

# Anti-proliferative properties of commercial *Pelargonium sidoides* tincture, with cell cycle G<sub>0</sub>/G<sub>1</sub> arrest and apoptosis in Jurkat leukemia cells.

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## Abstract

Context - *Pelargonium sidoides* DC (*Geraniaceae*) is an important medicinal plant indigenous to South Africa and Lesotho. Previous studies have shown root extracts rich in polyphenolic compounds with antibacterial, antiviral and immunomodulatory activities. Little is known regarding the anticancer properties of *Pelargonium sidoides* extracts.

Objective – This study evaluates the anti-proliferative effects of a *Pelargonium sidoides* radix mother tincture (PST).

Materials and methods – The PST was characterized by LC-MS/MS. Anti-proliferative activity was evaluated in the pre-screen panel of the National Cancer Institute (NCI-H460, MCF-7 and SF-268) and the Jurkat leukemia cell line at concentrations of 0-150 µg/mL. Effect on cell growth was determined with sulforhodamine B and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays after 72 h. Effect on cell cycle and apoptosis induction in Jurkat cells was determined by flow cytometry with propidium iodide and Annexin V: fluorescein isothiocyanate staining.

Results – Dihydroxycoumarin sulfates, gallic acid as well as gallo catechin dimers and trimers were characterized in PST by mass spectrometry. Moderate anti-proliferative effects with GI<sub>50</sub> values between 40 and 80 µg/mL observed in the NCI-pre-screen panel. Strong activity observed with

Jurkat cells with a GI<sub>50</sub> of 6.2 µg/mL, significantly better than positive control 5-fluorouracil (GI<sub>50</sub> of 9.7 µg/mL). The PST arrested Jurkat cells at G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle and increased the apoptotic cells from 9% to 21%, while the dead cells increased from 4% to 17%.

Conclusion - We present evidence that *Pelargonium sidoides* has cancer cell type specific anti-proliferative effects and may be a source of novel anticancer molecules.

*Keywords:* anticancer; cell cycle arrest; apoptosis; polyphenolics

## **Introduction**

Plant extracts have played important roles in anticancer drug discovery with several anticancer drugs in clinical use derived from plant extracts (Balunas & Kinghorn, 2005; Cragg & Newman, 2007). Much research has thus been devoted to analyzing plant extracts for anticancer activity. Inflammation and oxidative stress have been implicated in cancer pathogenesis and are not only initiators of tumorigenesis but also involved in driving the disease progression (Brown & Bicknell, 2001; Lucia & Torkko, 2004; Federico et al., 2007; Azad et al., 2008; Colotta et al., 2009; Grivennikov et al., 2010; Kundu & Surh, 2012).

Plant extracts or compounds that have anti-inflammatory and/or antioxidant activity may thus prove useful in cancer chemoprevention. Plant extracts with these properties are rich in phenolic compounds such as phenolic acids, flavonoids, flavonol glycosides, anacardic acids, proanthocyanidins, phenylcoumarins, theaflavins, cannabinoids, phenolic amides, curcuminoids, stilbene oligomers, xanthones, phenolic oils and flavonoligans (Anilkumar, 2010). Specific phenolic compounds include quercetin (Dajas, 2012), anacardic acid (Sun et al., 2006, Hsieh et al., 2011), epigallocatechin gallate (EGCg) (Khan & Muktar, 2008), gallic acid (Verma et al., 2013), proanthocyanidins (Nandakumar et al., 2008), curcumin (Goel et al., 2008), cannabinoids (Alexander et al., 2009), mangostin (Nakagawa et al., 2007; Johnson et al., 2012), gossypin (Kim et al., 2008; Shi et al., 2012), silymarin (Ramasamy & Agarwal, 2008), gingerols and shagoals (Lee et al., 2008; Sang et al., 2009). In addition, anticancer effects can also include inhibition of

cell growth, cell cycle arrest, induction of apoptosis, inhibition of topoisomerase enzymes and matrix metalloproteinases as well as angiogenesis. The green tea derived flavanol EGCG is currently being evaluated in clinical trials as a part of combinational anticancer drug therapy (Saba et al., 2014). Plant derived coumarins have been identified as important scaffolds for anticancer therapies, particularly as lead candidates for the treatment of hematological malignancies (Riveiro et al., 2008; Kaur et al., 2015).

*Pelargonium sidoides* DC (*Geraniaceae*) is indigenous to South Africa and Lesotho and is collected from the wild. Due to overharvesting, commercial farming of *Pelargonium sidoides* is being developed in South Africa as an alternative source of raw material (Brendler & Van Wyk, 2008, Moyo et al., 2013). Plant extracts of the tubers or rhizomes are rich in various polyphenolic compounds such as gallic acid, di- and trihydroxy and methoxycoumarins, coumarin sulfates, catechins and proanthocyanidins (Kolodziej, 2007). Its traditional uses include the treatment of diarrhea, dysentery, colic, anemia and tuberculosis and reported medicinal properties include antibacterial, antifungal, antiviral, and immunomodulatory activities (Brendler & Van Wyk, 2008). Based on these medicinal properties commercial formulations such as Linctagon and EPs® 7630 have been developed for the treatment of upper respiratory tract infections (Matthys et al., 2007; Brendler & Van Wyk, 2008).

Based on the previous reports on the types of polyphenolics found in *Pelargonium sidoides* root extracts it can be expected that *Pelargonium sidoides* will also have anticancer properties (Kolodziej, 2007). An extensive search of scientific literature only found a patent by Kong et al. (2009), claiming that a *Pelargonium sidoides* extract exhibited cytotoxicity towards GLC4 human lung cancer and COLO320 human colon cancer cell lines. The aim of this study was to evaluate the anti-proliferative effects of a *Pelargonium sidoides* radix mother tincture (PST) in the anticancer pre-screen panel of the National Cancer Institute (NCI) as well as the Jurkat cell line. This is the first report to our knowledge describing the anticancer activity of *Pelargonium sidoides* extracts.

