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Leishmanicidal, Trypanocidal, anti-fungal and anti-helmintic activities of extracts and isoquinoline isolated from *Monodora tenuifolia* Benth (Annonaceae)

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Abstract
The seeds of *Monodora tenuifolia* Benth are used for the treatment of skin diseases. The roots are used against dysentery and toothache. Little work has been done on this species, and to our knowledge, no activity has been attributed to the leaves of this plant. In this paper, we have evaluated Leishmanicidal, Trypanocidal, anti-fungal and anti-helmintic activities in order to attribute therapeutic properties to the plants growing in Côte d'Ivoire. Hot and cold methanol extracts were obtained from *M. tenuifolia*. Two compounds namely (-)-xylopinidine (1) and (-)-*N*-methylarmeparvine (2) were isolated for the first time from the leaves of cold methanol extract of *M. tenuifolia* (Annonaceae). Their structures were established from the spectral data obtained (Nuclear Magnetic Resonance; NMR, Mass Spectroscopy, MS, Infra-Red, IR and Ultra Violet Visible, UV Vis spectrocopies). Methanol extracts (hot and cold) and isolated compounds were screened against *Leishmania donovani*, *Trypanosoma brucei*, *Candida albicans*, *Aspergillus fumigatus* and *Caenorhabditis elegans*. The cold and hot methanol extracts from *M. tenuifolia*, exerted the most significant activity against *T. brucei* (LC100: 12.5 mg/L, cold methanol extract) and (LC100: 25 mg/L, hot methanol extract). This is the first report on the anti-protozoal (anti-parasitic), anti-fungal and anti-helmintic activities of these extracts and compounds. Our results review huge potentials for further studies of the cold extract for enhanced Trypanocidal activity.

Keywords: *Monodora tenuifolia*, trypanocidal, nuclear magnetic resonance, isoquinoline, leishmanicidal

1. Introduction
Traditional medicine is assumed to be a crucial health care option for poor households in the third- world countries. It relieves more than 70 % of the population of the countries [1] and 70 to 90 % of the population of Africa [2]. Because of the high cost of conventional medicine, associated with their increase of multi-resistant bacteria, there has been a renewed interest in African traditional medicine [3-5]. It is therefore, essential to look for new bioactive substances stemming from the traditional pharmacopoeia, effective, broad spectrum anti-infective agents with a low cost. Recent studies have shown that the *M. tenuifolia* seed extract possesses several biological effects such as anti-diarrhoeal, antioxidant and anti-microbial activities. In traditional medicine practice, it is widely used to relieve dermatitis, headache and as vermifuge [6]. The seeds are used as ingredient in herbal medicines in southern Nigeria and are aromatic. In food, they are used as flavour [7] and as a spice [8]. When roasted, the ground seeds are rubbed on the skin to treat various skin diseases [8]. In traditional medicine practice, the plant is used mainly for the treatment of dysentery, diarrhoea, dermatitis and toothache. It is also used as vermifuge. The reported antioxidant potential of the plant makes it important in the management of stress induced conditions such as depression [9]. The current study aimed to assess potential effects of isolated compounds (-)-xylopinidine (1) and (-)-*N*-methylarmeparvine (2), cold and hot methanol extracts, against *L. donovani*, *T. brucei*, *C. albicans*, *A. fumigatus* and *C. elegans*.

2. Material and Methods
2.1. Material
2.1.1. General
The NMR spectra were recorded on a Brüker Advance-400 operating at 400 MHz, using TMS as internal standard.

Chemical shifts were quoted in *d* ppm and coupling constant *J* was measured in Hertz (Hz). One-dimensional 1H and 13C spectra were acquired under standard conditions. Currently, 1H–1H homonuclear (COSY, NOESY) and 1H–13C heteronuclear (HSQC, HMBC) correlation techniques were routinely applied in field of constitutional analysis. Column chromatography was performed on silica gel (Kieselgel 60, particle size 0.040–0.063 mm) and Sephadex®LH-20. TLC was run on silica gel pre-coated glass plates (Merck silica gel 60 F254). IR spectra were measured on a Brüker Vector 22. Polarimeter Optical rotations were recorded on an Optical Activity PolAAr 32. Polarimeter using a sample concentration of 10 mg/ml, unless otherwise specified. ESIMS were obtained with ITQ 900 spectrometer using an Agilent DB-5HT (30 x 0.32 x 0.1) column. Gas chromatography was performed on TRACE GC ULTRA Thermo Scientific instrument. HR-ESIMS were run on a TOF LCT Premier WATERS coupling with HPLC Alliance 2695 (Waters) and also with micro TOFq Brüker.

