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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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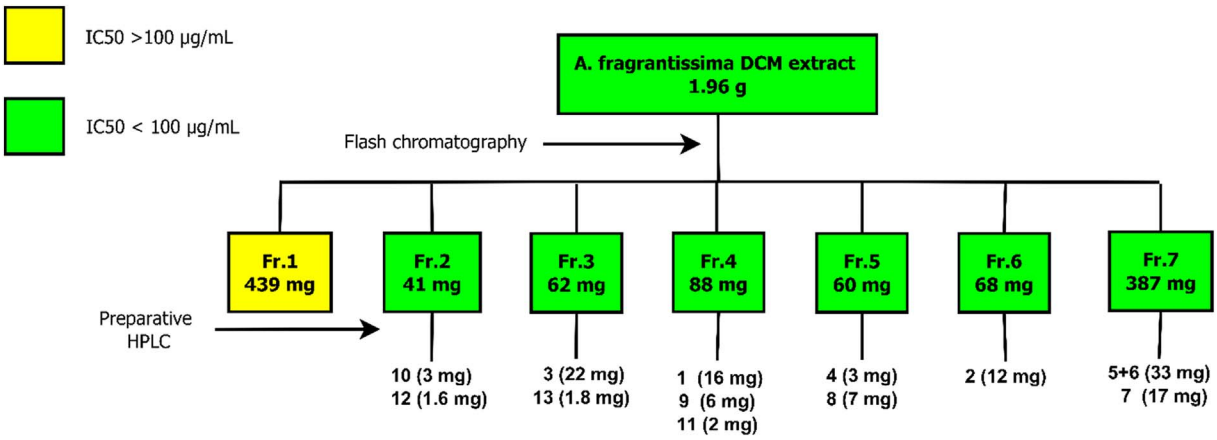


Fig. 1. Bioassay-guided fractionation of the DCM extract of *A. fragrantissima*.

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

light absorption using a MR 700 microplate reader (Dynatech Engineering Ltd., Willenhall, UK) at a wavelength of 550 nm with a reference wavelength of 630 nm.

2.4.2. Cytotoxicity assay

The experiments were carried out according to Hiltensperger et al. [2] The macrophage cell line J774.1 was maintained in complete Click RPMI medium. For the experimental procedures, cells were detached from the flasks using a rubber scraper and cell densities were adjusted to having 2×10^4 cells/mL. J774.1 macrophages were seeded into 96-well plates and were incubated (37 °C, 5% CO_2, 95% humidity) overnight to allow attachment and recovery. A set of increasing concentrations (1, 2.5, 5, 10, 25, 50, 75, and 100 μM) of the tested compounds (125 μL) and complete RPMI medium were transferred into the cell culture plates and allowed to incubate for 24 h. After adding 10 μL of the cell proliferation reagent WST-1, incubation was continued at the same conditions. The plates were read after 30 min and 2 h, respectively, at $\lambda = 440$ nm. Control experiments to evaluate the effect of cell density, incubation time, and DMSO concentration were performed. Absorbance in the absence of any compounds was set as 100% of growth control.

2.4.3. In vivo toxicity assay (Galleria mellonella larvae)

Toxicity assessment in *Galleria mellonella* was performed as described previously by Gibreel and Upton [11] with minor modifications. Larvae were used immediately upon arrival for toxicity evaluation experiments and were considered healthy if they were clear in color and free of any spots or pigmentation (weighing approximately 200–250 mg). Solutions of the test substances **26** and **27** having concentrations ranging from 1.25–20 mM were prepared in endotoxin-free PBS + 10% DMSO (vehicle). 20 μL of test substance was injected in the last left pro-leg of a larvae using insulin syringes (BD Micro-Fine™ + -Demi). Ten larvae were used per group. Four control groups were included in the experiment: one group received no injection, one group was injected 20 μL of endotoxin-free PBS, one group was injected 20 μL of vehicle control, and one group received 20 μL methanol as positive control. All groups of larvae were incubated in the absence of light at 37 °C in Petri dishes. Larval survival rates were recorded every 24 h for 120 h. Larvae not responding to touch were scored dead and *vice versa*. Finally, Kaplan-Meier survival curves were generated with GraphPad Prism v 6.04 and statistical significance of the curves was evaluated using the Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test utilizing the software; $p < 0.05$, statistically significant.