## **Methods**

### **Chemicals**

The PST prepared according to the German Homeopathic Pharmacopoeia HAB4a was purchased from Parceval Pharmaceuticals (Pty) Ltd., Wellington, South Africa. Positive controls, doxorubicin (DOX) and 5-fluoruracil (5FU) were obtained from Sigma-Aldrich Company, Atlasville, South Africa. All other reagents were obtained either from Sigma-Aldrich Company, Atlasville, South Africa or Merck, Johannesburg, South Africa.

### **Cell cultures**

The NCI-H460 human lung large cell carcinoma, SF-268 human gliomablastoma and the MCF-7 human breast adenocarcinoma cell lines were obtained from the NCI, Frederick, Maryland, USA. The Jurkat E6.1 Human leukemic T-cell lymphoblast cell line was obtained from the European Collection of Cell Cultures supplied by Sigma-Aldrich Company, Atlasville, South Africa. Cell lines were maintained in RPMI 1640 (Sigma-Aldrich Company, Atlasville, South Africa) and Penicillin/Streptomycin/Fungizone formulation (Highveld Biological, Lyndhurst, South Africa) and maintained at 37°C, 5% CO<sub>2</sub>.

### **Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis of PST**

Analysis of the PST was performed on a Waters Ultra Performance Liquid Chromatography (UPLC) Acquity system fitted with a Waters Acquity Photo Diode Array (PDA) detector and coupled to a Waters Synapt G2 mass spectrometer (Milford, Massachusetts, USA). The PST was analyzed with a Waters Acquity UPLC BEH C18 column, 2.1 x 100 mm with 1.7 µm particle size coupled with a Waters Acquity UPLC BEH C18 pre-column, 2.1 x 5 mm with 1.7 µm particle size. The column temperature was set at 55°C and the solvents used were HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B). Two µL of the PST was injected, and the flow rate was set at 0.2 mL/min. The chromatographic conditions were as follows: 5% B for first 5 min, 5% to 95% solvent B over 45 min, 95% solvent B was maintained for 5 min followed by column re-

equilibration over 5 min. The mass spectrometer was operated in the negative ionization mode and mass data was acquired from 50 to 1500 amu. Conditions were as follows: capillary voltage 2.5 kV, sampling cone voltage 15, desolvation temperature 275°C, desolvation gas flow 650 L/h and cone gas flow of 50 L/h. A low energy function with trap collision energy of 4 V and a high energy function with trap collision energy that ramped from 15 to 60 V were used to acquire the mass data. Data was processed with Waters MassLynx V4.1 software and compounds were tentatively identified based on their fragmentation patterns and previous literature.

## **Anticancer assays**

### **Anticancer activity**

The anticancer activity of the PST was evaluated with NCI-H460, SF-268, MCF-7 and Jurkat cell lines. Briefly, cells were plated in 96-well culture plates in 100  $\mu$ L of RPMI medium with 10% FBS at the following cell densities:  $7.5 \times 10^4$ /mL for the NCI-H460,  $1.5 \times 10^5$ /mL for the SF-268,  $1 \times 10^5$ /mL for the MCF-7 and  $2 \times 10^5$ /mL for the Jurkat cells. The cells were left to settle for 24 hours at 37°C and 5% CO<sub>2</sub>. Different concentrations of the PST was then added in 100  $\mu$ L of RPMI medium with 10% FBS and cells were left for a further 72 h at 37°C and 5% CO<sub>2</sub>. DOX and 5FU were used as positive controls while cells in the absence of any drug were used as the negative control. Following the 72 h exposure period, the anticancer activity was evaluated with the sulforhodamine B (SRB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

For the SRB assay, cells were fixed with 50  $\mu$ L of a 50% cold trichloroacetic acid solution (w/v) for 1 h at 4°C. Thereafter, plates were washed with water and left to dry. Plates were stained with a 0.4% SRB solution (w/v) in 1% acetic acid solution (v/v) for 10 min, washed with 1% acetic acid solution and left to dry. The dye was then solubilised with 200  $\mu$ L of a 10 mM Tris base solution pH 10.5, and the absorbance was determined at 550 nm with 690 nm as the reference with a Multiscan Ascent plate reader (AEC Amersham, Kelvin, South Africa).

For the MTT assay, 50  $\mu$ L of a 1 mg/mL MTT solution in phosphate buffered saline (PBS) was added to each well. Plates were allowed to react at 37°C and 5% CO<sub>2</sub> for 4 h. The medium was removed, and the dye was solubilised with 200  $\mu$ L of dimethylsulfoxide. Absorbance was determined at 550 nm with 690 nm as the reference with a Multiscan Ascent plate reader (AEC Amersham, Kelvin, South Africa).

The concentration that inhibits the cell growth by 50% (GI<sub>50</sub>) was calculated as follows:  $(T - T_0)/(C - T_0) \times 100 = 50$  where T is the absorbance of the cell growth in the presence of a drug concentration, T<sub>0</sub> the initial cell growth before any drugs were added, and C is the control cell growth (Boyd & Paull, 1995).

### **Flow cytometric analyses**

The effect of the PST on cell cycle and apoptosis was determined with the Jurkat cell line. Briefly, 4 x 10<sup>5</sup> Jurkat cells in 5 mL of RPMI medium with 10% FBS were plated in 25 cm<sup>2</sup> cell culture flasks and left to settle for 24 h at 37°C and 5% CO<sub>2</sub>. Cells were exposed to 10  $\mu$ g/mL of the PST for 72 h.

