Chemical and Bioactivity Studies on Salvia Africana-Lutea: Cytotoxicity and Apoptosis Induction by Abietane Diterpenes Isolated from Salvia Africana-Lutea


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Abstract

Re-investigation of the phytochemical constituents of Salvia africana-lutea L. methanolic extract, led to the purification and identification of six abietane diterpene (7-Methoxyrosmanol (1), rosmadial (2), rosmanol (3), carnosol (4), epiisorosmanol (5), epirosmanol (6)) and rosmaric acid methyl ester (7). The compounds 1 and 3-5 showed significant anti-proliferative effect on HT-29 and MCF-7 cells, additionally, 7-Methoxyrosmanol (1) found to induce apoptosis in colorectal adenocarcinoma cells. The apoptotic pathway is characterized by the activation of caspases cascade (viz caspase 9) which climaxcd in caspase-3 activation.


1. Introduction

The genus Salvia is the largest genus of the family Lamiaceae and encompasses 900 species worldwide of which 26 are found in southern Africa and many of them are used in local traditional medicine. One of the most important species used by traditional healers is Salvia africana–lutea (synonym: Salvia aurea) [1]. S. africana-lutea was used traditionally as an infusion to treat colds, tuberculosis, chronic bronchitis, influenza, gynaecological complaints [2], fever, headaches and digestive disorders [3].

S. africana-lutea extract showed good antimicrobial activity against Escherichia coli, Staphylococcus aureus and Microsporum audouinii. (MIC−39.06 μg/ml) [4]. The dichloromethane: methanol (1:1; v/v) extracts demonstrated strong inhibition of Fusarium verticillioides and F. proliferatum with MIC values of 31 and 63 μg/ml respectively [5]. S. africana-lutea collected from Western Cape showed antioxidant activity using ABTS assay (IC50 30.4 ± 2.97) and DPPH (47.6±2.61), with weak anti-inflammatory activity [6]. A water extract significantly inhibited acetic acid-induced writhing and also significantly delayed the time of reaction of mice to thermal stimulation produced by the hot plate. The plant also significantly reduced fever induced by LP. Which indicate the analgesic and antipyretic potential of the plant [3].

A chemical study on an ethanolic extract of the aerial parts of S. africana-lutea (S. Aurea) afforded 12-methoxycarnosic acid, carnosol, 7-methoxyrosmanol, rosmanol, and ursolic acid [7]. Our preliminary phytochemical study on S. Africana-lutea collected from South Africa resulted in the identification of three known compounds; carnosol, rosmadial and carnosic acid methyl ester. The later compound showed interesting anti-TB (MIC 28 μM), as well as cytotoxic activity against a breast (MCF-7) human cancer cell line with IC50 69 μM [8]. Additionally, the methanolic extract demonstrated interesting chemical profile on TLC with spots indicated several interesting abietane diterpenes. These factors in addition to the important traditional uses lead us to re-investigate the methanolic extract to identify more bioactive abietane diterpenes and evaluate them biologically.

2. Materials and methods

General Experimental Procedures.

1H and 13C NMR spectra were recorded in CDCl3 solution on a Jeol 600 spectrometer at 600 and 150 MHz, respectively. Chemical shifts are reported relative to the residual CHCl3 or CD3OD signals (δ 7.25/3.35) for protons and 77.0/49.3 for carbons. Merck Si gel (70-230 mesh and 230-400 mesh, for gravity flow chromatography) was used for column chromatography. Merck 5554 Kieselgel 60 F254 sheets were used for HPLC analysis. hexane, dichloromethane (DCM), methanol (MeOH) technical grade were used for column chromatography and MeOH HPLC grade for HPLC.

Plant Materials. Aerial parts of S. africana-lutea L. from the same plant community of last study[8], were collected in November 2010, at the Botanical Garden of the Department of Botany, University of Pretoria, South
Africa, and were identified by HGWJ Schweickerdt Herbarium of the University of Pretoria, where voucher specimens (registry numbers: S. africana-lutea, A. H. 095053) are preserved.

