Akinrinde et al., Afr J Tradit Complement Altern Med., (2018) 15 (4): 32-44
https://doi.org/10.21010/ajtcam.v15i4.5.

ANTI-PROLIFERATIVE ACTIVITIES OF THE AQUEOUS ROOT EXTRACT OF *DIANTHUS THUNBERGII SS HOOPER (CARYOPHYLLACEAE)*

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Article History
Received: Sept, 14. 2017
Revised Received: Apr. 4, 2018
Accepted: Apr. 4, 2018
Published Online: Oct. 16, 2018

Abstract

Background: The roots of *Dianthus thunbergii* SS Hooper are used traditionally in South Africa for the treatment of diabetes, wounds, colic, chest complaints and cancer. This study was aimed at investigating the potential anti-proliferative activities of the *D. thunbergii* in mammalian cancer cell lines.
Materials and Methods: Aqueous and ethanol extracts of *D. thunbergii* were tested *in vitro* on two cancer cell lines: human hepato-cellular carcinoma (HepG2) cells and murine insulinoma (INS-1) cells using the 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and crystal violet cell viability assays, as well as live-cell fluorescence imaging microscopy. A tentative profiling of the aqueous extract was also carried out using liquid chromatography-mass spectrometry (LC-MS).
Results: The aqueous extract (50-200µg/ml) exhibited significant ($p<0.05$) cytotoxicity in HepG2 cells ($IC_{50}<50$ µg/ml), while also significantly ($p<0.05$) decreasing the viability of INS-1 cells ($IC_{50}=36.0$ µg/ml), although no toxicity was evident in L6 myotubes. Hoechst 33342® and propidium iodide staining of INS-1 cells further revealed significant growth inhibition ($p<0.001$) of INS-1 cells by the aqueous extract. No meaningful toxicity was, however, obtained with the ethanol extract (IC_{50} = 204.0 µg/ml). Non-targeted LC-ESI-TOF/MS analysis of the aqueous extract revealed the putative identities of main compounds present in the aqueous root extracts, including some that may contribute to its anti-proliferative action.
Conclusion: Taken together, the results showed that the roots of *D. thunbergii* may represent a potential plant-based source of agents with anti-proliferative efficacy.

Keywords: *D. thunbergii*, Cytotoxicity, Caryophyllaceae, HepG2, INS-1, LC-MS

Abbreviations: HepG2- Human hepato-cellular carcinoma cells, MTT- 3-(4, 5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide, LC-MS- Liquid chromatography-Mass spectrometry, INS-1- Murine insulinoma cells, ESI- Electrospray ionization, TOF- Time of flight, DMSO- Dimethyl sulphoxide, PI- Propidium iodide.

Introduction

The effective treatment of cancer in humans and animals is commonly plagued by the fact that most synthetic chemotherapeutic agents present with a variety of toxic effects, mainly due to their non-selectivity in killing cells. There is, therefore, increasing attention towards development of natural products, especially of botanical origin, as novel prophylactic and therapeutic alternatives against cancer (Newman *et al.*, 2003; Newman, 2008). Indeed, as much as 60% of currently used anticancer drugs have been isolated from plant sources over the past several decades (Gordaliza, 2007). It must be noted, however, that herbs themselves can be toxic, especially at high concentrations and frequent use, with the possibility of deleterious herb-drug interactions (Fong, 2002). Nevertheless, from drug discovery and development perspectives, current efforts are in favor of more effective, affordable, less toxic and more target-based natural compounds that tend to inhibit selected molecular markers important for cancer growth and/or metastases (NCI, 2008).

Being naturally-derived, plant products are better tolerated by normal mammalian cells than chemically-derived synthetic anticancer agents. In addition, plants are more readily available, more affordable and less likely to cause dependency compared to synthetic drugs (Mahady, 2001; Unnati *et al*., 2013). The anticancer mechanisms of action of plant-derived compounds include the inhibition of cellular proliferation during tumorigenesis or the induction of apoptosis in cancer cells (Lian *et al.*, 2003). Others act by inhibiting telomerase activity (Baum *et al.,* 2006) or enhancing the immune system (Chen *et al.*, 2006).

The Caryophyllaceae is a large family of flowering plants containing about 88 genera used traditionally in several ethnic communities of the world to treat several diseases. They are especially well known in Chinese traditional medicine for the treatment of a wide variety of ailments (Chandra and Rawat, 2015). In ethno-medicinal terms, members of this family are renowned for their anticancer activities which are believed to be due to their rich content of structurally diverse saponins, benzenoids, phenyl propanoids and organo-nitrogen compounds (Chandra and Rawat, 2015; Gevrenova *et al*., 2014; Naghibi_*et al.*, 2014). Gevrenova *et al.* (2006) also reported the abundance of the group of glucuronide oleanane-type triterpene carboxylic acid 3, 28-bidesmosides (GOTCAB) saponins in the Caryophyllaceae. The genus *Dianthus*, popularly called carnation, consists of over 300 species distributed throughout Asia, Europe, Africa and North America. About 16 of these, including *Dianthus thunbergii* are found growing in South Africa, especially in the eastern part of the country (Pooley, 1998).

In South Africa, *D. thunbergii* SS Hooper (Hooper, 1961) (also called 'wild pink' from the color of its flowers) is included in a published list of Xhosa plants from the Eastern Cape, South Africa (Russell *et al*., 1987; Dold and Cocks, 1999). The mature plant is about 30 cm tall, possessing slender leaves, almost always opposed and with or without stipules. The flowers are pale pink with bracts about 4 cm long and blue grey leaves at the base. The root is the part most commonly used for medicinal purposes in the Eastern Cape Province of South Africa (Oyedemi *et al.,* 2009).

The anti-cancer properties of some *Dianthus* species have been described in other studies. The ethanol extract of *D. chinensis* was found to suppress HepG2 cell viability and to induce apoptosis in these cells via a down-regulation of the expression of anti-apoptotic Bcl-2 and Bcl-XL proteins (Nho *et al*., 2012). *D. caryophyllus* was shown to inhibit the growth of human colon cancer cell lines due to its content of a glycosylated flavonol, Kaempferol triglycoside (Martineti *et al*., 2010). To the best of our knowledge, there is as yet no record of any scientific evaluation of the anti-cancer properties of *D. thunbergii*. In the present study, we have examined the anti-proliferative potentials of extracts of *D. thunbergii* against two cancer cell lines: HepG2 and INS-1 cells, while also evaluating the possibility of its selectivity towards normal cells using normal rat skeletal muscle, L6 myotubes. The potential for herb-drug interactions was also determined in an *in vitro* assay of CYP 3A4 activity. In addition, we attempted an untargeted analysis to characterize the major compounds in the root extracts of *D. thunbergii* using HPLC-MS with a triple time-of-flight analyzer.

