Pharmacognosy Communications, 2023; 13(3):126-140.
https://www.phcogcommn.org

Original Article

Tasmannia lanceolata (Poir.) A.C.Sm. Berry and Leaf Extracts Inhibit Proliferation and Induce Apoptosis in Selected Human Carcinoma Cell Lines

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ABSTRACT

Background: *Tasmannia lanceolata* berries and leaves have high antioxidant capacities and high levels of therapeutic phytochemicals. Despite this, the anticancer activity of *T. lanceolata* extracts has not been adequately explored. **Materials and Methods:** This study examined the anti-proliferative of *T. lanceolata* berry and leaf extracts against a panel of human carcinoma cell lines using MTS assays. The apoptotic activities were examined using cell imaging and caspase-3 activity assays. LC-MS and GC-MS headspace analysis were used to identify noteworthy phytochemical components. **Results:** The methanolic, aqueous and ethyl acetate extracts inhibited the proliferation of HeLa, Caco-2, Jeg-3, JAR, MC3T3-E1 and MG63 cell lines. The aqueous leaf extract was a particularly potent inhibitor of proliferation against most cell lines (HeLa IC_{50} = 230µg/mL; Caco-2 IC_{50} = 150µg/mL; Jeg-3 IC_{50} = 488µg/mL; JAR IC_{50} = 450µg/mL; MC3T3 IC_{50} = 195µg/mL; MG63 IC_{50} = 315µg/mL). The berry and leaf ethyl acetate extracts were also good inhibitors of proliferation of the same cell lines, albeit with slightly higher IC_{50} values. Morphological features consistent with apoptosis were evident in Caco-2 cells exposed to the berry and leaf ethyl acetate extracts, and the aqueous leaf extract. Furthermore, exposure of Caco-2 to sub-lethal concentrations of these extracts induced significantly elevated levels of caspase 3, indicating that the extracts induced apoptosis in Caco-2 cells. **Conclusion:** All *T. lanceolata* berry and leaf extracts induced apoptosis in a panel of cancer cells and were nontoxic in the ALA toxicity bioassay and in a HDF cell viability assay, supporting their potential as cancer chemotherapies.

Keywords: Winteraceae, Tasmanian pepper, Mountain pepperberry, Anti-cancer activity, Anti-proliferative activity, Antioxidant capacity, Apoptosis.

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Received: 16-04-2023;
Revised: 29-04-2023;
Accepted: 07-05-2023.

INTRODUCTION

The role of Reactive Oxygen Species (ROS) in the maintenance of cellular redox state and their implications in carcinogenesis and cancer therapy has been extensively studied for several decades. In general, cancer cells have higher ROS levels than their non-cancerous cell counterparts and these higher levels of ROS have been linked with cancer etiology, progression, angiogenesis, metastasis and ultimately, patient survival.[1] However, at higher levels, ROS may also induce apoptosis. Therefore, modulation of the cellular redox state (and thus the levels of ROS) is the focus of multiple studies aimed at developing new cancer therapies.[2-4]

Regulation of the cellular redox state involves a coordinated balance between cellular (and exogenous) oxidants and antioxidants. High antioxidant therapies, the use of traditional/herbal therapies, or the ingestion of high antioxidant fruits and vegetables may all be useful in regulating the redox state of a cell.[4] The primary aim of using these high antioxidant therapies is to scavenge free radicals, thereby protecting cell constituents, including nucleic acids, proteins and lipids from accumulating damage.[4] Consuming high antioxidant plant extracts may reduce ROS induced oxidative damage and scavenge free radicals, blocking further damage. Thus, high antioxidant plant extracts may be useful for both preventing and treating cancer.

Several recent studies have screened high antioxidant plant extracts for anticancer properties and reported strong inhibition of proliferation. For example, plants of the genus *Terminalia* have been studied for anticancer activity due to their very

DOI: 10.5530/pc.2023.3.20

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high antioxidant contents and interesting phytochemistry.[5] *T. ferdinandiana* Exell. fruit extracts have potent cytotoxic effects on human HT29 colorectal adenocarcinoma and human HL60 polymyelocytic leukemia cell lines.[3] The extracts also inhibit proliferation in non-cancerous human cell lines, but did not significantly affect viability in AGS (human gastric adenocarcinoma) cells. The anti-proliferative effects of the *T. ferdinandiana* extract was associated with caspase-7, caspase-9 and poly (ADP-ribose) Polymerase (PARP) activation, indicating that the extracts induced apoptosis via intrinsic pathways. A different study also reported potent anti-proliferative activity against human Caco-2 colorectal, HeLa cervical, Jeg3 and JAR choriocarcinomas, as well as the human (MG63) and mouse osteosarcoma cell lines.[2] Other studies have screened human carcinoma cells against a variety of high antioxidant Asian,[6-9] Australian,[10-12] European[13] and African plants.[14] Those studies reported good anticancer activities for several plant species and correlated that activity with the antioxidant content. However, many other high antioxidant plant species are yet to be screened for anti-proliferative activity against human carcinomas.

Tasmannia lanceolata (Poir.) A.C. Sm. (family Winteraceae; commonly known as Tasmanian pepper, mountain pepperberry) is an endemic Australian shrub that is known for its exceptionally high antioxidant contents.[15,16] Indeed, *T. lanceolata* extracts have been reported to have antioxidant contents three times higher than those of blueberries and four times the levels in *Ocimum basilicum* L.,[16] both of which are considered to have high antioxidant contents. Furthermore, *T. lanceolata* berries and leaves have high levels of a variety of phytoconstituents with reported therapeutic properties. In particular, *T. lanceolata* is rich in flavonoids and flavonoid glycosides (including quercetin and rutin), as well as tannins (particularaly gallic acid) and terpenoids (including polygodial, eugenol).[16] Notably, that study also reported that several of those compounds have good anti-proliferative activity against multiple human cancers. Despite this, the effects of *T. lanceolata* extracts on human cancer cells remains largely unreported.

Our study was designed to screen *T. lanceolata* extracts against several important human cancer cell types. We also chose several carcinoma pairs with different properties with the aim of determining details of the anti-proliferative mechanisms induced by the extracts. Human colorectal carcinoma Caco-2 and the cervical carcinoma HeLa were selected for our study as both cell lines have been screened against many plant extracts and compounds, allowing for direct comparison of efficacies between studies. Furthermore, both cell lines have been extremely well characterised and their susceptibilities and resistances are well documented. All other cell line pairs selected for study were chosen due to their differential profiles of redox regulatory and apoptotic proteins. Using these panels of cell lines may provide mechanistic details for the anti-proliferative extracts. For example, the Jeg-3 and JAR choriocarcinoma cell pair produce similar levels of most apoptotic and redox proteins, yet vary significantly in the levels of thioredoxin that each cell line expresses. Jeg-3 cells secrete substantially higher levels of thioredoxin compared with JAR cells.[17] Therefore, it is expected that Jeg-3 cells would be better able respond to oxidative stress and would be more resistant to the induction of apoptosis than JAR cells. Additionally, JAR cells upregulate their production of Tumour Necrosis Factor (TNF) during proliferation, whereas the secretion of TNF by Jeg-3 cells remains relatively constant during proliferation.[18] Therefore, screening against this cell pair may indicate which pathways are affected by the *T. lanceolata* extracts. The MC3T3-E1 (mouse primary osteoblast cell line) and MG63 (human osteosarcoma) carcinoma pair were included in our study due to their differential responses to specific antioxidant compounds. In the presence of ascorbic acid, MC3T3-E1 cells over express collagen and will mineralise.[19] In contrast, collagen levels are substantially lower and relatively constant in MG63 carcinoma cells. Therefore, it is expected that the high antioxidant content (including increased ascorbic acid levels) in the *T. lanceolata* berry and leaf extracts would induce collagen and matrix formation by the MC3T3-E1 cells in comparison to the MG63 cells. Here, we report the anti-proliferative activity of *T. lanceolata* berry and leaf extracts against these cell lines.

MATERIALS AND METHODS

Plant source and extraction

The *Tasmannia lanceolata* (Poir.) A.C.Sm. berries and leaves used in this study were purchased from GoWild Harvest (Australia) as dried materials. The berries were further dehydrated in a Sunbeam food dehydrator until a constant mass was obtained upon repeated measurements. Voucher specimens (GUTPGW-2015-BE and GUTPGW-2015-LE for the berry and leaf respectively) are stored in the School of Environment and Science, Griffith University, Australia. Prior to extraction, the dried plant materials were ground into a coarse powder. Individual 1g masses of the powdered *T. lanceolata* berries and leaves were extracted separately for 24 hr at 4°C with 50mL of methanol, deionised water, ethyl acetate, chloroform or hexane. All solvents were AR grade and were purchased from Ajax Fine Chemicals, Australia. The *T. lanceolata* extracts were filtered through Whatman No. 54 filter paper and air dried at room temperature in the shade. The aqueous extracts were lyophilised at -50°C in a freeze dryer. The dried extracts were subsequently weighed and resuspended in 10mL deionised water (containing 1% DMSO) and stored at 4°C until use.

