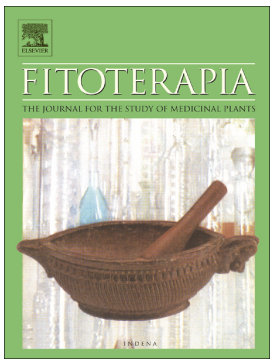

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Antiplasmodial and antileishmanial inhibitory activity of triterpenes and steroidal alkaloid from the leaves of *Funtumia elastica* (Preuss) Stapf (Apocynaceae).

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ABSTRACT

The phytochemical study of leaves of *Funtumia elastica* led to the isolation of three undescribed ursane derivatives, funtumic acids A, B and C (**1**–**3**), as well as one steroidal alkaloid, elasticine (**4**) and five other known compounds (**5**–**9**). Their structures were elucidated on the basis of NMR, MS, IR, UV spectroscopic data as well as by comparison with the literature. The compound 5-hydroxypyridine-3-carboxamide (**9**) was isolated for the first time from the Apocynaceae family. All the isolated compounds were evaluated for their antiparasitic effects against 3D7 and Dd2 strains of *Plasmodium falciparum* and promastigotes of *Leishmania donovani* (MHOM/SD/62/1S). Compounds **1**–**4** possessed good *in vitro* antimalarial activities against CQR Dd2 with IC_{50} values ranging from 4.68 to 5.36 µg/mL and moderate on CQS 3D7. Only compounds **1** and **2** showed leishmanicidal activities with IC_{50} values ranging between 10.49 and 13.21 µg/mL. In addition, crude extract exhibited potent antiplasmodial (IC_{50} 0.91 and 3.12 µg/mL) and antileishmanial (IC_{50} 3.32 µg/mL) activities, thus demonstrating their potential synergistic action.

Keywords: *Funtumia elastic*a; Apocynaceae; Ursane triterpenes; Steroidal alkaloids; Antiplasmodial activity; Antileishmanial activity.

1. Introduction

Leishmaniasis is among the top five of vector-bone diseases identified in the Global Health Estimates 2016 summary table of the World Health Organization (WHO). It is one of 20 neglected tropical diseases that infect one billion people in low socioeconomic populations in 149 countries [1]. The disease is characterized by four forms; visceral leishmaniasis, post-kala-azar dermal leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis [2]. Existing chemotherapeutics are not sufficient because of their extensive toxicity, their lack of efficacy, their expensive cost (amphotericin B) and the development of drug resistance [2,3]. Therefore, combination therapies are used as an alternative option to improve the efficacy of antileishmanial therapies which may also shorten the duration of treatment [4]. To further address these challenges, this work envisages to isolate promising compounds from selected plant species to investigate their potential as antileishmanial agents, based on prior reports demonstrating natural product as a strategy to overcome leishmaniasis [5]. Another source of tropical parasitic disease is Malaria. It is the major tropical disease due to parasites, responsible for significant morbidity and mortality in the world. It affects nearly 40% of the world population and is responsible for 1–2 million deaths each year [6]. Human malaria is endemic to 90 countries and is caused by protozoan parasites of the genus *Plasmodium*, mainly *Plasmodium falciparum*. Development of resistance to mainstay drugs like chloroquine, and controlled use of new artemisinin analogs have created an urgent need to discover new antimalarial agents.

In this context, investigating natural products, which are known to be used in folk medicine, appears as a suitable approach. Plants of the Apocynaceae family are used in Cameroon to treat parasitic and infection diseases [7,8]. The genus *Funtumia* is the poorest group of plants among the Apocynaceae family, with three taxons of *Funtumia* (*F. latifolia*, *F. africana* and *F. elastica*), mostly in central and west parts of Africa [9]. Species of this genus are not being used as ornamental but for commercial purposes [10]. They are also known to possess several applications in folk medicine against bacterial diseases, malaria, parasitic infections, asthma, antiseptic, haemorroids, venereal diseases, vermifuge and as analgesic [11-13]. In Cameroon, *Funtumia elastica* (Preuss) Stapf is traditionally used in the South and East regions by Baka pygmies to treat wounds, lumbago, snake bite and malaria [14]. The chemistry of plant species belonging to the genus *Funtumia* is not well documented when compared to that of the renowned *Rauvolfia* and *Tabernaemontana* genera (~76 and 110 species respectively) [15,16]. However, previous studies on *Funtumia* species report the isolation of various classes of secondary metabolites including alkaloids, sterols, acid

derivative (ascorbic acid) and an ursane triterpene (methyl ursolate) with potent, antiplasmodial activities [11, 17-19].

Despite the works carried out on this species [11], to be best of our knowledge, no investigation has focused on the evaluation of the antileishmanial properties of *F. elastica*. In our continuing search for new antileishmanial and antiplasmodial agents from Cameroonian medicinal plants, the MeOH extract of *F. elastica*, was found to exhibit interesting antileishmanial and antiplasmodial activities during preliminary screening *in vitro* against *L. donovani* 1S (MHOM/SD/62/1S) promastigotes and some strains of *P. falciparum* 3D7 and Dd2. We report herein the isolation and structure elucidation of four undescribed secondary metabolites as well as their antileishmanial, antiplasmodial and cytotoxicity activity.

