

Full Length Research Paper

Tulbaghia violacea* inhibits growth and induces apoptosis in cancer cells *in vitro

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Methanol extracts of *Tulbaghia violacea* leaves and bulbs inhibited growth of MCF-7, WHCO3, HT29 and HeLa cancer cell lines. At 250 µg/ml, bulb extracts exhibited higher growth inhibition than leaf extracts in MCF-7 (49.6 ± 2.6%), HT29 (26.0 ± 4.5%) and HeLa cells (54.7±5.9%) relative to untreated controls. In WHCO3, the leaf extract was more active, inhibiting growth by 30.3 ± 1.8%. The growth inhibitory activity of *T. violacea* was due to induction of apoptosis in all four cell lines. This was shown by the staining of cells with Hoechst 33342, indicating fragmented nuclear material and condensed chromatin. HeLa and MCF-7 cells treated with bulb extract had higher apoptotic indices than the other two cell lines (HeLa, 25.8 ± 3.9%; MCF-7, 19.0 ± 4.3%). Treated cells stained with Annexin V but not with propidium iodide (PI), indicating that the extract induced apoptosis and not necrosis. Using Western blotting, cleavage of Poly [ADP-ribose] polymerase-1 (PARP) was shown in HeLa cells upon exposure to *T. violacea* bulb extract. These findings provide evidence for anticancer activities in *T. violacea*. The induction of apoptosis by the extract is promising for anticancer therapy as it is desirable for anticancer agents to induce apoptosis.

Key words: anticancer, apoptosis, Poly [ADP-ribose] polymerase-1, *Tulbaghia violacea*.

INTRODUCTION

Medicinal plants play a major role in primary health care in many developing countries, and their use had increased in recent years. More than 1 200 species of plants are used for medicinal purposes (van Wyk et al., 1997). Scientific evidence has been documented for some plants, however further research is needed to ascertain the efficacy and safety of many others. *Tulbaghia violacea* is commonly known as wild garlic, wilde knoffel (Afrikaans), isihaqa (Zulu) or itswele lomlambo (Xhosa). *T. violacea* is indigenous to the Eastern Cape, South Africa and the leaves and bulbs are widely used as an herbal remedy for various ailments. Its medicinal uses include: fever and colds, asthma, tuberculosis, and stomach problems. The leaves of the plant are used to treat oesophageal cancer and may also be eaten as vegetables. The plant is also used as a snake repellent (van Wyk et al., 1997; van Wyk and Gericke, 2000).

Aqueous and ethanolic extracts of *T. violacea* tubers have previously been shown to have anthelmintic activity (McGaw et al., 2000). Angiotensin converting enzyme inhibition has also been demonstrated for aqueous and ethanolic extracts of leaves and roots, with leaf extracts being more active (Duncan et al., 1999). Aqueous and organic bulb extracts were more potent inhibitors of *Candida albicans* than leaf extracts (Motsei et al., 2003). No further scientific evidence could be found in literature to support the traditional use of *T. violacea*, but it has been postulated to have similar activities to garlic (*Allium sativum*), since both plants belong to the Alliaceae family (van Wyk et al., 1997). Numerous epidemiological, clinical and laboratory data have demonstrated that garlic and its various preparations have anticancer activities (Dirsh et al., 1998; Dorant et al., 1993; Hirsh et al., 2000; Knowles and Milner, 2001; Milner, 2001; Nakajawa et al., 2001; Sundaram and Milner, 1993).

In the present study, the inhibition of cell growth was tested against four cancer cell lines, namely HeLa (cervical cancer), HT29 (colon cancer), MCF-7 (breast cancer) and WHCO3 (oesophageal cancer). Induction of

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apoptosis was investigated by staining the cells with Hoechst 33342 dye to detect chromatin condensation and nuclear fragmentation and Annexin V-FITC/propidium iodide dual staining to differentiate between apoptosis and necrosis.

METHODOLOGY

Plant material

Tulbaghia violacea Harv. (Alliaceae) was authenticated by Prof. Eileen Campbell, Department of Botany, Nelson Mandela Metropolitan University. Fresh material was collected early in the morning, washed gently under running water to remove dust and soil, separated into leaves and bulbs and extracted immediately.

Extraction of plant material

Methanol extracts of *T. violacea* leaves and bulbs were prepared separately. Leaves and bulbs were separated and 10 g of each chopped and homogenized in a blender with 50 ml of methanol at 4°C. The crude extracts were incubated at 37°C for 15 min, followed by centrifugation at 1500 x *g* for 10 min at 4°C (Mohammad and Woodward, 1986). The supernatant was filtered using Whatman No 1 filter paper to remove residual plant material, dried under vacuum and stored at 4°C in the dark. On the day of the assay the dried extracts were redissolved in DMSO and diluted with culture medium to yield a final DMSO concentration of 0.25% (v/v). To eliminate batch to batch variation, enough extract was prepared in one batch to complete the study.

