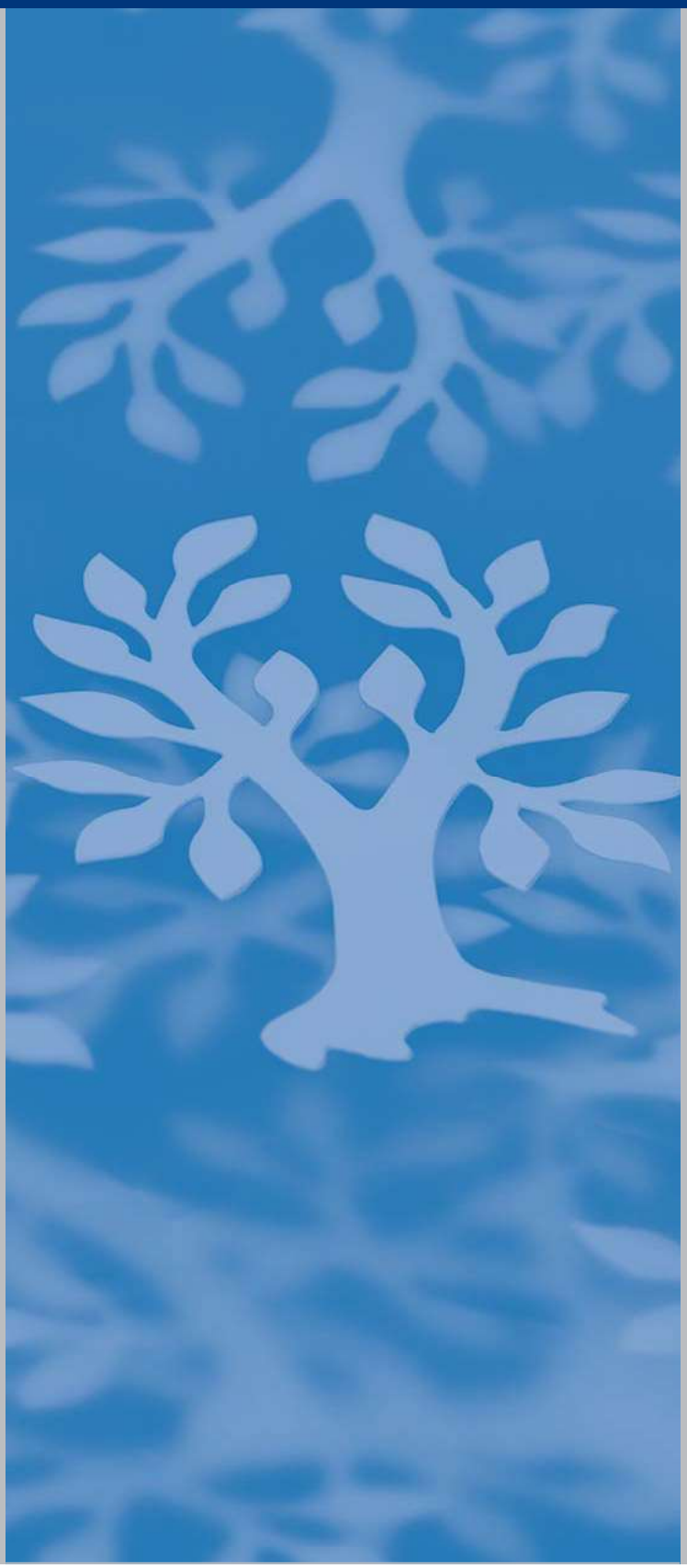

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Antiprotozoal activities of Triterpenic Acids and Ester Derivatives Isolated from the Leaves of *Vitellaria paradoxa*

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Vitellaria paradoxa, Sapotaceae, parasites, triterpenic esters, antitrypanosomal, natural compounds

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ABSTRACT

Leaves of *Vitellaria paradoxa*, also called "Shea butter tree", are used in traditional medicine to treat various symptoms including malaria fever, dysentery, or skin infections. Composition of the dichloromethane extract of *V. paradoxa* leaves possessing antiparasitic activities was investigated. Five pentacyclic triterpenic acids together with 6 ester derivatives were isolated and identified by standards comparison, MS and ^{1}H-NMR analysis. Corosolic, maslinic, and tormentic coumaroyl esters and their corresponding triterpenic acids were isolated from this plant for the first time. The antiparasitic activities of the 11 isolated compounds were evaluated *in vitro* on *Plasmodium falciparum, Trypanosoma brucei brucei*, and *Leishmania mexicana mexicana* and their selectivity determined by cytotoxicity evaluation on WI38 cells. None of the isolated compounds showed good antiplasmodial activity. The antitrypanosomal activity of individual compounds was in general higher than their antileishmanial one. One isolated triterpenic ester mixture in equilibrium, 3-*O*-*p*-*E*/*Z*-coumaroyltormentic acids, showed an attractive promising antitrypanosomal activity (IC_{50} = 0.7 µM) with low cytotoxicity (IC_{50}= 44.5 µM) compared to the corresponding acid. Acute toxicity test on this ester did not show any toxicity at the maximal cumulative dose of 100 mg/kg intraperitoneally on mice. *In vivo* efficacy evaluation of this compound, at 50 mg/kg by intraperitoneal route on a *T. b. brucei*-infected mice model, showed a significant parasitemia reduction on day 4 post-infection together with 33.3 % survival improvement. Further bioavailability and PK studies are needed along with mode of action investigations to further assess the potential of this molecule.