2. Experimental

2.1. General Experimental Procedure

Optical rotations were measured on JASCO P-2100 polarimeter. UV and visible spectra were recorded in MeOH at 25 ^{o}C using a Kontron Uvikon spectrophotometer. The IR spectra were measured on a PerkinElmer 1750 FTIR spectrometer. 1D NMR spectra (^{1}H and ^{13}C NMR) and 2D NMR spectra (HSQC, HMBC, ^{1}H-^{1}H COSY and NOESY) were recorded in $CDCl_3$ and/or MeOH-d_4 on a Bruker 500 MHz NMR Avance II spectrometer equipped with a cryoprobe, with TMS as an internal reference. Melting points of the isolated compounds were determined using an Electrothermal IA9000 Series digital melting point apparatus (Bibby scientific, Great Britain). Chemical shifts (δ) were expressed in ppm with reference to TMS and coupling constants (*J*) were given in Hz. MS data were measured on SHIMADZU LCMS-IT-TOF spectrometer (Kyoto, Japan). Analytical TLC was performed on precoated SiO_2 gel 60 F_{254} (Merck. 1.05735, Hohenbrunn, Germany) plates. After development (*n*-hexane/EtOAc at the different polarity), the dried plates were examined under short-wave (254 nm) or long-wave (366 nm) UV light and also, to check the purity of compounds, Liebermann-Burchard's test and Dragendorff 's spray reagent was used for the staining of compounds on TLC. Silica gel 60, 0.036-0.071 mm (215-400 mesh) and Sephadex LH-20 were used for CC with step gradients of *n*-hexane-EtOAc and pure MeOH as eluents respectively.

2.2. Plant Material

Whole plant of *F. elastica* (Preuss) Stapf, was collected from Touessong near Yaounde III Sub-Division, Yaounde in October 2019. The plant was identified by Mr. Tsambang Nole, Senior Scientific Officer, at the Institute of Medical Research and Medicinal

Plants Studies, Yaounde, Cameroon, where a voucher specimen (N°. 59012 HNC) was deposited.

2.3. Extraction and Isolation

The air-dried leaves (2.574 Kg) of *F. elastica* were powdered and extracted with MeOH (6 L) at room temperature for 72 h to obtain a crude extract (501 g, 19% yield) after filtration and evaporation in reduced pressure at 40°C. Part of this crude extract (250 g) was dissolved in cold distilled water (350 mL) and successively partitioned with n-hexane, AcOEt and BuOH respectively. The AcOEt extract (42.0 g) was subjected to a silica gel column (240 – 300 mesh) and eluted with n-Hex/AcOEt system (100:0 to 80:20, v/v) to afford seven major fractions: A (10 g), B (1.5 g), C (4.8 g), D (2.9 g), E (1.8 g), F (5.0 g) and G (13.0 g). Fraction B (1.5 g) was submitted to a silica gel CC over silica gel eluted with a gradient system n-Hex/AcOEt (30:70 to 100:0, v/v) to afford three subfractions (B1 to B3). Subfraction B3 (600 mg) was further purified over silica gel CC using a gradient of n-Hex/AcOEt system (75:25 to 90:10, v/v) to yield compounds **1** (18 mg), **2** (16 mg) and **3** (8.9 mg). Subfraction B2 (450 mg) was purified over silica gel CC with n-Hex/AcOEt (90:10 to 40:60, v/v) to afford compound **8** (22 mg). Fraction D (2.9 g) was subjected to silica gel CC and eluted with n-Hex/EtOAc (100:0 to 0:100 v/v) to obtain five major subfractions (D1– D5). Subfraction D5 (258 mg) was further purified over a Sephadex LH-20 CC with MeOH to provide compounds **6** (10.3 mg) and **7** (12.3 mg).

Fraction F (5.0 g) was purified on a silica gel CC using a gradient system of n-Hex/EtOAc (100:0 to 40:60 v/v) to obtain 4 sub-fractions (F1– F4). Subfraction F2 (400 mg) was further purified over a silica gel CC using the gradient system n-Hex/EtOAc (30:70 to 5:95 v/v) to produce compounds **4** (9.9 mg) and **5** (16 mg). Subfraction F3 (1100 mg) was further chromatographed on a Sephadex LH-20 CC using MeOH as mobile phase to afford compound **9** (13 mg).

2.3.1. 3*β-hydroxyurs -20(21)-en-29-oic Acid; **(1)***

White amorphous powder; $[\alpha_D^{21}]$ = +14.9 ($CHCl_3$ /MeOH, *c* 0.1); m.p. 236–238°C; UV (MeOH): λ_{max} in nm: 205; IR (KBr): 3219, 2978, 2881, 1652, 1603, 1494, 1386, 1204, 1175, 1044, 778 cm^{-1}. ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-ESI-MS: *m/z* 455.3504 ($[M–H]^-$, $C_{30}H_{47}O_3$, calcd. 455.3505).

2.3.2. 2*α*, 3*β -dihydroxyurs-20(21)-en-29-oic Acid; **(2)***

White amorphous powder; $[\alpha_D^{21}]$ = + 24.3 ($CHCl_3$ /MeOH, *c* 0.1); 239–241°C; UV (MeOH): λ_{max} in nm: 208; IR (KBr): 3200, 2979, 2881, 1653, 1604, 1573, 1451, 1386, 1278,

1175, 1091, 973, 565 cm^{-1}. ^{1}H- and ^{13}C-NMR: Tables 1 and 2. HR-ESI-MS: 471.3467 ($[M–H]^{-}$, calcd for $C_{30}H_{7}O_{4}$, 471.3470).

