

Full Paper

Leishmanicidal and Cholinesterase Inhibiting Activities of Phenolic Compounds from *Allanblackia monticola* and *Symphonia globulifera*

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Abstract: In a preliminary antiprotozoal screening of several Clusiaceae species, the methanolic extracts of *Allanblackia monticola* and *Symphonia globulifera* showed high *in vitro* leishmanicidal activity. Further bioguided phytochemical investigation led to the

isolation of four benzophenones: guttiferone A (**1**), garcinol (**2**), cambogin (**3**) and guttiferone F (**4**), along with three xanthenes: allanxanthone A (**5**), xanthone V₁ (**6**) and globulixanthone C (**7**) as active constituents. Compounds **1** and **6** were isolated from *S. globulifera* leaves, while compounds **2-5** were obtained from *A. monticola* fruits. Guttiferone A (**1**) and F (**4**) showed particularly strong leishmanicidal activity *in vitro*, with IC₅₀ values (0.2 µM and 0.16 µM, respectively) comparable to that of the reference compound, miltefosine (0.46 µM). Although the leishmanicidal activity is promising, the cytotoxicity profile of these compounds prevent at this state further *in vivo* biological evaluation. In addition, all the isolated compounds were tested *in vitro* for their anticholinesterase properties. The four benzophenones showed potent anticholinesterase properties towards acetylcholinesterase (AChE) and butylcholinesterase (BChE). For AChE, the IC₅₀ value (0.66 µM) of garcinol (**2**) was almost equal to that of the reference compound galanthamine (0.50 µM). Furthermore, guttiferone A (**1**) and guttiferone F (**4**) (IC₅₀ = 2.77 and 3.50 µM, respectively) were more active than galanthamine (IC₅₀ = 8.5) against BChE.

Keywords: *Allanblackia monticola*; *Symphonia globulifera*; Phenolic compounds; Leishmanicidal; Anticholinesterase

Introduction

The plants of the Clusiaceae family are used for the treatment of several parasitic diseases like leishmaniasis in Cameroon. Cutaneous leishmaniasis prevails mainly in the northern part of the country, in the area of Mokolo, whereas visceral leishmaniasis is more frequent in the area of Kousseri, in the north-east [1].

Clusiaceae are well known to biosynthesize bioactive benzophenones and xanthenes [2-8]. Xanthenes are known to show a wide range of biological activity such as antibacterial, antidiabetic, antiplasmodial, human cancer cell line growth inhibition, antihypertensive and vasorelaxing, cardiovascular protection, inhibition of HIV-1 reverse transcriptase and HIV-1 replication [9-16]. The leishmanicidal activity of several xanthenes were reported previously [17], but to the best of our knowledge, there are no reports on leishmanicidal activity of benzophenones of the guttiferone type. On the other side, polyprenylated benzophenones have been shown to possess different biological properties such as the cytoprotection against HIV-1 *in vitro*, antimicrobial properties, antioxidant activity, and cytotoxic activity [3-4, 18-19]. Recently garcinol has attracted considerable interest because of its associated beneficial health properties, including antiulcer activity, antiglycation activity and cancer chemopreventive activity [20-22]. These data show the wide range of activities of these compounds and their potential interest in other biological assays.

On the other hand, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) have been identified as attractive targets in the treatment of *Alzheimer's* dementia, myasthenia gravis and glaucoma. Any chemical that inhibits cholinesterase activity increases the availability of