For the cell cycle analysis, cells were harvested, washed with Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 1 mL of 70% cold ethanol solution (v/v) for 1 h at 4°C. Cells were pelleted and washed with DPBS staining solution containing 2% FBS and 0.01% sodium azide. Cells were resuspended in 1 mL of the DPBS staining solution and treated with 100  $\mu$ L of 1 mg/mL RNase solution for 30 min at 37°C. Cells were stained with 10  $\mu$ L of 1 mg/ml propidium iodide solution for 30 min at room temperature.

The apoptosis analysis was performed with Annexin V: fluorescein isothiocyanate (FITC) assay kit (AbDSerotec, Oxford, UK) and cells were treated as per the manufacturer's instructions. Three controls were used to determine the gates for the viable, dead and apoptotic cells. For the viable cell's gate, unstained cells were used. Cells were fixed with 70% cold ethanol for 1 h at 4°C and

stained with propidium iodide were used to gate for the dead cells. Cells treated with a 3% (v/v) formaldehyde solution in 1x DPBS for 30 min at 4°C were used to gate the apoptotic cells.

Cell cycle and apoptosis analysis was performed using the BD FACSAria flow cytometer (BD Biosciences, San Diego, USA). Cell cycle data was analysed with the FlowJo software version 7.6.5 while apoptosis data was analysed with the FACSDiva software version 6.1.3. Two independent experiments were conducted for cell cycle and apoptosis analysis, and results are expressed as means together with the standard error of the mean (SEM).

## Statistical analysis

The statistical significance of each sample's  $GI_{50}$  value was determined with the Tukey honestly significant difference test calculated with the JMP (version 9.0.0) software at the 95% level of confidence.

## Results

### LC-MS/MS analysis of PST

A commercial formulation prepared from the roots of *Pelargonium sidoides* was evaluated for its potential anticancer activity. This formulation is prepared according to German Homeopathic Pharmacopoeia standards namely procedure HAB 4a that involves the extraction of 10 parts fresh *Pelargonium sidoides* roots with 100 parts of alcohol, final alcohol concentration of 60%. The concentration was  $0.14 \pm 0.01$  g dry mass/10 mL. LC-MS/MS analysis of the PST (Figure 1) revealed that it was complex in nature, and many compounds could be tentatively identified based on fragmentation patterns, UV-vis spectrum and previous literature. Twenty-two phenolic compounds were identified, ten hydroxy, methoxy or sulfated coumarin derivatives, nine flavan-3-ols (catechins and proanthocyanidins) as well as the phenolic acid gallic acid and flavonoid derivative eriodictyol sulfate (Table 1). Three unknown compounds with molecular masses of 321 m/z, 305 m/z and 523 m/z were also found. The presence of the coumarin umckalin (peak 24) confirmed that the tincture was prepared from the roots of *Pelargonium sidoides* and not