*2.3.3. 3-O-β-D-[6'-O-acetylglucopyranosyl]-3β-hydroxyurs-20(21)-en-29-oic Acid; (**3**)*

White amorphous powder; $[\alpha_D^{21}]$ = + 19.9 ($CHCl_3$ /MeOH, *c* 0.1); 279–281°C; UV (MeOH): λ_{max} in nm: 208; IR (KBr): 3368, 2956, 2866, 1537, 1458, 1368, 1217, 1203, 1164, 1104, 1053, 1021 cm^{-1}. ^{1}H and ^{13}C NMR Tables 1 and 2. HR-ESI-MS: 659.4013 ($[M–H]^{-}$, calcd for $C_{38}H_{59}O_{9}$, 659.4014)

*2.3.4. 1α, 11α, 17α-trihydroxy-3β-(N-benzamido)-5, 6-dihydroantidysentericine (**4**)*

White amorphous solid; $[\alpha_D^{21}]$ = + 168 ($CHCl_3$ /MeOH, *c* 0.1); 123–125°C; UV (MeOH): λ_{max} in nm: 215, 270; IR (KBr); 3616, 3412, 1728, 1629, 1514, 1468, 1256, 1218, 1175 cm^{-1}. ^{1}H and ^{13}C NMR Tables 1 and 2. HR-ESI-MS: 481.2647 ($[M–H]^{-}$, calcd for $C_{38}H_{59}O_{9}$, 481.2646)

*2.3.5. 5-hydroxypyridine-3-carboxamide (**9**)*

White amorphous powder, m.p. 131–133°C, UV (MeOH): λ_{max} in nm 260; IR (KBr) 3400, 3200, 2950, 1690, 1560, 1455, 1195, 1035 cm^{-1}. ^{1}H NMR: 8.86 (1H, brs, OH), 8.70 (1H, brs,), 7.56 (1H, brs H-6), 7.20 (1H, brs, H-4), 5.42 (2H, brs, NH_2). ^{13}C NMR: 149.4 (C-2), 123.5 (C-3), 123.9 (C-4), 149.9 (C-5), 135.7 (C-6), 166.9 (C-7). HRESIMS *m=z* 139.848 ($[M+H]^{+}$, calcd for $C_6H_6O_2N_2$, 139.251).

2.4. SYBR green I-based fluorescence assay

The Chloroquine-sensitive (3D7-(MRA-102)) and Chloroquine-resistant (Dd2) of *Plasmodium falciparum* strains was cultured in fresh O^{+} human red blood cells at 4% haematocrit in complete RPMI 1640 medium [500 mL RPMI 1640 (Gibco, UK) supplemented with 25 mM HEPES (Gibco, UK), 0.50% Albumax I (Gibco, USA), 1X hypoxanthine (Gibco, USA) and 50 mg/mL gentamicin (Gibco, China)] and incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced with fresh complete medium daily to propagate the culture. Giemsa-stained thin blood smears were examined microscopically under immersion oil to monitor cell-cycle transition and parasitaemia evolution.

Before each experiment, synchronized ring stage parasite was obtained by 5% sorbitol (w/v) treatment in respect to the experiments [20]. It is important to note that, the use of synchronized cultures over mixed stage cultures can enable the test molecules to interact with all the three stages (ring, trophozoite and schizont) of the 48 hrs long life cycle of *P. falciparum* in culture. Moreover, starting the experiment with synchronized ring stage culture

provides the distinct advantage of observing growth inhibitory effects without a rise in parasitemia during the ring-trophozoite-schizont transitions. Drug sensitivity assay was carried on in 96-well microtitration plates using SYBR green I based fluorescence assay [21]**.** This assay is specifically based on the ability of SYBR green to give strong fluorescence only in the presence of parasite DNA during cell proliferation. The absence of nucleus in human red blood cells where the malarial parasite proliferates allows the use of SYBR green for the specific monitoring of the growth of malarial parasite. Sorbitol-synchronized ring stage parasites (haematocrit: 1%, parasitaemia: 2%, 90 µl) under normal culture conditions were incubated in the presence of pre-diluted extracts, fractions, isolated compounds and reference drug (10µL) followed by the incubation at 37°C for 72h. After incubation, 100 µL of SYBR Green I buffer [6 µL of 10,000 × SYBR Green I (Invitrogen) + 600µL of Red Blood Cells lysis buffer {Tris (25 mM; pH 7.5)} + 360µL of EDTA (7.5 n M) + 19,2µL of parasites lysis solution {saponin (0.012%; wt/vol) } and 28,8µL of Triton X-100 (0.08%; vol/vol)}] were added to each well, mixed twice with multi-channel pipette and incubated in dark at 37°C for 1h. Fluorescence was measured using a TECAN M 200 Microplate reader with excitation and emission at 485 and 538 nm, respectively. The fluorescence counts were plotted against the logarithme of sample concentration and the 50% inhibitory concentration (IC_{50}) was determined by analysis of dose–response curves using GraphPad Prism 5. Experiments were done in duplicate.

2.5. Antileishmanial assay

The *in vitro* anti-leishmanial activities were performed on both promastigotes and amastigotes of *L. donovani* 1S (MHOM/SD/62/1S) strain. The strain was graciously offered by Bei Resources and was maintained in continuous culture at the Laboratory for Phytobiochemistry and Medicinal Plant Studies, Antimicrobial and Biocontrol Agents Unit, at the department of biochemistry of the University of Yaoundé I in Cameroon. The evaluation of the inhibitory potential of extracts, fractions and isolated compounds on parasite growth has been done in the same laboratory.

The method used is based on the reduction of rezazurin (blue color) to rezorufin (pink color) by mitochondrial dehydrogenases present in leishmania parasite. The complex formed displays absorption at 570 nm by direct measurement of metabolic activity in viable parasites. The quantity of rezorufin produced is directly proportional to the quantity of viable parasites.

The promastigotes forms of *L. donovani* cultivated as mentioned above were taken in exponential growth phase by centrifugation at 2500 Rotation per Minute (RPM) for 10 minutes. The number of parasites was determined using the Neubauer Cell.