Qualitative phytochemical studies

Qualitative phytochemical screening of the extracts for the presence of alkaloids, anthraquinones, cardiac glycosides,

flavonoids, phenolic compounds, phytoysterols, saponins, triterpenoids, and tannins was conducted by standard assays.[20]

Screen for anticancer bioactivity

Cancer cell lines

Caco-2 colorectal and HeLa cervical carcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, USA). Mouse MC3T3-E1 osteoblast and MG63 human osteosarcoma cell lines were donated by Professor Ann McDonnell, Griffith University. Jeg-3 and JAR human choriocarcinoma cell lines were a gift from Dr Jenny DiTrapanni, Griffith University. Human primary dermal fibroblasts (HDF; ATCC PCS-201-012) were purchased from ATCC (Rockville, USA) and used as a control cell line. The Caco-2, HeLa, Jeg-3 and JAR cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), whilst MG63, MC3T3-E1, and HDF cells were cultured in Dulbecco/Vogt modified Eagle's Minimal Essential Medium (DMEM). DMEM and RPMI medias were both supplemented with 20mM HEPES, 10mM sodium bicarbonate, 50µg/mL streptomycin, 50 IU/mL penicillin, 2mM glutamine and 10% foetal calf serum (Invitrogen, Australia). All cell lines were grown at 37°C in a humidified 5% CO_2 environment.

Evaluation of cancer cell anti-proliferative activity

The anti-proliferative activity of the *T. lanceolata* extracts was assessed by standard anti-proliferative assays.[2] Briefly, 70µL of each carcinoma cell suspension (containing approximately 5000 cells), was added to the wells of a 96 well plate and 30µL of the extract dilutions, or cell media (for the negative control) was added. The cells were incubated at 37°C, 5% CO_2 for 12 hr in a humidified atmosphere. Cisplatin (50µg/mL) was purchased from Sigma, Australia and included on each plate as a positive control. Following the incubation, 20µL of Cell Titre 96 Aqueous One solution (Promega) was added to each well and the plates were incubated for a further 3 hr under the same conditions. Absorbances were measured at 490nm using a Molecular Devices, Spectra Max M3 plate reader. All tests and controls were performed three times, each with 3 internal replicates (n=9). Anti-proliferative activity was calculated and expressed as a percentage of the negative control using the formula:

$$\text{Proliferation (\% untreated control)} = (A_{ct}/A_{cc}) \times 100$$

A_{ct} is the corrected absorbance for the test extract (calculated by subtracting the absorbance of the test extract in media without cells from the extract cell test combination) and A_{cc} is the corrected untreated control (calculated by subtracting the absorbance of the untreated control in media without cells from the untreated cell media combination).

Cell imaging studies

Changes to cell morphology were visualised using bright field microscopic imaging without staining. Caco-2 cells were seeded into individual wells of a 96 well plate (5 x 10^3 cells/100 µL) and allowed to adhere overnight. All *T. lanceolata* extracts with IC_{50} values ≤250µg/mL against Caco-2 were tested at half the concentration of the IC_{50}. The Caco-2 cells were incubated for 24 hr at 37°C in a humidified 5% CO_2 atmosphere and the cell morphology was qualitatively evaluated using an inverted phase microscope coupled with an Olympus IX70 camera at an optical zoom of 20X. Three independent experiments were performed, each with internal triplicates (n=9). Characteristic cell images are reproduced in this study.

Determination of caspase 3 activity

Cellular caspase 3 activity following exposure to the *T. lanceolata* berry and leaf extracts was evaluated using a caspase 3 assay kit (Sigma Aldrich, Australia) as previously described.[2] *T. lanceolata* extracts were tested against Caco-2 cells at concentrations equivalent to the individual IC_{50} values for each extract. Following an incubation for 3 hr at 37°C, 5% CO_2 in a humidified atmosphere, the cells were lysed on ice for 20 min. The caspase activity of the resultant supernatants was assessed at 405nm using a Molecular Devices, Spectra Max M3 plate reader. Untreated Caco-2 cells were included in the assay as a negative control. All tests were performed three times, each with internal triplicates (n=9).

Toxicity screening

The toxicity of the *T. lanceolata* berry and leaf extracts was assessed by two methods: An *Artemia* Lethality Assay (ALA) provided rapid preliminary toxicity determinations, whilst cellular viability assays were used for a more accurate evaluation of cellular toxicity.

Artemia franciscana nauplii toxicity screening

Toxicity of the *T. lanceolata* extracts was quantified using a modified *Artemia franciscana* nauplii lethality assay.[21] A volume of 400µL of the diluted plant extracts were transferred to the wells with 400µL of seawater containing approximately 50 *Artemia* nauplii and incubated at 25±1°C under artificial light (1000 Lux). Potassium dichromate (AR grade, Chem-Supply, Australia) was prepared at 1mg/mL and included as a reference toxin. A negative control (artificial seawater) was also included on each plate. The percentage of dead nauplii in each well was determined following 24 hr exposure and LC_{50}'s with 95% confidence limits were calculated using Probit analysis. All treatments and controls were performed three times, each with internal triplicates (n=9).

Cellular viability assay

As a measure of cellular toxicity, the *T. lanceolata* extracts were screened against Human primary Dermal Fibroblasts (HDF) obtained from American Type Culture Collection (ATCC PCS-201-012). The cells were maintained and cultured in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10% foetal calf serum (Invitrogen), 50µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigma Aldrich, Australia). Cell viability was determined using standard methods.[2] Briefly, 70µL of resuspended HDF cells (containing approximately 5000 cells) was dispensed into the wells of a 96 well plate and 30µL of the extracts (200µg/mL) or cell media (for the negative control) were added to individual wells. The plates were incubated at 37°C, 5% CO_2 for 24 hr in a humidified atmosphere. To detect cell viability, 20µL of Cell Titre 96 Aqueous One solution (Promega) was added to all wells of the plate and incubated for a further 3 hr. Absorbances were measured at test and blank wavelengths of 540nm and 690nm respectively, using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed three times, each with internal triplicates (n=9). Triplicate controls were also included on each plate. The % cellular viability of each test was calculated using the following formula:

$$\%\ \text{cellular viability} = \frac{\text{Abs test sample} - (\text{mean Abs control} - \text{mean Abs blank})}{(\text{mean Abs control} - \text{mean Abs blank})}$$

Cellular viability ≤50% of the untreated control indicated toxicity, whereas extracts or controls with >50% untreated control viability were deemed to be nontoxic.

HPLC-MS QTOF fingerprint analysis

Separation, putative identification and determination of the relative abundance of the extract components was achieved using an Agilent 1290 HPLC system equipped with a Zorbax Eclipse C18 column (2.1 × 100mm, 1.8µm particle size) linked to an Agilent 6530 quadrupole time-of-flight mass spectrometer. The chromatographic procedure utilised previously optimised parameters developed for previous studies evaluating the phytochemistry of similar *T. lanceolata* extracts.[22] Mass spectra were obtained in the electron ionisation mode and analytes were recorded in Total Ion Count (TIC) mode for a duration of 45 min and screened against two accurate mass databases: a database of plant compounds of therapeutic importance generated specifically for this study (800 compounds), and the Metlin metabolomics database (24,768 compounds).

GC-MS Head space fingerprint analysis

Chromatographic separation, identification and quantification of the relative abundance of the *T. lanceolata* extract constituents was achieved using a Shimadzu GC-2010 plus (USA) chromatography system, linked to a Shimadzu MS TQ8040 (USA) mass selective detector system using previously optimised parameters.[23] The mass spectrometer was operated in the electron ionisation mode at 70eV and the analytes were recorded in Total Ion Count (TIC) mode. The TIC was acquired for a duration of 45 min utilising a mass range of 45-450m/z and screened against the ChemSpider database.

Statistical analysis

The data of all experiments is expressed as the mean ± SEM of three independent experiments, each with triplicate internal replicates (n=9). Statistical significance between the control and treated groups was calculated using one way ANOVA (p values < 0.01 were considered to be statistically significant).

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Individual extractions of 1g of dried *T. lanceolata* berry and leaf powders with solvents of varying polarity yielded dried extracts ranging from 9mg (*T. lanceolata* leaf hexane extract) to 295mg (aqueous *T. lanceolata* leaf extract) (Table 1). The higher polarity methanol and water solvents extracted substantially higher yields than the mid to lower polarity solvents. Qualitative phytochemical studies showed that methanol and water also extracted a substantially wider range and greater relative abundances of phytochemicals, compared to the lower polarity extracts (Table 1). Methanol and aqueous berry and leaf extracts contained high levels of polyphenolics and flavonoids, as well as low to moderate levels of saponins and tannins. The ethyl acetate berry and leaf extracts contained similar phytochemical classes, albeit generally at substantially lower levels. The chloroform and hexane extracts were much less complex.