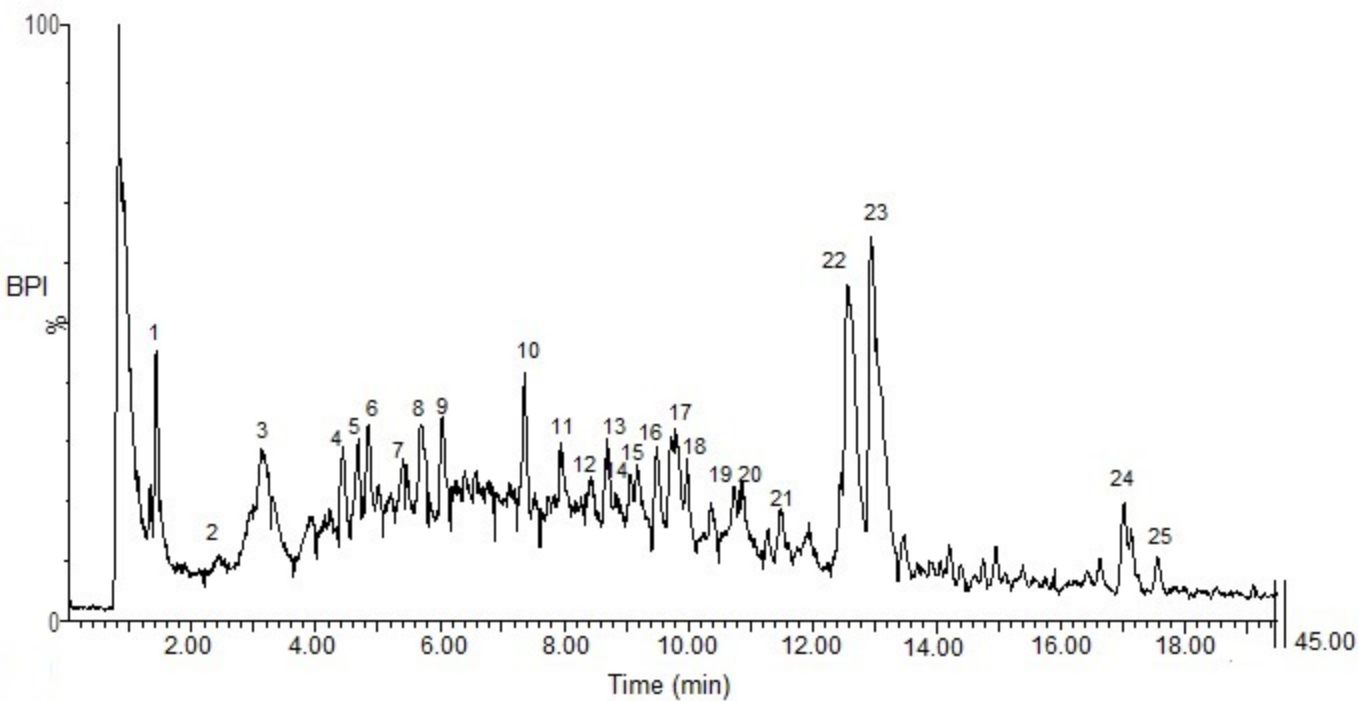


Figure 1



**Table 1. Proposed phenolic compounds identified from the PST.**

| Peak | RT<br>min | Accurate<br>mass [M-H] <sup>-</sup> | Proposed<br>formula                             | Error<br>ppm | MS/MS<br>fragments | Wavelength<br>nm | Proposed compound              | Reference                |
|------|-----------|-------------------------------------|---|--------------|--------------------|------------------|--------------------------------|--------------------------|
| 1    | 1.4       | 191.0190                            | C <sub>6</sub> H <sub>7</sub> O <sub>7</sub>    | -1.0         |                    | 270              | Citric acid                    | Philips et al., 2010     |
| 2    | 2.5       | 169.0134                            | C <sub>7</sub> H <sub>5</sub> O <sub>5</sub>    | -1.8         | 125                | 271              | Gallic acid                    | Kim et al., 2009         |
| 3    | 3.1       | 272.9701                            | C <sub>9</sub> H <sub>5</sub> O <sub>8</sub> S  | -1.5         | 193                | 324              | Dihydroxycoumarin sulfate      | Gödecke et al., 2005     |
| 4    | 4.4       | 331.1027                            | C <sub>14</sub> H <sub>19</sub> O <sub>9</sub>  | -0.6         | 169, 139           | 275              | Koaburaside                    | Chung et al., 1997       |
| 5    | 4.7       | 609.1240                            | C <sub>30</sub> H <sub>25</sub> O <sub>14</sub> | -0.7         | 423, 305, 177      | 273              | GC-GC dimer                    | Callemien & Collin, 2008 |
| 6    | 4.9       | 611.1426                            | C <sub>30</sub> H <sub>27</sub> O <sub>14</sub> | 4.1          | 423, 305, 167      | 270              | GC-GC dimer                    | Callemien & Collin, 2008 |
| 7    | 5.4       | 913.1785                            | C <sub>45</sub> H <sub>37</sub> O <sub>21</sub> | -4.6         | 609, 423, 305      | 271              | GC-GC-GC trimer                | Callemien & Collin, 2008 |
| 8    | 5.7       | 272.9696                            | C <sub>9</sub> H <sub>5</sub> O <sub>8</sub> S  | -1.1         | 193                | 339              | Dihydroxycoumarin sulfate      | Gödecke et al., 2005     |
| 9    | 6.0       | 272.9703                            | C <sub>9</sub> H <sub>5</sub> O <sub>8</sub> S  | -0.7         | 193                | 321              | Dihydroxycoumarin sulfate      | Gödecke et al., 2005     |
| 10   | 7.4       | 611.1411                            | C <sub>30</sub> H <sub>27</sub> O <sub>14</sub> | 1.6          | 423, 305, 167      | 270              | GC-GC dimer                    | Callemien & Collin, 2008 |
| 11   | 8.0       | 286.9855                            | C <sub>10</sub> H <sub>7</sub> O <sub>8</sub> S | -2.4         | 207, 192           | 301              | Hydroxymethoxycoumarin sulfate | Hauer et al., 2010       |
| 12   | 8.4       | 913.1915                            | C <sub>45</sub> H <sub>37</sub> O <sub>21</sub> | 9.6          | 609, 423, 305      | 270              | GC-GC-GC trimer                | Callemien & Collin, 2008 |
| 13   | 8.7       | 286.9864                            | C <sub>10</sub> H <sub>7</sub> O <sub>8</sub> S | 0.7          | 207, 192           | 275, 335sh       | Hydroxymethoxycoumarin sulfate | Hauer et al., 2010       |
| 14   | 9.1       | 271.9915                            | C <sub>10</sub> H <sub>7</sub> O <sub>7</sub> S | 1.1          | 191, 148           | 278, 335sh       | Methoxycoumarin sulfate        | Hauer et al., 2010       |