The antileishmanial assay was done in 96 well plates. For this purpose, $4x10^5$ promastigotes/mL/well were seeded in a 96 well microtiter plate and treated with 5-fold diluted concentrations of extracts (100, 20, 4, 0.8 and 0.16 µg/mL) for 72 h at 26°C. The viability rate of promastigotes positively correlated with the amount of pink resorufin that was produced through the reduction of blue resazurin by the dehydrogenase enzymes in the inner mitochondrial membrane of the living parasites. Briefly, promastigotes from a logarithmic phase culture (4×10^5 cells/mL; 90 µL) were seeded in 96-well microtiter plates and were treated with 10 µL of inhibitors at different triplicate concentrations ranging from 100 to 0.16 µg/mL for extracts and fractions and 50–0.08µg/mL for compounds for the final volume per well of 100 µL. Plates were incubated for 28 hours at 26°C, followed by the addition of 1mg/mL of resazurin. The sterility and negative control were M-199 10% without parasites and complete M-199 medium with parasites respectively. A positive control was amphotericin B (10–0.016µg/mL). After an additional incubation for 4 hours, plates were then read on a Magelan Infinite M200 fluorescence multi-well plate reader (Tecan) at excitation and emission wave lengths of 530 and 590 nm respectively.

For each sample, growth percentages inhibition were calculated on Microsoft Excel Software using the formula:

$$I(\%) = \frac{(\text{absorbance of untreated cells} - \text{absorbance of treated cells})}{\text{absorbance of untreated cells}} \times 100$$

Finally, dose–response curves were constructed to determine the 50% inhibitory concentration (IC_{50}) using the GraphPad Prism-version 5.0 software

2.6. *Cell Culture*

RAW 264.7 cells were procured from the RIKEN BioResource Centre Cell Bank in Japan. They were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin under 5% CO_2 and humidified atmosphere at 37°C.

2.7. Cytotoxicity activity

To determine selectivity indices of active compounds, a toxicological assessment was carried out against RAW cell lines using the resazurin assay [22]. From sub-confluent cultures in 75 cm^2 culture flasks, they were trypsinized, counted, suspended in respective medium and then seeded into triplicate wells of a 96-well plate (100 µL per well) at concentrations of 1.10^5 cells per mL and incubated. Cells were allowed to attach overnight and then treated in triplicate with 10 µL per well of 5-fold of serially diluted individual compound (3.2-2000

μg/mL) in the culture medium and incubated for 48 h. After incubation, 10 μL of 2.5 mM of resazurin solution were added to each well and then incubated for 4h at 37°C. Fluorescence signal was measured using the microplate reader at the excitation and emission wavelengths of 530 nm and 590 nm respectively. Growth control (0.1 % DMSO-100 % growth) and positive control wells (podophyllotoxin at 20 μM) were included in the experiment plates. Experiments were conducted in triplicate. The percent growth inhibition was calculated from relative absorbances to the negative control, and the concentration of extract that inhibited 50% cell (CC_{50} values) were determined. The selectivity index (SI) ratio (CC_{50} for macrophages/IC_{50} for amastigotes) was used to compare the toxicity of the compounds against the macrophages and their activity against the parasites.

2.7. *Statistical analysis*

Results were expressed as mean ± standard deviation (S.D). The IC_{50} and CC_{50} values were calculated fitting the data as a non-linear regression using a dose-response inhibitory model, in the GraphPad Prism 5.0 program.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as an amorphous powder, giving a positive Liebermann-Burchard's reaction [23]. Its molecular formula was determined as $C_{30}H_{48}O_3$, based on the HR–ESI–MS *quasi* molecular ion peaks at *m/z*= 455.3504 ($[M–H]^-$, $C_{30}H_{47}O_3$, calcd. 455.3505) requiring 7 degrees of unsaturation. The IR absorption bands at 3219, 1652 and 1603 cm^{-1} suggested the presence of hydroxyl(s) (OH), a carboxylic functional group (COOH) and an olefinic double bond (C=C) respectively. The ^{1}H-NMR spectrum of **1** (Table 1) displayed signals of six methyl groups at δ_H 0.76, 0.83, 0.87, 0.94, 0.96 and 0.97 (3H each, s, Me–24, Me–26, Me–28, Me–27, Me–25 and Me–23 respectively), one vinylic methyl at δ_H 1.12 (3H, s, Me–30), an olefinic proton atom at δ_H 5.24 (1H, m, H–21), one carbinolic proton at δ_H 3.14 (1H, dd, *J* = 8.2; 4.8 Hz, H–3) [24–26]. The typical ursanyl H$_\beta$-19 proton at δ_H 2.18 (1H, d, *J* = 12.0 Hz, H–19) was associated with C–19β proton interacting with C–18α and C–13β methine protons in an ursane-type carbon skeleton [27]. The ^{13}C–NMR spectra of compound **1** revealed 30 carbon signals which were sorted by DEPT ^{13}C–NMR (Table 2) as seven methyl groups (δ_C 14.6, 14.9, 16.2, 16.4, 20.2, 22.6 and 27.4), one oxygenated methine carbon (δ_C 78.3), two olefinic carbons (δ_C 125.7 and 138.4) and one carboxylic carbon (δ_C 180.2). By analyzing the above signals, especially the set of particular methine carbons [δ_C

