

# Antibacterial agents from the leaves of *Crinum purpurascens* herb (Amaryllidaceae)

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## Abstract

**Background:** Typhoid fevers and urogenital infections continue to be serious health problems in developing countries. In our search for therapeutic agents from natural sources with potential for the treatment of typhoid fevers and urogenital infections, extract and compounds were obtained from *Crinum purpurascens* and tested.

**Methods:** Two alkaloids (4,5-ethano-9,10-methylenedioxy-7-phenanthridone or hippadine (**1**) and 4,5-ethano-9-hydroxy-10-methoxy-7-phenanthridone or pratorimine (**2**)) and one steroid (  $\beta$  -D -glucopyranoside of sitosterol (**3**)) were isolated from the CH<sub>2</sub>Cl<sub>2</sub>/MeOH (1:1) leaf extract of *Crinum purpurascens* and screened for antibacterial activity using both agar diffusion and broth dilution techniques.

**Results:** For the CH<sub>2</sub>Cl<sub>2</sub>/MeOH extract, the MIC values obtained were 3 mg/ml (against *P. aeruginosa*), 4 mg/ml (against *E. coli*, *K. pneumoniae* and *S. aureus*) and 6 mg/ml (against *S. typhi* and *S. paratyphi* B), whereas the MBC values varied between 7 and 12 mg/ml. For compound **1**, the MIC values varied between 200 and 250  $\mu$ g/ml, whereas the MBC value was 300  $\mu$ g/ml against all the bacteria strains used. Compound **2** did not show any antimicrobial activity against these bacteria strains. For compound **3**, the MIC values varied between 250 and 300  $\mu$ g/ml, whereas the MBC values were 300  $\mu$ g/ml (against *S. typhi* and *S. paratyphi* B) and > 300  $\mu$ g/ml (against the other bacteria strains).

**Conclusion:** These data suggest that *C. purpurascens* leaf extract contains antibacterial agents which could be used in the treatment of typhoid fevers and urogenital infections.

**Keywords:** *Crinum purpurascens*., alkaloids, steroid, antimicrobial activity, *African Health Sciences* 2009; 9(4): 264-269

## Introduction

*Crinum purpurascens* herb (Amaryllidaceae), known in English as starry crinum (Morton), is a herbaceous plant of about 90 cm height, with bulbs and leaves, growing in humid regions of Africa <sup>1</sup>. It is often grown for ornamental purposes <sup>2</sup>. However, in Cameroonian folk medicine the macerated leaves are used as anti-poison or as antidote of mystical poisoning <sup>1</sup>. Also, an infusion of the leaves of *Crinum purpurascens* is used for the treatment of some microbial infections. Biologically active compounds have been detected within species of the genus *Crinum*; extracts and compounds obtained from some species of *Crinum* have been reported to exhibit antitumour, antifungal, antiparasitic and insecticidal activities <sup>2</sup>.

Typhoid, paratyphoid A and paratyphoid B fevers are caused by *Salmonella typhi*, *Salmonella paratyphi* A and *Salmonella paratyphi* B, respectively <sup>3</sup>. Typhoid fever continues to be a marked public health problem in developing countries in general and in sub-Saharan Africa in particular, where it is endemic <sup>4</sup>. Non specific urogenital infections are caused by a variety of bacteria whose habitat is not limited to the urogenital tract only. These bacteria are generally commensals which become pathogenic in debilitated persons or following a modification of their habitat <sup>5</sup>. Among the bacteria causing non specific urogenital infections are *Escherichia coli* <sup>6</sup>, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* <sup>7</sup> and *Staphylococcus aureus* <sup>5</sup>. Cases of resistance to the currently used antibiotics have been encountered with some strains of these bacteria <sup>5,8</sup>.