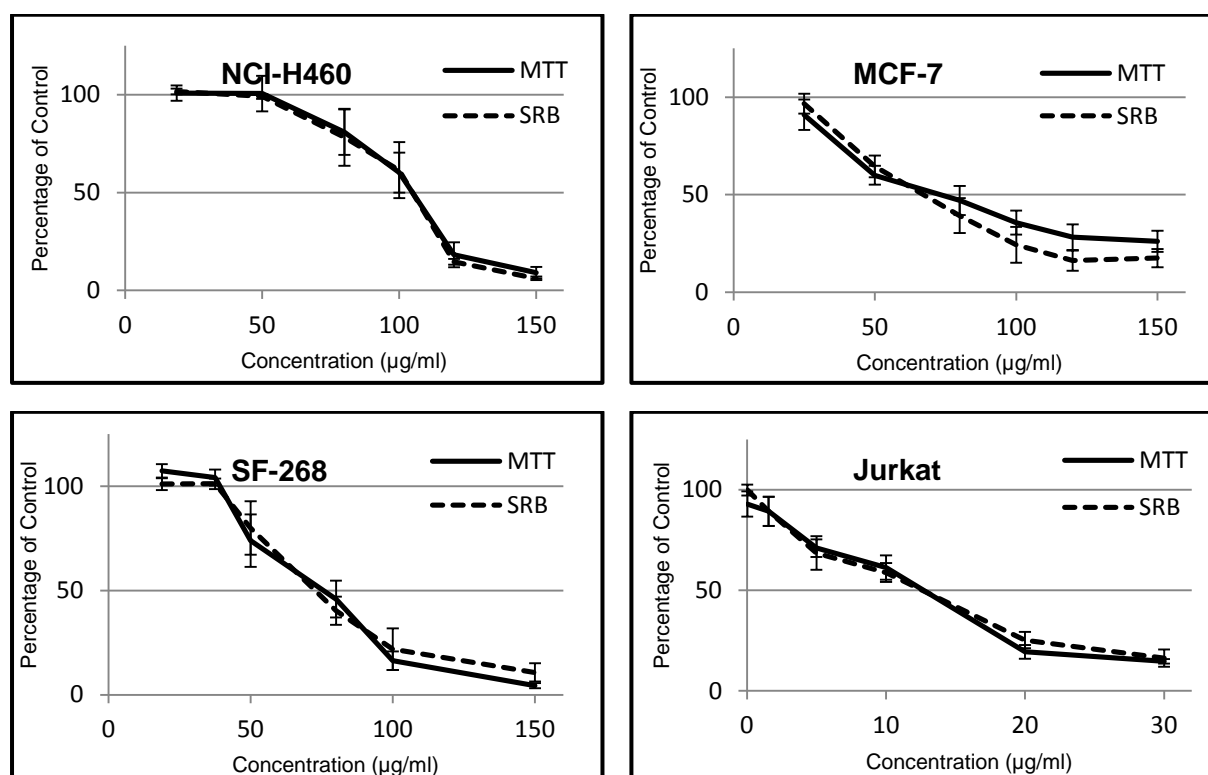
|    |      |          |   |      |               |     |                                  |                          |
|----|------|----------|---|------|---------------|-----|----------------------------------|--------------------------|
| 15 | 9.2  | 321.0281 | C <sub>11</sub> H <sub>13</sub> O <sub>9</sub> S  | 0.3  | 241, 167      | 275 | Unknown                          | -                        |
| 16 | 9.5  | 305.0695 | C <sub>12</sub> H <sub>17</sub> O <sub>7</sub> S  | -2   | 225           | 275 | Unknown                          | -                        |
| 17 | 9.8  | 383.0064 | C <sub>15</sub> H <sub>11</sub> O <sub>10</sub> S | -2.3 | 303, 177, 125 | 284 | GC/EGC sulfate                   | Schötz & Nölder, 2007    |
| 18 | 10.0 | 523.1667 | C <sub>21</sub> H <sub>31</sub> O <sub>15</sub>   | 0.8  | 477, 305, 293 | 275 | Unknown                          | -                        |
| 19 | 10.8 | 301.0031 | C <sub>11</sub> H <sub>9</sub> O <sub>8</sub> S   | 4.3  | 221, 191      | 275 | Dimethoxycoumarin sulfate        | Hauer et al., 2010       |
| 20 | 10.9 | 383.0079 | C <sub>15</sub> H <sub>11</sub> O <sub>10</sub> S | -0.5 | 303, 177, 125 | 287 | GC/EGC sulfate                   | Schötz & Nölder, 2007    |
| 21 | 11.5 | 367.0121 | C <sub>15</sub> H <sub>11</sub> O <sub>9</sub> S  | -0.8 | 287, 152      | 275 | Eriodictyol sulfate              | Medina et al., 2012      |
| 22 | 12.6 | 316.9976 | C <sub>11</sub> H <sub>9</sub> O <sub>9</sub> S   | 2.8  | 237           | 320 | Hydroxydimethoxycoumarin sulfate | Hauer et al., 2010       |
| 23 | 13.0 | 301.0020 | C <sub>11</sub> H <sub>9</sub> O <sub>8</sub> S   | 0.7  | 221, 191      | 296 | Dimethoxycoumarin sulfate        | Gödecke et al., 2005     |
| 24 | 17.0 | 221.0453 | C <sub>11</sub> H <sub>9</sub> O <sub>5</sub>     | 1.4  | 191, 163      | 327 | Umckalin                         | Kayser & Kolodziej, 1995 |
| 25 | 17.6 | 435.1292 | C <sub>21</sub> H <sub>23</sub> O <sub>10</sub>   | 0.2  | 273, 167      | 275 | Afzelechin glucoside             | Wen-Sheng et al., 1992   |

closely related species *Pelargonium reniforme* Curt. (*Geraniaceae*) that does not contain umckalin (Kolodziej, 2007).

## Anticancer activity

### Effect on cell growth and viability

The effect of 72 h exposure of PST on cell viability and number was determined with the SRB and MTT assays, respectively. Two known anticancer drugs, DOX and 5FU were included as positive controls (Figure 2). Good correlation was found between the SRB and MTT assays. Treatment of these cell lines with the PST showed a dose-dependent response effect over the concentration



**Figure 2**

range evaluated i.e. 0-150 µg/ml for the NCI-H460, MCF-7 and SF-268 cells, and 0-30 µg/ml for the Jurkat cell line. The  $GI_{50}$  of PST was compared to the positive controls (Table 2). The PST was the most effective in the Jurkat cell line with a  $GI_{50}$  of 6.2 µg/mL followed by the MCF-7 cell line with  $GI_{50}$  of 43 µg/mL. The SF-268 and NCI-H460 cell lines were more resistant with  $GI_{50}$  values of 60 and 80 µg/mL, respectively.

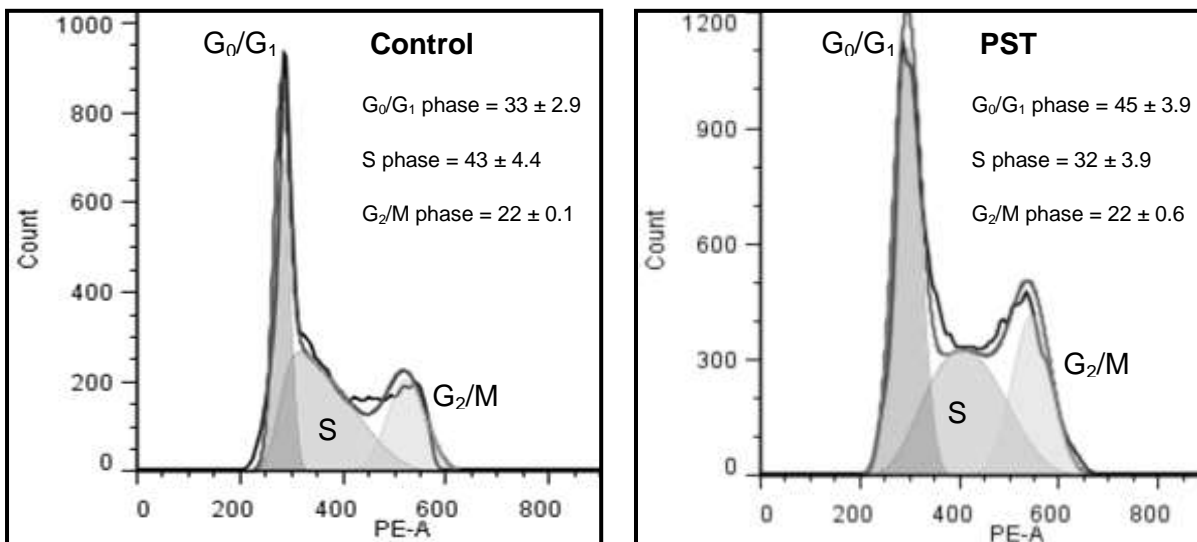
**Table 2. GI<sub>50</sub> values for PST and positive controls as determined with the SRB assay**

