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ORIGINAL ARTICLE

Antischistosomal Evaluation of Stem Bark's Extract and Chemical Constituents from *Anonidium mannii* against *Schistosoma mansoni*

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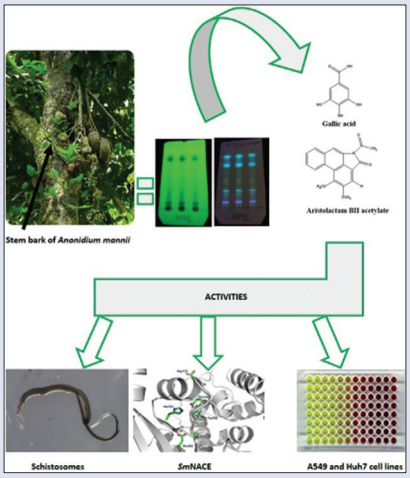
ABSTRACT

Context: *Anonidium mannii* (*Annonaceae*) has been traditionally used in Africa to treat stomach aches, schistosomiasis, and many other illnesses. However, few phytochemical study and no investigation on schistosomiasis have been conducted on this species. This neglected tropical disease, caused by a worm, comes second after malaria as the most devastating parasitical infection. **Aim:** The goal of this study was to evaluate the anti-*Schistosoma mansoni* activity of fractions and constituents from *A. mannii*'s stem bark and also to search efficient inhibitors of a recently discovered ectoenzyme of *S. mansoni* (*S. mansoni* nicotinamide adenine dinucleotide + catabolizing enzyme [*Sm*NACE]). **Materials and Methods:** The powdered stem bark of *A. mannii* was extracted with ethanol/distilled water (80:20). The extract was then subjected to a partial bioguided separation by chromatography means. The structures of compounds were elucidated using modern spectroscopic techniques. Furthermore, isolated and semisynthetic compounds were evaluated for their antischistosomal and cytotoxic activities. **Results:** Chemical investigation led to the isolation and identification of eight compounds, in the majority, obtained for the first time from this genus. In addition, acetylation reactions were carried out to afford a new semisynthetic derivative. Preliminary biological screening of the extracts and compounds showed very good activities from antiparasitic and enzymatic tests and also very good percentage of cell viability evaluation. **Conclusion:** Like praziquantel drug, gallic acid exhibited full anthelmintic activity at concentration of 100 μM. On the other hand, piperolactam D showed important inhibition on *Sm*NACE (IC_{50} 10 μM). Thus, standardization of bioactive fraction can help in improving traditional medicine. The optimization of those two compounds will enhance their selectivity/effectiveness and could be used as seed for the development of new remedies against schistosomiasis. Further, the study will be focus on other pathogens species of *Schistosoma* genus.

Key words: *Anonidium mannii*, antischistosomal (antiparasitic and enzymatic activity), aristolactam BII acetylate, cytotoxicity, gallic acid, semisynthetic reactions

SUMMARY

- The stem bark of *Anonidium mannii* was examined for their bioactive constituents. This study led to the isolation and identification of eight known compounds: β-sitosterol 1, stigmasterol 2, polycarpol 3, aristolactam BII 4, gallic acid 5, aristolactam AII 6, epicatechin 7, and piperolactam D 8. At the exception of compound 3, all these compounds were isolated and characterized from the genus *Anonidium* for the first time. Interestingly, semisynthetic reactions were carried out on two isolated compounds (4 and 6) to give aristolactam AII diacetylate 10 and BII acetylate 9, respectively, the latter being a new compound and described here for the first time. All those structures were elucidated by one-dimensional and two-dimensional nuclear magnetic resonance spectroscopic methods as well as mass spectrometry. The cytotoxic activities of the extract and some compounds were evaluated against A549 and Huh7 cell lines. Extract, natural products, and semisynthetic derivatives were also tested for antischistosomal (antiparasitic and enzymatic activity). As praziquantel (positive control), the only molecule used in Africa to cure schistosomiasis, compound 5 displayed very good worm-killing capacity on *Schistosoma mansoni*, compound 8 showed significant inhibition on *S. mansoni* nicotinamide adenine dinucleotide + catabolizing enzyme, which is an ectoenzyme newly discovered.



Abbreviations used: 1D: One dimension; 2D: Two dimension; ^{13}C: Carbon 13; ^{1}H: Hydrogen; A549: Human lung cancer cells line; *A. mannii*: *Anonidium mannii;* Calcd.: calculated; CH_3CN: Acetonitrile; DCM: Dichloromethane; DEPT: Distortionless enhancement by polarization transfer; DMEM: Dulbecco's modified Eagle medium; EtOAc: Ethyl acetate; H_2O: water; HPLC: High-performance liquid chromatography; HR-ESI-MS: High resolution-electrospray ionization-mass spectrum; HSQC: Heteronuclear single quantum coherence spectroscopy; Huh7: Human hepatocarcinoma cells line; MeOH: Methanol; NAD+: Nicotinamide adenine dinucleotide; NMR: Nuclear magnetic resonance; NOE: Nuclear overhauser effect; NOESY: Nuclear overhauser effect spectroscopy; OD: Optical density; RPMI: Roswell Park Memorial Institute Medium; *S.*: *Schistosoma; Sm*NACE: *Schistosoma mansoni* NAD+ catabolizing enzyme; TFA: Trifluoroacetic acid; UV: Ultraviolet.

