

Antimalarial and anticancer activities of selected South African *Salvia* species and isolated compounds from *S. radula*

G.P.P. Kamatou^a, R.L. Van Zyl^a, H. Davids^a, F.R. Van Heerden^b,
A.C.U. Lourens^b, A.M. Viljoen^{c,*}

^a Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, 2193, South Africa

^b School of Chemistry, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

^c Department of Pharmaceutical Sciences, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa

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Abstract

Extracts of seventeen *Salvia* species used in traditional medicine in South Africa were subjected to biological testing. The potential ability to inhibit the *in vitro* growth/proliferation of *Plasmodium falciparum* (FCR-3 strain) and the cytotoxic effects on three human cancer cells [breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29) and glioblastoma (SF-268)] and a human kidney epithelial cell line were investigated. The extracts displayed antimalarial activity with IC₅₀ values ranging from 3.91 to 26.01 µg/ml and *S. radula* displaying the most favorable activity. Two compounds were subsequently isolated from the active fraction of *S. radula* and identified as betulafolientriol oxide and salvigenin. The two compounds displayed similar or lower antimalarial activity (IC₅₀ values: 4.95 and 24.60 µg/ml, respectively) compared to the crude solvent extract. The concentration required to inhibit 50% of cancer cells ranged between 9.69 µg/ml and 43.65 µg/ml, and between 8.72 µg/ml and 59.12 µg/ml against the MCF-7 and SF-268 cell lines, respectively. The IC₅₀ values determined for the HT-29 cell line ranged from 17.05 to 57.00 µg/ml, with *S. lanceolata* being the most active. The samples also displayed some degree of toxicity when tested against the human kidney epithelial cells, with IC₅₀ values ranging from 12.12 to 53.34 µg/ml. The *in vitro* antimalarial and anticancer activities support the historic and present use of *Salvia* species in traditional medicine.

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Keywords: *Salvia*; Antimalarial activity; Anticancer activity; Toxicity; Betulafolientriol oxide; Salvigenin

1. Introduction

Many studies have been carried out on the antimalarial and anticancer activities of various plant species with some encouraging results (e.g. Schwikkard and Van Heerden, 2002; Boik, 2001). Two of the most potent antimalarial drugs originated from plants; quinine from *Cinchona* and artemisinin from *Artemisia annua* (Gessler et al., 1994). Plants have a reputable history of use in the treatment of cancer. In a review by Hartwell (1982), more than 3000 plant species are listed that have reportedly been used in the treatment of cancer, but in many instances, the “cancer” is undefined (Cragg and Newman, 2005). Over 60% of currently used anticancer agents are derived in one way or another from natural sources including plants,

marine organisms and micro-organisms (e.g. paclitaxel, topotecan and irinotecan) (Cragg et al., 1997; Cragg and Newman, 2005).

In South Africa, reports on plants used for the treatment of cancer are rare, and can be ascribed to a complex set of signs and symptoms associated with the disease (Steenkamp and Gouws, 2006). With this in mind, it is recommended that when selecting plants for potential anticancer activity, ethnopharmacological properties such as immune, inflammatory and skin disorders should be considered (Cordell et al., 1991; Popoca et al., 1998).

Salvia species have been used in traditional medicines in China, South Africa and many other countries against various infectious and inflammatory diseases and to treat malaria, hard swellings, abscesses, calluses, warts or cancer (Watt and Breyer-Brandwijk, 1962; Ulubelen et al., 1999; Shoemaker et al., 2005). In our previous investigation, we demonstrated that the essential oils of indigenous *Salvia* species exhibited various

* Corresponding author. Tel.: +27 12 382 6360; fax: +27 12 382 6243.

E-mail address: viljoenam@tut.ac.za (A.M. Viljoen).

biological properties such as anti-inflammatory, antimalarial and antibacterial activities (Kamatou et al., 2005, 2006). In a continuation to verify the efficacy of traditional medicines used in South Africa, the antimalarial and anticancer activities of the solvent extracts of seventeen *Salvia* species were investigated.

2. Materials and methods

2.1. Plant material

The aerial parts of 17 *Salvia* species were collected from various localities in South Africa between December 2003 and December 2004, predominantly from the Cape region (Table 1). The identity of each species was confirmed by the South African National Biodiversity Institute (Pretoria) and voucher specimens were deposited in the Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa. The air-dried plant material was ground to a fine powder, extracted with a mixture of methanol and chloroform (1:1, v/v) and the solvent evaporated under vacuum at 70 °C.