2.5. Synthesis procedures

2.5.1. General procedure A for synthesis of compounds 14–19 and 22–40

To a mixture of 1 equiv. of the corresponding carboxylic acid, 4 equiv. of trimethylamine, 1 equiv. of the corresponding amine in dichloromethane (10 mL), and 1 equiv. of propylphosphonic anhydride solution (T3P®) was slowly added at 0 °C under Argon atmosphere. The reaction mixture was allowed to warm to RT and was stirred for 5 h. The reaction was diluted with dichloromethane (20 mL) and saturated aqueous NaCl solution (30 mL). The aqueous layer was extracted 3 times with dichloromethane (90 mL). The combined organic layers were dried over Na_2SO_4. The solvent was removed *in vacuo*.

2.5.2. General procedure B for synthesis of compounds 20 and 21

1 equiv. of the corresponding carboxylic acid and 5 equiv. of 4-methylmorpholine (NMM) were dissolved in abs. dimethylformamide (10 mL) at RT under Argon atmosphere. After cooling to 0 °C, 4 equiv. of i-butyl chloroformiate were added and the mixture was stirred for 1 h. Then, 4 equiv. of the corresponding amine were added and the mixture was stirred for additional 2 h at RT. The solvent was removed *in vacuo*, and 20 mL of water were added. The aqueous solution was extracted 3 times with ethyl acetate (150 mL). The combined organic layers were dried over Na_2SO_4. The solvent was removed *in vacuo*.

2.5.3. Purification of synthesized compounds

The purification of all synthesized compounds **14–40** was performed by means of flash chromatography using a mixture of cyclohexane and ethyl acetate (70: 30) as solvent system over 20 min for compounds **14–19**, and dichloromethane and methanol gradient (100: 0 to 85: 15) over 35 min for compounds **20–40**.

The analytical and spectroscopic data of all compounds can be found in the supplementary data.

3. Results and discussion

Initially, a dichloromethane extract of *A. fragrantissima* collected in Palestine was screened using the corresponding *Alamar-Blue* assay [12] revealing a substantial anti-leishmanial activity. The question arose whether the extract would also show anti-trypanosomal activity towards *T.b. brucei*. Therefore, seeds of *A. fragrantissima* were collected in Homs, Syria, and planted in a greenhouse of the botanical garden of the University of Würzburg. The plants were harvested in summer and dried in shadow. The powdered aerial parts of *A. fragrantissima* were extracted with dichloromethane and the extract was tested for its antitrypanosomal activity. Since a substantial anti-infective activity was found, the extract was fractionated by means of flash chromatography to obtain 7 fractions. These fractions were again tested against *T.b. brucei*. The most active fractions 2 to 7 were further fractionated each. For the entire scheme of fractionation see Fig. 1.

3.1. Identification of isolated compounds

As can be seen in Fig. 2, three groups of natural products were found: seven sesquiterpene lactones **1–7**, two flavonoids **8** and **9**, and four alkamides **10–13** including a novel alkamide **13**.

Whereas the sesquiterpene lactones **1**, Achillolide-A **2**, **5**, **6**, and **7** were reported previously being found in *A. fragrantissima*[13–14], the natural products **3** and **4** have never been isolated from this plant though they were previously found in *A. afra*[15] and *A. luoviciana*[16], respectively. Compounds **5** and **6** were isolated as a mixture of two epimeric lactones which have a different configuration of the hydroxyl group at the carbon C-1 [17].

Flavonoids **8** and **9** were identified to be Chrysosplenol-D [18–19] and Chrysosplenetine [20], respectively. Chrysosplenol-D has a hydroxyl group at C-3′, whereas Chrysosplenetine carries a methoxyl group. This is the first report of isolation of Chrysosplenetine from *A. fragrantissima*.

Furthermore, four alkamides Pellitorine **10**, **11**, **12**[21–22], and the new alkamide **13** were isolated from the plant in small amounts. The alkamides group has never been reported being present in *A. fragrantissima*. However, the alkamides **10**, **11**, and **12** were previously found in *A. ptarmica* from the *Asteraceae* family [22].

The spectral data (NMR, MS, IR) of all isolated compounds, sesquiterpene lactones, flavonoids, and alkamides, were in accordance with their respective data reported in the literature.

