

Original Paper

Identification of compounds from the plant species *Alepidea amatymbica* active against HIV

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Abstract

As *Alepidea amatymbica* is commonly used and accepted as medicinal plant in South Africa for various indications, the scientific basis of its anecdotally described, putative anti-HIV properties was investigated. To this aim, we used an accelerated extraction-purification approach; extracts and therein sub-fractions of *A. amatymbica* were assessed in a cell-based assay targeting the replication of prototypic CXCR4-tropic (NL4-3) or CCR5-tropic (NL-AD87) HIV-1 strains.

Sub-fractions of the extracts were generated through semi-preparative high performance liquid chromatography (HPLC) fractionation into triplicates of 96-well microtitre plates; they were then separately subjected to biological analysis and ultra performance liquid chromatography (UPLC) time-of-flight (TOF) analysis. A correlation plot was generated between the biological and chemical data to identify the biologically active compounds in those fractions that showed significant selective anti-HIV activity. The results indicated that rosmarinic acid was present in the wells that showed promising anti-HIV activity *in vitro* indicating that this compound is at least in part responsible for the antiviral properties of the *A. amatymbica* extracts. However, compared to standard retroviral inhibitor the anti-HIV activity of the pure compound was found to be only quite moderate. Nevertheless, the accelerated approach described herein increases the efficiency of screens towards identifying drug candidates much earlier in the discovery stage.

Keywords

HIV, South Africa, plant-based inhibitor, herbal medicine, Traditional Medicine, Ethnobotany, Virus profiling, antiretroviral screen

Introduction

The human immunodeficiency virus (HIV) is globally currently one of the most deleterious pathogens causing disease and death associated with profound immunosuppression (1, 2). It is estimated that more than 20 million people with HIV/AIDS have died since the first AIDS cases were described in 1981. Sub-Saharan Africa is the hardest-hit region in the world. Approximately 22.5 million people in Sub-Saharan Africa live with HIV. Furthermore, in some parts of Sub-Saharan Africa, the seroprevalence of HIV in the general population is still at alarmingly high levels of up to 26% (3). The pandemic in these areas has reached levels where the economic development of entire societies is threatened or stopped. Current prevention strategies are still inadequate to stop the rapid spread of the epidemic and the search for safe, affordable treatment for HIV thus remains a major challenge and key towards controlling the disease.

South Africa with its rich plant biodiversity with close to 24,500 plants species represents an enormous potential source of antiviral compounds. However, until today only few isolated studies have investigated the anti-HIV properties of South African medicinal plants (1, 4). In response to this shortcoming, a collaborative research programme was initiated at the Council for Scientific and Industrial Research (CSIR) in South Africa that aimed at screening plant extracts and at identifying new anti-HIV drug leads. CSIR is approached on a regular basis by knowledge holders and Traditional Health Practitioners to undertake research on their plant based medicines. As one outcome from this effort the present report evaluates the activity of aqueous extracts of *Alepidea amatymbica* against the HIV virus as the plant was brought to the CSIR by a Traditional Health Practitioner to evaluate its beneficial properties to HIV infected patients (CSIR, confidential documentation on file).

A. amatymbica belongs to the family Apiaceae, and the approximately 28 species in the genus are predominantly found in Africa(5) and are endemic to grassland areas of eastern and southern Africa (5, 6). Rhizomes and roots of *A. amatymbica* are used in South Africa as a traditional medicine (7, 8). *A. amatymbica* is known under the Zulu name 'iKhataso' and is used for the treatment of colds and chest complaints (8) as well as for asthma, influenza, diarrhoea and abdominal cramps, sore throat and rheumatism (7). As the plant is used for treating diseases that affect the patients' "immune system" it

was added to our screening system for investigating in more detail its potential use in HIV therapy. Previous phytochemical investigation of rhizomes and roots of the plant have resulted in the isolation of several diterpenoid kaurene derivatives (9, 10), and more recently phenolic acids have been identified (11).

Materials and Methods

Plant material

All plant species were identified in Trenmore, South Africa (GPS 27° 59' 54 E, 311° 8' 27 S), and the whole plants (leaves, stems, and roots) were collected. A voucher specimen (No PRE577495.0) was deposited and identified at the South African National Biodiversity Institute (SANBI).

