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ORIGINAL RESEARCH PAPER

ANTIPLASMODIAL AND ANTISALMONELLA ACTIVITIES OF PHENOLIC COMPOUND FROM *ROUREA COCCINEA* BENTH (CONNARACEAE)

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Abstract: The leaves extract of *Rourea coccinea* was fractionated, following the bioguided method, to give one phenolic compound named 1-caffeoylquinic acid lactone (1-CQL) (**1**), isolated from this plant for the first time, and the 3-*O*-*β*-*D*-glucopyranoside of stigmasterol (**2**). The structure of these compounds was determined. The Minimum Inhibitory Concentrations (MIC) for antisalmonella activity was up to 2000 $\mu g \cdot mL^{-1}$ for extract and fractions, and up to 500 $\mu g \cdot mL^{-1}$ for compound **1**. The half-inhibitory Concentration (IC_{50}) values against *Plasmodium falciparum PfDd2* was 92.56 $\mu g \cdot mL^{-1}$ for extract and 31.24 to 77.21 $\mu g \cdot mL^{-1}$ for fractions. Compound **1** exhibited on the same strain an antiplasmodial activity with an IC_{50} value of 8.58 $\mu g \cdot mL^{-1}$. These results justify the use of this plant in Beninese pharmacopeia for the treatment of several diseases such as malaria.

Keywords: *1-caffeoylquinic acid lactone, column chromatography, ethanol extract, IC_{50}, leaves, MIC, spectroscopic method*

INTRODUCTION

Natural products coming from natural sources are nowadays use mainly by native population for their nutritional values and medicinal properties [1, 2]. These properties refer to their abundance in terms of secondary and primary metabolites [3]. However, population mainly from tropical countries continuous to suffer due the infection diseases, caused by pathogens microorganisms [4]. Despite the discovery and recommendation of drug against infection diseases, their monitoring poses a great problem in public health [5] because of drug resistance and side effect [6]. It is then essential to continuous to search for new alternative drug [7]. Regarding the secondary metabolites present in plants using in traditional medicinal, their exploration could be appropriate approach to discover new drugs for such treatment [8]. *Rourea coccinea* (Schumach & Thonn). Benth belonging to Connaraceae family [9] is widely used in traditional medicine worldwide, especially in Beninese pharmacopeia to fight against several ailments like malaria, male and female infertility, gonorrhea, and snakebite [9]. In Nigeria, leaves are used to treat diabetes, fever, diarrhea, injury, sexually transmitted infections and skin infections [1, 5]. The previous biological activity investigation has demonstrated the antimalarial, antileishmanial, antitrypanosomial [7], antioxidant, analgesic [10], antipyretic [11], hypotensive [12] antidiabetic, anti-microbial [13], anti-diarrhetic, anti-cancer [14], aphrodisiac and uretonic [15] properties of *Rourea coccinea*.
In this paper, we report the structural identification of a phenolic compound named 1-caffeoylquinic acid lactone (1-CQL) as well as the antiplasmodial and antisalmonella activity of crude extract, fractions and the phenolic compound.

MATERIALS AND METHODS

General experimental procedure

1D- and 2D-NMR spectra were recorded in deuterated solvents on either a Bruker ARX (Bruker, Germany) 500 or an AVANCE DMX 600 NMR spectrometer (proton at 500 MHz and carbon ^{13}C at 125 MHz). All chemical shifts (δ) were measured in parts per million (ppm) using a residual solvent signal as a secondary reference relative to tetramethylsilane as internal standard, while coupling constants (J) are given in Hz. Solvents were distilled prior to use. Analytical grade solvents were used for Liquid Chromatography Mass Spectrum (LCMS). Column chromatography was performed using Merck MN silica gel 60 M (75 × 4 cm, 0.04 - 0.063 nm) (Merck), and Thin Layer Chromatography (TLC) on aluminum silica gel 60 F254 (Merck) precoated plates (0.2 mm layer thickness). Compounds were visualized on TLC either by the use of an UV lamp (LAMAG UV Lamp 4, Switzerland; 254 and 366 nm) or by heating after spraying with 20 % H_2SO_4 (v/v) solution. Different mixtures of *n*-hexane (*n*-hex), ethyl acetate (EtOAc), methylene chloride (CH_2Cl_2), and methanol (MeOH) produce by ADER-Cameroon, were used as eluting solvents.
Plasmodium falciparum's strain is that chloroquine resistant *PfDd2* that was grown in human red blood cells The cells are from group O, Rhesus positive to 4 % hematocrit in full Roswell Park Memorial Institute (RPMI) medium 500 mL RPMI 1640 (Gibco, UK) supplement 25 mM HEPES (Gibco, UK), 0.50 % Albumax I (Gibco, USA),