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INTRODUCTION

Schistosomiasis, also known as bilharzia, is a disease caused by parasitic worms infecting people in subtropical areas in the world. In terms of socio-economic effect, this neglected tropical disease[1] represents the second global parasitosis after malaria. It is reported that this water-borne illness is responsible of 200 thousand deaths per year; 200 million people infections and 600 million people are at risk of contamination.[2] Those parasites were transmitted by specific freshwater mollusk intermediate hosts that depend on the species of the parasite concerned. Thus, the parasite's species mainly involved in the spread of human schistosomiasis are *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. Further, symptoms that usually observed are fever, headache, myalgia, respiratory issues, and often by painful hepato- and/or spleno-megaly. At this moment, vaccine is not yet available. In Africa, the only medication used for the treatment of schistosomiasis is based on the pyrazino isoquinoline derivative, praziquantel, known as the common brand Biltricide.[3] Unfortunately, this drug has displayed many side effects. There is a recent increase of the resistance of the parasites from this late cure.[4] With this setback, studies have shown prominent deficiency in the search for antagonists of schistosomes. Unfortunately, a literature study highlights pronounced deficiency in the search for antagonists of schistosomes. While aiming for new therapeutic targets, there is also an urgent need to find new, safe, and effective antischistosomal drugs for this reason, *S. mansoni* nicotinamide adenine dinucleotide (NAD)+ catabolizing enzyme (*Sm*NACE) is an ectoenzyme newly discovered. It has a favorable topology due to its expression as a constitutively active ectoenzyme in the tegument membrane of adult *S. mansoni*. To be precised, *Sm*NACE is scored high grades based on its accessibility of drugs and might represent a good drug target.[5-7]

As a part of our ongoing novel strategy for the search for bioactive constituents of plant origin against schistosomiasis disease,[8] we undertook the study of stem bark of *Anonidium mannii*. It is a tropical plant commonly known as *Junglesop*, belonging to *Annonaceae* family. This species is a fast-growing tree (8–30 m high), a girth of 2 m with leaves between 20 and 40 cm. Its flowers produce large fruits which can reach 15 kg.[9] Different parts of this plant are used in traditional medicine to relieve several ailments and symptoms such as dysentery, gastroenteritis, bronchitis, syphilis, diarrhea, malaria, and schistosomiasis.[10,11] Very few previous phytochemical investigations of the *A. mannii* species revealed the presence of alkaloids, phenols, polyphenols, saponins, tannins, and steroids.[12-14] Some of those compounds have been reported to possess antibacterial, immunosuppressive, and radical scavenging activities.[13,14] In this study, we report the isolation and identification of structure of eight constituents from stem bark of *A. mannii*. We also processed with acetylation reactions on two isolated compounds. For the first time, the viability on Huh7 and A549 cancer cell lines was vetted. Plus, the crude extract and some compounds were tested for their antischistosomal (antiparasitic and enzymatic) activities.

MATERIALS AND METHODS

General methods

Column chromatography was carried out using silica gel (Merck 60-120, 70-230, and 230-400 mesh). High-performance liquid chromatography (HPLC) was performed on a Dionex apparatus equipped with an ASI100, ultimate 3000 pump, a diode array detector UVD 340S, and a chromeleon software. A prepacked RP-C_{18} column (Phenomenex 250 × 15 mm, Luna 5 μ) was used for semipreparative HPLC. The eluting mobile phase consisted of H_2O with trifluoroacetic acid (0.0025%) and acetonitrile with a flow rate of 17 mL/min, and the chromatogram was monitored at 210 and 254 nm.

Thin layer chromatography was performed on percolated 0.5 mm thick Merck Si gel 60 F_{254} aluminum sheets. Separated compounds were visualized under ultraviolet (UV) light and by spraying with H_2SO_4-EtOH (1:9, v/v) followed by mild heating for about 2–3 min. The mass spectra were recorded on an Agilent MS instrument (Agilent Technologies 6520, Accurate mass Q-Tof). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DRX-500 instrument operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), using deuterated dimethyl sulfoxide (DMSO) or deuterated chloroform ($CDCl_3$) as solvent. Chemical shifts (δ) are quoted in parts per million from internal standard tetramethylsilane, and the coupling constants (*J*) are given in Hertz. Different mixtures of n-hexane, ethyl acetate (EtOAc), CH_2Cl_2, and methanol (MeOH) were used as eluting solvents. They were distilled before use. The masses of the isolates were taken using an electronic balance, Mark MELTER PC 2000.

