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Full Length Research paper

In vitro assays for bioactivity-guided isolation of antisalmonella and antioxidant compounds in *Thonningia sanguinea* flowers

J. D. N'Guessan[1, 3]*, A. P. Bidié[1], B. N. Lenta[2], B. Weniger[3], P. André[4], F. Guédé-Guina [1]

[1]Laboratoire de Pharmacodynamie Biochimique, Université de Cocody Abidjan, 22 BP 582 Abidjan 22, Côte d'Ivoire.
[2]Department of Chemistry, Higher Teacher Training College, University of Yaounde´ I, P. O. Box 47 Yaoundé, Cameroon.
[3]Laboratoire de Pharmacognosie et Molécules Naturelles Bioactives, UMR 7175-LC1, Faculté de Pharmacie, Université Louis Pasteur Strasbourg BP 60024, 67401 Illkirch cedex, France.
[4]Laboratoire de Bactériologie et de Cryptogamie, Faculté de Pharmacie, Université Louis Pasteur Strasbourg BP 60024, 67401 Illkirch cedex, France.

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Bioguided fractionation of the aqueous extract of *Thonningia sanguinea* flowers, used traditionally in the treatment of microbial diseases, led to the isolation of two phenolic compounds. The structure of these compounds was elucidated by ^{1}H, ^{13}C 1D NMR and mass spectrometry experiments. The antibacterial activity against *Salmonella* strains and antioxidant activity of the crude extract, fractions and isolated compounds was evaluated using the DPPH method. The isolated compounds identified as brevifolin carboxylic acid and gallic acid demonstrates moderate antibacterial activity against *Salmonella enteritidis, Salmonella typhimurium,* and *Salmonella abony*. The results indicated that the two isolated compounds, gallic acid (IC_{50} = 13.5 µM) and brevifolin carboxylic acid (IC_{50} = 18.0 µM) were mainly responsible for the good scavenging activity of the aqueous extract.

Key words: *Thonningia sanguinea*, antimicrobial activity, antioxidant activity, gallic acid, brevifolin carboxylic acid.

INTRODUCTION

Over the years, natural products have contributed enormously to the development of important therapeutic drugs used currently in modern medicine (Hostettmann et al., 1995; Cragg et al., 1997; Shu, 1998). In Africa and elsewhere, the use of indigenous plants plays an important role in the treatment of microbial diseases. For instance, the flowers of *Thonningia sanguinea* are largely used in Ivory Coast in the treatment of microbial diseases mainly the salmonellae diseases (Guédé-guina, 2003). In Africa, multidrug-resistant non-typhoidal salmonellae (NTS) are one of the leading causes of morbidity and high mortality in children under 5 years old (Kariuki et al., 2006). *T. sanguinea* is also known to possess antioxidant activity (Gyamfi et al., 1999; Atawodi, 2006).

In recent years, the studies on "oxidative stress" and its adverse effects on human health have become a subject of considerable interest. It is well documented fact that exposure of organism to exogenous and endogenous factors generates a wide range of reactive oxygen species resulting in homeostatic imbalance (Halliwell et al., 1992). Many efforts have been made to discover new antimicrobial and antioxidant compounds from various kinds of sources such as micro-organisms, animals, and plants. Systematic screening of folk remedies is another strategy in the discovery of novel effective compounds (Walter and Memory, 1995).

Previous study on *T. sanguinea* reported the isolation of two antioxidant ellagitannins from the stem bark (Ohtani et al., 2000). As part of our continuing search for biologically active compounds from Ivorian plants, we have examined the aqueous extract of flowers of *T. sanguinea*.

*Corresponding author. E-mail: nguess100jd@yahoo.fr. Tel: (00225) 05-785-789.

Table 1. ^{1}H (300 MHz) and ^{13}C (200 MHz) NMR data for compounds 1 and 2 in CD_3OD

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	Compound 1			Compound 2	
n°	**^{13}C (δ)**	**^{1}H (δ)**	**N°**	**^{13}C (δ)**	**^{1}H (δ)**
1	175.4		1	170.3	
2	41.5	4,5 (dd) (1.2; 7.5)	2	121.9	
3	37.7	2,6 (dd) (1.2; 18.8)	3	110.3	7.05 (d) (2.9)
		3,0 (dd) (7.5; 18.8)			
4	194.6		4	146,3	
4a	149.7		5	139,5	
4b	115.4		6	146,3	
5	-------		7	110,3	7.05 (d) (2.9)
6	161.5				
6a	114.0				
7	108.3	7.3 (s)			
8	143.9				
9	140.9				
10	146.3				
10a	140.1				

We report here the anti-salmonella and antioxidant activity of the aqueous extract, fractions and isolated compounds from the flowers of *T. sanguinea*.