3.2. Structure elucidation of compound 13

The new compound **13** was isolated from fraction 3 as a white solid substance. NMR data exhibits the structure of an alkamide similar to the known Pellitorine (**10**) (Fig. 2). MS-data (*m/z*: 238.10) hinted to an additional oxygen atom. The ^{13}C NMR spectrum displays a signal at $\delta = 202$ ppm which is not visible in DEPT, indicating the presence of a ketone group. ^1H NMR and COSY diagrams point to a conjugated diene moiety at $\delta = 7.25$ ppm (m, 2H, H-3, 4), 6.51 ppm (d, $J = 14.9$ Hz, 1H, H-5), and 6.44 ppm (d, $J = 14.5$ Hz, 1H, H-2). The coupling constants of 14.9 Hz and 14.5 Hz indicate a *trans*-configuration of the protons of each double bond. Furthermore, the analysis of the HMBC data (Fig. 3)

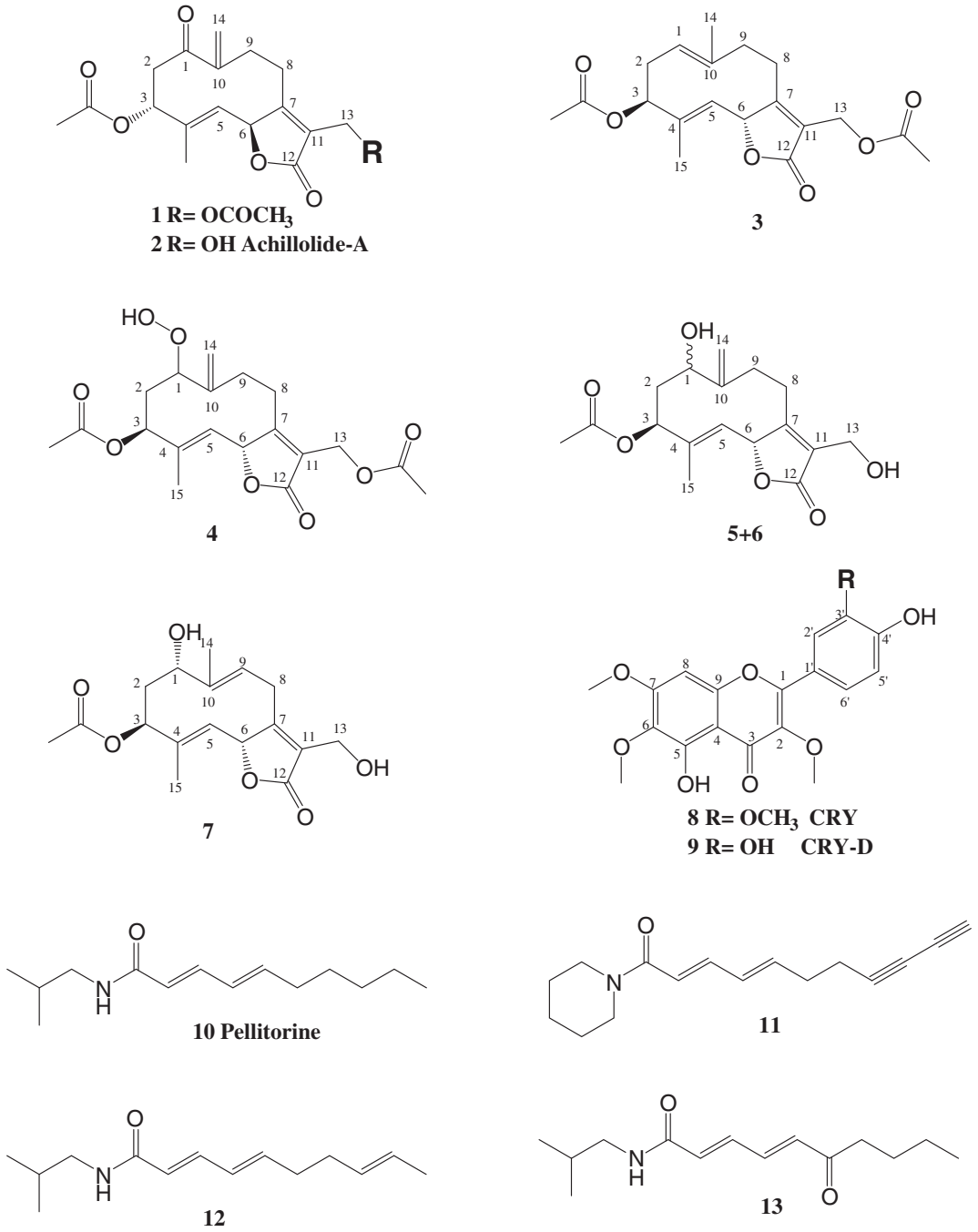


Fig. 2. Isolated compounds from dichloromethane extract of *A. fragrantissima*.

