Antiparasitic activities of triterpenic acids and ester derivatives isolated from the leaves of *Vitellaria paradoxa*

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ABSTRACT. *Vitellaria paradoxa* C.F. Gaertn. ssp. *paradoxa* (Sapotaceae) also called "Shea butter tree", is used in traditional medicine to treat various symptoms including malaria fever, dysentery or skin infections. Compounds from the leaves dichloromethane extract were investigated, and 5 pentacyclic triterpenic acids together with 6 ester derivatives were isolated and identified by standards comparison, MS and ^{1}H-NMR analysis, respectively. These esters, as well as three triterpenic acids were isolated from this plant for the first time. The antiparasitic activities of the eleven isolated compounds were evaluated *in vitro* on *Plasmodium falciparum* , *Trypanosoma brucei brucei* and promastigotes of *Leishmania mexicana mexicana* while their cytotoxicity was determined on WI38 cells using colorimetric methods. None of the isolated compounds showed good antiplasmodial activity. The antitrypanosomial activity of individual compounds was mostly higher than their antileishmanial one. Among these activities, moreover, one isolated triterpenic ester, 3-O-*p*-coumaroyltormentic acid, showed an attractive promising antitrypanosomal activity (IC_{50}=0.75 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 tested intraperitoneally (ip) on mice. *In vivo* efficacy evaluation of this compound, at 50 mg/kg by ip route on a *Trypanosoma brucei brucei* infected mice model, showed a significant parasitemia reduction together with a survival improvement. Further bioavailability and PK studies are needed along with mode of action investigations to further assess the potential of this molecule.

INTRODUCTION.

Vitellaria paradoxa C.F. Gaertn. (Sapotaceae) (VP), 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 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 non-saponifiable compounds[2]. While shea stearin is used as cocoa butter equivalent, improver or replacer in chocolate manufacture, shea butter is also increasingly popular as component of skin care products and cosmetic product formulations[3,4] Anti-inflammatory properties of shea butter were also reported[5], as well as for the nut triterpene-rich extract[6]. The leaves, fruits or bark of VP 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[7-11]. Stem bark of VP also showed significant antifungal effect[6]: Antimicrobial potential of its stem bark and leaves extracts against Gram positive and Gram negative bacteria such as *Staphylococcus aureus* and *Escherichia coli* was also shown[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 causative agents of sleeping sickness, leishmaniasis and malaria respectively, three parasitic diseases with high public health concerns[9–11]. With the increase in the resistance of *Plasmodium falciparum* to many of the first-line treatments[12,13], morbidity and mortality due to malaria remain an important health problem in many developing countries. The different forms of leishmaniasis require expensive treatments, and the currently available drugs generally have immunomodulatory effect together with toxicity and adverse side effects that can lead to death[14]. Human African trypanosomiasis (HAT) is a vector-borne parasitic disease which is causing major health and economic problems in rural sub-Saharan Africa[15]. The five current available drugs include old treatments based on melarsoprol, pentamidine, suramin, eflornithine, and nifurtimox. 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[16,17]. Strong limitations are associated to all these drugs such as major side effects, toxicity, variable efficacy and increasing parasite resistance[18,19]. In an effort to discover new lead compounds , our research group screened several extracts from African plants for their antiparasitic activities[19,20–22]. The aim of the present study was to evaluate the *in vitro* antiplasmodial, antitrypanosomal and antileishmanial activities of the leaves dichloromethane extract of VP, analyze its composition and determine the antiparasitic activities of its identified triterpenic constituents. The one showing the best significant antitrypanosomal activity and good selectivity index has then been tested *in vivo* for its acute toxicity and antiparasitic activity in a *Trypanosoma brucei brucei* infected mice model.

MATERIALS AND METHODS.

Plant material. Leaves of *Vitellaria paradoxa* C.F. Gaertn. (syn. *Butyrospermum parkii*) were collected in Benin (Parakou) in August 2012. A voucher specimen was identified and deposited at the Herbier National of Abomey-Calavi University in Benin, identified as #AP2130.