Plant material

Stem bark of *A. mannii* was collected in January 2016 at Mount Kalla (Latitude 3° 30'. North, Longitude 11° 13'. East), from MBankomo locality, Mefou and Akono Subdivision, Center Region of Cameroon, and identified by M. Victor Nana, a botanist of National Herbarium, Yaounde, Cameroon. A voucher specimen was deposited under the voucher number 45582HNC in the National Herbarium of Yaounde, Cameroon.

Extraction and isolation

Plant material was chopped and air-dried until a constant mass was obtained. Dried sample was ground into a fine powder and stored in desiccators until extracted.

Air-dried and powdered stem bark of *A. mannii* (3.2 kg) was extracted with 25 L of a mixture of MeOH/distilled water (8:2) for 72 h at room temperature. The hydroalcoholic extract was evaporated and freeze-dried to give a crude extract (71.15 g). 65.5 g of the extract was subjected to flash CC on silica gel eluting with a gradient solvent system of hexane-EtOAc (1:0-0:1) and EtOAc (1:0)-MeOH (8.5:1.5) to obtain five fractions F_A–F_E. Fraction F_B (14.8 g) was further purified by successive column chromatography (CC) on silica gel eluting with hexane-EtOAc (1:0-3:2) to afford compounds 1 (30.6 mg) and 2 (11 mg), respectively. Fraction F_C (12.6 g) resulting from hexane-EtOAc (2.5:7.5) was chromatographed by successive CC on silica gel using hexane-EtOAc (9.5:0.5-0:1) to afford compounds 3 (21.9 mg) and 4 (15 mg). Fraction F_D (15.2 g) resulting from hexane-EtOAc (0:1) was chromatographed by successive CC on silica gel using hexane-EtOAc (9.5:0.5–0:1) to afford compounds 5 (11 mg), 6 (13 mg), and 7 (16 mg). Fraction F_E (15.2 g) resulting from hexane-EtOAc (0:1) was chromatographed by successive CC on silica gel using dichloromethane (DCM)-MeOH (9.5:0.5-8:2) to afford compounds 6 (10.3 mg) and 8 (11.2 mg).

General procedure for the acetylation of compounds 4 and 6

Compounds 4 (3.12 mg, 11.2 μmol) and 6 (3.84 mg, 14.5 μmol) were dissolved, respectively, in dry pyridine (0.5 mL). The resulting solution was cooled down to 0°C before acetyl chloride (0.5 mL) was added dropwise. After stirring at 0°C for 10 min, the ice bath was removed and the mixture was allowed to stir at room temperature for 16 h (0.5 mL of pyridine was added after warming up to room temperature to dissolve the reaction mixture that had solidified). The resulting deep brown solution was then diluted with diethyl ether (10 mL), transferred to a separatory funnel, and washed three times with 1.0 M HCl (20 mL in total). The aqueous layers were extracted two times with diethyl ether (20 mL in total) before the

organic layers were combined, dried over sodium sulfate, and filtered and concentrated under reduced pressure. The resulting crude was purified by flash chromatography (silica gel, 100% DCM or 1:1 petroleum ether 35–60/DCM, respectively) to afford compound 9 (3.20 mg, 9.96 μmol, 89%, R_f 0.86 in 100% DCM) as a yellowish solid and compound 10 (3.70 mg, 10.6 μmol, 73%, R_f 0.53 in 100% DCM) as a light orange solid, respectively.

Bioassay

In vitro antischistosomal activity against adult Schistosoma mansoni

The *in vitro* investigation of the adult's blood fluke *S. mansoni* was conducted on the extract of *A. mannii* and some of its compounds. *S. mansoni* (NMRI strain from Puerto Rico) worms were maintained in *Biomphalaria glabrata* snails (albino variety from Brazil) as intermediate hosts and *Mesocricetus auratus* hamsters (Janvier Labs; Le Genest-Saint-Isle, France) as definitive hosts at the Laboratory of Ecology and Evolution Interactions (IHPE, Perpignan, France). Female hamsters were infected by percutaneous exposition to 400 cercariae following the standard procedures.[15] After 40 days, adult *S. mansoni* specimens were recovered from each hamster by hepatic perfusion technique.[16] Adult living schistosomes were washed in Roswell Park Memorial Institute Medium (RPMI) 1640 medium (supplemented with 5% inactivated fetal calf serum [FCS] and 100 U/mL penicillin and 100 μg/mL streptomycin [Invitrogen, Carlsbad, CA, USA]) at 37°C in an atmosphere of 5% CO_2. Adult worm pairs (10–12 [male and female]) were incubated in a 24-well culture plate. All compounds were initially added to the culture at a concentration of 100 μg/mL, using a 10 mg/mL stock solution in RPMI 1640 containing DMSO (final concentration of DMSO: 0.2). The final volume in each well was 2 mL. The control worms were assayed in RPMI 1640 medium and DMSO in medium as negative control and praziquantel as positive control. All the experiments were performed in duplicate or triplicate. The parasites were maintained for 6 h and monitored every hour using a light microscope to evaluate their body contractility and movement. Parasite death was defined as no movement for at least 30 s of examination.[17]

Ethic statement

The experiment's laboratory was approved by the "Ministère de l'Enseignement supérieur de la Recherche et de l'Innovation (France)" (permission A66040 from for animal experimentation). Experimenters hold a certificate for animal experimentation (authorization 007083, decree 87-848 and 2012201-0008). Animal care (housing, breeding,…) was strictly in accordance with National and European requirements.

