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Antitrypanosomal and Antileishmanial Activities of *Tacca leontopetaloides* Tubers and *Zanthoxylum zanthoxyloides* Stem Bark

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Abstract: Phytochemical screening of extracts of *Tacca leontopetaloides* tubers, has afforded the isolation of two novel chalcones, tarkalynins A and B, along with taccalonolide A and its 12-propanoate. Screening of *Zanthoxylum zanthoxyloides* stem bark yielded taraxerol acetate, dihydrochelerythrin and fagaramide. These compounds were obtained through column and thin-layer chromatography and identified using NMR and LC-HRMS. The compounds were tested against *Trypanosoma brucei brucei* s427 and its multi-drug-resistant clone B48, against *T. evansi*, *T. equiperdum*, *T. congolense*, and against *L. mexicana*. Cytotoxicity was tested against the human HEK293 cell line. The highest activities were observed with dihydrochelerythrin and fagaramide against *T. b. brucei* s427 and B48, *T. evansi*, and *L. mexicana* with EC_{50} values of 0.5, 0.9, 0.4, and 1.9 μg/mL and of 4.4, 2.7, 2.7, and 3.3 μg/mL, respectively. In addition, tarkalynin A and taraxerol acetate displayed promising activity against *T. equiperdum* (EC_{50} = 7.1 and 9.98 μg/mL, respectively). None of these compounds showed significant cross-resistance with existing trypanocides (RF ≈ 1; P>0.05). The compounds displayed low toxicity to human cells, with most exhibiting no growth inhibition at concentrations of 100 or 200 μg/mL. This report provides further evidence of the potential of natural products for combating parasitic diseases.

Keywords: *Tacca leontopetaloides*; tarkalynin A; tarkalynin B; taccalonolides; antitrypanosomal; Antileishmanial activity; *Zanthoxylum zanthoxyloides*; dihydrochelerythrin; fagaramide

1. Introduction

African trypanosomes are the etiological agents of a wide range of diseases and are usually transmitted by insect vectors feeding on humans (Human African Trypanosomiasis or sleeping sickness) and animals (African Animal Trypanosomiasis or AAT) [1]. This mode of transmission is true for all pathogenic trypanosomes, except *Trypanosoma equiperdum*, which is transmitted sexually

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by copulation in horses and other equids, causing a wasting disease known as *dourine* [2]. Sleeping sickness, caused by *T. gambiense* and *T. rhodesiense*, is progressing towards control and elimination [3] but animal trypanosomiasis cases continue to grow in number and geographically [4–6]. AAT is commonly known as *nagana* in sub-Saharan Africa, where it is mostly transmitted by tsetse flies, and is classified as a neglected tropical veterinary disease. It is also referred to as *surra* in North Africa and Asia, *mal de caderas* and *Derrengadera* in South America and as *dourine* for the sexually-transmitted form in equines [2,6,7]. The main species responsible for nagana are *T. brucei brucei*, *T. congolense,* and *T. vivax*, whereas *T. evansi* causes surra and together with *T. vivax* contributes to *mal de caderas* in South America [6–9]. Mutations in *Trypanosoma sp.* have led to the emergence of different drug-resistant strains to the current drugs used at different treatment stages [10,11]. Moreover, most of the available drugs are only effective against the hemolymphatic stage of the disease, before the infection spreads to the central nervous system [12]. There is no effective chemotherapy for several of the animal trypanosomiasis infections, most notably dourine, but drug resistance threatens the treatability of surra and nagana as well [6,13]. This implies that AAT can no longer be effectively treated, and this situation is affecting livelihoods, economies, and food security [2,6].

Leishmaniasis, caused by protozoan parasites of up to 20 different *Leishmania* species is also a public health and veterinary concern in many countries in the tropics and subtropics, with about one million new human cases annually [14]. It is transmitted through the bite of an infected female phlebotomine sandfly [15]. Current chemotherapies depend on treatment with drugs of various efficacies, availability, and toxicities coupled with resistance as the greatest challenge [16].

Plants produce diverse active substances used in many fields of medicine with proven templates for new drug development [17,18]. Likewise, propolis, sourced by bees from local plants or trees, has proved to be a treasure trove of anti-parasite compounds [19–21]. Even simple derivatives of agricultural waste products can yield highly active new antiparasitics [22,23]. *Tacca leontopetaloides,* commonly referred to as Polynesian arrow or bat flower, *Amura* or *Tarayaya* (in Hausa), *Gbache* (in Tiv), or *Andru* (in Idoma) [24], is a perennial herbaceous plant commonly found in the North Central and Western parts of Nigeria [25]. It is a flowering plant with a highly bitter and starchy bulb-like tuber which is used as a staple food and as a source of starch in North Central Nigeria [25–27]. Several compounds of pharmaceutical importance have been isolated from the plant with ethnopharmacological activities, including antimicrobial and antioxidant [28,29], anti-inflammatory and anti-pyretic [30,31] and anti-cancer properties [32]. Most of these compounds are taccalonolides, a class of highly oxygenated pentacyclic triterpenes. In cancer research, they have been shown to stabilize microtubules, similar to the anticancer agents paclitaxel and epothilone, and are active against cell lines resistant to those drugs [33]. The diversity in structure, unique mechanism of action, and low toxicity of taccalonolides have attracted interest for drug discovery. Consequently, several taccalonolides [27,33,34] have been isolated, as well as taccabulins A-E, evelynin A [35] and evelynin B [36]. The initial anti-protozoan report on taccalonolides [34] showed these compounds to possess a range of activities from promising to moderate ($0.76 \leq EC_{50} \leq 12.2$ μg/mL) and demonstrated their potential as drugs against *Trypanosoma* and *Leishmania* species.

