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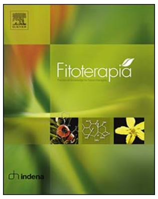

Improving anti-trypanosomal activity of alkamides isolated from *Achillea fragrantissima*

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ABSTRACT

In previous studies the aerial parts of *Achillea fragrantissima* were found to have substantial antileishmanial and antitrypanosomal activity. A bioassay-guided fractionation of a dichloromethane extract yielded the isolation of the essential anti-trypanosomal compounds of the plant. Seven sesquiterpene lactones (including Achillolide-A), two flavonoids, chrysosplenol-D and chrysosplenetine, and four alkamides (including pellitorine) were identified. This is the first report for the isolation of the sesquiterpene lactones **3** and **4**, chrysosplenetine and the group of alkamides from this plant. Bioevaluation against *Trypanosoma brucei brucei TC221 (T.b brucei)* using the *Alamar-Blue* assay revealed the novel alkamide **13** to have an IC_{50} value of 40.37 μM. A compound library, derived from the alkamide pellitorine (**10**), was synthesized and bioevaluated in order to find even more active substances. The most active compounds **26** and **27** showed activities in submicromolar concentrations and selectivity indices of 20.1 and 45.6, respectively, towards macrophage cell line *J774.1*. Toxicity of **26** and **27** was assessed using the greater wax moth *Galleria mellonella* larvae as an *in vivo* model. No significant toxicity was observed for the concentration range of 1.25–20 mM.

1. Introduction

Trypanosomiasis, also known as African sleeping sickness, is a vector-borne protozoal disease caused by the trypanosoma subspecies *Trypanosoma brucei* (*T.b*) *gambiense* and *T.b. rhodesiense*[1]. The infection is transmitted to humans mainly by the bite of an infected tsetse fly (*Glossina* genus), particularly in Sub-Saharan Africa [1–2]. The World Health Organization (WHO) reported three major *T.b.* epidemics, the first between 1896 and 1906, the second in the 1920s, and the third in the 1970s [3–4]. A report published in January 2017 estimated the number of actual cases to be below 20,000 with further 65 million people being at risk of infection [3].

The disease is characterized by two stages; the hemolymphatic stage called stage 1, being a peripheral extracellular infection associated with non-specific clinical symptoms, and stage 2 where the parasite crosses the blood-brain barrier (BBB) and invades the central nervous system (CNS). It causes severe symptoms including mental impairment, fever, headache, and chronic encephalopathy. This is followed by somnolence and death if left untreated [5–6]. Currently available treatments of trypanosomiasis depend on the subspecies of the parasite and the stage of infection. During the first stage, pentamidine is the drug of choice to treat *T.b. gambiense,* while suramin is used against *T.b. rhodesiense*. Both substances are not able to cross the BBB; therefore, they are ineffective during the second stage of the disease. In addition, they are associated with significant adverse effects such as exfoliative dermatitis and renal failure for suramin and diabetes mellitus and nephrotoxicity for pentamidine [5].

In the late stage of the infection, three therapeutic options are available. Melarsoprol, an organo-arsenic compound, is effective against both subspecies. It has, however, many undesirable side effects and can lead to fatal encephalopathic syndromes (3% to 10%) [3,5]. Eflornithine is a second therapeutic option and less toxic than melarsoprol. Unfortunately, it is only effective against *T.b. gambiense*. Both compounds have to be administered intravenously. Nowadays, the WHO recommends the application of a nifurtimox and eflornithine combination therapy (NECT), because it simplifies the treatment regime of eflornithine alone by reducing the treatment period and the number of daily doses. However, this combination is not effective against *T.b. rhodesiense*[5] and consequently, new effective and less toxic drugs are in demand [4,7].

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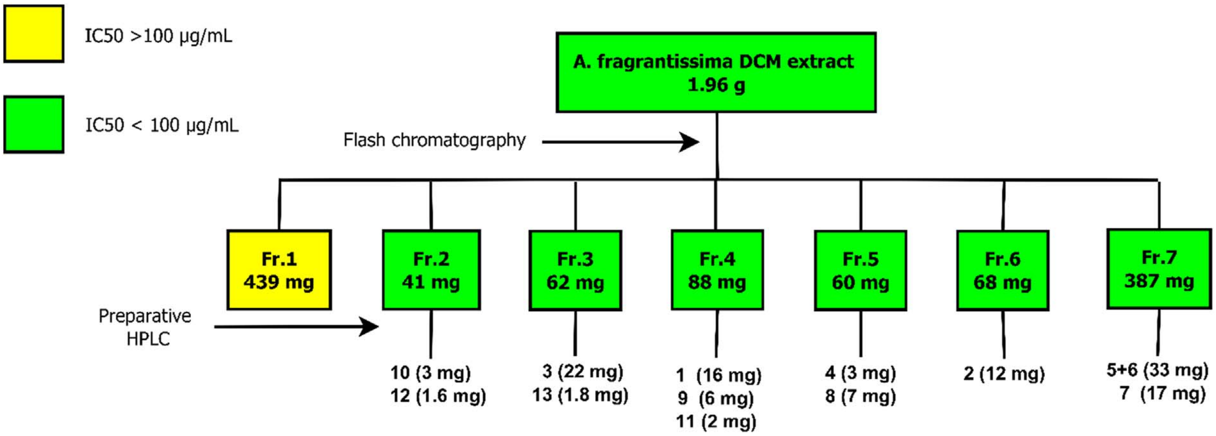

Fig. 1. Bioassay-guided fractionation of the DCM extract of *A. fragrantissima*.

