumoniae	11.33±0.40	-	-	-	13.33±0.4	-	13.33±0.8	-	-	-	-	-	-	31.6±0.4
S. aureus	13.66±0.40	-	-	-	13.16±0.2	-	12.50±0.7	-	-	-	-	-	-	32.3±0.4
S. typhi	12.33±0.40	-	-	-	12.66±0.9	-	12.16±0.2	-	-	-	-	-	-	30.3±0.9
S. flexneri	15.16±0.62	-	-	-	14.33±0.4	-	14.00±0.8	-	-	-	-	-	-	24.0±0.8
C. freundi	13.33±0.40	-	-	-	15.00±0.0	-	14.50±0.7	-	-	-	-	-	-	22.0±0.8
M. morganii	11.00±0.80	-	-	-	12.33±0.4	-	11.50±0.7	-	-	-	-	-	-	23.6±0.4
E. cloake	12.50±0.70	-	-	-	14.5±1.08	-	13.50±0.4	-	-	-	-	-	-	22.6±0.4
P. aeruginosa	11.66±0.4	-	-	-	11.00±0.0	-	13.60±0.9	-	-	-	-	-	-	26.6±0.4
P. vulgaris	12.50±0.40	-	-	-	12.16±0.6	-	11.80±0.6	-	-	-	-	-	-	32.0±0.8

THRG: Crude extract, THRG1: Friedelinol, THRG2: Betulenic acid, THRG3: Lupeol, THRG4: 2,5-dihydroxyxanthone, THRG5: 1,3,6,7-tetrahydroxyxanthone, THRG6: 1,3,5,6-tetrahydroxyxanthone or Norathyriol, THRG7: 5,7,3,4-tetrahydroxyflavanone, THRG8: Friedelin, THRG9: 1,7-dihydroxyxanthone, THRG10: 5-hydroxy-2-methoxyxanthone, GM: Gentamycin, (-): Absence of inhibition zones

Table 2: Minimal inhibition concentrations and minimal bactericidal concentrations (μg mL^{-1}) of *H. roeperanum* extract and active compounds in the microdilution assays comparable to gentamycin

	MIC				MBC			
Bacterial stains	THRG	THRG4	THRG6	GM	THRG	THRG4	THRG6	GM
E. coli	600	37.5	75.0	10	600	75	150.0	20
K. pneumoniae	300	75.0	75.0	10	600	150	150.0	20
S. aureus	300	150.0	75.0	10	600	150	150.0	20
S. typhi	150	75.0	37.5	20	600	150	75.0	40
S. flexneri	150	18.7	18.7	20	300	75	37.5	40
C. freundi	600	75.0	75.0	20	600	150	150.0	40
M. morganii	300	75.0	37.5	20	300	150	75.0	40
E. cloake	600	75.0	37.5	10	1200	150	75.0	20
P. aeruginosa	300	75.0	75.0	10	600	150	75.0	20
P. vulgaris	600	150.0	75.0	10	1200	150	150.0	20

MIC: Minimal inhibition concentration, MBC: Minimal bactericidal concentration

ranging from 18.7 to 75 μg mL^{-1} and 18.7 to 37.5 μg mL^{-1}, respectively observed on S. flexneri. MIC and MBC obtained with gentamycin varied from 10 to 40 μg mL^{-1}.

DISCUSSION

The antibacterial effect is the results of the presence of xanthone in this plant. However, this activity is related to the structure, mainly to the position of the hydroxyl group present in these compounds. The active compounds became inactive after acetylation. The antibacterial effect of crude extract and active compounds was found to be half comparable to the antibacterial activity exhibited by gentamycin. The antibacterial activity 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 (Gnanamani *et al*., 2003). It was considered that if the extract display a MIC less than 100 μg mL^{-1}, the antimicrobial activity was good, from 100 to 500 μg mL^{-1}, the antimicrobial activity was moderate, from 500 to 1000 μg mL^{-1}, the antimicrobial activity was weak, over 1000 μg mL^{-1} the extract was considered inactive (Holetz *et al*., 2002). These results showed that 2,5-dihydroxyxanthone and 1,3,5,6-tétrahydroxyxanthone exhibited a good activity against all the strains tested and crude extract exhibited a moderate activity. In all the cases, the ratio MBC/MIC suggested that *H. roeperanum* has a bactericidal effect.

CONCLUSION

The results obtained from the present study suggested that ethylacetate extract of *H. roeperanum* possess significant antibacterial property. This activity is the results of the presence of 1,3,5,6-tétrahydroxyxanthone (Norathyriol) and 2,5-dihydroxyxanthone which can be used as antidiarrhoeal agents in new drugs for therapy of infectious disease.

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