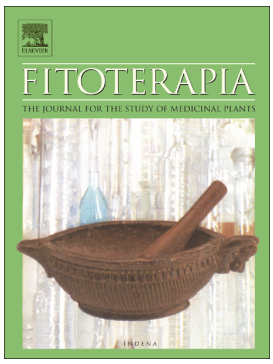

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Identification of Tagitinin C from *Tithonia diversifolia* as antitrypanosomal compound using bioactivity-guided fractionation

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Tithonia diversifolia (Asteraceae), is used as traditional medicine in tropical countries for the treatment of various diseases, including malaria. Although numerous studies have assessed the antimalarial properties, nothing is known about the effect of *T. diversifolia* extracts on trypanosomiasis. In this study extracts of *T. diversifolia* aerial parts were evaluated for their bioactivity against *Trypanosoma brucei*. The activity was studied against bloodstream forms of *T. brucei* (TC221), as well as against mammalian cells (BALB/3T3 mouse fibroblasts), as a counter-screen for toxicity. Both methanolic and aqueous extracts showed significant effects with IC_{50} values of 1.1 and 2.2 µg/mL against *T. brucei* (TC221) and 5.2 and 3.7 µg/mL against BALB/3T3 cells, respectively. A bioassay-guided fractionation on the methanolic extract yielded in identification of active fractions (F8 and F9) with IC_{50} values of 0.41 and 0.43 µg/mL, respectively, against *T. brucei* (TC221) and 1.4 and 1.5 µg/mL, respectively, against BALB/3T3 cells,. The phytochemical composition of the extracts and the purified fractions were investigated using HPLC-ESI-MS/MS and 1D and 2D NMR spectra showing the presence of sesquiterpene lactones that in turn were subjected to the isolation procedure. Tagitinin A and C were rather active but the latter presented a very strong inhibition on *T. brucei* (TC221) with an IC_{50} value of 0.0042 µg/mL. This activity was 4.5 times better than that of the reference drug suramin. The results of this study shed light on the antitrypanosomal effects of *T. diversifolia* extracts and highlighted tagitinin C as one of the possible responsible for this effect. Further structure activity relationships studies on tagitinins are needed to consider this sesquiterpenes as lead compounds for the development of new antitrypanosomal drugs.

Keywords: BALB/3T3 mouse fibroblasts; Mexican sunflower; HPLC-ESI-MS/MS; NMR; Tagitinins A and C; Sesquiterpene lactones

Abbreviations:

1D and 2D NMR: One and two-dimensional nuclear magnetic resonance

BALB//3T3 = Mouse fibroblasts

CH_2Cl_2/MeOH: Methylene chloride/Methanol

DMSO: Dimethylsulfoxide

HAT: Human African Trypanosomiasis

NTDs: Neglected tropical diseases

SAR: Structure-activity relationship

SI: Selectivity index

STLs : Sesquiterpene lactones

T. brucei: *Trypanosoma brucei*

TC221: *T. brucei* bloodstream-form parasites

TLC: Thin layer chromatography

WHO: World Health Organization

Introduction

Neglected tropical diseases (NTDs) are classified as a subgroup of infectious illnesses affecting more than a billion people worldwide and causing significant public health problems, particularly in marginalized populations of rural developing countries (Benelli and Mehlhorn, 2016). People suffering from NTDs live in close contact with infectious vectors and domestic livestock and represent an unattractive market to pharmaceutical industry investments. For this reason, in 2010 the World Health Organization (WHO) released its first report on the global treat posed by NTDs. In this report WHO prioritized 17 NTDs. Of these 17, the three major protozoan diseases, namely human African trypanosomiasis (HAT), Chagas disease and leishmaniasis, have been accounted as life-threatening infections (Mehlhorn 2015).

African trypanosomiasis, which includes African sleeping sickness in humans (HAT) and "Nagana" in cattle, are fatal vector-borne diseases caused by the kinetoplastid protozoan pathogen *Trypanosoma brucei* (*T. brucei*). HAT is endemic in 36 sub-Saharan African countries and is transmitted to the host by the bites of tsetse flies (*Glossina* spp., Glossinidae). The disease progresses through two stages, *i.e.* from a haemolymphatic first stage to a meningoencephalitic second stage, in which the parasites enter the central nervous system. HAT is caused by two subspecies of the parasite *T. brucei*: *T. b. gambiense* (West Africa) and *T. b. rhodesiense* (East Africa). *T. b. rhodesiense* as well as other African trypanosomes such as *T. congolense*, *T. vivax* and *T. brucei brucei* infect wild and domestic animals causing "Nagana", which has a significant impact on socioeconomic development in many parts of rural Africa. In

about eleven million square kilometres of farming land infested by the tsetse flies, cattle represent an important zoonotic reservoir for human infections.

There is a pressing need to develop novel and cost-effective drugs to treat HAT, especially at later stages when the parasites infect the brain. The lack of potential vaccine candidates together with the uncertainty for a successful chemotherapy against HAT add impetus to explore naturally occurring compounds as inspiring leads for designing future trypanocidal drugs (Scotti et al., 2016).