Anti-proliferative activity

Caco-2 and HeLa cell lines

The *T. lanceolata* berry and leaf extracts were screened for anti-proliferative activity against Caco-2 and HeLa cell lines (Figure 1). The methanolic, aqueous and ethyl acetate berry extracts were particularly potent inhibitors of HeLa proliferation (Figure 1a). Indeed, the *T. lanceolata* methanolic berry extract inhibited 81% HeLa cellular proliferation, whilst the aqueous fruit extract inhibited 94% proliferation. The *T. lanceolata* ethyl acetate berry extract was also a good inhibitor of HeLa proliferation, with 69% inhibition recorded. Interestingly, the lower polarity *T. lanceolata* chloroform and hexane fruit extracts did not inhibit HeLa proliferation. Instead, these extracts induced proliferation, by approximately 10% and 15% respectively compared to the negative control. All of the berry extracts also significantly inhibited Caco-2 proliferation (with the exception of the hexane

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the *T. lanceolata* extracts.

	Berry extracts					Leaf extracts				
	M	W	E	C	H	M	W	E	C	H
Yield (mg)	171	144	57	47	11	144	295	17	37	9
Extract concentration (mg/mL)	111	11	6	5	1	14	30	2	4	1
Total phenolics	+++	+++	+	-	-	+++	+++	+	+	-
Water soluble phenolics	+++	+++	+	-	-	+++	+++	+	-	-
Water insoluble phenolics	+	+	++	-	-	+	+	++	-	-
flavonoids	+++	+++	++	-	-	+++	+++	++	+	+
Phytosterols	-	-	-	-	-	-	-	-	+	-
Saponins	++	+	+	-	-	+++	++	-	-	-
Triterpenoids	+	-	++	-	-	+	-	+	+	+
Cardiac glycosides	-	-	-	-	-	-	-	-	-	-
Tannins	+	+	-	-	-	++	++	+	-	-
Alkaloids (Mayer)	-	-	-	-	-	-	-	-	-	-
Alkaloids (Wagner)	-	-	-	-	-	-	-	-	-	-
Anthraquinones (Kumar)	-	-	-	-	-	-	-	-	-	-
Athraquinones (Aiayeoba)	-	-	-	-	-	-	-	-	-	-

+++ = large response; ++ moderate response; + = low response; - = absence of phytochemical class in the extract; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.

berry extract). The methanolic, aqueous and ethyl acetate berry extracts were particularly strong inhibitors of Caco-2 proliferation, inhibiting proliferation by approximately 98%, 94% and 66% respectively (Figure 1a). The *T. lanceolata* chloroform berry extracts inhibited Caco-2 by approximately 31%, whilst the low polarity hexane berry extract was devoid of inhibitory activity. Instead, the *T. lanceolata* hexane extract induced Caco-2 proliferation by approximately 5% above the level of the untreated control. Therefore, the *T. lanceolata* methanolic, aqueous and ethyl acetate berry extracts were deemed to be effective inhibitors of both HeLa and Caco-2 cellular proliferation.

A similar trend was noted for the *T. lanceolata* leaf extracts. The methanolic, aqueous and ethyl acetate *T. lanceolata* leaf extracts were potent HeLa inhibitors, inhibiting HeLa cell proliferation by approximately 93%, 95% and 87% respectively (Figure 1b). The lower polarity *T. lanceolata* chloroform and hexane leaf extracts also inhibited HeLa cell proliferation, albeit to a lesser degree (10 and 18% respectively). The *T. lanceolata* methanolic, aqueous and ethyl acetate leaf extracts also strongly inhibited Caco-2 cell proliferation (by approximately 95, 97 and 53% respectively). The chloroform leaf extract was a less potent inhibitor of Caco-2 cell proliferation, (approximately 10% inhibition compared to the negative control cell proliferation). In contrast, the *T. lanceolata* leaf hexane extract did not inhibit Caco-2 cell proliferation, but and instead induced Caco-2 proliferation (by 13% compared to the untreated control). Thus, the *T. lanceolata* leaf methanolic, aqueous and ethyl acetate extracts were also deemed to be effective inhibitors of Caco-2 proliferation.

Jeg-3 and JAR cell lines

The methanolic, aqueous and ethyl acetate *T. lanceolata* berry extracts also strongly inhibited Jeg-3 proliferation (Figure 2a). Indeed, a decrease in Jeg-3 cell proliferation below the baseline level was noted for the *T. lanceolata* methanolic and aqueous berry extracts (-2.4 and -4.7% proliferation compared to the untreated control respectively), suggesting that the anti-proliferative effects were at least partly due to cytotoxic mechanisms. *T. lanceolata* ethyl acetate and chloroform berry extracts also inhibited Jeg-3 cell proliferation (by 57% and 39% respectively) (Figure 2a), whilst the *T. lanceolata* hexane berry extract also induced minor inhibition of Jeg-3 cells. The *T. lanceolata* methanolic and aqueous berry extracts were also particularly potent inhibitors of JAR cell proliferation. Notably, the aqueous berry extract induced decreases in JAR proliferation to negative values, providing further support that cytotoxic mechanisms were induced. Lower, albeit significant inhibition was also recorded for the chloroform extract against Jeg-3 proliferation, whilst the hexane berry extract was ineffective against Jeg-3 cells. Similarly, JAR cell proliferation was substantially inhibited by the higher polarity *T. lanceolata* berry extracts, and to a lesser extent, by the mid polarity and lower polarity extracts. The methanolic berry extract inhibited 96% JAR cell proliferation. Furthermore, negative JAR proliferation

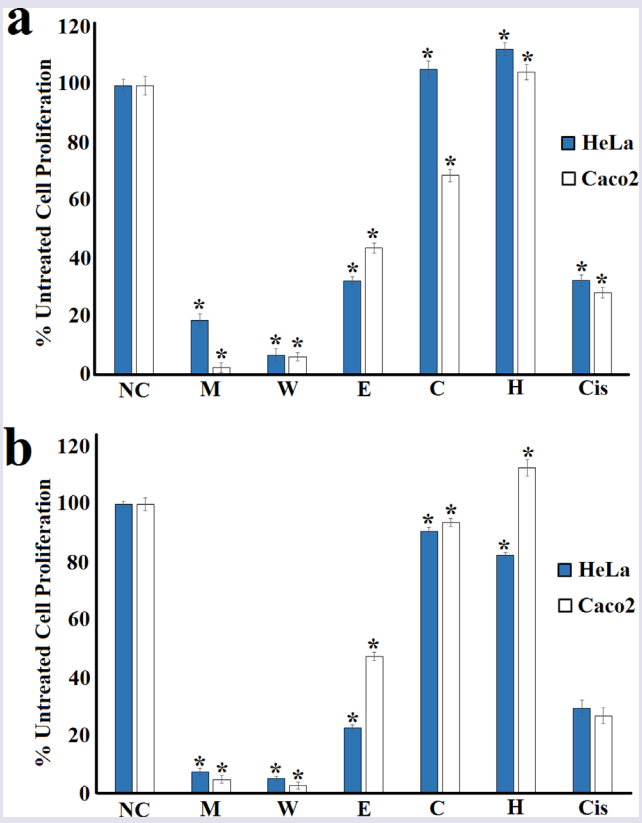

Figure 1: Anti-proliferative activity of the *T. lanceolata* (a) berry and (b) leaf extracts against Caco-2 and HeLa cancer cell lines measured as percentages of the untreated control cells. NC = untreated control; M = methanolic *T. lanceolata* extract; W = aqueous *T. lanceolata* extract; E = ethyl acetate *T. lanceolata* extract; C = chloroform *T. lanceolata* extract; H = hexane *T. lanceolata* extract; Cis = cisplatin control (50 µg/mL). Results are expressed as mean percentages ± SEM of three independent experiments, each with internal triplicates (n=9). * Indicates results that are significantly different to the untreated control (p<0.01).

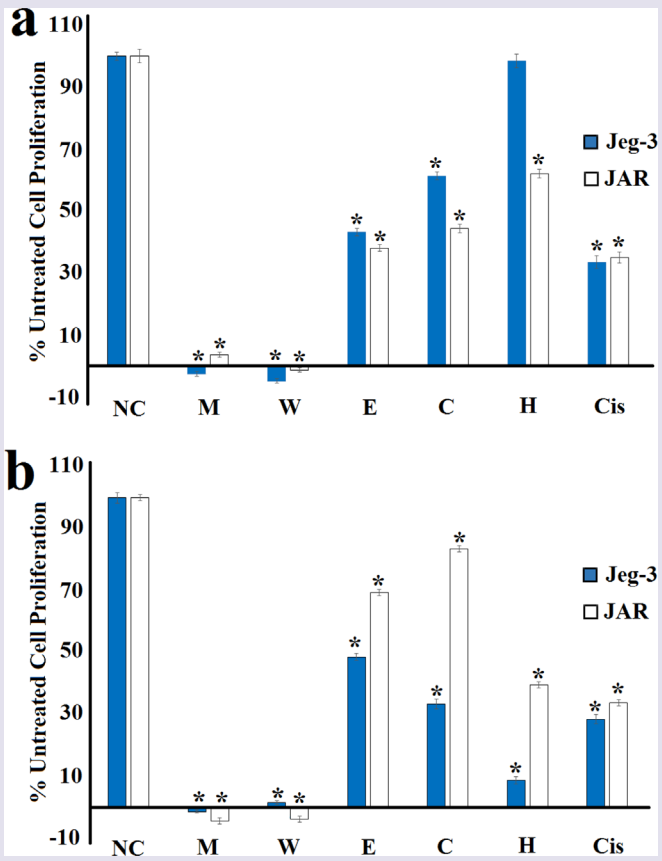

Figure 2: Anti-proliferative activity of the *T. lanceolata* (a) berry and (b) leaf extracts against Jeg-3 and JAR cancer cell lines measured as percentages of the untreated control cells. NC = untreated control; M = methanolic *T. lanceolata* extract; W = aqueous *T. lanceolata* extract; E = ethyl acetate *T. lanceolata* extract; C = chloroform *T. lanceolata* extract; H = hexane *T. lanceolata* extract; Cis = cisplatin control (50 µg/mL). Results are expressed as mean percentages ± SEM of three independent experiments, each with internal triplicates (n=9). * Indicates results that are significantly different to the untreated control (p<0.01).

was noted for the aqueous *T. lanceolata* berry extract, suggesting possible cytotoxic activity (Figure 2a). The mid polarity ethyl acetate, as well as the lower polarity chloroform and hexane berry extracts, also inhibited JAR proliferation by 61%, 55% and 39% respectively compared to the untreated control. In summary, the *T. lanceolata* methanolic, aqueous and ethyl acetate berry extracts were the most potent inhibitors of Jeg-3 and JAR proliferation.