Maintenance of cell cultures

HT-29 (colon adenocarcinoma), HeLa (cervical carcinoma) and MCF-7 (breast carcinoma) cell lines were purchased from Highveld Biological (Johannesburg, South Africa). The WHCO3 (oesophageal cancer) cells were a gift from Professors Thornley and Veale, Department of Zoology, University of the Witwatersrand, Johannesburg, South Africa. All the cancer cells were cultured in 10 cm dishes in the following antibiotic-free growth medium: RPMI 1640 (Sigma, Germany) containing 10% heat inactivated foetal calf serum (Highveld Biological, Johannesburg SA) and 2 g/l NaHCO₃. The cells were incubated in a humidified 5% CO₂ incubator at 37°C. They were fed every 48 h and subcultured by trypsinisation at about 70% confluence.

Sulforhodamine B (SRB) assay

Sulforhodamine B (SRB) dye (Sigma, Germany) was used to test the effects of methanol extracts of *T. violacea* bulbs and leaves on cell growth and viability of HeLa, HT29, MCF-7 and WHCO3 cells. The dried extracts were dissolved in DMSO before diluting with growth medium to a final DMSO concentration of 0.25% (v/v). Melphalan (Sigma, Germany) was used as a positive control in all experiments. The method was performed as described by Monks et al. (1991) and Skehan et al. (1990). The cancer cells were seeded into 96 well plates in growth medium at 6 000 cells/well. After 24 h the medium was replaced with fresh growth medium containing the extracts and the cells were incubated for a further 48 h. The cells were fixed with TCA by gently adding 50 µl TCA (50%) to each well, to a final TCA concentration of 10% and incubating for 1 h at 4°C. The plates were then washed five times with tap water

and air dried. The dried plates were stained with 100 µl of 0.4% (w/v) SRB prepared in 1% (v/v) acetic acid for 10 min at room temperature. The plates were rinsed quickly four times with 1% acetic acid to remove unbound dye and air dried until no moisture was visible. The bound dye was solubilised in 1 mM Tris base (100 µl/well) for 5 min on a shaker. Optical densities were read on a microplate reader (Labsystem Multiscan MS, 665 Dosimat) at 540 nm.

Apoptosis assay methods

The general morphological features of apoptosis (chromatin condensation and nuclear condensation) were examined by phase-contrast microscopy and fluorescence microscopy.

Hoechst 33342 staining and calculation of apoptotic index: A modified method of Darzynkiewicz et al. (1994) was used for Hoechst 33342 (HO342) staining. Cells were seeded in growth medium on 13 mm glass cover slips (BDH Laboratory Supplies, England) in 24 well plates (Nunc, Denmark). They were incubated for 24 h before addition of extract at 250 µg/ml in growth medium; the control cells were treated with growth medium containing 0.25% DMSO. After treatment for 6, 12, 24 or 48 h as indicated, each well was rinsed with 500 µl of phosphate buffered saline (PBSA). The HO342 (Sigma, Germany) stock solution (0.1 mg/100 µl in PBSA) was prepared in advance and stored at 4°C. The working reagent consisted of 10 µl stock solution: 1000 µl PBSA. Two hundred microlitres of working reagent was added to each well, followed by 10 min incubation at room temperature in the dark. The cover slips were placed upside down on microscope slides and viewed under a fluorescent microscope (Olympus, BX60) and photographs were taken with a Nikon camera (Japan). The percentage apoptotic cell was determined by counting 150 cells and expressing the number of apoptotic cells as a percentage of the total counted.

Combination of HO342 and PI: The method was followed as indicated above, except that working reagent consisted of 10 µl HO342 stock: 5 µl PI: 1000 µl HEPES buffer (PI and HEPES were included in the Annexin-V-FLUOS staining kit, Roche diagnostics, Germany).

Annexin-V- FLUOS and PI: The method was followed as indicated for HO342 staining except that the working reagent consisted of 10 µl Annexin-V-fluorescein labeling reagent: 5 µl PI: 1000 µl HEPES buffer, all included in the Annexin-V-FLUOS staining kit (Roche diagnostics, Germany).

Analysis of PARP cleavage: For preparation of cell extracts, a modified method of Boucher et al. (2001) was used. Cells were seeded into 6 cm culture dishes (400 000 cells/dish) in growth medium. After 24 h, the cells were treated with extract for 48 h at 250 µg/ml in growth medium; control cells were treated with growth medium containing 0.25% DMSO. After treatment, the cells were harvested and centrifuged for 10 min at 500 x *g* at 4°C. The pellet was resuspended in 500 µl of ice cold phosphate buffered saline, pH 7.4. An aliquot of 50 µl was kept for protein determination (using the BCA assay). The remaining cell mixture was re-centrifuged at 500 x *g* at 4°C, for 5 min. The cell pellet was lysed in 100 µl lysis buffer [62.5 mM Tris-HCl, pH 6.8; 6 M deionized urea; 10% glycerol; 2% sodium dodecyl sulphate (SDS); 0.3% bromophenol blue; 5% β- mercaptoethanol (freshly added)]. The cell extract was sonicated on ice for 15 s (Sonoplus, Bandelin, HD, 2200, MS 73 probe). The samples were stored at -80°C or directly incubated at 65°C for 15 min before they were analysed on SDS-PAGE (Boucher et al., 2001). Low- and high molecular weight markers (Sigma) were run with each gel to assist in identification of the bands of interest.