We also assess the anti-parasite properties of *Zanthoxylum zanthoxyloides*, popularly known as *Fagara, Candlewood, zanthoxylum* and to some natives of Nigeria as *Akenaka* or *Ayer* (in Tiv), *Ufu otachacha* (in Igede) and *Faschuari* (in Hausa) [24]. It is a shrub, spiny and rather scandent, up to 6-8 m tall, belonging to the family Rutaceae. It has been reported to contain α-pinene, citronellol, geraniol, limonene, β-myrcene [37], tannin, saponins, flavonoids, phenol, alkaloids, terpenoids, essential oils and coumarins, and to possess several medicinal activities including antinociceptive, antimalarial, cytotoxic, antiproliferative, anthelminthic, antiviral and antifungal, antioxidant, analgesic, anti-inflammatory, antimicrobial, wound healing, larvicidal, trypanocidal, uterine contraction, antitumor and hepatoprotective properties [38–41]. Here, we report further investigation of the plant extracts to isolate compounds possessing higher activity against parasites causing HAT, AAT or leishmaniasis, with possible different modes of action and no cross-resistance to existing treatments.

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2. Results and Discussion

2.1. Isolation and Structural Characterization

Purification of compounds from the ethyl acetate extracts of *Tacca leontopetaloides* tubers afforded a novel chalcone, tarkalynin A (**1**), belonging to the taccabulin class of compounds. Three previously known compounds were also identified in the *T. leontopetaloides* extracts. First, a methylenedioxy dihydrochalcone (**2**) from the combined hexane and ethyl acetate extracts of the *Tacca* peels in fraction TPHE 26 was identified as 1-(benzo[d][1,3]-dioxol-5-yl)-β-(2′-hydroxy-4′,6′-dimethoxyphenyl)propan-1-one (tarkalynin B), and we here report its isolation as a natural compound for the first time; it has previously only been produced synthetically, as an intermediate to the synthesis of some taccabulins [42]. Second, taccalonolide A (**3**) was obtained from the combined hexane and ethyl acetate fractions of the *Tacca* tubers (TTHE 70-76) and peels (TPHE 62-74), and from the methanol fractions of the peels (TPM 49-69). Thirdly, taccalonolide A 12-propanoate (**4**) was isolated from the tuber hexane and ethyl acetate fraction TTHE 73 and peel methanol extract fractions TPM 20-37. Taraxerol acetate (**5**) was obtained from ethyl acetate fraction of *Zanthoxylum zanthoxyloides* (ZSE-06), dihydrochelerythrin (**6**) was isolated from ethyl acetate fraction of *Z. zanthoxyloides* (ZSE-42) and fagaramide (**7**) from *Z. zanthoxyloides* stem bark hexane fraction ZSH-44.

Compound **1** was obtained from the ethyl acetate fractions (TTHE 48) of *Tacca* tubers as a brown solid. Its LC-HRMS spectrum yielded a molecular ion $[M+H]^+$ at m/z 333.1336 (calculated 333.1338, $C_{18}H_{21}O_6$), corresponding to the molecular formula $C_{18}H_{20}O_6$, with seven degrees of unsaturation. This was confirmed by its ^{1}H-NMR spectrum (Table 1) which showed four aromatic proton signals at δ_H (ppm) 8.09 (d, J = 8.9 Hz, H-2 and 6), 7.04 (d, J = 9.0 Hz, H-3 and H-5), 6.23 (d, J = 2.3 Hz, H-3′), 6.05 (d, J = 2.4 Hz, H-5′) and a set of methylene signals at δ_H (ppm) 3.46 (dd, 14.9, 2.9, H-αa), 2.86 (dd, 14.9, 7.6, H-αb). Integration of the proton spectrum showed that the signals at 7.04 and 8.09 ppm are due to two protons each, indicating symmetry in the aromatic ring. The spectrum also showed that the methylene protons are coupled to an oxymethine proton at 5.18 (dd, J = 7.6, 2.9, H-β). Three methoxy singlet signals were observed at δ_H (ppm) 3.95, 3.79, and 3.61. The ^{13}C-MNR spectrum showed a total of eighteen signals including two quaternary aromatic carbons at δ_C (ppm) 125.9 (C-1) and 105.1 (C-1′); four aromatic tertiary carbons at δ_C (ppm) 164.4 (C-4), 160.4 (C-4′), 158.3 (C-6′) and 157.7 (C-2′); six aromatic CH at 131.5 (C-2 and C-6), 114.0 (C-3 and C-5), 94.8 (C-3′) and 91.3 (C-5′) indicating the presence of a disubstituted and a tetrasubstituted aromatic ring. A characteristic signal at δ_C 197.9 ppm was indicative of an open-chain saturated ketone. The three signals at δ_C (ppm) 55.6, 55.3 and 55.4 confirmed the presence of three methoxy carbons substituted on the aromatic rings. Two aliphatic signals at δ_C (ppm) 75.6 and 30.5 were attributed to an oxygenated carbon and a methylene carbon respectively. The structure was fully deduced through its 2D NMR (COSY, HSQC, HMBC) spectra. Its COSY spectrum gave correlations between the aromatic protons at 8.09 (H-2 and H-6) and 7.04 (H-3 and H-5) indicating they were ortho coupled. There were also correlations between the oxymethine proton at 5.18 (H-β) and the methylene protons at 3.46 (H-αa) and 2.86 (H-αb). The HMBC spectrum showed long-range correlations between the aromatic protons at 7.04 (H-3 and H-5) to the quaternary carbon at 125.9 (C-1) while the protons at 8.09 (H-2 and H-6) had correlations to the quaternary carbon at 164.4 (C-4) and the carbonyl carbon at 197.9. Long-range correlations from the protons at 8.09 (H-2 and H-6) to the carbonyl carbon at 197.9 and from the methylene proton at 2.86 (H-αb) to two aromatic quaternary carbons at 105.1 (C-1′) and 157.7 (C-2′) confirmed the propanone chain was substituted by an aromatic ring at both ends. The three methoxy groups were identified by their HSQC correlations to their respective carbons: 3.95 (δ_C 55.6), 3.79 (δ_C 55.3) and 3.61 (δ_C 55.4). The methoxy-bearing carbons were identified by the HMBC correlations of the methoxy protons: 3.95 (δ_C 164.4), 3.79 (δ_C 160.4), and 3.61 (δ_C 158.3). Thus, compound **1** was characterized as 1-(4-hydroxy)-β-(2′-hydroxy-4′,6′-dimethoxyphenyl)propan-1-one with a common name tarkalynin A (Figure 1).

