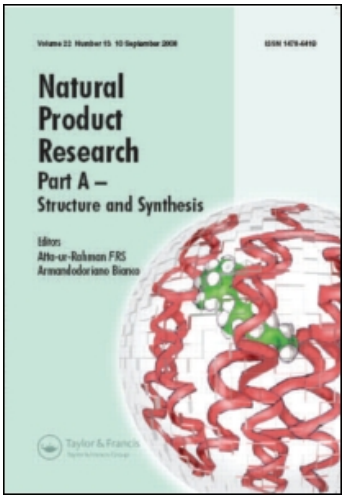

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Antimicrobial and antileishmanial xanthones from the stem bark of *Allanblackia gabonensis* (Guttiferae)

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The phytochemical study of stem bark of *Allanblackia gabonensis* has resulted in the isolation and characterisation of one new xanthone derivative, named allanxanthone D, together with 10 known compounds, including 6 xanthones derivatives, allanxanthone A, 1,5-dihydroxyxanthone, 1,7-dihydroxyxanthone and 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone, forbexanthone, 6-deoxyisojacareubin, one polyisoprenylated benzophenone, guttiferone F, one flavanol, epicathechin, two phytosterols, β-sitosterol and campesterol. The structures of these compounds were established on the basis of one- and two-dimensional NMR homo- and heteronuclear evidence. These compounds were evaluated for their activity against *Leishmania amazonensis in vitro* and antimicrobial activities against a range of Gram negative and Gram positive bacteria.

Keywords: Guttiferae; *Allanblackia*; Allanxanthone D; Xanthones; Antileishmania and antimicrobial activities

1. Introduction

According to estimations of the World Health Organisation (WHO), 350 million individuals are living at risk with *Leishmania* parasite (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm). Leishmaniasis incidence increases especially in Africa, Asia and Latin America; and this illness becomes a more and more important cause of death.

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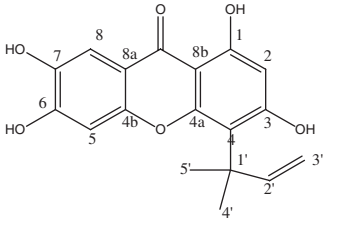
Antileishmanial drugs are few, expensive and often toxic. Most of them (except miltefosine) are only usable by IV routes, and the resistance of the parasites are increasing. Thus, the search of new compounds as safe and efficient drugs for this disease became urgent. Natural substances could be an interesting source of such drugs and in particular, natural products from plants as drug candidates and lead compounds against leishmaniasis and trypanosomiasis [1].

Plants of the genus *Allanblackia* are intriguing targets for phytochemical investigation not only because of their great structural variability, but also because of the diverse biological activities of their secondary metabolites amongst which xanthones, benzophenones, biflavonoids and triterpenoids [2–6]. *Allanblackia gabonensis*, locally named 'agnoumé', is a high sub-mountain dweller tree widely distributed in Centre Province of Cameroon [7,8], where it is used as a medicinal plant against infections like dysenteries, cold and tooth aches [9,10].

This article described the isolation and structural elucidation of a new xanthone derivative named Allanxanthone D (**1**); together with 10 known compounds, Allanxanthone A (**2**) [6], 1,5-dihydroxyxanthone (**3**) [11], 1,7-dihydroxyxanthone (**4**) [12] and 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone (**5**) [13], forbexanthone (**6**) [14], 6-deoxyisojacareubin (**7**) [15], one polyisoprenylated benzophenone, guttiferone F (**8**) [16], one flavanol, epicathechin (**9**) [17], two phytosterols, β-sitosterol (**10**) and campesterol (**11**). Antileishmanial activity against *Leishmania amazonensis* and antimicrobial activities against a range of Gram negative and Gram positive bacteria of some of the isolated compounds are also reported.



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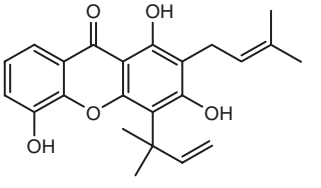

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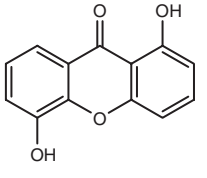

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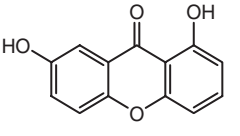

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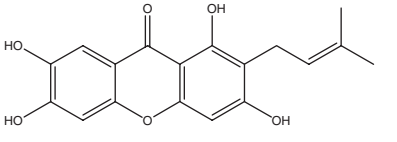