2.2. Preparation of samples

Stock solutions of the solvent extracts and reference compounds [5'-fluorouracil (Merck), chloroquine diphosphate (Sigma), quinine sulfate (Fluka)] were prepared in dimethyl sulfoxide (Saarchem) at a concentration of 10 mg/ml and stored at -20 °C. The dilutions were prepared with appropriate experimental medium on the day of the experiment.

2.3. In vitro maintenance of *Plasmodium falciparum* and the antimalarial assay

The chloroquine-resistant *P. falciparum* FCR-3 strain was continuously cultured according to the procedure described by Trager and Jensen (1976) with modifications as described by Van Zyl and Viljoen (2002). The antiplasmodial activity was assessed using the [³H]-hypoxanthine method (Desjardins et al., 1979; Van Zyl and Viljoen, 2002) against a single cycle (48 h) of growth. Dilutions of the test samples were plated out in triplicate in a 96-well plate before parasitized red blood cells (0.5% parasitaemia and 1% haematocrit) were added and incubated for 24 h at 37 °C in a candle jar. The [³H]-hypoxanthine (Amersham) was then added to the plate and incubated for a further 24 h. The amount of the [³H]-hypoxanthine incorporated into the parasite DNA was determined with beta scintillation counting (Wallac®). The inhibitory concentration which killed 50% of parasites as indicated by the *in vitro* uptake of [³H]-hypoxanthine (IC₅₀ value) was calculated. Chloroquine diphosphate and quinine sulphate were used as reference antimalarial drugs.

2.4. In vitro cancer cell line maintenance and sulforhodamine B assay

Cells lines representing the most common human cancers (WHO, 2006) were obtained from the National Cancer Institute (NCI). These included the breast adenocarcinoma (MCF-7), the glioblastoma (SF-268) and the colon adenocarcinoma (HT-29).

Table 1
The IC₅₀ values (mean±S.D., µg/ml) of antimalarial activity and cytotoxic effects of solvent extracts of indigenous *Salvia* species

Species	Location	Antimalarial activity (n=3)	Cytotoxic effects on various human cell lines (n=3)			
			HT-29	MCF-7	SF-268	Kidney cells
<i>S. africana-caerulea</i>	SWC ^a	22.68±2.80	27.10±1.08	23.36±4.20	8.72±1.52	14.38±3.39
<i>S. africana-lutea</i>	SWC ^a	15.86±5.04	20.00±3.41	43.65±8.38	nc	25.01±1.94
<i>S. albicaulis</i>	KBG ^b	15.83±1.94	25.33±1.11	35.25±5.66	27.50±0.89	37.29±0.58
<i>S. aurita</i>	KBG ^b	8.92±2.63	24.58±6.41	17.28±1.71	44.87±2.48	28.31±4.94
<i>S. chamelaeagnea</i>	KBG ^b	8.71±1.96	29.53±5.14	18.12±1.67	34.98±4.07	24.76±7.60
<i>S. disermas</i>	KBG ^b	24.17±4.10	26.87±0.57	38.56±1.28	59.12±2.75	53.34±3.90
<i>S. dolomitica</i>	Ex Manning	7.62±1.44	nc	37.05±2.10	nc	40.26±9.07
<i>S. garipensis</i>	KBG ^b	13.95±3.76	nc	39.44±3.90	nc	42.44±3.03
<i>S. lanceolata</i>	SWC ^a	26.01±2.95	17.05±3.50	26.15±2.15	nc	26.71±6.41
<i>S. muirii</i>	KBG ^b	11.87±2.13	55.63±1.30	39.07±2.86	nc	37.00±5.41
<i>S. namaensis</i>	Swartberg	25.38±2.11	24.39±3.42	36.36±3.07	nc	21.91±2.89
<i>S. radula</i>	Road to Derby	3.91±0.52	32.10±2.93	9.69±0.92	27.55±4.52	20.12±4.02
<i>S. repens</i>	KBG ^b	8.25±2.09	43.62±5.17	23.36±2.16	nc	23.24±4.72
<i>S. runcinata</i>	Klerkskraal Dam	16.61±3.33	55.37±2.10	43.42±5.46	nc	22.00±3.75
<i>S. schlechteri</i>	KBG ^b	17.51±2.05	57.00±11.67	18.37±0.47	54.40±4.42	28.16±4.69
<i>S. stenophylla</i>	East of Clarens	6.50±1.37	17.41±2.65	23.74±1.96	43.86±3.43	12.12±2.02
<i>S. verbenaca</i>	De Rust	23.97±1.10	50.04±5.39	31.50±13.70	nc	20.85±2.59
5'-FU		–	7.00±2.02	1.11±0.31	nc	>100
Chloroquine		0.06±0.01	–	–	–	>100
Quinine		0.14±0.03	–	–	–	>100
Betulafolientriol oxide	(1)	4.95±2.00 ^c	–	–	–	>100
Salvigenin	(2)	24.60±1.38 ^c	–	–	–	>100