Materials and Methods

Plant material, collection and preparation of extracts

Whole plant samples of *D. thunbergii* were obtained from localities within Alice and Grahamstown, Eastern Cape Province of South Africa in June 2015. Specifically, the plant can be found on the rocky flats between Army base and Gowies Kloof, Grahamstown, Eastern Cape, South Africa (33° 17.353´S 26° 36.944´E). The plant was identified and authenticated by Prof. D.S. Grierson and a voucher specimen was deposited at the Giffen herbarium, Department of Botany, University of Fort Hare, South Africa (Voucher number: CRY-2502). The roots were separated from the rest of the plant, washed in clean tap water and oven-dried at 30°C for 24 hours and milled with an electric blender (Commercial Blender type GB27, Hamilton Beach Brands, Inc. China) to coarse powder. The extraction was done by marceration of the plant material separately in distilled water or ethanol (99.99%). These were gently rotated on an orbital shaker (Labcon laboratory service Pty, South Africa) for 24 hours. Filtrates from the aqueous extract were dessicated for about 48 hours using a freeze dryer, after initial freezing in an acetone chilling machine at -40°C. The ethanol extract was concentrated using a rotary evaporator (Heidolph Laborata 4000, Heidolph instruments, GmbH & Co, Germany). The extracts were either dissolved in DMSO for cell culture experiments or the respective solvents as required.

HPLC-MS analysis

Putative identification of metabolites in the aqueous extract of *D. thunbergii* was carried out using a high performance Agilent 1260 Infinity Liquid chromatography (LC) system, equipped with an AbSciex 5600 Triple TOF hybrid mass spectrometer (MS) (Applied Biosystems Sciex, USA) and operated in the negative electrospray ionization (ESI) mode. The LC system consisted of a 4.6 x 50 mm reverse phase column (ProShell 120; EC-C18) with diameter 7 µm. The mobile phase for gradient elution consisted of two solvents: Solvent A: Water with 0.1% formic acid and Solvent B: Acetonitrile with 0.1% formic acid. The linear elution gradient used for the elution of bound compounds was as follows: 75% Solvent A (2 min), 75% Solvent A (6 mins), 25% Solvent A (4 min), 25% Solvent A (3 min) and 75% Solvent A (2 min), giving a total run time of 17 minutes. The injection volume was 5.0 µl and the flow rate was 1 ml/minute. TOF-MS parameters were as follows: The Declustering potential (DP) was 60 V, while collision energy (CE) was 35 V.

Product ion parameters were as follows: Ion Spray voltage floating (ISVF): 4500; Ion Source Gas 1 (GS 1): 45 psi; Ion Source Gas 2 (GS 2): 45 psi and Temperature (TEM): 450. TOF masses were acquired using the Analyst Software for masses ranging from 100–1000 Da, while spectra were recorded in the ESI negative mode between m/z 50 and 1000.

For data treatment, XCMS online software (https://xcmsonline.scripps.edu) (Smith *et al*., 2006) was used for pre-processing of all scanned data (3361 potential peaks) to extract potential molecular features, whose isotopic distribution were defined with peak spacing tolerance of *m/z* 0.01 and mass accuracy of 10 ppm. Adduct formation in negative mode using two ions (-H and +Cl) were included to identify features with same potential metabolites. The resulting molecular features (298 peaks) were tentatively identified by searching the MS and MS/MS information in the Food Database (http://foodb.ca/) Version 1.0, with an error limit of 5 ppm.

Cell culture conditions

HepG2 cells were purchased from Highveld Biological, (Johannesburg, South Africa). INS-1 cells were a gift from Prof Guy Ritter (University of Bristol, England). L6 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). HepG2 cells and INS-1 cells were maintained in EMEM and RPMI 1640 medium respectively, supplemented with 10% fetal bovine serum and sub cultured every 2-3 days in the presence of penicillin-streptomycin. L6 myoblasts maintained in DMEM supplemented with 10% fetal bovine serum were allowed to differentiate into myotubes by culturing the cells in 2% horse serum for 7 additional days. All cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

MTT assay

HepG2 cells were seeded at a density of 6 000 per well at a volume of 100 µl per well. The cells were treated by adding 100 µl of the aqueous and ethanol extracts of *D. thunbergii* prepared at final concentrations of 50, 100 and 200 µg/ml in complete culture medium. The treatments were allowed for 48 hours at 37°C in a humidified 5% CO2 incubator, after which all spent media were removed by aspiration. The medium was replaced with 100 µl EMEM containing 10% fetal calf serum and 0.5 mg/ml MTT. The plates were gently rocked and then incubated at 37°C for 3 hours. At the end of incubation, the medium was aspirated and 200 µl of DMSO was added to solubilize the formazan crystals formed in the cells. The absorbance of the wells was read at 540 nm with a multi-plate reader (BioTek® PowerWave XS spectrophotometer). Concentrations of the extracts that were toxic to HepG2 cells were also tested in L6 myotubes using MTT assay as described. Cell viability was expressed as a percentage relative to the untreated control.

Crystal violet assay

INS-1 cells were seeded at a density of 8 000 cell/well in 96-well micro-titer plates with 100 µl of complete medium (RPMI 1640: 10% fetal bovine serum). Aqueous extracts of *D. thunbergii* suspended in DMSO (0.25%, v/v) were added to the wells already containing complete medium to reach concentrations of 12.5, 25 and 50 µg/ml of the extracts. The plates were incubated for about 48 hours at 37ºC in a humidified incubator and 5% CO2. DMSO (0.25%, v/v) served as the vehicle (untreated) control. All spent culture medium were removed and replaced with 100 µl of formaldehyde in PBS (10 %, v/v) to fix the cells. After about 1 hour, the fix solution was removed and 100 µl crystal violet solution (0.1%, w/v) was added to each well, followed by incubation of the plates at room temperature for about 30 minutes. Excess dye was removed by washing the plates with distilled water and the plates were dried by tapping on a paper towel. The dye taken up by the cells was extracted with 10% acetic acid (100 µl/ well). The absorbance of the wells was read at 595 nm using a BioTek® PowerWave XS spectrophotometer. Cell viability was expressed as a percentage relative to the untreated control.

Hoechst® 33342 and Propidium iodide staining for live cell imaging

INS-1 cells were maintained and treated as for the crystal violet assay with aqueous extracts of *D. thunbergii* (12.5–50µg/ml) with DMSO as control. Hoechst stock solution was prepared as a 10 mg/ml solution in distilled water. The stock solution was further diluted 1:2 in PBS to a working concentration of 5µg/ml. All medium was removed from the cultured cells and replaced with 100 µl of the Hoechst solution. The plates were incubated for about 20 minutes at room temperature (~ 24°C). Propidium iodide (PI) (50 µg/ml) was then added to the cells and incubated for 5 minutes before acquiring the images with a Molecular Devices ImageXpress® Micro XLS microscope (CA, USA) using the red and blue filters and 40× objective.

CYP 3A4 inhibition assay

The ability of the aqueous extract to inhibit the activity of human cytochrome P450 reductase was determined using a Vivid® CYP 3A4 fluorescent screening kit consisting of CYP 3A4 Baculosomes® Plus reagent, reaction buffer, BOMR substrate, Red fluorescent standard, a regeneration system and NADP+. The assay is based on the metabolism (oxidation) of the BOMR substrates by the specific CYP 450 enzyme, in the presence of NADP+, into products that are highly fluorescent in aqueous solution. The assay was performed according to the manufacturer's instructions. Briefly, 40 µl of 2.5X solutions of the aqueous extract (100 µM), the positive control Ketoconazole (92 µM and 230 µM) and solvent control were added to respective wells of a 96-well plate in four replicates. 50 µl of the Master pre-mix (containing CYP 3A4 Baculosomes® Plus reagent and Vivid® regeneration system in the Vivid® reaction buffer) was then added to each well, after which the plate was incubated for 10 minutes at room temperature to allow the test compounds and Ketoconazole to interact with the CYP 3A4 enzyme. The reaction was started by the addition of 10 µl of the substrate/NADP+ mixture. In an end-point assay format, the plate was incubated for 30 minutes at room temperature, after which the reaction was stopped by the addition of 50 µl of 0.5 M Tris base. The fluorescence was measured at an emission of 590 nm following excitation at 550 nm using a Biotek® Synergy MX fluorimeter. CYP 3A4 inhibition was calculated as a percentage relative to the solvent control as follows: % CYP 3A4 inhibition = 1 – (test fluorescence/average fluorescence of solvent control) X 100.