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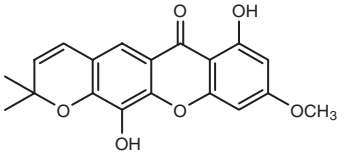

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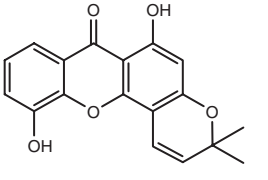

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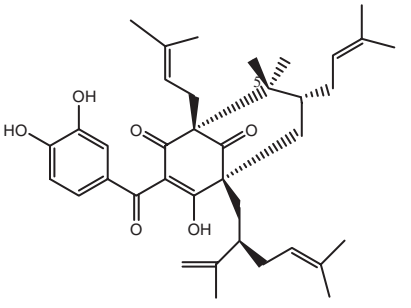

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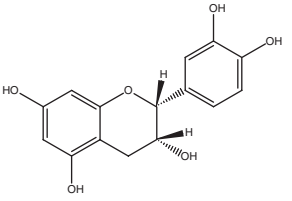

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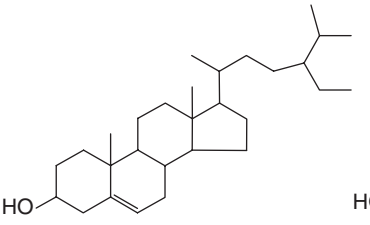

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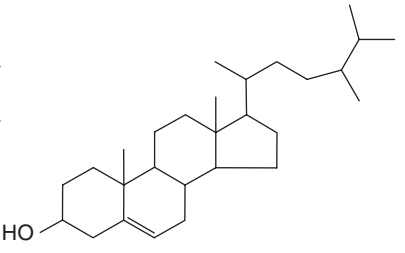

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2. Results and discussion

Compound (**1**), Allanxanthone D, m.p. 238–240°C was obtained as an orange powder and reacted positively to the Gibbs and $FeCl_3$ reagent indicating the presence of phenolic group. The high resolution ESI–TOF mass spectrum showed a $[M+H]^+$ at m/z 329, 0945 corresponding to a molecular formula $C_{18}H_{16}O_6$, indicating 11 degrees of unsaturation.

The UV spectrum of **1** displayed the maxima of absorption (MeOH) at 253 and 280 nm. The data obtained from the IR spectrum showed free hydroxyl (ν_{max} 3456 cm^{-1}), chelated hydroxyl (ν_{max} 3290 cm^{-1}), conjugated carbonyl (ν_{max} 1646 cm^{-1}) and aromatic ring (ν_{max} 1620, 1585 cm^{-1}). All these absorptions were consistent with the presence of a xanthone skeleton tetraoxygenated [5].

The broad-band decoupled ^{13}C NMR spectrum of **1** showed 18 carbons signals, which were attributed by APT, DEPT and HSQC techniques as 2 methyls, 1 methylene, 4 methines and 10 quaternary carbons including a carbonyl (δ 181.2).

The ^1H NMR spectrum (acetone, table 1) of compound **1** analysed by ^1H–^1H COSY, indicated three singlets of aromatic protons at δ 6.26; δ 6.91 and δ 7.51, whose positions remain to be established. The high deshielding of this last proton at δ 7.51 suggested that it is located in the paramagnetic anisotropy cone of carbonyl i.e. at C-8 position of xanthone skeleton. Futhermore, the ^1H and ^{13}C NMR spectra also displayed an ABC system set of signals comprising a singlet of six protons at δ 1.67 (s)/δ_c 29.5 and three

Table 1. Anti-*Leishmania* tests.

Tested samples	MeOH extract	9	5	1	4	Amphotericin B
IC_{50} $\mu g\,mL^{-1}$	14.7	NA	13.3	13.9	NA	0.2 μM

doublet of doublets each of one-proton at δ 4.91 (1H, dd, 10.5 Hz; 1.05 Hz)/δ_c 108.0 at δ 5.03 (1H, dd, 17.6; 1.05 Hz)/δ_c 108.0 and δ 6.35 (1H, dd, 10.5 Hz; 17.6 Hz)/δ_c 151.5, suggesting the presence of a 1,1-dimethylallyl substituent. In addition; we observe a free hydroxyl signal at δ 8.10, a chelated hydroxyl signal at δ 13.47 and two chelated hydroxyl protons at δ 9.10 and δ 9.16.