MATERIALS AND METHODS

The present study was performed according to international, national and institutional rules considering biodiversity rights.

Extraction procedure

T. sanguinea flowers were collected in Adzopé, Ivory Coast and identify by Pr Aké-Assi of the Department of Botany, University of Cocody- Abidjan. A voucher specimen (Voucher n° 14162) is deposited in the herbarium of "Centre National de Floristique" of Abidjan.

The freshly collected flowers of the plant were air dried at room temperature for 7 days and powdered. Briefly 20 g of powder was soaked in 500 ml distilled water for 24 h with constant stirring. The suspension was further filtered through Whatmann (N°1) filter paper. The filtrate was concentrated in vacuo using a rotary evaporator to obtain the aqueous extract. The aqueous extract (20 g) was extracted successively with cyclohexane, ethyl acetate and butanol to yield three main fractions labelled F1 (0.18 g), F2 (0.8 g), F3 (1.5 g) and a residue (14.8 g). The disc diffusion assay was used to perform bioguided fractionation (Table 1). The active fraction F3 (1.5 g) was fractionated by column chromatography on Sephadex LH20 (25 cm x 2.2 cm diameter) using methanol as eluent.

Twenty sub-fractions of 20 mL were collected and pooled on the basis of their TLC profiles. Two main sub-fractions labelled F1-8 (Fb1) and F9-20 (Fb2) were obtained. The active sub-fraction Fb2 (210 mg) was purified by preparative HPLC (H2O-ACN; 95 - 5% to 0 - 100% in 35 min, flow rate = 5.0 mL/min) to afford compound (1) (Rt = 10.5 min, 17 mg).

The ethyl acetate fraction F2 was fractionated on Sephadex LH20 using MeOH as eluent. Twenty fractions of 20 mL were collected and pooled on the basis of their TLC profiles. Three main sub-fractions Fa1 to Fa3 were obtained. The active sub-fraction Fa1 was purified by preparative HPLC (H_2O-ACN; 95 - 5% to 0 - 100% in 35 min, flow rate = 5.0 mL/min) to afford compound (2) (Rt = 7.9 min, 20 mg).

Disc diffusion assay

Base plates were prepared by pouring 10 mL Mueller-Hinton (MH) agar into sterile Petri dishes (9 cm) and allowed to set. Molten Filter paper discs (Watmann no 1; 6 mm) were impregnated with the aqueous extract and the different fractions (5 mg/disc). The discs were air-dried and placed on the top layer of the agar plates. Each extract was tested in triplicate with a chloramphenicol (30 µg/disc) as reference or positive control. Water saturated disc (air-dried) were used as negative controls. The sensitivity was recorded by measuring the clear zone of growth inhibition on agar surface around the discs (Bauer et al., 1966).

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined according to Wilkinson and Gentry (1981). Two-fold dilutions (six) of test substances (extract, fractions, isolated compounds) were carried out starting from the concentration of 5 mg/mL. The tubes were inoculated with a microorganism suspension at a density of 10^5 cells/mL. Each tube was incubated at 37°C for 24 h. The lowest concentration of the tube which did not show any visible growth after macroscopic evaluation was considered as the MIC. Gentamycin was used as positive control.

DPPH free radical scavenging activity

The free radical scavenging activity of the extracts, fractions and pure compounds were measured from the bleaching of purple coloured methanol solution of DPPH. This spectrophotometric assay uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent (Cuendet et al., 1997). Five hundred (500) µL of various concentrations using serial 2-fold dilutions of the extract, the fractions and pure compounds in methanol was added to 1 mL of a solution of DPPH (0.02 mg/mL). After a 20 min incubation period at room temperature, the absorbance was read against a blank at 517 nm.