2.1.2. Plant material
The leaves of *M. tenuifolia* were collected in August 2015 in Adiopodoumé (South of Côte d'Ivoire). They were identified by Pr. Ipou Ipou Joseph (Centre National de Floristique-Université Félix HOUPHOUËT-BOIGNY). A voucher specimen (n° MT-KABLAN-Diapodoumé 2015-2) is deposited at the Herbarium of the Botanic Laboratory (Université Félix HOUPHOUËT-BOIGNY).

2.1.3. Biological assay
2.1.3.1. Chemicals & Laboratory Materials
Sterile distilled water (IN HOUSE), Dimethyl sulfoxide "DMSO" (VWR, France), Sterile disks of blotting paper (Pratdumas, France), MOPS and RPMI media (SIGMA and GIBCO, France), Petri dishes (Monolab S.N.C, Italy), Sterile test tubes (LAB-BOX, France), Microplates (Deltalab, Spain), Optical microscope (Optika®, B-383 PLI, Italy), Melarsoprol "standard Trypanocidal drug" (Arsobal®, Sanofi-Aventis, France), Pentamidine "standard Leishmanicidal drug" (Pentacarinat®, Sanofi-Aventis, France), Mebendazole "standard anthelminthic drug" Amphotericin B "standard, anti-fungal drug" (Fungizone®, Bristol-Myers Squibb, France).

2.1.3.2. Tested Microorganisms
2.1.3.2.1. Parasites
Trypanosoma brucei, *Leishmania donovani* and *Caenorhabditis elegans*

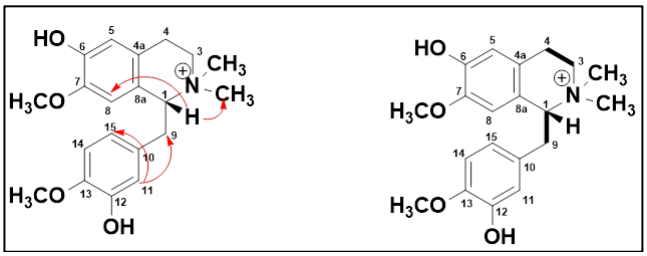
2.1.3.2.2. Fungi
Aspergillus fumigatus and *Candida albicans*

2.2. Methods
2.2.1. Extraction Procedure
Cold extraction: The cold methanol extract of *M. tenuifolia* leaves were prepared in methanol (100 g dry powder material in 1 L; room temperature, under magnetic stirring, 24 hours). After filtration twice on white cotton and once on whatman filter paper No 3, the extract were dried under reduced pressure at 45 °C temperature using a rotary evaporator (Heidoph RZ 2.5). Extraction yield was 11.16 g, corresponding of a percent recovery of 11.16 %. The extracted powder were stored in a glass container covered with parafilm and kept in at 7-8 °C temperature.

Reflux extraction: The dried powder of *M. tenuifolia* (100 g) were extracted using a reflux apparatus with 1000 mL of solvent for 30 minutes. After the extraction and filtration process, the solvents were removed using a rotary evaporator. We obtained 12.7 g (12.7 % percent recovery) of a dry residue.

2.2.2. Isolation
The cold methanol extract was fractionated on silica gel column chromatography, eluting with CH_2Cl_2-methanol gradient systems. Seven fractions (F-1'' to F-7'') were obtained. Fraction F-6'' was purified on column chromatography of Sephadex®LH-20 [(CH_2Cl_2/MeOH (2:1) and CH_2Cl_2/MeOH (1:1)] to yield respectively compound 1 (8.9 mg) and compound 2 (7.7 mg).