Among several medicinal plants traditionally used in Africa to treat different diseases and having potential as a source of trypanocidal compounds, our attention has been attracted by *Tithonia diversifolia* (Hemsl.) A. Gray. This plant, belonging to the Asteraceae family, is also known as Mexican sunflower, being native to central America. It is distributed in tropical areas of South America, Asia and Africa where it can be considered as a weed of cultivated and non-cultivated lands (Chagas-Paula et al., 2012; Ayeni et al., 1997). Given its abundance, *T. diversifolia* enjoys a long use as a traditional remedy in tropical regions (Heinrich, 2000). In particular, the plant leaves are used in Africa to treat malaria (Heinrich et al., 1998; Njoroge and Bussman, 2006). Noteworthy, this use was then confirmed by scientific studies (Goffin et al., 2002; Elufioye and Agbedahunsi, 2004; Oyewole et al., 2008) where the bioactive compounds were identified as germacranolide-type sesquiterpene lactones called tagitinins, with tagitinin C as the lead compound (Goffin et al., 2002; Ferreira et al., 2005). These sesquiterpene lactones (STLs) were also proven to exhibit anti-inflammatory/analgesic (Owoyele et al., 2004), antiproliferative (Liao et al., 2011), antifeedant (Ambrósio et al., 2008) and insecticidal activities (Castaño-Quintana et al., 2013). Other secondary

metabolites characteristics of *T. diversifolia* are diterpenoids, flavonoids, chlorogenic acid derivatives and essential oils (Orsomando et al., 2016).

Given the potential of *T. diversifolia* secondary metabolites to interact with protozoan parasites (Goffin et al., 2002), here we have investigated the effect of a *T. diversifolia* methanolic leaf extract against *T. brucei* using a bioassay-guided fractionation approach, leading to the identification of tagitinin A and C as main active constituents. To our knowledge, nothing is known about either the anti-trypanosomal activity of *T. diversifolia* extracts or the components responsible for this effect.

Different techniques were applied for the identification of phytoconstituents. Methanolic extract was initially studied by ^{1}H NMR and 2D NMR to obtain information about the main compounds. The total extract was subsequently fractionated by flash chromatography in 19 fractions of increasing polarity which were tested against *T. brucei in vitro*. A few fractions showed antitrypanosomal activity. Active fractions were analysed by HPLC MS-MS and by NMR to find the phytoconstituents responsible for activity towards the protozoan parasite. The two most abundant sesquiterpene lactones in the active fractions were finally purified and assessed for their antitrypanosomial activity.

Material and Methods

Chemicals and reagents

LC–MS grade acetonitrile (ACN) and methanol (MeOH) were obtained from J. T. Baker (Phillipsburg, USA). HPLC-grade formic acid was purchased from Dikma

Tech. Inc. (Beijing, China). Cycloexane was obtained from Scharlau. Deuterated chloroform was purchased from Sigma-Aldrich. Water (H_2O) was purified by a Milli-Q system (Millipore, Billerica, MA, USA) in our laboratory. Other reagents were of analytical grade.

Plant material

Leaves of *T. diversifolia* (Asteraceae) were collected in the city of Dschang, western region of Cameroon (N 05°26'18", E 10°04'07", 1450 m a.s.l) by Saague T. Maximiliene and Prosper C. Biapa Nya in January 2016 during the dry season. Botanical authentication was performed by plant taxonomist Mr. Nana of the National Herbarium, Yaoundé, Cameroon, where a voucher specimen coded 10196/HNC was deposited.

Preparation of plant extracts

T. diversifolia leaves were air-dried in the shade at room temperature (≈25°C) for 3 days and conserved in wrapping papers before extraction. Fifty g of dry leaves were reduced into powder using a blender MFC DCFH 48 IKA-WERK (D-Staufen) equipped with sieves of 2-mm size in diameter. The powder was subsequently macerated in 500 ml of methanol (MeOH) for 24 h and filtered. The filtrate was concentrated under reduced pressure at 30°C with a rotary evaporator and freeze-dried to obtain a crude MeOH extract (2.64 g, 5.3% yield, Tith-MeOH). Using the same protocol, an aqueous extract was obtained by maceration of 5 g of leaves in 50 mL of

deionized water to get 0.42 g (8.4%, Tith-H_2O) of extract. The extracts were kept in glass vials protected from light at -20°C before chemical analysis and anti-trypanosomal experiments.

Apparatus and chromatographic conditions

Detection and quantification of the compounds were performed on an LC–MS system. The LC–MS equipment (Varian Inc.) contained a chromatographic system (Varian LC-212) coupled with a Varian 500-MS (ion trap) mass spectrometer fitted with an ESI source (Varian). MS conditions were the following: needle potential −5.0 kV, shield 600 V, spray chamber temperature 50°C, drying gas pressure 10 psi, drying gas temperature 350°C, capillary voltage 80 V, RF loading 100, and MS range 150–2000 Da. MS^n spectra were obtained during the chromatography run by using the turbo-dds (tdds) utility of the instrument. HPLC-DAD analysis was carried out by an Agilent 1100 series liquid chromatograph equipped with an Agilent 1100 Diode Array (DAD). An Eclipse XDB-C8, 5 µm, 4.6 x 150 mm column (Agilent Technologies) was used. The mobile phase consisted of (A) aqueous formic acid (0.1 %) and (B) acetonitrile. Gradient conditions were: 0–30 min, linear gradient from 10 % to 100 % of B; 30–35 min, isocratic conditions at 100 % of B; 35–36 min, linear gradient from 100% to 10% of B; 36-40 min, isocratic conditions at 10% of B. Flow rate: 1 mL/min. Calibration curves were obtained by standard solutions of rutin for flavonoid derivatives (UV detection at 350 nm), chlorogenic acid for caffeoylquinic derivatives (UV detection at 330 nm) and gallic acid for small phenols (UV detection at 280 nm). The concentration ranges were 11.7–117, 13.2–132, and 14.6-146 µg/mL for chlorogenic acid, rutin and

gallic acid, respectively. The limits of detection (LOD) and quantification (LOQ) were 1.5 and 4.0 μg/ml and 0.5 and 1.5 μg/ml for chlorogenic acid and rutin, respectively.