Enzymatic activity *in vitro*

The enzymatic activity on *Sm*NACE was conducted *in vitro* on the stem bark's extract of *A. mannii* and some of its compounds. The activity was determined by a fluorometric assay using 1, N^6-etheno NAD$^+$ (ε-NAD+, Sigma) as substrate. This assay consists in measuring the appearance of the reaction product ε-ADP-ribose by the increase of fluorescence at λem = 410 nm (λexc = 310 nm) at 37°C in 10 mM potassium phosphate buffer, pH 7.4, containing 0.05% (w/v) emulphogen (1 mL final volume) in a quartz tank.

Briefly, in a spectrofluorimeter (*Shimadzu RP-5301 PC*), substrate (with final concentration 20 μM) and inhibitors were added successively to the buffer contained in the tank at 37°C and with stirring. To determine the initial fluorescence F_0, the tracing was performed before the introduction of the enzyme. The reaction was started by the addition of the enzyme and followed for 2 min, measurement time. The kinetics was analyzed at the end of this time using a nonlinear regression program (GraphPad, Prism) for the determination of catalytic activity. All the inhibitors were initially tested at a concentration of 100 mM diluted in DMSO (less than 2% added). The concentrations were decreased in case of the fluorescence changes (quenching or increase of fluorescence). Finally, 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM were the concentrations used. Each test was performed twice.

The approximate IC_{50} values were determined based on concentrations giving approximately 50% inhibition.

Cytotoxicity activity

The *in vitro* cytotoxicity of the stem bark's extract of *A. mannii* and some of its isolated compounds against Huh7 and A549 cell lines was measured by a MTS assay.

Huh7 cells (human hepatocarcinoma cells) were cultured in Dulbecco's modified Eagle medium (DMEM) 1 g/L glucose (SIGMA), 2 mM glutamine, and 10% FCS (SIGMA). The A549 cells (human lung tumor cells) were cultured in HamF12 medium, 2 mM glutamine, and 10% FCS.

The extract and compounds were dissolved in DMSO and diluted with culture medium with serum. They were added, each at a time, at a rate of 20 μL/well to obtain the desired final concentration. The cells, seeded in a 96-well plate (Nunc Edge 2.0, ThermoScientific), were treated with different concentrations (25 μM, 50 μM, 100 μM, 150 μM, 200 μM, and 250 μM) of stem bark's extract of *A. mannii* and its compounds at a density of 4000 cells/100 μL per well (Huh7) and 6000 cells/100 μL per well (A549) and then incubated for 24 h at 37°C in 5% CO_2 incubator. 48 h after the addition of the extract/compounds, 20 μL of MTS solution (CellTiter 96R Aqueous One Cell Proliferation Assay Solution, Promega) was added to each well. After 1 h of incubation at 37°C, the absorbance was read at 490 and 700 nm (Safas, Monaco). All tests were carried out in triplicate.

The optical density (OD) or absorbance of the extract/compounds to be assayed (490 nm) was analyzed after deducting the OD at 700 nm, the OD of the plastic, and the OD of the cells alone.

The positive and negative controls used were praziquantel and DMEM, respectively.

Characterization of compound 9

Aristolactam BII acetylate (compound 9)

Yellow powder. ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C120.2 (C-1), 113.4 (C-2), 153.5 (C-3), 151.9 (C-4), 133.8 (C-4a), 118.1 (C-4b), 126.5 (C-5), 127.1 (C-6), 126.4 (C-7), 129.6 (C-8), 126.3 (C-8a), 109.0 (C-9), 131.7 (C-10), 124.0 (C-10a), 166.3 (C = O), 170.5 ($NCOCH_3$), 25.4 ($NCOCH_3$), 56.3 (MeO-3), 59.8 (MeO-4). ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H 7.76 (1H, s, H-2), 9.18 (1H, d, J = 6.5 Hz, H-5), 7.57 (2H, m, H-6 and H-7), 7.87 (1H, d, J = 6.5, H-8), 8.26 (1H, s, H-9), 2.75 (3H, s, $NCOCH_3$), 4.04 (3H, s, MeO-3), 4.09 (3H, s, MeO-4). High resolution-electrospray ionization-mass spectrum (HR-ESI-MS) *m/z*: =322.1083 [M + H] + (calculated [calcd] for $C_{19}H_{15}NO_4$, 322.1079).