1X hypoxanthine (Gibco, USA), gentamicin (Gibco, China), and incubator which consists of 92 % of N_2, 5 % of CO_2 and 3 % of O_2 provided by Pasteur Center of Cameroon (CPC). The medium was replaced daily with full RPMI medium to facilitate the growth of pests in cultivation. Subsequently, thin blood smears were carried out and stained with Giemsa and then observed under the microscope at the 100 X objective with immersion oil in order to follow all the steps of the cell cycle and evaluate the parasitemia.

Preparation of plant material

The leaves of *Rourea Coccinea* were harvested in central Benin (Avogbanna) in Mars 2021. The specimen was taken to UAC botanical garden where the plant was identified by comparing with the reference specimen numbered YH761/HNB. The collected plant was brough to the laboratory of Physic and Synthesis Organic Chemistry (LaCOPS) of University of Abomey-Calavi to be chemically investigate. The leaves were dried at room temperature for two weeks. After drying, they were further ground into fine powder and followed by extraction. The leaves extracts were obtained by maceration, with the help of percolator, of 2.7 kg of powder in 20 L of ethanol (95 %). After 72 hours, the resulting solution was then filtered. The filtrate obtained was concentrated using a rotavapor (BUCHI Rotavapor R-100) and air-dried in the oven (VWR, VENTI-Line VL 112 Prime, Poland) at 50 °C to eliminate the remaining solvent. The crude extracts obtained were weighted (300 g) and kept at 4 °C for the chemical and bioguided studies.

Strains and growth conditions

The clinical isolates Salmonella namely *Salmonella enteritidis, Salmonella typhi, salmonella typhimurium* from "*Centre Pasteur du Cameroun*" (CPC) were used to assess the anti-salmonella properties. The strains were cultured in Salmonella-Shigella Agar to confirm the identity and kept in Muller Hinton agar sloping in test tube. The strains were revived 24 hours at 37 °C prior to each assay. For the antiplasmadial assay, Chloroquine *Plasmodium falciparum PfDd2* resistant culture in 60 mm sterile petri dishes containing complete Roswell Park Memorial Institute 1640 medium (Sigma Aldrich) supplemented with Human red blood cell strains of O Rh^+ was used.

Preparation of stocks solution and reference antibacterial

The stock solution of extracts, fractions and compounds were prepared at 100 $mg \cdot mL^{-1}$ and 2 $mg \cdot mL^{-1}$ respectively dissolving 100 mg of extracts, fractions and 2 mg of compounds in 1 mL of absolute DMSO. The ciprofloxacin used as positive control was prepared in the same conditions dissolving 1 mg of powder into 1 mL of acidified distilled water. The stocks solutions were kept at 4 °C for the studies.

Anti-salmonella activity

The antisalmonella activity was performed according to M09A7 protocol of CLSI, 2012 with minor modifications using microdilution method coupled with resazurin based assay [16]. The tests were performed in sterile 96 well microplate. In fact, the leaves crudes extract and ciprofloxacin were prepared using two-fold serial dilution to have a final

concentration ranged from 2000 to 31.25 µg·mL^{-1}and 1.95 to 0.015 µg·mL^{-1} respectively. 100 µL of bacteria suspension prepared at 10^6 CFU·mL^{-1} from 24 hours culture (using standard 0.5 McF) were introduced into each well excepted those of sterility control. The negative control was constituted by bacteria suspension and Muller Hinton Broth (MHB), the positive control was constituted by ciprofloxacin, MHB and bacteria suspension while the sterility control was just MHB. The final bacteria suspension was $5 \cdot 10^5$ CFU·mL^{-1} with 200 µL as final volume into each well. The plates were incubated at 37 °C for 24 hours. At the end of this incubation period, 20 µL of freshly prepared resazurin (0.15 mg·mL^{-1}) were added into each well follow by the incubation in the same previous conditions for 30 min. The MIC was defined as the smallest concentration in which any change of color (from blue to pink) was observed corresponding to the visible bacteria growth.