Achillea fragrantissima (*Af*; Asteraceae) or Qaysum (Arabic name), respectively, is a desert plant that has been used in traditional medicine in the Middle East region since many years for the treatment of respiratory diseases and gastrointestinal disturbances [8]. Bedouins use the plant for preparing anti-diuretic drinks for the treatment of stomach ailments and various infections, *e.g.* of the urinary tract [9]. Since a preliminary screening of the dichloromethane extract of the aerial parts of *Achillea fragrantissima* revealed anti-leishmanial and anti-trypanosomal activity, a bioassay-guided fractionation was performed to unravel the active compounds. One of these compounds could be identified as pellitorine, an alkamide having a moderate antitrypanosomal activity. Thus, a small library of alkamides was synthesized in order to improve activity of this substance and to decrease cytotoxicity.

2. Experimental

2.1. General

All starting materials and reagents where purchased from Sigma Aldrich, Schnelldorf, Germany, and TCI Chemicals, Eschborn, Germany. NMR spectra were recorded on a Bruker Avance 400 Ultra Shield™ spectrometer (Bruker Biospin, Ettlingen, Germany) and a Bruker DMX 600 (Bruker, Karlsruhe, Germany) instrument which were calibrated using the residual undeuterated solvent as an internal reference (DMSO-*d6*: ^{1}H 2.5 ppm, ^{13}C 39.52 ppm; methanol-*d4*: ^{1}H 3.31 ppm and 4.78 ppm, ^{13}C 49.00 ppm, cChloroform-*d1*: ^{1}H 7.24 ppm, ^{13}C 77.23 ppm). Coupling constants (*J*) are given in Hertz.

For determining the purity of all compounds, analytical HPLC was conducted on a Shimadzu system (Hilden, Germany) equipped with a DGU-20A3R degassing unit, a LC20AB liquid chromatograph, and a SPD20A UV/Vis detector. The stationary phase was a Synergi fusion-RP (150 × 4.6 mm, 4 µm) column (Phenomenex, Aschaffenburg, Germany). The following gradient elution was applied: solvent A: water with 0.1% formic acid, solvent B: MeOH with 0.1% formic acid. Solvent A from 0% to 100% in 13 min, then 100% A for 5 min, from 100% to 5% A in 1 min, and 5% A for 4 min. The flow rate was set to 1.0 mL/min. UV detection was performed at 254 nm. ESI mass spectral data were acquired on a Shimadzu LCMS-2020 instrument (Hilden, Germany). IR spectra were recorded on a Jasco FT/IR-6100 spectrometer with an ATR unit (Groß-Umstadt, Germany) at room temperature. Flash column chromatography was performed on an Interchim Puri-Flash 430 instrument (Ultra Performance Flash Purification) connected to an Interchim Flash ELSD (Montluçon, France). Preparative HPLC was performed using an Agilent 1100 preparative HPLC instrument (Waldbronn, Germany) utilizing a semi-preparative Synergy 4 µm MAX-RP 80A column 150 × 10 mm (Phenomenex, Aschaffenburg, Germany), fraction collector, and a multiple wavelength detector. The following gradient was applied: solvent A: water, solvent B: acetonitrile. Separation method: solvent B 10% for 2 min, 10% to 30% B in 1 min, then to 100% B over 22 min and again to 10% B over 2 min.

Galleria mellonella at the final larval stage were purchased from Mouse Live Bait (Balk, The Netherlands).

2.2. Plant material

Seeds of *Achillea fragrantissima* were obtained from a suburb near Homs, Syria. 100 seeds were planted in spring in a greenhouse of the garden. The fully-grown plants were collected in summer and dried at room temperature in shade for 20 days until constant weight (harvest approx.700 g). The identity of the seeds and the grown plants was verified by Dr. Hildebrandt and Dr. Vogg at the botanical garden, University of Würzburg.

2.3. Extraction and isolation

The aerial parts of *A. fragrantissima* were powdered using a laboratory grinder. The pulverized plant material (100 g) was extracted for 24 h at room temperature with dichloromethane by stirring. The extract was filtered, followed by complete drying *in vacuo* to give a crude residue (1.96%). Fractionation of the extract was performed using flash chromatography with a hexane/ethyl acetate gradient eluent, yielding 7 major fractions. After bio-evaluation of each fraction *in vitro* against *T.b. brucei* using the *Alamar-Blue* assay [10], the active fractions (F2–F7) (IC_{50} $<$ 100 µg/mL) were sub-fractionated by means of preparative HPLC yielding 13 compounds (Fig. 1). The structures of isolated compounds were elucidated by means of NMR, infrared (IR), and mass spectroscopy (MS).

2.4. Biological assays

2.4.1. Anti-trypanosomal assay

The fractions, the extracts, as well as isolated and synthesized compounds were tested towards trypanosoma according to Räz et al. [10] Trypomastigote forms of *T.b. brucei* laboratory strain TC221 were cultivated in Balz medium. A defined number of parasites (10^{4} trypanosomes per mL) was tested in 96-well plates against different concentrations of the test substances in a final volume of 200 µL (1% DMSO in Balz medium). Positive (trypanosomes added to culture medium) and negative controls (test substance without trypanosomes) were run with each plate. The plates were incubated at 37 °C in an atmosphere of 5% CO_2. After 24 h additional 20 µL of *Alamar-Blue* was added to each well. A reading was done at 48 h. Any effect of the test substances was quantified as IC_{50} values by linear interpolation of three different measurements. The activity of the test substances was measured by