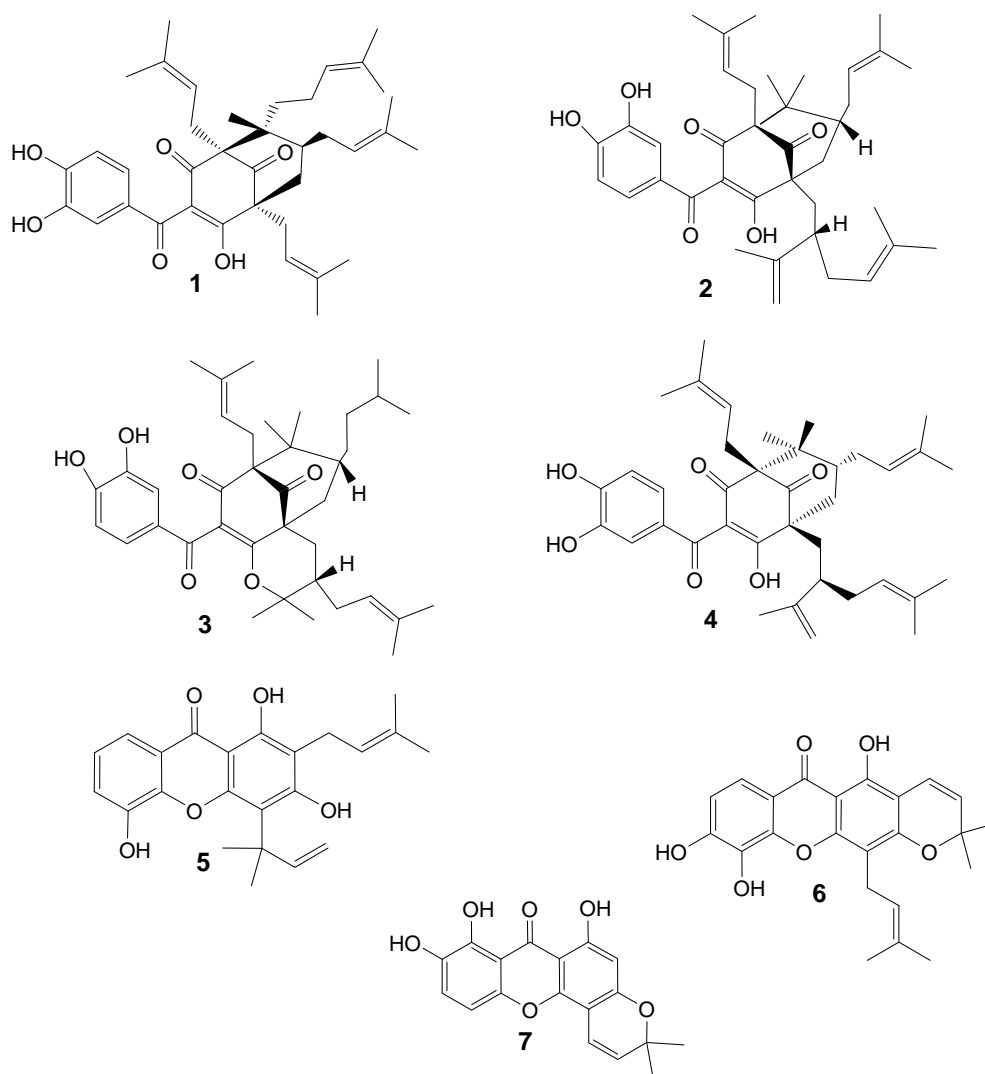
acetylcholine to sustain nerve cell communications and boost cholinergic neurotransmission [23-24]. The exact physiological role of BChE is still elusive, but it is generally viewed as a backup for the homologous AChE [25]. Hence, the search for the new cholinesterase inhibitors is an important strategy to introduce new drug candidates against *Alzheimer's* disease and related dementias. Several natural products, like atropine and related alkaloids or huperzine-A, have shown promising cholinesterase inhibiting activities both *in vivo* and *in vitro* [26].

Thus, in our continuing interest in the search for bioactive metabolites from Cameroonian medicinal plants, we performed a bioguided fractionation of fruit extracts of *Allanblackia monticola* and leaf extracts of *Symphonia globulifera* in order to isolate and characterize bioactive compounds with antileishmanial activity and/or anticholinesterase properties.

Results and Discussion

In the preliminary evaluation of the antiprotozoal activity of crude extracts from Cameroonian medicinal species, *A. monticola* and *S. globulifera*, all belonging to the *Clusiaceae* family, the methanolic extracts were found to have significant activity against *Leishmania donovani* axenic amastigotes [27].

Figure 1.



Bioguided investigation of these extracts resulted in the isolation and characterization of four benzophenones, guttiferone A (**1**) [3], garcinol (**2**), cambogin (**3**) [2] and guttiferone F (**4**) [4], along with three xanthenes, allanxanthone A (**5**) [5], xanthone V₁ (**6**) and globulixanthone C (**7**) [6] (Figure 1). Compounds (**1**) and (**6**) were isolated from *S. globulifera* leaves, as compounds (**2-5**) were obtained from *A. monticola* fruits.

All these compounds were evaluated for *in vitro* leishmanicidal activity to check whether or not they contribute to activity. Compounds were first screened at two concentrations (0.8 and 4.8 µg/mL), at which parasite growth inhibition was measured, to identify the most active compounds (Table 1). Compounds for which parasite growth inhibition was greater than 50% at the concentration of 0.8 µg/mL were subsequently assayed for IC₅₀ determination and cytotoxicity evaluation against L6 cells. IC₅₀ values and selectivity indexes (ratio of cytotoxic to leishmanicidal activity) are presented in Table 2.

Table 1. Leishmanicidal screening of the isolated compounds.

Compounds	Parasite growth inhibition (%) tested at:	
	0.8 µg/mL	4.8 µg/mL
Guttiferone A (1)	82.1	98.3
Guttiferone F (4)	58.2	98.2
Allanxanthone A (5)	13.3	57.4
Xanthone V ₁ (6)	99.6	71.9
Globulixanthone C (7)	98.7	49.7
miltefosine ^a	57.6	95.0

Each compound was assayed in duplicate at 7 final concentrations

Compounds for which parasite growth inhibition was greater than 50% were assayed for IC₅₀ determination and cytotoxicity evaluation

^a Standard used.

