ANTIMICROBIAL ACTIVITY OF THE EXTRACT FROM THE TWIGS OF *DORSTENIA ELLIPTICA* (MORACEAE)

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Summary

The crude extract from the twigs of *Dorstenia elliptica* (DET) as well one of the five compounds isolated from this extract namely O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)-3-hydroxybutyl]bergaptol (**5**) were tested for their antimicrobial activity against bacteria and fungi. The Agar disc diffusion test was used to determine the sensitivity of the tested samples while the well micro-dilution was used to determine the minimal inhibition concentrations (MIC) and the minimal microbicidal concentration (MMC) of the active samples. The results of the disc diffusion assay showed that DET prevented the growth of all the 22 tested microbial species while the two tested compounds showed selective activity. At the tested MIC interval ranged from 1.22 to 156.25 µg/ml for the crude extracts and 0.08 to 39.06 µg/ml for the compounds, the activity of DET was noted on 20 of the 22 microbial species. The antimicrobial activities of DET as well as that compound **5** are being reported for the first time. The overall results provide promising baseline information for the potential use of the crude extract in the treatment of bacterial and fungal infections.

Keywords: *Dorstenia elliptica,* Moraceae, O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)-3-hydroxybutyl]bergaptol, antimicrobial activity.

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Introduction

It is well known today that the use of medicinal plants contributes significantly to primary health care for about 80 % of Africans. Many plants are used in Cameroon in form of crude extracts, decoction, infusion or percolation to treat infections. Numerous of these plants are without any scientific evidence of efficacy. In our research group, the investigation of the pharmacological properties of such plants includes the evaluation of the activity of the crude organic extracts as well as that of the isolated compounds. We have focus our interest of many plants families such Guttiferae, Apocynaceae, Moraceae, Ebenaceae, solanaceae, etc. Within the Moraceae family, plants of the genus *Dorstenia* are mostly represented. We have previously reported the interesting antimicrobial activity of *Dorstenia angusticornis* as well as compounds isolated from this plant such as Stipulin, Angusticornin B, Gancaonin Q and Bartericin A (1). In our continuing search of antimicrobial drugs from this genus, we undertook to evaluate the antibacterial and antifungal activity of the crude extract of *Dorstenia elliptica* Bureau as well as that of compounds isolated from this extract, a plant used to traditionally treat eyes infections (2, 3).

Methods

Plant material

The twigs of *Dorstenia elliptica* Bureau were collected in March 2000 at Kribi, South province of Cameroon. The botanical identification of the plants was done by the National Herbarium in Yaounde, where the voucher specimens were conserved under the reference number 44018/HNC.

Isolation and General procedures

The air-dried and powdered twigs of *D. elliptica* Bureau (2.1 kg) were soaked in CH_2Cl_2-MeOH (1:1) for 24 h, followed by MeOH for 2 h. The filtrates were concentrated under reduce pressure then combined after TLC analysis to give a dark- green residue or crude extract (DET) (115 g). A part of DET (70 g) was chromatographed over a silica gel 60 (200 g) column eluting with hexane followed by hexane-EtOAc (3:1, 1:1, 1:3) mixtures and then EtOAc to give 40 fractions, A-D of 250 ml each. The fractions A (sub-fractions 1-6; 5 g), eluted with hexane examined on TLC (hexane-EtOAc; 95:5 v/v) contained mainly mixtures of hydrocarbons and phytosterols, and were not investigated further. The fractions B (sub-fractions 7-12; 4 g), obtained with (3:1 v/v) hexane-EtOAc mixtures, crystallized in the same solvent system to give Psoralen (**1**, 28 mg, MW: 186, m.p: 163-164) (4) and O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)butyl] bergaptol or Dorstenin (**2**) (650 mg, MW: 368, mp: 140) (4-5). The filtrate (3.2 g) was re-chromatographed over silica gel 60 column using a mixture of hexane-EtOAc as eluent to afford, Bergapten (**3**, 65 mg, MW: 216, 189-190) (4). The combined fractions C (sub-fractions 13-30; 23 g) obtained from the (1:1) to (1:3) hexane-EtOAc mixtures were passed through Sephadex LH-20 column and eluted with a mixture of $CHCl_3$-MeOH (2:1). The post-chlorophyll fractions were combined and subjected, successively to silica gel CC and preparative TLC to yield 3-(3,3-dimethylallyl)-4,2',4'-trihydroxylchalcone (**4**, 24 mg, MW: 324) (Abegaz et al., 2004) and O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)-3-hydroxybutyl]bergaptol (**5**), 65 mg, MW: 384, mp: 144-145) (5). The fractions D (sub-fractions 31-40; 10 g) eluted with EtOAc were purified over silica gel 60 column, eluting with solvent mixtures of increasing polarity, (from CH_2Cl_2 to 96:4 (v/v) CH_2Cl_2-MeOH) to afford **2** (12 mg), **3** (28 mg) and **5** (17 mg).