Fractionation of the extract, isolation of Tagitinin A and C

The crude methanolic extract of *T. diversifolia* leaves (1 g) was dissolved in methanol and adsorbed on silica gel (40 mesh, 2 g). Subsequently, the solvent was removed under vacuum to obtain a dried powder. This powder was packed in a pre-column, which was fixed on a Silica column (Buchi Sepacore® silica 12 g). Separation was performed on a Varian Intelliflash Flash chromatograph. The eluent was initially cyclohexane followed by an increasing percentage of methanol up to 30 % in 120 min. The flow rate was 1 mL/min and the chromatogram was monitored at 220 and 340 nm. Eluted fractions were checked by TLC and collected in 19 groups. The solvent was removed under vacuum and the weight of each fraction obtained was as follows: 1 (25.8 mg), 2 (15.0 mg), 3 (17.1 mg), 4 (18.5 mg), 5 (23.1 mg), 6 (26.7 mg), 7 (13.9 mg), 8 (16.7 mg), 9 (19.4 mg), 10 (18.0 mg), 11 (8.9 mg), 12 (5.3 mg), 13 (7.0 mg), 14 (4 mg), 15 (104.6 mg), 16 (128.4 mg) 17 (78.7 mg), 18 (59.7 mg), 19 (38.2 mg).

For tagitinin isolation 250 g of dried plant material was extracted using dichloromethane (550 mL x 3 times) using ultrasound bath. Liquids were collected, filtered and pooled and solvent was evaporated under vacuum. The crude extract (9.5 g) was used for chromatographic separation. A silica gel column (10 x 100 cm) was packed using hexane as solvent. Extract was charged in the column and hexane was used for elution, then hexane/ethyl acetate with an increasing ratio, starting from 6/1 to 1/1, was used as eluent system. Fifty-five fractions were pooled on the basis of their

chromatographic behaviour. Further separations were achieved on Sephadex LH20 column (5 x 40 cm) eluting with dichloromethane. Tagitinin A and tagitinin C were isolated as pale oil (600 and 250 mg, respectively). Their spectral data were compared with those of literature (Baruah et al., 1979).

NMR analysis was obtained on a Bruker AVANCE III spectrometer operating at 400.13 MHz for ^{1}H-NMR. 2D spectra, HSQC-DEPT, HMBC, COSY and TOCSY were used for identification of compounds occurring in mixtures. Samples were dissolved in deuterated chloroform and used for analysis. For quantitative purposes, previously published conditions were used (Comai et al., 2010).

T. brucei and mammalian cell culture

The cell culture conditions and the growth inhibition assay on T. brucei and Balb/3T3 cells were performed as described before (Petrelli et al., 2016). *T. brucei* bloodstream forms (TC221) were cultured at 37 °C with 5 % CO_2 in HMI-9 medium supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (Gibco). Mouse BALB/3T3 fibroblast (ATCC no CCL-163) were cultivated at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, glutamine (0.584 g/L) and 10 mL/L of 100 × penicillin-streptomycin (Gibco BRL) (Hirumi and Hirumi, 1989).

Growth inhibition assay on _T. brucei_ and BALB/3T3 cells

The methanolic extract and its fractions were dissolved in dimethyl sulfoxide (DMSO). They were serially diluted with growth medium to concentrations ranging

from 2×10^{-5} to 200 µg/mL in 96-wells microtiter plates (100 µL/well). Subsequently, 100 µL of *T. brucei* or mammalian cell culture was added to each well (20,000 cells/well). After 48 h incubation, the plates were treated for 24 h with 20 µL of 0.5 mM resazurine (Sigma-Aldrich). They were subsequently quantified by fluorescence (540 nm excitation and 590 nm emission) using an Infinite M200 microplate reader (Tecan Group, Ltd.). The IC_{50} values were calculated by fitting the data to a log inhibitor vs. response curve (variable slope, four parameters) using the GraphPad Prism 5.04 software.

Results and Discussion

Characterization of *T. diversifolia* extracts by NMR and HPLC-MS^n

In order to establish the phytochemical composition of the Tith-MeOH extract, we decided to use a multi-technique approach. As a first step the Tith-MeOH extract was dissolved in deuterated chloroform (final concentration 20 mg/mL) and different spectra were acquired namely ^{1}H-NMR, HSQC-DEPT, HMBC and COSY experiments. Diagnostic signals of sesquiterpene sp^2 protons were detected, and assigned on the basis of the ^{1}H, HSQC, HMBC and COSY data as compared with the literature (Baruah et al., 1979). These results are reported in Table 1. A representative portion of a HSQC-DEPT spectrum is shown in Figure 1 indicating the assigned positions.