Similar but stronger anti-proliferative effects were noted for the *T. lanceolata* leaf extracts against Jeg-3 and JAR cells (Figure 2b). The methanolic, aqueous and hexane *T. lanceolata* leaf extracts were particularly strong inhibitors of Jeg-3 proliferation. Indeed, negative Jeg-3 proliferation was noted for the *T. lanceolata* methanolic leaf extract, suggesting that the anti-proliferative activity may be due to cytotoxic mechanisms. The aqueous and hexane extracts also inhibited Jeg-3 proliferation substantially by >90% each. Whilst less potent inhibitors of Jeg-3 proliferation, the ethyl acetate and chloroform leaf extracts inhibited Jeg-3 proliferation by approximately 51 and 67% compared to the negative control. The anti-proliferative activity against JAR cells followed similar trends to those of the Jeg-3 cells. The methanolic and aqueous *T. lanceolata* leaf extracts strongly inhibited JAR proliferation to negative values (Figure 2b). This may indicate that inhibition of proliferation may be (at least in part) cytotoxic. The *T. lanceolata* ethyl acetate and the lower polarity chloroform and hexane leaf extracts also inhibited JAR proliferation by 31, 16 and 60% respectively compared to the negative control. Therefore, all *T. lanceolata* leaf extracts were classed as strong inhibitors of Jeg-3 and JAR proliferation.

MC3T3-E1 and MG63 cell lines

The methanolic, aqueous and ethyl acetate *T. lanceolata* berry extracts were also potent inhibitors of MC3T3-E1 cell proliferation

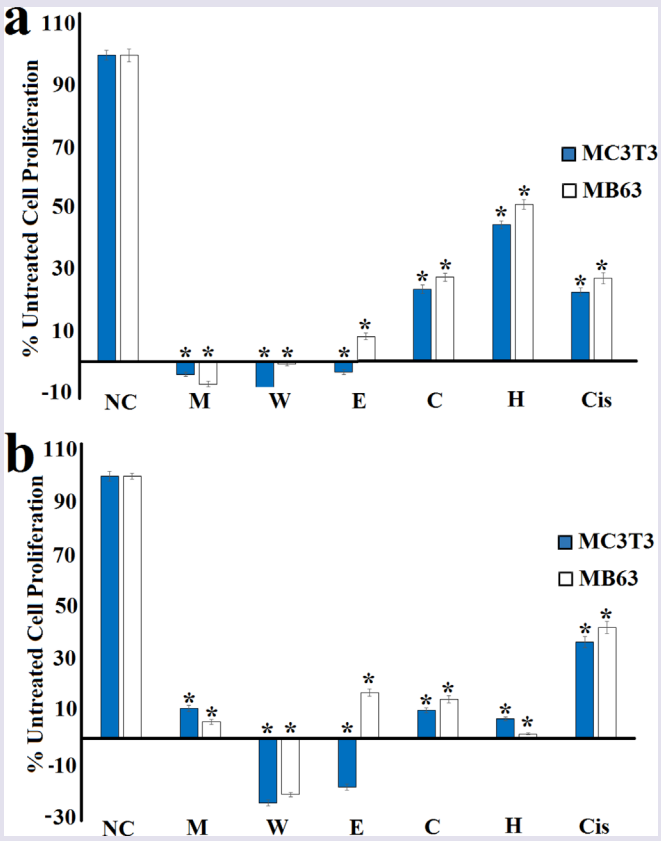

Figure 3: Anti-proliferative activity of the *T. lanceolata* (a) berry and (b) leaf extracts against MC3T3-E1 and MG63 cancer cell lines measured as percentages of the untreated control cells. NC = untreated control; M = methanolic *T. lanceolata* extract; W = aqueous *T. lanceolata* extract; E = ethyl acetate *T. lanceolata* extract; C = chloroform *T. lanceolata* extract; H = hexane *T. lanceolata* extract; Cis = cisplatin control (50 µg/mL). Results are expressed as mean percentages ± SEM of three independent experiments, each with internal triplicates (n=9). * Indicates results that are significantly different to the untreated control (p<0.01).

(Figure 3a). Indeed, negative MC3T3-E1 proliferation was noted for each of these extracts, suggesting that their anti-proliferative activity may have been cytotoxic in nature. Interestingly, the lower polarity chloroform and hexane berry extracts were also good inhibitors of MC3T3-E1 proliferation (by approximately 76 and 65% respectively). Similar trends were also observed for MG-63 cells (Figure 3a). The methanolic, aqueous and ethyl acetate *T. lanceolata* berry extracts were also strong inhibitors of MG-63 proliferation. Negative MG-63 proliferation was noted for the *T. lanceolata* methanolic and aqueous berry extracts, suggesting that the anti-proliferative activity of these extracts may be cytotoxicity. The *T. lanceolata* ethyl acetate extract was also a strong inhibitor of MG-63 cellular proliferation, with an approximate 92% decrease noted. The chloroform and hexane berry extracts also significantly inhibited MG-63 cellular proliferation (approximately 72 and 49% decreases compared to the untreated control respectively). In summary, the *T. lanceolata* methanolic, aqueous and ethyl acetate berry extracts were potent inhibitors of both MC3T3-E1 and MG-63 cellular proliferation, whilst the chloroform and hexane extracts had lower (although significant) activity.

Interestingly, (with the exception of the methanolic extract) the *T. lanceolata* leaf extracts were more potent inhibitors of cellular proliferation than the berry against the MC3T3-E1 and MG-63 cell lines. Indeed, negative MC3T3-E1 proliferation was recorded for the aqueous and ethyl acetate *T. lanceolata* leaf extracts (124.6 and -18.5% respectively) (Figure 3b), suggesting cytotoxic mechanisms. The methanolic, chloroform and hexane leaf extracts were also potent inhibitors of MC3T3-E1 proliferation, albeit with lower potency (89, 89 and 92% decreases in cell proliferation respectively). Similar trends were noted for the *T. lanceolata* leaf extracts against MG-63 proliferation (Figure 3b). The aqueous extract was again the strongest inhibitor of MG-63 proliferation, with a decrease in proliferation substantially below the baseline level (approximately -21% proliferation). The methanolic, ethyl acetate, chloroform and hexane leaf extracts were also strong inhibitors of MG-63 growth, inhibiting proliferation by approximately 94, 83, 85 and 98% respectively, compared to the untreated control proliferation. Therefore, all *T. lanceolata* leaf extracts were deemed to be good inhibitors of both MC3T3-E1 and MG-63 proliferation, although the activity of the aqueous, ethyl acetate and hexane leaf extracts was particularly noteworthy.

IC_{50} Determination

The anti-proliferative potency of the *T. lanceolata* extracts was further quantified by determination of their IC_{50} values (Table 2). The *T. lanceolata* leaf extracts were notably more potent inhibitors of cellular proliferation than the corresponding berry extracts against all carcinoma cell lines. With some notable exceptions, the *T. lanceolata* aqueous leaf extract was the most potent inhibitor of proliferation, with IC_{50} values of 230, 150 488, 450, 195 and 315µg/mL against HeLa, Caco-2, Jeg-3, JAR, MC3T3-E1 and MG-63 cells respectively. The ethyl acetate leaf extract was also a potent inhibitor of most cell lines, with IC_{50}'s of 800, 225, 200 and 525µg/mL against HeLa, Caco-2, MC3T3-E1 and MG-63 cells respectively. Several exceptions were evident. Whilst the methanolic leaf extract had moderate anti-proliferative activity against HeLa, Caco-2 and Jeg-3 cells, it was substantially more potent against JAR, MC3T3-E1 and MG-63 cells. Indeed, the greatest potency against any cell line was recorded for the methanolic leaf extract against MG-63 cells (53µg/mL). The ethyl acetate berry extract was also a strong inhibitor of proliferation against HeLa, Caco-2 and MG-63 cells, with IC_{50} values of 755, 146 and 275µg/mL respectively. All other extracts displayed only moderate to low anti-proliferative activity against the tested cell lines.