Aluminium sheet pre-coated with silica gel 60 GF_{254} Merck was used for thin layer chromatography and the isolated spots were visualized using both ultra-violet light (254 and 366 nm) and 50 % H_2SO_4 spray reagent. The chemical structure of each of the isolated compound was determined on the basis of spectral data produced by one and two-dimensional nuclear magnetic resonance (NMR), recorded on Brüker DRX-400 instrument. This spectrometer was equipped with 5-mm, ^1H- and ^{13}C-NMR probes operating at 400 and 100 MHz, with tretramethylsilane as internal standard. Mass spectra were recorded on a API QSTAR pulsar mass spectrometer. The chemical structures of the compounds isolated from *D. elliptica* are given on figure1.

Microbial strains

Twenty two of microorganisms namely *Bacillus cereus* LMP0404G, *Bacillus megaterium* LMP0204G, *Bacillus stearothermophilus* LMP0104G, *Bacillus subtilis* LMP0304G, *Staphylococcus aureus* LMP0206U, *Streptococcus faecalis* LMP0207U (Gram-positive bacteria), *Escherichia coli* LMP0101U, *Shigella dysenteriae* LMP0208U, *Proteus vulgaris* LMP0103U, *Proteus mirabilis* LMP0504G, *Shigella flexneri* LMP0313U, *Klebsiella pneumoniae* LMP0210U, *Pseudomonas aeruginosa* LMP0102U, *Salmonella typhi* LMP0209U, *Morganella morganii* LMP0904G, *Enterobacter aerogens* LMP1004G, *Citrobacter freundii* LMP0804G, *Enterobacter cloacae* LMP1104G (Gram negative bacteria), *Candida albicans* LMP0204U, *Candida gabrata* LMP0413U, *Microsporum audorium* LMP0725D, *Trichophyton rubrum* LMP0723D (fungi) were used in this study. 'Institut Appert de Paris' provided three Bacillus species, while the A.F.R.C Reading Laboratory of Great Britain provided Bacillus cereus. Other strains were clinical isolates from 'Centre Pasteur of Cameroon', Yaoundé. The microbial isolates were maintained on agar slant at 4°C in the Laboratory of Applied Microbiology and Molecular Pharmacology (LMP) (Faculty of Science, University of Yaoundé I) where the antimicrobial tests were performed. The strains were sub-cultured on a fresh appropriate agar plate 24 hours prior to any antimicrobial test.

Antimicrobial assays

Culture media

Nutrient Agar (NA) (Oxoid) containing Bromocresol purple was used for the activation of *Bacillus* species while NA was used for other bacteria. Sabouraud Glucose Agar (Oxoid) was used for the activation of the fungi. The Mueller Hinton Agar (MHA)(Oxoid) was used in sensitivity assay. Nutrient broth containing 0.05 % phenol red and supplemented with 10 % glucose (NBGP) was used for MIC and MMC determinations.