RESULTS AND DISCUSSION

The hydroalcoholic extract of stem bark of *A. mannii* was fractionated and purified by successive silica gel CC, affording eight known compounds: β-sitosterol 1,[18,19] stigmasterol 2,[19] polycarpol 3,[20] aristolactam BII 4,[21] gallic acid 5,[22] aristolactam AII 6,[23] epicatechin 7,[24] and piperolactam D 8[25] [Figure 1]. Interestingly, all these compounds, except polycarpol, have been isolated from the genus *Anonidium* for the first time. In addition, two semisynthetic derivatives were obtained by acetylating compounds 4 and 6 [Scheme 1]: aristolactam BII acetylate 9 and aristolactam AII diacetylate 10,[26] respectively. The former is described here for the first time.

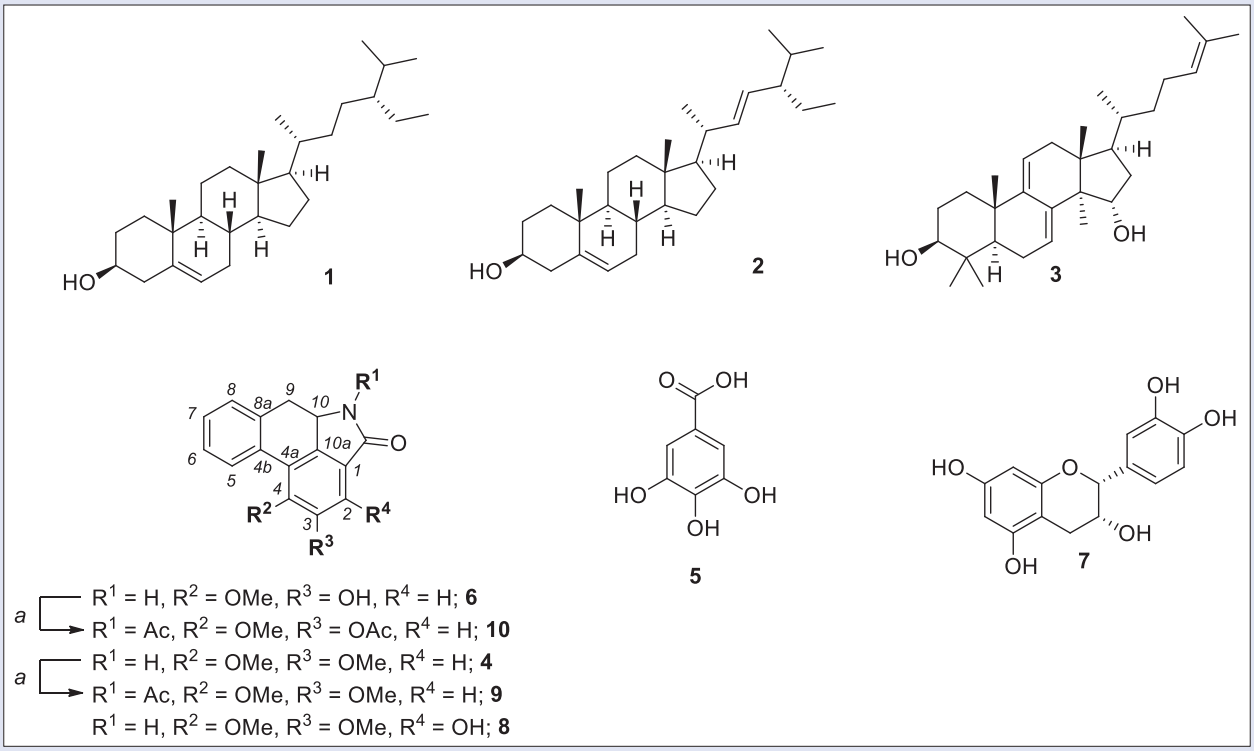

Figure 1: Constituents isolated from the stem bark of *Anonidium mannii* (compounds 1–8) and semisynthetic derivatives (9 and 10); conditions *a*: Ac_2O, pyridine, room temperature, 16 h (9: 89%; 10: 73%)