It remained to establish at this stage of the discussion, not only the position of the 1,1-dimethylprop-2-enyl group, but also those of the three others groups of hydroxyls on the xanthone skeleton. Thus, the fact that in the HMBC spectrum, the chelated hydroxyl proton at δ 13.41 displayed cross peaks with the carbons at δ 99.6 (C-2); δ 156.8 (C-4a) and δ 163.9 (C-1) in addition to cross peaks between the proton at δ 6.26 (H-2) and carbons δ 162.2 (C-3); δ 163.9 (C-1), suggested that this aromatic proton is localised in C-2 position. In the same way, correlations observed between the proton at δ 6.26 with carbons δ 162.2 (C-3), δ 111.7 (C-4) and those observed between the protons of the gem-methyl group at δ 1.67 with carbons δ 41.8 (C-1′); δ 111.7 (C-4) indicated that the, α-dimethylallyl group is located in C-4 position. Moreover, the fact that the H-8 proton appears as singlet indicate that it is in para position compared to the other aromatic proton at δ 6.91 i.e. in C-5 position. This assumption is consolidated by the analysis of the NOESY spectrum, on which we correlated peaks between this aromatic proton at δ 6.91 and one of the hydroxyls slightly chelated at δ 9.10 as well as the interactions with the proton in peri position i.e. C-8 and the other hydroxyl slightly chelated at δ 9.16. On the basis of the above analysis, the structure of allanxanthone D **1** was assigned to be 1,3,6,7-tetrahydroxy-4-(1,1-dimethylprop-2-enyl)xanthone.

The result of antileishmanial activity (table 1) of the compounds **1**, **4**, **5** and **9** may be related to some of their structural features. Xanthones have showed to be more active than flavonoids. Amongst xanthones, tetraoxygenated prenylated xanthones were more active than dioxygenated simple xanthones and their activities may be attributed to the presence of prenyl group at position 2 or 4.

Results of the antimicrobial assay demonstrated that compounds **1**, **2**, **5** exhibited a wide spectrum of antimicrobial activity with important inhibition either on Gram positive and Gram negative bacteria, yeasts and mycelial fungus. The MIC values range from 1.22–39.06 $\mu g\,mL^{-1}$ (3.72–119.1 μM) on Gram negative bacteria, 1.22–4.88 $\mu g\,mL^{-1}$ (3.72–14.88 μM) on Gram positive bacteria, 1.22–19.53 (3.72–59.54 $\mu M\,mL^{-1}$) on *Candida* species, while the MIC obtained on the mycelial fungi was 1.22 $\mu g\,mL^{-1}$ (3.72 $\mu M\,mL^{-1}$) (table 2). The activity of compound **1** is greater than that of reference antibiotics on all Gram positive and some of the tested Gram negative bacteria as well as against *Candida krusei*. Xanthones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to the inactivation of proteins and loss of function [18]. This appeared to be the possible mechanism by which compounds **1**, **2** and **5** exhibited their antimicrobial effects.

Table 2. Minimum inhibition concentration[a] ($\mu g\,mL^{-1}$ and μM) of compounds **1**, **2**, **5** and reference antibiotics.