Extraction

Aerial parts and roots of the plant were cut into small pieces and dried in an oven at 30-60 °C for 48 hours. Dried material was ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction. The powdered plant material (500 g) was extracted with purified water. The aqueous extract was filtered and concentrated by freeze-drying and stored at -20°C prior to analysis.

General experimental procedures

Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Romil Pure Chemistry (Cambridge, UK). HPLC grade water was obtained from a TKA Ultra Pure water purification system (Niederelbert, Germany). Formic acid (98.0-100.0%) was purchased from Sigma Aldrich (South Africa). Reference materials rosmarinic acid (>98.0% purity) and caffeic acid (>98.0% HPLC purity) were purchased from Tocris Bioscience (R&D Systems Europe, UK) and Sigma Aldrich (Basel, Switzerland), respectively.

Semi-preparative purification

The extract was fractionated using an Agilent 1200 semi preparative HPLC system consisting of auto sampler, high pressure mixing pump, column oven, and diode array detector (DAD). Semi-preparative purification was carried out using an Atlantis Preparative T3 column (C18, 5µm, 10 X 250mm, Waters) and the following gradient conditions: 0-13 min, from 5 to 90% aqueous acetonitrile; 13-15

min, a plateau at 90% aqueous acetonitrile; 15-16 min, increase to 100% acetonitrile; 16-17.8 min, 100% acetonitrile. Column was developed at a flow rate of 5 mL/min; absorption spectrum (190-700 nm) was recorded in real-time. Five hundred μL of sample at a concentration of 25 mg/mL in water were injected and fractions were collected every 0.2 min in a 96-well microtitre plate.

Residual solvent was evaporated from the collection 96-well microtitre plates using a GeneVac EZ-2 Plus evaporator at 40°C with pre-installed "HPLC fractions" vacuum settings. Four injections per plate were completed and this process was repeated 3 times thus generating 3 identical microtitre plates.

UPLC-Mass spectrometry analysis

UPLC-MS data were obtained with a Waters Acquity UPLC system connected in series to a Waters photodiode array (PDA) detector as well as a SYNAPT G1 HDMS QTOF (4KDa) mass spectrometer. The system was controlled through MassLynx v4.1 SCN639. UPLC separation was performed using an Acquity HSS T3 column (C18, 1.8 μm , 150 x 2.1mm, Waters) at 40°C. Chromatographic solvents were ultra-pure water containing 0.1% v/v formic acid (Solvent A) and UPLC grade acetonitrile (Solvent B). The following gradient conditions were used: 5% (v/v) acetonitrile in 0.1% (v/v) aqueous formic acid increasing to 90% acetonitrile (v/v) over 13 min followed by a 2-min plateau at 90% (v/v) acetonitrile. Flow rates used were 0.35mL/min (0-13min) and 0.45mL/min (13-15min). Full scan absorption spectra (200-500 nm, 1.2nm resolution) were recorded in real time for each analysis with a runtime of 20 minutes. Five μL of sample at a concentration of 1 mg/mL in water were injected. Fractions of interest were analysed by electrospray ionisation-quadrupole time-of-flight mass spectrometry (ESI-QTOF MS) using a scanning range of 100 to 1200 Da. The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray ionisation mode. The mass spectrometer was operated in negative mode with a capillary voltage of 2.5 kV, the sampling cone at 30 V and the extraction cone at 5 V. The scan time was 0.1 seconds covering the 100 to 1200 Dalton mass range. The source temperature was 120 °C and the desolvation temperature was set at 400 °C. Nitrogen gas was used as the nebulisation gas at a flow rate of 800 L/h. The trap collision energy was set at 6V while the transfer optics collision energy was set to 4V. The Synapt mass spectrometer was

calibrated using a formic acid solution in acetonitrile to obtain a mass accuracy below 5 mDa for the scan range of 100 to 1200 Da. A Lockmass interface was used to maintain mass accuracy and utilised a 50 pg/mL leucine enkephalin solution infused at 10 uL/min.