Statistical analysis

Values were expressed as mean ± standard deviation. Statistical analysis was carried out using Graph Pad prism Version 5.01 and the test of significance was done using Student's t-test (two-tailed). Significant differences between controls and the test compounds were defined at p values ranging from $p<0.001$ to $p<0.05$. IC50 values were determined using MS Excel software.

Results and Discussion

Cytotoxicity of aqueous and ethanol extracts in HepG2 and INS-cells

In this study, we investigated the anti-proliferative activities of *D. thunbergii* based on ethno-medicinal claims for its use in the treatment of cancer and evidence of reported cytotoxic activities reported for its closely related species. We employed different *in vitro* assays in different cell lines in order to establish preliminary data on the potential cytotoxic activities of the aqueous and ethanol extracts of *D. thunbergii*.

Rat insulinoma (INS-1) cells, a benign pancreatic cancer cell line and human hepato-cellular carcinoma (HepG2) cells, a largely malignant cell line, have been used to examine the cytotoxicity of *D. thunbergii* in this study. The viability of HepG2 cells exposed to the aqueous and ethanol extracts (50-200 µg/ml), as determined by MTT assay, is shown in Fig. 1. Significant anti-proliferative activity against HepG2 cells was observed for the aqueous extracts, with IC_{50} predicted to be much less than the lowest concentration tested. The ethanol extract though showed very little toxicity (IC_{50} = 204.0 µg/ml) to HepG2 cells and was thus eliminated from further study. According to the criteria by the American National Cancer Institute (Suffness and Pezzuto, 1990) for preliminary screening of cytotoxic crude plant extracts, promising candidates for further purification for anticancer therapy are those with IC50 ≤ 30 µg/ml, after exposure for at least 72 hours. As the cells were exposed for only 48 hours in this study, the aqueous extract was considered promising for further cytotoxicity evaluation.

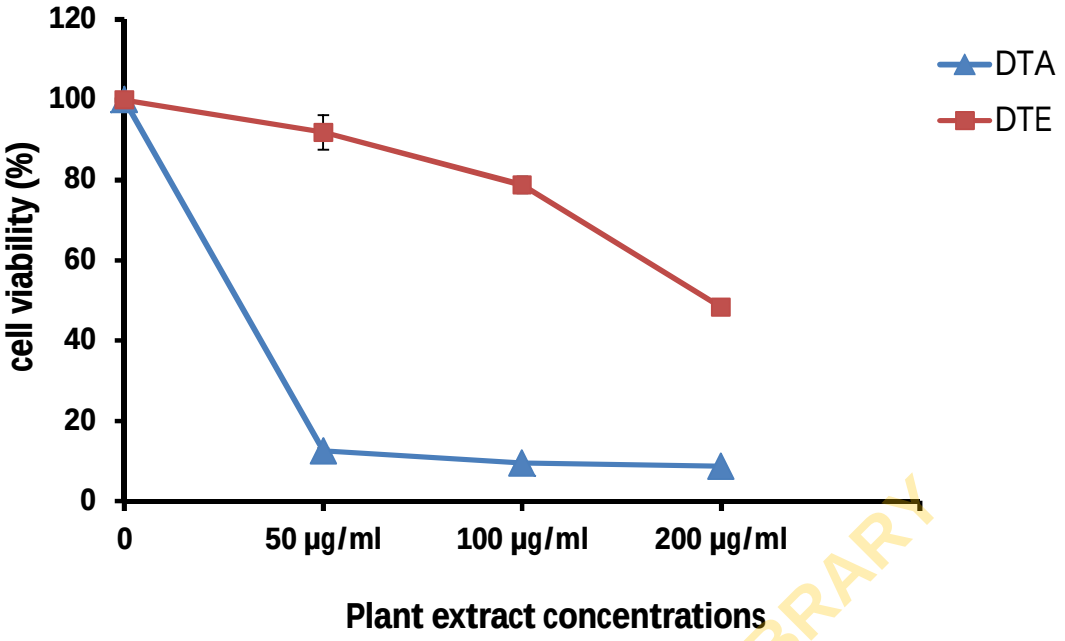

Figure 1: Dose response assay of cytotoxicity of aqueous and ethanol extracts of *D. thunbergii* in HepG2 cells, after 48 hours exposure, as determined by MTT reduction. Data are expressed as % of control ± standard deviation (n = 4). DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract. The IC50 values of the extracts are: DTA (< 50 µg/ml) and DTE (204.0 µg/ml).

Further assessment of cell viability was carried out in INS-1 cells, utilizing lower concentrations of the aqueous extract (12.5, 25 and 50 µg/ml), as it was obvious from MTT assay that concentrations lower than 50 µg/ml produced significant cell death. Results from crystal violet staining revealed a significant ($p < 0.05$) and concentration dependent decrease in the viability of INS-1 cells, with IC_{50} detected at 36.0 µg/ml (Fig. 2a). From the results of this study, cell viabilities at 50 µg/ml of the aqueous extract were 39.9±5.1% in INS-1 cells and only 12.5±0.5% in HepG2 cells. It may, thus, be reasonable to suggest that INS-1 cells may be more resistant than HepG2 cells to the cytotoxic effect of the extract.

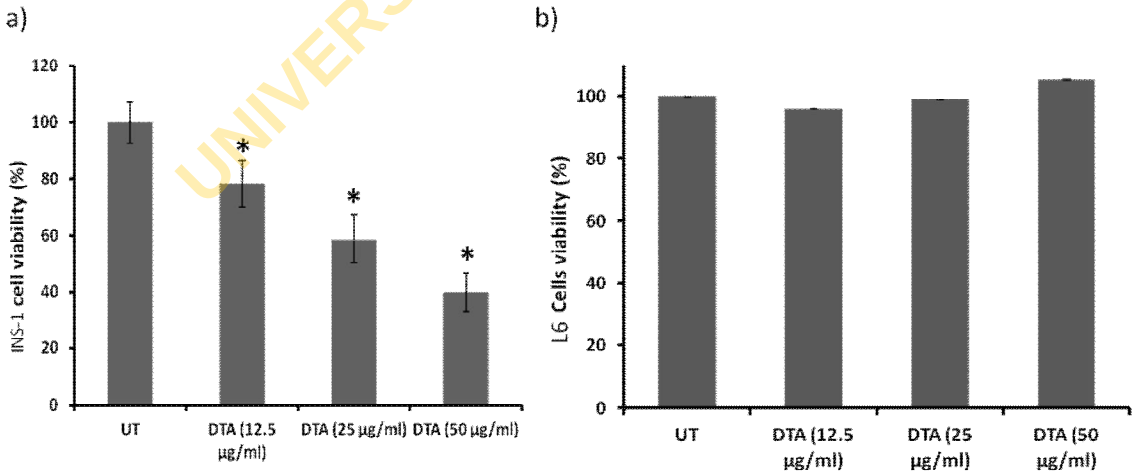

Figure 2: Viability of a) INS-1 and b) L6 cells after exposure to aqueous extract of *D. thunbergii* (for 48 hours). Values are expressed as mean (% of control) ± standard deviation (n = 8); UT = untreated control; DTA = *Dianthus thunbergii* aqueous extract; Glu = Glucose (11.1 mM). IC50 value for the extract is 36.0 µg/ml. * Significant differences at $p < 0.05$, compared to the untreated control.