Many other signals were detected and may be linked to other phytoconstituents. For this reason, HPLC-MS^n analysis was performed in order to identify other secondary metabolites occurring in the crude extract. The fragmentation pathways for the main

sesquiterpenes of *T. diversifolia* were also studied and are reported in Figure 2. Six different sesquiterpenes were detected and their structures were determined on the basis of HPLC-MS^n analysis. Limited information is available related to ESI/MS behaviour of these sesquiterpenes. Abea et al. (2015) reported proposed fragmentation pathways of tagitinin F. In our analysis, we observed that the tirotundin derivatives (compounds **4** and **5**, Figure 3) are characterized in MS^2 by three main fragments ascribable to the loss of an ester side chain, a water molecule and an ether bridge or lactone ring with rearrangement at *m/z* 265, 247 and 229, respectively (Figure 2). The same fragments are detectable in the MS^2 spectra of compound **6** at *m/z* 279, 261 and 243 (Figure 2) presenting additional 14 Da due to the presence of a methyl group. Polyphenol derivatives, mainly caffeic acid derivatives and flavonoids, were also identified. Table 2 reports the different constituents identified. Phytochemical analysis showed that the *T. diversifolia* extract is complex and contains sesquiterpenes, phenylpropanoids and flavonoid derivatives as main constituents.

***In vitro* evaluation of antiprotozoal activity of bioactive fractions, tagitinin A and tagitinin C**

Two extracts (Tith-MeOH and Tith-H_2O) were prepared from the aerial parts of *T. diversifolia* to assess their *in vitro* antitrypanosomal activity (Table 3). The methanolic extract was two times more active against *T. brucei* than the aqueous extract (IC_{50} = 1.08 and 2.18 µg/mL, respectively) and was therefore chosen for further fractionation. The methanolic extract was subjected to chromatographic separations on silica gel and the fractions with increasing polarity collected after each purification step (F1-F19) were tested against the TC221 cells with the purpose of following the

antitrypanosomal activity. The bioassay-guided purification of the Tith-MeOH extract gave two fractions (F8 and F9, Table 3) showing significantly higher antitrypanosomal effects in our cell-based assay (IC_{50} = 0.41 and 0.43 μg/mL, respectively). Bioassays performed on the fractions from Silica-based chromatography of Tith-MeOH extracts showed the effect on *T. brucei vs.* mammalian cell proliferation (Table 3).

^{1}H-NMR of the 16 assayed fractions (Figure 4) clearly shows the presence of signals ascribable to sesquiterpene derivatives as well as other aromatic compounds in the most active mixtures, namely F7 to F11. In all the fractions, the signals ascribable to phenolic and phenylpropanoid derivatives were not detectable or poorly detectable. We decided to consider the most active fractions, thus the ones showing IC_{50} <1.6 μg/mL, i.e. fractions 8-11, which were analysed for the contents of tagitinins A and C. Due to the non-commercially availability of tagitinins as reference compounds, the quantification of the main derivatives, namely tagitinins A and C, was performed using an ^{1}H-NMR approach using caffeine as internal standard (Comai et al., 2010) and the amounts of these STLs in the active fractions were reported in Table 4. Results showed that bioactivity of the obtained fractions can be in part ascribed to STLs constituents. Isolated tagitinin A and C were also tested. As reported in Table 5, tagitinin C resulted by far the most active compound compared with active fractions. Notably, tagitinin C was 4.5 times more active than the reference compound suramin. Furthermore, selective index for tagitinine C was 3 showing preferential effect on target cells compared with tagitinin A (1.3). The large difference in bioactivity observed for the tagitinin C and A indicates the need for the evaluation of other tagitinine derivatives in order to explore possible structure activity relationships.

Potent and selective antitrypanosomal activity of different plant sequiterpenes such as α-eudesmol, hinesol, nardosinone and 4-peroxy-1,2,4,5-tetrahydro-α-santonin (Otoguro et al. 2011) has been previously reported, as well as the possible usefulness of sesquiterpenes as lead compounds for the development of new antitrypanosomal drugs (Saeidnia et al. 2013).

As regards the mode of action of *T. diversifolia* STLs on trypanosome cell, some considerations can be made. As reported by Schimdt et al. (1999), STLs possessing an α,β-unsaturated moiety in the form of α-methylene-γ-lactone can act as a Michael acceptor, reacting with nucleophiles such as free thiol groups (R-SH) in proteins leading to macromolecular dysfunction, oxidative stress and genetic mutations as a result of the oxidative stress. The presence of one potentially reactive α,β-unsaturated carbonyl group as a pharmacophore is often associated with significant antiprotozoal and cytotoxic activities. Tagitinin A has α,β-unsaturated carbonyl group in the γ-lactone ring, whereas tagitinin C has a carbonyl group conjugated with two double bonds in the germacrene ring and an α,β-unsaturated carbonyl group in the γ-lactone ring. The measured activity of the two compounds showed the importance of the carbonyl group conjugated with two double bonds because of the greater antitripanosomal activity of tagitinin C. The mechanism of these compounds against trypanosomes may be related to the capability to form thiol adducts with components found in the cellular environment such as trypanothione, glutathione and SH-groups in proteins. The lack of reduced trypanothione and other SH-group containing substances may in turn lead to that the parasite cells become more vulnerable to oxidative stress (Wink, 2008). The same

mechanism of action may occur in the mammalian fibroblasts (BALB/ 3T3), where glutathione depletion renders the cells prone to oxidative stress.