**Plant collection and extraction:**

Fresh aerial parts left to dry at room temperature (~27 °C) for three weeks. 500 g of the plant powder were extracted with methanol (7.0 L X 3) at room temperature. The combined extracts were concentrated under reduced pressure at 45 °C using rotary evaporator. The total extract (50 g) was fractionated on a silica gel column (6x120 cm) eluted with hexane (3L), followed by a gradient of hexane: dichloromethane (DCM) up to 100% DCM and DCM-MeOH gradient up to 15% MeOH. Similar fractions were pooled together according to the TLC to yield 13 main fractions. Fraction 4-6 and 6 showed signals of abietane diterpene (according to NMR analysis). Fraction four was further purified by HPLC, Column, YMC-Pack ODS-A (250X10 mm i.d.), flow rate 8 ml/min, eluted with MeOH: H2O (10:30) to afford SAHH1-1 (10 mg), SAHH 2-1 (11 mg), SAHH-3 (7 mg) and SAHH (9 mg). Fraction five was also purified by HPLC under the same conditions, to afford SAHH-4 (12 mg). Fraction six was further purified by HPLC to afford compound SAHH5-13 (15 mg). Fraction seven was purified by HPLC eluted with MeOH-H2O (50:50) to afford SAHH-6 (15 mg).

**Cell culture**

The HT29 (colon adenocarcinoma) cells were kindly provided by Prof Denver Hendricks (Department of Clinical and Laboratory Medicine, University of Cape Town - South Africa). The Caco-2 (human colorectal adenocarcinoma) and the MCF7 (breast adenocarcinoma) cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in a 37°C humidified incubator with 5% CO2 in a shaker and the absorbance of the samples measured at 450 nm (reference wavelength was 750 nm) using a Promega Micro-plate (Madison, WI, USA). Cytotoxicity was expressed as a percentage of the absorbance measured in control untreated cells. IC50 values were calculated using Prism Graph pad software. Triplicates experiment and the results expressed as mean ± SEM.

**APOPercentage™ assay**

The induction of apoptosis was assessed using the APOPercentage assay (Biocolor Ltd., UK). The cells (HT-29 and MCF-7) were plated in 24 well culture plates and treated for 48 h with 50 µg/ml of the compound H1 and evaluated for caspases 9 and Caspase 3/7 activity. All treatments were done in triplicate. The results were confirmed using flow cytometry on a Becton Dickinson FACSscan instrument (BD Pharmingen™, USA) as described by [9].

**Caspase 3/7and 9 assay**

Caspases 9 and 3/7 activity was measured using the Caspase-Glo® 9 and Caspase-Glo® 3/7 assays (Promega Corp., USA) according to the method described by [10] with slight modifications. The cells were plated in 96-well culture plates and treated for 6, 12 and 24 h with 6.25-25 µg/ml of the compound H1 and evaluated for caspases 9 and 3/7 activities. After treatments, the cells were lysed and the cleavage of the substrate by caspases was measured by the generated luminescent signal with a 96 multi-well Glomax luminometer (Promega Corporation, USA).

**Statistical analysis**

Data were represented as mean ± SEM of at least three independent experiments. Data were analyzed using Prism Graph Pad software (San Diego, USA). Statistic test two-way ANOVA and Bonferroni post hoc test were conducted for pairwise comparisons. P value less than 0.05 was considered statistically significant.