Live cell imaging in INS-1 cells offered further confirmation of the cytotoxicity of the aqueous extract as revealed by the nuclear stains Hoechst and propidium iodide. Treatment of INS-1 cells with the aqueous extract produced marked growth inhibition of INS-1 cells with significant ($p < 0.001$) and concentration-dependent reduction in the percentage total cell counts of viable cells when compared to control values (Fig. 3). This was corroborated by significant ($p < 0.05$) increases in the percentage dead cell counts as revealed by propidium iodide. Hoechst® 33342 nucleic acid stain is a popular cell-permeant nuclear counter-stain that emits blue fluorescence when bound to dsDNA, while Propidium iodide (PI) is a popular red-fluorescent nuclear and chromosome counter-stain, and being non-permeant to live cells, it is used to detect dead cells in a population (Kelter *et al.*, 2007), as presented in the images in Fig. 3. The results here further affirm the observations made with Crystal Violet assay in INS-1 cells. The consistent growth-inhibitory properties of this extract may, therefore, find application for controlling cancer progression, via reduction in cell proliferation.

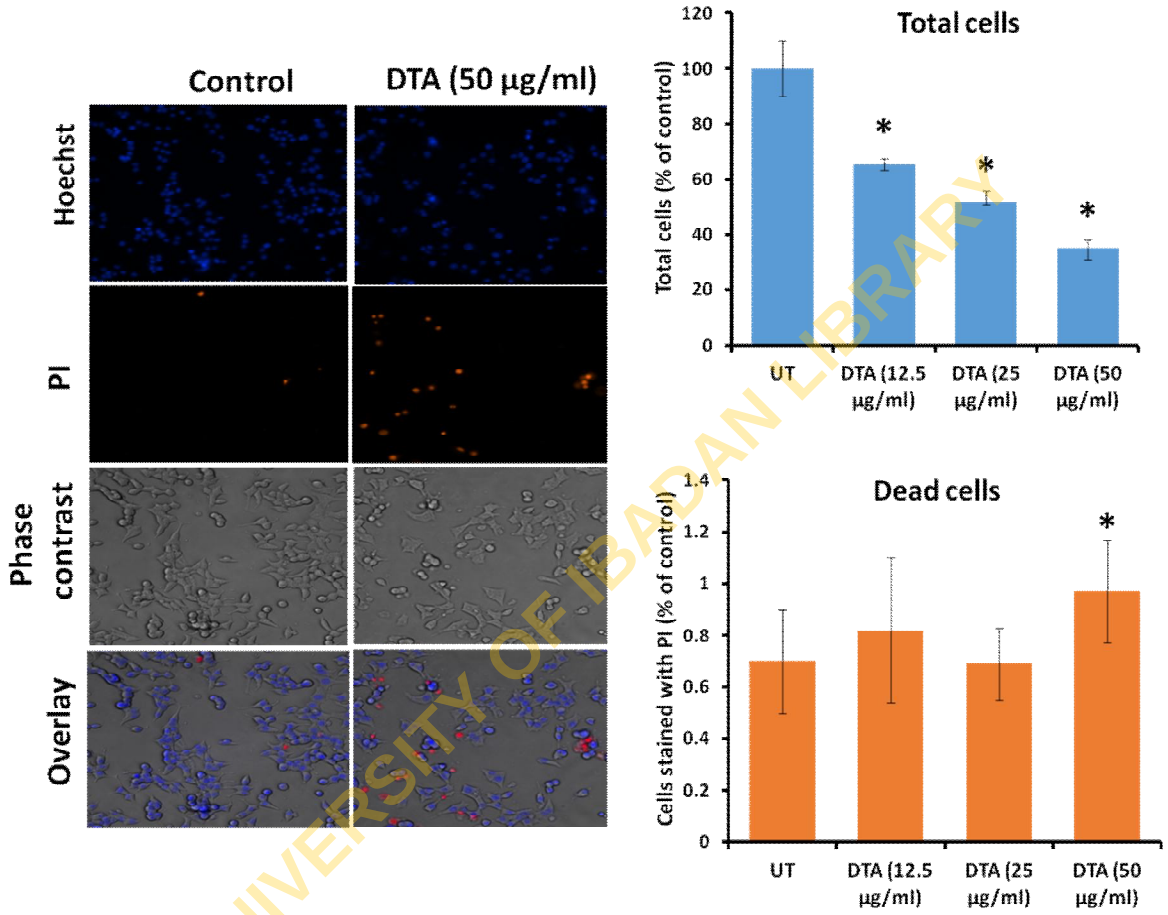

Figure 3: Image cytometry analysis of the anti-proliferative effects of the aqueous extract of *D. thunbergii* on INS-1 **cells.** Values in the charts are expressed as mean (% of control) ± standard deviation (n = 8). *Significant differences at $p < 0.001$ (total cell counts) and $p < 0.05$ (dead cells), compared to the untreated control (UT); DTA = *Dianthus thunbergii* aqueous extract; PI = Propidium iodide.

Absence of Cytotoxicity in L6 myotubes and lack of CYP 3A4 inhibition

The promise for practical application of the anti-proliferative potentials of a drug in cancer therapy depends on its ability to demonstrate considerable toxicity to cancer cells, while sparing non-cancerous cells (Lindholm *et al.*, 2002; Hu *et al.*, 2010). In addition, promising anticancer agents are required to demonstrate very little potentials for herb-drug interactions as frequent concomitant use of herbal products with conventional anticancer drugs by cancer patients may elicit adverse clinical effects or therapeutic failure due to the inhibition or induction of drug metabolism (Boullata *et al.*, 2005; Chavez *et al.*, 2006; Izzo and Ernst, 2009). In this study, MTT assay carried out on L6 myotubes revealed no alterations in percentage cell viabilities, when compared with the untreated control (Fig. 2b). The absence of toxicity to L6 myotubes suggest that the aqueous extract was not deleterious to normal cells However, it is important to also note that the L6 cells were confluent cultures (non-proliferating cells) and therefore, cytostatic effects may not be apparent.