2.2.3. Identification of compounds 1 and 2 (-)-xylopinidine (1): Amorphous brown solid; 1H and 13C NMR (400 MHz) data in Table 1, [□]D24 (O) = -21.2 ; c = 6.8 mg/ml in MeOH; IR ($CHCl_3$): max (cm^{-1}) = 2921 ; 1518 ; 1261; UV (MeOH): max (nm) = 221.4 ; 268.5 ; 301.6 ; ESI-MS (*m/z*) = 344.0 [M]+ (Fig. 1).



HMBC **COSY**

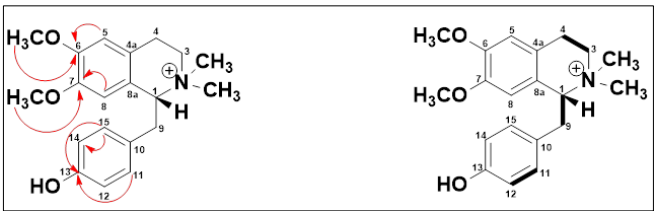
Fig 1: (-)-xylopinidine (1)

Table 1: NMR ^1H and ^{13}C of (-)-xylopinidine (1) in MeOD

Atom number	^{13}C (δ, ppm)	^1H (δ, ppm; *m*; *J* en Hz)
1	74.0	4.67; 1H; *m*
3	56.1	3.61; 1H; *m* 3.77; 1H; *m*
4	24.4	3.16; 1H; *m* 3.16; 1H; *m*
4a	120.5	---
5	116.2	6.83; 1H; *s*
6	147.7	---
7	146.2	---
8	112.7	5.96; 1H; *s*
8a	123.9	---
9	38.7	2.84; 1H; *m* 3.58; 1H; *m*
10	129.3	---
11	117.8	6.57; 1H; *s*
12	148.4	---
13	149.7	---
14	112.4	6.84; 1H; *d* (8.4)
15	122.4	6.51; 1H; *dd* (8.4; 2.0)
OCH_3(C-13)	56.4	3.85; 1H; *s*
OCH_3(C-7)	56.4	3.86; 1H; *s*
N-CH_3(1)	52.8	3.43; 1H; *s*
N-CH_3(2)	51.6	3.04; 1H; *s*

(-)-*N*-méthylarmeparvine (**2**): Amorphous brown solid; 1H and 13C NMR (400 MHz) data in table 1, [α]D21(O) = -32.0;

c = 6.0 mg/ml in MeOH; IR ($CHCl_3$): max (cm-1) = 2920; 2336; 1260; 718; UV (MeOH): max (nm) = 221.4; 268.5; 301.6; HR-ESI-MS (*m/z*) = 328.1946 [M]+ (molecular formula $C_{20}H_{26}NO_3$; calc. 328.1947 mDa = 0.1), SMIE: *m/z* (%) 328 [M]+(17 %), 283[M-H-N(CH3)2]+(22 %), 268[M-H-$N(CH_3)_2$-CH3]+(13 %), 252[M-H-$N(CH_3)_2$-CH3-OH]+(22%), 237[M-H-$N(CH_3)_2$-$2CH_3$-OH]+(8 %) (Fig. 2).



HMBC **COSY**

Fig 2: (-)-*N*-methylarmeparvine (2)

Table 2: NMR ^1H and ^{13}C of (-)-*N*-methylarmeparvine (2) in MeOD

Atom number	^{13}C (δ, ppm)	^1H (δ, ppm, *m*, *J* en Hz)
1	74.3	4.61; 1H; *dd* (10.8; 3.2)
3	55.7	3.67; 1H; *dt* (12.4; 4.0) 3.84; 1H; *m*
4	24.4	3.24; 1H; *m* 3.25; 1H; *m*
4a	121.8	---
5	113.3	6.86; 1H; *s*
6	150.9	---
7	148.3	---
8	112.4	5.71; 1H; *s*
8a	123.1	---
9α 9β	38.2	2.89; 1H; *t* (12.4) 3.67; 1H; *dt* (12.4; 4.0)
10	127.1	---
11	132.5	6.75; 1H; *d* (8.4)
12	116.6	6.87; 1H; *d* (8.4)
13	158.1	---
14	116.6	6.87; 1H; *d* (7.6)
15	132.5	6.75; 1H; *d* (7.6)
OCH_3(6)	56.4	3.84; 3H; *s*
OCH_3(7)	55.9	3.40; 3H; *s*
N-$(CH_3)_1$	51.4	3.17; 3H; *s*
N-$(CH_3)_2$	53.0	3.46; 3H; *s*

