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Anti-plasmodial and antioxidant activities of constituents of the seed shells of *Symphonia globulifera* Linn f.

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Abstract

A xanthone derivative, named gaboxanthone (**1**), has been isolated from the seed shells of *Symphonia globulifera*, together with known compounds, symphonin (**2**), globuliferin (**3**), guttiferone A (**4**), sistosterol, oleanolic acid and methyl citrate. The structure of the compound was assigned as 5,10-dihydroxy-8,9-dimethoxy-2,2-dimethyl-12-(3-methylbut-2-enyl) pyrano [3,2-b]xanthen-6(2H)-one, by means of spectroscopic analysis. The anti-plasmodial and antioxidant activities of the phenolic compounds were evaluated, respectively, in culture against W2 strain of *Plasmodium falciparum* and using the free radical scavenging activity of the DPPH radical, respectively. Compounds **1**–**4** were found to be active against the *Plasmodium* parasites (IC_{50} of 3.53, 1.29, 3.86 and 3.17 μM, respectively). Guttiferone A (**4**) showed a potent free radical scavenging activity compared to the well-known antioxidant caffeic acid.

Keywords: *Symphonia globulifera*; Xanthones; Seed shells; *Plasmodium falciparum*; Anti-plasmodial; Antioxidant

1. Introduction

Despite countless efforts to eradicate or control malaria, it represents the most deadly parasitic human disease. Each year, it threatens around 40% of the world's population, infects over 200 million people, and claims 2 million lives, primarily children under five years of age (WHO, 2002). In the particular case of Cameroon, it is the leading cause of morbidity and mortality, where it accounts for 40–50% of medical consultations, 30% of hospitalizations, 40% of deaths among children under five years, and 35–40% of hospital deaths (National Malaria Control Program of Cameroon, 2002). Treatment of malaria is becoming increasingly more difficult due to the emergence of multi-drug-resistant strains of *Plasmodium falciparum*, the causative agent of the most severe form of the disease. In Cameroon, poverty for the most part has driven people into self-medication, which is partly responsible for the widespread drug resistance, with 67% in the particular case of chloroquine in some sentinel sites (National Malaria Control Program of Cameroon, 2002).

As a result, there is an urgent need to search for and develop novel antimalarial agents. In Africa and elsewhere, the use of indigenous plants still plays an important role in malaria treatment (Gessler et al., 1994). These plants are indeed an interesting source of new anti-plasmodial compounds, among which xanthones showed potent activity. Polyhydroxyxanthones, oxygenated and prenylated xanthones, bixanthones and xantholignoids have been reported

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(Hay et al., 2004; Dua et al., 2004; Likhitwitayawuid et al., 1998; Winter et al., 1996, 1997) as a novel class of antimalarial compounds with good activity against multi-drug-resistant *Plasmodium* parasites. It has been demonstrated that selected hydroxyxanthones form soluble complexes with heme and prevent the precipitation of heme in aqueous solution under the mildly acidic conditions of the digestive vacuole at pH 5.2 (Ignatushchenko et al., 1997, 2000).

Symphonia globulifera, a plant from the Guttiferae family, is a large tree (about 110 ft. high and 6 ft. girth, with ellipsoid to depressed globose, reddish brown fruits) found in Cameroon, where it is used as a medicinal plant to cure several diseases such as stomach and skin aches. It is also used as laxative for pregnant women and as a general tonic (Irvine, 1961; Aubreville, 1950). In the North western province of Cameroon, the bark is used by traditional healers to treat malaria. Polyprenylated xanthones and benzophenones isolated from this plant were found to have various biological activities (Nkengfack et al., 2002; Peres and Nagem, 1997; Gustafson et al., 1992), but very few studies have been carried out on their anti-plasmodial activity. Previously, we reported the isolation of the xanthones symphonin and globuliferin from the seeds of *S. globulifera* (Lenta et al., 2004; Ngouela et al., 2005). In our continuing interest in searching for new biologically active metabolites, we investigated the seed shells of this plant. In this paper, we report the isolation and the structural elucidation of a new xanthone, namely gaboxanthone, and the anti-plasmodial and antioxidant activities of its phenolic constituents.