Twenty micrograms of protein of the cell extract was loaded on a 10% acrylamide gel. After separation, the proteins were transferred to a PVDF membrane in a transblot apparatus (Bio-Rad) with pre-

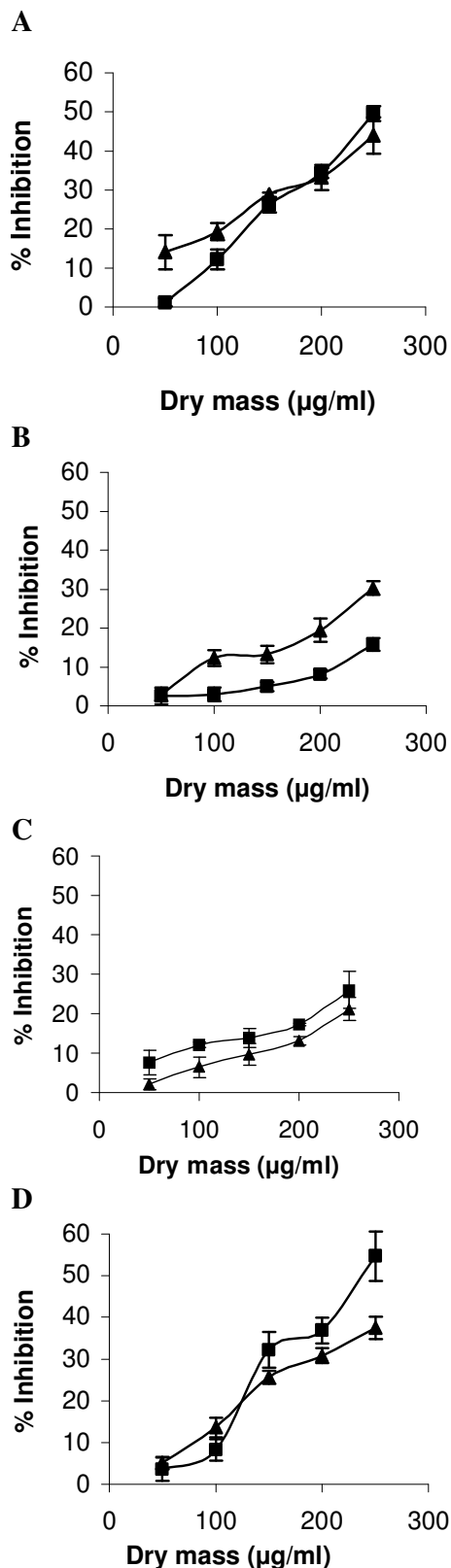


Figure 1. Cell growth inhibition of (A) MCF-7, (B) WHCO3, (C) HT-29 and (D) HeLa cancer cell lines by methanol extracts of *T. violacea* leaves (▲) and bulbs (■). (Mean \pm SD, three independent experiments, each performed in quadruplicate).

cooled transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). The transfer of proteins was performed at 4°C with stirring at 30 V for 16 h. After the transfer was complete, the PVDF membranes were soaked for an hour at room temperature in a blocking buffer (5% non-fat powdered milk in TBST buffer [20 mM Tris, pH 7.6; 137 mM NaCl and 0.015% Tween 20]). The membranes were washed with two changes of wash buffer (TBST). The membranes were incubated with the primary antibody (diluted 1:1000 in TBST) for a minimum of two hours at room temperature. The primary antibody, monoclonal rabbit anti-PARP (Boehringer Mannheim, Germany) recognizes intact PARP as well as the cleaved 89 kDa fragment. Afterwards the membranes were washed with 3 x 10 min changes of TBST (Boucher et al., 2001). The secondary antibody (ECL Western blotting analysis system, Amersham), anti-mouse coupled to horse-radish peroxidase was added (1:2000 dilution) for 1 h at room temperature. The PVDF membranes were washed with 3 x 10 min changes of TBST. Finally the protein was visualized by using the ECL detection kit (Amersham) and exposing the membranes to X-ray film (HYPERFILM™, Amersham), for periods between 1 and 10 min (Boucher et al., 2001).

Statistical analysis

Results were compared using two-tailed Student's t-test with $p < 0.05$ considered significant.

RESULTS

Growth inhibition of cancer cells by *T. violacea* extracts

Methanol extracts of *T. violacea* bulbs and leaves were tested for growth inhibition against four cancer cell lines: HT-29 (colon cancer), HeLa (cervical cancer), MCF-7 (breast cancer) and WHCO3 (oesophageal cancer) using the SRB assay. The growth inhibition of cells treated with methanol extracts of *T. violacea* leaves and bulbs was calculated as a percentage of control cell growth and plotted against the concentration of the plant extract used (Figure 1). Both the bulb and the leaf extracts exhibited growth inhibition in all four cancer cell lines tested. At 250 µg/ml of the bulb extract the growth was inhibited by $49.6 \pm 2.6\%$ in MCF-7, $15.8 \pm 1.6\%$ in WHCO3, $26.0 \pm 4.5\%$ in HT29 and $54.7 \pm 5.9\%$ in HeLa cells relative to the untreated control cells ($p < 0.001$ compared to control in all four cell lines). At the same concentration the leaf extracts exhibited the following growth inhibition values: $43.9 \pm 4.7\%$ in MCF-7, $30.3\% \pm 1.8$ in WHCO3, $21.16 \pm 2.9\%$ in HT29 and $37.5 \pm 2.7\%$ in HeLa cells ($p < 0.001$ for all cell lines). It was interesting to find that the leaf extract was more active on WHCO3 cells than the bulb extract (where a twofold difference of $15.8 \pm 1.6\%$ for the bulbs compared to $30.3 \pm 1.8\%$ for the leaves at a 250 µg/ml concentration was obtained, $p < 0.001$). This correlates well to reports by van Wyk and Gericke (2000) that the leaves of *T. violacea* are used as traditional medicine for treating cancer of the oesophagus. For all three other cell lines, the differences between the activities of the bulb and leaf extracts were much smaller,