Chemicals

Nystatin (Maneesh Pharmaceutic PVT. Ltd., Govandi, Mumbai, 400 043 India) and Gentamicin {Jinling Pharmaceutic (Group) corp., Zhejang Tieng Feng Pharmaceutic Factory, No. 11 Chezhan Road, Huzhou city, Zhejang, China) were used as reference antibiotics (RA) against fungi and bacteria respectively. The Dimethylsulfoxide (DMSO)(SIGMA) was used as solvent for the tested samples.

Sensitivity test: agar disc diffusion assay

Preparation of discs

Whatmann filter paper (N°1) discs of 6 mm diameter were impregnated with 10µl of the solution of crude extract (at 20 mg/ml) or isolated compound (at 5 mg/ml) prepared using DMSO. The discs were evaporated at 37°C for 24 hours. The RA discs (gentamycin for bacteria and nystatin for fungi) were prepared as described above using the appropriate concentrations to obtain discs containing 50 µg of drug. Two discs were prepared for each sample.

Diffusion test

The antimicrobial diffusion test was carried out as described by Berghe and Vlietinck (6) using a cell suspension of about 1.5 10^6 CFU/ml obtained from a McFarland turbidity standard N° 0.5. The suspension was standardised by adjusting the optical density to 0.1 at 600 nm (SHIMADZU UV-120-01 spectrophotometer) (7). This was used to inoculate by flooding the surface of MHA plates. Excess liquid was air-dried under a sterile hood and the impregnated discs applied at equidistant points on top of the agar medium. A disc prepared with only the corresponding volume of DMSO was used as negative control. The plates were incubated at 37°C for 24 hours. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc. The assay was repeated twice and the results expressed using signs as follows: (-) for no activity and (+) for samples with IZ >6mm.

MIC and MMC determinations

The MICs of test samples and RA were determined as follows: the test sample was first of all dissolved in DMSO. The solution obtained was added to the NBGP to a final concentration of 156.25 µg/ml for the crude extracts and 39.06 µg/ml for the compounds and RA. This was serially diluted two fold to obtain concentration ranges of 1.22 to 156.25 µg/ml for the crude extracts and 0.08 to 39.06 µg/ml for the compounds and RA. 100 µl of each concentration was added in a well (96- wells microplate) containing 95µl of NBGP and 5µl of the standard inoculum. The final concentration of DMSO in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO/NBGP affected neither the growth of the test organisms nor the change of colour due to this growth). The negative control well consisted of 195 µl of NBGP and 5 µl of the standard inoculum (8-9). The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37°C for 24 hours. The assay was repeated trice. Microbial growth was determined by observing the change of colour in the wells (red when there is no growth and yellow when there is growth). The lowest concentration showing no colour change was considered as the MIC.

For the determination of MMC, a portion of liquid (5 µl) from each well that showed no change in colour was plated on MHA and incubated at 37°C for 24 hours. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC.

Results

The structures of the isolated compounds were established using spectroscopic analysis, especially, NMR spectra in conjunction with 2D experiments, COSY, HMQC and HMBC, and direct comparison with published information and with authentic specimens obtained in our research group for some cases.

The compounds isolated from the twigs of *D. elliptica* were found to be coumarins known as Psoralen (**1**), O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)butyl]bergaptol or Dorstenin (**2**), Bergapten (**3**), O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)-3-hydroxybutyl]bergaptol (**5**), and a chlacone identified as 3-(3,3-dimethylallyl)-4,2',4'-trihydroxychalcone(**4**). In this study, the antibacterial and antifungal activities of the crude extract and compounds **5** were evaluated and the results are reported in Table 1.

From the results of the disc diffusion assay (Table 1), it appeared that the crude extracts from *D. elliptica* tested at 200 μg/disc prevented the growth of all the 22 tested microbial species. Compounds **5** was active on 3 (13.6 %) of the 22 tested microorganisms respectively.

The MIC values ranging from 19.53-156.25 μg/ml (Table 1) were obtained with the crude extract from *D. elliptica* on 20 of the 22 microbial species. For the compound **5** tested, it's appeared from the results of this study that, the MIC values were also noted on 3 (13.6 %) of the tested microbial strains.