ABBREVIATIONS

CA	corosolic acid
MA	maslinic acid
OA	oleanolic acid
SI	selectivity index
TA	tormentic acid
UA	ursolic acid

Introduction

Vitellaria paradoxa C. F. Gaertn., (syn *Butyrospermum parkii*), Sapotaceae, also called "shea butter tree", is a tree that grows naturally up to 14 m high in the dry savannah belt of West Africa and is considered a sacred tree by many communities and ethnic groups [1]. This tree is generally protected because of the economic value of the shea butter, the fat extracted from the fermented kernels consisting of olein and stearin fractions along with nonsaponifiable compounds [2]. While shea stearin is used as an equivalent of cocoa butter in chocolate production [3], shea butter is also used

▶ **Table 1** IC_{50} of standard drugs in µg/mL (µM) ± SD: camptothecin (WI38) 0.04 ± 0.01; pentamidine (*Lmm*) 0.1 ± 0.1; artemisinin (*Pf*) 0.01 ± 0.003; suramine (*Tbb*) 0.03 ± 0.00.

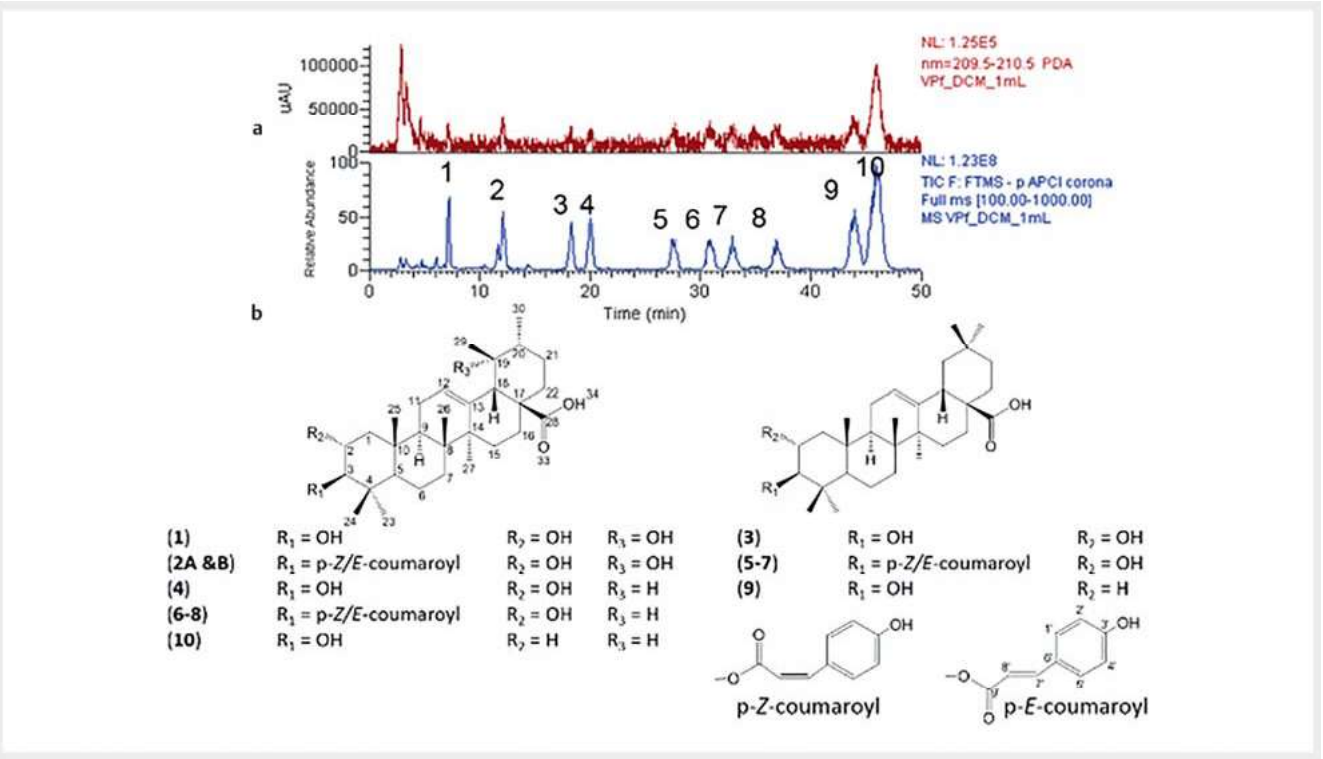
	WI38 (IC_{50} µg/mL (µM))	*Leishmania mexicana mexicana (Lmm)* (IC_{50} µg/mL (µM))	SI	*Plasmodium falciparum (Pf)* (IC_{50} µg/mL (µM))	SI	*Trypanosoma brucei brucei (TB)* (IC_{50} µg/mL (µM))	SI
VPf DCM	7.2 ± 2.9	8.9 ± 0.3	0.8	30 ± 3.6	0.2	3.4 ± 0.1	2.1
1 (TA)	26.5 ± 0.3 (54.2 ± 0.7)	27.1 ± 1.5 (55.5 ± 3.1)	0.9	> 45 (> 92)	< 0.5	7.5 ± 0.3 (15.3 ± 0. 7)	3.5
2A-2B	21 ± 3.2 (44.5 ± 6.9)	11.6 ± 1.5 (18.3 ± 2.4)	1.8	23.6 ± 6.6 (37.2 ± 10.4)	0.9	0.5 ± 0.4 (0.7 ± 0.6)	43.8
3 (MA)	20.8 ± 4 (44.1 ± 8.4)	9 ± 0.4 (19.1 ± 0.9)	2.3	> 45 (> 95)	< 0.5	4.4 ± 0.6 (9.4 ± 1.3)	4.7
4 (CA)	9.3 ± 2.5 (19.8 ± 5.2)	3.1 ± 0.2 (6.7 ± 0.4)	3.0	32.4 ± 6.6 (68.6 ± 14.1)	0.3	3.4 ± 0.3 (7.3 ± 0.6)	2.7
5–7	5.9 ± 1 (9.5 ± 1.6)	5.2 ± 0.7 (8.4 ± 1.2)	1.1	13.1 ± 2.2 (21.2 ± 3.5)	0.4	2.9 ± 0.4 (4.7 ± 0.6)	2.0
6–8	9 ± 3.1 (14.6 ± 5)	> 50	< 1	37.4 ± 4.4 (60.4 ± 7.2)	0.2	3.4 ± 0.2 (5.4 ± 0.3)	2.7
9 (OA)	28.9 ± 0.3 (63.3 ± 0.7)	9 ± 0.92 (19.7 ± 2)	3.2	30.7 ± 4. 6 (67.4 ± 10)	0.9	2.7 ± 0.3 (5.8 ± 0.7)	10.9
10 (UA)	51 ± 0.1 (11.1 ± 0.2)	3.2 ± 0.2 (7 ± 0.5)	1.6	31.5 ± 5 (69 ± 11)	0.2	1.1 ± 0.1 (2.4 ± 0.2)	4.7
Coumaric acid	> 100	–	–	–	–	> 40	–