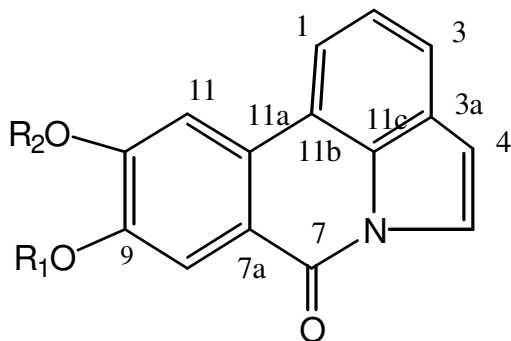
In a continuation of our search for therapeutic agents from natural sources with potential for the treatment of typhoid fevers and urogenital infections <sup>3,4,9,10,11,12,13,14</sup>, two alkaloids (4,5-ethano-9,10-methylenedioxy-7-phenanthridone or hippadine (**1**) and 4,5-ethano-9-hydroxy-10-methoxy-7-phenanthridone or pratorimine (**2**)) and one steroid (  $\beta$  -D -glucopyranoside of sitosterol (**3**)) were obtained from the extract of the leaves

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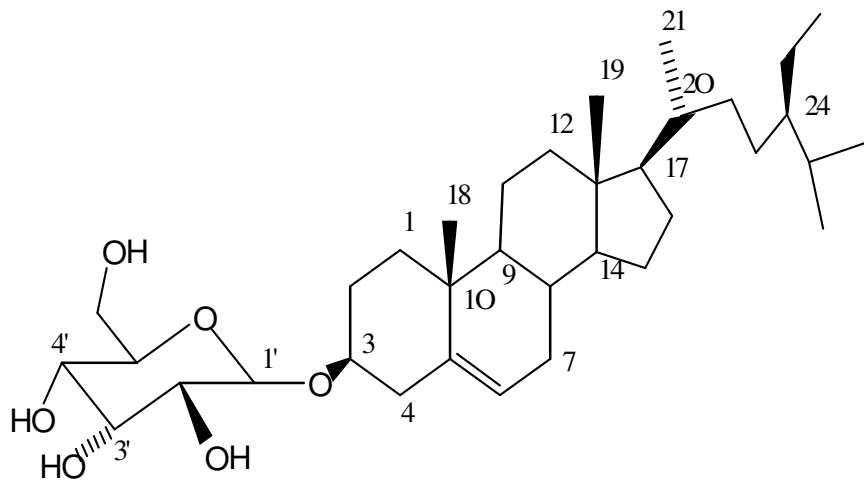
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of *Crinum purpurascens* through bioassay-guided fractionation. *C. purpurascens* was selected based on the claims of some traditional healers and users consulted in the area of collection, and on the results

of the preliminary tests. The extract and the above three compounds were tested for their antimicrobial activity. Compounds **1** and **2** were isolated for the first time from *C. pratense* by Ghosal et al.<sup>15</sup>.



**1** :  $R_1 + R_2 = -CH_2-$   
**2** :  $R_1 = H$  ;  $R_2 = Me$



**3**

## Materials and Methods

### Plant material

The leaves of *Crinum purpurascens* Herb. were collected in the West province of Cameroon, in the month of May 2006. The authentication was carried out at the Cameroon National Herbarium in Yaoundé, where a voucher specimen (N° 97/SRF/CAM) is deposited.

### Test bacteria and culture media

The bacteria strains, including *Salmonella typhi*, *Salmonella paratyphi* B, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, were obtained from the Medical Bacteriology Laboratory of the Pasteur Centre, Yaoundé, Cameroon. The culture media used, namely Salmonella-Shigella agar (used for the isolation of

the *Salmonella* species and for the screening of contaminants when preparing the inoculums), Mueller Hinton agar, selenite broth and tryptose phosphate broth (used for antibacterial tests), were obtained from International Diagnostics Group PLC, UK.

### Extraction and isolation

The leaves of *C. purpurascens* were chopped, dried, ground (600 g of powder) and extracted with a cold mixture of methylene chloride/methanol (1:1) for 3 days. Filtration and concentration on a rotavapor afforded 91 g (15.16%) of extract. This extract was dissolved in distilled water, followed by acidification with H<sub>2</sub>SO<sub>4</sub> (pH 3-4) and filtration. The residue was then further extracted with *n*-butanol to obtain *n*-butanol extract (14 g). The acidic filtrate was extracted with ethyl acetate to produce two phases which were separated by decantation to obtain a neutral extract and an acidic extract. The acidic extract obtained was basified with 10% NH<sub>4</sub>OH (pH 8-10) and further extracted with ethyl acetate to produce an aqueous phase and an organic extract. The organic extract was conserved while the aqueous phase was further extracted with a mixture of ethyl acetate/methanol (2:3) to yield an aqueous and another organic phase which was then added to the previous organic phase conserved which constituted the alkaloid-containing fraction (9 g).