| Drug | NCI-H460<br>µg/mL (µM)                    | MCF-7<br>µg/mL (µM)                      | SF-268<br>µg/mL (µM)                     | Jurkat<br>µg/mL (µM)                        |
|------|---|--|--|---|
| PST  | 80 ± 9.4 <sup>a</sup>                     | 43 ± 7.2 <sup>b</sup>                    | 60 ± 9.2 <sup>a</sup>                    | 6.2 ± 0.5 <sup>b</sup>                      |
| DOX  | 0.03 ± 0.003 ( <b>0.06</b> ) <sup>b</sup> | 0.16 ± 0.02 ( <b>0.29</b> ) <sup>c</sup> | 0.15 ± 0.01 ( <b>0.27</b> ) <sup>b</sup> | 0.006 ± 0.0003 ( <b>0.01</b> ) <sup>c</sup> |
| 5FU  | 0.26 ± 0.03 ( <b>2.0</b> ) <sup>b</sup>   | 10 ± 0.8 ( <b>80</b> ) <sup>c</sup>      | 15 ± 2.4 ( <b>117</b> ) <sup>b</sup>     | 9.7 ± 1.4 ( <b>75</b> ) <sup>a</sup>        |

Each value is an average of three independent experiments. Means were compared separately for each cell line. Values marked with different letters, in each column, are statistically different at the 95% level of confidence. Values in bold parentheses represent concentrations in µM.

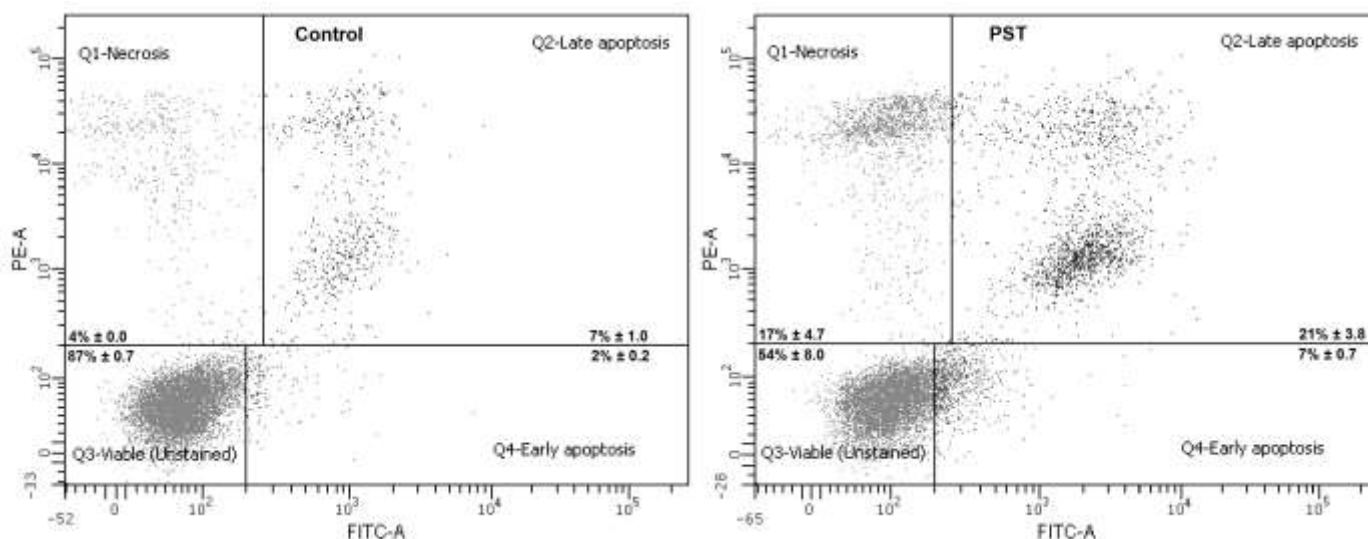
### Effect on cell cycle and apoptosis induction

Jurkat cells were exposed to 10 µg/mL PST for 72 h (Figure 3) and in the control cell population, 33%, 43% and 22% of cells were in the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phase of the cell cycle, respectively. In the presence of PST, an increased accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> to 45% with a concomitant decrease in the S phase to 32% was observed. This indicated that treatment of Jurkat cells with PST results in a G<sub>0</sub>/G<sub>1</sub> cell cycle arrest.



**Figure 3**

The ability of the PST to induce apoptosis and cell death at 10 µg/mL in the Jurkat cells was also evaluated (Figure 4). The number of viable cells decreased from 87% in the control cells to 54% in the treated cells while the number of cells undergoing apoptosis following treatment increased 3-fold from 9% to 21% (early and late apoptosis). The percentage dead cells also increased from 4% in the control to 17% in the treated cells. The PST not only disrupts the cell cycle but also induces apoptosis and cell death of Jurkat cells.