in skin care products due to its effects on several skin properties [4]. The leaves, fruits, or bark of *V. paradoxa* are used in traditional medicine to treat several diseases such as skin infections, jaundice, diarrhea, stomach ache and ulcers, diabetes, dysentery, inflammation, malaria, and breast cancer [5, 6]. Antimicrobial potential of its stem bark and leaves extracts against Gram-positive and Gram-negative bacteria was also shown alongside significant antifungal effect for *V. paradoxa* stem bark [7, 8]. Parasitic protozoan diseases constitute one of the world's most widely spread human health problems. *Trypanosoma brucei, Leishmania* species, and *Plasmodium falciparum* are the causative agents of sleeping sickness, leishmaniasis, and malaria, respectively. Malaria morbidity and mortality remain high due to an increase in *P. falciparum* resistance to many first-line treatments [9, 10]. The different forms of leishmaniasis require expensive treatments, and the currently available drugs have some immunomodulatory effects, toxicity, and side effects possibly leading to death [11]. Human African trypanosomiasis (HAT) is a vector-borne parasitic disease causing health and economic problems in rural sub-Saharan Africa [12]. The 5 current available drugs include old treatments based on melarsoprol, pentamidine, suramin, eflornithine, and nifurtimox. Strong limitations are associated with these drugs such as major side effects, toxicity, variable efficacy, and increasing parasite resistance [13]. In January 2020, fexinidazole, a 10-day once-a-day oral treatment received the marketing authorization for HAT treatment but only for *T. b. gambiense* infections, and resistance can also develop [14]. In an effort to discover new lead compounds, our research group screened several extracts from African plants for their antiparasitic activities [15]. The aim of the present study was to evaluate the *in vitro* antiparasitic activities of the dichloromethane extract of *V. paradoxa* leaves, identify its main constituents, and analyze their antiparasitic potential. The one showing the best significant activity together with a good SI has then been tested *in vivo* for acute toxicity and antitrypanosomal activity.