Vacuum liquid chromatography of the neutral fraction on alumina gel (activity III) using a gradient of methanol in methylene chloride (100:0; 98:2; 95:5; 90:10; 80:20; 70:30; 60:40; 40:60; 20:80 and 0:100) gave forty-seven fractions (10 ml each), which were concentrated and combined on the basis of TLC profiles. Compound **1** (34 mg; colourless solid) was obtained from the residue of fraction 2 (0.1 g) under further recrystallisation in pure methanol. The residue obtained from fractions 3-7 (0.2 g) was purified on a silica gel (60 g) column eluted with CH<sub>2</sub>Cl<sub>2</sub> (5 ml for each fraction) to yield compound **2** (17 mg; colourless solid).

Vacuum liquid chromatography of *n*-butanol fraction (14 g) on silica gel using a gradient of MeOH in CH<sub>2</sub>Cl<sub>2</sub> (100:0; 98:2; 96:4; 90:10; 85:15; 80:20; 60:40; 20:80 and 0:100) gave one hundred and forty fractions (100 ml each), which were concentrated and combined on the basis of TLC profiles. Chlorophyll was removed from fraction 67-91 (obtained with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (85:15 and 80:20) by gel permeation through Sephadex LH-20 (70 g), (Hex/CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7:4:2) 10 ml for each

elution). The remaining residue of the fraction 67-91 previous was purified on a silica gel (150 g) column using a gradient of EtOAc in *n*-hexane (50 ml for each fraction) to yield compound **3** (23 mg, colourless solid). The structure of compounds **1** and **2** were successfully determined using a combination of one dimensional NMR techniques (<sup>1</sup>H and <sup>13</sup>C), homo and heteronuclear two-dimensional NMR techniques (<sup>1</sup>H-<sup>1</sup>H COSY, HMBC, HMQC spectra), and other spectroscopy methods (IR and mass spectra). Compound **3** was identified by comparative co-chromatography with authentic sample available in our laboratory.

### Antimicrobial bioassay

The antibacterial activity was determined using both agar diffusion and broth dilution techniques as previously described by Gatsing et al. <sup>3</sup>.

Agar diffusion susceptibility testing was done using both the well and the disc methods. The well method was used to test the CH<sub>2</sub>Cl<sub>2</sub>/MeOH (1:1) extract, whereas the disc method was used to test the isolated pure compounds, chloramphenicol and ciprofloxacin. On each plate containing the appropriate agar medium already inoculated with the test organism (100 µl of the bacteria suspension in the broth, at the concentration of 5.10<sup>5</sup> cfu/ml), equidistant wells (of 6 mm diameter) were bored using a cork borer. The bottom of each well was sealed with a drop of molten agar. The wells were filled with 100 µl of the solution (40 mg/ml) of the extract dissolved in DMSO. For the disc method, a disc of blotting paper was impregnated with 10 µl of a 20 mg/ml solution of each pure compound dissolved in DMSO. Thus, the disc potency was 200 µg for each compound. Ciprofloxacin (5 µg) and chloramphenicol (30 µg) were used as the standard drugs for urogenital infection bacteria and typhoid fever bacteria, respectively. After drying, the disc was placed on a plate of sensitivity testing agar inoculated with the appropriate test organism. The petridishes were left at room temperature (25 ± 2 °C) for about 45 min to allow the extract or the compounds to diffuse from the well/disc into the medium. They were then incubated at 37 °C for 24 h, after which the zones of no growth were noted and their diameters recorded as the zones of inhibition.