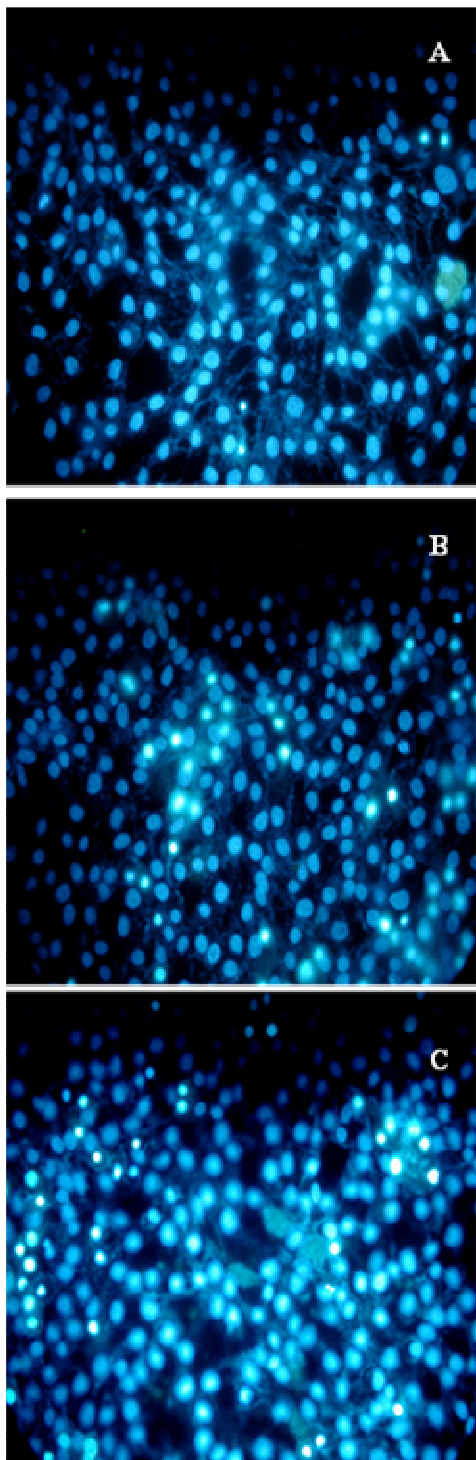


Figure 2. Staining of MCF-7 cells with Hoechst 33342. Control cells (A) and cells treated with 250 µg/ml leaf (B) or bulb extract (C) of *T. violacea*.

with the bulbs being slightly more active in all three cases. These differences were, however, not statistically significant ($p > 0.05$).

Induction of apoptosis

It has been shown that the mechanism of action of many anticancer drugs is based on their ability to induce apoptosis (Melendez-Zajgla et al., 1996; Sen and D'Incalci, 1992). Based on this it was desirable that cancer cells treated with *T. violacea* undergo apoptosis as their mode of cell death.

Analysis of nuclear chromatin condensation and apoptotic index: MCF-7, WHCO3, HT29 and HeLa cells were treated with *T. violacea* bulb and leaf extracts. At the end of the incubation period the cells were stained with Hoechst 33342 dye and the cells were observed under the microscope for chromatin condensation. One of the characteristics of cells undergoing apoptosis is nuclear chromatin condensation. The DNA in condensed chromatin stain strongly with fluorescent dyes allowing non apoptotic cells to be discriminated from apoptotic ones (Darzynkiewicz et al., 1994). The results for MCF-7 are shown in Figure 2 (the photographs for the other three cell lines looked very similar and are therefore not shown here). Apoptotic cells can be differentiated from non-apoptotic cells as the former are bright and their nuclei condensed. The nuclear condensation can be clearly seen in Figures 2B and C.

The apoptotic indices for the four cell lines as a function of time was calculated from photographs of cells stained with Hoechst 33342 after exposure to extract (Figure 3). Very few apoptotic cells were seen after 6 and 12 h in all cell lines tested. After 24 h a significant number of apoptotic cells could be seen and this increased greatly at 48 h (after exposure to bulb extract for 48 h: MCF-7: 23.0 ± 3.9 ; WHCO3: 7.6 ± 2.9 ; HT-29: 11.8 ± 2.8 ; HeLa: 29.8 ± 3.8). The leaf extracts showed few apoptotic cells compared to the bulb, except for WHCO3 where the opposite was seen, confirming the results obtained with the SRB assay (Figure 3).