Extract Preparation. Air dried and powdered leaves (100 g) of *Vitellaria paradoxa* were extracted using a soxhlet apparatus with 700 mL of hexane and subsequently with 700 mL of dichloromethane (DCM) for 8h 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

Ursolic (UA) , tormentic (TA), corosolic (CA), maslinic (MA), and oleanolic (OA) acids were bought from AvaChem, San Antonio, TX. In the antiplasmodial and cytotoxicity assays, Alamar Blue was obtained from Thermo Fisher Scientific (Merelbeke, Belgium); tetrazolium salt (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and all other chemicals were purchased from Sigma-Aldrich (Bornem, Belgium).

HPLC-UV-MS/MS analysis. 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 x 4.6 mm packed with 5µm particles. The flow rate was 1mL/min using an isocratic binary solvent system: solvent A (20%), H_2O pH 6 (CH_3COONH_4 0.02M); 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. High-resolution mass spectra 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. Tormentic **(1)**, maslinic **(3)**, corosolic **(4)**, oleanolic **(9)** and ursolic **(10)** acids were identified from the leaf DCM extract by comparing retention times, mass spectra and fragmentations with standard compounds (AvaChem Scientific, CA, USA).

Compounds isolation and NMR analysis. The DCM extract was prepared from 1 kg of powdered leaves as cited above. Two grams of the extract were solubilized in 400 mL of hexane-methanol-water (10:8:2) and a L/L extraction was realized three times with 200 mL of methanol-water (8:2)[23]. This was repeated nine times for the rest of the extract. The lower phases (φMeOH) were pooled, evaporated and the residue (10.47g) was submitted to fractionation (5 x 2g) through polyamide columns (Machery Nagel, SC6 0.05-0.16mm) eluted with 450mL respectively of methanol and ethyl acetate leading to fractions FDM and FDA. Vacuum liquid chromatography (VLC) (Si 60 0.040-0.063mm) of the FDM fraction (9g) was performed using a Buchner funnel (5.0 x 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 x 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 210nm and 310nm 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 ^{13}C at 125 MHz), with TMS as an internal standard.

Parasites, cells and media. Parasites used were *Trypanosoma brucei brucei* (*Tbb*) bloodstream forms (BSF strain 427); promastigotes of *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) (*Lmm*), *Plasmodium falciparum*, strain 3D7 (*Pf*). Cells used were human normal fibroblasts line, WI38. *Tbb* BSF were cultured *in vitro* at 37°C with 5% CO_2 in HMI9 medium containing 10% heat-inactivated fetal bovine serum, β-mercaptoethanol (20mM) and L-cysteine (150mM). *Lmm* were cultivated *in vitro* at 28°C with 5% CO_2 in a semi-defined medium supplemented with 15% heat-inactivated fetal bovine serum and haemin (5 mg/L). *Pf* 3D7 asexual erythrocytic stage were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen at 37°C and under an atmosphere of 5% CO_2, 5%O_2 and 90% N_2.[24] The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/L glucose (Sigma-Aldrich), 44 mg/mL hypoxanthin (Sigma-Aldrich), 100 mg/L gentamycin (Gibco) and 10% human pooled serum (A or O Rh+). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitemia and 1% haematocrit.

Cytotoxicity assays. The cytotoxicity of compounds was evaluated on J774 and WI38 cells using the MTT colorimetric assay[25]. 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 – 0.04mg/L. The solvent cytotoxicity was tested in parallel and found to be negligible at the highest tested concentration (0.5%). Each extract was tested in eight 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 Alamar-Blue assay while the effect on *Pf* was determined by the lactate dehydrogenase assay[26]. Stock solutions of the crude extract and isolated compounds were prepared at a concentration of 10mg/mL in DMSO. The solutions were further diluted in medium to give 0.1 or 0.5 mg/mL stock solutions. Extracts and compounds were tested in eight serial three-fold dilutions (final concentration range: 50–0.02 mg/L) in 96-well microtiter plates except for *Pf*, where two-fold dilutions were performed (final concentration range: 50-0.39 mg/L). Pentamidine isethionate salt (an antileishmaniasis drug), suramine sodium salt (an antitrypanosomal drug), and artemisinin (an antiplasmodial drug) were used as positive controls (tested concentration ranges: 10 000-4.6, 5000-2.3 and 100-0.78 µg/L respectively). IC_{50} values were calculated by linear regression on Excel Software based on the sigmoid curves obtained from the mean (2 wells/concentration) of the eight concentrations, and tested at least in triplicate.