Drug susceptibility testing

The anti viral properties of the extracts were assessed in a cell-based replicative assay. The assay was carried out exactly as described in(12) using either a CXCR4-tropic (NL4-3) or a CCR5-tropic (NL-AD87) wild-type reference viruses. The assay makes use of the reporter cell line HeLa-SxR5 which stably expresses the CD4 receptor, the CCR5 and CXCR4 chemokine receptors. It furthermore contains stably integrated into the genome an HIV-1 Long Terminal Repeat fused upstream of the bacterial reporter gene LacZ coding for β -Galactosidase. HEK293T cells were transfected with reference proviruses using Lipofectamine2000 (Invitrogen) as lipofectant. Following transfection cells were dispensed into 96-well plates and incubated for 4 days in the presence of HeLa-SxR5 as reporter cells as well as the respective test substances. At the end of the incubation period β -galactosidase activity was determined in each well. Data are expressed in per cent viral inhibition following normalisation of data with positive and negative control wells included in each 96-well plate (13, 14). Data were processed and analysed as described elsewhere (12).

Results

In an effort to identify new antiviral lead compounds of natural origin active against HIV, the aqueous extract of *A. amatymbica* was screened in a cell-based infection assay designed to detect inhibitors blocking any step of the viral life cycle. Two HIV-1 laboratory strains differing by their receptor tropism namely, NL4-3 for CXCR4-usage and NL-AD87 for CCR5-usage, were used for the assessment. The EC₅₀ values of the aqueous extracts were determined as 22 μ g/mL against the HIV-1 strain NL4-3 and 85 μ g/mL against NL-AD87. The extract was found to be non-toxic as judged by observing cell density and morphology.

In order to optimise the process of identifying active compounds, an accelerated purification and identification route was adopted (Figure 1). An HPLC fractionation protocol was established and a

total of 150 mg of extract was separated using reverse phase semi-preparative HPLC (see materials and methods). A maximum of 4 cycles of HPLC fractionation was performed per 96-well microtitre plate with the generation of three replicate plates. The first plate was used to identify fractions inhibiting HIV replication; the second plate was analysed using UPLC QTOF, and the third plate was stored as a reference plate. This approach speeds up the development time as correlation to bioactive fractions can be made within a much shorter timeframe than with a standard sequential method.

Selection of active fractions was based on two criteria: a threshold of more than 50% inhibition of viral replication for dilution 1:20 (Figure 2) and greater than 20% for the dilution 1:200 (data not shown) in addition to a lack of overt cellular toxicity. Based on these criteria, fractions B11 to D4 were considered the most active ones and were selected for further analysis. In order to ascertain if the fractions contained the same or a common compound, the base peak intensity (BPI) chromatogram (negative mode) of fractions B11, C6 and C12 were analysed in more detail (Figure 3).

Fraction C12 showed the presence of a single peak with a retention time of 5.36 min. Significantly, this peak was present in all selected active fractions but in varying concentrations. Based on this observation, the identified activity was attributed to the peak eluting at a retention time of 5.36 min. The ESI-QTOF MS (negative mode) data of fraction C12 (Figure 4) showed a pseudo molecular ion with a m/z 359.0726 $[M-H]^-$ corresponding to a mass of 360.0845, and the UV spectrum showed maxima at 242, 290(sh) and 329 nm. An evaluation of the accurate mass data was done using the following search criteria; C 5-30, H 1-100, O 0-10; mass tolerance < 5 mDa and using three (3) mass ions to calculate the iFit value. The iFit value is also called the Isotopic fit value and is an indication of how well the measured isotopic ion ratio compares to the theoretical isotopic ion ratio. These search criteria produced an empirical formula of $C_{18}H_{15}O_8$ with an iFit value of 0.0 and a DBE value of 11.5. The calculated values were submitted to ChemSpider (www.chemspider.com) and resulted in 117 hits with rosmarinic acid as the first one. Further analysis of the daughter ions through MS/MS fragmentation showed a caffeoyl group (m/z 197.0411 $[(M-H) - C_9H_6O_3]^-$). A search of the Dictionary of Natural Products based on the MS, UV and MS/MS fragmentation data identified the compound as rosmarinic acid, which has previously been reported to occur in *A. amatymbica* [11]. To definitely ascertain that the compound is identical to rosmarinic acid, the pure standard was obtained from a

commercial source. The retention time and mass fragmentation of the rosmarinic acid standard, when analysed by UPLC QTOF confirmed it is the major compound in the most active fractions. The activity of the pure compound against HIV replication was then assessed as described above. EC₅₀ values were 30µM and 47µM against NL4-3 and NL-AD87, respectively (Figure 5).