confirms the position of the carbonyl carbon $\delta = 202$ ppm being at C6. The IR spectrum shows one band at 3358 cm^{-1} indicating a secondary amine and two bands at 1658 cm^{-1} (m) and 1632 cm^{-1} (s) indicating two carbonyl groups corresponding to the amide group at position 1 and the ketone group at position 6, respectively. Therefore, the structure was assigned to (2*E*,4*E*)-*N*-isobutyl-6-oxodeca-2,4-dienamide (Fig. 3).

3.3. Bio-evaluation of the isolated compounds

All isolated compounds were subjected to the evaluating their *in vitro* activity against *T.b. brucei* using the corresponding *Alamar-Blue* assay and Pentamidine as a reference [10]. The bioevaluation data of all isolated compounds is reported in Table 1.

Sesquiterpene lactones **1**, **2**, **3**, and **4**, were quite active with IC_{50} values between 3 and 27 μM. The epimeric mixture of **5** and **6** showed weak activity, whereas compound **7** was not active at all. Inspecting structures of the active sesquiterpene lactones revealed that the combination of two acetoxy groups and an enone Michael system is important for the activity of **1**. In absence of either the Michael system (*cf.***3** and **4**), or of the acetoxy group at C-13 (see **2**, **5**, and **6**) and of both the Michael system and the acetoxy group, the activity is substantially reduced or completely lost as in **7**.

This is the first study on the anti-trypanosomal activity of these particular sesquiterpene lactones. However, sesquiterpene lactones are widely known to exhibit antiprotozoal activity [23–24]. Their biological activity is usually accompanied with cytotoxicity and can mostly be explained by a reactive Michael system being prone to show the

PAINS problem [25]. Since this structural feature determines both anti-trypanosomal and cytotoxic activity, [23] we did not consider the sesquiterpene lactones for further investigations or synthetic optimizations.

The isolated flavonoids Chrysosplenetine **8** and Chrysosplenol-D **9** represented moderate activities with two digit micromolar anti-trypanosomal activity. Both flavonoids were never reported for their anti-trypanosomal activity. However, flavonoids are generally known to exhibit anti-protozoal activity [26] in addition to various other bioactivities [27]. They are also well known as strong antioxidants being able to effectively protect against radical toxicity. This is due to the catechol moiety which can be easily oxidized to the quinones which often are present in flavonoids. However, quinones deliberately react with the nucleophilic thiol residues in proteins resulting in cytotoxicity [28].

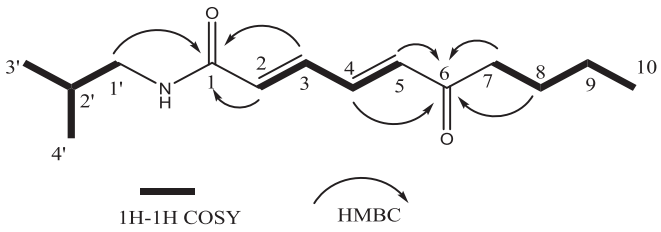
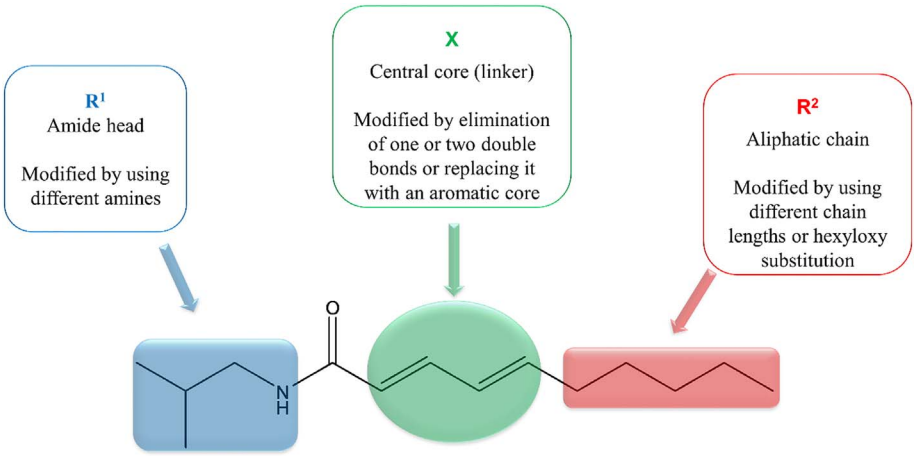