Thus, it is important to clarify that the absence of any anti-proliferative effect in L6 cultures may not imply cancer cell specificity, but probably that the extract targets proliferating cells, which is a feature common to many of the current anti-cancer drugs. In effect, the promise of probable selective toxicity demonstrated by the extract requires further investigation. The potential for herb-drug interactions by *D. thunbergii* was examined by studying its effect on CYP 3A4, a CYP450 isoenzyme renowned for its involvement in the metabolism of up to 50% of currently prescribed drugs. Fig. 4 shows the single point inhibitory effect of the aqueous extracts on the activity of CYP 3A4. The extract produced 27.5 ± 1.4 % inhibition of the CYP 3A4, a value which was lower than that exhibited by both concentrations of the positive control, Ketoconazole. This result, in itself, suggests that aqueous extract may not likely produce significant interactions when administered concurrently with drugs metabolized by CYP 3A4. However, considering that Ketoconazole is a pure compound, compared to the crude extract and given that a concentration of 100 µg/ml used in this study, should be easily achieved as a practical dosage, the inhibition produced by the extract may well be considered physiologically relevant

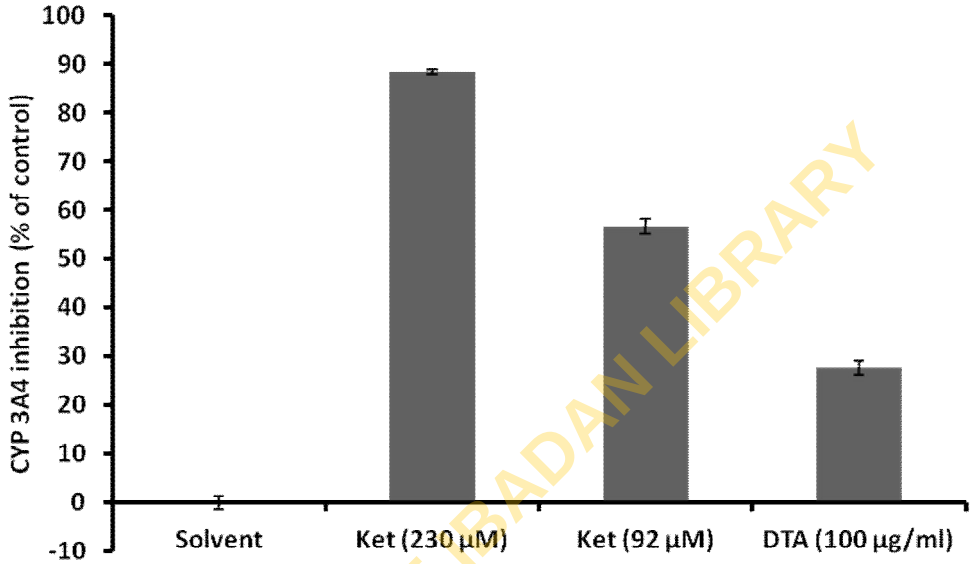

Figure 4: CYP 3A4 inhibitory activities of the aqueous extracts *Dianthus thunbergii* roots. Values are expressed as mean (% of control) ± standard deviation (n = 5) Ket = Ketoconazole; DTA = *Dianthus thunbergii* aqueous extract.

Tentative identification of compounds in D. thunbergii roots

Accurate mass measurements of metabolites using liquid chromatography (LC) coupled to electrospray ionization (ESI) techniques and time-of-flight mass spectrometry (TOF/MS) is widely accepted for verification of the elemental composition of compounds in complex matrices such as plant extracts (Lacorte and Fernandez-Alba, 2006; Heng *et al.*, 2013). These techniques incorporate high mass accuracy (< 5 ppm) which can provide initial or 'putative' identification of unknown peaks by searching metabolite databases (Prakash *et al.*, 2007). As no previous studies exist on the chemical composition of *D. thunbergii*, we have attempted a tentative characterization of the aqueous extracts of *D. thunbergii* using LC-ESI-TOF/MS.

A total of fifty three metabolites were tentatively identified in the aqueous root extract using accurate mass measurements, and, where necessary the fragmentation patterns obtained in MS/MS spectra (Table 1). These have been classified into different families along with the main parameters that support their identification, including their masses (Da), m/z values (Da), retention times (minutes), adduct types and the delta values (ppm), which aided the selection of metabolites for peaks corresponding to different isomers. Figure 5 shows the MS total ion chromatogram of the analysis scanned in negative mode. The negative mode was selected as it is reported to be more sensitive in detecting polyphenols, especially flavonoids, and also has lower limits of detection (Fabre *et al.*, 2001; De Rijke *et al.*, 2003). Pre-processing was performed using the XCMS software which is currently the most widely used open source algorithm for defining molecular features from signatures of ionized metabolites (Smith *et al.*, 2006; Tautenhahn *et al.*, 2008). Among compounds identified, the majority of those with reported anti-proliferative activities were flavonoids and their derivatives as well as terpenoids (Table 1).

In total, 18 phenolic compounds were tentatively identified, including 13 flavonoids and flavonoid derivatives. Of these, 8 flavonoids were identified as the $[M-H]^-$ adduct form with high accuracy. These were 7-chloro-3,3´,4´,5,6,8-hexamethoxyflavone (*m/z* 435.09), Irilone 4´-glucoside (*m/z* 459.09), Hyperoside (*m/z* 463.09), Myricetin 3(4´-acetyl rhamnoside) (*m/z* 505.10), 6´´-caffeoylastragalin (*m/z* 609.13), Kaempferol 3-gentiobioside (*m/z* 609.15),

alpha viniferin (*m/z* 677.18) and Peonidin 3-sophoroside 5-glucoside (*m/z* 786.22). Other phenolic compounds were in the family of lignans, phenolic glycosides, tannins and coumaric acid derivatives. Anti-proliferative effects have been attributed to hyperoside by Liu *et al* (2016) against lung cancer by inhibition of transcription of NF-κB transcriptional activity, activation of Caspase-9/Caspase-3 apoptotic pathway and cell cycle arrest. Alpha viniferin, a derivative of resveratrol, has been found to exhibit inhibitory properties against colorectal cancer via anti-inflammatory activities involving reduced expression of COX-2 and pro-apoptotic pro-apoptotic caspase-3 expression (Kwak *et al*, 2015). In addition, isoacteoside, a coumaric acid derivative, detected by the formation of the $[M+Cl]^-$ adduct at Rt 8.35 has been reported to exhibit anticancer properties (Wang and Nixon, 2001).

Terpenoid saponins are known for their anticancer properties and are found in rich quantities in the Caryophyllaceae (Gevrenova *et al.*, 2014; Bottger and Melzig, 2010). It is remarkable, therefore, that a number of them were tentatively identified in the aqueous extract of *D. thunbergii*, including 4 triterpenes, 2 terpenoids and a monoterpenoid, all of which were detected in the chromatographic interval 9-12 minutes. Among these, Centellasaponin B (*m/z* 827.4435) was detected by the formation of the $[M-H]^-$ adduct at Rt 9.21 min. It is a triterpenoid glycoside belonging to the group of ursane- and oleanane-type triterpene oligoglycosides originally isolated from *Centella asiatica* (Matsuda *et al.*, 2001). This compound, along with other related saponins, including centellasaponins C and D are believed to be responsible for the cytotoxic activities exhibited by *Centella asiatica* (Pitella et al., 2009)). Another triterpene saponin, Soyasaponin γg (*m/z* 957.46) was observed at Rt 9.76 by the formation of the $[M+Cl]^-$ adduct. This metabolite and a variety of other soyasaponins have been characterized in soy bean extracts and are reported to exhibit anti-mutagenic activities in Chinese hamster ovary cells (Berhow *et al.*, 2000).