For the broth dilution susceptibility testing, the solutions (maximum concentration) of the extract and pure compounds were prepared in DMSO, diluted following an arithmetic progression

by the amount of 1 (for the extract) and 25 (for the compounds), and 0.5 ml of each dilution was introduced into a test tube containing 4.4 ml of selenite broth or tryptose phosphate broth; then 0.1 ml of bacteria suspension ( $5.10^5$  cfu/ml) was added and the mixture was homogenized. The total volume of the mixture was 5 ml, with the extract concentrations in the tube ranging from 20 to 1 mg/ml, those of the test-compounds ranging from 300 to 25  $\mu$ g/ml, and those of the standard drugs, i.e. ciprofloxacin and chloramphenicol, ranging from 20 to 1  $\mu$ g/ml. After 24 h of incubation at 37 °C, the MIC was reported as the lowest concentration of antimicrobial that prevented visible growth. The MBC was determined by sub-culturing the last tube showing visible growth and all the tubes in which there was no growth on already prepared plates containing Mueller Hinton agar medium. The plates were then incubated at 37 °C for 24 h and the lowest concentration showing no growth was taken as the MBC.

## Results

The  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  extract was initially tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi* and *Salmonella paratyphi* B. This extract showed a remarkable activity against *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *E. coli*, *S. typhi* and *S. paratyphi* B, as shown by the diameters of zones of inhibition. Thereafter the  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  extract was fractionated and purified to yield mainly two alkaloids (4,5-ethano-9,10-methylenedioxy-7-phenanthridone or hippadine (**1**) and 4,5-ethano-9-hydroxy-10-methoxy-7-phenanthridone or pratorimine (**2**)) and one steroid ( $\beta$ -D-glucopyranoside of sitosterol (**3**)) which were also tested against all the six bacteria strains. The data obtained from the diffusion sensitivity test showed that compound **1** (at 200  $\mu$ g) was active against all except *P. aeruginosa* and *K. pneumoniae*, whereas compound **3** was active against all the six bacteria strains. Compound **2** did not show any antimicrobial activity against the bacteria strains used (Table 1).

**Table 1: Diameters of inhibition of the bacteria by the  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  extract and compounds isolated from *Crinum purpurascens* leaves**

Extract/compound	Well/disc	Bacteria and diameters of zones of inhibition (mm)						
		Potency*	EC	PA	KP	SA	ST	SPB
$\text{CH}_2\text{Cl}_2/\text{MeOH}$ extract	4 mg	20	17	17	17	25	22	
Compound <b>1</b>	200 $\mu$ g	7	NA	NA	7	8	7	
Compound <b>2</b>	200 $\mu$ g	NA	NA	NA	NA	NA	NA	
Compound <b>3</b>	200 $\mu$ g	9	7	8	7	8	8	
Ciprofloxacin	5 $\mu$ g	22	21	22	23	NT	NT	
Chloramphenicol	30 $\mu$ g	NT	NT	NT	NT	18	20	

\*The  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  extract was tested using the well method, whereas the compounds isolated (**1**, **2** and **3**), chloramphenicol and ciprofloxacin were tested using the disc method.

EC= *Escherichia coli*; PA=*Pseudomonas aeruginosa*; KP=*Klebsiella pneumoniae*; SA= *Staphylococcus aureus*; ST=*Salmonella typhi*; SPB=*Salmonella paratyphi* B; NT = Not tested; NA = Not active.

The antimicrobial activity of the extract and pure compounds was further studied using broth dilution technique in order to determine the MIC and MBC values (Table 2). For the  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  extract, the MIC values were 3 mg/ml (against *P. aeruginosa*), 4 mg/ml (against *E. coli*, *K. pneumoniae* and *S. aureus*) and 6 mg/ml (against *S. typhi* and *S. paratyphi* B), whereas the MBC values varied between 7 and 12 mg/ml. For compound **1**, the MIC values were 200  $\mu$ g/ml (against *K. pneumoniae* and *S. aureus*) and 250  $\mu$ g/ml (against *E. coli*, *P. aeruginosa*, *S. typhi* and *S. paratyphi* B), whereas the MBC value was 300  $\mu$ g/ml against all the bacteria strains used. Compound **2** still did not show any antimicrobial activity against the bacteria strains tested. For compound **3**, the MIC

values were 250  $\mu$ g/ml (against *K. pneumoniae*, *S. typhi* and *S. paratyphi* B) and 300  $\mu$ g/ml (against *E. coli*, *P. aeruginosa* and *S. aureus*), whereas the MBC values were 300  $\mu$ g/ml (against *S. typhi* and *S. paratyphi* B) and > 300  $\mu$ g/ml (against *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus*).