Compound **2** was isolated from the combined hexane and ethyl acetate extracts of the *T. leontopetaloides* peels in column fraction 26 (TPHE 26) as a brown solid with the molecular formula

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$C_{18}H_{18}O_6$ from its LC-HRMS spectrum which gave a molecular ion $[M+H]^+$ at m/z = 331.1168 (calculated 331.1182, $C_{18}H_{19}O_6$) with eight degrees of unsaturation. Its ^{1}H-NMR spectrum gave signals (Table 1) for five aromatic protons at δ_H (ppm) 7.63 (dd, J = 8.3,1.8 Hz, H-2), 7.47 (d, J = 1.8 Hz, H-6), 6.84 (d, J = 8.3 Hz, H-3), 6.20 (d, J = 1.8 Hz, H-3′) and 6.07 (d, J = 2.5 Hz, H-5′) indicating the compound has two aromatic rings and was similar to compound **1**. The only difference was a methylenedioxy group attached to one of the aromatic rings and the presence of neighbouring methylene protons at 3.33 (H-α) and 2.95 (H-β) as indicated by the COSY correlations between them. From correlations in its 2D spectra, it was identified as 1-(benzo[d][1,3]-dioxol-5-yl)-β-(2′-hydroxy-4′,6′-dimethoxyphenyl)propan-1-one and named tarkalynin B. The spectral data agreed with the literature reports [42].

Compound **3** was isolated from the hexane and ethyl acetate extract fractions of the *T. leontopetaloides* tubers (TTHE 70-76) and peels (TPHE 62-74), and the methanol extract fractions of the peels (TPM 49-69) as a brown solid. It was identified as taccalonolide A by comparison of its spectral data with literature reports [34]. Its LC-HRMS spectrum gave a molecular ion $[M+H]^+$ at m/z 703.2973 (calculated 703.2966, $C_{36}H_{47}O_{14}$), corresponding to the molecular formula $C_{36}H_{46}O_{14}$.

Compound **4** was identified as Taccalonolide A 12-propanoate by comparison of its ^{1}H-NMR data with literature reports [34]. It was also obtained as a brown solid from the hexane and ethyl acetate fractions of the *T. leontopetaloides* Tacca tubers (TTHE 73) and the peel methanol fractions (TPM 20-37). Its LC-HRMS spectrum yielded a molecular ion $[M+H]^+$ at m/z 717.3156 (calculated 717.3122, $C_{37}H_{49}O_{14}$), corresponding to the molecular formula $C_{37}H_{48}O_{14}$.

Compound **5** was obtained from the ethyl acetate extract fraction ZSE-06 of *Z. zanthoxyloides* stembark as a white solid and identified as taraxerol acetate by comparison of its ^{1}H-NMR data with literature reports [43].

Compound **6** was also obtained from the ethyl acetate extract fraction ZSE-42 of *Z. zanthoxyloides* stembark as a white solid and identified as dihydrochelerythrin by comparison of its spectral data with literature reports [44].

Compound **7** was obtained from the hexane extract fraction ZSH-44 of *Z. zanthoxyloides* stembark as a white solid and identified as fagaramide by comparison of its spectral data with earlier reports [45].