Combination of Hoechst and Annexin V with PI: The dual staining of cells with Hoechst 33342 and PI or Annexin V and PI enables one to distinguish the nuclear and membrane changes of the cells during apoptosis simultaneously. This assay was used to confirm the results obtained with Hoechst 33342 staining and to also investigate if any cells died by necrosis. The latter was achieved by simultaneously staining the cells with Annexin V-FITC and PI. No cells stained with PI at 48 h (Figure 4A), ruling out the possibility of cells dying by necrosis. Apoptotic cells expose PS on the outer layer of the membrane which binds with Annexin V and results in a green fluorescence (Figure 4B). Since this assay was used to confirm the Hoechst results the cells were only exposed to the bulb extracts for 48 h, and the leaf extracts were not tested.

After 72 h of exposure to extract, the cells stained with both Annexin V and PI as shown in Figure 4C. According to Darzynkiewicz et al. (1994), this is a sign of late apoptosis or secondary necrosis, a phenomenon obser-

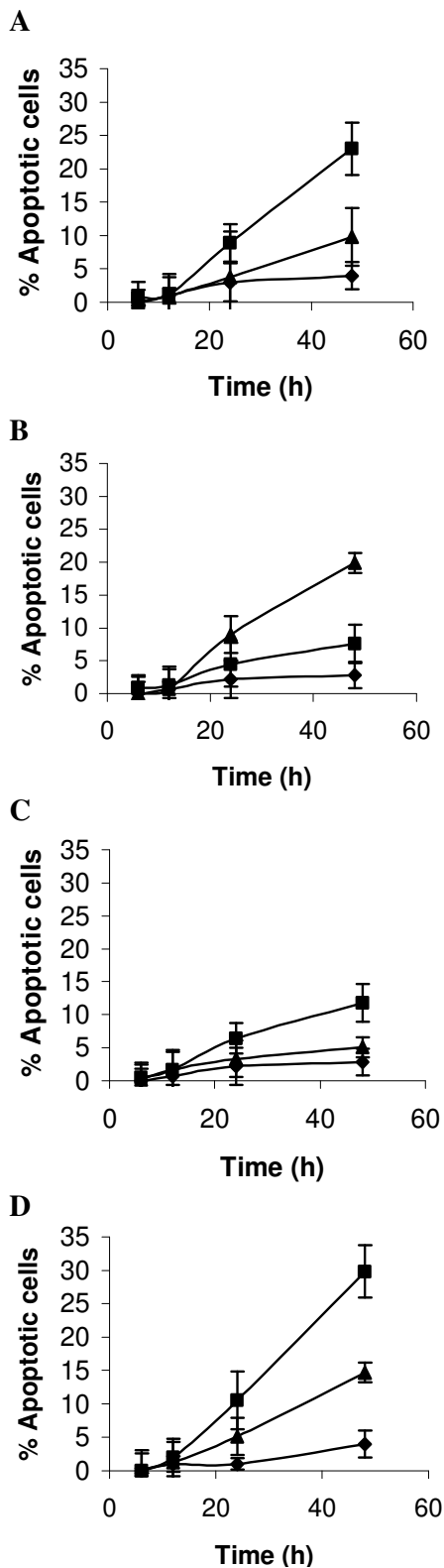


Figure 3. Apoptotic indices of (A) MCF-7, (B) WHCO3, (C) HT29 and (D) HeLa cells treated with 250 $\mu\text{g/ml}$ of *T. violacea* bulb (■) and leaf (▲) extracts over 6, 12, 24 and 48 hour time intervals and untreated control cells (◆). (Mean \pm SD, triplicate assays from one experiment. The experiment was repeated three times, with similar results).

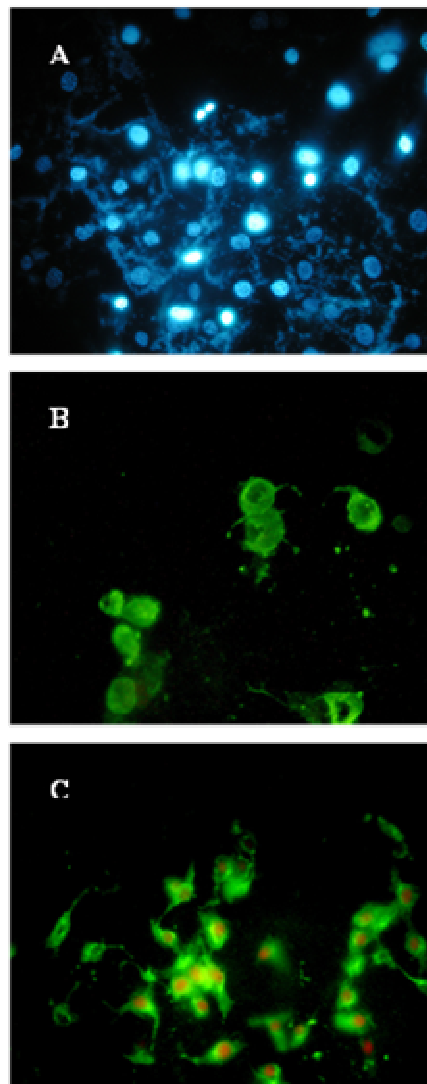


Figure 4. Dual staining of HeLa cells with (A) Hoechst 33342/PI and (B and C) Annexin V-FITC/PI. The cells were treated with 250 $\mu\text{g/ml}$ bulb extract of *T. violacea* for 48 hours (for A and B) before staining, for C the cells were treated for 72 hours before staining.

ved under *in vitro* conditions due to the absence of immune cells to engulf the cell fragments (Kasof et al., 1999).