Compound 9, soluble in DCM, was obtained as a yellow powder from the acetylation reaction of compound 4. The molecular formula of this compound inferred to be $C_{19}H_{15}NO_4$ based on its molecular ion at *m/z* = 322.1083 [M + H]+ in positive ion mode HR-ESI-MS (calcd. 322.1079) [Supplementary Figure 1]. The UV spectrum in MeOH exhibited absorptions characteristics of phenanthrene chromophores with absorption at 208, 242, 260, 288, 331, and 394 nm[27-29] [Supplementary Figure 2S]. The ^{13}C, distortionless enhancement by polarization transfer, and ^{1}H-^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) NMR spectra [Supplementary Figures 3-5, respectively] showed resonance of 19 carbons consisting of eight quaternary aromatic carbons at δ_C 153.5 (C-3); 151.9 (C-4); 133.8 (C-4a); 120.2 (C-1); 131.7 (C-10); 126.3 (C-8a); 124.0 (C-10a); 118.1 (C-4b), along with two carbonyl at δ_C 166.3 (acetyl group) and 170.5 (amide moiety); six sp^2 carbons bearing one hydrogen each at δ_C 126.5 (C-5); 129.6 (C-8); 127.1 (C-6); 126.4 (C-7); 113.4 (C-(2); 109.0 (C-9); and three methyl carbons at δ_C 25.4 (CH_3CON); 56.3 (CH_3O-3); and 59.8 (CH_3O-4). ^{1}H NMR spectrum [Supplementary Figure 6] indicated six aromatic proton signals at δ_H 9.18 (1H, d, *J* = 6.5 Hz, H-5), δ_H 7.57 (2H, m, H-6 and H-7), and δ_H 7.87 (1H, d, *J* = 6.5 Hz, H-8), showing also AMX coupling pattern and two singlets appearing at δ_H 7.76 (1H, s, H-2) and δ_H 8.26 (1H, s, H-9). In addition, this spectrum confirmed the existence in nine of three methyl groups at δ_H 2,75 (3H, s, CH_3CON); 4.04 (3H, s, CH_3O-3); and 4.09 (3H, s, CH_3O-4). The connectivities between proton and carbon atoms were supported by the Heteronuclear Multiple Bond Correlation (HMBC) spectrum [Supplementary Figure 7].

Thus, we observed Key correlations,[29] first between hydrogen in position 2 with carbons C-3, C-4, C-10a, carbonyl (δ_C 166,3); also between hydrogen H-5 with carbons in position 4a, 4b, 7; between hydrogens in position 6 and 7 with carbons in 5, 8; between H-8 with carbons in position 7, 9; and between H-9 which correlated with C-8, C-8a, C-10, C-10a [Figure 2].

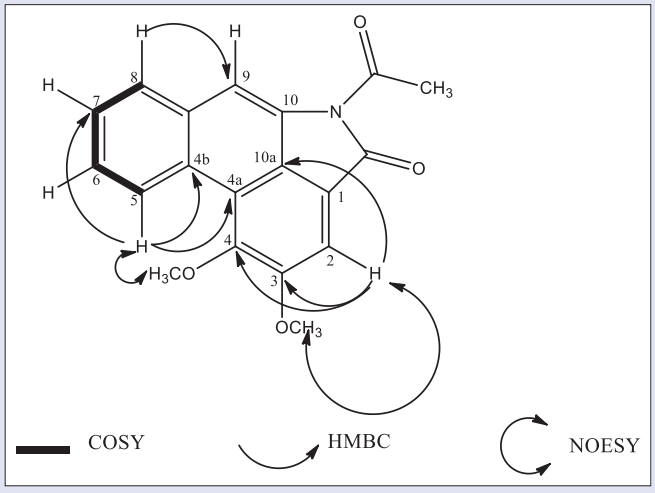

Figure 2: Key HMBC, COSY, and nuclear overhauser effect spectroscopy correlations of compound 9

Moreover, examination of nuclear overhauser effect spectroscopy spectrum [Supplementary Figure 8] showed that the following pairs of protons are close to each other in space δ_H 4.04 CH_3O-3 and δ_H 4.09 CH_3O-4; δ_H 4.04 CH_3O-3 and δ_H 7.76 H-2; and δ_H 4.09 CH_3O-4 and δ_H 9.18 H-5, which helped confirm the position the positions of methoxyl groups at 3 and 4, as it was observed in studies of the 3,4-dimethoxyphenanthrene lactam.[30,31] Besides the acetyl moiety observed in the ^{1}H and ^{13}C spectrum of compound 9 and the absence of

the characteristic proton of –NH group of lactam, all the spectral data were similar to those of aristolactam BII described in the literature.[21,27,32] All the above information and data previously mentioned in the literature allowed us to identify the compound 9 as aristolactam BII acetylate (10-acetylamino-3, 4-dimethoxyphenanthrene-1-carboxylic acid lactam): a new derivative of aristolactam BII described for the first time.

The compound 9 together with compounds 4, 5, 6, 7, and 8 was evaluated for their antischistosomal (antiparasitic and/or enzymatic) and/or cytotoxic activities.

Antiparasitic assay *in vitro*

The extract and compounds 4, 5, 6, 8 and 9 were tested for their antischistosomal activity against *S. mansoni*. The stem bark extract showed interesting worm-killing capacity at concentration of 100 µg/mL with parasite rate survival of 81.8%. In addition, all compounds were less efficient than praziquantel (positive control) except gallic acid 5, which exhibited same activity as the positive control after 6 h at the concentration of 100 µM [Table 1]. To the best of our knowledge, this is the first time that gallic acid showed to be a potent antagonist against *S. mansoni*; on the contrary, piperolactam D 8 showed no activity at this concentration. On the other hand, the new compound aristolactam BII acetylate 9 displayed weak worm-killing capacity with a rate of 40% at the concentration of 100 µM [Figure 3]. Meanwhile, the study of structure–activity relationship of lactam compounds revealed that the most active was 6 at the concentration of 100 µM. This activity decreased when the hydroxyl group borne by carbon C-4 of 6 was replaced by a methoxyl (compound 4) and when carbon C-2 was substituted by a hydroxyl group in 4. However, an increase in activity was observed upon acetylation of the lactam nitrogen in 6 (compound 9). All compounds, for which an antiparasitic activity had been determined, were further subjected to enzymatic and cytotoxic assays.