**Results and discussion;**

A detailed phytochemical study on a methanolic extract of S africana-lutea using prep-HPLC leads to the purification six known abietane diterpenes (1 - 6) and rosmarinic acid methyl ester (7). The compounds were identified based on NMR intensive analysis.
Effect of the isolated compounds on the Cell viability of Caco-2, HT-29 and MCF-7 cells

We examined the effect of compounds 1 and 3-5 on cell viability using WST-1 assay. The tested compounds decreased cell viability of Caco-2, HT-29 and MCF-7 cells. The cells were treated with different concentrations of the compounds; their viability was determined by the uptake of the formazan dye and expressed as percentage of untreated control cells. The compounds induced a dose-dependent increase in viable formazan accumulating cells after the treatment (Fig. 2A-C). The 50% growth inhibition concentration IC\textsubscript{50} obtained after 24 h of incubation are shown in Table 1.
Fig (2): Effect of compounds 1 and 3-5 on the viability of Caco-2, HT-29 and MCF-7 cells. (A) Cells were treated with various concentrations of 1 and 3-5; the relative cell viability was assessed by WST-1 assay for 24 h on Caco-2 cells. (B) Cells were treated with various concentrations of 1 and 3-5; the relative cell viability was assessed by WST-1 assay for 24 h on HT-29 cells. (C) Cells were treated with various concentrations of 1 and 3-5; the relative cell viability was assessed by WST-1 assay for 24 h on MCF-7 cells. The results represent the mean ± SEM of three independent experiments.
Table (1): IC_{50} values of compounds H1-H5

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<tr>
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<th>Caco-2</th>
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<td>1</td>
<td>16.0</td>
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<td>6</td>
<td>22.7</td>
<td>&lt;6.25</td>
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Apoptosis induction

The effect of 1 and 3-5 on HT-29 and MCF-7 cell growth was assessed using the APOPercentage™ dye which detects apoptosis at the stage of phosphatidylyserine externalization and it’s specific for the quantitation of apoptosis. The cells were treated for 24 h with different concentrations of the compounds and stained with APOPercentage™ dye and then analyzed by flow cytometry. The tested compounds induced substantial levels of apoptosis in a dose-dependent manner as illustrated in (Fig.3A&B).

Fig (3): Effect of compounds 1 and 3-5 on apoptosis in HT-29 and MCF-7 cells. (A) Flow cytometry analysis of APOPercentage™ dye staining after exposure of compounds 1 and 3-5 treated with various concentrations on HT-29 cells for 24 h. (B) MCF-7 cells were treated with increasing concentrations of 1 and 3-5; apoptosis was assessed by APOPercentage™ assay for 24 h as determined by flow cytometry. The results represent the mean ± SEM of three independent experiments.

Caspase 3/7 and 9 inductions

Caspase activation which mediates apoptosis was analysed by the exposure of HT-29 and MCF-7 to the compounds 1 and 3-5. The expressions of caspase 3/7 and 9 activities were measured in cells exposed to 6.25µg/ml.
and 25 µg/ml of the compounds and incubated for 6-24 h. The levels of caspase activation in the cells were compared with untreated control cells arbitrarily set to 1.0; the results showed that the compound 6 significantly increase caspase 3/7 activation at the lowest dose 6.25 µg/ml and the highest dose of 25 µg/ml after 24 h. It is noteworthy that the caspase 3/7 activity reached a maximum (1.78-fold increase) at 24 h when HT-29 cells were treated with 25 µg/ml (Fig.4A). Our result in Fig. 4B further confirmed that MCF-7 cells lacked caspase 3 activities. Similarly, caspase 7 was not activated at the concentration used in the assay. The treatment on HT-29 cells showed that after 24 h, significant activation of caspase 9 was observed at the lowest dose 6.25 µg/ml used in the assay. No activation was observed at 25 µg/ml, a significant drop in the caspase 9 activity was also noted after 24 h, see Fig. 5A. From Fig. 5B, it can be observed that MCF-7 caspase activity was dose dependent and it was significant at 6.25 µg/ml for 6 and 24 h, but highly significant at 12 h (1.50-fold increase). No activation was observed at 25 µg/ml for 6-24 h.