Results and Discussion

Given the use of *V. paradoxa* leaves to treat fever and malaria and the research interests of our laboratory, we first assessed the antiparasitic activities of the dichloromethane extract of these leaves. The results are given in ▶ **Table 1** and show promising activities on *L. m. mexicana* and *T. b. brucei* but low selectivity, while the effect on *P. falciparum* was lower. As compounds responsible for the antiparasitic and cytotoxic effects may be different, we analyzed its composition by LC-UV-MS. This allowed us to propose the presence of 1: TA, 3: MA, and 4: CA, together with the previously reported 9: OA and 10: UA by comparison of retention times, mass spectra, and fragmentations with standards. Analysis of the HPLC-UV chromatogram allowed us to determine UA as the major triterpene in the DCM extract (about 21%), followed by OA (about 6%), TA and CA (about 2.5%), and MA (about 1.5%). Although betulinic acid has previously been identified in *V. paradoxa* leaves [16, 17], it was not detected in this study. This method likewise detected 5 triterpenic esters (▶ **Fig. 1**). The high resolution APCI-MS of (2) and (5–8) in the negative ion mode $[M - H]^-$ showed molecular peaks at *m/z* 633.37865 and 617.38561, corresponding to $C_{39}H_{54}O_7$ and $C_{39}H_{54}O_6$ respectively. Both MS/MS mass spectra showed a loss of a *m/z* 164 fragment, forming ions at *m/z* 469.34744 and 453.34337 corresponding respectively to $[C_{30}H_{47}O_4, M - 164]^-$ and $[C_{30}H_{47}O_3, M - 164]^-$. The loss of the *m/z* 164 fragment observed in all these MS/MS spectra corresponds to $C_9H_8O_3$, a coumaroyl moiety.

Several triterpenoids have already been isolated from *V. paradoxa* kernels or stem bark while no compounds were identified as yet from its leaves, with the exception of OA, UA, betulinic acids, and sitosterol cinnamate, an ester of cinnamic acid and β-sitosterol [16, 17]. Noteworthy, 2 isomers of TA (or 2α, 3β, 19α-trihydroxyurs-12-en-28-oic acid) namely euscaphic acid (or 2α, 3α, 19α-trihydroxyurs-12-en-28-oic acid) and 2-epi-tormentic acid (2β,3β,19α-trihydroxyurs-12-en-28-oic acid) have already

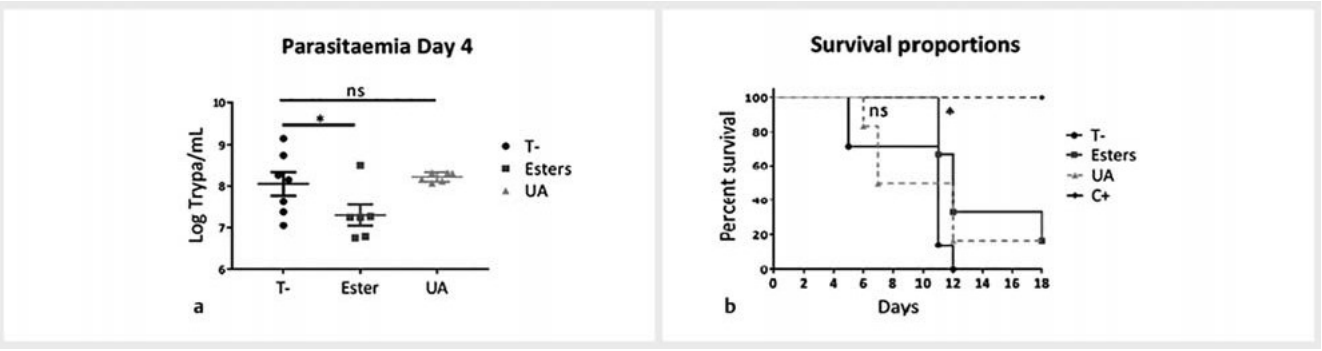


▶ **Fig. 1** LC-UV-PDA (**a**) and LC-MS in TIC negative mode (**b**) of V. paradoxa crude leaf dichloromethane extract and structures of the 11 identified compounds

been isolated from *V. paradoxa* kernels and stem bark respectively [18, 19]. Triterpenic acids (1, 3–4) and coumaroyl-triterpenic esters (2, 5–8) were purified for further structures determination. They were identified as tormentic (1), maslinic (3) and corosolic (4) acids by comparison of NMR data with standards and literature data [20]. Inspection of the ^{1}H and ^{13}C NMR downfield region of (2) indicates the presence of splitted signals for all the characteristic positions of the coumaroyl moiety, which appears to be present as a *Z/E* mixture (2A–2B) in equilibrium [21]. Isomers could not be separated by the developed HPLC method.