2.2.4. Biological assay

2.2.4.1. Trypanocidal activity

Trypanocidal activity was studied, using GVR 35 (Glasgow Veterany Research) strain of *T. brucei brucei*. Extracts and compounds were evaluated for their activity against bloodstream forms of *T. brucei brucei* (CMP fast strain) as described in previous research [10, 11, 12]. This method is based on the observation of the motility of cultivated parasites, followed by the inoculation to naive. The strain was maintained at a mouse by transplanting in syringe at least three days before the test. The parasites got back by aseptic taking of the blood of the vein of the tail of a strongly infected mouse, were diluted in an adequate way by means mice of the solution of immobile parasites with the LC100 of the tested compounds and extracts. No infection confirms the trypanocidal effect for LC100 of the culture medium to obtain 200 000 trypomastigotes/mL. The circulating forms of the parasite were cultivated *in vitro* without loss of their infectious power, during 24 hours at 37 °C in an atmosphere of air containing 5 % CO_2. The parasites were distributed in microplate of 96 wells of 200 µL at the rate of 2.105/mL. Then, 5 µL of the adequate dilution of tested products (extract and Melarsoprol as standard drug) in the DMSO, were added in triplicate. Wells control received only 5 µL of solvent. After 24 hours of incubation, the viability of trypanosomes was estimated by direct observation in optical microscope.

2.2.4.2. Leishmanicidal activity

The anti-leishmanial activity of the isolated compounds and the extracts were tested *in vitro* against *Leishmania donovani* (WHO designation: MHOM/ET/1967/L82), according to previous report [13]. This method is based on a dying agent specific of died parasites, thus allowing the measurement of an EC_{50} value. *Leishmania*'s, at promastigote stadium, were maintained in culture in flasks of 25 mL containing 5 mL of RPMI medium. Tested products (extract and pentamidine as standard drug) were diluted at first in DMSO, then in RPMI to reduce the final concentration in solvent under 2 % (v/v) during the tests, such a percentage of DMSO not affecting the viability of the parasites. In each of 96 wells of the microplate, 195 µL of culture medium containing 2.105 promastigotes were put down. The microplates were then placed in a steam room at 27 °C for 1 hour. After this incubation, 5 µL of tested substances were added in triplicate for 4 hours. The evaluation of leihmanicide activity was realized by direct reading in optical microscope and by quantitative colorimetric dosage by MTT. The starting concentration of screened compounds was 100 µg/mL. Pentamidine was used as reference compounds [12].

2.2.4.3. Anti-fungal activity

Strains of *Aspergillus fumigatus* and *Candida albicans* were provided by Pasteur Institute. *A. fumigatus* conidia were collected from a culture of 7 days in malt agar at 35 °C, then cleansed by centrifugation in 1500 rpm and by washing with saline buffer containing 0.01 % of Tween 20®. For yeasts, cultures of 48 hours of *C. albicans* on Sabouraud Dextrose Agar were used. Both strains were studied in MOPS medium and the inhibition of *fungi* culture was measured by the method of dilution in broth. Amphotericin B was used as reference compound [12].

2.2.4.4. Anti-helmintic activity

Caenorhabditis elegans strain assessed for anti-helminthic activity was provided by Pasteur Institute. The experience was led according to method described by [14]. The tests were realized in microplate of 24 wells, each well receiving 10-15 parasites in 0.5 mL. After 7 to 20 days of incubation with tested products (extracts, isolated compounds and Mebendazole as standard drug), the optical density of each well was measured, by reading in binocular microscope. Thus, the percentage of motionless worms, considered as "dead" were determined [12].