Discussion

In our previous study (10), we have also reported the antimicrobial activity of compounds **1**, **2** **3**and **4**. Regarding the degree of activity of the tested samples, the crude extract could mostly be considered as very active in the perspective of the development of plant drugs. The lowest MIC values of 39.06 μg/ml for the crude extract of D. *elliptica* are 32 fold greater than the corresponding value (1.22 μg/ml) for both gentamycin and nystatin used as the RA. Table 1 also showed that MMC values were obtained with the crude extract of *D. elliptica* on 9 (40.9 %) of the 22 tested microorganisms showing that the cidal effects of tested samples on many of the tested microorganisms could be expected (11). Looking for the structure-activity relationship, it has latterly been demonstrated that the methoxylation of a three-cycle coumarin (compound **1** to give compound **3**) generally decreases the antimicrobial activity (10). Our previous study has also indicated that substitution of the $-CH_3$ group of compound **3** by the O-[3-(2,2-dimethyl-3-oxo-2H-furan-5-yl)butyl] group to yield compound **2** decreases the antimicrobial activity (10). This comment could also be assigned to the transformation of compounds **3** in **5**. However, from our lately reports (10), the results of this study also confirmed that the substitution of the $-CH_3$ group of compound **3** by O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)-3-hydroxybutyl] group to give compound **5** decreases the antimicrobial activity. To the best of our knowledge, the antibacterial and the anticandidal activities of *D. elliptica* as well as that of compound **5** is being reported for the first time. Nevertheless, the antimicrobial properties of Psoralen (**1**), O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)butyl]bergaptol (**2**) and Bergapten (**3**) and 3-(3,3-dimethylallyl)-4,2',4'-trihydroxychalcone (**4**) was previously reported (10). The known antimicrobial mechanisms associated to each class of chemical to which the isolated compounds belong, may explain the antimicrobial potency of the crude extracts and compounds from *D. elliptica.* The interaction with eucaryotic DNA leading to growth inhibition (12) could be the possible mechanism by which coumarin-like compounds (**1**, **2**, **3** and **5**) exhibit their anticandidal action. The activity of flavonoids such as compound **4** might be due to their ability to complex with bacterial cell wall (12) and therefore, inhibiting the microbial growth. The results of this study provide an important basis for the use of extract from *Dorstenia elliptica* for the treatment of infections associated to the studied microorganisms under reserve of the pharmacological and toxicity studies currently going on in our laboratory.