39.0, 39.1, 52.9 (C–13, C–18 and C–19 respectively)] and olefinic methine [δ_H 5.24 (1H, m, H–21)], the spectroscopic data of **1** were comparable with other ones previously reported in the literature [28]. More specifically, NMR spectroscopic features of **1** were very closed to those of a related known compound pseudotaraxasterol [27,29], which suggest both compounds basically exhibit a similar skeleton. All the positions of the substituents were deduced using the COSY and HMBC techniques (Fig. 2). The HMBC correlations between H–19 (δ_H 2.18) and C–13, C–17, C–18, C–20, C–21, C–29 and CH_3–30, H–21 (δ_H 5.24) and C–19, C–20, C–22 and CH_3–30, CH_3–30 (δ_H 1.12) and C–19, C–20, C–21, of CH_3–28 (δ_H 0.87) to C–18 allowed location of the double bond at C(20)–C(21) and to confirm the occurrence of the carboxyl group at C–29 (Fig. 2). In addition, *gem* methyl groups (CH_3–23 and CH_3–24) are noticeable through correlations between CH_3–23/CH_3–24 (δ_H 0.97/0.76) to C–3 and C–5 [30]. Based on the above evidence, the structure of **1** was suggested to be an urs-20(21)-en-29-oic acid type triterpene with one hydroxyl group. Relative configurations of compound **1** could be established based on coupling constants and literature data [27,29]. The proton H–3 coupling constant value (4.8 Hz) allowed the *β* and equatorial assignments [26]. Large coupling constants of H–18 and H–19 (~12.0 Hz, *J*ax−ax) confirmed the diaxial orientation of these protons and placement is the same for H–13 and H–18 (9.2 Hz, *J*ax−ax) (Table 1). Additionally, the downfield shifts of C–3 (δ_C 78.3), C–5 (δ_C 55.3), C–13 (δ_C 39.0) and C–18 (δ_C 39.0) respectively, are in accordance with reported values [29–33]. Those observations confirmed that the stereochemistry of **1** should be consistent with analogues reported from other genera [27,33]. Therefore, compound **1** was a new *pseudo*taraxasterol-type triterpenoid, determined to be (3*β*, 18*α*, 19*α*)-3*β*-hydroxyurs-20(21)-en-29-oic acid and named funtumic acid A (Fig. 1).

Compound **2** was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{30}H_{48}O_4$ from the molecular ion peak at *m/z* 471.3467 ($[M–H]^-$, calcd for $C_{30}H_{47}O_4$, 471.3470) from the HR-ESI-MS, indicating seven degrees of unsaturation. The IR spectrum displayed strong absorption bands at 3200 and 1653 cm^{-1} indicative of hydroxyl and carboxylic acid functional groups. A careful analysis of the ^{13}C–NMR data of **2** (Table 2) suggested that they were very closed to those of compound **1**, except for δ_C 68.2 ppm which evolved from the occurrence of one additional hydroxyl group, hence, a 16 amu increase in molecular weight of **2** compared to **1**. This was confirmed by the ^{1}H–^{1}H COSY correlations between H–2 (δ_H 3.66) and H–3 [δ_H 2.99 (1H, d, *J* = 9.5 Hz)] and H–2 and H–1a/b (δ_H 2.03/0.96); HMBCs between H–2 (δ_H 3.66) with C–3, C–4 and C–10; between H–3 (δ_H 2.99) with C–2, C–4 and C–5 and between H–19 (δ_H 3.66) with C–18, C–20, C–29 (Fig. 2).

Additionally, the hydroxyl group at C–2 was axial (α) according to an axial–axial coupling between H–2β and H–3 (9.5 Hz, *J*ax−ax). Therefore, compound **2** was found as 2α, 3β-dihydroxyurs-20(21)-en-29-oic acid, trivially named funtumic acid B.

Compound **3** was also obtained as a white amorphous powder, and its molecular formula was deduced as $C_{38}H_{60}O_9$ based on its negative-ion HRESIMS at *m/z* 659.4013 ([M–H]$^-$, calcd for $C_{38}H_{59}O_9$, 659.4014). The ^{1}H– and ^{13}C–NMR data of **3** were in good agreement with those of **1** except for the presence of the signals of a sugar moiety in **3,** which showed resonances for a glucose [δ_H 4.39 (1H, d, *J* = 8.0 Hz, H–1′), 3.19–3.54 (4H, m, H–2′, 3′, 4′, 5′), 4.23 (1H, dd, *J* = 12.0, 5.6 Hz, H–6′a) and 4.35 (1H, dd, *J* = 12.0, 2.4 Hz, H–6′b); δ_C 101.1 (C–1′), 73.5 (C–2′), 76.6 (C–3′), 70.2 (C–4′), 79.2 (C–5′) and 63.4 (C–6′)] (Table 1). The configuration of the glucose moiety was determined to be β according to its coupling constant (*J* = 8.0 Hz). In addition, the presence of a signal of an acetyl group at δ_H 2.07 (3H, s, H–2″)/ δ_C 19.8 (C–2″) was recognized, suggesting an acetylation of glucose. This was strengthened by the HMBC (Fig.2) correlations between H–6′a (δ_H 4.23)/ H–6′b (δ_H 4.35) with C–1″ (δ_C 171.7). Else, location of the glucopyranosyl moiety at C–3 was achieved through correlations between H–3 (δ_H 3.17) to C–1′ and with H-1′ (δ_H 4.39) to C–3 from the same HMBC spectrum, suggesting the connection through C–3 of **1** to C–1′ of the glucopyranosyl unit. Therefore, compound **3** was characterized as 3-*O*-β-D-[6′-*O*-acetylglucopyranosyl]-3β-hydroxyurs-20(21)-en-29-oic acid, trivially named funtumic acid C.