3. Result and discussion

3.1. Result

3.1.1. Chemical study

Compound 1 was isolated as a colourless amorphous powder. Its ESIMS showed a peak at *m/z* 344.0 [M]+ consistent with the molecular formula $C_{20}H_{26}NO_4$.

1H and 13C NMR spectra (Table 1) and UV of 1 resembled those of (+)-tembetarine, suggesting that this molecule is a 6, 7, 12, 13-tetrasubstituted tetrahydrobenzylisoquinoline alkaloid [15]. Measuring its various 2D-NMR spectra provided further support for the structure of compound 1. All methyl, methylene and methine protons and carbons were assigned

from analysis of the HSQC, 1H-1H COSY and HMBC spectra. The presence of two singlet protons (δH 6.83 and δH 5.96) (Table 1) and an HMBC correlation from one of the protons (δH 5.96) to C-1 (δC 74.0), and from another proton (δH 6.83) to C-4 (δC 24.4) revealed that they were bound to C-5 and C-8, respectively. An HMBC correlation was also observed between H-9α (δH 2.84 ppm) and two aromatic carbons (δC 117.8 and δC 122.4) (Fig. 1). The presence of three aromatic protons combined with C-11, C-14 and C-15 was noted having an ABX spin system. Therefore, two hydroxyl and two methoxyl groups are substituents at [C-6, C-12], [C-7, C-13]. From the HMBC and NOESY experiments, signals at δH 3.86 and δH 3.85 were assigned to two methoxyl groups on C-7 and C-13, respectively; hence two hydroxyl groups must bind to C-6 and C-12. Accordingly, the structure of compound 1 was elucidated as that of (-)-xylopinidine (1) (Fig. 1). This was confirmed by its optical rotation which was negative, indicating that it has either a very optical rotation. Its physical and spectral data are consistent to those reported by literature [16]. To our knowledge, it was isolated from *Xylopia parviflora* and *Monodora crispata*.

Compound 2 was obtained as an amorphous brown powder. Its UV visible spectrum showed maximum absorption bands at λmax 221.4 nm, 268.5 nm and 301.6 nm. HR-ESI-MS showed the pseudo-molecular ion fragment [M]+ at *m/z* 328.1946 corresponding to the molecular formula $C_{20}H_{26}NO_3$ (calc. 328.1947 mDa = 0.1). The 13C NMR spectrum of 2 (Table 2) showed characteristic signals at δC 113.3 ppm (C-5), 112.4 ppm (C-8), 38.2 ppm (C-9) and 74.3 ppm (C-1) corresponding to a 6, 7, 13-trisubstituted benzyl tetrahydroisoquinoline alkaloid [17]. The significant differences between compounds 1 and 2 were observed on the ring C. The 1H NMR spectrum of 2 (Table 2) exhibited characteristic signals of four aromatic protons at δH 6.75 ppm (H-11, H-15) and 6.87 ppm (H-12, H-14). The HMBC correlations confirmed the position of two methoxyl groups on the aromatic ring B and one hydroxyl group on the aromatic ring C (Fig. 2). In consequence, compound 2 was identified as *N,N*- dimethyl benzyl tetrahydroisoquinoline [16, 17]. The absolute configuration of the asymmetric carbon C-1 was also determined according to its [α]D value ([α]D21(O)= -32.0) to be *R* form. The compound 2 was identified as (-)-*N*-methylarmeparvine (Fig. 2). This compound had previously been isolated from *Monodora brevipes* and *M. crispata* [18, 19].

3.1.2. Biological activity

Compounds 1, 2 and extracts were evaluated for their antiprotozoal, anti-fungal and anti-helmintic properties. The results are discussed below:

- **Antiprotozoal, anti-fungal and anti-helmintic potentials**

The cold and hot extracts of these compounds showed Trypanocidal activity with LC_{100} of 12.5 mg/mL and 25.0 mg/mL respectively. The cold extracts showed lesser activity compared to the hot extracts (Table 3). The two extracts have no anti-fungal and anti-helmintic activities (Table 4).