2. Results and discussion

The air-dried and ground seed shells of *S. globulifera* were extracted at room temperature with a mixture of CH_2Cl_2–MeOH (1:1). The extract was concentrated to dryness under vacuum and the residue subjected to repeated column chromatographic separation to yield gaboxanthone (**1**) along with symphonin (**2**), globuliferin (**3**), guttiferone A (**4**), oleanolic acid, sistosterol and methyl citrate.

Compound (**1**) was isolated as a yellow powder, m.p. 222–223 °C and gave a positive reaction with ferric chloride, indicating its phenolic nature. Its elemental composition $C_{25}H_{26}O_7$, with 12 degrees of unsaturation, was deduced from its HR-EIMS (*m*/*z* 438.1666). The broad band decoupled ^{13}C NMR spectrum (Table 1) of compound (**1**) showed 25 carbon signals which were sorted by Jmod and HSQC techniques as four methyls, one methylene, four methines, two methoxyls and 14 quaternary carbons including a carbonyl group at δ 180.0. The IR spectrum of (**1**) exhibited strong absorption bands due to phenolic hydroxyl (*s*) (3280 cm^{-1}), chelated hydroxyl group (3265 cm^{-1}) and conjugated carbonyl (1640 cm^{-1}). Its UV spectrum showed specific absorptions [λ_{max} (237, 253, 285, and 350 nm)] for a xanthone nucleus (Hostettmann and Hostettmann, 1989) similar to that of symphonin. In the ^1H NMR spectrum, a chelated hydroxyl group at δ_H 13.35 and a D_2O exchangeable broad singlet at δ_H 5.84 due to a free phenolic hydroxyl group were observed. The ^1H NMR spectrum of (**1**) also showed the characteristic resonances of a 3,3-dimethylallyl moiety [δ_H 1.70 (3H, *s*); 1.89 (3H, *s*); 3.52 (2H, *d*, $J = 7.2$ Hz) and 5.30 (1H, *m*)], together with signals for two methoxyl groups [δ_H 3.94 (3H, *s*) and 4.06 (3H, *s*)] and the dimethylpyran ring at δ_H 1.55 (6H, *s*), 5.65 (1H, *d*, $J = 10$ Hz) and 6.65 (1H, *d*, $J = 10$ Hz). The presence of the dimethylpyran ring was further supported by the set of signals at δ_C 28.4, 78.1, 115.8 and 127.4 in the ^{13}C NMR spectrum (Nkengfack et al., 2002). Comparison of the ^{13}C NMR shifts of **1** with those of symphonin **2** (Table 1) revealed some differences. C-5, C-4a, and C-12a were shielded by 2–7 ppm while C-12 and C-10b were deshielded (3–7 ppm) relative to those of **2** suggesting a difference in the location of the pyran ring and the 3,3-dimethylallyl group. All the remaining carbon signals in the two compounds were very similar. This similarity was further confirmed by examination of the ^1H NMR spectrum of the two compounds. Correlations in the HMBC spectrum of **1** showed that the 3,3-dimethylallyl group was attached to C-12 and the pyran ring to C-4a and C-12a of ring A. Thus the methylene proton at δ_H 3.52 (*d*, $J = 7.2$ Hz) showed cross-peaks with C-12a ((δ_C 155.6), C-12 (δ_C 107.5), C-10b ((δ_C 153.0) and the protons of the AB system of the pyran ring at δ_H 6.65 (*d*, $J = 10$ Hz) and 5.50 (*d*, $J = 10$ Hz) correlated to C-5 ((δ_C 158.0), C-4a (105.0)

Table 1
^1H (400.1 MHz) and ^{13}C (100.6) NMR chemical shift assignments for gaboxanthone **1**

No.	^{13}C ($CDCl_3$)	^1H ($CDCl_3$) (*m*) *J* (Hz)	HMBC correlations
2	78.1	–	–
3	127.4	5.65 (*d*, $J = 10$ Hz)	C-4a
4	115.8	6.65 (*d*, $J = 10$ Hz)	C-2, C-4a, C-5
4a	105.0	–	–
5	158.0	–	–
5a	103.3	–	–
6	180.0	–	–
6a	116.0	–	–
7	96.6	7.23 (*s*)	C-6, C-9
8	149.4	–	–
9	141.1	–	–
10	140.0	–	–
10a	138.0	–	–
10b	153.0	–	–
12	107.5	–	–
12a	155.6	–	–
1′	21.6	3.52 (*d*, $J = 7.2$ Hz)	C-10b, C-3′
2′	122.3	5.30 (*t*, $J = 7.2$ Hz)	C-12
3′	131.7	–	–
4′	25.8	1.89 (*s*)	C-2′, C-3′, C-5′
5′	17.9	1.70 (*s*)	
2(2-Me)	28.4	1.55	C-2, C-3
8-OMe	56.2	3.94 (*s*)	C-8
9-OMe	61.4	4.06 (*s*)	C-9
5-OH	–	13.35 (*s*)	C-4a, C-5a
10-OH	–	5.85 (*s*)	C-9