Conclusions

Overall our results, although preliminary, confirmed the usefulness of *T. diversifolia* as an effective herbal remedy in the African traditional medicine and highlighted the opportunity to use the tagitinins as inspiring leads for designing future trypanocidal drugs. Further studies are needed in particular to increase the knowledge about the mode of action and the possible molecular targets related to the antitrypanosomal activity of tagitinins. The opportunity to study different derivatives as well as strictly related sesquiterpene can be a good opportunity to explore structure activity relationships. Also synthetic or semisyntetic derivatives can be produced in order to follow a rational approach for the development of more active compounds. Finally further investigation will be needed in order to assess the real efficacy *vs.* toxicity through *in vivo* assays.

Conflict of interest

The authors declare no competing interests.

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Table 1. Diagnostic assignments of tagitinins A and C from NMR spectra of Tith-MeOH extract. Assignments were obtained comparing HSQC-DEPT, HMBC and COSY data with the literature (Baruah et al., 1979).

	Tagitinin C			Tagitinin A	
Position	δH	δC	Position	δH	δC
1	6.93 d *J*=13.5	159.6	1	4.23	78.4
2	6.21	129.5	6	4.55	81.8
13	6.36-5.81	124.0	8	5.59	69.9
5	5.88	137.6	13	6.25-5.53	121.7

Table 2. HPLC-MS constituents identified in *T. diversifolia* methanolic extracts.

Ret. time	Compound	Polarity	$[M-H]^-$ or $[M+H]^+$	Fragments
2.53	Quinic acid[a]	Negative	191	85
12.0	Chlorogenic acid[a]	Negative	353	191-179
13.9	Rutin[a]	Negative	609	301-271-255-179-151
14.5	Quercetin-glucuronide	Negative	477	301-179-151
14.6	1,5-Dicaffeoyl quinic acid[a]	Negative	515	353-191-173-127
14.8	3,4-Dicaffeoyl quinic acid	Negative	515	353-191-179-173
15.7	3,5-Dicaffeoyl quinic acid[a]	Negative	515	353-191-173-127-111-85
18.8	Isorhamnetin	Negative	315	300-272-228
19.5	Tagitinin A	Negative	367	279-261-235
		Positive	369	281-263-245
19.7	Tirotundin 3-*O*-methylether	Positive	367	279-261-243-233-215
		Negative	365	277-233-215-191-176
20.8	Hispidulin	Negative	299	284-256-227-212
24.6	Tagitinin C	Positive	349	261-243-215-

				173
27.4	1-β-Methoxydiversifolin	Positive	395	293-275-261-199-181
26.8	8-β-O-(2-methylbutyroyl)-Tirotundin or 8-β-O-(isovaleroyl)-Tirotundin	Positive	367	265-247-229

[a]confirmed by the injection of a reference compound

Table 3. Activity of *T. diversifolia* leaves extracts and fractions 1-19 against *T. brucei* TC221 and BALB/3T3.

Samples	**IC_{50} (µg/mL)**		**Selectivity Index (SI)**
	***T. brucei* (TC221)**	**BALB/3T3**	
Crude methanol extract (Tith-MeOH)	1.05 ± 0.18	5.15 ± 0.82	4.9
Crude aqueous extract (Tith-H_2O)	2.18 ± 0.06	6.71 ± 0.48	3
***Fractions from methanol extract* (Tith-MeOH)**			
Fraction 1	62.05 ± 20.4	-	-
Fraction 2	13.69 ± 1.8	52.16 ± 3.1	3.8
Fraction 3	8.29 ± 0.79	17.52 ± 2.9	2.1
Fraction 4	8.28 ± 0.56	12.85 ± 1.1	1.5
Fraction 5	4.37 ± 1.98	7.65 ± 0.85	1.7
Fraction 6	1.66 ± 0.66	4.37 ± 0.61	2.6
Fraction 7	1.67 ± 0.51	2.78 ± 0.03	1.6
Fraction 8	0.41 ± 0.22	1.44 ± 0.04	3.3
Fraction 9	0.43 ± 0.09	1.47 ± 0.04	3.4
Fraction 10	1.09 ± 0.37	2.36 ± 0.17	2.1
Fraction 11	1.20 ± 0.21	2.66 ± 0.18	2.2
Fraction 12	2.34 ± 0.55	4.24 ± 0.49	1.8
Fraction 13	3.16 ± 0.89	6.03 ± 1.86	1.9
Fraction 14	2.02 ± 0.88	5.10 ± 0.64	2.5
Fraction 15	1.63 ± 0.61	3.17 ± 0.14	1.9
Fraction 16	7.65 ± 1.61	19.34 ± 2.76	2.5
Fraction 17	>100	-	-
Fraction 18	>100	-	-
Fraction 19	>100	-	-
Pure compounds	**µg/mL (µM)**	**µg/mL (µM)**	

Tagitinin A	0.356 ± 0.17 (0.97)	0.467 ± 0.21 (1.27)	1.3
Tagitinin C	0.0042 ± 0.0005 (0.0121)	0.012 ± 0.001 (0.036)	3
Reference drug	**μg/mL (μM)**	**μg/mL (μM)**	
Suramin	0.0191 ± 0.002 (0.0147)	n.d.*	n.d.*

*n.d. not determined

Table 4. Amount of tagitinins in the bioactivity-guided selected fractions. The amount is shown as the percentage of the total volume, IC_{50} are expressed in µg/mL.