nc: IC₅₀ values not calculated because the percentage inhibition at 100 µg/ml was less than 80%. –: not determined.

^a SWC: South Western Cape.

^b KBG: Kirstenbosch Botanical Garden.

^c Mean±s.e. (n=1) due to the small quantity of the isolated compound.

The three cell lines were cultured in either RPMI-1640 or DMEM (Highveld Biological) culture medium and supplemented with 10% fetal bovine serum.

The colourimetric sulforhodamine B (SRB) assay was performed according to Monks et al. (1991) and Wu et al. (1993). Cells were seeded at 15 000 cells/well and incubated for 24 h. Thereafter, different sample dilutions were added to the appropriate microtitre wells and the plate incubated for a further 48 h. At the end of the 48 h incubation period, cells were fixed with 50% (w/v) trichloroacetic acid (TCA; Saarchem) for 1 h at 4 °C. TCA-fixed cells were stained with 0.4% (w/v) SRB solution (Sigma®) for 10 min at room temperature. Bound dye was solubilised with 10 mM tris (hydroxymethyl) aminomethane (Merck) and the absorbance was read at 492 nm against a tris (hydroxymethyl) aminomethane blank on an automated spectrophotometer plate reader (Labsystems iEMS reader MF) connected to the Ascent® version 2.4 software. 5'-Fluorouracil (5'-FU) was used as positive control.

2.5. *In vitro* maintenance of human kidney cells and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, USB) colourimetric assay was performed on transformed human kidney (Graham) cells according to Mosmann (1983), Van Zyl and Viljoen (2002). Cells were seeded at 45 000/well and exposed to test extracts at 37 °C for 44 h. The MTT solution (0.5%, w/v) was then added and the plate incubated for a further 4 h before the absorbance was read at the test wavelength of 540 nm and 690 nm (as a reference) using a spectrophotometer plate reader (Labsystems iEMS reader MF) connected to the Ascent® version 2.4 software. The toxicity of the plant extracts was compared to 5'-FU.

2.6. Isolation of compounds by column chromatography

As *S. radula* exhibited the best antimalarial activity among the plants screened, it was selected for the bioassay-guided isolation of the active compound(s). The residue obtained (22.4 g) from the methanol/chloroform extract (1:1; v/v) was mixed with silica 60 (0.063–0.2 mm/70–230 mesh ASTM), dried to a fine powder. The extract was eluted with hexane:dichloromethane (9:1), dichloromethane:methanol (6:1) and finally with 100% methanol. Five fractions were collected and tested for antimalarial activity. Fraction 3 (12.5 g) was found to display the best activity and was selected for further purification. It was re-chromatographed with toluene:ethyl acetate (10:3). A total number of 222 subfractions in 3 ml test tubes were collected and spotted onto a TLC plate developed in with toluene:ethyl acetate (10:3). The separated spots were viewed under UV (254 nm and 366 nm) and also sprayed with 0.5% anisaldehyde (v/v) sulfuric acid reagent (Fluka®) in glacial acetic acid:methanol:sulfuric acid (10:85:5). Subfractions with similar R_f values were combined and concentrated to afford 7 major fractions (1–7). Two of the major fractions were selected based on the results of their biological activity. Fraction 3 eluted with toluene:ethyl acetate (10:3) resulted in the

isolation of compound 1 (29.4 mg). Compound 2 (24.1 mg) was obtained after repeated column chromatography of fraction 5 with toluene:ethyl acetate (10:2). The structures of the two compounds were determined using the ^1H NMR and ^{13}C NMR data recorded on a Varian Inova 2000, 300 MHz spectrometer. All spectra were recorded at 25 °C in deuterated chloroform and the chemical shifts were recorded in ppm referenced to tetramethylsilane (TMS). Electron ionization MS of the isolated compounds were performed by direct inlet at 70 eV on a GC-MS QP 2010 gas chromatography-mass spectrometer. Structures were confirmed by comparison of the ^1H NMR and ^{13}C NMR data of the isolated compounds with literature values (Voinin, 1983; Harraz et al., 1995; Rouf et al., 2001).

