Antileishmanial activity of 12-methoxycarnosic acid from *Salvia repens* Burch. ex Benth. (Lamiaceae)

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Abstract

In South Africa, *Salvia repens* is used traditionaly to treat sores, stomach ache and diarrhoea. The high performance liquid chromatography (HPLC)-based activity profiling of *S. repens* whole plant extract showed an active abietane diterpene which was identified as 12-methoxycarnosic acid (**1**) which showed antiprotozoal activity against axenically grown *L. donovani* amastigotes with IC_{50} of 0.75 µM with marginal cytotoxicity against the L6-cells (IC_{50}, 17.3 µM).

Keywords: Antileishmanial activity; *Salvia repens*; *Leishmania donovani*, 12-methoxycarnosic acid, abietane diterpene, HPLC profiling

Abbreviations: VL = Visceral leishmaniasis; HIV = Human Immunodeficiency virus; SANBI = South African National Biodiversity Institute; HPLC = high performance liquid chromatography; DMSO =dimethyl sulfoxide; PBS = phosphate buffer solution; FBS = fetal bovine serum; PDA = Photo Diode Array; NMR = Nuclear Magnetic Resonances; MS = Mass Spectrometry; SI = selectivity index; DHT = dihydrotestosterone; AR = androgen receptor; SM = simple monophasic.

1. Introduction

Visceral leishmaniasis (VL), also known as "Kala azar" is caused by the intracellular protozoan *Leishmania donovanii* and is transmitted by the bite of infected phlebotomine sandflies. It has been estimated that the annual global burden of VL is 500 000 new cases with more than 59 000 associated deaths (Den Boer et al., 2011). The control of VL relies entirely on chemotherapy due to lack of antileishmanial vaccines. In poor countries, however, the use of leishmanicidal drugs is hampered by high costs, toxicity and the emergence of resistance (Sundar et al., 2007). In addition, the emergence of the Human Immunodeficiency virus/Acquired Immunodeficiency disease (HIV/AIDS) has severely compromised the control of VL (Alvar et al., 2008). Therefore, there is an urgent need for the development of new leishmanial therapeutics.

The genus *Salvia* (sage) is the largest in the Lamiaceae family with over 900 species worldwide (Tan et al., 2002). Approximately 30 *Salvia* species occur in southern Africa of which 26 are found in South Africa especially in the Cape region (Codd, 1985). *Salvia* species are used in folk medicine for the treatment of asthma, eczema, psoriasis and tuberculosis (Salimpour et al., 2011). In South Africa, *Salvia* species are used against fever, headache and digestive disorders (Amabeoku et al., 2001), and to treat sores (Auge et al., 2003). A decoction of the roots is also used in South Africa for the treatment of stomach ache and diarrhoea (Auge et al., 2003). Several kinds of tanshinone-type diterpenoids (Ślusarczyk et al., 2011) abietane and icetexane diterpenoids and triterpenoic acids (Nieto et al., 2000; Tan et al., 2002; Kabouche et al., 2007), phenolic acids, phenolic glycosides, flavonoids and anthocyanins (Walker and Sytsma, 2007) have been reported from *Salvia* species. These constituents have shown various biological properties including antiviral (Tada et al., 1994), antiprotozoal (Sairafianpour et al., 2001;

Ślusarczyk et al., 2011; Farimani et al., 2012) antimalarial (Achenbach et al., 1992), antileishmanial (Tan et al., 2002) activities.

In our previous study, the dichloromethane/methanol (1:1) extract of the whole plant of *Salvia repens* showed promising antiprotozoal activity with a IC_{50} value of 5.4 µg/ml against axenically grown *Leishmania donovani* amastigotes (MHOM/ET/67/L82) (Mokoka et al., 2011). In this paper we report the isolation and identification of the active component and its antileishmanial activity.

2. Materials and methods

2.1. Plant collection

Salvia repens whole plant material was collected from the Eastern Cape Province near Aliwal-North on January 2002. The identification of plant material was done at the South African National Biodiversity Institute (SANBI, Pretoria) where the voucher specimen (BP00998) was deposited. The wet plant material was placed in an oven at 40°C for three days to dry, then milled to a fine powder and kept at room temperature in the dark before extraction.

2.2. Extract preparation

Finely ground plant material of *S. repens* (100 g) was extracted with (2 × 1 L) of a mixture of dichloromethane and methanol (1:1, v/v) overnight while occasionally aggitating. The mixture was filtered and concentrated using a rotavapour at 40 °C and left under a stream of cold air to dry.