Compound **4** was isolated as white amorphous powder and reacted positively to Dragendorff's reagent. It had the molecular formula, $C_{28}H_{38}N_2O_5$, as determined by a *quasi*molecular ion peak from HR-ESI-MS spectrum at 481.2647 ([M–H]$^-$, calcd for $C_{28}H_{37}N_2O_5$, 481.2646) with eleven degrees of unsaturation. Four unsaturations were accounted for a pentacyclic structure related to conenine [34], five to a benzoyl moiety and two for a five membered cyclic amide. These findings were confirmed by the absorption bands at 1728 and 1639 cm^{-1} in the IR spectrum [35]. Additionally, a secondary amino stretching absorption at 3412 cm^{-1} was also observed. The investigation of ^{1}H–NMR spectrum (Table 1) revealed, apart from the presence of signals of benzene protons at δ_H 7.90 (2H, dd, *J* = 8.0, 2.0 Hz, H–3′/7′), 7.44 (1H, dd, *J* = 8.0, 2.0 Hz, H–6′), and 7.39 (2H, dd, *J* = 8.0, 7.5 Hz, H–4′/6′), one tertiary methyl at δ_H 1.15 (3H, s, Me–19) and one secondary methyl at δ_H 1.38 (3H, d, *J* = 6.5 Hz, Me–21). In addition, we noticed the presence of three oxymethine protons at δ_H 4.27 (1H, m, H–11), 3.97 (1H, m, H–1), and 3.94 (1H m, H–20). Moreover, methylene protons between 1.22 – 2.67 could characterized a steroidal skeleton [11,36,37]. The ^{13}C–NMR and DEPT spectra of **4** displayed 28 carbon signals including two

methyl (δ_C 18.2 and 19.1), seven methylene (δ_C 22.9, 28.9, 29.2, 33.7, 33.9, 37.7 and 42.7), eight methine (δ_C 35.2, 48.0, 49.0, 51.8, 52.6, 66.6, 67.8 and 70.2), three quaternary (δ_C 46.3, 57.9 and 76.5) and two amide carboxyl groups (δ_C 174.5 and 179.8). Additionally, the carbon signals at δ_C 127.5, 128.9, 130.5 and 136.6 emphasized the occurrence of an aromatic ring, whereas the aforementioned signal at δ_C 174.5 is was consistent with the carboxylic carbon of the benzoyl moiety. (Table 1) These signals were those expected for the conanine derivative especially of antidysentericine [34,37] except for the occurrence of a benzoyl moiety and oxymethines and absence of a double bond in B ring of **4** compared to analogues previously cited. The correlations between Me–21/H–20, H–6/H–7/H–8 and H–11/H–12 were assigned through the ^{1}H–^{1}H COSY spectrum. The placement of the amide carboxyl group at C–18 was fully supported by HMBC correlations between Me–21 with C–17 and C–20, H–12a/b with C–11, C–17 and C–18 and H–14 with C–17 and C–18. In addition, the attachment of hydroxyl groups at C–1, C–11 and C–17 was supported by correlations between H–19 (δ_H 1.15) with C–1, H–5 (δ_H 1.12) with C–1, H–12a/b (δ_H 2.67/2.34) with C–11 and H–14/H–15/H–16 and Me–21 with C–17. Carbon atoms at above positions (C–1, C–11 and C–17) appear at δ_C 37.6, 24.2 and 54.6 for dihydroconessine and conanine [34]. Two downfield protons at δ_H 8.50 (1H, s) and 7.88 (1H, overl) revealed the presence of two N–H groups which were attached to two methine amino carbons at δ_H 66.6 and 51.8 respectively from HSQC experiment. Thus, confirming the absence of the *N*-Me group in cyclopentamide moiety of **4**. In addition, the lower field methine proton at δ_H 3.74 (1H, brs, H–3) was assigned to *N*-substituted carbon atom C–3 [36,38]. The benzoyl unit was attached at C–3 (δ_C 51.8) through HMBC correlation between H–3 (δ_H 3.74) with C–1′ and its ^{13}C data were consistent with those from literature [36,38]. The assignments were further supported when comparing chemical shifts/coupling constants with the reported data [34,36,38–40]. Furthermore, the stereochemistry of the hydroxyl groups was inferred after comparing NMR spectra with previously reported data which indicated that those groups were almost biogenetically at the *α*-face [41]. Hence, compound **4** is 1*α*, 11*α*, 17*α*-trihydroxy-3*β*-(*N*-benzamido)-5, 6-dihydroantidysentericine named elasticine.

Compound **9** was obtained as a white amorphous powder. Its molecular formula was deduced as $C_6H_6O_2N_2$ from the molecular ion peak at *m/z* 139.848 ($[M+H]^+$, calcd for $C_6H_6O_2N_2$, 139.831) in the ESI–MS, hence five degrees of unsaturation. The IR spectrum displayed strong absorption bands at 3300 and 1700 cm^{-1} in accordance with hydroxyl and carboxamide functional groups. The ^{13}C-NMR data of **9** was similar to that of nicotinamide, except for the existence of one additional hydroxyl group at δ_H 8.86 (1H, s, H–2) /(δ_C 149.9),

the carboxamide functional group at δ_C 166.9 and three methine carbon atoms at δ_C 149.4, 135.7 and 123.9. Based on the above evidence, compound **9** was identified as 5-hydroxypyridine-3-carboxamide already synthesized [42]. This is its first report from the Apocynaceae family.

Extensive chromatographic purifications of the MeOH extract of the leaves of *F elastica* on silica gel and Sephadex LH-20 afforded four new compounds (**1**–**4**), notably three new pentacyclic triterpene derivatives and one new steroidal alkaloid. Additionally, the following known compounds were also isolated: pseudotaraxasterol (**5**) [27], taxifolin (**6**), quercetin (**7**) [43], holarrhetine (**8**) [11], and 5-hydroxypyridine-3-carboxamide (**9**) [42] (Fig. 1).