2.7. Data analysis

The IC_{50} values (concentration at which 50% of cells/parasites were killed) are reported as mean \pm standard deviation of three independent experiments. The IC_{50} values against the human cancer cell lines were only calculated for the solvent extracts exhibiting at least 80% inhibition when tested at a concentration of 100 $\mu\text{g/ml}$. One-way analysis of variance (ANOVA) and Student *t*-tests were used to compare data using Statistica version 5.0 software at a 95% confidence limit. A correlation between the anticancer activity and toxicity profile was assessed using Pearson's correlation coefficient.

3. Results and discussion

3.1. The antimalarial activity of *Salvia* species

The *in vitro* inhibitory effects of the *Salvia* extracts against the chloroquine-resistant *P. falciparum* (FCR-3) are shown in Table 1. The antimalarial activity of the extracts (IC_{50} values) ranged from 3.91 ± 0.52 to 26.01 ± 2.95 $\mu\text{g/ml}$, with the extract of *S. radula* being the most active ($p < 0.05$). One-way analysis of variance showed that there was no difference in the antimalarial activity of the extracts of *S. aurita*, *S. chamelaeagnea*, *S. dolomitica*, *S. muirii*, *S. repens* and *S. stenophylla* ($p > 0.05$). Similarly, the activity of *S. africana-lutea*, *S. albicaulis*, *S. garipensis*, *S. runcinata* and *S. schlechteri* did not differ ($p > 0.05$). The extract of *S. africana-caerulea*, *S. disermas*, *S. lanceolata*, *S. namaensis* and *S. verbenaca* also exhibited comparable activity ($p > 0.05$). All the extracts were less potent than the two antimalarial reference drugs chloroquine diphosphate and quinine sulfate ($p < 0.05$).

In this study, the solvent extracts from *Salvia* species exhibited antimalarial properties against the chloroquine-resistant *P. falciparum* FCR-3 strain, with all IC_{50} values lower than 30 $\mu\text{g/ml}$. Clarkson et al. (2004) also demonstrated that *S. repens* displayed antimalarial activity against the chloroquine-sensitive D10 strain of *P. falciparum* (IC_{50} value: 10.8 $\mu\text{g/ml}$) using the pLDH assay, which is similar to that reported in the current study (IC_{50} value: 8.25 ± 2.09 $\mu\text{g/ml}$).

The isolated compounds from *S. radula* and the presence of betulafolientriol oxide (IC_{50} value: 5 $\mu\text{g/ml}$) in all the investigated species (Kamatou, 2006), may have contributed to the overall

activity of the solvent extracts. The inhibitory activity is probably due to betulafolientriol oxide acting alone or in combination with other compounds present in the solvent extract.

3.2. Identification, characterisation and antimalarial activity of the isolated compounds

Compound 1 was isolated as a white powder. From the mass spectrum, compound 1 has an M^+ at m/z 476, which corresponds to a molecular formula of $C_{30}H_{52}O_4$. This compound, a diterpene, was identified as betulafolientriol oxide. Compound 2 was isolated as a yellow powder and was identified as 5-hydroxy-6,7,4'-trimethoxyflavone (salvigenin), with a molecular formula of $C_{18}H_{16}O_6$. The structures of the two compounds are presented in Fig. 1. The two isolated compounds were tested for antimalarial activity. Betulafolientriol oxide exhibited the best activity compared to the flavonoid (salvigenin), with 4.95 ± 2.00 $\mu\text{g/ml}$ killing 50% of parasites (Table 1). The two compounds exhibited comparable or lower antimalarial activity than the solvent extract of *S. radula* and the reference antimalarial drugs (Table 1). In theory, fractionation leading to the isolation of individual compounds should result in aliquots having higher activity than the original extract. However, in this study, the activity of the isolated compounds was not greater than that of the crude extract. This loss of activity may be explained by the possibility of decomposition of the active compounds during the fractionation procedure (Houghton et al., 2007). Decomposition of constituents to less active substances may take place due to the reaction with solvents used. The loss of activity may also be due to the fact that synergy might account for the better activity of mixture than isolated compounds. This has been claimed by many herbalists, but it is only recently that it has received much scientific attention, partly because it is now possible to demonstrate it experimentally (Houghton et al., 2007).