2.3. HPLC activity profiling

The method described by Adams et al (2009) was used for the profiling of *S. repens* extract. Briefly, 10 mg/ml sample in dimethyl sulfoxide (DMSO) was prepared, filtered through 0.45 µm sterile filters for HPLC fractionation. Gilson 215 liquid handler with Gilson 819 injection module and 50 µl loop was used as the autosampler, connected to an HPLC system consisting of a 1100 series low-pressure mixing pump with degasser module, column oven, and a 1100 series PDA detector (Agilent, Waldbronn, Germany). The mobile system used was made up of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Separation conditions: Sunfire RP-18 column (3.5 µm, 3 mm × 150 mm i.d.; Waters GmbH, Eschborn, Germany), water (A) 90-0% in acetonitrile (B) over 30 min, 100% acetonitrile (B) for 5 min. The flow rate was 0.5 ml/min, and the injection volume was 35 µl (350 µg extract in DMSO). Thirty-five one-minute fractions were collected in 96 deep well plates during the run (Screenmates 96 well, Matrix Technology, Hudson, USA) and dried in a GeneVac EZ-2 Plus evaporator (Genevac Ltd., Ipswich, UK). The samples were redissolved in 5 µl of DMSO and diluted with 95 µl phosphate buffer solution (PBS) buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) for microfraction antileishmanial screening.

2.4. *In vitro* antileishmanial activity evaluation of the microfractions and isolated compound

The anti-leishmanial activity of the microfractions and isolated compound was evaluated by the method described by Adams et al. (2009) at two different concentrations (5 µg/ml) and (0.8

µg/ml) against *L. donovani* amastigotes (MHOM/ET/67/L82) in 96-well microtiter plate. The plate was read in a spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Synnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm. Flouresence development was measured and expressed as a percentage of the control. The IC_{50} value was determined from the sigmoidal inhibition curve and miltefosine was used as a positive control.

2.5. Cytotoxicity evaluations of 12-methoxycarnosic acid against L6-cells

Assays were performed in 96-well microtiter plates, as previously discussed by Adams et al (2009). The plates were incubated for 72 hours and thereafter inspected under an inverted microscope to assure growth of the controls and sterile conditions. A volume of 10 µl of resazurin was then added to each well and the plates incubated for a further 2 hours. The plates were then evaluated as described above. The IC_{50} value was calculated from the sigmoidal inhibition curve using SoftmaxPro software and podophylotoxin was used as a positive control.

2.6. Isolation of the active compound

The semi-preparative HPLC purification of the DCM/MeOH (1:1) crude extract of *Salvia repens* whole plant was performed with an HPLC 1200 series consisting of a low-pressure mixing pump with degasser module, a column oven and a PDA detector (Agilent, Waldbronn, Germany). A 100 mg/ml sample in DMSO was prepared and filtered through a 0.45 µm Millipore (Bedford, MA, USA) membrane filter and several repititions of 400 µl injections of the sample were made.

The separation was performed on a Waters Sunfire RP-18 column (10 × 150 mm i.d.; 10 µm; Waters GmbH, Ireland). The mobile system used was similar to that used for the HPLC activity profiling of the extract and made up of water (A) and acetonitrile (B). The elution gradient was linear from A:B (90:10) to (0:100) in 30 min at a flow rate of 6.0 ml/min followed by washing and returning to the initial elution conditions (90:10) over 5 min. The separation of the active component was detected at 280 nm. The targeted fraction containing the compound of interest was collected and dried in a Genevac evaporator overnight to afford compound **1**. The structure of this compound was determined by ^{1}H and ^{13}C nuclear magnetic resonances (NMR) spectroscopy and mass spectrometry (MS) analysis as well as by comparison with the published data and was identified as 12-methoxycarnosic acid (**1**) (Richheimer et al., 1996). Using 800 mg of crude extract 10 mg of pure compound **1** was isolated. The purity was >97% as was shown by HPLC.

3. Results and discussion

In our effort to identify potential antileishmanial compound/s from the DCM/MeOH (1:1) extract of *Salvia repens* whole plant, an analytical HPLC-based activity profiling technique (Adams et al., 2009) was used to fractionate the *S. repens* extract in a 96 deep-well microtiter plate. The antileishmanial activity was in fraction 27 (figure **1**). The HPLC chromatogram at 280 nm depicted in figure **1** made it possible to identify the active peak responsible for the observed antileishmanial activity.