During HPLC analyses, the phenomenon of partial isomerization was also observed for the pair (5–7) and (6–8), indicating equilibrium between the paired structures. NMR data of both pairs (5–7) and (6–8) indicated the presence of *Z/E*-coumaroyl substructure, explaining the equilibrium observed with the isomerization of the double bond geometry with light [15]. Analysis of all ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC allowed the identification of 6 triterpenoid esters as 3-*O*-*p*-(*Z/E*)-coumaroyltormentic acid (2A and 2B), 3-*O*-*p*-(*Z/E*)-coumaroylmaslinic acid (5–7), and 3-*O*-*p*-(*Z/E*)-coumaroylcorosolic acid (6–8). The ^{13}C and ^{1}H NMR spectra are similar to those reported in literature [22, 23]. Compounds (2A) and (2B) were earlier reported from various plants, including *Perilla frutescens* var. acuta (Labiatae), *Eriobotrya japonica* (Rosaceae), and *Osmanthus fragrans* fruits [21, 22, 24]. Compounds (5–7) as well as (6–8) have been previously reported together in the leaves of *Prinsepia utilis royle* (Rosaceae), in the fruits of *Zizyphus jujuba* (Rhamnaceae), and for (6–8) in *Myrtus communis* Linn. (Myrtaceae) [25–27], but this is the first identification in *V. paradoxa*. The antiparasitic activities and cytotoxicity of isolated compounds are reported in ▶ **Table 1**.

Hydroxylations (2α and/or 19α) of triterpenic acids appear to decrease the antiplasmodial activity, as MA (3) is less active than OA (9) and TA (1) < CA (4) < UA (10). Results are in accordance with Filho et al. reporting an IC_{50} of 3.2 µg/mL for CA, higher than UA (1 µg/mL) against the chloroquine-sensitive *P. falciparum* D6 strain [28]. Concerning the antiplasmodial activity of acid derivatives, a 3-*O*-esterification of the coumaroyl moiety appeared to increase the activity of TA and MA while it did not seem to have an effect on CA. The SIs of these derivatives remained low. Regarding the antileshmanial activity, UA (9) and OA (10) are the most studied triterpenoids amongst the tested constituents. However, this is the first report of their activity against *L. m. mexicana*. UA appears to be more active than OA, which confirms the tendency observed by several authors on other *Leishmania* species like *L. major, L. amazoniensis* or *L. donovani* [29, 30]. A similar activity for MA (3) and OA (9) was observed on *L. m. mexicana* though higher activity of MA was reported on both *L. infantum* and *L. amazoniensis* [31]. Similar activity was observed for CA (4) and UA (10), while CA was reported to be less active against *L. amazoniensis* [28]. Impact of a 2α-hydroxylation on the antileishmanial activity of ursane and oleanane acids needs further investigation. TA exhibited higher IC_{50} than UA on *L. m. mexicana*, as also observed on *L. amazoniensis* (IC_{50} = 95 and 5 µg/mL respec-



▶ **Fig. 2** **a** *In vivo* parasitemia (logarithm of the trypanosomes number/mL) on day 4 post-infection in mice infected by *Trypanosoma brucei brucei* (*: $p < 0.05$; ns: $p > 0.05$). **b** Survival. T-, vehicle treated mice; Esters, 3-O-p-(Z/E)-coumaroyltormentic acids; UA, ursolic acid; C+, suramine (*: $p < 0.05$; ns: $p > 0.05$).

tively) [28]. Therefore, a third hydroxylation of the E ring seems deleterious for the antileishmanial activity. According to their skeleton, the addition of the 3-coumaroyl moiety enhances (TA and MA) or suppresses (CA) the activity. It was previously shown that 3-*O*-acetylation of UA reduced activity against *L. amazoniensis* promastigotes (IC_{50} from 9.4 to > 11.4 µg/mL) as well as on another strain of the same species (IC_{50} from 360.3 to 406.5 µM), while the same structural modification increased the activity of OA on the same strain (IC_{50} from > 11.4 to 2.3 µg/mL). These modifications did not induce any change in the antileishmanial activity against another *Leishmania* strain (*L. infantum*) [32]. None of the *V. paradoxa* major constituents exhibit an $IC_{50} \leq 1$ µg/mL with a good selectivity.