Other compounds, belonging to other families tentatively identified in the extracts were also found with reported anti-proliferative activities. The naphthoquinone, Neodiospyrin (*m/z* 409.05) detected as the $[M+Cl]^-$ adduct reportedly slows cancer metastasis by inhibiting 12(S)-HETE, a metabolite of 12-lipooxygenase (Wube *et al.*, 2005). Mangiferin (*m/z* 421.08) tentatively identified at Rt 7.24 as the $[M-H]^-$ is a xanthone, originally isolated from *Mangifera indica* and is known to exhibit promising anticancer properties (Khurana *et al.*, 2016). Also, cytotoxic activities in HeLa cells have been reported for the alkaloid Bis (5-hydroxynoracronycine) (Weniger *et al.*, 2001), which was tentatively identified in this study at Rt 3.02 as the $[M+Cl]^-$ adduct. Apart from the groups of compounds already discussed, our analysis also detected compounds in *D. thunbergii* roots, including lipids and lipid-like molecules, oligosaccharides, organo-heterocyclic compounds, organo-nitrogen and organo-oxygen compounds. However, most of these compounds have been poorly studied with regards to their anti-proliferative activities.

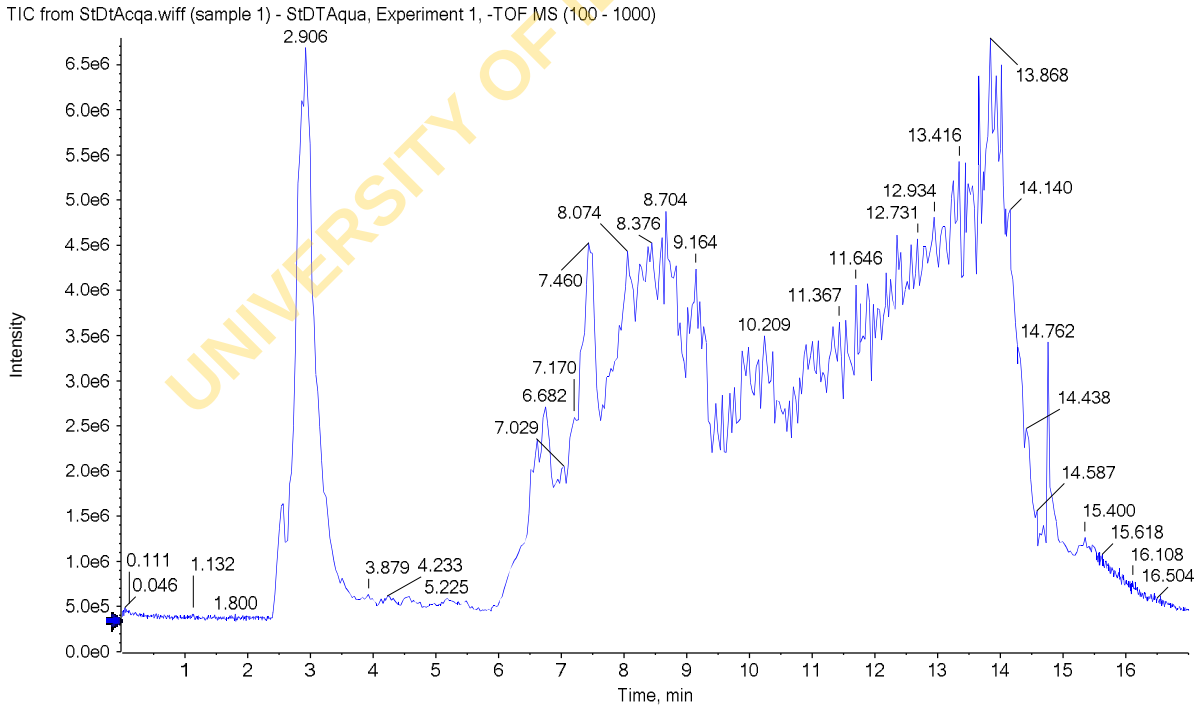

Figure 5: MS total ion chromatogram from the aqueous extract of *D. thunbergii* roots in negative ionization mode.

Table 1: Putative identification of compounds in the aqueous extracts of *D. thunbergii* showing the main parameters for their identification

Class of compound	Putative identification	Mass	*m/z*	Adduct	RT (min)	δ (ppm)	Previous report of anti-proliferative activity
Phenolic Compounds							
Flavonoids and derivatives							
	Peonidin 3-Sophoroside 5-glucoside	787.2297	786.2224	M-H	2.73	0.0002	
	Albanin H	840.3146	875.2674	M+Cl	2.86	0.0028	
	5',8,8''-Trihydrohy-3',3''',4',4''',5''',7',7''-heptamethoxy-5,5''-biflavan	676.2520	711.2214	M+Cl	2.93	0.0000	
	Pelargonidin 3-arabinoside	403.1029	438.0723	M+Cl	7.37	0.0079	
	7-chloro-3,3',4',5,6,8-hexamethoxyflavone	436.0925	435.0852	M-H	7.45	0.0071	
	Isoeriotricin	596.1741	631.1435	M+Cl	7.53	0.0090	
	Alpha-Viniferin	678.1890	677.1817	M-H	7.79	0.0045	[36]
	Kaempferol 3-gentiobioside	610.1534	609.1461	M-H	7.90	0.0012	
	Hyperoside	464.0955	463.0882	M-H	8.48	0.0005	[35]
	Pinotin A aglycone	463.1029	498.0723	M+Cl	8.96	0.0011	
	Myricetin 3(4'-acetyl rhamnoside)	506.1060	505.0988	M-H	9.37	0.0003	
	Irilone 4'-glucoside	460.1006	459.0933	M-H	10.24	0.0001	
	6''-Caffeoylastragalin	610.1323	609.1250	M-H	11.04	0.0005	
Lignans							
	Lappaol H	750.2888	749.2815	M-H	6.56	0.0081	
Phenolic glycosides							
	Nb-trans-Feruloylserotonin glucoside	514.1951	549.1645	M+Cl	3.02	0.0033	
	Zeanoside C	385.1009	420.0703	M+Cl	7.44	0.0034	
Tannins							
	6-Cinnamoyl-1,6-digalloyl-beta-D-glucopyranose	614.1272	613.1199	M-H	8.12	0.0041	
Coumaric acid							