In vivo acute toxicity and antitrypanosomal activity. The assessment of the highest tolerated dose was based on a DNDi protocol[27] and adapted from Beaufay C., et al.[28]. Briefly, 3-O-*p*-coumaroyltormentic acid or UA was given intraperitoneally every 2h 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 48h 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 50mg/kg/day in accordance with the DNDi drug screening protocol for antiparasitic compounds[29]. Interestingly, this dose is two times lower than the one usually used to assess the efficacy of antiparasitic compounds[30]. Mice were randomly divided into 3 groups: 6 mice for the 3-*O*-p-coumaroyltormentic acid and UA, 4 for the positive control (suramine) and 7 for the negative control, and were infected intraperitoneally with 10^4 *Tbb*. All compounds were solubilized in water-tween 80-ethanol, and administered intraperitoneally. 3-*O*-p-coumaroyltormentic acid was 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[31]. Remaining mice were euthanized on day 19 post-infection. All *in vivo* experiments performed during this work were approved by the Ethical Committee for animals use at the Health Sciences Sector of the Catholic University of Louvain (2014/UCL/MD/002).

Statistical analyses. Data were analysed by Graphpad Prism7.01 statistical software and presented as the mean ± standard error. Differences between independent groups results obtained for *in vivo* assay were analyzed by the non-parametric Mann–Whitney test in one-tailed and by the log-rank test to compare survival curves. Statistical significance between treatments was set at p-value < 0.05.

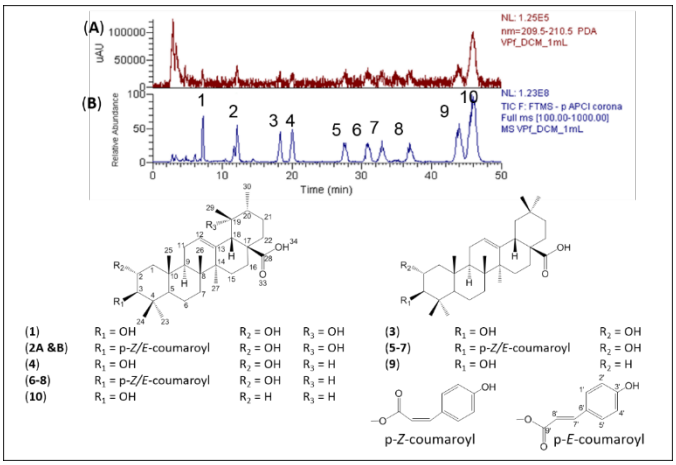
RESULTS AND DISCUSSION

Crude DCM extract HPLC-UV-MS/MS analysis. LC-UV-MS analysis of the leaves DCM crude extract allowed the identification for the first time in the leaves of *Vitellaria paradoxa* of tormentic **(1)**, maslinic **(3)** and corosolic acids **(4)** by comparison of retention times, mass spectra and fragmentations with standard compounds. These three triterpenic acids were identified together with oleanolic **(9)** and ursolic acids **(10)** which were previously reported by our team to be present in these leaves[32]. Betulinic acid was shown to be present in VP leaves but we did not detect it in our samples[33]. This method also detected 5 triterpenic esters but the lack of standards did not allow their direct identification (figure 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 $C_9H_8O_3$ in accordance to a coumaroyl moiety. Several triterpenoids have already been isolated from the kernels or the stem bark of *Vitellaria paradoxa* while no compounds were identified up to now from its leaves, with the exception of oleanolic, ursolic and betulinic acids[34–37] and sitosterol cinnamate, an ester of cinnamic acid and β-sitosterol[38]. Noteworthy, two isomers of tormentic acid (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 been isolated from *Vitellaria paradoxa* kernels[39] and stem bark[40] respectively. Purification of the corresponding peaks was thus necessary to confirm the structure and stereochemistry of the different acids as well as to identify esters present in the extract.