3.3. Anticancer activity of *Salvia* species

The inhibition of tumour cell proliferation by the solvent extracts of the 17 indigenous *Salvia* species investigated are displayed in Table 1. All the extracts were significantly less

potent than 5'-FU ($p < 0.05$). The best cytotoxic activity against the HT-29 cell line was obtained with the extracts of *S. lanceolata* (IC_{50} value: 17.05 ± 3.50 $\mu\text{g/ml}$); *S. stenophylla* (IC_{50} value: 17.41 ± 2.65 $\mu\text{g/ml}$) and *S. africana-lutea* (IC_{50} value: 20.00 ± 3.41 $\mu\text{g/ml}$), *S. aurita* (IC_{50} value: 24.58 ± 6.41 $\mu\text{g/ml}$) and *S. namaensis* (IC_{50} value: 24.39 ± 3.42 $\mu\text{g/ml}$), showed no significant difference in their activity ($p > 0.05$). Six species, viz. *S. africana-caerulea*, *S. albicaulis*, *S. aurita*, *S. chamelaeagnea*, *S. disermas* and *S. namaensis*, did not differ significantly from one another in their inhibitory effects ($p > 0.05$).

S. radula exhibited the most favourable activity (IC_{50} value: 9.69 ± 0.92 $\mu\text{g/ml}$) ($p < 0.05$), while *S. africana-lutea* and *S. runcinata* were among the least active against the MCF-7 cells (Table 1). Two species, *S. dolomitica* and *S. garipensis*, showed a degree of selectivity, as they were not active against the HT-29 (IC_{50} value > 100 $\mu\text{g/ml}$) and SF-268 (IC_{50} value > 100 $\mu\text{g/ml}$) cell lines, but active against the MCF-7 cell line (IC_{50} value < 39 $\mu\text{g/ml}$) (Table 1).

In contrast, *S. africana-caerulea*, with an IC_{50} value of 8.72 ± 1.52 $\mu\text{g/ml}$, was the most active extract against the SF-268 cells ($p < 0.05$). The cell proliferative inhibition by *S. africana-lutea*, *S. dolomitica*, *S. garipensis*, *S. lanceolata*, *S. muirii*, *S. namaensis*, *S. repens*, *S. runcinata* and *S. verbenaca* tested at a concentration of 100 $\mu\text{g/ml}$ exhibited less than 80% inhibition of the cell growth. The IC_{50} values were thus not calculated for these species (Table 1). The IC_{50} values of nearly all the species were above 30 $\mu\text{g/ml}$. No significant difference in the activity of *S. albicaulis* and *S. radula* against the SF-268 cells ($p > 0.05$) was observed.

Treatments with *Salvia* extracts resulted in growth inhibition in the majority of cases. For the three cell lines examined, MCF-7 cells were the most sensitive, as it was inhibited by all the solvent extracts, while the SF-268 cell line was the least sensitive with approximately 53% (9/17) of the samples exhibiting less than 80% inhibition at 100 $\mu\text{g/ml}$ (Table 1).

In the screening of the anticancer activity of plants, species which need to be considered for further analysis are those that show activity against certain cell lines and are not toxic to others. In this study, cell line selectivity was observed. The solvent extracts of *S. dolomitica*, *S. garipensis*, *S. lanceolata*, *S. muirii*, *S. namaensis*, *S. repens*, *S. runcinata* and *S. verbenaca*