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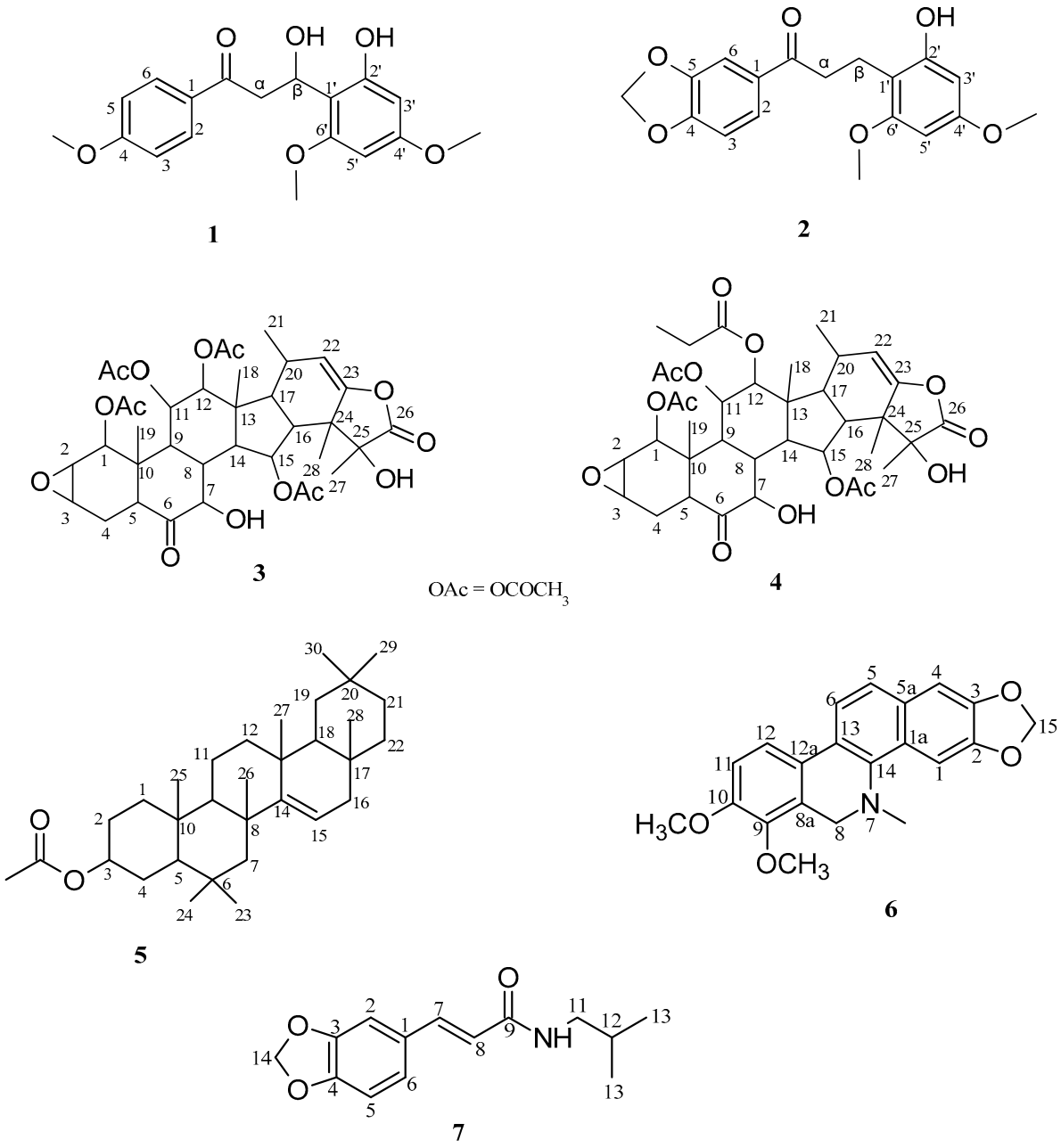

Figure 1. Structures of isolated compounds: 1, Tarkalynin A; **2**, Tarkalynin B; **3**, Taccalonolide A; **4**, Taccalonolide A 12-propanoate; **5**, Taraxerol acetate; **6**, Dihydrochelerythrin; **7**, Fagaramide.

Table 1. NMR data (400Hz, in $CDCl_3$) (δ, ppm) for compounds **1** and **2.**

Position	Compound 1		Compound 2	
	^{1}H (δ ppm, m, *J* (Hz))	**^{13}C (m)**	**^{1}H (δ ppm, m, *J* (Hz))**	**^{13}C (m)**
1	-	125.9 (C)	-	131.4 (C)
2	8.09 (d, 8.9)	131.5 (CH)	7.63 (dd, 8.3, 1.8)	125.0 (CH)
3	7.04 (d, 9.0)	114.0 (CH)	6.84 (d, 8.3)	107.9 (CH)
4	-	164.4 (C)	-	152.3 (C)
5	7.04 (d, 9.0)	114.0 (CH)	-	147.7 (C)
6	8.09 (d, 8.9)	131.5 (CH)	7.47 (d, 1.8)	108.1 (CH)
1′	-	105.1 (C)	-	108.7 (C)
2′	-	157.7 (C)	-	156.3 (C)
3′	6.23 (d, 2.3)	94.8 (CH)	6.20 (d, 2.4)	94.7 (CH)
4′	-	160.4 (C)	-	159.9 (C)

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5′	6.05 (d, 2.4)	91.3 (CH)	6.07 (d, 2.5)	91.3 (CH)
6′	-	158.3 (C)	-	159.3 (C)
α	3.46 (dd, 14.9, 2.9) 2.86 (dd, 14.9, 7.6)	30.5 (CH_2)	3.33 (m)	38.8 (CH_2)
B	5.18 (dd, 7.6, 2.9)	75.3 (CH)	2.95 (dd, 6.6,4.4)	16.6 (CH_2)
-C=O	-	197.9 (C)	-	201.4 (C)
O-CH_2-O	-	-	6.02 (s)	101.9 (CH_2)
4-OCH_3	3.95 (s)	55.6 (CH_3)	-	-
4′-OCH_3	3.79 (s)	55.3 (CH_3)	3.77 (s)	55.3 (CH_3)
6′ -OCH_3	3.61 (s)	55.4 (CH_3)	3.81 (s)	55.5 (CH_3)
2′-OH	6.13 (s, br)	-	8.80 (s, br)	-

2.2. Effect of the Isolated Compounds on Trypanosomes

The *in vitro* activities of six of the isolated compounds were carried out against bloodstream forms of *T. b. brucei* (s427 wild-type), multi-drug resistant-resistant *T. b. brucei* (B48), *T. evansi* (WT) and *T. equiperdum* (WT), *T. congolense* (WT) promastigotes using a resazurin-based assay. All values are displayed in Table 2.