Enzymatic assay *in vitro*

In 2010,[7] *Sm*NACE was used as a model in the context of screening for inhibitors, comprising thousands of molecules including natural products, using a high-throughput screening assay easily applicable in the laboratory. The high-throughput screening revealed a more or less marked inhibitory effect of natural products on *Sm*NACE (although cyanidin proved to be the best, but no specific). It was therefore interesting to test our extract and compounds on the activity of *Sm*NACE without presuming their action.

Results of enzymatic activity of some compounds are presented in Table 2. The stem bark extract was active on *Sm*NACE with an inhibition rate of 51.08% at 100 µg/mL. For isolated compounds, we noticed that only piperolactam D 8 presented a comparable activity to that of the reference *Sm*NACE inhibitors cyanidin and delphinidin, while the rest of evaluated molecules were shown to possess less IC_{50} values.

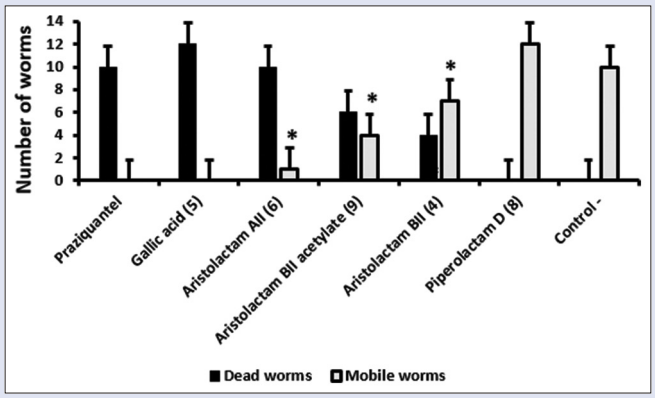

Figure 3: Survival of *Schistosoma mansoni* adult worms treated with the praziquantel (positive control). Worms were treated with compounds 4, 5, 6, 8, and 9 at 100 µM. Each test was performed in duplicate or triplicate. Parasites were subsequently observed for body contractility and movement each hour for 6 h. *$P < 0.05$

Cytotoxicity assay

The cytotoxicity assay was carried out with the same subset of extract and compounds (4, 5, 6 and 8) against Huh7 and A549 cells line. The human lung cancer (A549) and the human hepatocarcinoma (Huh7) cells line were chosen because lungs and liver are the focal points of pathogenic insult and subsequent pathological damage in schistosomiasis.[33] The results are consigned in Figure 4.

It showed that stem bark extract (AME) was not tested on Huh7 cells but showed toxicity on A549 cells line (with a percentage of cell viability of 37%). For isolated compounds, their percentage of cell viability on

Table 1: *In vitro* evaluation of the antischistosomal activity of compounds 1, 2, 3, 5, 8, and 10 at various concentrations; unless otherwise indicated, experiments were done in triplicates; for each experiment, 10-12 worms were used with equilibrated sex ratio

Extract/compound (number)	Concentration (µM)	Mobile worm after 6 h of incubation (%)
Stem bark of *Anonidium manniia*	100	81.8
Gallic acid (5)	100*	0
	50*	53.6
	10*	92
Aristolactam AII (6)	100	9.1
	50	66.7
	10	71
Aristolactam BII (4)	100	62.5
	50	77.3
	10	94.4
Aristolactam BII acetylate (9)	100	40
	50	95
	10	100
Piperolactam D (8)	100	100
	50	Not done
	10	Not done
Praziquantel	100	0
	50	7
	10	Not done
Control (RPMI)	-	100

*Experiments were performed in duplicates, [a]Concentration expressed in µg/mL. RPMI: Roswell Park Memorial Institute Medium

Table 2: IC_{50} values for the inhibition of *Schistosoma mansoni* nicotinamide adenine dinucleotide+ catabolizing enzyme by compounds

Compound (number)	IC_{50} (µM)
Aristolactam BII acetylate (9)	NT
Aristolactam AII (6)	>100
Aristolactam BII (4)	>100
Gallic acid (5)	>100
Piperolactam D (8)	10-20
Cyanidin*	2.3
Delphinidin*	6.0

*Cyanidin and Delphinidin are the natural products reference *Sm*NACE. NAD+: Nicotinamide adenine dinucleotide; *Sm*NACE: *Schistosoma mansoni* NAD+ catabolizing enzyme; NT: Not Tested