Evaluation of antiplasmodial activity

The antiplasmodial activity test of the leaves crude extract, fraction, compounds and positive controls was performed in 96 flat-bottom microplates based on the fluorescence of SYBR green I [17]. The ability of SYBR green I to produce high fluorescence only in the presence of DNA is the basis for its use in cell proliferation assessment. The absence of a cell nucleus within the human red blood cells in which the parasite proliferates allows for specific monitoring of plasmodial growth using SYBR green I. Artemisinin and Chloroquine were used as positive controls for the antiplasmodial test.

Fractionation and isolation

Part of this extract (200 g) was diluted in distilled water (500 mL) and fractionated by liquid-liquid partitioning using successively solvents with increasing polarity such as *n*-hexane (4 × 500 mL), ethyl acetate (EtOAc; 4 × 500 mL) and *n*-butanol (*n*-BuOH; 4 × 500 mL) to yields four fractions namely *n*-hexane fraction (20 g), ethyl acetate fraction (35 g), *n*-butanol fraction (55 g) and the aqueous fraction (80 g of residue). These fractions were then subjected to the antimicrobial assay and the ethyl acetate fraction which were the most active fraction than orders were chemically investigated. The ethyl acetate fraction (35 g) was then subjected to column chromatography (3.5 × 75 cm) over silica gel (0.04 - 0.063 nm) and eluted with *n*-hexane/ethyl acetate as solvent system with a gradient of polarity (100:0, 1500 mL; 95:5, 1500 mL; 90:10, 1500 mL; 80:20, 1500 mL; 70:30, 1500 mL; 60:40, 1500 mL; 50:50, 1500 mL; 40:60, 1500 mL; 30:70, 1500 mL; 20:80, 1500 mL; 0:100, 1500 mL) to yield 3-*O*-*β*-*D*-glucopyranoside of stigmasterol (34 mg) and a phenolic compound named 1-caffeoylquinic acid lactone (14 mg).

Statistical analysis

The resulting fluorescence values were used to calculate inhibition percentages using Microsoft Excel software. Subsequently, the 50 % inhibitory concentrations (IC_{50}) were determined using the concentration-response curves obtained from the logarithm plot of the concentration as a function of percent inhibition using the Graphpad Prism 5 software provided by Pasteur Center of Cameroon.

RESULTS AND DISCUSSION

Evaluation of antisalmonella activity of extract and fractions of *Rourea coccinea*

Analysis of the results shows that the minimum inhibitory concentrations (MICs) of ethanolic extract of leaves was greater than 2000 $\mu g \cdot mL^{-1}$. Our extract would not be active, based on the evaluation criteria of our *Salmonella* work. It was shown by Ahmadu and collaborators [18] that the ethyl acetate extract from leaves causes considerable inhibition with a MIC values of 200 $mg \cdot mL^{-1}$. In the same work of Ahmed, the ethyl acetate fraction from the ethanolic extract of leaves of the same plant showed an activity against *Salmonella typhi* with a MIC value of 1.75 $mg \cdot mL^{-1}$. The results obtained corroborate with those obtained by Ahmadu et *al*. [19] (Table 1) and show that the antisalmonella activity of the leaves is low. The same authors showed that the plant has activity with other strains such as *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus* but the ethyl acetate fraction has better activity with a MIC 1.75, 0.88 and 0.44 $mg \cdot mL^{-1}$ for *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus* respectively [19]. In addition, Ezeh et *al* in 2019 showed that the methanol extract of the leaves of *Rourea coccinea* harvested in Nigeria has an interesting antifungal activity against isolated strains of *Escherichia coli* and *Bacillus subtilis* with a MIC value of 6.25 $mg \cdot mL^{-1}$. However, the extract is not active against *Salmonella typhi* and *Klebsiella pneumonia*. In addition, it has been reported by Nwoko *et al* that the plant harvested in Nigeria exhibits interesting antifungal activity against *C. tropicalis* strains, 25 (31.6 %) and *C. krusei*, 10 (12.7 %) especially in people suffering from malaria [20].