derivatives							
	Isoacteoside	624.2054	659.1748	M+Cl	8.35	0.0086	[45]
Terpenoids							
	Centellasaponin B	828.4507	827.4435	M-H	9.21	0.0014	[40]
	Alpha-Amyrin tetratriacontanoate	916.8975	915.8903	M-H	9.53	0.0059	
	Soyasaponin gamma-G	922.4926	957.4620	M+Cl	9.76	0.0020	[41]
	Sanguisorbin E	792.4660	827.4354	M+Cl	9.95	0.0008	
	Auxin a	328.2250	327.2177	M-H	10.02	0.0003	
	Licorice saponin C2	806.4089	805.4089	M-H	10.44	0.0018	[37]
	Beta-Citraurin epoxide	448.2978	483.2672	M+Cl	11.37	0.0060	
Lipids and Lipid-like molecules							
	Maltobionic acid	358.1111	357.1039	M-H	2.96	0.0000	
	Lactodifucotetraose	634.2320	633.2248	M-H	6.62	0.0002	
	Corchonisoside E	860.4042	895.3736	M+Cl	8.32	0.0065	
	Lactosamine	682.2644	681.2571	M-H	8.38	0.0090	
	Endomorphin-2	571.2795	606.2489	M+Cl	8.45	0.0045	[46]
	Lyciumin B	896.3817	895.3755	M-H	9.02	0.0046	
	Betavulganoside 1	954.4461	989.4155	M+Cl	9.44	0.0009	
	22-Deoxocurcubitacin D	502.3294	501.3222	M-H	12.22	0.0001	
Glycerophospholipids							
	PS (14:1(9Z)/14:1(9Z))	675.4111	710.3805	M+Cl	9.98	0.0082	
	LPA(18:2(9Z, 12Z)/0:0)	434.2433	433.2361	M-H	11.77	0.0005	
Oligosaccharides							
	Maltohexose	990.3275	989.3202	M-H	2.83	0.0036	
	Inulin	504.1690	503.1618	M-H	2.99	0.0001	
Organo-heterocyclic compounds							
	Thiamine monophosphate	344.0708	379.0402	M+Cl	7.01	0.0055	
	Norrubrofusarin 6-gentiobioside	582.1585	617.1279	M+Cl	8.19	0.0087	
	3'-N-acetyl-4'-O-(9-octadecenoyl) fusaro chromanone	598.3982	633.3676	M+Cl	11.01	0.0022	
	1,3,5-Trihydroxy-10-methyl acridone	257.0688	256.0615	M-H	12.65	0.0005	

	Citrusine II	287.0794	286.0721	M-H	12.88	0.0006	
	Cyanidin 3-(diferyloyl sophoroside) 5-glucoside	274.2045	309.1739	M+Cl	13.46	0.0006	
Organo-nitrogen and Organo-oxygen compounds							
	Azimsulfuron	424.1026	423.0953	M-H	7.31	0.0011	
	4-Phenylbutyl glucosinolate	451.0971	450.0898	M-H	7.50	0.0053	
	6'-Sialyllactosamine	632.2276	631.2203	M-H	8.63	0.0023	
	9-Decenoylcholine	256.2277	291.1971	M+Cl	11.10	0.0032	
Other classes							
Naphthoquinones							
	Neodiospyrin	374.0790	409.0484	M+Cl	6.78	0.0037	[42]
Xanthones							
	Mangiferin	422.0849	421.0776	M-H	7.24	0.0036	[43]
Diarylheptanoids							
	Kuwanon Y	582.1890	617.1584	M+Cl	7.47	0.0031	
Benzenoids							
	Aloinoside A	564.1843	599.1537	M+Cl	8.02	0.0091	
	N-Undecylbenzenesulfonic acid	312.1759	311.1686	M-H	11.82	0.0007	
Alkaloids							
	Bis (5-hydroxynoracronycine)	646.2315	681.2009	M+Cl	3.06	0.0098	[44]

RT = retention time in minutes; δ = delta value (Query mass – Adduct mass); *m/z* = mass-to-charge (adduct mass). For peaks with isomers, the metabolites with the lowest delta value were selected as their tentative identities.

Conclusion

The current study proved the *in vitro* anti-proliferative activity of *D. thunbergii* aqueous root extract. The selective toxicity demonstrated by the extract suggests the plant may serve as a prospective material for the development of novel anti-proliferative agents against cancer cells. Profiling of compounds present in the extract by LC-TOF-MS tentatively revealed a total of 53 compounds, mainly on the basis of accurate mass measurements of the metabolites, while some compounds with reported anti-proliferative activities have been identified and discussed. However, more detailed analysis of the chemical composition of this plant is required for the confirmation of the actual identities of the unknown peaks via incorporation of retention time and/or mass spectral data of reference standards in further experiments.

Conflict of Iinterests: The authors declare no conflicts of interest.

Acknowledgements: The authors thank the Govan Mbeki Research and Development Centre, University of Fort Hare, South Africa for their research support. Dr Akinrinde AS received financial support from the Tertiary Education Fund (TetFund), Nigeria, under the Academic Staff Training and Development Fund.

Author contributions: A.S. and G.B. conceived and designed the study. A.S. wrote the first draft of the manuscript. A.S., T.K. and M.V. performed the experiments and analyzed the data. T.K., M.V. and G.B. revised the manuscript. All authors approved the final draft of the manuscript.