Table 2: The IC_{50} of the *T. lanceolata* berry and leaf extracts against the cancer cell lines and detection and quantification of toxicity LC_{50} values (µg/mL) of the *T. lanceolata* berry and leaf extracts.

	M	Berry extracts					Leaf extracts				
		W	E	C	H	M	W	E	C	H	
Carcinoma cell IC_{50}'s (ug/mL)	HeLa	1910	3573	755	NA	NA	810	230	800	NA	NA
	Caco-2	3070	2665	146	NA	NA	1030	150	225	NA	NA
	Jeg-3	2952	2882	4680	NA	NA	900	488	1800	3203	3265
	JAR	4293	1950	4290	NA	NA	450	450	NA	NA	1980
	MC3T3	1400	536	1365	3810	NA	245	195	200	1188	858
	MG63	200	1192	275	3810	NA	53	315	525	843	990
Toxicity	*Artemia* nauplii LC_{50}'s (ug/mL)	3285	1970	NA	NA	NA	NA	NA	NA	NA	NA
	HDF viability assay	-	-	-	-	-	-	-	-	-	-

Values indicate the mean IC_{50} for triplicate determinations. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NA = an IC_{50} or LC_{50} value was not achieved as inhibition of proliferation did not exceed 50% at any concentration tested; - indicates that the extract was nontoxic in the assay as cellular viability was >50% of the untreated control cell viability.

Cell imaging studies

The anti-proliferative studies demonstrated that several extracts (particularly the methanolic, aqueous and ethyl acetate leaf and berry extracts) have profound effects on the proliferation of multiple carcinoma cell lines. Indeed, not only did these extracts block cell proliferation, but a decrease in cellular proliferation below the baseline level was recorded against several cell lines. Such large proliferation decreases indicates that these extracts may not only have cytostatic effects via cell cycle inhibition, but may instead (or in addition) inhibit proliferation by cytotoxic/apoptotic mechanisms. Therefore, cell imaging studies were used to search for apoptotic cell morphologies. The Caco-2 cell line was chosen for these studies as it has been used for similar microscopic studies,[2] allowing for comparisons. It also has well defined morphological features and has a relatively rapid doubling time. Furthermore, Caco-2 cells were sensitive to the *T. lanceolata* extracts, making it ideal for this study.

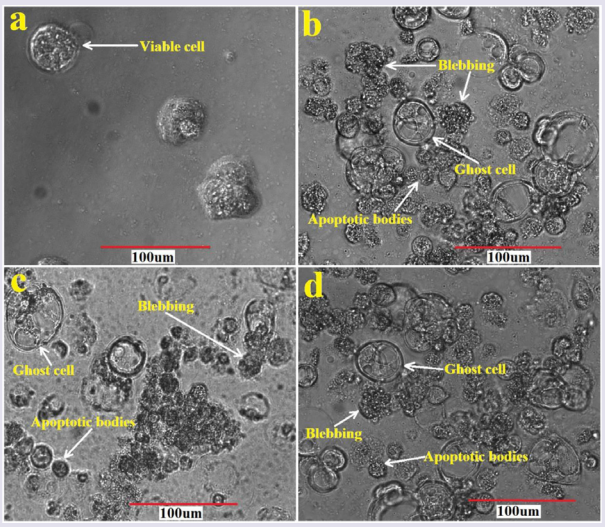

Figure 4: Morphological effects of viable Caco-2 cells (a) without treatment, and cells exposed to (b) *T. lanceolata* berry ethyl acetate extract, (c) *T. lanceolata* aqueous leaf ethyl acetate extract, (d) *T. lanceolata* leaf ethyl acetate extract.

Changes to the morphology of the nucleus, cytoplasm, vacuoles and cell membrane are characteristic for stressed cells undergoing apoptosis. All of these organelles are clearly visible at 20X optical magnification in the untreated control cell images (Figure 4a). The outline of these cells was clear, distinct and compact, with no apparent abnormalities in size or density. Furthermore, stress granules were not evident within the cells. In contrast, typical apoptotic phenomena were readily apparent in Caco-2 cells tested against the berry ethyl acetate extract (Figure 4b), as well as the leaf aqueous (Figure 4c) and ethyl acetate (Figure 4d) extracts at sub-lethal concentrations. Notably, these images support the trends observed in the cell proliferative assays, which showed that berry ethyl acetate extract, as well as the aqueous and ethyl acetate leaf extracts, were potent inhibitors of Caco-2 proliferation. Cells undergoing apoptosis generally have characteristic morphologies, including the presence of an intact membrane, marginalisation of the cytoplasm and chromatin condensation.[24] These morphologies were clearly visible in the Caco-2 cells treated with the *T. lanceolata* berry ethyl acetate (Figure 4b), and the leaf aqueous (Figure 4c) and leaf ethyl

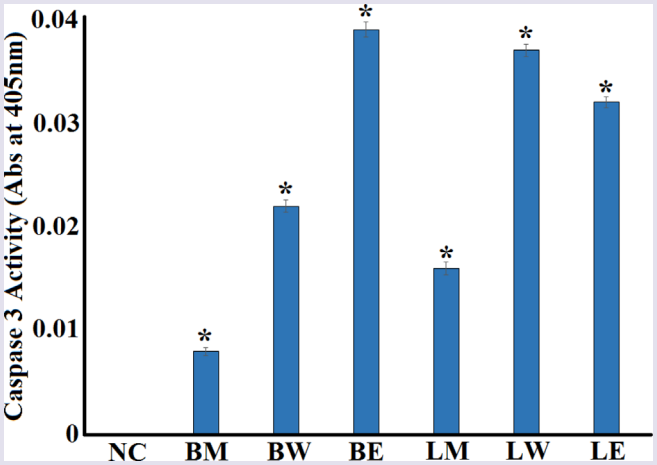

Figure 5: Caspase 3 activity of Caco-2 cells treated with half IC_{50} concentrations of *T. lanceolata* berry and leaf methanolic aqueous and ethyl acetate, extracts. NC = untreated control; BM = berry methanolic extract; BW = aqueous berry extract; BE = berry ethyl acetate extract; LM = leaf methanolic extract; LW = aqueous leaf extract; LE = leaf ethyl acetate extract. Results are expressed as mean absorbance at 405nm ± SEM of three independent experiments, each with internal triplicates (*n*=9). * Indicates results that are significantly different to the untreated control (p<0.01).

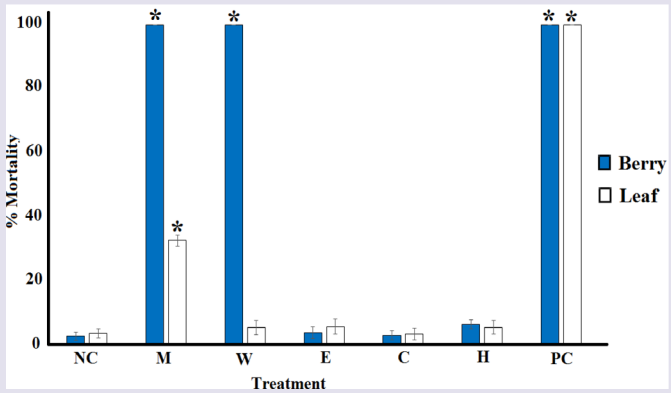

Figure 6: The lethality of the *T. lanceolata* extracts (2000µg/mL) and the potassium dichromate control (1000µg/mL) towards *Artemia* nauplii following 24 hr exposure. NC = negative control; M = methanolic *T. lanceolata* extract; W = aqueous *T. lanceolata* extract; E = ethyl acetate *T. lanceolata* extract; C = chloroform *T. lanceolata* extract; H = hexane *T. lanceolata* extract; PC = positive control (1000µg/mL potassium dichromate). Shaded bars represent fruit extracts and open bars represent leaf extracts. Results are expressed as mean ± SEM of three independent experiments, each with internal triplicates (*n*=9). * Indicates results that are significantly different to the negative control (p<0.01).

acetate extracts (Figure 4c). During apoptosis, marginalisation of the cytoplasm produces a large central vacuole, resulting in "ghost cells". The central vacuoles are clearly visible in the populations of cells exposed to the *T. lanceolata* extracts, but were lacking in the untreated control cells. Stress granules were also apparent in the treated cells, further indicating that the cells are undergoing stress. Another characteristic feature of cells undergoing apoptosis is the presence of blebbing on the cell membrane surface. Notably, blebbing was apparent in all of the cells treated with the *T. lanceolata* extracts, but was absent in the untreated control cells. A loss of membrane integrity was also apparent in the cells exposed to the *T. lanceolata* extracts. These cells generally lacked a clear cell outline, had extensive chromatin condensation, and numerous vacuoles were apparent in the cytoplasm. Furthermore, cell ghosts were also apparent within all of the *T. lanceolata* extract treated cell populations. All of these morphological changes are indicative of apoptosis induction.