Samples	**Tagitinin A %**	**Tagitinin C %**	***T. brucei* (TC221) IC_{50} (µg/mL)**
Crude methanol extract (Tith-MeOH)	0.83 ± 0.01	1.10 ± 0.01	1.05 ± 0.18
***Active fractions from methanol extract* (Tith-MeOH)**			
Fraction 8	10.8 ± 0.01	9.39 ± 0.01	0.41 ± 0.22
Fraction 9	8.36 ± 0.01	8.18 ± 0.01	0.43 ± 0.09
Fraction 10	7.03 ± 0.01	7.09 ± 0.01	1.09 ± 0.37
Fraction 11	7.07 ± 0.01	6.51± 0.01	1.20 ± 0.21

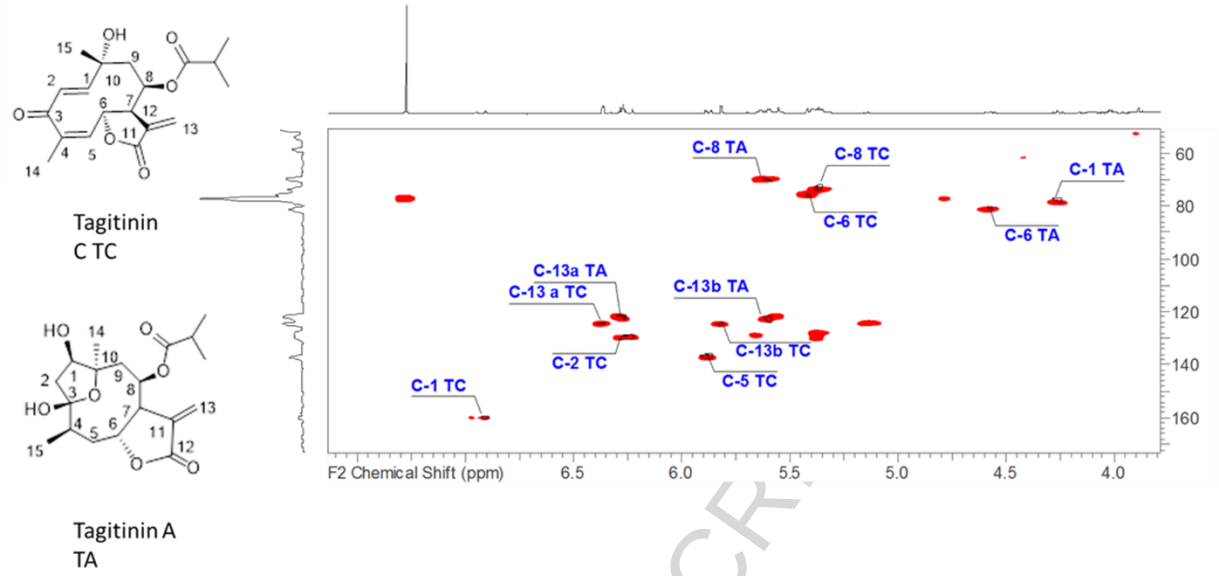
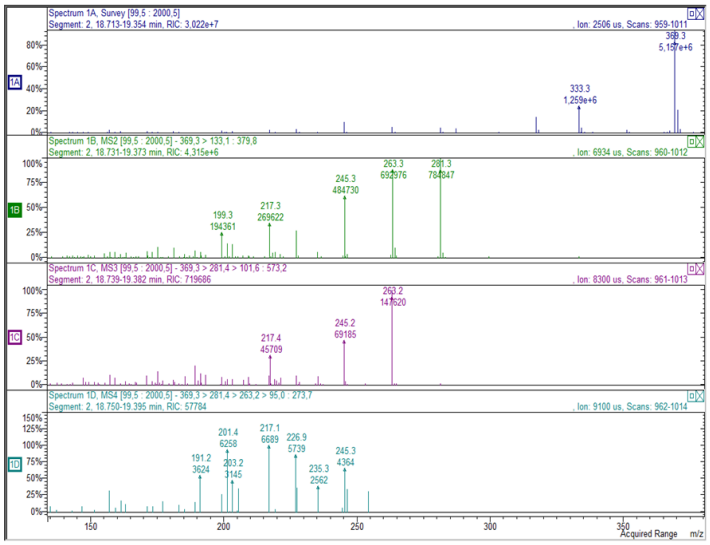
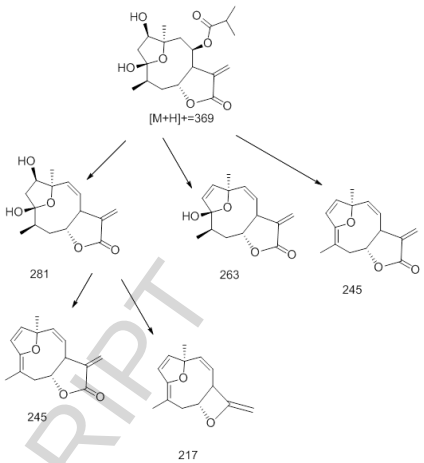
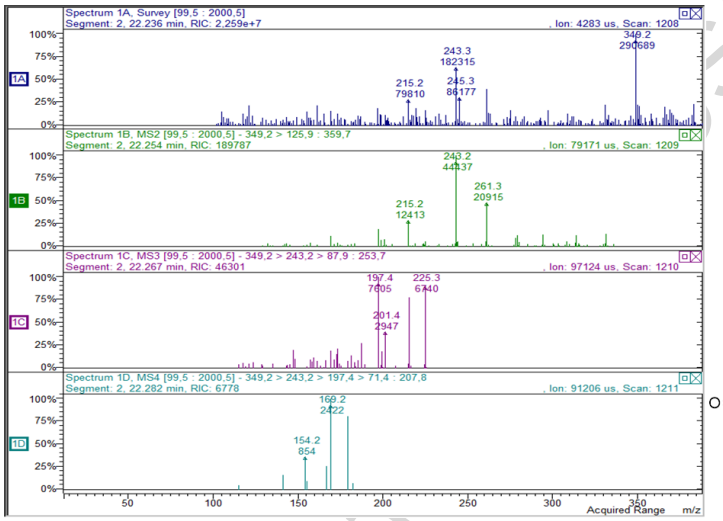