Dihydrochelerythrin **(6)** exhibited a very high activity with EC_{50} values between 0.48 to 1.08 µg/mL against all the trypanosomes of the *Trypanozoon* subgenus (i.e. *T. b. brucei*, *T. evansi* and *T. equiperdum*), including the multidrug-resistant clone B48 ($P > 0.05$ relative to s427). Against *T. congolense* (subgenus *Nannomonas*) the activity was significantly ($P < 0.001$) lower, although still promising with an EC_{50} value of 2.9 ± 0.2 µg/mL. Since toxicity against HEK 293 was low, the *in vitro* selectivity index (SI) was good, especially for the *Trypanozoon* species (30.4 < SI < 87). Fagaramide (**7)** also displayed good activity against all trypanosome species (2.7 < EC_{50} < 8.5 µg/mL), and was less selective for the *Trypanozoon* subgenus, as the small difference between *T. b. brucei* s427 and *T. congolense* was not statistically significant (P >0.05). With toxicity against the human cell line above 90 µg/mL, the SI values ranged from 10.6 to 33.8. This confirms a previous report by [45], who also reported an interestingly high antitrypanosomal activity of fagaramide against *T. b. brucei* s427, with EC_{50} = 7.3 µM and no toxicity to normal cell lines (macrophages RAW264.7; EC_{50} = 214.96 µM). This compares to the activity displayed by our compound **7** (fagaramide) with an EC_{50} 4.4 µg/mL (~17.8 µM) against s427. *Z. zanthoxyloides* has been shown to possess anticancer [46,47] and antibacterial [39] properties.

Of the *T. leontopetaloides*-derived compounds, the novel compound Tarkalynin A (**1**) displayed the most promising activity, but again with a highly significant separation between the *Trypanozoon* and *Nannomonas* subgenera (P <0.001), as EC_{50} values against the former ranged between 7.1 µg/mL (*T. equiperdum*) and 15.3 µg/mL for *T. evansi*, whereas the compound was almost inactive against *T. congolense* (EC_{50} = 81.7 µg/mL). No EC_{50} could be determined against HEK 293 because it was inactive even at the highest concentration tested, 100 µg/mL.

Taccalonolide A (**3**) and its 12-propanoate (**4**) displayed highly similar but moderate activities against the various trypanosome species. The small change from acetate to propanoate did not significantly impact its anti-trypanosomal properties. Although the compounds were not toxic to HEK 293 cells to the limited tested, their antiparasite activity was not sufficiently promising for them to be considered as lead compounds. Similarly, Taraxerol acetate (**5**) performed relatively poorly against most trypanosome species (EC_{50} > 25 µg/mL), with only the EC_{50} for *T. equiperdum* marginally below 10 µg/mL. Compounds **4** and **5** were not toxic to HEK 293 cells at the highest concentrations tested (100 and 200 µg/mL, respectively).

Cross-resistance with existing trypanocides of the diamidine class (e.g. pentamidine, diminazene, furamidine) and melaminophenyl arsenical class (melarsoprol, cymelarsan) was tested by side-by-side comparison of the standard drug-sensitive strain *T. b. brucei* s427 and clone B48. This clone was derived of s427 by sequentially deleting the TbAT1 drug transporter [48] and in vitro exposure to pentamidine [49]; it is highly resistant to all these important drugs against HAT and AAT

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[49,50]. None of the compounds here tested exhibited significant resistance in B48, nor as much as a 2-fold higher EC_{50}, while resistance to pentamidine was approximately 50-fold ($P < 0.001$). The diamidine and arsenical resistance in *T. brucei*, *T. evansi* and *T. equiperdum* is associated with the functional loss of two drug transporters: the aminopurine transporter P2/TbAT1 and the aquaporin TbAQP2 [51–54], as has been demonstrated for B48 [55]. Thus, the trypanocidal action of the compounds used in this study is not dependent on these crucial, common drug transporters.

In general, the compounds displayed lower activity against *T. congolense* than against *Trypanozoon* group trypanosome species. This is also observed with several important trypanocides such as diminazene, pentamidine, other mitochondrion-targeting drugs, and the arsenicals [13,56]. This is a problem for the treatment of nagana since *T. brucei* and *T. congolense* incidence almost completely overlaps geographically, as they are transmitted by the same vectors, and therefore, the infecting species of a particular animal is rarely known [6]. However, dourine, in horses and other equids, is invariably caused by *T. equiperdum* and surra, from North Africa to South Asia and the Philippines, is caused by *T. evansi* only [2,57]. In this context, it is important that **6** and **7** displayed the highest activity against *T. evansi* and almost as promising activity against *T. equiperdum*, and that new treatments for dourine and surra are urgently required.

The promising antitrypanosomal ability of dihydrochelerythrin and fagaramide may be due to the presence of the methylene-dioxy moiety. A report by [47] had it that a derivative of dihydrochelerythrin: 6-Acetonyl-5, 6-dihydrochelerythrine, isolated from *Zanthoxylum leprieurii* showed no significant activity against s427 and the structure-activity relationships of **6** should therefore be carefully studied.