Determination of caspase 3 activity

The *T. lanceolata* extracts were tested for the ability to activate caspase 3 activity as a further confirmation that the anti-proliferative activity reported here is mediated via apoptosis. Activation of caspase 3 was selected as a measure of apoptosis as it is activated via both extrinsic and intrinsic apoptotic pathways. Therefore, caspase 3 activity would be elevated relative to the untreated control, regardless of which apoptotic pathway is activated. The methanolic aqueous and ethyl acetate *T. lanceolata* berry and leaf extracts substantially elevated caspase 3 activity following a 3 hr exposure (Figure 5). The leaf extracts generally induced substantially higher caspase 3 activity than the corresponding berry extracts. The ethyl acetate extracts of both plant parts, as well as the aqueous leaf extract, induced substantially greater levels of caspase 3 activity than the other extracts.

Quantification of toxicity

As an initial measure of toxicity, all *T. lanceolata* extracts were screened at 2000µg/mL in the *Artemia* nauplii assay (Figure 6). Potassium dichromate (1000 µg/mL) was also included in the bioassay as a positive control. The methanolic and aqueous berry extracts displayed >50% mortality at 24 hr and were thus deemed to be toxic. All other berry extracts, and all of the *T. lanceolata* leaf extracts, induced substantially <50% mortality in the ALA assay and were therefore considered to be non-toxic. To further quantify the toxicity, the methanolic and aqueous berry *T. lanceolata* extracts were tested across a range of concentrations in the ALA bioassay. LC_{50} values of 3285 and 1970µg/mL were determined for the *T. lanceolata* methanolic and aqueous berry extracts respectively (Table 2). As plant extracts with LC_{50} values >1000µg/mL have previously been defined as nontoxic in this assay,[21] all of the *T. lanceolata* extracts were determined to be non-toxic. The cellular based HDF assay confirmed these results. All *T. lanceolata* extracts were non-toxic towards normal HDFs, with cellular viability for all tests substantially >50% of the untreated control.

Phytochemical fingerprint analysis

As the ethyl acetate berry, and the aqueous and ethyl acetate leaf extracts displayed substantially more potent anti-proliferative activity than the other *T. lanceolata* extracts, they were selected for LC-MS and GC-MS headspace phytochemical fingerprint analysis. In recent studies from our group, we used similar techniques to identify and highlight several notable phytochemical constituents in similar *T. lanceolata* extracts using LC-MS and GC-MS headspace metabolomics analysis to highlight tannin and flavonoid components,[22] as well as the monoterpenoid, sesquiterpenoid and diterpenoid components[23] in those extracts. Optimised HPLC-MS and GC-MS parameters developed for those projects were utilised in the current study to screen the ethyl acetate berry extract, and the aqueous and ethyl acetate leaf extracts for the presence of these compounds, and to quantify their relative abundances in these extracts (Table 3). LC-MS analysis (Table 3 and Figure S1) showed that the extracts contained relatively few tannins, although those identified were detected in high relative abundances. The levels of 2-ethylhexyl-gallic acid ester were particularly noteworthy. This compound was present in relatively high abundance in the aqueous leaf extract (1.36%), although the presence of approximately 0.7% in both ethyl acetate extracts is also noteworthy. The aqueous leaf extract also contained lower (but still substantial) levels of pyrocatechol (0.43% relative abundance). The flavonoids rutin and myricetin were also present in all of the tested extracts, with the highest abundance in the leaf ethyl acetate extract. Of further interest, several stilbenes were also detected in the *T. lanceolata* extracts. Of particular interest, several combretastatins (including combretastatin a1) have been reported to have strong anti-proliferative activity.[25] Similarly, the glycosylated stilbene piceid also inhibits the proliferation of some cancer cell lines.[26] Thus, their detection in our study is particularly noteworthy.

Of further interest, a diversity and relative abundance of monoterpenoids (eucalyptol, linalool, camphor, terpineol, myrtenol, cymen-7-ol and chrysanthenol) were detected in all of the tested extracts, although the levels were generally substantially higher in the ethyl acetate extracts than in the aqueous leaf extract (Table 3 and Figure S2). The levels of eucalyptol, linalool and terpineol were especially noteworthy in the leaf ethyl acetate extract (7.3, 3.2 and 3.7% relative abundances respectively). The *T. lanceolata* extracts also contained a diversity of sesquiterpenoids. Polygodial was present in particular high relative abundance in the aqueous leaf extract (7.3%), whilst caryophyllene oxide was the main sesquiterpenoid identified in the berry ethyl acetate extract. A very different sesquiterpenoid composition was present in the leaf ethyl acetate extract, with decahydro-1H-cycloprop[e]azulen-7-ol and 5-azulenemethanol, 1, 2, 3, 3a, 4, 5, 6, 7-octane (3.2 and 1.4% relative abundances respectively) predominating. Azulene sesquiterpenoids were also present in relative abundance in the berry ethyl acetate extract (2.1 and 1.6% for decahydro-1H-cycloprop[e]azulen-7-ol and decahydro-1,5,5,8a-tetramethyl-1,2,4-methenoazulene respectively). In contrast, these compounds were either completely absent, or were in substantially lower abundances in the aqueous leaf extract.

DISCUSSION

Plants are an important source of new compounds for drug discovery and they have been a particularly good source of new anticancer drugs. Many of the anticancer drugs in clinical usage were either originally isolated from plants, or are semi-synthetic analogues of plant derived compounds. Indeed, 74% of all anticancer agents introduced for clinical use between 1981 and 2002 are plant compounds or semi-synthetic analogues based on plant compounds.[27] Drugs such as paclitaxel (derived from taxol isolated from *Taxus brevifolia* Nutt.) and vincristine and vinblastine (isolated from *Catharanthus roseus* (L.) G.Don.) are perhaps the best know examples of plant-dervied anticancer drugs. Unfortunately, many of the current retinue of cancer chemotherapeutics are inadequate for the treatment of a myriad of aggressive cancers. For example, pancreatic cancer often requires a concurrent high dosage of chemotherapeutics and radiotherapy to arrest angiogenesis and metastasis. This is ultimately detrimental to the patient as these treatments are highly toxic and patients may suffer from acute chemical poisoning from the treatment. Furthermore, continuous exposure to cancer chemotherapeutics may induce carcinoma cells to adapt and develop resistances to bypass the drug's effects.[28] Therefore, there is a need to develop new cancer drugs to provide a continued supply of effective therapies.

T. lanceolata was selected to screen for anti-proliferative activity against human carcinomas due to its high antioxidant content.[15] Furthermore, previous studies have highlighted the interesting phytochemistry profile of this plant, which includes multiple compounds with potential anticancer properties.[16] *T. lanceolata* extracts were initially screened against HeLa human cervical cancer and Caco-2 colorectal cell lines to establish their anti-proliferative properties. The aqueous and ethyl acetate *T. lanceolata* extracts prepared from both the berry and leaf were good inhibitors of HeLa and Caco-2 proliferation. The aqueous leaf extract was particularly potent, with IC_{50} values of 230 and 150µg/mL against HeLa and Caco-2 respectively. The berry and leaf ethyl acetate extracts were also good inhibitors of Caco-2 proliferation, with IC_{50} values of 146 and 150µg/mL respectively. The ethyl acetate extracts were also good inhibitors of HeLa proliferation, albeit with substantially higher IC_{50} values (755 and 800µg/mL respectively). Whilst these IC_{50} values are several folds higher than the aqueous leaf extract, they still indicate good anti-proliferative activity.

Table 3: Qualitative HPLC-MS QTOF and GC-MS headspace analysis of the *T. lanceolata* berry ethyl acetate, and leaf aqueous and ethyl acetate extracts, elucidation of empirical formulas and putative identification (where possible) of the compounds.

LC-MS Analysis						
Retention Time (min)	Molecular Weight	Empirical formula	% Area			Putative identification
			BE	LW	LE	
3.81	110.037	$C_6H_6O_2$	-	0.43	-	Pyrocatechol
5.56	610.096	$C_{27}H_{30}O_{16}$	0.16	0.11	0.33	Rutin
5.9	318.038	$C_{15}H_{10}O_8$	0.16	0.11	0.33	Myricetin
6.94	332.125	$C_{18}H_{20}O_6$	0.16	0.11	0.33	Combretastatin a1
7.02	404.147	$C_{21}H_{24}O_8$	0.22	0.85	0.73	Piceid
7.27	334.14	$C_{18}H_{22}O_6$	0.82	0.79	0.21	Combretastatin
7.46	282.144	$C_{15}H_{22}O_5$	0.73	1.36	0.68	Gallic acid, 2-ethylhexyl ester
7.97	248.142	$C_{15}H_{20}O_3$	0.65	0.34	0.79	Salutarisolide
8.52	252.172	$C_{15}H_{24}O_3$	0.58	0.16	0.74	Punctaporin
GC-MS Headspace Analysis						
Retention Time (min)	Molecular Weight	Empirical formula	% Area			Putative identification
			BE	LW	LE	
14.14	154.249	$C_{10}H_{18}O$	-	1.43	7.25	Eucalyptol
16.308	154.249	$C_{10}H_{18}O$	0.25	0.21	3.16	Linalool
17.631	152.237	$C_{10}H_{16}O$	-	0.23	0.3	Camphor
18.659	154.249	$C_{10}H_{18}O$	-	0.36	3.66	α-Terpineol
19.292	152.237	$C_{10}H_{16}O$	-	-	0.3	Myrtenol
22.164	150.218	$C_{10}H_{14}O$	-	0.62	0.1	*p*-Cymen-7-ol
30.351	220.35	$C_{15}H_{24}O$	2.82	-	0.67	Caryophyllene oxide
31.236	222.3663	$C_{15}H_{26}O$	2.12	0.05	3.15	Decahydro-1H-cycloprop[e]azulen-7-ol
32.597	204.357	$C_{15}H_{24}$	0.29	0.45	1.43	5-Azulenemethanol, 1,2,3,3a,4,5,6,7-octa
32.734	220.35	$C_{15}H_{24}O$	1.13	0.45	0.44	Spathulenol
34.439	204.357	$C_{15}H_{24}$	1.59	-	0.19	Decahydro-1,5,5,8a-tetramethyl-1,2,4-methenoazulene
34.599	222.372	$C_{15}H_{26}O$	0.75	-	0.19	Ledol
35.27	206.37	$C_{15}H_{26}$	-	-	0.16	Longipinane
35.287	234.334	$C_{15}H_{22}O_2$	0.32	7.33	0.16	Polygodial
36.636	152.23	$C_{10}H_{16}O$	0.89	-	0.1	Chrysanthenol

The relative abundance expressed in this table is a measure of the area under the peak expressed as a % of the total area under all chromatographic peaks.