Fig. 3. 1H-1H COSY and HMBC correlations for 13.

Table 1
Bioevaluation of isolated compounds.

Compound	Chemical group	IC_{50} [μM] *T.b. brucei*
1	Sesquiterpene lactones	3.03 ± 0.06
2	Sesquiterpene lactones	10.97 ± 0.47
3	Sesquiterpene lactones	10.97 ± 0.11
4	Sesquiterpene lactones	27.03 ± 0.13
5 + 6	Sesquiterpene lactones	60.92 ± 0.76
7	Sesquiterpene lactones	> 80
8	Flavonoids	30.35 ± 0.23
9	Flavonoids	47.27 ± 0.39
10	Alkamides	30.76 ± 0.08
11	Alkamides	31.05 ± 0.13
12	Alkamides	36.98 ± 0.64
13	Alkamides	40.37 ± 0.21
Pentamidine	Dibenzamidine	0.007 ± 0.00

Fig. 4. Modified regions in Pellitorine 10 for SAR study.



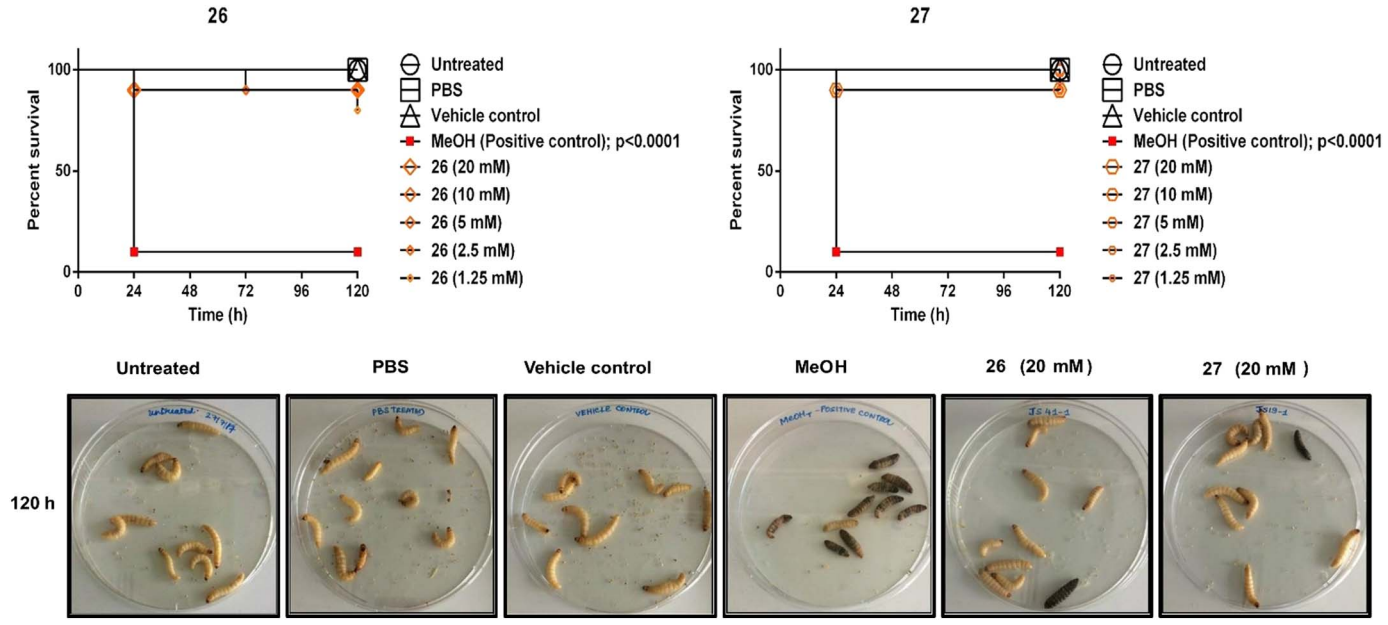
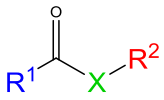
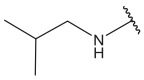
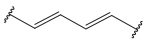
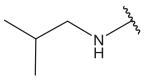
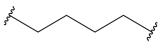
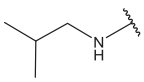
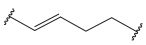
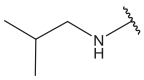
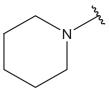
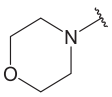
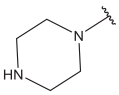
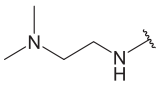
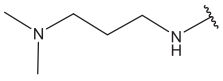
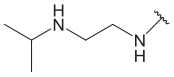
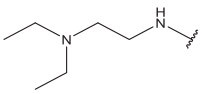
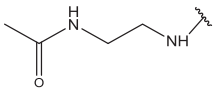
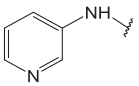
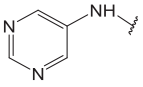
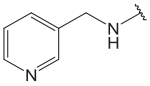
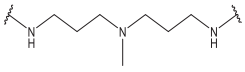