Using semipreparative HPLC a known compound was isolated and identified as 12-methoxycarnosic acid (**1**) from its ^{1}H and ^{13}C NMR spectra and by comparison of the NMR and

MS data with that published in the literature (Richheimer et al., 1996). The compound has been isolated previously from *S. microphyllla* (Aydoğmuş et al., 2006) and *Rosmarinus officinalis* (Richheimer et al., 1996, Oluwatuyi et al., 2004).

The antileishmanial activity of 12-methoxycarnosic acid (**1**) was evaluated against axenic *Leishmania donovani* amastigotes and showed an IC_{50} value of 0.75 µM. Miltefosine (the positive control) had an IC_{50} of 0.19 µM. The selectivity index (SI), calculated as the ratio between IC_{50} value for cytotoxicity and IC_{50} value for *L. donovani* amastigotes for compound (**1**) was 23.2.

Previous reports have shown that **1** has very interesting biological activities. Murata and co-authors (2012) reported that, **1** resulted in a 66.7% binding inhibition of 5α-dihydrotestosterone (DHT) to the androgen receptor (AR) at 5 µM and the inhibition of 5α-reductase (IC_{50}, 61.7 µM), thereby playing a significant role in promoting hair growth. To the best of our knowledge this is the first report on the antileishmanial activity of 12-methoxycarnosic acid (**1**).

4. Conclusion

The use of HPLC-based activity profiling made it possible to identify the active compound responsible for antileishmanial activity. Compound (**1**), 12-methoxycarnosic acid isolated from *Salvia repens* showed in *vitro* antileishmanial activity against axenic *L. donovani* amastigotes. In addition, compound **1** has good selectivity. Further research into the structure of this compound could result in the development of new analogues with much more improved activity against this parasite.

Acknowledgements

The authors would like to thank the Council of Scientific and Industrial research (CSIR), and the Swiss Confederation for financial support under the Swiss South African Joint Research Programme (grant JRP 03), and the South African National Biodiversity Institute (SANBI) for the identification of plant species.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legend

Figure 1. Antileishmanial activity trace of a DCM/MeOH (1:1) extract of the whole plant of *Salvia repens* fractions and UV trace (280 nm) of the one minute fractions tested against axenically grown *L. donovani* amastigotes at lower concentration (0.8 µg/ml) and the Chemical structure of 12-methoxycarnosic acid (**1**)

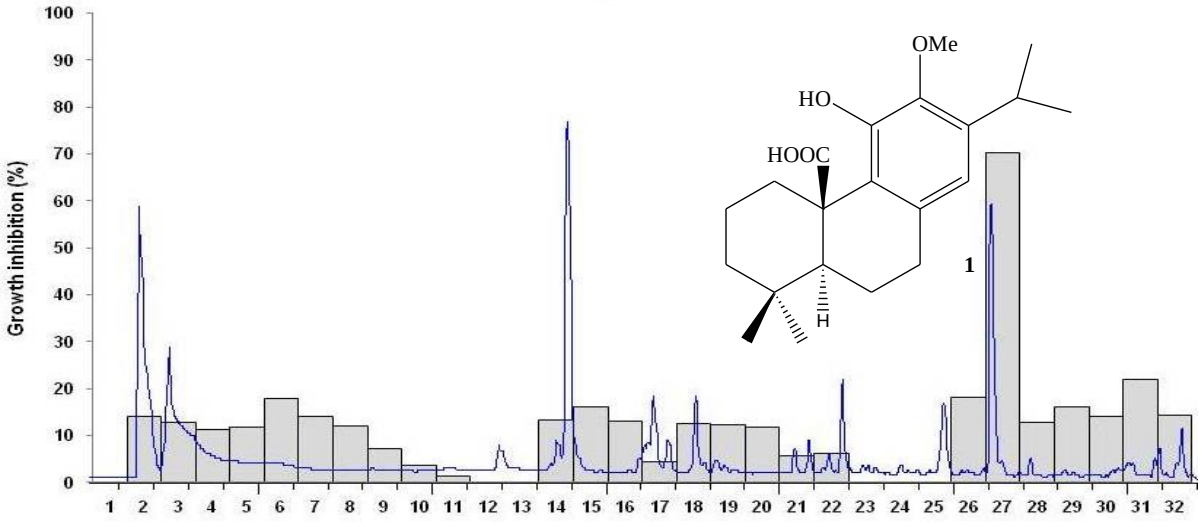
Growth inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
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Figure 1