A previous work reported the antiplasmodial activities of one class of compounds from this species, steroidal alkaloids, and that study attributed these activities to its alkaloid contents [11]. Antiplasmodial and antileishmanial activities of **1**–**3** were of interest because of the close relationship in structure of the three triterpenoids.

The new compounds (**1**–**4)** were tested for their antimalarial activities against *Plasmodium falciparum* 3D7 and Dd2 strains. The results revealed that all the triterpenes showed significant *in vitro* antimalarial activities against both strains with IC_{50} values of 4.68−25.82 µg/mL. The presence of an additional hydroxyl group at the C–2 position seems to enhancing the activity of pseudotaraxasterol type triterpene as shown for compound **2** with IC_{50} 4.68 µg/mL against CQR Dd2. However, the presence of an osidic group at C–3 and hydroxyl groups at C–1, C–11 and C–17 for either **3** or **4** slightly decreases antiplasmodial activities compared to compound **2** with its adjacent hydroxyl groups at C–2 and C–3 (Table 3). In parallel, compound **4** is slightly more active than compounds **1**–**3** against CQS 3D7 as demonstrated by previous works [44]**.** as a conclusion, the contribution of triterpenoids to the antiplasmodial activity of the plant species seems to be high, previous works reported the efficiency of compounds and extracts against CQR strain FcB1 [11].

Regarding the *in vitro* antileishmanial assay, the crude extract of *F. elastica* displayed an IC_{50} value of 3.32 µg/mL (Table 3). Among all the isolated compounds, **1** and **2** exhibited the highest antileishmanial activities, leading to IC_{50} values of 10.49 and 13.21 µg/mL respectively. In addition, **3** and **4** exhibited IC_{50} values of 19.27 and 22.54 µg/mL against *L. donovani* respectively, while amphotericin B, used as positive control, showed IC_{50} values of 0.05. µg/mL. when comparing the antileishmanial activities of compounds **1**–**3**, there is evidence that the hydroxyl groups at C–2 and C–3 significantly improve the *in vitro* activity against *L. donovani*, since 3*β*-hydroxyurs -20(21)-en-29-oic acid (**1**) and 2*α*, 3*β* -

dihydroxyurs-20(21)-en-29-oic acid (**2**) were more active than 3–*O*–β–D-[6′–*O*–acetylglucopyranosyl]-3*β*-hydroxyurs-20(21)-en-29-oic acid (**3**). These findings are similar to results of Torres-Santos et al. [45]: they got antileishmanial activities against *L. amazonensis* for analogous compounds and suggested that the hydroxylation pattern of ursane triterpenoids derivatives, mainly at C–3, could be important for such activity.

In a previous report, antileishmanial activities of various pentacyclic triterpenes of the ursolic class recommended that the presence of the carboxyl group at C–28 might be of interest [46]; unfortunately, there is no evidence to get the same conclusion according to the location of the said functional group at C–29 from the present study.

Moreover, it is important to point out that all evaluated samples showed no cytotoxicity against RAW 264.7 murine macrophage cells in the maximum dose tested (CC_{50} value of >200 µg/mL) (Table 3).

We noticed that the activity of the crude extract was better than that of the most active compounds from the plant. This should result from a concerted action between two or several molecules. Their separation thus weakened the activity. The antiplasmodial and antileishmanial activities of all these compounds were evaluated for the first time from the present study.

4. Conclusion

Three new pseudotaraxasterol triterpenoid derivatives and one antidysentericine alkaloid type were successfully isolated and characterized from the leaves of *F. elastica*, and their structures were elucidated. The antileishmanial and antiplasmodial activities of compounds **1**–**4** were assessed. Moreover, the plasmodicidal and leismanicidal activities of these triterpenoids and alkaloid were reported, adding and contributing new insight and knowledge towards the pharmacology properties of the secondary metabolites from this plant species.

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Declaration of Competing Interest

The authors report no declarations of interest

Appendix A. Supplementary data

Supplementary data to this article can be found online

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 1. Compounds isolated from *F. elastica*.

Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations of **1**–**3** and **4**.

Table 1. ^{1}H-NMR Spectroscopic data for **1** – **4** (measured in 500 MHz; δ in ppm, J in Hz).

Position	**1**[a]	**2**[a]	**3**[a]	**4**[b]
1a	1.55 m	2.03 m	1.54 m	3.97[c]
1b	1.34 m	0.96[c]	1.36 m	
2	1.69[c]	3.66 m	1.89 m	2.43 brs
			1.08 m	
3	3.14 dd (8.2, 4.8)	2.90 d (9.5)	3.17 m	3.74 brs
4	-	-	-	2.41 brs
5	0.75 brs	0.92[c]	0.97[c]	1.12 m
6a	1.56[c]	1.60 m	1.54 m	1.22 m
6b	1.40[c]	1.41[c]	1.42[c]	
7	1.58 m			1.24 m
8	-	-	-	1.17 m
9	1.55 m	1.62 m	1.60 m	0.94 m
10	-	-	-	-
11a	1.76 m	1.71 m	2.03 m	4.27 brt
11b	1.22[c]		1.64 m	
12a	1.58 m	1.72 m	1.20	2.67 dd (12.0, 4.8)
12b	-			2.36 dd (12.0, 8.0)
13	0.99 m	1.03 m	0.98 m	-
14	-	-	-	1.86[c]
15a	1.48 m	1.58 m	1.50 m	1.88[c]
15b	1.29 m	1.42 m	1.34 m	-
16a	1.66 m	1.70 m	1.97 m	2.02 m
16b	-	-	1.61 m	1.89[c]
17	-	-	-	-
18	1.38 dd (11.8, 9.2)	1.44 dd (11.8, 8.9)	1.37 dd (11.2, 9.0)	-
19	2.18 d (12.0)	2.29 d (11.0)	2.19 d (9.2)	1.15 s