Pure compounds were more active than the extracts from which they were isolated (IC₅₀ of 2.1 ± 0.8 for *S. globulifera* and 1.8 ± 0.5 for *A. monticola*). Of the seven compounds tested, guttiferone A (**1**) and guttiferone F (**4**) exhibited a prominent leishmanicidal effect. Moreover, IC₅₀ value determination showed that **1** and **4** are more potent than the reference compound miltefosin (0.16 µM, 0.2 µM and 0.47 µM, respectively). Trypanocidal activity has been described for guttiferone A (**1**) [28] but, to the best of our knowledge, leishmanicidal activity has not been reported yet for guttiferones.

In order to determine the selectivity of their antileishmanial activity, the pure compounds were also tested on rat skeletal myoblasts (L6 cells). Although the two guttiferones exhibited a cytotoxicity / leishmanicidal ratio (selectivity index) >10, implying some selectivity of the concentration used for the leishmanicidal activity evaluation, these compounds are toxic to mammalian L6 cells (IC₅₀ values against L6 cells <10 µM). Given their strong leishmanicidal activity, these compounds could nevertheless be used effectively as lead compounds in the synthesis of novel potent leishmanicidal agents.

Compounds **1-5** were also tested against the cholinesterases AChE and BChE which represent the most attractive target for drug design and discovery of mechanism-based inhibitors for the treatment of neurone degenerative disorders such as *Alzheimer's* disease [29]. The percentage of inhibition was first determined at 0.1 mM. Compounds for which parasite growth inhibition was greater than 50% were subsequently assayed for IC₅₀ determination. All four benzophenones showed good AChE and BChE activities (Table 3). For AchE, the IC₅₀ of garcinol (**2**) was almost equal to the one of the reference drug galanthamine. Against BchE, guttiferone A (**1**) and guttiferone F (**4**) were two to three times more active than galanthamine. The anticholinesterase properties of this class of metabolites are reported here for the first time. These interesting results highlight the interest of this class of secondary metabolites which commonly occur in several genera such as *Symphonia*, *Garcinia*, *Allanblackia* and *Clusia* of the Clusiaceae family.

Table 2. IC₅₀ determination for leishmanicidal activity and cytotoxicity of the most active compounds.

Compounds	<i>L. donovani</i>	L6 cells	SI ^c
	IC ₅₀ (μM)	IC ₅₀ (μM)	
Guttiferone A (1)	0.16	7.3	46
Garcinol (2)	0.82	-	2.7
Camboginol (3)	0.33	-	6.1
Guttiferone F(4)	0.20	5.4	27
Xanthone V ₁ (6)	1.40	18.0	13
Standard	0.47	0.048	-

Standards used: miltefosine (*L. donovani*) and podophyllotoxin (L-6 cells, cytotoxicity). Data shown are values from two replicate experiments. SI: selectivity index (ratio of cytotoxicity to leishmanicidal activity).

Table 3: AChE and BChE inhibitory activities of compounds **1-5** (IC₅₀, μM)

Compounds	AChE±SEM ^a	BChE±SEM ^a
Guttiferone A (1)	0.88 ± 0.04	2.77 ± 0.02
Garcinol (2)	0.66 ± 0.02	7.39 ± 0.23
Cambogin (3)	1.13 ± 0.06	8.30 ± 0.01
Guttiferone F (4)	0.95 ± 0.01	3.50 ± 0.15
Allanxanthone A	95 ± 0.30	19.10 ± 0.90
Galanthamine ^b	0.5 ± 0.01	8.5 ± 0.01

^a Standard error of mean of five assays.

^b Positive control used in the assays.

Data shown are values from triplicate experiments.

Experimental

General

Melting points were determined on a Büchi-540 melting point apparatus. Optical rotations were measured in CHCl_3 solution on a Jasco digital polarimeter (model DIP-3600). IR spectra were determined on Jasco Fourier Transform IR spectrometer. UV spectra were determined on a Spectronic Unicam spectrophotometer. ^1H - and ^{13}C -NMR spectra were run on a Bruker spectrometer equipped with 5mm ^1H and ^{13}C probes operating at 300 and 75 MHz respectively, with TMS as internal standard. Silica gel 230-400 mesh (Merck) and silica gel 70-230 mesh (Merck) were used for flash and column chromatography, while percolated aluminium silica gel 60 F₂₅₄ sheets were used for TLC with different mixtures of petrol ether, cyclohexane, ethyl acetate, and acetone as eluents; spots were visualised under UV lamps (254 nm) and (365 nm) or by MeOH–H₂SO₄ reagent. The structures of compounds were established by spectroscopic means and comparison of their data to those reported in literature.