Isolation and structure identification. Triterpenic acids **(1, 3-4)** and coumaroyl-triterpenic esters **(2, 5-8)** were purified for further structures determination. The structure of compound **(1)** was confirmed as tormentic acid on the basis of its 1D/2D NMR and HRMS analysis and by comparison with the data reported in the literature[41,42]. The configuration of the hydroxyl groups as 2α-OH and 3β-OH, respectively, was deduced from the characteristic coupling constant of 9.8 Hz observed between H-2 and H-3, with H-2 (δ 3.60, ddd, J = 10.8, 9.8, 4.1 Hz) and H-3 (δ 2.90, d, J = 9.8 Hz).

Figure 1 LC-UV (A) and LC-MS in negative mode (B) of crude leaf dichloromethane extract and structures of the 11 identified compounds



Indeed, euscaphic acid having 2α-OH/3α-OH on the same side of the molecule is expected to display a lower coupling constant H-2/H-3 of 2.5 Hz [43] while 5 Hz was found for 2-epi-tormentic acid (2β-OH/3β-OH)[44]. We further confirmed the identification of **(3)** and **(4)** as maslinic and corosolic acids by comparison of NMR data with standard compounds. Surprisingly, inspection of the downfield region of the ^1H and ^{13}C NMR spectra of **(2)** indicates the presence of splitted signals for all the characteristic positions of a coumaroyl moiety, which appears to be present as a mixture of a *Z* and *E* **(2A-2B)** forms in equilibrium[45]. Indeed, *Z* or *E* could not be separated as independent molecules using the developed HPLC method. However, single signals were clearly observed for the ursan core of the aglycon, except positions 1 to 4, also splitted by the influence of the *Z* or *E* configuration of the coumaroyl group which esterifies the position 3 of the triterpenic core. During HPLC analyses of isolated compounds (**5-8**), we detected a lack of stability of compound (**5**) which partially isomerized into **(7)**. The same was observed for **(7)** isomerizing into **(5)**, indicating equilibrium between both structures. An identical phenomenon was observed for the pair **(6-8)**. Similarly to compounds (**2A**) and (**2B**), NMR data of both pairs **(5-7)** and **(6-8)** indicated the presence of a *E*- or *Z*-coumaroyl substructure explaining the equilibrium previously observed, corresponding to a *Z/E* modification of the double bond geometry which is known to isomerize with light[20,46]. The ^1H signals of the triterpene residues formed a

similar pattern to that of **(3)** and **(4)** respectively. Analysis of all ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC allowed us to identify the six 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)**[42,47,48]. Compounds (**2A)** and **(2B)** were earlier reported from various plants, among which *Perilla frutescens* var. acuta (Labiatae)[47] or *Eriobotrya japonica* (Rosaceae)[42] and *Osmanthus fragrans* fruits[45]. Compounds **(5-7)** as well as **(6-8)** have been previously reported together in the leaves of *Prinsepia utilis* Royle (Rosaceae), as well as in the fruits of *Zizyphus jujuba* (Rhamnaceae) and *Myrtus communis Linn.* (Myrtaceae)[49] for the second pair, but it is the first time they are identified in *Vitellaria paradoxa*.