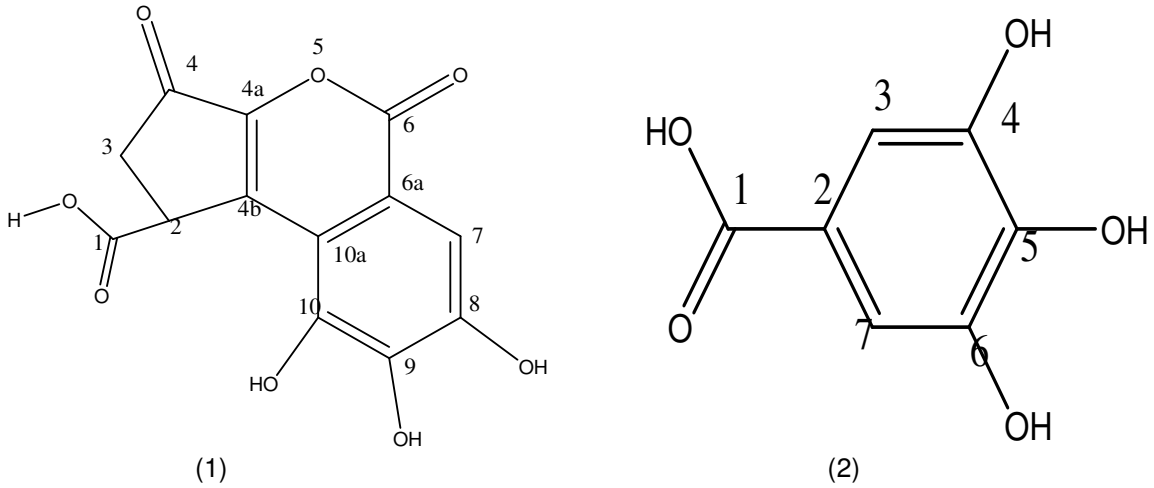

Figure 1. Structures of compounds isolated from the flowers of *Thonningia sanguinea*.

The tests were carried out in triplicate. The inhibitory percentage of DPPH was calculated according to the following equation: Scavenging effect (%) = $[(A_0-A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of samples and standard (quercetol). The amount of sample in µg/ml at which the absorbance at 517 nm decreases to half its initial value is used as the IC_{50} value.

Structure elucidation

The approach for structural elucidation was based on spectroscopic methods, including mass spectra (MS) using HR-ESI-TOF mass spectrometry (MICRO TOF, Bruker Daltonique), ^1H and ^{13}C nuclear magnetic resonance (NMR) experiments recorded on Bruker Avance 300 (300 MHz for ^1H and 200 MHz for ^{13}C). For NMR analysis, compounds 1, 2 were dissolved in 0.50 mL of CD_3OD. The structure of compounds 1 and 2 were determined by comparison of their spectral data with those reported in literature.

RESULTS

Structure of isolated compounds

Compound (1) was isolated as yellow powder, mp 200 - 201°C, and gave a positive reaction with ferric chloride, indicating its phenolic nature. Its elemental composition $C_{13}H_8O_8$ with 10 degrees of insaturation in the molecule was determined according to the high resolution electrospray ionisation mass spectrometry (ESI-TOF-MS), ^1H and ^{13}C RMN spectroscopy. These data permitted us to identify (1) with brevifolin carboxylic acid (Figure 1), previously isolated from *Erodium cicutarium* (Fecka et al., 2001).

Colouring reagents, UV-light fluorescence (254 and 365 nm), comparison of ^1H and ^{13}C NMR data and comparison with a known standard (Sigma) permitted us to identify (2) with gallic acid (Figure 1) (Ohtani et al., 2000). The NMR data of the two compounds are indicated in Table 1. Assignments were confirmed by COSY, HMBC and DEPT experiments.

Antibacterial and antioxidant activities

The aqueous extract of *T. sanguinea* dried flowers was tested for its antibacterial activity against *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella abony*, and its antioxidant activity using the DPPH radical scavenging activity. The results showed bacterial inhibition (MIC = 5 mg/mL; Table 2) and radical scavenging activity (CI_{50} = 19.0 µg/mL; Table 3). The aqueous extract was successively fractionated with cyclohexane, ethyl acetate and butanol. Brevifolin carboxylic acid, isolated by bioguided fractionation from the butanolic fraction (MIC = 2.5 mg/mL; Table 2), exhibited weak antibacterial activity against *S. enteritidis, S. typhimurium* and *S. abony* (MIC > 1600 µg/mL; Table 1) while gallic acid, isolated from the ethyl acetate fraction, showed mild activity (MIC = 1600 mg/mL; Table 2).