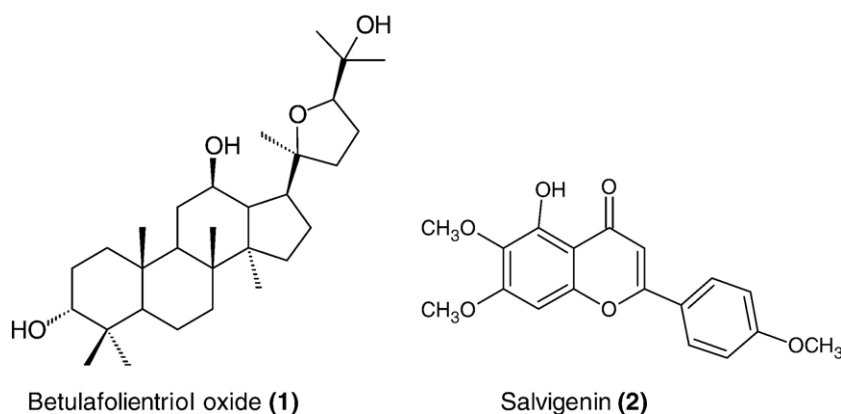


Fig. 1. Structures of compounds isolated from *S. radula*.

showed some degree of activity against MCF-7, but were, however, not active against the SF-268 and/or HT-29 cell lines (Table 1).

The solvent extracts also exhibited varying degrees of toxicity against the human kidney epithelial cells with the IC_{50} values ranging from 12.12 ± 2.02 $\mu\text{g/ml}$ to 53.34 ± 3.90 $\mu\text{g/ml}$. The solvent extracts of *S. africana-caerulea* and *S. stenophylla* were similar ($p > 0.05$) and were the most toxic, while *S. disermas* was the least toxic in comparison to the other extracts ($p < 0.05$). The 5'-FU was significantly less toxic than all the solvent extracts ($p < 0.05$) with the safety indices indicating the solvent extracts being 3 to 11 times more toxic than 5'-FU. The isolated compounds, namely betulafolientriol oxide and salvigenin from *S. radula* exhibited low toxicity against the human kidney epithelial cells, with IC_{50} values greater than 100 $\mu\text{g/ml}$.

In this study, there may be compounds in *Salvia* species extracts that can induce a cytotoxic action against various cancer cell lines and initiate cell death. The American National Cancer Institute guidelines set the limit of activity for crude extracts at a 50% inhibition (IC_{50} value) of proliferation of less than 30 $\mu\text{g/ml}$ (Suffiness and Pezzuto, 1990). The plants exhibited various responses depending on the cell line used. The percentage of plant extracts with IC_{50} values less than 30 $\mu\text{g/ml}$ was 17, 47 and 53% against the SF-268, MCF-7 and HT-29 cells, respectively. *S. africana-caerulea* was the only species that exhibited anticancer activity against all three cell lines, with an IC_{50} value of less than 30 $\mu\text{g/ml}$ (Table 1).

The extract of *S. hypargeia* has been investigated by Ulubelen et al. (1999) against a panel of cell lines, including the human colon cells (COL-2), breast cancer cells (BC-1) and lung cancer cells (LU-1). Results indicated that the plant possesses anticancer activity with IC_{50} values < 20 $\mu\text{g/ml}$, which in general, were lower than those obtained in this study when tested against the MCF-7 breast cancer cell line (Table 1).

Flavonoids are biosynthesised by plants and have strong anti-oxidant activity for scavenging free radicals which are involved in cell damage and tumour promotion. Indigenous *Salvia* species also possess flavonoids (Kamatou, 2006) and the presence of flavonoids may have contributed to their anticancer activity. The flavonoid isolated from *S. radula* (salvigenin) was tested against the MCF-7 cells and it exhibited moderate activity (IC_{50} value: 67.78 ± 3.78 $\mu\text{g/ml}$), although not significantly comparable to the activity of 5'-FU. Testing could not be conducted on the HT-29 and SF-268 cell lines as the amount of flavonoid isolated was limited.

Many alkaloids are poisonous to both animals and humans. Some of the species investigated, such as *S. chamelaeagnea*, *S. namaensis* and *S. runcinata*, tested positive for alkaloids (Raffauf, 1996). A poor correlation between the anticancer activity and the toxicity of the extracts was observed. It was, however, interesting to note that the extracts of *S. lanceolata*, *S. radula* and *S. stenophylla* displaying anticancer activity, also exhibited some degree of toxicity. In these cases, it could be speculated that the same compound(s) may be responsible for both the anticancer activity and the "general" toxicity profile of the solvent extracts.

This is the first extensive *in vitro* investigation on the antimalarial and anticancer activities of indigenous *Salvia* species. The investigated species have shown their ability to inhibit cellular proliferation of three different types of human cancer.

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