In vitro antiparasitic activity. The antileishmanial, antiplasmodial and antitrypanosomal activities of *Vitellaria paradoxa* leaf dichloromethane crude extract and isolated compounds are reported in Table 1, together with their cytotoxicity on WI38 cell line. In line with previous results on plant extracts, antiparasitic activities can be classified as follow: $IC_{50} \leq 15$ mg/L: promising activity; IC_{50} = 15-50 mg/L: moderate activity; $IC_{50} > 50$ mg/L: weak activity and $IC_{50} > 100$mg/L: inactivity[32]. In the present work, a moderate activity for the VP leaf dihloromethane extract on *Plasmodium falciparum* 3D7 strain ($IC_{50} = 30.03 \pm 3.60$ mg/L) is shown although cytotoxicity was observed at much lower concentration (SI= 0.2). The antiplasmodial activity of UA and OA have been extensively studied on various *Plasmodium* strains with a general better activity for UA than OA [23,50,51]. Hydroxylations (2α and/or 19α) seem to decrease the antiplasmodial activity and we can class the derivatives activity results as MA < OA and TA < CA < UA. Our results are in accordance with Filho et al. who reported an IC_{50} of 3.2 mg/L for CA, higher than the one of UA (1mg/L) against the chloroquine-sensitive *Plasmodium falciparum* D6 strain[52]. While TA (**1**) was found here to be inactive against the tested *Pf* strain 3D7, it was previously reported to have an IC_{50} of 15.2 mg/L against the chloroquine-resistant W2 strain[53].Concerning the antiplasmodial activity of acid derivatives, we observe here for the first time, that the 3-*O*-esterification with a coumaroyl moiety increased the activity of TA and MA while it did not induce any significant effect for CA derivatives. However, the selectivity indexes of these derivatives remain low. It has been shown in the literature that the 3-*O*-esterification with a short alkyl chain improved the antiplasmodial activity[54–56] and *p*-coumarate moieties at the 27-position contributed to the antiplasmodial activity observed[23,57]. Even if the IC_{50} obtained for 3-*O*-coumaroyl derivatives are higher than those obtained for 27-*O* coumaroyl derivatives, it is difficult to attribute the decrease of activity to the ester position as our derivatives are hydroxylated in position 2α or/and 19α and we showed above the negative impact of these hydroxylations on the antiplasmodial activity. Concerning the antitrypanosomal and antileishmanial activities, some activities of VP hexane[58] and aqueous[59] extracts were already reported, while we report here for the first time the promising potential of its dichloromethane extract (IC_{50}<15 mg/L), though with quite general low selectivity indexes.

Regarding the antileshmanial activity, ursolic (**9**) and oleanolic (**10**) acids are the most studied triterpenoid compounds amongst the tested constituents. However, this is the first report of their activity against *Leishmania mexicana 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*[60–62]. In 2014, Sifaoui et al. studied

Table 1. Antiparasitic activities, cytotoxicity and selectivity indexes of the crude VP DCM leaf extract and isolated compounds (IC50 mg/L (*μ*M))

	WI38 (IC_{50} mg/L (μM))	Lmm (IC_{50} mg/L (μM))	SI[a]	Pf (IC50 mg/L (μM))	SI[a]	Tbb (IC_{50} mg/L (μM))	SI[a]
VPf DCM	7.17 ± 2.93	8.88 ± 0.32	0.8	30.03 ± 3.60	0.2	3.45 ± 0.13	2.1
1	26.48 ± 0.33 (54.22 ± 0.7)	27.10 ± 1.51 (55.5 ± 3.1)	0.9	>45 (>92)	<0.5	7.49 ± 0.33 (15.33 ± 0.67)	3.5
2A-2B	21.04 ± 3.24 (44.5 ± 6.9)	11.58 ± 1.53 (18.3 ± 2.4)	1.8	23.61 ± 6.61 (37.2 ± 10.4)	0.9	0.48 ± 0.36 (0.75 ± 0.57)	43.8
3	20.85 ± 3.97 (44.15 ± 8.4)	9.03 ± 0.41 (19.1 ± 0.9)	2.3	>45 (>95)	<0.5	4.45 ± 0.64 (9.4 ± 1.3)	4.7
4	9.34 ± 2.47 (19.8 ± 5.2)	3.15 ± 0.19 (6.7 ± 0.4)	3.0	32.45 ± 6.65 (68.6 ± 14.1)	0.3	3.45 ± 0.28 (7.3 ± 0.6)	2.7
5-7	5.89 ± 0.96 (9.5 ± 1.6)	5.22 ± 0.75 (8.44 ± 1.2)	1.1	13.13 ± 2.16 (21.2 ± 3.5)	0.4	2.90 ± 0.37 (4.7 ± 0.6)	2.0
6-8	9.04 ± 3.11 (14.6 ± 5.0)	>50	< 1	37.38 ± 4.44 (60.4 ± 7.2)	0.2	3.39 ± 0.21 (5.4 ± 0.3)	2.7
9	63.26 ± 0.74 (138.5 ± 1.6)	9.01 ± 0.92 (19.74 ± 2.03)	7.0	27.1* (59.4)		5.8 ± 0.7 (12.7 ± 1.5)	10.9
10	5.08 ± 0.10 (11.14 ± 0.22)	3.2 ± 0.2 (7.03 ± 0.46)	3.4	14.8* (32.4)		1.08 ±0.11 (2.38 ± 0.24)	4.9
Coumaric acid	>100	-	-	-	-	> 40	-