Inhibitory Concentration 50 (IC_{50}) of extract, fractions and compound

The obtained crude extracts, fractions and isolated compounds were assessed for their ability to stop chloroquine resistant *PfDd2* and the results are presented in Table 1 expressed in terms of IC_{50}. The crude extract exhibited an antiplasmodial activity with the IC_{50} value of 92.52 $\mu g \cdot mL^{-1}$ and further fractionate. Most interesting the fractions also exhibited good antiplasmodial activity with the IC_{50} values ranged from 77.21 to 31.24 $\mu g \cdot mL^{-1}$. The ethylacetate fraction was the most active with an IC_{50} of 31.24 $\mu g \cdot mL^{-1}$ followed by *n*-butanol fraction and *n*-hexane fraction with the IC_{50} values of 35.67 and 45.58 $\mu g \cdot mL^{-1}$ respectively. In the similar way, compounds **1** obtained from ethyl acetate fraction strongly arrest the growth of *PfDd2* with an IC_{50} value of 8.58 $\mu g \cdot mL^{-1}$ (Table 1). This moderate activity on multi-resistant strains appears to be due to the secondary metabolites contained in ethyl acetate fraction. Indeed, according to Akindele and Adeyemi [12], phytochemical screening of the plant extract reveals the presence of flavonoids, tannins, saponines, anthraquinones, glucosides and sugars [12]. The results obtained corroborate with those reported by Bero and collaborators [2]. These authors showed that dichloromethane extract and the ethanolic extract of the leaves of *Rourea coccinea* had moderate activity with an IC_{50} values of 41.6 and 54.7 $\mu g \cdot mL^{-1}$, respectively. The work of other authors revealed a better antiplasmodial activity of *Rourea coccinea* compared to ours on other *Plasmodium falciparum* strains.

Table 1. *Results of the evaluation of antisalmonella activity and antiplasmodial activity of extract, fractions and compound 1*

	MICs [µg·mL^{-1}]			**IC_{50} [µg·mL^{-1}]**
	Clinical isolates of *Salmonella* genus			**Parasite strains of the *Plasmodium* genus**
	SE CPC	**STM CPC**	**ST CPC**	***PfDd2***
Extract	>2000	>2000	>2000	92.52 ± 2.56
n-hexane fraction	>2000	>2000	>2000	45.58 ± 0.07
Ethylacetate fraction	>2000	>2000	>2000	31.24 ± 0.25
n-butanol fraction	>2000	>2000	>2000	35.67 ± 0.65
Residue	>2000	>2000	>2000	77.21 ± 0.37
Compound **1**	>500	>500	>500	8.58 ± 0.62
Ciprofloxacin	0.3	0.3	0.63	-
Chloroquine	-	-	-	0.03 ± 0.02
Artemisinin	-	-	-	0.7 ± 0.22

MICs: Minimal Inhibitory Concentrations; **IC_{50}**: Inhibitory Concentrations 50; **SE**: *Salmonella enteritidis*; **STM**: *Salmonella typhimurium*; **ST**: *Salmonella typhi*; **CPC**: *Centre Pasteur du Cameroun.*

Characterization of compound 1

Silica gel column chromatography (CC) of the ethanol extract of leaves of *Rourea coccinea* led to the isolation of a new phenolic compound named 1-caffeoylquinic acid lactone (**1**), along with the 3-*O*-*β*-*D*-glucopyranoside of stigmasterol (**2**). The structures of these compounds were determined based on the analysis of their spectroscopic data, which showed complete agreement with those reported in the literature (Figure 1).
Compound **1** was obtained as a pink powder in the *n*-hex/EtOAc (9:1 v/v) fraction. It reacted positively both with the iron chloride III test and with 2,4-ditrophenylhydrazine test, suggesting its phenolic nature and the presence in its structure of a carbonyl function.