Figure 1. HSQC-DEPT (MeOD) of a *T. diversifolia* extract. TA and TC indicate signals of Tagitinin A and Tagitinin C, respectively.

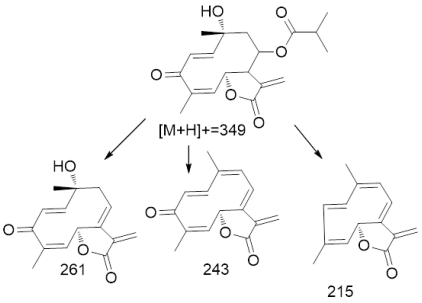


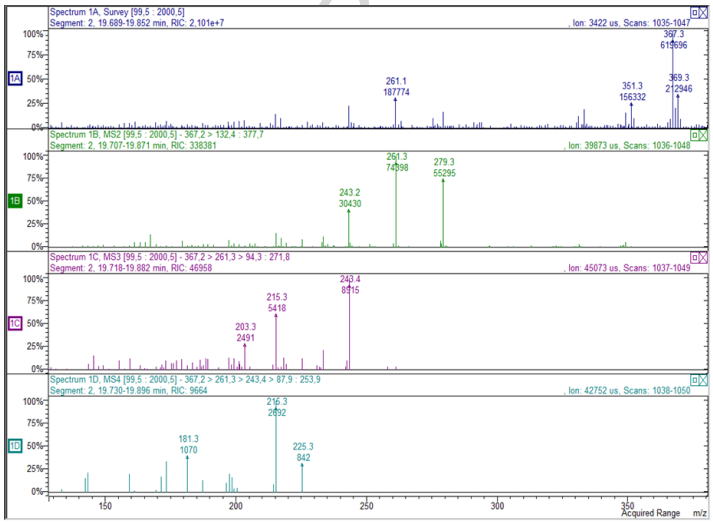
Tagitinin A



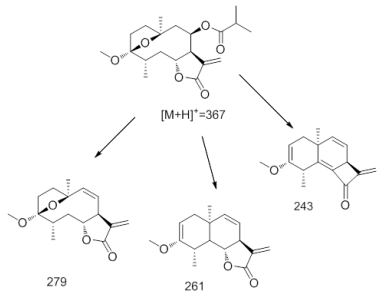


tagitinin C





Tirotundin 3-methyleter



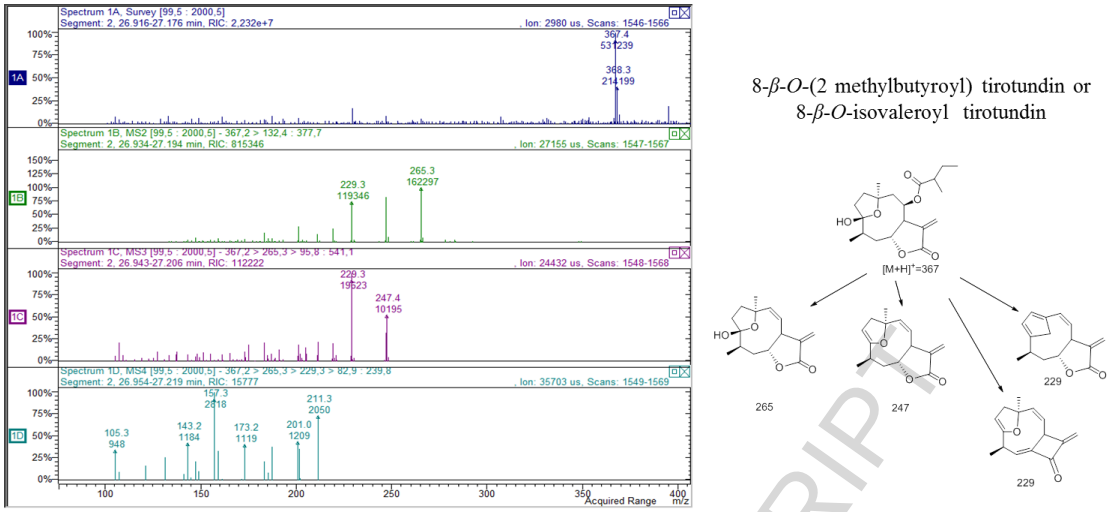
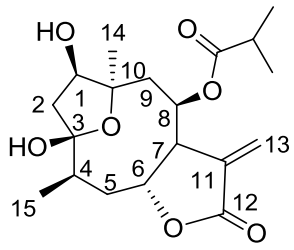
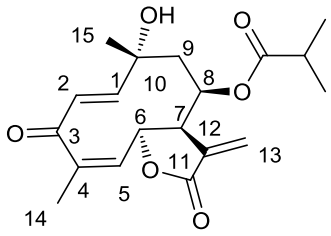


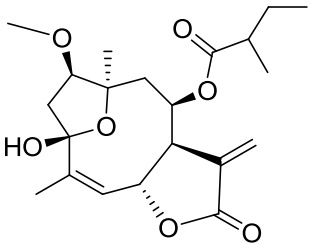
Figure 2. Mass spectra of the main sesquiterpenoids of *Tithonia diversifolia* and proposed fragmentation pathways.