**Figure 4**

## Discussion

Inflammation and oxidative stress have been implicated in cancer pathogenesis and thus plant extracts or compounds that have anti-inflammatory and/or antioxidant activity may thus prove useful in cancer chemoprevention (Brown & Bicknell, 2001; Lucia & Torkko, 2004; Federico et al., 2007; Azad et al., 2008; Colotta et al., 2009; Grivennikov et al., 2010; Kundu & Surh, 2012). Extracts prepared from the roots of *Pelargonium sidoides* have shown in previous studies that they are rich in polyphenolic compounds, and have antioxidant and immunomodulatory activities (Brendler & Van Wyk, 2008; Moyo et al., 2013). This suggests that *Pelargonium sidoides* should also show anticancer activity.

Mass spectrometry analysis revealed that the PST was rich in sulfated coumarins and flavonoids that were identified based on the loss of 80 mass units to yield major fragments of 193m/z for dihydroxycoumarin (Gödecke et al., 2005), 303m/z for gallocatechin (GC)/ epigallocatechin (EGC) and 287m/z for eriodictyol (Callemien & Collin, 2008; Sudjaroen et al., 2005). Sulfated coumarins were also found by Lattè et al. (2000) and Gödecke et al. (2005) in extracts prepared with 80% acetone from the dried roots and aerial parts of *Pelargonium sidoides*, respectively. These sulfated coumarins were 5,6-dimethoxycoumarin 7-sulfate, 6-hydroxy-5,7-dimethoxycoumarin 8-sulfate, 8-hydroxy-5,7-dimethoxycoumarin 6-sulfate and 6,7-dihydroxycoumarin 8-sulfate. Two pyranocoumarin sulfates and a furanocoumarin sulfate have been isolated from an aqueous extract of the roots of *Seseli libanotis*, a perennial herb of the Umbelliferae family (Lemmich & Shabana, 1984). Sulfated flavanols have previously been described by Schötz & Nölder (2007) in oligomeric fractions prepared from *Pelargonium sidoides* medicine Umckaloabo. Sulfated flavonoids appear to be more widespread in nature and can be found in plants such as *Flaveria bidentis*, *Polygonum hydropiper*, *Brickellia californica* and *Malva sylvestris* (Barron et al., 1988). Sulfation is an important element of the mammalian metabolic pathways, however, little is known about the bioactivity of these molecules compared to the parent compound. This is an aspect that should be addressed in future research.

Standardized evaluation of molecules and plant extracts involves initial screening using the NCI-pre-screen panel consisting of the NCI-H460 human lung large cell carcinoma, SF-268 human gliomablastoma and the MCF-7 human breast adenocarcinoma cell lines (Takimoto, 2003). The NCI adopted this pre-screen panel since a large number of compounds submitted to the 60 cell line panel showed very little activity. Only compounds that can inhibit the growth of the pre-screen panel are then further evaluated in the 60 cell-line panel. A limitation in this pre-screen panel is that it excludes cancer cell types that represent the hematological system and therefore, the Jurkat T lymphocyte leukemia cell line was included in this study. The SRB assay is the standard cytotoxicity assay employed by the NCI to evaluate anticancer activity of compounds/plant extracts (Takimoto, 2003), the MTT assay was included as this assay is widely employed by various

authors to assess anticancer activity and as a result data generated in this study can be compared to other studies. Two positive controls were included in this study namely DOX and 5FU. The anticancer activity of DOX is via the generation of hydrogen peroxide and the subsequent formation of reactive oxygen species and the inhibition of DNA topoisomerase II (Mizutani et al., 2005). Positive control, 5FU is an anti-metabolite that prevents DNA synthesis by inhibiting the enzyme thymidylate synthetase (Pinedo and Peters, 1988).

The anticancer activity observed for the PST was found to be similar to that of other polyphenolic rich plant extracts. Green tea extracts have shown anticancer activity ranging from 5-70  $\mu\text{g}/\text{mL}$  on lung, colon and T-cell leukemia cancer cells (Yang et al., 1998; Li et al., 2000). Grape seed extracts on different lung, breast and colorectal cancer cells have shown activity ranging from 30-100  $\mu\text{g}/\text{mL}$  (Sharma et al., 2004; Kaur et al., 2008; Akhtar et al., 2009). Berry extracts also rich in polyphenolic compounds have shown anticancer activity on oral, colon, breast and prostate cancer cells ranging from 30-200  $\mu\text{g}/\text{mL}$  (Seeram et al., 2006).

The PST was most effective in inhibiting cancer cell growth in the Jurkat cell line with a  $\text{GI}_{50}$  of 6.2  $\mu\text{g}/\text{mL}$ . Polyphenolic compounds similar to those found in the PST have shown anti-proliferative effects in leukemia type cancer cells. Gallic acid was observed to dose-dependently inhibit the growth of HL-60 promyelocytic leukemia cells *in vitro* with an  $\text{IC}_{50}$  of 24  $\mu\text{M}$  (4.1  $\mu\text{g}/\text{mL}$ ) (Madlener et al., 2007). Similarly, dihydroxycoumarins esculetin (6,7-dihydroxycoumarin) and 7,8-dihydroxycoumarin has been shown to inhibit the growth of U937 leukemia cells with  $\text{IC}_{50}$  values of 31  $\mu\text{M}$  (5.5  $\mu\text{g}/\text{mL}$ ) and 48  $\mu\text{M}$  (8.6  $\mu\text{g}/\text{mL}$ ), respectively (Riveiro et al., 2008).