The differential anti-proliferative activity of the *T. lanceolata* extracts against Jeg-3 and JAR cells was examined to determine whether high cellular antioxidant levels affect the anti-proliferative activity of the *T. lanceolata* extracts. Jeg-3 cells produce substantially higher thioredoxin levels than JAR cells, although the cells are similar in most other ways.[17] Therefore, differential effects of the *T. lanceolata* extracts between Jeg-3 and JAR cells may indicate the involvement of redox-related pathways. Interestingly, similar anti-proliferative was noted against the Jeg-3 than JAR cells (488 and 450 µg/mL for the aqueous leaf extract against Jeg-3 and JAR respectively). Similarly, the methanolic leaf extract had similar anti-proliferative potencies against Jeg-3 and JAR cells (900 and 450µg/mL respectively). This was a surprising finding as it was anticipated that the higher thioredoxin levels in the Jeg-3 cells may have protected those cells against the induction of apoptosis. However, this was not seen in our study and instead, Jeg-3 proliferation was inhibited to a similar extent to JAR cells. Notably, JAR cells produced higher levels of TNF compared to

Jeg-3 cells[18] and the elevated TNF levels may have stimulated JAR proliferation in those cells. Similar effects have been reported for other high antioxidant plant extracts.[2] Indeed, extracts produced from *T. ferdinandiana* fruit extract in that study had significantly greater anti-proliferative effects against Jeg-3 cells compared with JAR cells. That study determined that the high antioxidant contents of the extracts negated the effects of the increased thioredoxin levels in the Jeg-3 cells. It is possible that the elevated levels of TNF in the JAR cells may have promoted cellular proliferation and may indicate that the induction of apoptosis in these cells may be independent of the extrinsic pathways. Instead, the induction of apoptosis in JAR cells may be reliant on intrinsic pathways, although this is yet to be verified.

The *T. lanceolata* berry and leaf extracts were also good inhibitors of proliferation of the MC3T3-E1/MG-63 cell pair. However, the anti-proliferative mechanisms may be substantially different to those induced in the Jeg-3/JAR cell pair. Notably, ascorbic acid can stimulate the proliferation of MC3T3-E1 cells.[19] Interestingly, previous studies have reported that *T. lanceolata* extracts have high antioxidant capacities,[15] and that ascorbic acid contributes to these high antioxidant levels.[12] It is likely that the high antioxidant capacities of the *T. lanceolata* extracts would stimulate MC3T3-E1 proliferation. However, a trend towards increased proliferation was not apparent in our study. Indeed, the *T. lanceolata* berry and leaf extracts inhibited MC3T3-E1 proliferation to a similar extent to the MG-63 cells. Interestingly, the aqueous and ethyl acetate extracts induced negative proliferation in MC3T3-E1 and MG-63 cells, indicating that the decreased proliferation may be due (at least in part) to the induction of cytotoxic mechanisms. It is noteworthy that MC3T3-E1 cells are highly sensitive to TNF.[29] Therefore, *T. lanceolata* extract components may have induced apoptosis by upregulating TNF production. However, the levels of TNF were not examined in our study and further work is required to verify the role of TNF in the anti-proliferative effects of the extracts on the MC3T3-E1 cells. In contrast, MG-63 cells do not have the same TNF susceptibility[30] and apoptosis in these cells may follow intrinsic pathways, or alternatively, extrinsic Fas-mediated pathways. Hence, inhibition of proliferation may be mediated by different pathways and by different compounds in the MC3T3-E1 and MG-63 cells.

The Caco-2 cellular imaging studies reported here demonstrate that exposure to *T. lanceolata* berry and leaf ethyl acetate extracts, or the aqueous leaf extract, induces morphological changes consistent with apoptosis. Furthermore, exposure of Caco-2 cells to these extracts also significantly elevates cellular caspase-3 in the culture media. Caspase-3 secretion may be elevated via extrinsic and intrinsic apoptotic pathways.[2] Our study has not differentiated which of these pathways (or perhaps both) was induced by exposure to the extracts. Furthermore, whilst the cell imaging and caspase-3 studies demonstrate that the *T. lanceolata* extracts possess cytotoxic activity, they did not examine whether the extracts also have cytostatic properties. To the best of our knowledge, *T. lanceolata* berry and leaf extracts have not previously been examined for cell cycle inhibitory activity. As the *T. lanceolata* extracts screened in our study are crude mixtures containing complex phytochemical profiles, they may illicit multiple anti-proliferative mechanisms simultaneously. Therefore, whilst it is apparent from our study that the *T. lanceolata* extracts induce apoptosis, it is possible that extract components may also inhibit cell cycle progression and therefore have cytostatic effects. However, this is yet to be tested and studies screening for inhibition of cell cycle progression are warranted.

The presence of high relative abundances of gallic acid esters in the aqueous leaf extract and in both ethyl acetate extracts was particularly noteworthy. Similar tannins can profoundly affect the intracellular redox state[5] and may therefore contribute to anti-proliferative effects reported here. It is likely that the *T. lanceolata* tannins may also have had more direct effects on cellular proliferation. Indeed, gallotannins (similar to the gallic acid, 2-ethylhexyl ester detected in our study) are potent inhibitors of cell proliferation via multiple mechanisms, including binding to several cell surface proteins,[31] and by inhibiting the activity of cellular glucosyltransferase enzymes.[32] Furthermore, multiple studies have reported high tannin contents in other plant species that have been used in traditional medicine to treat cancer.[5,33]

Two flavonoids (rutin and myricetin) were also identified in the leaf and berry ethyl acetate, and the aqueous leaf extracts. Several studies have reported good anti-proliferative activity for several flavonoids. Indeed, rutin (which was detected in relative abundance in our study) has been reported to be a potent inhibitor of several carcinomas via multiple mechanisms. Rutin can induce apoptosis in human colon cancer cells via mitochondrial-dependent pathways.[34] A different study reported that rutin upregulates TNF-α production, thereby promoting apoptosis in A549 human lung carcinoma cells.[35] Rutin has dual effects in MCF-7 estrogen responsive human breast cancer cells: it induces apoptosis via p53-dependent pathways and inhibits cell cycle progression via G2/M arrest.[36] Furthermore, quercetin also has good anti-proliferative activity against human HT29 and HCT-116 colorectal cancer cells, LNCaP and DU145 prostate cancer cells, and CAL-27 and KB oral carcinoma cell lines.[37] As rutin is a glycosylated form of quercetin, it is likely that quercetin would be liberated from rutin *in vivo*. However, that study tested a single quercetin concentration (100μg/mL) and IC_{50} values were not determined, making comparisons between the efficacies of different compounds between studies impossible. Quercetin is also a potent inhibitor of the activity of glutathione S-transferase P1-1 and the GS-X pump in MCF7 cells (IC_{50} values between 0.8 and 8 μM).[38] Therefore, quercetin may also be useful for potentiating apoptosis in multidrug resistant cells. A variety of other flavonoids also induce apoptosis in human MDA-MB-231

breast cancer and LNCaP prostate cancerinoma cells by inhibiting fatty acid synthesis.[39]