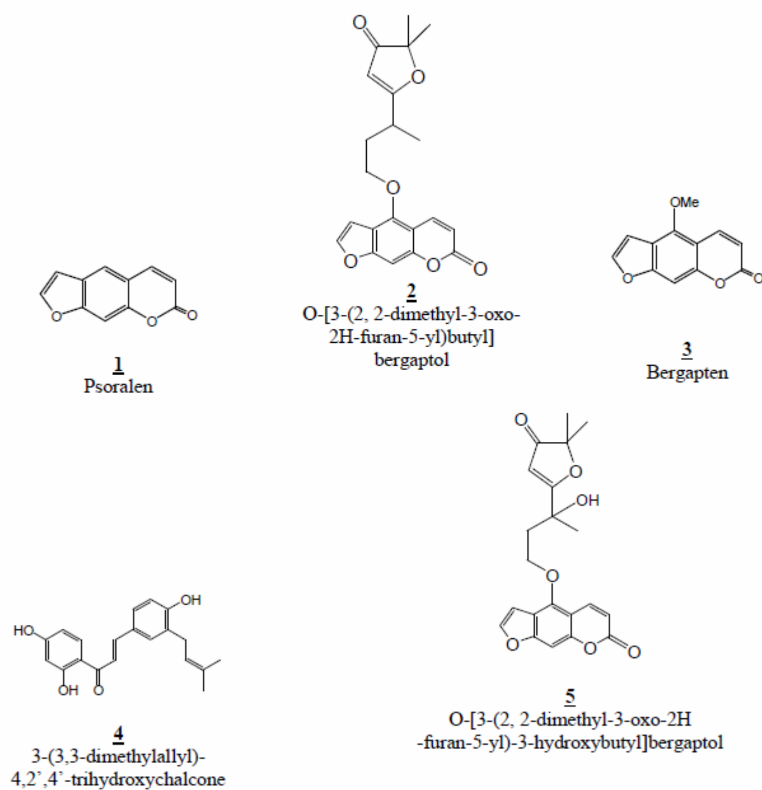

Figure1. Chemical structures of compounds isolated from the twigs of *D. elliptica* Bureau

Table1

Antimicrobial activity of the crude extract compounds **5** isolated from the twigs of *D. elliptica* and reference antibiotics.

Microorganisms	**Tested parameters**								
	Inhibition zones[a]			**Minimal Inhibition concentration[b] (µg/ml)**			**Minimal Microbicidal concentration[c] (µg/ml)**		
	DET[d]	**5**	**RA[e]**	**DET[d]**	**5**	**RA[e]**	**DET[d]**	**5**	**RA[e]**
Gram-negative bacteria									
Citrobacter freundii	+	-	+	39.06	-	4.88	156.25	-	9.76
Enterobacter aerogens	+	-	+	39.06	-	9.76	78.12	-	19.53
Enterobacter cloacae	+	-	+	78.12	-	4.88	156.25	-	9.76
Escherichia coli	+	+	+	156.25	39.06	1.22	nd	nd	4.88
Klebsiella pneumoniae	+	-	+	156.25	-	2.44	nd	-	2.44
Morganella morganii	+	-	+	156.25	-	2.44	nd	-	2.44
Proteus mirabilis	+	-	+	39.06	-	2.44	78.12	-	2.44
Proteus vulgaris	+	-	+	39.06	39.06	1.22	78.12	nd	4.88
Pseudomonas aeruginosa	+	+	+	156.25	39.06	4.88	nd	nd	9.76
Shigella dysenteriae	+	+	+	nd	-	2.44	nt	-	2.44
Shigella flexneri	+	-	+	78.12	-	2.44	156.25	-	2.44
Salmonella typhi	+		+	156.25	-	2.44	nd	-	2.44
Gram-positive bacteria									
Streptococcus faecalis	+	-	+	78.12	-	4.88	nd	-	9.76
Staphylococcus aureus	+	-	+	156.25	-	2.44	nd	-	2.44
Bacillus cereus	+	-	+	156.25	-	1.22	nd	-	4.88
Bacillus megaterium	+	-	+	39.06	-	2.44	156.25	-	2.44
Bacillus stearothermophilus	+	-	+	39.06	-	4.88	156.25	-	9.76
Bacillus subtilis	+	-	+	156.25	-	1.22	nd	-	4.88
Fungi									
Candida albicans	+	-	+	39.06	-	2.44	78.12	-	2.44
Candida gabrata	+	-	+	156.25	-	2.44	nd	-	2.44
Microsporum audorium	+	-	+	nd	-	4.88	nt	-	9.76
Trichophyton rubrum	+	-	+	156.25	-	1.22	nd	-	4.88

[a]Antimicrobial activity: crude extract were tested at 200 µg/disc and compounds at 50 µg/disc; (-): Not active; (+): Active;

[b]Minimal inhibition concentration: (-):(-): Not tested because samples were not active by disc diffusion

[c]Minimal microbobicidal concentration: (nd): Not determined because MIC was greater than 78.12 µg/ml for crude extracts and 39.06 µg/ml for isolated compounds; (-): Not tested because samples were not active by disc diffusion

[d] Crude extract from the twigs of *D. elliptica* (DET)

[e]RA: Reference antibiotics [Gentamycin (G) for bacteria, Nystatin (N) for yeasts];

5: O-[3-(2, 2-dimethyl-3-oxo-2H-furan-5-yl)-3-hydroxybutyl]bergaptol.

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