and C-2 (78.1), respectively. Thus **1**, gaboxanthone, is 5,10-dihydroxy-8,9-dimethoxy-2,2-dimethyl-12-(3-methylbut-2-enyl) pyrano [3,2-b]xanthen-6(2H)-one, an isomer of symphonin (**2**) (see Fig. 1).

In addition to (**1**), six known compounds were isolated and identified as symphonin (**2**), globuliferin (**3**), guttiferone A (**4**), oleanolic acid, sistosterol and methyl citrate (Lenta et al., 2004; Ngouela et al., 2005; Gustafson et al., 1992; Mahato and Kundu, 1994; Ando et al., 1972).

Compounds **1**–**4** were tested for their anti-plasmodial activity against the W2 strain of *P. falciparum*, which is resistant to chloroquine and other antimalarials (Table 2).

All the three xanthones (**1**–**3**) and guttiferone A **4** (benzophenone) were found to exhibit good to moderate activity relative to chloroquine, with symphonin **2** demonstrating the best potency (1.29 μM). Previous studies showed that the anti-plasmodial activity of xanthones involves complexation with heme, inhibiting the hemozoin formation (Kelly et al., 2002). From the structure–activity relationship, it appears that the cyclisation of one of the isopentenyl groups (positions 2 and 4) to give a pyran ring increases the potency of xanthones. The best result is obtained when the dimethylpyran ring is attached to positions 3 and 4 of the xanthone nucleus as in **2**. In our case, the activity is multiplied by a factor of ~3 from compound **3** to **2**. These results are in accordance with those reported previously (Kelly et al., 2002; Hay et al., 2004).

The antioxidant activity of compounds **1**–**4** was also evaluated using the free radical scavenging activity of DPPH. Guttiferone A, a polyhydroxybenzophenone showed very good radical scavenging activity (89% of inhibition) relative to the antioxidant caffeic acid (58% of inhibition). The other compounds were found to be less active, with 23%, 28% and 54% inhibition, respectively, for **2**, **1** and **3** (Table 3).

Table 3
Antioxidant activity (% inhibition) of compounds **1**–**4**

Compound	% Inhibition of DPPH free radical
Gaboxanthone (**1**)	28 ± 0.5
Symphonin (**2**)	23 ± 0.4
Globuliferin (**3**)	54 ± 0.7
Guttiferone A (**4**)	89 ± 0.5
Caffeic acid	58 ± 0.6

The results obtained from the four tested compounds for both anti-plasmodial and antioxidant activities are of interest. In fact, it has been proven that the *Plasmodium*-infected erythrocyte is under constant oxidative stress. This is caused by exogenous reactive oxidant species and reactive nitrogen species produced by the immune system of the host, and by endogenous production of reactive oxidant species generated during the digestion of host cell haemoglobin and concomitant biochemical reactions. Therefore, the defense system is not fully effective since the fingerprints of oxidative stress are discernable in the membrane of the infected red blood cells such as clustering and increased levels of lipid peroxides, and furthermore serious effects on the recovery of the host system (Giribaldi et al., 2001; Simões et al., 1992). This is why compounds exhibiting both anti-plasmodial and antioxidant activities could be very interesting as leads in the search for new antimalarial drugs (see Fig. 2).

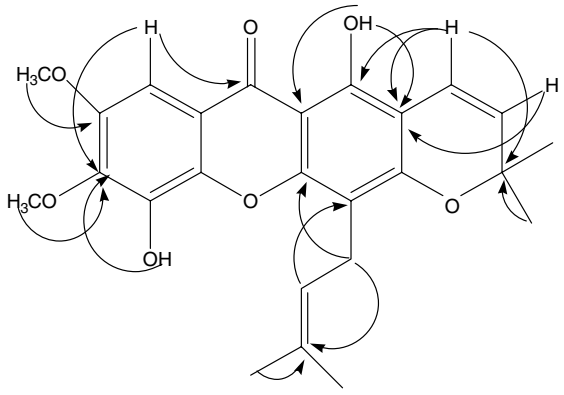

Fig. 1. Significant HMBC correlations for compound **1**.