In addition, as rosmarinic acid was only found to be a minor component of fraction C4, the latter was analysed in greater detail. Using the exact same methodology as described above, the major component of this fraction was identified as caffeic acid (not shown). However, when tested in our bioassays, the commercial standard of caffeic acid only displayed a strong cytotoxic activity (not shown).

Discussion

The accelerated approach for identification of potentially new anti-HIV leads from complex plant extracts using our semi-preparative HPLC fractionation procedure was proven to be successful. This was demonstrated through the dereplication of aqueous extract of *A. amatymbica* which has led to the identification of rosmarinic acid, a compound already known to possess a weak inhibitory activity for HIV. This physico-chemical process was achieved within a period of 2 months including the time for biological screening. It has been reported (15) that the classical bioassay-guided approach for the dereplication of extracts typically takes much longer time (several months to even years) while often the realization that the active compounds are published or patented happens at a much later stage resulting in wastage of time and resources. The accelerated approach allows natural product drug discovery scientists to make a go/no go decision on the further development of the active compounds much earlier in the research development programme. In addition, the combination of medium-throughput screening methodology for activity using 96-well microtitre plates is essential for the success of this accelerated approach.

Caffeic acid, a key intermediate in lignin biosynthesis found in numerous plants, has been described as a weak inhibitor of HIV replication *in vitro* (16). However, in our bioassay this compound proved to be only cytotoxic. Some of its natural derivatives such as caffeic acid phenethyl ester are more potent inhibitors and target the integrase enzyme of the virus (17). Similarly, rosmarinic acid, which derives

from caffeic acid, has a superior anti-HIV activity with higher potency in our assay. This molecule is known to bind in the catalytic core of purified HIV-1 integrase and thereby blocks with equal potency both activities of this enzyme namely, 3'-processing and strand transfer (18). However, this desired antiviral property is counterbalanced by a poor uptake *in vivo* (19, 20) and by rapid degradation by gut flora (21). Therefore, due to these unfavourable properties, rosmarinic acid cannot be considered a suitable candidate for an anti-HIV drug, and for these reasons, the potential efficacy of aqueous extracts of *A. amatymbica* against AIDS cannot be ascribed to a direct antiviral activity.

On the other hand, but departing from the direct use of a herbal extract for HIV therapy, a medicinal chemistry approach towards simple synthetic modifications of rosmarinic acid could be undertaken with the view not only to render it bioavailable but also to produce more active analogues. Such an approach has been already described for caffeic acid, which has been used as a pharmacophore and modified with the aim to improve its bioactivities (22-24).

In conclusion, although the active ingredient identified in the aqueous extract of *A. amatymbica* does not support a direct application of this plant extract for treating HIV infection, it may form the starting point towards simplified plant-based chemical optimization of HIV inhibitors. In addition the methodology represents a highly suitable tool for the identification and antiviral profiling of new biologicals with pharmacological potential originating from complex, plant-based or microbiological preparations.