	Tested samples			
Microorganisms	1	2	5	RA[b]
Gram-negative bacteria				
C. freundii	2.44 (7.44)	4.88 (12.84)	39.06 (119.1)	4.88 (9.0)
E. aerogens	39.06 (119.1)	39.06 (102.79)	> 78.12 (> 238.17)	9.76 (18)
E. cloacae	2.44 (7.44)	> 78.12 (> 205.58)	9.76 (29.77)	4.88 (9.0)
E. coli	2.44 (7.44)	> 78.12 (> 205.58)	19.53 (59.54)	1.22 (2.25)
K. pneumoniae	2.44 (7.44)	2.44 (6.42)	4.88 (14.89)	2.44 (4.5)
M. morganii	39.06 (119.1)	> 78.12 (> 205.58)	19.53 (59.54)	2.44 (4.5)
P. mirabilis	4.88 (14.88)	> 78.12 (> 205.58)	19.53 (59.54)	2.44 (4.5)
P. vulgaris	1.22 (3.72)	4.88 (12.84)	> 78.12 (> 238.17)	1.22 (2.25)
P. aeruginosa	1.22 (3.72)	> 78.12 (> 205.58)	4.88 (14.89)	4.88 (9.0)
S. dysenteriae	4.88 (14.88)	> 78.12 (> 205.58)	39.06 (119.1)	2.44 (4.5)
S. flexneri	19.53 (59.54)	39.06 (102.79)	78.12 (238.17)	2.44 (4.5)
S. typhi	2.44 (7.44)	19.53 (51.37)	39.06 (119.1)	2.44 (4.5)
Gram-positive bacteria				
S. faecalis	1.22 (3.72)	39.06 (102.79)	9.76 (29.77)	4.88 (9.0)
S. aureus	1.22 (3.72)	9.76 (25.68)	2.44 (7.44)	4.88 (9.0)
B. cereus	1.22 (3.72)	> 78.12 (> 205.58)	> 78.12 (> 238.17)	2.44 (4.5)
B. megaterium	2.44 (7.44)	39.06 (102.79)	2.44 (7.44)	4.88 (9.0)
B. stearothermophilus	2.44 (7.44)	4.88 (12.84)	19.53 (59.54)	4.88 (9.0)
B. thurengiensis	4.88 (14.88)	39.06 (102.79)	> 78.12 (> 238.17)	9.76 (18)
B. subtilis	2.44 (7.44)	> 78.12 (> 205.58)	9.76 (29.77)	2.44 (4.5)
Fungi				
C. albicans	19.53 (59.54)	78.12 (> 205.58)	> 78.12 (> 238.17)	4.88 (5.21)
C. gabrata	1.22 (3.72)	2.44 (6.42)	2.44 (7.44)	4.88 (5.21)
C. krusei	19.53 (59.54)	39.06 (102.79)	> 78.12 (> 238.17)	9.76 (10.42)
Absidia sp.	1.22 (3.72)	> 78.12 (> 205.58)	> 78.12 (> 238.17)	1.22 (2.11)

Notes: [a]Minimum Inhibition Concentration: lowest concentration at which there was 100% growth inhibition of the tested pathogens in $\mu g\,mL^{-1}$ or μM (in parentheses); No effect of the DMSO used as dilution solvent was observed on the tested microbial strains.
[b]RA: Reference antibiotics (Gentamycin for bacteria, Nystatin for *Candida* species and amphotericin B for *Absidia* sp.); The others compounds were in small quantities for the tests.

3. Experimental

3.1. *General experimental procedures*

The melting points were measured using a Büchi apparatus (Büchi melting points B-540) or on microscope with heating platinum of Reicheirt and are uncorrected. The optical activities were given at room temperature in methanol on a Perkin-Elmer 341 polarimeter. The optical rotation of the light polarisation caused by the dissolves products in MeOH were measured in a 10 cm tank length. The D line (559 nm) of sodium was used as source of incidental light. The IR spectra were recorded using an infrared spectrophotometer in Fourrier Transformer's of Nicolet 400 type on KBr pellet. Positions of the absorption bands are given in cm^{-1}. UV spectra were recorded in MeOH solution on a Kontron-Uvikon 932 spectrophotometer. The mass spectra were obtained in positive mode on the spectrometers of APCI QSTSAR^{TM} type, equipped with an analyser, in time of flight and using the electrospray like the ionisation technique. The NMR experiments were carried out in various deutereted solvents

(MeOH, acetone, pyridine, DMSO, $CDCl_3$) on a Brucker spectrometer of NMR (AC300 with of fourier's transformed for the experiments 1D (300.13 MHz for ^1H and 75.47 MHz for the ^{13}C) and on a Brucker 400 spectrometer in fourier's transformed for the experiments 2D (400.13 MHz for the ^1H and 100.71 for the ^{13}C). The chemical shifts of the ^1H are expressed in ppm and the coupling constants of J in Hz. The direct spectra of correlation ^1H ^{13}C, HSQC were carried out to 400.13 MHz in two dimensions (dimension proton) and to 100 in carbon dimension. HMBC experiments were recorded to 400.13 MHz on an average frequency of 4401 MHz. NOESY spectra were recorded with a time of mixing of 500 ms. The column chromatographies were carried out on variable diameters of columns by using like stationary phase silica gel with granulometry, 60 Merk between 70–230 mesh and 230–400 mesh. The flash chromatographies were done on a short column (12 × 10 cm). The preparative and analytical on thin layer chromatographies were carried out on aluminium plate covered with G.60 silica of GF_{254} (Merck, Darmstadt, Germany) type. The spots in CCM are visualised on UV light or by pulverisation with means of the sulphuric acid (H_2SO_4) or revealed with iodine. The various mass obtained were measured on the electronic balance of type 'Sartorius'. The various fractions were concentrated on an evaporator of the type Heidolph VV 200.