Table 2
Anti-plasmodial activity of compounds **1**–**4**

Compound	IC_{50}[a] (μM)
Gaboxanthone (**1**)	3.53
Symphonin (**2**)	1.29
Globuliferin (**3**)	3.86
Guttiferone A (**4**)	3.17
Chloroquine	0.13

[a] IC_{50} concentration 50% of parasites relative to negative control.

3. Experimental

3.1. General methods

Melting points were determined on Buchi melting point apparatus B-545. UV spectra were determined on Shimadzu-265 spectrophotometer. ^{1}H and ^{13}C NMR spectra were run on a Bruker spectrometer equipped with a 5 ml ^{1}H and ^{13}C probe operating at 400.1 and 100.6 MHz, respectively, with TMS as internal standard.

3.2. Plant material

The seeds of *S. globulifera* were collected in January 2002 at Fundong in the North-West province of Cameroon. The plant was identified by Dr. Achoundong of the National Herbarium of Cameroon where a voucher specimen No. 50788 has been deposited.

3.3. Extraction and isolation

One kilogram of the air-dried seeds of *S. globulifera* was shelled. The air-dried powdered seed shells (0.4 kg) was extracted with MeOH–CH_2Cl_2 (1:1) at room temperature

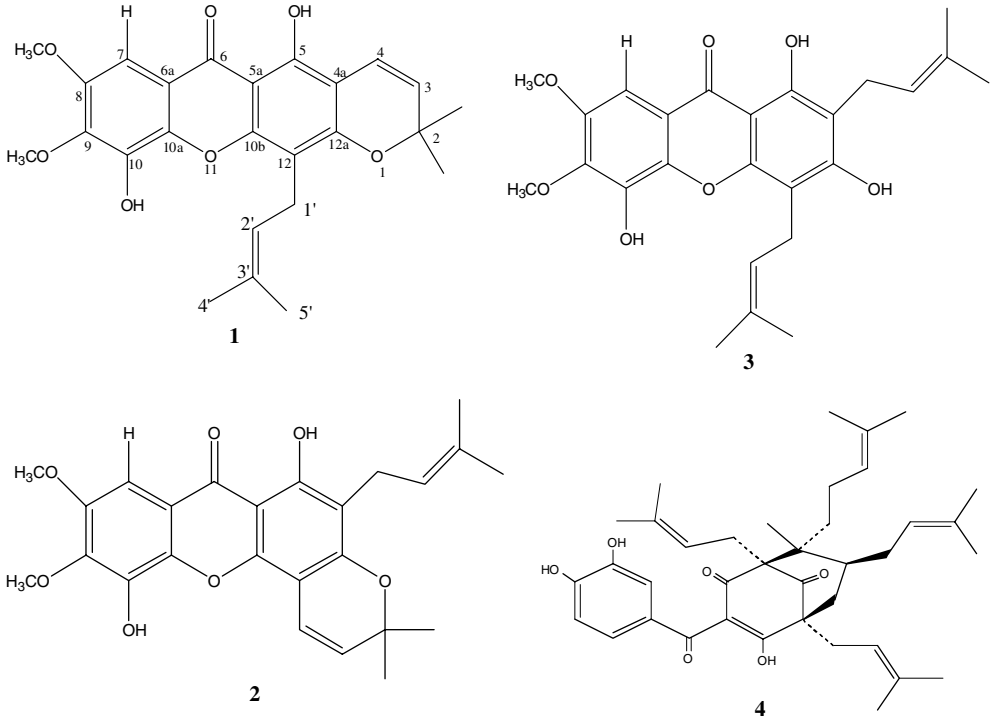

Fig. 2. Chemical structures of the tested compounds.

and the extract concentrated to dryness to obtain a viscous residue (80.0 g). This residue was then subjected to flash column chromatography over silica gel (230–400 mesh) as stationary phase eluting with Hexane–EtOAc of increasing polarity. Eighty-six fractions of 300 ml each were collected and grouped on the basis of TLC analysis to yield two main fractions labelled G_1 and G_2.