Plant material

The selected plant materials were collected in the Cameroon Western Province in September 2004 in their natural habitats, *Allanblackia monticola* Mildbr. ex Engl. in Bazou and *Symphonia globulifera* L. f. in Bangangté. Botanical identification was performed by M. Nana Victor, botanist at the National Herbarium of Cameroon (NHC), where the voucher specimens are deposited under the number 61168/HNC for *A. monticola* and 32192/HNC for *S. globulifera*.

Isolation and characterisation

Air-dried and ground fruit of *A. monticola* (800 g) was extracted successively with *n*-hexane and MeOH at room temperature and concentrated to dryness under vacuum. The methanolic residue (60 g) was subjected to flash chromatography over silica gel (70-230 mesh, Merck) eluting with *n*-hexane-EtOAc of increasing polarity to yield four main fractions labelled A-D. Separations of fraction A and B by repeated column chromatography on silica gel (70- 230 mesh, Merck) eluted with *n*-hexane - EtOAc mixture of increasing polarity yield garcinol (**2**, 63 mg), and cambogin (**3**, 110 mg), guttiferone F (**4**, 21 mg) and allanxanthone A (**5**, 57 mg).

Air-dried and ground leaves of *S. globulifera* (850 g) were extracted at room temperature with MeOH and concentrated to dryness under vacuum. The residue (65 g) was extracted successively in water with hexane, CH_2Cl_2 , EtOAc and butanol to yield 23 g, 16 g, 12.5 g and 8 g of hexane, dichloromethane, ethyl acetate and butanolic extract respectively. 12.5 g of ethyl acetate residue was subjected to successive column chromatography to yield guttiferone A (**1**, 12 mg), xanthone V₁ (**6**, 25 mg) and globulixanthone C (**7**, 6 mg).

In vitro leishmanicidal activity

50 μL of culture medium, a 1:1 mixture of SM medium [30] and SDM-79 medium [31] at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum (FBS), was added to each well of a 96-well microtiter plate (Costar, USA). Serial extracts dilutions in duplicates were prepared covering a range from 30 to 0.041 $\mu\text{g}/\text{mL}$. Then 105 axenically grown *Leishmania donovani* amastigotes (strain MHOM/ET/67/L82) in 50 μL medium were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Resazurin solution (12.5 mg resazurin dissolved in 100 mL distilled water, 10 μL) were then added to each well and incubation continued for a further 2-4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm [32]. Fluorescence development was measured and expressed as percentage of the control. Miltefosin (Zentaris GmbH, Germany) was used as a positive reference. Each assay was run in duplicate. The values given in Table 2 are means of two independent assays.

Cytotoxicity

The cytotoxicity assay of the examined extracts and compounds were done following the method of Pagé *et al.* [33] with the modification of Ahmed *et al.* [34]. Cell line L-6 (rat skeletal muscle myoblasts) were seeded in 96-well Costar microtiter plates at 2×10^3 cells/100 μL , 50 μL per well in MEM supplemented with 10% heat inactivated FBS. A three-fold serial dilution ranging from 90 to 0.13 $\mu\text{g}/\text{mL}$ of extracts in test medium was added. Plates with a final volume of 100 μL per well were incubated at 37 °C for 72 h in a humidified incubator containing 5% CO_2 . Resazurin was added as viability indicator according to Ahmed *et al.* [34]. After an additional 2 h of incubation, the plate was measured with a fluorescence scanner using an excitation wavelength of 536 nm and an emission wavelength of 588 nm (SpectraMax GeminiXS, Molecular Devices). Podophyllotoxin (Polysciences Inc., USA) was used as a positive reference. Each assay was run in duplicate. For the calculation of IC_{50} values, data were transferred into the graphic programme Softmax Pro (Molecular Devices) which calculated IC_{50} values from the sigmoidal inhibition curve.

In vitro cholinesterase inhibition assay and determination of IC_{50}

Acetylcholinesterase (Electric-eel EC 3.1.1.7), butyrylcholinesterase (horse-serum E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5'-dithiobis[2-nitrobenzoic-acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO, USA). Buffers and other chemicals were of analytical grade. Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured according to a slightly modified spectrophotometric method [35]. Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. 5,5'-Dithiobis[2-nitrobenzoic-acid] (DTNB) was used for the measurement of cholinesterase activity. 100 mM sodium phosphate buffer (pH 8.0, 140 μL), DTNB (10 μL), test compound solution (20 μL) and acetylcholinesterase or butyrylcholinesterase solution (20 μL) were mixed and incubated for 15 minutes (25° C). The reaction was then initiated by the addition

of acetylthiocholine or butyrylthiocholine (10 μ L), respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). Test compounds and control were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro title plates and monitored in a *SpectraMax 340* (Molecular Devices, USA) spectrometer. The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst, USA*).

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Sample Availability: Contact the authors.