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

Stefania Sut[a], Stefano Dall'Acqua[a,*], Valeria Baldan[a], Stephane L. Ngahang Kamte[b], Farahnaz Ranjbarian[c], Prosper C. Biapa Nya[d], Sauro Vittori[b], Giovanni Benelli[e], Filippo Maggi[b], Loredana Cappellacci[b], Anders Hofer[c,**], Riccardo Petrelli[b]

[a] *Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy*
[b] *School of Pharmacy, University of Camerino, Camerino, Italy*
[c] *Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, Sweden*
[d] *Laboratory of Medicinal Plant Biochemistry, Food Science and Nutrition, Department of Biochemistry, Faculty of Sciences, University of Dschang, Dschang, Cameroon*
[e] *Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy*

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ABSTRACT

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.

1. 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 [5]. 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 [16].

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

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

* Correspondence to: Stefano Dall'Acqua, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy.
** Correspondence to: Anders Hofer, Department of Medical Biochemistry and Biophysics, Umeå University, SE-90187 Umeå, Sweden.
E-mail addresses: stefano.dallacqua@unipd.it (S. Dall'Acqua), anders.hofer@medchem.umu.se (A. Hofer).

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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 [23].

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 [3,7]. Given its abundance, *T. diversifolia* enjoys a long use as a traditional remedy in tropical regions [12]. In particular, the plant leaves are used in Africa to treat malaria [13,17]. Noteworthy, this use was then confirmed by scientific studies [9,11,19] where the bioactive compounds were identified as germacranolide-type sesquiterpene lactones called tagitinins, with tagitinin C as the lead compound [10,11]. These sesquiterpene lactones (STLs) were also proven to exhibit anti-inflammatory/analgesic (Owoyele et al., 2004), antiproliferative [15], antifeedant [2] and insecticidal activities [6]. Other secondary metabolites characteristics of *T. diversifolia* are diterpenoids, flavonoids, chlorogenic acid derivatives and essential oils [18].

Given the potential of *T. diversifolia* secondary metabolites to interact with protozoan parasites [11], 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.

2. Material and methods

2.1. 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.

2.2. 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 10,196/HNC was deposited.

2.3. 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.

2.4. 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 × 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.

2.5. 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