PARP cleavage: HeLa cells were exposed to 250 $\mu\text{g/ml}$ of the bulb extract of *T. violacea* for 48 h. The protein from the cells was extracted, electrophoresed and transferred onto a PVDF membrane. The membrane was then immunoblotted with anti-PARP antibody which allows the detection of PARP cleavage. Uncleaved PARP is about 113 kDa and is cleaved to produce fragments of 89 and 24 kDa in a wide variety of cells undergoing apoptosis (Boucher et al., 2001). The monoclonal antibody used was specific for the cleaved and uncleaved (113 and 89 kDa fragments respectively) form of PARP.

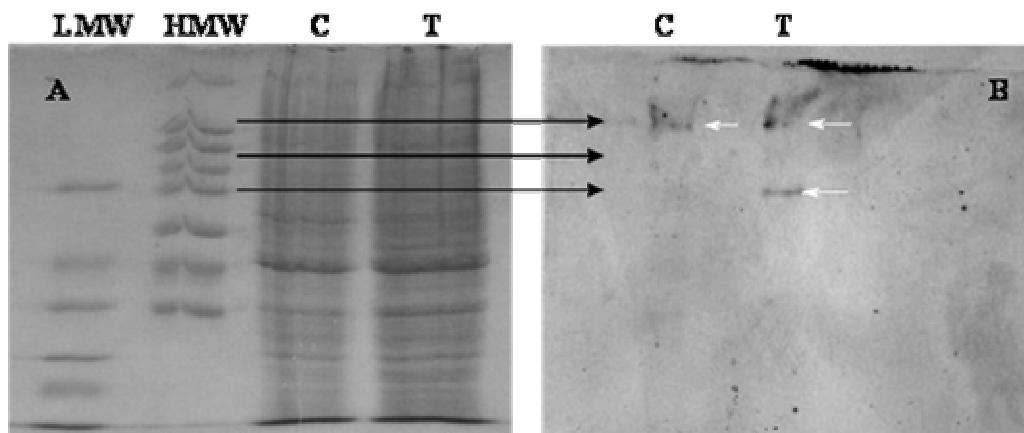


Figure 5. (A) SDS-PAGE pattern of HeLa cells treated with 250 µg/ml bulb extract of *T. violacea* for 48 hours. (B) The immunoblot showing PARP cleavage. LMW: low molecular weight markers; HMW: high molecular weight markers; C: control cells; T: treated cells. Sizes of the molecular weight markers, indicated by black arrows are (top to bottom) 116, 97 and 84 kDa respectively. The sizes of proteins in the immunoblot, indicated by white arrows (top to bottom) are 116 and 84 kDa respectively.

In Figure 5A, the SDS-PAGE gel, the three arrows indicate the positions of molecular weight markers of 116, 97 and 84 kDa from top to bottom. In Figure 5B, the immunoblot, the lane marked "C" are the control cells and there is only one band around the 116 kDa region, indicating that PARP remains uncleaved in control cells. However, in the cells treated with the bulb extract two bands can be seen (Lane "T", Fig 5B); a faint one around 116 kDa and a second, more intense band around 84 kDa where the cleaved fragment is expected. The X-ray film shows that no non-specific binding of the antibody occurred as bands are only seen at the 116 and 84 kDa regions. In most cases of cells undergoing apoptosis, PARP is cleaved by caspase 3 (Cohen, 1997; Kwon et al., 2002; Oommen et al., 2004); therefore these results indicate that *T. violacea* might be inducing a caspase 3-dependant apoptotic pathway in HeLa cells.

DISCUSSION

Screening of medicinal plants for potential anticancer properties has increased greatly over the past five decades, for example the US National Cancer Institute has implemented a large-scale project of acquisition and screening of compounds isolated from medicinal plants. The medicinal plants are identified based on ethnopharmacological, chemosystemic and ecological information. There is still a need for more effective anticancer agents since the most common tumours of the adult are resistant to available anticancer drugs and the majority of the available drugs have limited anti-solid tumour activity (Mans et al., 2000).

T. violacea belongs to the same family as garlic and has been postulated to have the same activities as the

latter. Several studies have been reported on garlic's anticancer activities and all the activities appear to be due to organosulphur compounds. The present study demonstrates, for the first time, that *T. violacea* exhibits antiproliferative and pro-apoptotic effects in human cancer cell lines.