For crude plant extracts to be considered for further evaluation, they need to have a  $\text{GI}_{50}$  value of 30  $\mu\text{g}/\text{mL}$  or less (Itharat et al., 2004; Steenkamp & Gouws, 2006). As the  $\text{GI}_{50}$  for the PST in the Jurkat cell line was less than 30  $\mu\text{g}/\text{mL}$ , the effect of PST on the cell cycle and the ability of PST to induce apoptosis were further evaluated. Cancer cells proliferate uncontrollably and bypass cell-cycle checkpoints (Stewart et al., 2003). Molecules that block the cell cycle of cancer cells and

prevent their uncontrollable proliferation are considered to be promising cancer chemotherapeutics (Owa et al., 2001).

The PST was observed to arrest Jurkat cells in the  $G_0/G_1$  phase of the cell cycle. Abnormal  $G_1$  phase regulation has been implicated as a crucial factor in tumor development and progression (Owa et al., 2001).  $G_1$  phase block will prevent the cancer cells from undergoing S phase DNA synthesis and subsequent cell division. Progression through the  $G_1$  phase is regulated by the cyclin D/cyclin-dependent kinase (CDK) 4, 6 complexes. Grape seed proanthocyanidins were shown to induce a  $G_1$  phase block of the cell cycle in human epidermoid carcinoma A431 cells (Meeran & Katiyar, 2006). The block was associated with a decrease in cyclin D1, D2 and E as well as CDKs 2,4 and 6. An increase in CDK inhibitors p21 and p27 was also observed. Treatment of human leukemia HL-60 cell with dihydroxycoumarin esculetin was also associated with a  $G_1$  phase block in a study by Wang et al. (2002). Decreased levels of cyclin D1 and E, CDK 4 and hyperphosphorylated retinoblastoma protein were observed as well as increased levels of p27. Huang et al. (2012) reported that *Toona sinensis* derived gallic acid caused  $G_1$  arrest in HL-60 human promyelocytic cells. The PST was shown to contain gallic acid, proanthocyanidins as well as dihydroxycoumarins, and it is suspected that these compounds may be involved in the  $G_0/G_1$  phase block of the cell cycle observed in the Jurkat cell line.

Apoptosis maintains the balance between cell proliferation and cell death and eliminates all unnecessary, old, damaged and infected cells from tissues (Schulze-Bergkamen & Krammer, 2004). Cancer cells have developed mechanisms to avoid apoptosis and continue proliferating uncontrollably. This is due to alterations in p53 function, upregulation of anti-apoptotic proteins, increased activation of the NF- $\kappa$ B and the PI3K/AKT pro-survival pathway as well as the down regulation of death receptors and of pro-apoptotic proteins. Induction of apoptosis by anticancer drugs is an important mechanism to eliminate cancer cells. The PST was observed to induce both apoptosis and necrosis in the Jurkat cell line. The increase in the number of dead cells was most likely due to a dose-dependent effect. At low concentrations apoptosis will predominately be seen while at higher concentrations ( $GI_{50}$  and above) both apoptosis and necrosis will be observed. The



results reflect the treatment of the Jurkat cells with the GI<sub>50</sub> concentration of the extract. Similar results have been observed with platinum-containing compounds (Pèrez et al., 2003) and piplartine (Bezerra et al., 2007). It has been suggested that the pathways leading to apoptosis and necrosis are interconnected and that factors like energy availability as well as metabolic condition of the cells may play a role in deciding apoptotic or necrotic cell death (Fuertes et al., 2003). Further studies are needed to evaluate the mechanisms of apoptosis and necrosis induction by PST in leukemia cells.

Polyphenolics like gallic acid, proanthocyanidins and dihydroxycoumarins when evaluated individually have been observed to induce apoptosis in cancer cells with little effect on normal cells. These compounds can trigger apoptosis through either the intrinsic mitochondrial pathway or the death receptor extrinsic pathway depending on the cancer cell type (Chu et al., 2001; Mantena et al., 2006; Meeran & Katiyar, 2006; Kok et al., 2009; Ji et al., 2009; You et al., 2010).

Synergism between drugs can increase efficacy, reduce toxicity and resistance and measured effects are a function of cell and target and pathway specificity, concentration and bio-availability. Synergistic effects have been reported between drugs and polyphenolics such that reported between EGCg, green tea catechins and anticancer compounds/drugs (Fujiki et al., 2014). Likewise, in plant extracts synergistic bioactive effects between polyphenolics can occur. Del Follo-Martinez et al. (2013) reported that resveratrol and quercetin in combination had increased anticancer activity in colon cancer cells.

Commercial medicinal formulations of *Pelargonium sidoides* are based on the reported antibacterial, antioxidant and immunomodulatory activities of *Pelargonium sidoides* (Brendler & Van Wyk, 2008) and is clinically used to treatment of upper respiratory tract infections (Matthys et al., 2003, 2007; Brendler & Van Wyk, 2008). Inflammation and oxidative stress are two important elements in cancer pathogenesis (Brown & Bicknell, 2001; Lucia & Torkko, 2004; Federico et al., 2007; Azad et al., 2008; Colotta et al., 2009; Grivennikov et al., 2010; Kundu & Surh, 2012). Taken

in conjunction with the findings of this study, the anticancer effect of PST would be multi-factorial and includes anti-inflammatory, antioxidant activity as well as the inhibition of cellular proliferation.

## **Conclusion**

A commercial extract of *Pelargonium sidoides* reduces the cell growth and viability of human cancer cell lines, in particular leukemia type cells. In the Jurkat leukemia cell line, the anti-proliferative effects are associated with G<sub>0</sub>/G<sub>1</sub> cell cycle arrest and the induction of apoptosis. The polyphenolic compounds identified in the *Pelargonium sidoides* extract are likely responsible for the observed activity. Further studies will be needed to isolate and identify these bio-active compounds as well as their mechanisms of action. The added scientific information provided by this study enables further development and identification of PST as a chemo-preventative product or as a source of new and novel anticancer molecules. It also further highlights the importance of *Pelargonium sidoides* as a commercial medicinal plant in South Africa.

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## **Declaration of interest**

All the authors declare no conflict of interest.

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