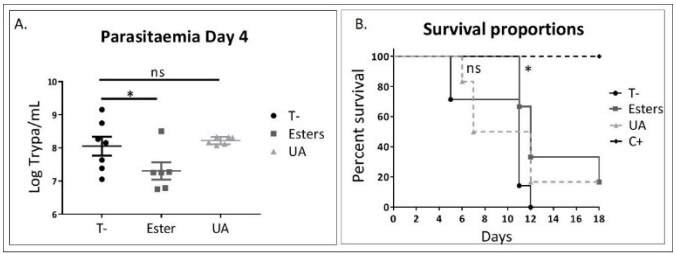
IC_{50} of standard drugs in mg/L (or μM) +-/ standard deviation: camptothecin (WI38) 0.04±0.01, pentamidine (Lmm) 0.11±0.06, artemisinin (Pf) 0.009±0.003, suramine (Tbb) 0.03±0.00. [a] Selectivity indexes are calculated by SI = IC_{50}(WI38)/ IC_{50}(*Tbb*, *Lmm* or *Pf* respectively).*[23]

for the first time the antileishmanial activity of maslinic acid (**3**). They report a higher activity for MA (IC_{50} = 9.32 ± 1.65 and 12.46 ± 1.25 mg/L) than for OA (IC_{50} = 17.25 ± 0.65 and 20.62 ± 0.75 mg/L) against *L. infantum* and *L. amazoniensis* respectively. They also highlighted a possible synergy between OA and MA, as a mixture of both exhibited a higher antileishmanial activity than each pure compound tested alone[63]. Here we observe a similar activity for both compounds. Another study reported corosolic acid (**4**) (IC_{50} = 19.0 mg/L) as less active than UA (IC_{50} = 3.7 mg/L) against *L. amazoniensis*[52], while we observed a similar activity. Further studies are thus needed to understand the impact of a 2α-hydroxylation on the antileishmanial activity of ursane and oleanane acids. Tormentic acid **(1)** corresponding to 19α-hydroxylated-CA is less active than CA itself, and also than UA. While it is the first report of the antileishmanial activity of TA against *L. mexicana mexicana*, it was reported to exhibit a much higher IC_{50}. than UA on *L. amazoniensis* (IC_{50}= 95 and 5 mg/L respectively)[61], in accordance with our results. A third hydroxylation of the E ring seems thus deleterious for the antileishmanial activity. Noteworthy, three pairs of 3-*O*-p-*Z/E*-coumaroyl derivatives of TA, MA and CA were tested here for the first time against *Leishmaniam mexicana mexicana*. According to their skeleton, the addition of the coumaroyl moiety enhances (TA and MA) or suppresses (CA) the antileishmanial activity. It has been previously shown that the 3-*O*-acetylation of UA appears to reduce its activity against *L. amazoniensis* promastigotes (IC_{50} from 9.41 to > 11.4 mg/L) 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 2.28 mg/L)[55,64]. These modifications did not induce any change in the antileishmanial activity against another *Leishmania* strain (*L. infantum*)[64]. None of the *Vitellaria paradoxa* major constituents exhibit an $IC_{50} \leq 1$ mg/L with a good selectivity. Even if some pentacyclic triterpenoids seem to be promising antileishmanial compounds, assays on a larger number of UA and OA derivatives are necessary.