There are no anti-protozoal activity reports for compounds obtained from *Tacca leontopetaloides* except for our initial report on the anti-trypanosomal activity of compounds and fractions from the plant [34], where activity of 3.13 μg/mL was displayed by taccalonolide A 12 propanoate and 11.42 μg/mL by taccalonolide A against *T. b. brucei* s427. Although taccalonolide A showed a moderate EC_{50} (31.9 – 46.6 μg/mL), no cross-resistance (RF ≤ 1) was observed with the multi-drug resistant B48, *T. evansi* and *T. equiperdum*. Taccalonolide A 12 propanoate showed the least activity but exhibited moderate activity against s427 with an EC_{50} of 38.2 ± 6.2 μg/mL (53.4 μM) and poor activity against B48, *T. evansi* and *T. equiperdum* in the range 53.8 – 59.3 μg/mL. Generally, the tested compounds were poorly active against *T. congolense* (EC_{50} > 50 μg/mL).

2.3. Effect of the ISOLATED Compounds on Leishmania Mexicana

Dihydrochelerythrin **(6)** and fagaramide (**7)** showed significant activity, (EC_{50} = 1.9 ± 0.2 μg/mL (5.5 μM) and EC_{50} 3.3 ± 0.1 μg/mL (13.3 μM) respectively against *L. mexicana* and thus showed genuine and broad anti-kinetoplastid activity. Tarkalynin A showed poor activity (EC_{50} = 193 μg/mL) against *L. mexicana*. Neither EC_{50} nor SI values for taccalonolide A and its propanoate against *L. mexicana* could be obtained due to low activity.

2.4. Effect of the Isolated Compounds on HEK

The compounds at a concentration of 100 μg/mL did not show toxicity to mammalian (HEK) cells as the observed EC_{50} values were above 90 μg/mL except for dihydrochelerythrin, which showed a moderate toxicity with EC_{50} = 33.0 μg/mL.

Table 2. Effect of isolated compounds on trypanosomes and leishmania. EC_{50} values are average ± SEM of at least three independent determinations. RF = Resistance factor, being EC_{50} (parasite)/ EC_{50}(s427WT). SI = Selectivity index, being EC_{50} (HEK)/ EC_{50}(parasite).

Compound	*T. b. brucei* s427		*T. b. brucei B48*			*T. evansi*			*T. equiperdum*			*T. congolense*			*L. mexicana*		HEK 293
	EC_{50} (µg/mL)	SI	EC_{50} (µg/mL)	RF	SI	EC_{50} (µg/mL)	RF	SI	EC_{50} (µg/mL)	RF	SI	EC_{50} (µg/mL)	RF	SI	EC_{50} (µg/mL)	SI	EC_{50} (µg/mL)
1	11.8 ± 1.2	>8.5	16.7 ± 5.0	1.4	>6.0	15.3 ± 0.8	1.3	>6.5	7.1 ± 1.3	0.60	>14	81.7 ± 1.7***	6.9	>1.2	193 ± 1***	0.52	>100
3	45.5 ± 14.9	>2.2	31.9 ± 2.9	0.7	>3.1	46.6 ± 6.7	1.0	>2.2	39.2 ± 0.0	0.86	>2.6	73.9 ± 28.9	1.6	>1.4	>200	ND	>100
4	38.2 ± 6.2	>2.6	53.8 ± 8.8	1.4	>1.9	59.3 ± 5.3	1.6	>1.7	55.7 ± 7.5	1.5	>1.8	>100	>2.8	ND	>200	ND	>100
5	64.5 ± 28.5	>3.1	59.8 ± 2.1	0.75	>3.4	29.5 ± 0.7	0.4	>6.8	9.98 ± 0.12	0.13	>20	83.6 ± 6.7	1.1	2.4	>200	ND	>200
6	0.48 ± 0.15	68.0	0.89 ± 0.10	1.8	37.0	0.38 ± 0.02	0.8	87.0	1.08 ± 0.18	2.2	30.4	2.9 ± 0.2***	6.0	11.4	1.9 ± 0.2**	18	33.0 ± 8.1
7	4.4 ± 1.8	20.4	2.7 ± 0.1	0.6	33.8	2.7 ± 0.1	0.6	33.0	6.70 ± 0.03	1.5	13.4	8.5 ± 1.4	1.9	10.6	3.3 ± 0.1	27	90.1 ± 10.8
PMD **(µM)**	[a]0.0066 ± 0.0001 [b]0.0052 ± 0.0006	-	[a]0.312 ± 0.0349*** [b]0.288 ± 0.05***	[a]47.3 [b]55.4		[a]0.016 ± 0.004** [b]0.0025 ± 0.0003**			[a]0.0081 ± 0.001 [b]0.0033 ± 0.001			ND			[a]1.10 ± 0.03*** [b]0.76 ± 0.05***		ND
DA **(µM)**	ND		ND			ND			ND			[a]0.51 ± 0.01 [b]0.46 ± 0.15			ND		ND
PAO **(µM)**	ND		ND			ND			ND			ND			ND		[a]0.17 ± 0.01 [b]0.048 ± 0.011

Control: PMD = Pentamidine isethionate, DA = Diminazene aceturate, PAO = Phenyl arsine oxide. ND = not determined. **, P <0.01; ***, P <0.001. **a** = EC_{50} of control for **1**, Tarkalynin A; **3**, Taccalonolide A; **4**, Taccalonolide A 12-propanoate; **b** = EC_{50} of control for **5**, Taraxerol acetate; **6**, Chelerythrine; **7**, Fagaramide.