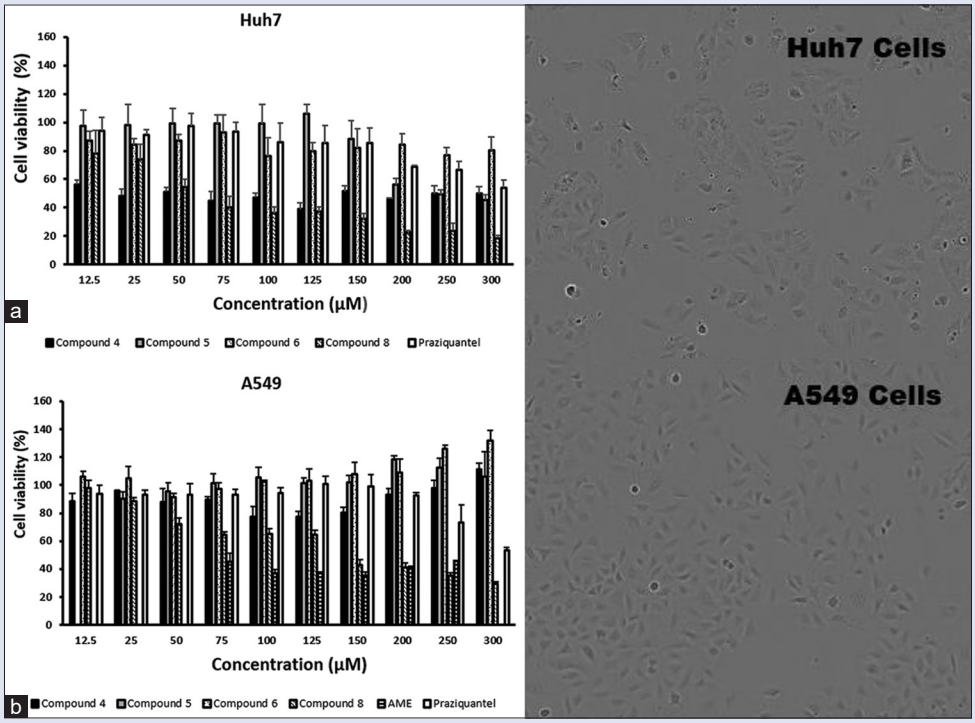

Figure 4: The cell viability rate of AME, compounds 4, 5, 6, 8 and praziquantel assayed by MTS. Notes: These two cell lines were continuously treated with different concentrations (12.5 μM, 25 μM, 50 μM, 75 μM, 100 μM, 125 μM, 150 μM, 200 μM, 250 μM, 300 μM) of AME, compounds 4, 5, 6, 8 and praziquantel for 48 h. (a) Huh7 cell line and (b) A549 cell line. Cell viability was then determined by MTS assay

Huh7 cells were 47% for 4, 99% for 5, 76% for 6, and 36% for 8 compared to reference drug (praziquantel, 86%). This revealed that gallic acid 5, which was a good antagonist against *S. mansoni*, did not exhibit toxicity on Huh7 cells line contrary to praziquantel (reference drug). Similarly, on A549 cells line, the percentage of cell viability of the tested compounds was 77% for 4, 100% for 5, 100% for 6, and 65% for 8 compared to drug (praziquantel, 94%). These results showed also that gallic acid 5 exhibited very good viability (100%) on A549 cell line.

CONCLUSION

The chemical investigation of stem bark of *A. mannii* led to the isolation and identification of eight compounds: β-sitosterol 1, stigmasterol 2, polycarpol 3, aristolactam BII 4, gallic acid 5, aristolactam AII 6, epicatechin 7, and piperolactam D 8. At the exception of compound 3, all these compounds were isolated and characterized from the genus *Anonidium* for the first time. Meanwhile, acetylation reactions were carried out on isolated compounds 4 and 6 to afford two semisynthetic derivatives: one known derivative aristolactam AII diacetylate 10 and one new aristolactam BII acetylate 9. Antischistosomal (antiparasitic and enzymatic) activity and cytotoxicity of *A. mannii* extract and some isolated compounds were also evaluated *in vitro* for the first time. As praziquantel (positive control), the only molecule used in Africa to cure schistosomiasis, gallic acid 5 was exhibited very promising worm killing capacity on *S. mansoni* after 6 h at the concentrations of 100 μM (all schistosomes were killed). Meanwhile, enzymatic activity testing on *Sm*NACE revealed that compound 8 showed a significant inhibition with IC_{50} around 10 μM compared to the cyanidin (IC_{50} = 2.3 μM), reference *Sm*NACE inhibitor. Moreover, the stem bark extract of *A. mannii* which was no tested on Huh7 cells showed less toxicity on A549 cells line all tested isolated compounds (4, 5, 6, and 8) showed little or no cytotoxicity activity on Huh7 cells and A549 cells line. Optimization of those two more bioactive compounds (5 and 8) could further improve their selectivity/effectiveness and so could be used as seed for the development of new remedies as well as the standardization of the stem bark extract of *A. mannii* which could be used to improved traditional medicine. In the future, we plan to biologically evaluate those extract and compounds on the two other pathogens species of *Schistosoma*: *S. haematobium* and *S. japonicum*.

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Conflicts of interest

There are no conflicts of interest.

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