![Graph A](image1.png)

**HT-29**

![Graph B](image2.png)

**MCF-7**

Fig (4): Compounds 1 and 3-5 apoptosis is mediated by caspase 3/7 activation. (A) Treatment of cells with various concentration of 1 and 3-5 for 6-24 h on HT-29 cells. (B) Cells were treated with increasing concentrations of 1 and 3-5 for 6-24 h on MCF-7 cells. Data are presented as mean ± SEM, ***P< 0.001, **P< 0.01 and *P< 0.05 compared with control.
3. Discussion
Cancer continues to be one of the major causes of death worldwide [11]. Cancer chemoprevention with natural products, specifically the phytochemicals present in various edible and non-edible medicinal plants, has received significant attention over the last few years [12,13]. Our previous paper described the cytotoxic properties of some of these diterpenoids on two cell lines[8]. However, their mechanisms of action were yet to be identified. The present study is to evaluate the potential anticancer effects of the compounds 1 and 3-5. Our results revealed that the compounds were cytotoxic on Caco-2, HT-29 and MCF-7 cells while 6 was apoptotic on HT-29 and MCF-7 cells in a dose dependent manner. Compound 6, also activated both caspases 3/7 and 9 at a significant level. Several diterpenoids have been reported for their anticancer activity. For instance; the three diterpenes isolated from Jatropha plants (J. gossypifolia and J. curcas), identified as jatrophone, curcusone B and jatropholone A has shown cytotoxic potency toward HeLa and WiDr cell lines. Jatrophonewas found to show better potency than curcusone B and jatropholone A. Precisely, jatrophonewas found to be more active than anticancer standard against HeLa and WiDr cell lines with IC50 values 5.13 and 8.97 µM respectively [14]. Diterpenes from Jatropha curcas have also been shown to be cytotoxic against five human cancer cells [15]. Similarly, isolated diterpenoids from the twigs and leaves of Fokienia hodginsii has been reported to be cytotoxic against some panel of cancer cell lines. Compound 9 showed moderate cytotoxicity against HL-60 and SMMC-7721 cell lines, with IC50 values of 9.10 and 7.50 µM, respectively [16]. Similarly, some diterpenoids have also demonstrated apoptosis in some cancer cells. The bioactivity evaluation shows that cyathin Q has a strong anticancer activity against HCT116 cells and Bax-deficient HCT116 in vitro and in vivo. This compound induced hallmarks of apoptotic events in HCT116 cells, including caspase activation, cytochrome c release, poly

Fig (5): Measurement of caspase 9 activity by 1 and 3-5. (A) Treatment of cells with various concentrations of 1 and 3-5 for 6-24 h on HT-29 cells. (B) Cells were treated with increasing concentrations of compounds 1 and 3-5 for 6-24 h MCF-7 cells. Data are presented as mean ± SEM, **P < 0.001, **P < 0.01 and *P < 0.05 compared with control.
(ADP-ribose) polymerase (PARP) cleavage, and depolarization of the mitochondrial inner transmembrane potential [17]. Carnosol has been reported to possess anticancer and its potential ability in blocking the growth of human breast, ovarian, and intestinal tumor cell lines [18]. Diterpenes 7-ethoxyrosmanol (1) and carnosol (2) isolated from the extracts of Hyptis dilatata had been reported to be cytotoxic against a panel of three cancer cell lines (MCF7, HeLa and HT29) using curcumin as positive control. Carnosol showed cytotoxic activity against all three cell lines having a similar response as the positive control with an IC50 approximately of 15-20 μg/ml in each case. 7-ethoxyrosmanol showed lower activity than carnosol, with an IC50~ 20-30 μg/ml for the three cancer cell lines [19].

4. Conclusion
The compounds 1 and 3-5 showed significant anti-proliferative effect on HT-29 and MCF-7 cells. To the best of our knowledge, this is the first report on the induction of apoptosis in colorectal adenocarcinoma cells for 7- β-methoxyrosmanol (6). The apoptotic pathway is also characterized by the activation of caspases cascade (vis caspase 9) which climaxed in caspase 3 activation.

Diterpenoidcyathin Qh have been reported to induce apoptosis by a similar mechanism [17]. However, additional work is required especially on other possible mechanisms like caspase 8, ROS, cell cycle and others. However, we hope that our discoveriesmay possibly contribute to the development of the compounds as anticancer agents.

References