Fraction G_1 was subjected to column chromatography over Si gel (70–230 mesh) eluting with a *n*-hexane–ethyl acetate gradient of increasing polarity resulting in the collection of 40 fractions (F_1–F_{40}) of 250 ml each which were combined on the basis of TLC analysis. Fractions F_1–F_{23} contained only oils. Fractions F_{24}–$F_{40'}$ eluted with a mixture of *n*-hexane–ethyl acetate (9:1) afforded sistosterol (53 mg) and oleanolic acid (263 mg).

Fraction G_2 was column chromatographed over Si gel (70–230 mesh), eluting with *n*-hexane–ethyl acetate mixture with increasing polarity. A total of 30 fractions of 100 ml each were collected and combined on the basis of TLC analysis in two series A–B. Fraction A was eluted with a mixture of *n*-hexane–ethyl acetate (7:3) to give off globulifern (7 mg) and symphonin (10 mg). Fraction B eluted with a mixture of *n*-hexane–ethyl acetate (6:4) afforded gaboxanthone (13 mg) and guttiferone A (1.80 g) and methyl citrate (11 mg).

3.4. Gaboxanthone (*1*)

Yellow powder, m.p. 222–223 °C; ^{13}C (100.6 MHz, $CDCl_3$) and ^1H (400.13 MHz, $CDCl_3$) NMR: see Table 1; EIMS: *m/z* (rel. int.): 438 $[M]^+$ (44), 423 [M − 15] (100), 383 (50), 373 (31), 369 (37), 227 (74), 195 (74). HR-EIMS: *m/z* 438.1666 (calcd. 438.1671 for $C_{25}H_{26}O_7$).

3.5. Evaluation of anti-plasmodial activity

P. falciparum strain W2, which is resistant to chloroquine and other antimalarials (Singh and Rosenthal, 2001), was cultured in sealed flasks at 37 °C, in a 3% O_2, 5% CO_2 and 91% N_2 atmosphere in RPMI 1640, 25 mM HEPES, pH 7.4, supplemented with heat inactivated 10% human serum and human erythrocytes to achieve a 2% hematocrit. Parasites were synchronized in the ring stage by serial treatment with 5% sorbitol (Sigma) (Lambros and Vanderberg, 1979) and studied at 1% parasitemia.

Compounds were prepared as 10 mM stock solutions in DMSO, diluted as needed for individual experiments, and tested in triplicate. The stock solutions were diluted in supplemented RPMI 1640 medium so as to have at most 0.2% DMSO in the final reaction medium. An equal volume of 1% parasitemia, 4% hematocrit culture was thereafter added and gently mixed thoroughly. Negative controls contained equal concentrations of DMSO. Positive controls contained 1 μM chloroquine phosphate (Sigma). Cultures were incubated at 37 °C for 48 h (1 parasite erythrocytic life cycle). Parasites at ring stage were thereafter fixed by replacing the serum medium by an equal volume of 1% formaldehyde in PBS. Aliquots (50 μl) of each culture were then added to 5 ml round-bottom polystyrene tubes containing 0.5 ml 0.1% Triton X-100 and 1 nM YOYO nuclear dye (Molecular Probes) in PBS. Parasitemias of treated and control cultures were compared using

a Becton-Dickinson FACSort flow cytometer to count nucleated (parasitized) erythrocytes. Data acquisition was performed using CellQuest software. These data were normalized to percent control activity and 50% inhibitory concentrations (IC_{50}) calculated using Prism 3.0 software (GraphPad) with data fitted by non linear regression to the variable slope sigmoidal dose–response formula $y = 100/[1 + 10^{(\log IC_{50} - x)H}]$, where H is the hill coefficient or slope factor (Singh and Rosenthal, 2001).

3.6. Chemical stable radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was measured according to the procedure described by Christov et al. (2005). The compounds were dissolved in ethanol (0.21 mg/ml). The solutions analyzed (250 μl) were diluted to 2 ml with ethanol and 1 ml of 0.02% DPPH/ethanol solution was added. The resulting mixture was thoroughly mixed and the absorbance measured at 517 nm after 30 min. The scavenging activity was determined by comparison of the absorbance with blank (100%), containing only DPPH and solvent. Caffeic acid has been chosen as positive control.

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