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References

1. Bessong PO & Obi CL (2006) Ethnopharmacology of human immunodeficiency virus in South Africa—a minireview. *African J Biotech* 5:1693-1699.
2. Singh IP, Bharate S, & Bhutani KK (2005) Anti-HIV natural products. *Current Science* 89(2):269-290.
3. UNAIDS (2010) UNAIDS report on the global AIDS epidemic. *Global Report*.
4. Bessong PO, *et al.* (2005) Evaluation of selected South African medicinal plants for inhibitory properties against human immunodeficiency virus type 1 reverse transcriptase and integrase. *Journal of ethnopharmacology* 99(1):83-91.
5. Burt BL (1991) Umbelliferae of southern Africa: an introduction and annotated checklist. *Edin J Bot* 48:133-282.
6. Townsend CC (1989) Umbelliferae. *Flora of Tropical East Africa*, ed Polhill RM (Balkema, A. A., Rotterdam), pp 1-127.
7. Hutchings A (1996) Zulu medicinal Plants. (Natal University Press, Pietermaritzburg), pp 223-224.
8. Watt JM & Breyer-Brandwijk MG (1962) *The medicinal and poisonous plants of Southern and Eastern Africa* (Livingstone, London).
9. Rustaiyan A & Sadjadi AS (1987) Kaurene derivatives from *Alepidea amatynsia* (A. amatymbica). *Phytochemistry* 26:2106-2107.
10. Somova LI, Shode FO, Moodley K, & Govender Y (2001) Cardiovascular and diuretic activity of kaurene derivatives of *Xylopiya aethiopica* and *Alepidea amatymbica*. *Journal of ethnopharmacology* 77(2-3):165-174.
11. Olivier D, Van Wyk B, & Van Heerden F (2008) The chemotaxonomic and medicinal significance of phenolic acids in *Arctopus* and *Alepidea* (Apiaceae subfamily Saniculoideae). *Biochem Systematics Ecol* 36:724-729.
12. Vidal V, *et al.* (2012) Library-based discovery and characterization of daphnane diterpenes as potent and selective HIV inhibitors in *Daphne gnidium*. *Journal of natural products* 75(3):414-419.
13. Klimkait T, Stauffer F, Lupo E, & Sonderegger-Rubli C (1998) Dissecting the mode of action of various HIV-inhibitor classes in a stable cellular system. *Archives of virology* 143(11):2109-2131.
14. Louvel S, *et al.* (2008) Detection of drug-resistant HIV minorities in clinical specimens and therapy failure. *HIV medicine* 9(3):133-141.
15. Clarkson C, Staerk D, Hansen SH, Smith PJ, & Jaroszewski JW (2006) Identification of major and minor constituents of *Harpagophytum procumbens* (Devil's claw) using HPLC-SPE-NMR and HPLC-ESIMS/APCIMS. *Journal of natural products* 69(9):1280-1288.
16. Kreis W, Kaplan MH, Freeman J, Sun DK, & Sarin PS (1990) Inhibition of HIV replication by *Hyssop officinalis* extracts. *Antiviral research* 14(6):323-337.
17. Fesen MR, Kohn KW, Leteurtre F, & Pommier Y (1993) Inhibitors of human immunodeficiency virus integrase. *Proceedings of the National Academy of Sciences of the United States of America* 90(6):2399-2403.

18. Mazumder A, *et al.* (1997) Curcumin analogs with altered potencies against HIV-1 integrase as probes for biochemical mechanisms of drug action. *Journal of medicinal chemistry* 40(19):3057-3063.
19. Baba S, Osakabe N, Natsume M, & Terao J (2004) Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid. *Life sciences* 75(2):165-178.
20. Lu T, *et al.* (2008) Plasma and urinary tanshinol from *Salvia miltiorrhiza* (Danshen) can be used as pharmacokinetic markers for cardiotoxic pills, a cardiovascular herbal medicine. *Drug metabolism and disposition: the biological fate of chemicals* 36(8):1578-1586.
21. Baba S, *et al.* (2005) Absorption, metabolism, degradation and urinary excretion of rosmarinic acid after intake of *Perilla frutescens* extract in humans. *European journal of nutrition* 44(1):1-9.
22. Bodiwala HS, *et al.* (2011) Design and synthesis of caffeoyl-anilides as portmanteau inhibitors of HIV-1 integrase and CCR5. *Bioorganic & medicinal chemistry* 19(3):1256-1263.
23. Ma CM, *et al.* (2010) Synthesis, anti-HIV and anti-oxidant activities of caffeoyl 5,6-anhydroquinic acid derivatives. *Bioorganic & medicinal chemistry* 18(2):863-869.
24. Touaibia M, Jean-Francois J, & Doiron J (2011) Caffeic Acid, a versatile pharmacophore: an overview. *Mini reviews in medicinal chemistry* 11(8):695-713.