Concerning the antitrypanosomal activity of OA and UA, our results are in accordance with previously published ones on the same strain, showing UA more active than OA. In our study, no clear effect of the 2α-hydroxylation was observed: both derivatives showing similar antitrypanosomal effects. TA antitrypanosomal activity resulted to be lower than CA, indicating that a further E ring hydroxylation might be deleterious for the antitrypanosomal activity. Concerning the 3-ester derivatives, only a few literature data are available [33, 34]. A single C-3 acetylation did not improve or decrease the activity [35]. In the present study, the 3-*O* esterification seems to enhance the activity against *T. b. brucei* with a slight increase in the cytotoxicity against human normal fibroblasts WI38. Noteworthy, the mixture of 3-*O*-*p*-(*Z*/*E*)-coumaroyltormentic acids is the only one corresponding to the hit selection criteria proposed by Pink et al., with $IC_{50} \leq 1$ µg/mL and a SI > 20.7. By testing coumaric acid (IC_{50} > 40 µg/mL), we showed that this activity was not due to this part of the molecule, opening further *in vivo* studies for the compound (2) considering its promising antitrypanosomal potential. We also tested UA, which was the most active isolated triterpenic acid. Pentacyclic triterpenes are often associated with toxicity due to their cytostatic properties [36]. In the present case, no acute toxic symptom was observed in each group (UA and 3-*O*-*p*-(*Z*/*E*)-coumaroyltormentic acid) after repeated injections during the acute toxicity test, which did not affect the weight or hematocrit. As autopsy of treated mice did not reveal any macroscopic sign of toxicity, and organ weight was normal; the total cumulative highest tolerated doses were evaluated at 100 mg/kg for both compounds. Both compounds were then tested intraperitoneally at 50 mg/kg/day on a mice model infected with *T. b. brucei*. Interestingly, this tested dose is 2 times lower than the one usually used to assess the efficacy of antiparasitic compounds [36]. ▶ **Fig. 2a** shows that the mixture of 3-*O*-*p*-(*Z*/*E*)-coumaroyl TAs isolated from the leaves of *V. paradoxa* exhibits a significant decrease of the parasitemia on day 4 post-infection. This triterpenic esters mixture was more active than UA, which did not show any effect on parasitemia (▶ **Fig. 1a**).

Concerning survival analyses, the ester treatment significantly improved the survival of infected mice in comparison to the untreated group, contrarily to UA for which no significant difference was observed (▶ **Fig. 2b**). Positive control mice survived during all the experiment period while all mice died after 12 days in the negative control group, and in both treated groups only 1 mouse survived till the end of the experiment. On day 12 post-infection, survival increases of 16.7% and 33.3% were observed for UA- and esters-treated mice respectively. Interestingly, the oral administration of the UA and OA (50 mg/kg/day) resulted respectively in 79% and 76% parasitemia reduction on *T. cruzi*-infected mice, while intraperitoneal administration of the same concentrations was not effective [37]. In accordance with these results, in the present study, UA did not show any antitrypanosomal activity in *T. b. brucei* infected mice in intraperitoneal route at the same reported dose (50 mg/kg/day). Considering the immune system participation in the parasitic clearance, the immunosuppressive effect described at high doses of UA and OA could perhaps explain the lack of efficiency observed in controlling the infection [38]. However, in our case, esters hydrolysis would lead to TA release, described as less effective *in vitro* without immunosuppressive activity. Further research is needed to assess and characterize the antitrypanosomal *in vivo* efficacy of tested esters. The observed parasitemia decrease could be due to a direct antitrypanosomal effect or to an immune system-mediated effect.

In conclusion, *V. paradoxa* is used in traditional medicine to treat several ailments including malaria, fever, dysentery, or skin infections. Among the tested antiparasitic activities, the dichloro-

methane extract of *V. paradoxa* leaves showed a more promising activity against *T. b. brucei* though with a low SI. This is also the first report of the presence of TAs, MAs, and CAs, as well as their coumaroyl derivatives in *V. paradoxa*. By testing the antiparasitic activities of the 11 pentacyclic triterpenic constituents of this extract, it was shown that, compared to UA and OA, hydroxylations at 2*α* and 19 do not have a positive effect on antiparasitic activities, while esterifications at 3-OH by coumaric acid may have a positive one. Among all tested compounds, only a mixture of isomers, namely 3-*O*-*p*-(Z/E)-coumaroyltormentic acids, exhibited a significant *in vitro* antitrypanosomal activity ($IC_{50} \leq 1$ µg/mL) with a high SI (> 20). After having verified the safety of this mixture in an acute model, we showed a significantly reduced parasitemia at day 4 as well as an improved survival rate of mice infected by *T. b. brucei*. This is the first report of the *in vitro* as well as *in vivo* antitrypanosomal activity of this potential hit compound. Further studies on the promising mixture of 3-*O*-*p*-(Z/E)-coumaroyltormentic acid are required to assess its bioavailability, pharmacokinetic profile, and mechanism(s) of action.

Materials and Methods

Plant material

Leaves of *V. paradoxa* were collected in Benin (Parakou) in August 2012. Plant material was collected and identified by Mr Agbani (botanist of University of Abomey-Calavi, Cotonou, Benin). A voucher specimen was deposited at the Herbier National of Abomey-Calavi University in Benin, identified as #AP2130.

Extract Preparation

Air dried and powdered leaves (100 g/extraction) of *V. paradoxa* were extracted using a soxhlet apparatus with 700 mL of hexane and subsequently with 700 mL of dichloromethane (DCM) for 8 h each. The DCM extract (yield = 1.83 %) was then evaporated to dryness under reduced pressure with a rotary evaporator at a temperature of 30 °C.