Sulphur compounds have been previously extracted from *T. violacea*. These are 2,4,5,7-tetrathiooctane-2, 2-dioxide from hexane extract and 2,4,5,7-tetrathiooctane from methanol extract (Burton, 1990) and a sulphur containing amino acid, (R(S) R(C))-S-(methylthiomethyl) cysteine-4-oxide (Kubek et al., 2002). Induction of apoptosis by garlic's organosulphur compounds; diallyl disulfide (DADS), diallyl sulfide (DAS), S-allyl ethylcysteine (SAC), S-allyl methylcysteine (SAMC), diallyl trisulfide (DATS), allicin and ajoene have been investigated in a range of cancer cells *in vitro*. Some of them reported inhibition of proliferation of cancer cells, while others reported induction of apoptosis in tumour cells of different tissue origin (Kwon et al., 2002; Oommen et al., 2004; Rashmi et al., 2003; Smith et al., 2004; Srivastava and Singh, 2004; Sundaram and Milner, 1993, 1996). For example, in a recent study by Oommen et al. (2004), activation of caspases-3, -8 and -9 and cleavage of poly (ADP-ribose) polymerase in HeLa (cervical cancer), SiHa (cervical cancer), SW-480 (colon cancer) and L-929 cells (murine fibrosarcoma) were induced by allicin.

The mechanism of action of many anticancer drugs is based on their ability to induce apoptosis (Melendez-Zajgla et al., 1996; Sen and D'Incalci, 1992). Based on this it was desirable that cancer cells treated with *T. violacea* induce apoptosis as their mode of cell death. The mode of cell death induced by the compounds on the cells was investigated by studying morphological features (nuclear chromatin condensation, fragmentation of nucle-

ar material and exposure of phosphatidyl serine on the outer layer of the membrane) and biochemical features (PARP cleavage). The apoptotic indices (Figure 3) calculated from photomicrographs of Hoechst 33342 stained cells (Figure 2) were low compared to the growth inhibition results (Figure 1) [for example with MCF-7 (growth inhibition of $49.6 \pm 2.6\%$ for bulbs and $43.9 \pm 4.7\%$ for leaves, versus apoptotic indices of $23.0 \pm 3.9\%$ for bulbs and $9.8 \pm 4.3\%$ for leaves) and HT-29 (growth inhibition of $26.0 \pm 4.5\%$ for bulbs and $21.1 \pm 2.9\%$ for leaves, versus apoptotic indices of $11.8 \pm 2.8\%$ for bulbs and $2.2 \pm 1.9\%$ for leaves)] (Figures 1A; 1C; 3A and 3C). Bursch et al., (1990) reported that 3% of apoptotic cells can result in tissue regression of 25% over several days if not balanced by proliferation. Therefore these results are significant. Cells undergoing apoptosis also expose PS on the outer layer of their membranes. To ascertain this, the cells were stained with Annexin V-FITC and PI (Figure 4). After 48 h of exposure to the extract, the cells only stained with Annexin V and not with PI, confirming that the mechanism of cell death was apoptosis and not necrosis.

Studies by Oommen et al. (2004) and Kwon et al. (2002) have shown that ajoene, a garlic sulphur compound induces PARP cleavage in cancer cells *in vitro*. The same result was seen in this study after exposure of HeLa cells to *T. violacea* extract (Figure 5). In the apoptotic pathway PARP is cleaved by caspase 3 (Cohen, 1997; Kwon et al., 2002; Oommen et al., 2004); therefore these results suggest that *T. violacea* is inducing a caspase 3 dependant apoptotic pathway in HeLa cells.

This study shows that *T. violacea* bulb and leaf extracts inhibit growth of four cancers of different tissue origin. This is an indication that the inhibitory compounds are likely to modify common metabolic events that are not tissue specific. For future studies, cytotoxicity tests need to be done on untransformed cells to test the selectivity of *T. violacea* extracts. Ajoene, a garlic sulphur compound has been shown to induce apoptosis in a human promyeloleukemic cell line (HL-60) but not in proliferating and non-proliferating peripheral blood mononuclear cells of healthy human donors (Dirsch et al., 1998). Their findings indicate that garlic is selective in its activity. Furthermore, an assessment of the exact molecular targets of *T. violacea* could be beneficial as the molecular mechanisms underlying the tumour cytotoxicity of garlic substances are poorly defined (Oommen et al., 2004).

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REFERENCES

Boucher C, Gobeil S, Samejima K, Earnshaw WC, Poirier GG (2001). Identification and analysis of caspase substrates: proteolytic