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4. Materials and Methods

4.1. Plant Collection

The tubers of *T. leontopetaloides* and the stem bark of *Z. zanthoxyloides* were collected from Kusuv (Buruku LGA, Benue State) and Bunu Tai (Tai LGA, Rivers State) in Nigeria, respectively. The plants were identified by the Forestry and Wildlife Department, Joseph Sarwuan Tarka University, Makurdi, Benue State, and the Department of Forestry and Environmental Studies, Rivers State University, Port Harcourt, with voucher specimen numbers, UAM/FH/0469 and BSU/2017/ZZ-56. The tubers were rinsed with water, the bark scraped off, air-dried, ground, and sieved while the stem bark was air-dried and ground to powder.

4.2. Extraction and Isolation of Constituents

The dried and ground tubers (6.9 kg) and peel (4.0 kg) of *T. leontopetaloides* and 1 kg stem bark of *Z. zanthoxyloides* were each macerated and extracted with, successively, hexane, ethyl acetate, and methanol (2500 mL, 48 h). The filtrates were concentrated on a rotary evaporator at 40 °C and air-dried to obtain the hexane, ethyl acetate and methanol extracts. These extracts were subjected to TLC on pre-coated silica gel plates and visualized using anisaldehyde-H_2SO_4 reagent. The hexane and ethyl acetate extracts of *T. leontopetaloides* showed similar patterns on TLC and were therefore combined. Thus, four crude extracts were obtained from this plant: *Tacca* Tuber Hexane/Ethyl acetate (TTHE), *Tacca* Tuber Methanol (TTM), *Tacca* Peel Hexane Ethyl acetate (TPHE), and *Tacca* Peel Methanol (TPM). The maceration of *Z. zanthoxyloides* yielded three crude extracts: *Zanthoxylum* Stem Bark Hexane (ZSH), *Zanthoxylum* Stem Bark Ethyl acetate (ZSE), and *Zanthoxylum* Stem Bark Methanol (ZSM). The extracts were subjected to column chromatography over silica gel 60 (0.063-0.200 mm for CC, Merck, Germany). Each extract was pre-adsorbed on silica gel, loaded onto a wet-packed silica gel column, and eluted gradient-wise using hexane, ethyl acetate, and methanol. Ten fractions of ~20 mL were collected with 200 mL each of increasing ratios of ethyl acetate in hexane and then methanol in ethyl acetate to obtain a total of between 150 and 170 fractions for each extract. Fractions with similar constituents were combined based on their TLC profiles.

4.3. Spectroscopic Analysis

The spectral analysis was carried out at the Institute of Organic Chemistry, University of Glasgow, Scotland. One and two-dimensional NMR spectra of the compounds were obtained on a Bruker AVIII (400 MHz) spectrophotometer using deuterated chloroform ($CDCl_3$) or acetone $(CD_3)_2CO$) as solvents. The spectra were processed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain), and chemical shifts were referenced against residual solvent peaks. Mass spectra were acquired on a QTOF high-resolution Agilent 6545 mass spectrometer coupled to an Agilent Infinity 1290 UHPLC system.

4.4. Parasite Culture

In vitro cultures of *T. b. brucei*, *T. evansi* and *T. equiperdum*: Bloodstream trypomastigotes of *T. b. brucei* (s427 wild-type) [58], multi-drug-resistant *T. b. brucei* (B48) [49], *T. evansi* (AnTat 3/3) [59] and *T. equiperdum* (BoTat1) [51] were cultured in HMI-9 medium (Invitrogen, UK) supplemented with 3.0 g/L $NaHCO_3$, 14.3 µL/L β-mercaptoethanol, adjusted to pH 7.4, 10% (v/v) heat-inactivated Foetal Bovine Serum (FBS; Gibco Life Technologies, Paisely, UK) and incubated at 37 °C in a 5% CO_2 atmosphere as described. The cells were passaged every 72 h.

In vitro cultures of *T. congolense*: Bloodstream forms of *Trypanosoma congolense* (IL3000) were cultured as described [60] in Tc-BSF3 medium at 34 °C in a 5% CO_2 atmosphere. The basal medium (1 L) was prepared using 9.60 g MEM (Sigma M0643), 5.96 g HEPES, 2.20 g $NaHCO_3$, 1 g D-glucose, 110 mg sodium pyruvate (Sigma P3662), 10.68 mg adenosine, 14 mg hypoxanthine, 4 mg thymidine,

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14.10 mg bathocuproinesulfonic acid (Sigma B1125). The basal medium (150 mL) was then supplemented with 40 mL heat-inactivated fresh goat serum (GIBCO, UK), 10 mL heat-inactivated serum plus (Sigma-Aldrich, UK), 2.8 µL β-mercaptoethanol, 1.6 mL glutamine and 2 mL penicillin/streptomycin solution to obtain 200 mL of Tc-BSF-3 medium [61]. The cells were passaged every 72 h.

In vitro Cultures of L. mexicana: Leishmania mexicana promastigotes of strain MNY/BZ/62/M379 [62] were cultured in HOMEM medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution (Gibco Life Technologies) at 27 °C as described [63]. The cells were passaged every 72 h.