Reference compounds

UA (purity > 98 %), TA (purity > 93 %), CA (purity > 99 %), MA (purity > 98 %), and OA (purity > 99 %) were purchased from AvaChem Scientific. In the antiplasmodial and cytotoxicity assays, Alamar Blue was obtained from Thermo Fisher Scientific; tetrazolium salt (MTT) and all other chemicals were purchased from Sigma-Aldrich.

HPLC-UV-MS/MS analysis and triterpenic acids quantification

DCM crude extract was analyzed using a LC-UV-MS/MS system consisting in a Thermo Accela pump, autosampler, photodiode array detector, and Thermo Scientific LTQ orbitrap XL mass spectrometer. The column used was a Phenomenex Luna C18, 250 × 4.6 mm packed with 5 µm particles. The flow rate was 1 mL/min using an isocratic binary solvent system: solvent A (20 %), H_2O pH 6 (CH_3COONH_4 0.02 M); solvent B (80 %), acetonitrile/methanol 40 : 35. The DCM extract solution was prepared by dissolving 10 mg of extract in 10 mL of HPLC grade methanol. The injection volume was 20 µL. Signals were monitored at 210 nm. HRMS were measured with the APCI source in the negative mode. The following inlet conditions were applied: capillary temperature 250 °C; APCI vaporizer temperature 400 °C; sheath gas flow 20.00 u. a.; auxiliary gas flow 5.00 u. a.; sweep gas flow 5.00 u. a. Data acquisition and processing were performed with Xcalibur software. Quantitative data were obtained only for the compounds for which standards were available by comparing the surfaces of the standards with those in the extract.

Compounds isolation and NMR analysis

Two grams of the DCM extract were solubilized in 400 mL of hexane-methanol-water (10 : 8 : 2), and a L/L extraction was realized 3 times with 200 mL of methanol-water (8 : 2) [15]. This was repeated 9 times for the rest of the extract. The lower phases (*ϕ* MeOH) were pooled and evaporated, and the residue (10.47 g) was submitted to fractionation (5 × 2 g) through polyamide columns (Machery Nagel, SC6 0.05–0.16 mm) eluted with 450 mL respectively of methanol and ethyl acetate leading to fractions FDM and FDA. Vacuum liquid chromatography (VLC) (Si 60 0.040–0.063 mm) of the FDM fraction (9 g) was performed using a Buchner funnel (5.0 × 113 cm^2) and eluted with cyclohexane/ethyl acetate (8 : 2). Interest fractions were submitted to preparative HPLC-UV consisting of a Shimadzu LC-20AP pump and a Spd-20AV detector. The column used was a Phenomenex Luna C18, 250 × 30 mm^2 packed with 5 µm particles. The flow rate was 42 mL/min of acetonitrile/methanol/water 45 : 35 : 20. Ten peaks were collected using detections at 210 nm and 310 nm yielding compounds 1–10. 1D and 2D NMR spectra of (1), (2), (3), (4), and (5–8) were recorded in deuterated methanol on a Bruker DRX 500 spectrophotometer (1H at 500 MHz and 13C at 125 MHz), with TMS as an internal standard. Extraction scheme is available in **Fig. 1S** (Supporting Information).

Parasites, cells, and media

T. b. brucei stock strain 427 (Molteno Institute in Cambridge, UK) bloodstream forms were cultured *in vitro* at 37 °C with 5 % CO_2 in HMI9 medium containing 10 % heat-inactivated FBS, *β*-mercaptoethanol (20 mM), and L-cysteine (150 mM). Promastigotes of *L. m. mexicana* (MHOM/BZ/84/BEL46 from Prof. P. A. M. Michels of De Duve Institute, UCLouvain) were cultivated *in vitro* at 28 °C with 5 % CO_2 in a semi-defined medium (SDM-79) supplemented with 15 % heat-inactivated FBC and hemin (5 µg/mL). *P. falciparum* chloroquine-sensitive strain 3D7 (from Prof. Grellier of Museum d'Histoire Naturelle, Paris-France) asexual erythrocytic stage were cultivated as described by Trager and Jensen at 37 °C and under an atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2. [39] Parasites were subcultured every 3–4 days with initial conditions of 0.5 % parasitemia and 1 % hematocrit. The host cells were human red blood cells (A or O Rh+). The human cells used were the noncancer fibroblast cell line WI38 (ATCC Number CCL-75 from LGC Standards) cultivated in RPMI 1640 medium (Gibco-Thermo Fisher Scientific) supplemented with 10 % FBS.