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[65,66]. No previous study reported the *in vitro* activity of TA, MA or CA nor the ester derivatives isolated here against *T. brucei brucei*. Of note, in 2006, Cunha et al., reported that a mixture of MA + CA was less active than a mixture of OA + UA against one strain of *Trypanosoma cruzi* (IC_{50} = 48.5 and 5.4µM respectively)[67]. In our study, we did not observe any clear effect of the 2α-hydroxylation, both derivatives showing similar antitrypanosomal effects. We further show here that the antitrypanosomal activity of TA is lower than CA indicating that hydroxylation of the E ring might be deleterious for the antitrypanosomal activity. Concerning the ester derivatives, only a few literature data are available[42,68–72]. It was shown that a single C-3 acetylation did not improve activity or decreased it[73,74]. In the present study, the 3-*O* esterification seems to enhance the activity against *T. brucei brucei* but it is also accompanied by a slight increase in the cytotoxicity against human normal fibroblasts WI-38. Noteworthy, the mixture of 3-*O*-p-(*Z/E*)-coumaroyltormentic acids are the only one corresponding to the hit selection criteria proposed by Pink et al., with a $IC_{50} \leq$ 1mg/L and a SI > 20.[75] By testing coumaric acid (IC_{50} > 40 mg/L), we showed that this activity was not due to this part of the molecule, opening further *in vivo* studies. Thus compound **(2)** was chosen for its promising antitrypanosomal potential for further *in vivo* studies.

In vivo acute toxicity and antitrypanosomal activity. Pentacyclic triterpenes are often associated with toxicity due to their cytostatic properties[76]. In the present case, no acute toxic symptom was observed in each group (UA and 3-*O*-p-(*Z/E*)-coumaroyltormentic acid) after the repeated injections of the treatment which did not impact neither weight nor haematocrit. As autopsy of treated mice did not reveal any macroscopic sign of toxicity and organs 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 *Trypanosoma brucei brucei.* Figure 2A shows that the mixture of 3-*O*-p -(Z/E)-coumaroyl tormentic acids isolated from the leaves of *Vitellaria paradoxa* exhibits a significant decrease of the parasitemia on day 4 post-.

Figure 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).



This triterpenic esters mixture was more active than UA which did not show any effect on parasitemia (figure 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 (figure 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 one 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[77]. In accordance with these results, in the present study, UA did not show any antitrypanosomal activity in *Tbb* infected mice for the intraperitoneal route at the same reported dose (50mg/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[92]. However, in our case, esters hydrolysis would lead to TA release, described as less effective *in vitro* but without of immunosuppressive activity[78]. Further researches are needed to assess and characterize the antitrypanosomal *in vivo* efficacy of tested esters. Indeed, the observed parasitemia decrease could be due to a

direct antitrypanosomal effect or to an immune system mediated effect. as

CONCLUSION

Vitellaria paradoxa (VP) is used in traditional medicine to treat several ailments including malaria, fever, dysentery or skin infections. This is the first report of the presence of tormentic, maslinic, corosolic acids as well as their coumaroyl derivatives in the leaves of *Vitellaria paradoxa*. Among the tested antiparasitic activities, the leaf dichloromethane extract showed a more promising activity against *T. brucei brucei* though with a low selectivity index. By testing the antiparasitic activities of 11 pentacyclic triterpenic constituents of this extract, it was shown that compared to UA and OA, hydroxylations at 2α and 19 does not in general have a positive effect on antiparasitic activities, while esterifications at 3-OH by coumaric acid may have positive effects. Among all tested compounds, only one mixture of ester isomers, namely 3-*O*-p-(*Z/E*)-coumaroyltormentic acid, exhibited a significant *in vitro* antitrypanosomal activity ($IC_{50} \leq 1$mg/L) with a high selectivity index (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. brucei 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 as well as its mechanism(s) of action.

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