- cleavage of Poly (ADP-ribose) polymerase and DNA fragmentation factor 45. *Methods Cell Biol.* 66:289-306.
- Bursch W, Paffe S, Barthel G, Schulte-Hermann R (1990). Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. *Carcinogenesis* 11:847-853.
- Burton GB (1990). A chemical investigation of *Tulbaghia violacea*. MSc thesis, Department of Chemistry, Rhodes University, Grahamstown, South Africa.
- Cohen GM. (1997). Caspases: the executive of apoptosis. *Biochem. J.* 326:1-16.
- Darzynkiewicz Z, Li X, Gong J (1994). Assays of cell viability: discrimination of cells dying by apoptosis. *Methods Cell Biol.* 41:15-37.
- Dirsh VM, Gerbes AL, Vollmar AM (1998). Ajoene, a compound of garlic, induces apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor κB. *Mol. Pharmacol.* 53:402-407.
- Dorant E, van-den-Brandt PA, Goldbohm RA, Hermus RJ, Sturmans F (1993). Garlic and its significant prevention of cancer in humans: a critical review. *Br. J. Cancer* 67:424-429.
- Duncan A, Jager AK, Van Staden J (1999) Screening of Zulu medicinal plants for angiotensin converting enzyme (ACE) inhibitors. *J. Ethnopharmacol.* 68:63-70.
- Hirsh K, Danilenko M, Giant J, Miron T, Rabinkov A, Wilchek M, Mirelman D, Levy J, Sharoni Y (2000). Effect of purified allicin, the major ingredient of freshly crushed garlic on cancer cell proliferation. *Nutr. Cancer* 38:245-254.
- Kasof G, Degenhardt K, Perez D, Thomas A, White E (1999). Overview: A matter of life and death. In: D. Watters and M. Lavin (Eds.), *Signalling pathways in apoptosis*. Volume 5. Harwood Academic Publishers, Australia, pp. 1-31.
- Knowles LN, Milner JA (2001). Possible mechanisms by which allyl sulfides suppress neoplastic cell proliferation. *J. Nutr.* 131:1061S-1066S.
- Kubek R, Velisek J, Musah RA (2002). The amino acid precursors and odor formation in society garlic (*Tulbaghia violacea* Harv). *Phytochemistry* 60:21-25.
- Kwon K, Yoo S, Ryu D, Yang J, Rho H, Kim J, Park J (2002). Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. *Biochem. Pharmacol.* 63:41-47.
- Mans DR, Da Rocha BA, Schwartzmann G (2000). Anti-cancer drug discovery and development in Brazil: target plant as a rational strategy to acquire candidate anti-cancer compounds. *The Oncologist* 5:185-198.
- McGaw LJ, Jager AK, Van Staden J (2000). Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. *J Ethnopharmacol.* 72:247-263.
- Melendez-Zajgla J, Garcia C, Maldonado V (1996). Subcellular redistribution of Hsp 72 protein during cisplatin-induced apoptosis in HeLa cells. *Biochem. Mol. Biol. Int.* 40:253-261.
- Milner JA (2001). A historical perspective on garlic and cancer. *J. Nutr.* 131:1027S-1031S.
- Mohammad SF, Woodward SC (1986). Characterisation of a potent inhibitor of platelet aggregation and release reaction isolated from *Allium sativum* (Garlic). *Thrombosis Res.* 44:793-806.
- Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Viagro-Wolff AI, Gray-Goodrich C, Mayo J, Boyd M (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. *J. Nat. Cancer Inst.* 83:757-765.
- Motsei ML, Lindsey KL, Van Staden J, Jager AK (2003) Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. *J. Ethnopharmacol.* 86:235-241.
- Nakajawa H, Tsuta K, Kuichi K, Senzaki H, Tanaka K, Hioki K, Tsubura A (2001). Growth inhibitory effects of diallyl disulfide on human breast cancer cell lines. *Carcinogenesis* 22:891-897.
- Oommen S, Anto RJ, Srinivas G, Karunagaran D (2004). Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. *Eur. J. Pharmacol.* 485:97-103.
- Rashmi R, Santhosh Kumar TR, Karunagaran D (2003). Human colon cancer cells differ in their sensitivity to curcumin induced apoptosis

- and heat shock protects them by inhibiting the release of apoptosis-inducing factor and caspases. *FEBS Lett.* 538:19–24.
- Sen S, D'Incalci M (1992). Apoptosis, biochemical events and relevance to cancer chemotherapy. *FEBS Lett.* 307:122-127.
- Skehan P, Storeng R, Sudiero D, Monks A, McMahon J, Vistica D, Warren JT, Kenney S, Boyd MR (1990). New colorimetric cytotoxicity assay for anticancer drug screening. *J. Nat. Cancer Inst.* 82:1107-1113.
- Smith TK, Lund EK, Parker ML, Clarke RG, Johnson IT (2004). Allyl-isothiocyanate causes mitotic block, loss of cell adhesion and disrupted cytoskeletal structure in HT29 cells. *Carcinogenesis* 25:1409–1415.
- Srivastava SK, Singh SV (2004). Cell cycle arrest, apoptosis induction and inhibition of nuclear factor kappa B activation in anti-proliferative activity of benzyl isothiocyanate against human pancreatic cancer cells. *Carcinogenesis* 25:1701–1709.
- Sundaram SG, Milner JA (1993). Impact of organosulfur compounds in garlic on canine mammary tumour cells in culture. *Cancer Lett.* 74:85–90.
- Sundaram SG, Milner JA (1996). Diallyl disulphide inhibits the proliferation of human tumour cells in culture. *Biochim. Biophys. Acta* 1315:15-20.
- Van Wyk B-E, Van Oudtshoorn B, Gericke N (1997). *Medicinal plants of South Africa*, 1st Ed, Brizza Publications, Pretoria, p 138.
- Van Wyk B-E, Gericke N (2000). *People's plants: a Guide to Useful Plants of Southern Africa*, 1st Edition, Brizza Publications, Pretoria, p. 138.