Cytotoxicity assays

Compounds citotoxicity was evaluated on WI38 cells using the MTT colorimetric assay [40]. Camptothecin was used as a positive

cytotoxic reference. Stock solutions of crude extract/pure compounds at 10 mg/mL in DMSO were diluted in medium with a final concentration range of 100–0.04 µg/mL. The solvent cytotoxicity was tested in parallel and found to be negligible at the highest tested concentration (1 %). Each extract was tested in 8 serial dilutions in 96-well plates.

In vitro antitrypanosomal, antileishmanial, and antiplasmodial activities

The crude extract and isolated compounds were evaluated for their antiparasitic *in vitro* activities. Antileishmanial and antitrypanosmal activities were assessed *in vitro* using AlamarBlue assay while the effect on *P. falciparum* was determined by the lactate dehydrogenase assay [41]. Stock solutions of the crude extract and isolated compounds were prepared at a concentration of 10 mg/mL in DMSO. The solutions were further diluted in medium to give 0.1 or 0.5 mg/mL stock solutions. The highest concentration of DMSO after serial dilution with complete culture medium was 1 %, which was shown to be nontoxic. Extracts and compounds were tested in 8 serial 3-fold dilutions (final concentration range: 50–0.02 µg/mL) in 96-well microtiter plates except for *P. falciparum*, where 2-fold dilutions were performed (final concentration range: 50–0.39 µg/mL). Pentamidine isethionate salt (reference antileishmaniasis drug), suramine sodium salt (reference antitrypanosomal drug), and artemisinin (reference antiplasmodial drug) were used as positive controls (tested concentration ranges: 10,000–4.6, 5000–2.3, and 100–0.78 ng/mL respectively).

In vivo acute toxicity and antitrypanosomal activity

The assessment of the highest tolerated dose was based on a DNDi protocol and adapted from Beaufay et al. [42]. 3-*O*-*p*-(Z/E)-coumaroyltormentic acids or UA was given intraperitoneally every 2 h to 2 mice using increasing doses: 10–15–25–50 mg/kg from stock solutions of 10 mg/mL in a mixture of filtered water/tween 80/ethanol (90 : 7 : 3). Mice were controlled for any health problem symptoms or behavioral changes and monitored for weight and hematocrit after each injection and every day during 48 h after administration. Main organs (heart, liver, spleen, lungs, and kidneys) of treated mice were observed and weighed wet during autopsy. Control group received the vehicle. For antitrypanosomal assays, tested dose was 50 mg/kg/day in accordance with the DNDi drug screening protocol for antiparasitic compounds [43]. Mice were randomly divided into 3 groups: 6 mice for the 3-*O*-*p*-(Z/E)-coumaroyltormentic acids and UA, 4 for the positive control (suramine), and 7 for the negative control, and were infected intraperitoneally with 10^4 *T. b. brucei*. All compounds were solubilized in water-tween 80-ethanol and administered intraperitoneally. 3-*O*-*p*-(Z/E)-coumaroyltormentic acids were administered at 50 mg/kg at day 3 after infection and then every day until day 7 post-infection. From day 3 post-infection, blood was collected each day from the mouse-tail to assess the parasitemia [44]. Remaining mice were euthanized on day 19 post-infection. For *in vivo* tests, NMRI female mice (6–8 w, 28.2 ± 3.6 g) obtained from Envigo laboratories were used. All *in vivo* experiments performed were approved by the Ethical Committee for animals use at the Health Sciences Sector of the Université catholique de Louvain (2017/UCL/MD/017), July 24, 2017.

Statistical analyses

In vitro results are expressed as the mean ± standard deviation (SD) of at least 3 independent experiment each performed in duplicate. IC_{50} values were calculated on Excel Software as described by Hills et al. [45]. The SI values were calculated using the formula:

SI = IC_{50} on mammalian cells/IC_{50} on protozoan parasites.

In vivo data were analyzed by Graphpad Prism 7.01 statistical software and presented as the mean ± error of the mean. Differences between independent groups results obtained for *in vivo* assay were analyzed by the nonparametric Mann-Whitney test in 1-tailed and by the log-rank test to compare survival curves. Statistical significance between treatments was set at p-value < 0.05.

Supporting Information

Supporting information presents the extraction scheme leading to compounds isolation as well as the structure of isolated compounds and NMR data used for esters identification. Furthermore, the data concerning the dose-activity relationship of a sample compound in antiparasitic and cytotoxicity assays are presented.

Contributors' Statement

Data collection: L. Catteau, C. Beaufay; C. Girardi, MF. Hérent; design of the study: L. Catteau, C. Beaufay, J. Quetin-Leclercq; statistical analysis: L. Schioppa, L. Catteau, C. Beaufay; analysis and interpretation of the data: L. Schioppa, L. Catteau, C. Beaufay, J. Quetin-Leclercq; drafting the manuscript: L. Schioppa, L. Catteau; critical revision of the manuscript: L. Schioppa, L. Catteau, C. Beaufay, M. Frédérich, J. Quetin-Leclercq.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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