## Discussion

Among the three compounds isolated from *C. purpurascens*  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  leaf extract, **1** and **3** were the only compounds found to exhibit antimicrobial activity against the bacteria used. Compound **2** did not show any antimicrobial activity against these bacteria strains. From the structures of **1** and **2**, it can be seen that compound **2** differs from

**1** only by the opening of methylenedioxy cycle (in C<sub>9</sub> and C<sub>10</sub>) which yields the methoxy group in C<sub>10</sub> and hydroxyl group in C<sub>9</sub>. Therefore, the activity of compound **1** may be attributed to the methylenedioxy (-O-CH<sub>2</sub>-O-) cycle in its structure.

Antimicrobial substances are considered as bactericidal agents when the ratio MBC/MIC ≤ 4 and bacteriostatic agents when the ratio MBC/MIC > 4.<sup>3,4,13,14,16</sup> For the extract, compounds **1** and **3**, the ratio MBC/MIC ≤ 4 (Table 2), suggesting that they

may be classified as bactericidal agents. Besides, the compounds **1** and **3** showed activity against both Gram-negative rods (e.g. *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *S. typhi* and *S. paratyphi* B) and Gram-positive cocci (e.g. *S. aureus*), indicating that these substances may be large spectrum antibacterials.

Compounds **1** and **3** were, however, about 150 times less active than ciprofloxacin and about 100 times less active than chloramphenicol (Table 2).

**Table 2: MIC and MBC values of the CH<sub>2</sub>Cl<sub>2</sub>/MeOH extract and the isolated compounds against the bacteria used**

Extract/compound	Parameters	Bacteria strains					
		EC	PA	KP	SA	ST	SPB
CH <sub>2</sub> Cl <sub>2</sub> /MeOH extract	MIC (mg/ml)	4	3	4	4	6	6
	MBC (mg/ml)	8	7	10	7	12	10
	MBC/MIC	2	3.5	2.5	1.75	2	1.66
Compound <b>1</b>	MIC (µg/ml)	250	250	200	200	250	250
	MBC (µg/ml)	300	300	300	300	300	300
	MBC/MIC	1.2	1.2	1.5	1.5	1.2	1.2
Compound <b>2</b>		NA	NA	NA	NA	NA	NA
Compound <b>3</b>	MIC (µg/ml)	300	300	250	300	250	250
	MBC (µg/ml)	>300	>300	>300	>300	300	300
	MBC/MIC	ND	ND	ND	ND	1.2	1.2
Ciprofloxacin	MIC (µg/ml)	2	2	2	2	NT	NT
	MBC (µg/ml)	5	5	8	5	NT	NT
	MBC/MIC	2.5	2.5	4	2.5	ND	ND
Chloramphenicol	MIC (µg/ml)	NT	NT	NT	NT	2.5	2.5
	MBC (µg/ml)	NT	NT	NT	NT	20	20
	MBC/MIC	ND	ND	ND	ND	8	8

EC= *Escherichia coli*; KP=*Klebsiella pneumoniae*; ST=*Salmonella typhi*; PA=*Pseudomonas aeruginosa*; SA= *Staphylococcus aureus*; SPB=*Salmonella paratyphi* B; NT = Not tested; NA= Not active; ND= Not determined; MIC= Minimum Inhibitory Concentration; MBC= Minimum Bactericidal Concentration

## Conclusion

All these results suggest that the *C. purpurascens* CH<sub>2</sub>Cl<sub>2</sub>/MeOH leaf extract, compounds **1** and **3** have antibacterial properties, and could be developed into remedies for the treatment of typhoid fevers and urogenital infections. Also, the activity of compound **1** may be attributed to the methylenedioxy (-O-CH<sub>2</sub>-O-) cycle in its structure. However, *in vivo* antityphoid properties and the toxicity of these extract and compounds should be evaluated in a further study to ascertain the therapeutic efficacy and the safety of these natural products.

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