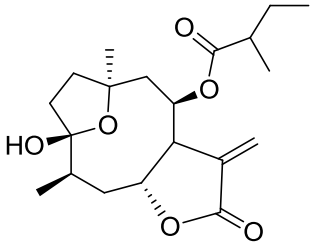
Tagitinin A
MW=368



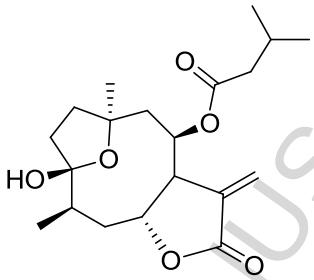
Tagitinin C
MW=348



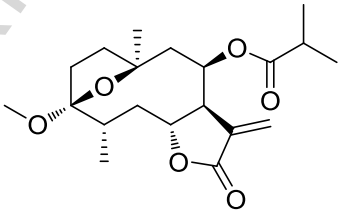
1β-methoxydiversifolin
MW=394



8β-O-(2-methylbutyroyl)-
tirotundin
MW=366



8β-O-(isovaleroyl)tirotundin
MW=366



tirotundin-3-O-methyleter
MW=366

Figure 3. Structures of main sesquiterpenes identified in *T.diversifolia* extracts.

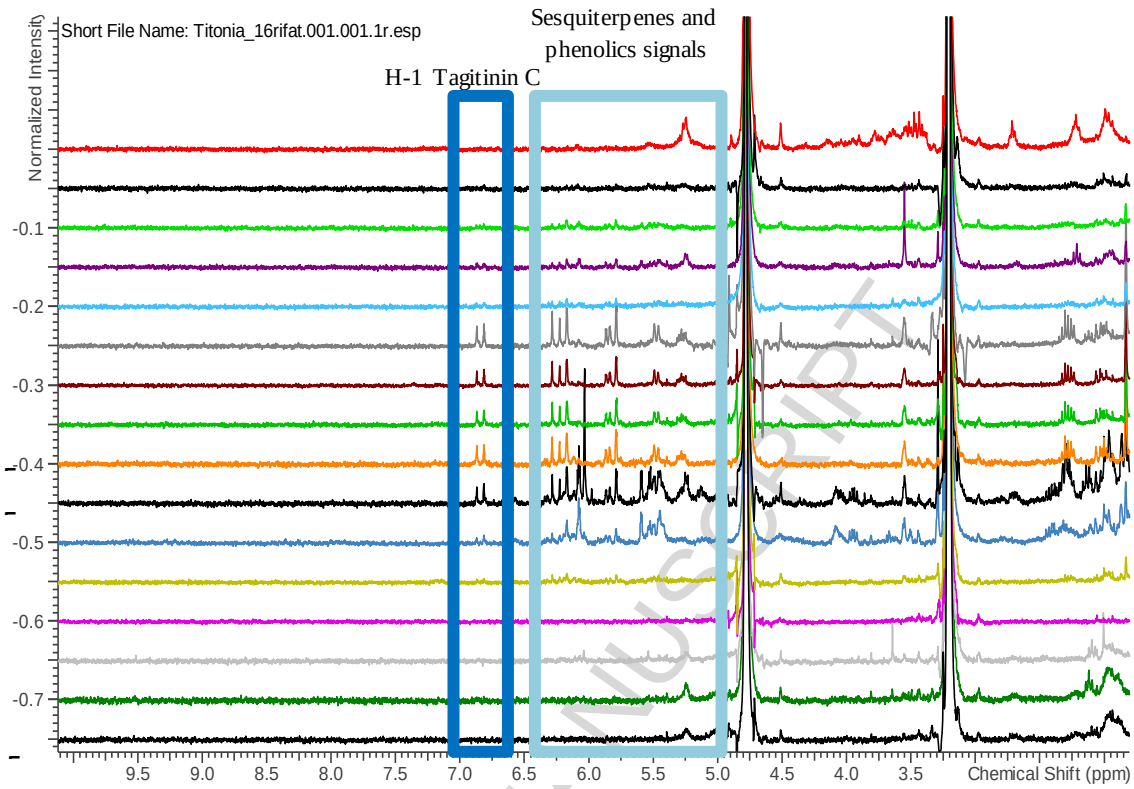


Figure 4. ^{1}H-NMR of *T. diversifolia* fractions 1-16, diagnostic signals indicating the presence of sesquiterpene and phenolics are highlighted with coloured rectangles.

Grafical abstract

Identification of Tagitinin C from *Tithonia diversifolia* as antitrypanosomal compound using bioactivity-guided fractionation