3.2. *Plant material*

The stem bark of *A. gabonensis* were collected with the Kala mount located in the district of Mbankomo, Mefou Afamba division, Centre province of Cameroon in October 2003 by M. Nana, botanist at the National Herbarium, Yaoundé, where a voucher specimen for documenting was deposited under reference number 23255/HNC.

3.3. *Extraction and isolation*

Ground, air dried, stem bark of *A. gabonensis* were extracted with CH_2Cl_2/MeOH at room temperature. The extracts concentrated under reduced pressure, submitted on flash chromatography afforded four series A (17 g) B (8 g) C (34 g) D (42 g). By repeated column chromatography on silica gel of serie A, a novel derivative compound: Allanxanthone D **1** together with 10 known compounds, including 4 xanthones derivatives allanxanthone A **2**, 1,5-dihydroxyxanthone **3**, 1,7-dihydroxyxanthone **4** and 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone **5**, forbexanthone **6**, 6-deoxyisojacareubin **7**, one polyisoprenylated benzophenone, guttiferone F **8**, one flavanol: epicathechin **9**, two phytosterols, β-sitosterol **10**, and campesterol **11**.

From Serie A eluted with a mixture of hexane ethyl acetate, 150 fractions were collected. Fractions 1–5 re-chromatographed on column silica gel, gave yellow greenish 0.03 g of guttiferone F **8**. Fractions 13–18 left at the room temperature, precipitated allanxanthone A **2** (1.02 g) in the form of soluble yellow powder, which dissolved in acetone. From fractions 20–26, a mixture of β-sitosterol **10** and campesterol **11** precipitated at ambient temperature in the form of white spangle soluble in acetone. From fractions 29–35 left at room temperature 1, 5-dihydroxyxanthone **3** (0.096 g) was obtained as an yellow powder soluble in acetone; also, from fractions 40–50 left at room

temperature, 1,7-dihydroxyxanthone **4** (0.06 g) was isolated as an yellow powder soluble in acetone. Fraction 139 precipitated at room temperature in the form of orange powder soluble in acetone, allanxanthone D **1** (1.05 g). From fractions 117–120 left at ambient temperature, epicathechin **9** (0.075 g) was isolated as shining crystals soluble in MeOH. From fractions 142–145 left at room temperature, precipitated an yellow powder soluble in acetone and corresponding to 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone **5** (0.75 g). Serie B was column chromatographed over silica gel (230–400 mesh). Left at room temperature, from fractions 41–64 on the one hand and fractions 65–80 on the other hand, precipitated a soluble yellow powder in acetone in the form of a mixture. Purification of this mixture was made possible by means of preparative chromatography, using for eluting the mixture hexane ethyl acetate (4 : 1). With three successive migrations, the most polar bands are recovered. The extraction and evaporation allowed us to obtain two pure amorphous compounds forbexanthone **6** (0.078 g) and 6-deoxyisojacareubine **7** (0.03 g).

The series C and D contained very polar compounds difficult to separate.

3.4. *Antimicrobial tests*

3.4.1. Microbial strains. A total of 23 microbial cultures showing resistance to the commonly used antibiotics and belonging to seven Gram positive bacterial species (*Bacillus cereus* LMP0404G, *Bacillus megaterium* LMP0204G, *Bacillus stearothermophilus* LMP0104G, *Bacillus thurengiensis* LMP9901E, *Bacillus subtilis* LMP0304G, *Staphylococcus aureus* LMP0206U, *Streptococcus faecalis* LMP0207U), 12 Gram negative bacteria (*Escherichia coli* LMP0101U, *Shigella dysenteriae* LMP0208U, *Proteus vulgaris* LMP0103U, *Proteus mirabilis* LMP0504G, *Shigella flexneri* LMP0313U, *Klebsiella pneumoniae* LMP0210U, *Pseudomonas aeruginosa* LMP0102U, *Salmonella typhi* LMP0209U, *Morganella morganii* LMP0904G, *Enterobacter aerogens* LMP 1004G, *Citrobacter freundii* LMP0904G, *Enterobacter cloacae* LMP1104G), three yeasts from Candida species (*Candida albicans* LMP0204U and *Candida krusei* LMP0311U and *Candida gabrata* LMP0416U) and one mycelial fungus (*Absidia* sp. LMP9903) were used in this study. Three of the four *Bacillus* species were provided by 'L'Institut Appert de Paris' while, *B. cereus* was provided by the A.F.R.C Reading Laboratory of Great Britain. The other strains were clinically isolated from patients in the Centre Pasteur de Yaoundé-Cameroon (health institution). They were then maintained on agar slant at 4°C in the Laboratory of the Applied Microbiology and Molecular Pharmacology (Faculty of Science, University of Yaoundé I), where the antimicrobial tests were performed. The strains were activated at 37°C for 24 h on nutrient agar (NA) (bacteria) or Sabouraud glucose agar (fungi). The nutrient broth (NB) was used to determine the minimal inhibition concentration (MIC) of compounds **1, 2** and **5** against the tested pathogens.