References

1. Berhow, M.A., Wagner, E.D., Vaughn, S.F., Plewa, M.J. (2000). Characterization and antimutagenic activity of soybean saponins. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 448(1): 11–22.
2. Bottger, S., Melzig, M.F. (2010). Triterpenoid saponins of the Caryophyllaceae and Illecebraceae family. Phytochemistry Letters 4: 59–68.
3. Boullata, J. (2005). Natural health product interactions with medication. Nutrition in Clinical Practice 20: 33–51.
4. Chandra, S., Rawat, D.S. (2015). Medicinal plants of the family Caryophyllaceae; a review of ethno-medicinal uses and pharmacological properties. Integrative Medicine Research 4: 123–131.
5. Chavez, M.L., Jordan, M.A., Chavez, P.I. (2006). Evidence-based drug-herbal interactions. Life Sciences 78: 2146–2157.
6. Chen, X., Hu, Z.P., Yang, X.X., Huang, M., Gao, Y., Tang, W., Chen, S.Y., Dai, X., Ye, J., Ho, P.C., Duan, W., Yang, H.Y., Zhu, Y.Z., Zhou, S.F. (2006). Monitoring of immune responses to herbal immune-modulator in patients with advanced colorectal cancer. Immunopharmacology 6(3): 499–508.
7. De Rijke, E., Zappey, H., Ariese, F., Gooije, C., Brinkman, U. (2003). Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. Journal of Chromatography A 984: 45–48.
8. Dold, A.P., Cocks, M.L. (1999). Preliminary list of Xhosa plant names from the Eastern Cape, South Africa. Bothalia 29: 267–292.
9. Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. (2001). Determination of flavone, flavonol and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. Journal of the American Society of Mass Spectrometry 12: 707–715.
10. Fong, H.H. (2002). Integration of herbal medicine into modern medical practices; issues and prospects. Integrative Cancer. Therapy 1(3): 287–293.
11. Gevrenova, R., Joubert, O., Mandova, T., Zaiou, M., Chapleur, Y., Henry, M. (2014). Cytotoxic effects of four Caryophyllaceae species extracts on macrophage cell lines. Pharmaceutical Biology. 52(7): 919–925.
12. Gevrenova, R., Voutquenne-Nazabadioko, L., Harakat, D. , Prost E, Henry M. (2006). Complete 1H and 13C NMR assignments of saponins from roots of *Gypsophila trichotoma*. Wend. Magnetic Resonance in Chemistry 44: 686–691.
13. Gordaliza, M. (2007). Natural products as leads to anticancer drugs. Clinical and Translational Oncology 9(12): 767–776.
14. Heng, M.Y., Tan, S.N., Yong, J.W.H., Ong, E.S. (2013). Emerging green technologies for the chemical standardization of botanicals and herbal preparations. TrAC Trends in Analytical Chemistry 50: 1–10.
15. Hooper, S.S. (1961). *Dianthus* L. (Caryophyllaceae). Flora zambesiaca 1: 355–358.
16. Hu, X., Zhang, X., Qiu, S., Yu, D., Lin, S. (2010). Salidroside induce cell cycle arrest and apoptosis in human breast cancer cells. Biochemical and Biophysical Research Communications 398: 62–67.
17. Izzo, A.A., Ernst, E. (2009). Interactions between herbal medicines and prescribed drugs. Drugs. 69: 1777–1798.
18. Kelter, G., Schierholz, J. M., Fischer, I. U., Fiebeg, H.H. (2007). Cytotoxicity activity and absence of tumour growth stimulation of standardized mistletoe extracts in human tumour models *in vitro*. Anticancer Research. 27(1): 223–234
19. Khurana, R.K., Kaur, R., Lohan, S., Singh, K.K., Singh, B. (2016). Mangiferin: a promising anticancer bioactive. Pharmaceutical Patent Analyst 5(3): 169–181.
20. Kwak, D.H., Shin, S.K., Youm, S.Y., Kim, T.W., Kim, Y., Ahn, B. (2015). Chemopreventive effect of alpha-viniferin in azoxymethane-induced mouse colorectal tumor and Caco-2 cells. Journal of Biomedical Research16 (2): 60–66.
21. Lacorte, S., Fernadez-Alba, A. (2006). Time of flight mass spectrometry applied to the liquid chromatographic analysis of pesticides in water and food. Mass Spectrometry Reviews 25: 866-880
22. Lian, Z., Niwa, K., Gao, J., Tagami, K., Mori, H., Tamaya, T. (2003). Association of cellular apoptosis with anti-tumor effects of the Chinese herbal complex in endocrine-resistant cancer cell line. Cancer Detection and Prevention 27(2): 147–154.
23. Lindholm, P., Gullbo, J., Claeson, P., Goranson, U., Johansson, S., Backlund, A., Larsson, R., Bohlin, L. (2002). Selective cytotoxicity evaluation in anti-cancer drug screening of fractionated plant extracts. Journal of Biomolecular Screening 7: 333–340.
24. Liu, Y.H., Liu, G.H., Mei, J.J., Wang, J. (2016). The preventive effects of hyperoside on lung cancer in vitro by inducing apoptosis and inhibiting proliferation through caspase-3 and p53 signaling pathway. Biomedicine and Pharmacotherapy 83: 381–391.
25. Mahady, G.B. (2001). Global harmonization of herbal health claims. Journal of Nutrition 131(3s): 1120S–1123S.
26. Martineti, V., Tognarini, I., Azzari, C., Carbonell Sala, S., Clematis, F., Dolci, M. Lanzotti V, Tonelli F, Brandi ML, Curir P. (2010). Inhibition of *in vitro* growth and arrest in the GO/G1 phase of HCT 8 line human colon cancer cells by Kaempferol triglycoside from *Dianthus caryophyllus*. Phytotherapy Research 24(9): 1302-1308.
27. Matsuda, H., Morikawa, T., Ueda, H., Yoshikawa, M. (2001). Medicinal foodstuffs XXVII. Saponin constituents Gotu kola (2): Structure of new ursane- and oleanane-type triterpene oligoglycosides, centellasaponin B, C and D, from *Centella asiatica* cultivated in Sri Lanka. Chemical and Pharmaceutical Bulletin 49: 1368–1371.

28. Naghibi, F., Irani, M., Hassapour, A., Pirani, A., Hamzeloo-Moghadam, M. (2014). Cytotoxic effects of selective species of Caryophyllaceae in Iran. Research Journal of Pharmacognosy 1(2): 29–32.
29. National Cancer Institute. Targeted Cancer Therapies. http://www.cancer.gov/cancertopics/factsheet/therapy/targeted. 2008.
30. Newman, D.J. (2008). Natural products as leads to potential drugs; an old process or the new hope for drug discovery? Journal of Medicinal Chemistry 51(9): 2589–2599.
31. Newman, D.J., Cragg, G.M., Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. Journal of Natural Products 66(7): 1022–1037.
32. Nho, K.J., Chun, J.M., Kim, H.K. (2012). Ethanol extract of *Dianthus chinensis* L. induces apoptosis in human hepatocellular carcinoma HepG2 cells *in vitro*. Evidence Based Complementary and Alternative Medicine doi: 10.1155/2012/573527.
33. Oyedemi, S.O., Bradley, G., Afolayan, A.J. (2009). Ethnobotanical survey of medicinal plants used for the management of diabetes mellitus in the Nkonkobe municipality of South Africa. Journal of Medicinal Plants Research 3(12): 1040–1044.
34. Pitella, F., Dutra, R.C., Junior, D.D., Lopes, M.T.P., Barbosa, N.R. (2009). Antioxidant and cytotoxic activities of *Centella asiatica* (L) Urb. International Journal of Molecular Sciences 10(9): 3713–3721.
35. Pooley, E. (1998). A field guide to wild flowers of KwaZulu-Natal and the Eastern Region, edn 1. Natal Flora Publications Trust, Durban.
36. Prakash, C., Shaffer, C., Nedderman, A. (2007). Analytical strategies for identifying Drug metabolites. Mass Spectrometry Reviews 26: 340–369.
37. Russell, G. G. E., Welman, W.G.M., Retief, E., Immelman, K.L., Germishuizen, G., Pienaar, B.J., Van Wyk, M., Nicholas, A. (1987). List of species of southern African plants. Memoirs of Botanical Surveys South Africa **2**(1–2), 1–152(pt. 1), 1–270(pt. 2).
38. Smith, C.A., Want, E.J., O'Maille, G., Abayan, R., Siuzdak, G. (2006). XCMS: processing mass spectrometry data for metabolite profiling using non-linear peak alignment, matching and identification. Analytical Chemistry 78: 779–787.
39. Suffness, M; Pezzuto, JM. Assays related to cancer drug discovery. In Methods in Plant Biochemistry: Assays for Bioactivity; Hostettmann, K, Ed.; Academic Press: London, UK, 1990; Volume 6, pp. 71–133"
40. Tautenhahn, R., Bottcher, C., Neumann, S. (2008). Highly-sensitive feature detection for high resolution LC/MS. BMC Bioinformatics. 9: 504-520.
41. Unnati, S.; Ripal, S; Sangeev, A; Niyati, A. (2013). Novel anticancer agents from plant sources. Chinese Journal of Natural Medicines 11(1): 0016–0023.
42. Wang, Z.Y., Nixon, D.W. (2001). Licorice and Cancer. Nutrition and Cancer. 39(1): 1-11.
43. Weniger, B., Um, B., Valentin, A., Estrada, A., Lobstein, A., Anton, R., Maille, M., Sauvain, M. (2001). Bioactive alkaloids from *Swinglea glutinosa*. Journal of Natural Products 64: 1221–1223.
44. Wube, A.A., Streit, B., Gibbons, S., Asres, K., Bucar, F. (2005). In vitro 12(S)-HETE inhibitory activities of naphthoquinones isolated from the root bark of *Euclea racemosa* ssp. schimperi. Journal of Ethnopharmacology14: 191-196.