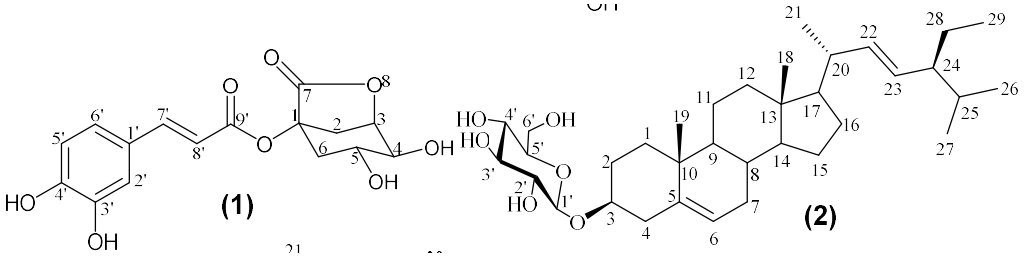

Figure 1. *Structures of compound 1 and 2*

The analysis of the ^{1}H (500 MHz, DMSO-d_6) NMR spectrum showed three signals of ABX system corresponding to three aromatic protons including one doublet of doublet at δ_H 7.05 (H-6', dd, J = 8.2, 2.1 Hz); one doublet at δ_H 7.01 (H-2' J = 2.1 Hz) and another doublet at δ_H 6.77 (H-5' J = 8.2 Hz,) characteristic of protons of a trisubstituted aromatic nucleus [21]. Were also observed two signals of two doublets at δ_H 6.26 (H-8', d, J = 15.9 Hz) and 7.54 (H-7', d, J = 15.9 Hz) corresponding to two non-chemically equivalent

olefinic protons whose high coupling constant value indicates that they are in trans [22]. Seven reasoning signals between δ_H 1.92 and δ_H 4.69 corresponding to the protons of a lactone bicyclic moieties (Table 2).

Table 2. *^{1}H (500 MHz, DMSO-d_6) and ^{13}C (125 MHz, DMSO-d_6) NMR spectral data, COSY and HMBC correlations of compound **1***

Positions	Spectral Data of compound 1			
	δ_C	Mult	δ_H (mH ; mult ; *J* in Hz)	COSY
1	71.9	C	-	
2	37.0	CH_2	2.22 (1H, m)	
			2.38 (1H, d, *J* = 11.5)	
3	76.2	CH	4.69 (1H, m)	
4	63.1	CH	4.16 (1H, d, *J* = 4.6)	
5	68.9	CH	4.72 (1H, m)	H_5-H_4
6	36.0	CH_2	1.92 (1H, t, *J* = 11.5)	
			2.03 (1H, m)	
7	177.5	C	-	
1'	125.9	C	-	
2'	115.3	CH	7.01 (1H, d, *J* = 2.1)	
3'	146.1	C	-	
4'	149.0	C	-	
5'	116.3	CH	6.77 (1H, d, *J* = 8.2)	$H_{5'}$-$H_{6'}$
6'	121.9	CH	7.05 (1H, dd, *J* = 8.2, 2.1)	
7'	145.0	CH	7.54 (1H, d, *J* = 15.9)	$H_{7'}$-$H_{8'}$
8'	114.2	CH	6.26 (1H, d, *J* = 15.9)	
9'	166.2	C	-	

Its ^{13}C NMR spectrum exhibited signals amount which, one signal at δ_C 177.5 (C-7) corresponding to carbonyl of lactone [23], one signal at δ_C 166.1 (C-9') corresponding to the conjugated carbonyl one signal at δ_C 166.1 (C-9') corresponding to the conjugated carbonyl [24]. Were also observed six signals appearing at δ_C 149.0 (C-4'), δ_C 146.1 (C-3'), δ_C 125.9 (C-1'), δ_C 121.9 (C-2'), δ_C 116.3 (C-5') and at δ_C 115.3 (C-6') which correspond to the carbons of aromatic ring trisubstituted at C-1', C-3' and C-4'. Six other signals appearing between δ_C 36.0 and δ_C 76.2 correspond to the lactone bicyclic moieties (Table 2).

The above analysis of spectroscopic data (^{1}H and ^{13}C NMR) in conjunction with 1D- and 2D- and with those reported in the literature allowed us identified compound **1** as 1-caffeoylquinic acid lactone which have been reported previously.

CONCLUSION

The present work which consisted of the investigation of the leaves of *Rourea coccinea* through bioguided method, allowed us to isolate a phenolic compound named 1-caffeoylquinic acid lactone (**1**) newly isolated from this specie, along with the 3-*O*-*β*-*D*-glucopyranoside of stigmasterol (**2**). *In vitro* biological studies have shown that the leaves of the plant have a weak antisalmonella activity. It was also showed that the

ethanol leaves extract as well as the hexane, ethyl acetate and *n*-butanol fractions are actives on the multi-resistant *Plasmodium falciparum PfDd2* strain. A 1-caffeoylquinic acid lactone was active against *Plasmodium falciparum*. These results justify the traditional use of the leaves of this plant in the treatment of malaria.

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