Fig. 5. Survival rates of the larvae for compound **26** and **27**.

Table 2
Anti-trypanosomal activity, cytotoxicity, and selectivity indices of compounds 14–40.



Compound	R^1	X	R^2	IC_{50} [μM] *T.b. brucei*	IC_{50} [μM] J 774.1	Sel. Index[a]
14			Methyl	> 40	> 100	–
15			Pentyl	> 40	> 100	–
16			Pentyl	36.64 ± 0.08	> 100	> 2.7
17		C_6H_4	Pentyl	34.37 ± 0.10	> 100	> 2.9
18		C_6H_4	Pentyl	30.73 ± 0.03	> 100	> 3.3
19		C_6H_4	Hexyloxy	19.82 ± 0.07	81.5	4.1
20		C_6H_4	Pentyl	> 40	–	–
21		C_6H_4	Hexyloxy	> 40	–	–
22		C_6H_4	Pentyl	3.90 ± 0.01		24.1
23		C_6H_4	Hexyloxy	2.16 ± 0.02	48.6	22.5
24		C_6H_4	Pentyl	3.11 ± 0.01	49.5	15.9
25		C_6H_4	Hexyl	3.24 ± 0.02	29.5	9.1
26		C_6H_4	Heptyl	0.72 ± 0.00	14.4	20.1
27		C_6H_4	Hexyloxy	0.72 ± 0.00	32.9	45.7
28		C_6H_4	Pentyl	3.74 ± 0.01	48.6	13
29		C_6H_4	Hexyl	3.31 ± 0.24	18.2	5.5
30		C_6H_4	Heptyl	2.94 ± 0.02	23.5	8
31		C_6H_4	Hexyloxy	3.01 ± 0.01	23.8	7.9
32		C_6H_4	Pentyl	3.36 ± 0.01	44.7	13.3
33		C_6H_4	Hexyloxy	3.41 ± 0.06	19.1	5.6
34		C_6H_4	Pentyl	3.89 ± 0.01	50.2	12.9
35		C_6H_4	Pentyl	31.74 ± 0.29	> 100	> 3.2
36		C_6H_4	Pentyl	20.77 ± 0.07	49.8	2.4
37		C_6H_4	Pentyl	32.49 ± 0.07	48.7	1.5
38		C_6H_4	Pentyl	13.61 ± 0.1	19.1	1.4
39		C_6H_4	Pentyl	0.48 ± 0.00	5.1	10.6
40		C_6H_4	Butoxy	0.71 ± 0.00	4.8	6.8

[a] Sel. Index = (IC_{50} against microphages J744.1)/(IC^{50} against *T.b. brucei*).