3.5. *Antimicrobial assays*

MICs of compounds **1, 2** and **5** were evaluated against the pathogens. The inocula of microorganisms were prepared from 12 h broth culture and the suspensions were

adjusted to 0.5 Mc Farland turbidity. Compounds were first dissolved in dimethyl sulfoxide (DMSO) to the highest dilution (78.12 $\mu g\,mL^{-1}$), and serial two-fold dilutions were made in a concentration ranged from 0.31 to 78.12 $\mu g\,mL^{-1}$ in the 96 wells microplate containing NB. MIC values of the tested compounds against pathogens were determined based on the microdilution method, as the lowest concentration at which there was 100% growth inhibition of the tested pathogens [19]. Gentamycin (bacteria), Nystatin (yeasts) and Amphotericin B (*Absidia* sp.) diluted prior in water were also used as reference antibiotics. The final concentration of DMSO in the well was <1% (preliminary analyses with 1% (v/v) DMSO do not affect the growth of the test organisms).

3.6. *Antileishmanial assays*

The antileishmanial activity of the molecules were evaluated against axenic amastigote of Leishmania amazonensis clone 1 (MHOM/BR/76/LTB-012) by a colorimetric method based on the reduction of tetrazolium salt, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The culture of axenic amastigote of *L. amazonensis* was maintained at $32 \pm 1^{\circ}C$ by sub-passages using MAA medium (medium for axenically grown amastigote), supplemented with 20% heat-inactivated foetal calf serum [20]. To estimate 50% inhibitory concentrations (IC_{50}), parasites from late log phase of growth (axenic amastigote) were seeded in 96-well flat-bottom microplates in a volume of 100 µL [21]. All tests were performed in triplicate. After 72 h of incubation at 32°C, 10 µL of MTT (10 $mg\,mL^{-1}$) was added to each well and plates were incubated for 3.5 h more. Enzyme reaction was stopped by addition of 100 µL of 50% isopropanol–10% SDS. The plates were shaken at room temperature for an additional 30 min. The optical density at 600 nm was read using a 96-well scanner. IC_{50} were determined graphically. Amphotericin B was used as the inhibition control. Each experiment was performed three times.

Allanxanthone D (1): (1,3,6,7-tetrahydroxy-4-(1,1-dimethylprop-2-enyl)xanthone) Orange powder; +HRESI-TOF-MS *m/z* 329, 0945 ($C_{18}H_{16}O_6$). IR (KBr) cm^{-1}: 3456, 3290, 1646, 1620, 1585, 1470, 1421, 1243, 1140, 1116. UV λ^{Et0H}max nm: 248, 253, 311, 342; (+NaOH): 261, 292, 352; (+$AlCl_3$): 260, 336, 397; (+NaOAc): 291, 344. For ^{1}H-NMR 13.41 (1H, s, OH-1); 9.16 (1H, brs, OH-7); 9.10 (1H, brs, OH-6); 8.10 (1H, brs, OH-3); 7.41 (1H, s, H-8); 6,91 (1H, s, H-5); 6.35 (1H, dd, 2.7; 17.6 Hz, H-2′); 6.26 (1H, s, H-2); 5.03 (1H, dd, 1.05; 10.5 Hz, H-3′); 4.91 (1H, dd, 1.05; 17.6 Hz, H-3′); 1.67 (3H, s, H-4′, H-5′) and ^{13}CNMR, 181.0 (C-9); 163.9 (C-1); 162.2 (C-3); 156.8 (C-4a); 154.2 (C-6); 152.3 (C-4b); 144.0 (C-7); 133.4 (C-3′); 123.5 (C-2′); 113.3 (C-5); 111.7 (C-4); 108.8 (C-8); 103.7 (C-8b); 99.6 (C-2);41.8 (C-1′); 29.5 (C-4′, C-5′).

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