In addition to antimicrobial evaluation, the isolated compounds were evaluated for their antioxidant activity using the free radical scavenging activity of the DPPH radical. The good activity observed with gallic acid (IC_{50} 2.3 µg/mL = 13.5 µM) and brevifolin carboxylic acid (IC_{50} 5.2 µg/mL = 18.0 µM) indicated that these compounds are mainly responsible for the free radical scavenging activity of the aqueous extract of dry flowers of this species (IC_{50} 19.0 µg/mL) (Table 2).

DISCUSSION

Salmonellosis and Typhoid fever are always public health concern problem in several countries around the world. These are mostly low-or middle-income countries with inadequate sanitation and hygiene, particularly regarding food, water and disposal of human excreta (Jesudason and Sivakumar, 2006).

In order to provide scientific supports to the use of the flowers of *T. sanguinea*, a *Balanophoraceae* used in Ivory Coast by Traditional practitioner to treat diarrhoeal disea-

Table 2. Minimum Inhibitory Concentration (MIC) values of active extracts, fractions, isolated compounds and gentamycin.

Salmonella sp.	Minimum inhibitory concentration (MIC: mg/mL)				
	Aqueous Extract	Butanolic Fraction	Brevifolin carboxylic acid	Gallic acid	Gentamycin
S. enteritidis	5	2.5	>1.6	1.6	8.10^{-3}
S. abony	5	2.5	>1.6	1.6	8.10^{-3}
S. typhimurium	5	2.5	>1.6	1.6	8.10^{-3}

Table 3. IC_{50} values of the aqueous extract and isolated compounds.

Sample	DPPH radical scavenging activity	
	IC_{50} (µg/mL)	IC_{50} (µM)
Aqueous extract	19.0	-
Ethyl acetate fraction	19.0	-
Fa1	15.5	-
Fa2	23.0	-
Fa3	30.2	-
Butanolic fraction	11.0	-
Residue	35.0	-
Brevifolin carboxylic acid	5.2	18.0
Gallic acid	2.3	13.5
Quercetol	1.0	3.3

se and salmonellosis, we have tested its aqueous extract against *S. enteritidis, S. abony*, and *S. typhimurium.* We have also isolated two compounds identified as gallic acid and brevifolin carboxylic acid.

Surprisingly, the bioguided fractionation led to the isolation of compounds with weak antibacterial activities. Gallic acid is known to possess moderate antibacterial activity against *Salmonella cholaerasius* (Kubo et al., 2002) and there is no scientific report concerning brevifolin carboxylic acid antibacterial activity. The presence of gallic acid in the aqueous extract of *T. sanguinea* flowers may partly explain the traditional use of this plant against Salmonella previously described by Vangah-Manda et al. (1994). The presence of tannins in this species, some of which are known to possess potent antibacterial activity (Scalbert, 1991), suggest that the antimicrobial activity of the aqueous extract of dry flowers of *T. sanguinea* is probably due to the synergistic activity of several polyphenolic compounds.

Plant compounds are routinely classified as “antimicrobial” on the basis of susceptibility tests that produce MICs in the range of 100 to 1000 µg/mL, orders of magnitude weaker than those of typical antibiotics produced by bacteria and fungi (MICs, 0.01 to 10 µg/mL). A secondary metabolite or compound which shows little activity in an *in vitro* susceptibility test is not necessarily an antimicrobial. Such a substance might have a regulatory function (Tegos et al., 2002).

The importance of reactive oxygen species (ROS) and free radicals has attracted increasing attention over the past decade. ROS which include free radicals such as superoxide anion radicals, hydroxyl radicals and non-free radical species such as oxygen peroxide and singlet oxygen, are various forms of activated oxygen. These molecules exacerbate factors in cellular injury and aging process (Halliwell and Gutterigde, 1989). The harmful action of free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organisms.

In this context, we have also investigated the antioxidant properties of the aqueous extract of *T. sanguinea* and the isolated compounds. The results showed high antioxydant properties of the extract and the two isolated compounds.

Gallic acid is well known as a potent antioxidant compound (Lu et al., 2006). The antioxidant activity of brevifolin carboxylic acid is probably related with the presence of various acidic phenolic protons. These results may be related with those of Gyamfi et al. (1999) who showed that the root extract of *T. sanguinea* possesses antioxydant activity. Brevifolin carboxylic acid is isolated for the first time from *T. sanguinea*.

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