Pellitorine **10** and the alkamides **11**, **12**, and **13** exhibited anti-trypanosomal activity with an IC_{50} range of 30 to 40 μM. Of note, pellitorine **10** as well as **11** and **12** were previously isolated from *A. ptarmica*[22] and reported to have anti-trypanosomal activity against *T.b. rhodesiense*.

3.4. Study of structure-activity relationship (SAR) of alkamides

Whereas flavonoids and sesquiterpene lactones are widely known to have a variety of biological effects [29–30] due to reactive moieties and their SARs as anti-protozoal agents were explored previously [23,26], the anti-trypanosomal activity of alkamides remains almost unknown. Hence, a library of 27 alkamides was synthesized and subjected to biological testing in order to improve the antitrypanosomal activity and to derive SAR. The compounds were varied at three key regions of the structure, namely the amide head, the central core, and the aliphatic chain (Fig. 4). All 27 compounds were biologically assessed for anti-trypanosomal activity against *T.b. brucei* and for cytotoxicity against macrophages J774.1. Selectivity indices were calculated as the ratio of IC_{50} against macrophages J774.1 and the IC_{50} against *T.b. brucei.* The biological data are summarized in Table 2.

Comparing the IC_{50} values of the compounds differing in length of the aliphatic chain (R^2) revealed the importance of the number of C atoms for the activity. Compound **14**, having a terminal methyl group, was found to be inactive ($IC_{50} > 40$ μM), while both Pellitorine **10** and the alkamide **16** having a pentyl chain were found to be active at a concentration level of approx. 30 μM. The presence of one or two double bonds at positions 1 and/or 2, or an aromatic system, respectively, in the linker region X is also essential for the activity. Compound **15**, having a completely saturated chain, was found to be inactive.

Compounds possessing a basic amine moiety in region R^1, *i.e.***22–34**, were highly potent anti-trypanosomal substances with IC_{50} values in the range of 0.72 to 3.90 μM. Compounds **26** and **27**, having heptyl and hexyloxy chains, respectively, in region R^2 possess the highest activities in the submicromolar concentration range along with the highest selectivity indices of 20.1 and 45.6, respectively. Interestingly, compounds carrying a morpholine at R^1 were found to be inactive; compound **35** having an acetamide moiety has a moderate activity and a low cytotoxicity, whereas compounds **36–38** bearing aromatic heterocyclic amines have a moderate activity, but a remarkable cytotoxicity and thus, low selectivity indices. The two bis-amino compounds **39** and **40** exhibited high activities in the submicromolar concentration range but again a high cytotoxicity was observed.

The toxicity of the most active compounds **26** and **27** was assessed using *Galleria mellonella* larvae as an *in vivo* model. The greater wax moth *Galleria mellonella* larvae have been increasingly used as an *in vivo* model for assessing the toxicity and efficacy of antimicrobial agents and studying microbial infections [11,31]. Their ability to survive at physiological temperatures (30 °C or 37 °C), low maintenance costs, easy handling, and absence of any ethical concerns are some of the most important advantages of using these larvae for toxicity evaluation and pharmacokinetics studies [32]. The survival rates of the larvae are represented in Fig. 5, demonstrating that none of the tested concentration levels (1.25–20 mM) of both compounds induced toxic effects on the individuals. Also, the occurrence of few deaths observed with the tested concentration levels was not statistically significant, whereas methanol treatment (positive control) significantly led to death of the larvae ($p < 0.0001$). Because of the apparent absence of an *in vivo* toxicity and the relatively high trypanocidal activity, **26** and **27** can be regarded as lead compounds.

4. Conclusion

Three groups of natural products having an anti-trypanosomal activity were identified in *A. fragrantissima* including sesquiterpene lactones, flavonoids, and alkamides. Since the anti-trypanosomal activity and the SAR of the alkamides were not exclusively studied, a series of systematically varied alkamides was synthesized. Within this series, the compounds having heptyl or hexyloxy, respectively, in region R^2 and a basic amine moiety in region R^1 were found to be highly active with high selectivity indices. Notably, no toxicity was observed in an *in vivo* model utilizing *Galleria mellonella* in concentrations up to 20 mM. These compounds can be regarded as new leads. However, further work has to be performed to elucidate the mode of action of these compounds.

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Supplementary data

The characterization data including NMR, MS, IR data, as well the yield and purity for all compounds are available as supplementary data.

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