Table 3: Antiprotozoal, anti-fungal and anti-helmintic activities of Extracts (Cold and hot extract of *M. tenuifolia*)

	T. brucei brucei (LC_{100})	*L. Donovani* (EC_{50})	*C. elegans* (EC_{50})	*C. albicans* (EC_{50})	*A. fumigatus* (EC_{50})
Cold Extract	12.5	50.00	>100	>100	>100
Hot Extract	25.0	>50.00	>100	>100	>100
Melarsoprol	0.4				
Pentamidine		7.7			
Mebendazole			2.6		
Amphotericin B				0.064	

It is seen that compounds **1** and **2** (isoquinoline) have no antiprotozoal, anti-fungal and anti-helmintic activities.

Table 4: Antiprotozoal, anti-fungal and anti-helmintic activities of compounds isolated from *M. tenuifolia*

	T. brucei brucei (LC_{100})	*L. Donovani* (EC_{50})	*C. Elegans* (EC_{50})	*C. albicans* (EC_{50})	*A. fumigatus* (EC_{50})
(-)-xylopinidine	> 363.37	> 363.37	> 363.37	> 363.37	> 363.37
(-)-*N*-methylarmeparvine	762.20	> 762.20	762.20	762.20	762.20
Melarsoprol	0.4				
Pentamidine		7.7			
Mebendazole			2.6		
Amphotericin B				0.064	

3.2. Discussion

Two isoquinolines were isolated from the leaves of *M. tenuifolia.*. Compound 2 was identified only in *Monodora* genus. It was only obtained from three *Monodora* species (*M. crispata*, *M. brevipes* and *M. Tenuifolia*). Compound 1 has been described in *Xylopia parviflora* and *Monodora crispata* (Annonaceae) [17]. However, this family rarely yields *N,N*-dimethyl benzyl tetrahydroisoquinoline, while frequently accumulating secondary and tertiary amino-counterparts.

In this study, our aim was also to assess potential Leishmanicidal, Trypanocidal, anti-fungal and anti-helmintic activities of extracts and compounds isolated from *Monodora tenuifolia* leaves, an Ivorian folk herbal plant used for the treatment of dysentery, diarrhoea, dermatitis and toothache the local population. Our results reveal that compounds 1 and 2 did not show any effect on *Leishmania donovani*, *Trypanosoma brucei brucei*, *Candida albicans*, *Aspergillus fumigatus* and *Caenorhabditis elegans* being superior to 363 µM. In addition, the compounds do not show any interesting anti-parasitic, anti-fungal and anti-helminths activities based on the different biological tests used These results are in complete agreement with data obtained from previous literature [12-14, 19]. Indeed, isoquinoline alkaloids are known for their moderate sedative property and their depressant effect on the cardiac system. As future work, compounds 1 and 2 isolated from *M. tenuifolia* could be tested for the inhibition of monoamine oxidase and DOPA-decarboxylase to assess their activity on the central nervous system or on the cardiovascular system.

The cold and hot extracts of the studied compounds have an effect on *L. donovani* (LC100: 12.5 mg/L, cold methanol extract) and (LC100: 25 mg/L, hot methanol extract). We also noticed that the activities of the extracts were maintained, even though reduced by continuous heating.

4. Conclusion

The phytochemical investigation of the extracts of the leaves from *M. tenuifolia* was successfully perfomed, leading to the isolation and identification of two *N*,*N*-dimethyl benzyl tetrahydroisoquinoline compounds; 1 and 2. The complete structures were proposed based on the spectroscopic studies; 1H and 13C NMR, COSY, HSQC, HMBC, UV visible, IR and spectrometric (ESI-MS) data. The *N*,*N*-dimethyl benzyl tetrahydroisoquinoline derivatives were identified as (-)-xylopinidine (1) and (-)-*N*-methylarmeparvine (2). The proposed structures are in agreement with those previously reported [12-14]. Furthermore, our study did not reveal any anti-protozoal (anti-parasitic), anti-fungal and anti-helmintic activities of the isolated isoquinolines, but the cold extracts showed activity against *Leishmania donovani* and *Trypanosoma brucei brucei*. This can be classified as Trypanocidal properties or activities [12-14, 19].

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