In vitro Cultures of HEK 293 cells: The Human Embryonic Kidney cell line HEK 293 strain was grown in a standard medium containing 500 mL Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), 50 mL of heat-inactivated FBS (GIBCO, UK), 5 mL penicillin/streptomycin solution [64]. All constituents were mixed under sterile conditions in the DMEM bottle and stored at 4 °C before use. The cells were incubated at 37 °C and 5% CO_2 and passaged twice a week in a vented flask until 80-85% confluence.

4.5. Antitrypanosomal and Antileishmanial Assays

The *in vitro* drug sensitivity assay in bloodstream forms of trypanosomes and *Leishmania* and the toxicity to mammalian cells were carried out at the School of Infection and Immunity, College of Medical, Veterinary and Life Sciences, University of Glasgow, Scotland according to the Resazurin (Alamar blue) assay methods used in recent reports [52,56]. The assay is based on the reduction of the blue, non-fluorescent dye resazurin sodium salt (Sigma) by living, but not dead cells, to the red, fluorescent metabolite resorufin [65]. Stock solutions of isolated compounds were prepared at 10 mg/mL in dimethyl sulfoxide (DMSO (Merck)) from which stocks of 200 µg/mL (400 µg/mL for *L. mexicana*) were prepared in the appropriate respective medium for each strain according to the standard protocol [56,66]. Exactly 200 µL of each stock was added to the first well of each row on a 96-well microplate, setting up for doubling dilutions of seven different drugs per plate, each over one row of a 96-well plate. Similarly, 200 µL of current trypanocides (positive control: diminazene aceturate for *T. congolense,* pentamidine for all other species) prepared at appropriate concentrations for each cell was added to the first well and included in each of the triplicate experiments. Next, 100 µL medium was pipetted using a multichannel micropipette into all remaining wells, and 100 µL of the drug was taken by a multichannel pipette from the first column and mixed gently with the medium in the wells of the second column, then another 100 µL from second column wells are added to third column wells and so on to the last-but-one column, creating a doubling dilution of 11 points. The last column of the plate was the drug-free negative control. Cell counts were performed using a haemocytometer and cell density was adjusted to the desired concentration of cells/mL (2×10^5 for *T. b. brucei* s427, B48, *T. equiperdum*; 2×10^6 for *L. mexicana,* 4×10^5 for *T. evansi*, 5×10^5 for *T. congolense*) of which 100 µL was added to all the wells in the plate, making a final top concentration of 100 µg/mL drug (200 µg/mL for *L. mexicana*). This was followed by incubation of the plates at 37 °C/5% CO_2 (s427, B48, *T. evansi* and *T. equiperdum*), 34 °C/5% CO_2 (*T. congolense*) or 27 °C (*L. mexicana*) for 48 h (72 h for *L. mexicana*) before the addition of the resazurin dye (20 µL of 125 mg/L), and a further incubation under the same conditions for 24 h (48 h for *L. mexicana*). The extended incubation period for *L. mexicana* is due to the slower metabolism of the dye by *Leishmania* promastigotes [52]. The fluorescence of the wells was read using a FLUOstar OPTIMA Fluorimeter (BMG Labtech, Durham, NC, USA) at wavelengths 544 nm for excitation and 590 nm for emission and a gain of 1250. The fluorescence values were analysed using GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA, plotting the data to an equation for a sigmoidal dose-response curve with variable slope and the EC_{50} (half maximal effective concentration of isolated compounds or control drug that induces a response halfway between the baseline and maximum after 72 h exposure time) values were determined. P-values were calculated using the unpaired Student's t-test. All experiments were performed on at least three different, fully independent occasions.

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4.6. Toxicity Assays

The toxicity assay of the isolated compounds to mammalian cells was carried out on human embryonic kidney (HEK 293) cells, essentially as previously described [67]. Exactly 100 µL (3×10^5 cell/mL) of a cell suspension grown in a vented flask (~80% confluence) at incubation conditions of 37 °C/5% CO_2 was added to each well of a 96-well plate. The plate was incubated for 24 h cytoadherence, after which 100 µL of a serially diluted drug was added (prepared in a separate sterile plate over one row). Phenylarsine oxide (PAO (Sigma)) was used as the positive control. The cells were then incubated for a further 30 h before the addition of 10 µL sterile Alamar Blue solution (125.0 mg/mL resazurin sodium salt (Sigma) in distilled water), followed by a further 24 h incubation. Fluorescence measurements and data analysis were performed as for the anti-parasite assays. The selectivity index (SI) was calculated as EC_{50} (HEK)/EC_{50} (parasite). All experiments were performed on at least three different, fully independent occasions.

5. Conclusions

Tarkalynin A and B, taccalonolide A and its propanoate were isolated from *Tacca leontopetaloides*, while taraxerol acetate, dihydrochelerythrin and fagaramide were isolated from *Zanthoxylum zanthoxyloides*. Dihydrochelerythrin (**6)** showed the highest anti-kinetoplastid activity across the board (all EC_{50} <3 µg/mL), and the highest selectivity index values. Furthermore, fagaramide (**7)** also displayed broad anti-kinetoplastid activity, albeit somewhat less potently (all EC_{50} < 8.5 µg/mL). Tarkalynin A (**1)** and taraxerol acetate (**5)** also displayed activity with EC_{50} below 10 µg/mL against *T. equiperdum*. No loss of activity was observed towards the multi-drug-resistant *T. b. brucei* clone B48. The study shows that compounds from *Z. zanthoxyloides*, in particular, have genuine anti-kinetoplastid properties, and these should now be studied in more detail.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: title; Table S1: title; Video S1: title.

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