20	-	-	-	3.94[c]
21	5.24 m	5.30 m	5.25 m	1.38 d (6.5)
22a	1.90 dd (11.8, 4.8)	2.03[c]	1.97 dd (12.0, 5.0)	-
22b	-	1.94 dd (11.8, 4.8)		
23	0.97 s	1.01 s	1.02 s	-
24	0.76[a]	0.86[a]	0.79 s	-
25	0.96 s	1.01 s	0.96 s	-
26	0.83 s	0.85[a]	1.00 s	-
27	0.94 s	1.06 s	1.02 s	-
28	0.87 s	0.96	0.89 s	-
29	-	-	-	-
30	1.12 s	1.17 s	1.12 s	-
1′			4.39 d (8.0)	-
2′			3.19 m	
3′			3.37 m	7.90 dd (8.0, 2.0)
4′			3.30 m	7.39 dd (8.0, 7.5)
5′			3.34 m	7.44 dd (8.0, 2.0)
6′			4.25a dd (12.5, 4.6)	7.39 dd (8.0, 7.5)
			4.23b dd (12.8, 5.8)	
7′				7.90 dd (8.0, 2.0)
1″				
2″			2.07 s	

[a]Measured in $CDCl_3$. [b]Measured in CD_3OD. [c]Overlapped signals are reported without designating multiplicity.

Table 2. ^{13}C-NMR Spectroscopic data for **1** - **4** (measured in 125 MHz; δ in ppm).

Position	**1**[a]	**2**[a]	**3**[a]	**4**[b]
1	32.9	46.9	32.7	67.8
2	27.9	68.2	27.8	33.9
3	79.3	82.9	78.3	51.8
4	38.3	38.5	38.4	33.7
5	55.3	55.4	56.5	48.0
6	18.1	20.8	18.2	28.9
7	36.7	33.2	37.8	29.2
8	38.6	38.4	38.6	35.2
9	47.5	47.6	47.4	52.6
10	38.6	36.9	38.4	46.3[d]
11	22.9	24.5	23.9	70.2
12	26.5	28.2	25.6	42.7
13	39.0	39.1	38.9	57.9
14	39.4	38.7	39.4	49.0
15	30.4	30.6	32.8	22.9
16	36.7	36.7	36.7	37.7
17	41.8	41.8	41.3	76.5
18	39.0	39.1	38.9	178.2
19	52.9	53.1	52.8	18.2
20	138.4	138.5	138.3	66.6
21	125.7	125.0	125.4	19.1
22	47.2	47.2	46.6	-

23	27.4	28.1	27.9	-
24	14.9	15.1	15.1	-
25	20.2	20.8	20.2	-
26	16.4	16.6	18.6	-
27	14.6	15.6	15.8	-
28	16.2	16.9	16.3	-
29	180.2	177.6	180.1	-
30	22.6	23.3	22.8	-
1′	-	-	101.1	174.5
2′	-	-	73.5	136.6
3′	-	-	76.6	128.9
4′	-	-	70.2	127.5
5′	-	-	79.2	130.5
6′	-	-	63.4	127.5
7′	-	-	-	128.9
1″	-	-	171.7	-
2″	-	-	19.5	-

[a]Measured in $CDCl_3$. [b]Measured in CD_3OD. [d]Obtained from the HMBC spectrum

Table 3. The *In vitro* antiplasmodial and antileishmanial activities of compounds **1**–**4**.

Extracts/ compounds	*P. falciparum* (µg/mL)		*L. donovani* (µg/mL)	Cytotoxicity (RAW 264.7 cells)	SI[c]
	3D7 clone[a]	Dd2 clone[b]	IC_{50} ± SD		
	IC_{50} ± SD	IC_{50} ± SD		CC_{50} ± SD (µg/mL)	
Crude extract	3.12 ± 0.13	0.91 ± 0.12	3.32 ± 0.08	56.71± 0.39	17.08
1	25.82 ± 0.22	5.33 ± 0.45	10.49 ± 0.15	NC	ND
2	25.25 ± 0.09	4.68 ± 0.28	13.21± 0.16	NC	ND
3	22.66 ± 0.17	6.36 ± 0.13	19.27 ± 0.21	NC	ND
4	17.56 ± 0.11	5.18 ± 0.19	22.54 ± 0.13	NC	ND
Chloroquine (nM)	272.2 ± 0.02	21.65 ± 0.25	-	-	-
Artemisin (nM)	17.76 ± 0.25	23.73 ± 0.24	-	-	-
Amphotericin B	-	-	0.05 ± 0.01	ND	ND
Podophyllotoxin (µM)	-	-	-	0.75± 0.09	ND

[a]Chloroquine-sensitive clone.
[b]Chloroquine-resistant clone.
NC = Not cytotoxic (up to the maximum dose tested, i.e > 200)
ND = Not determined
[c]SI (Selective Index) of bioactive compounds was determined as a measure of their toxicity against RAW cells lines macrophages. SI = CC_{50} against macrophages/IC_{50} against promastigotes; SD = Standard deviation.

Graphical abstract

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Highlights

- Isolation of four undescribed compounds from the leaves of *Funtumia elastica.*
- They possess ursane type triterpenoid and dihydroconessine derivative type alkaloid.
- Their structures were elucidated using IR, MS, ^{1}H and ^{13}C NMR, 1D and 2D
- *In vitro* evaluation of these compounds for antiplasmodial and antileishmanial activities
- Compounds **1** and **2** were the most potent antitplasmodial and antileishmanial agents