Several stilbenes were also detected in the *T. lanceolata* extracts. Interestingly, some stilbenes are useful in the prevention and treatment of cancer. Resveratrol is a potent inhibitor of NF-κB activation via its induction by TNF-α and IL-1β.[40] Thus, resveratrol treatment blocks cytokine production via inhibition of NF-κB activation. As inhibition of NF-κB potentiates apoptosis.[41,42] resveratrol is useful in the prevention and treatment of some cancers. Our LC-MS analysis of the *T. lanceolata* extracts detected the resveratrol glycoside piceid (2-[3-hydroxy-5[(E)-2-(4-hydroxyphenyl) ethenyl] phenoxy]-6-(hydroxymethyl) oxane-3, 4, 5-triol) in the *T. lanceolata* extracts. Glycosylated stilbenes may be hydrolysed *in vivo* to remove glucose. Thus, the presence of piceid in the extract is likely to result in the release of the resveratrol moiety *in vivo*. Other stilbenes (combretastatin, combretastatin A-1) were also detected in the *T. lanceolata* extracts. Combretastatins are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation.[43] Combretastatins function via a similar mechanism to that of colchicine (N-[(7S)-1, 2, 3, 10-tetramethoxy-9-oxo-5, 6, 7, 9-tetrahydrobenzo[a]heptlen-7-yl] acetamide). They bind to the colchicine binding site on the tubulin peptide and inhibit polymerisation.[43] A number of other stilbenes with anticancer activities have also previously been reported in other plant species. For example, 2, 3, 4, 5-tetrahydroxystilbene-2-O-β-D-Glucoside (TSG) suppresses the induction of pro-inflammatory mediators by reducing NF-κB binding to DNA and thus has potential as a cancer therapeutic.[44]

GC-MS headspace analysis also detected a diversity and relative abundance of terpenoids in the *T. lanceolata* extracts. The monoterpenoids linalool (3.2% relative abundance in the leaf ethyl acetate extract), α-terpineol (3.7% relative abundance in the leaf ethyl acetate extract), as well as the sesquiterpenoids polygodial (7.3% in the aqueous leaf extract), caryophyllene oxide (2.8% relative abundance in the berry ethyl acetate extract) and several azulene sesquiterpenoids (at levels up to 3.2%) were detected in the *T. lanceolata* extracts. The high relative abundance of polygodial in the aqueous leaf extract is in agreement with previous studies which frequently cite polygodial as a major component in other *T. lanceolata* extracts. Indeed, polygodial may account for nearly 40% of commercial *T. lanceolata* essential oil components.[16] Polygodial has been reported to inhibit the proliferation of HT-29, MDA-MB231, DHF, MCF-7, PC-3, DU-145 and CoN carcinoma cells, with IC_{50} values as low as 12.5 μM.[45] Polygodial is also a potent inhibitor of Ehrlich ascites tumour cells and mouse lymphocytic leukaemia-derived L1210 cells.[46] Interestingly, extracts with high polygodial contents that were prepared from several other plants have also been reported to have good anticancer properties.[47-49]

Similarly, caryophyllene oxide inhibits proliferation and induces apoptosis in human PC-3 prostate and MCF-7 breast cancer cells.[50] That study determined that caryophyllene oxide's anticancer effects are mediated by 2 mechanisms: an inhibition of PI3K/AKT/mTOR/S6K1 signalling cascade, and by activating cellular ERK, JNK and p38 MAPK. Caryophyllene oxide exposure induces increased levels of mitochondrial ROS, loss of mitochondrial membrane potential, release of cytochrome c, activation of caspase-3 and cleavage of PARP. Furthermore, caryophyllene oxide down-regulates the expression of cyclin D1, bcl-2, bcl-xL, survivin, IAP-1 and IAP-2, whilst stimulating the expression of p53 and p21. Caryophyllene oxide can also potentiate the apoptotic effects of other sesquiterpenoids, including humulene and isocaryophyllene.[51]

The use of plant extracts containing high antioxidant contents to treat and prevent cancer is an attractive option for the prevention and treatment of some cancers. However, oxidative stress is not the only factor that contributes to cancer etiology and development and the regulation of cellular redox state may not be an effective anticancer therapy alone. Various genetic and environmental factors may also induce some cancers and/or contribute to their development. Despite this, an examination of traditional plant-based medicines, especially those with high antioxidant capacities, is a promising area of research that may provide useful new cancer drug leads. Indeed, many of the current anticancer chemotherapeutic drugs in clinical use are either plant derived compounds, or are semi-synthetic analogues of plant compounds. The potent anti-proliferative/apoptotic activities described here for the *T. lanceolata* berry and leaf ethyl acetate extracts, and the aqueous leaf extract indicate that these extracts may prove to be useful anticancer therapies in their own right, and may also provide novel new anticancer compounds.

CONCLUSION

The results of this study demonstrate the potential of the ethyl acetate and aqueous *T. lanceolata* berry and leaf extracts to block proliferation of some cancer cells. Furthermore, the *T. lanceolata* extracts were nontoxic, providing further support for their potential in the prevention and treatment of some cancers. Further elucidation of the anti-proliferative mechanism(s) is required to evaluate the potential of *T. lanceolata* components as cancer chemotherapeutics.

ACKNOWLEDGEMENT

We are grateful to Professor Ann McDonnell for providing the MG63 and MC3T3-E1 cell lines used in this study. We are also thankful to Dr. Jenny Di Trapani for the gift of the Jeg-3 and JAR cells. Financial support for this work was provided by the Environmental Futures Research Institute, Griffith University.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALA: Brine-shrimp lethality assay; **DMSO:** Dimethyl sulfoxide; **DMEM:** Dulbecco/Vogt modified Eagle's minimal essential medium; **GC-MS:** Gas chromatography-mass spectrometry; **HDF:** Human primary dermal fibroblasts; **IC_{50}:** Dose that induces a 50% reduction compared to the untreated control; **LC_{50}:** Dose of sample necessary to have a lethal effect on 50% of test organisms or cells; **LC-MS:** Liquid chromatography-mass spectrometry; **ROS:** Reactive oxygen species; **RPMI:** Roswell Park Memorial Institute media.

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Cite this article: Shalom J, Cock IE. *Tasmannia lanceolata* (Poir.) A.C.Sm. Berry and Leaf Extracts Inhibit Proliferation and Induce Apoptosis in Selected Human Carcinoma Cell Lines. Pharmacognosy Communications. 2023;13(3):126-40.

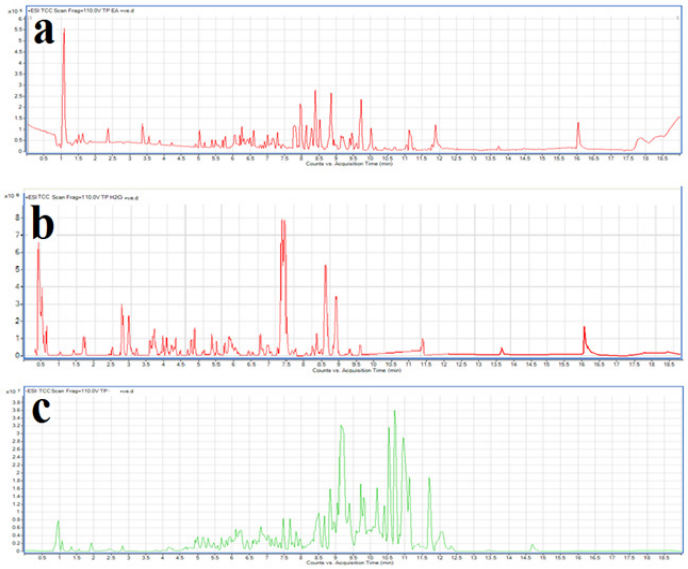

Figure S1: RP-HPLC total compound chromatograms of 2 µL injections of T. lanceolata (a) berry ethyl acetate, (b) aqueous leaf and (c) ethyl acetate leaf extracts run in positive ionisation mode.

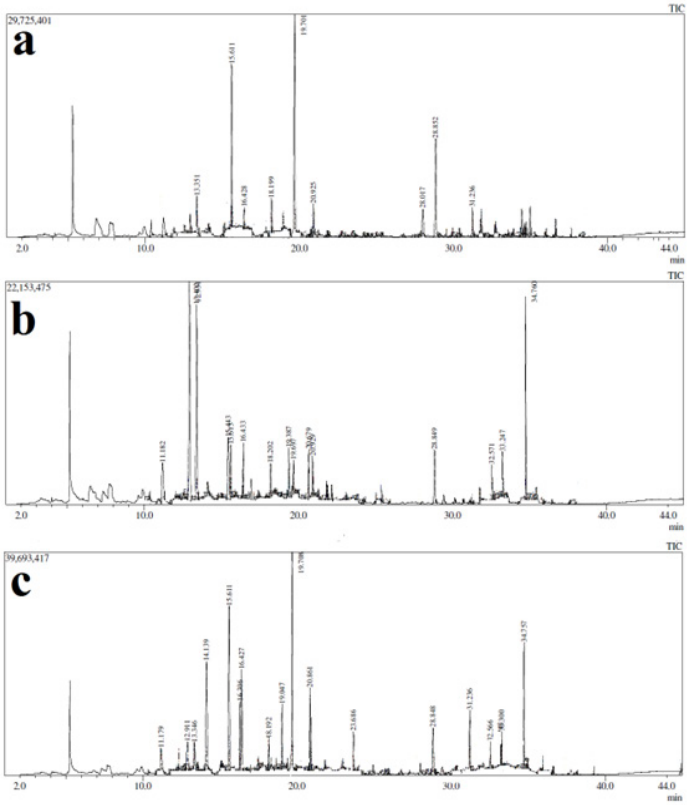

Figure S2: Head space gas chromatogram of 0.5µL injections of *T. lanceolata* a) berry ethyl acetate, (b) aqueous leaf and (c) ethyl acetate leaf extracts.