***Terminalia ferdinandiana* Exell. fruit and leaf extracts
inhibit proliferation and induce apoptosis in selected human
cancer cell lines**

Joseph Shalom^{a,b}, Ian E. Cock^{a,b*}

^aEnvironmental Futures Research Institute, Nathan Campus, Griffith University, 170
Kessels Rd, Nathan, Queensland 4111, Australia

^bSchool of Natural Sciences, Nathan Campus, Griffith University, 170 Kessels Rd,
Nathan, Queensland 4111, Australia

*Author of correspondence: Dr. I. E. Cock: E-mail: I.Cock@griffith.edu.au

ABSTRACT

Terminalia spp. are characterised by their high antioxidant contents and several species have anticancer activity. This study examined *T. ferdinandiana* fruit and leaf extracts for antiproliferative and apoptotic activities against a panel of human carcinoma cell lines. All extracts inhibited Caco2, HeLa, Jeg-3, JAR, MC3T3-E1 and MG63 proliferation. The leaf ethyl acetate extract was the most potent inhibitor of proliferation (MC3T3-E1 IC_{50} = of 6 $\mu\text{g/mL}$; Caco2 IC_{50} = 102 $\mu\text{g/mL}$). Furthermore, IC_{50} 's <500 $\mu\text{g/mL}$ were determined against all cell lines tested against that extract. The methanolic leaf extract was also a potent inhibitor of cell proliferation (Jeg-3 IC_{50} = 147 $\mu\text{g/mL}$; MC3T3-E1 IC_{50} = 40 $\mu\text{g/mL}$). The fruit extracts were also good inhibitors of carcinoma cell proliferation. Cell imaging studies detected morphological features consistent with apoptosis in Caco2 cells exposed to the ethyl acetate, methanolic and aqueous extracts. Caspase 3 activity was significantly elevated in Caco2 cells exposed to these extracts, indicating that apoptosis was induced. The leaf ethyl acetate extract contained a high diversity and relative abundance of tannins and flavonoids. All *T. ferdinandiana* fruit and leaf extracts displayed either no toxic or low toxicity in the *Artemia franciscana* bioassay and in a HDF viability assay.

Keywords: Combretaceae, Kakadu plum, gubinge, anticancer activity, antiproliferative activity, antioxidant capacity, apoptosis, DPPH.

Introduction

The maintenance of cellular redox state is required for the normal functioning of all living organisms. This involves a regulated balance between cellular oxidants and

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antioxidants. Cells use a variety of enzymatic (superoxide dismutase, catalase, thioredoxin, thioredoxin reductase, glutathione peroxidase and glutathione peroxidase) and non-enzymatic antioxidants (glutathione, vitamins A, C and E) to help maintain their redox state (1, 2). A deregulation of this redox balance is implicated in numerous chronic diseases including cancer (1, 2), autoimmune disease and chronic inflammation (3), atherosclerosis, diabetes, cirrhosis and neurodegeneration (4). The relationship between cellular redox state and cancer progression has been particularly well studied and the induction of oxidative stress has been linked with several types of cancer (5, 6). Thus, it is possible that the high antioxidant contents of many plants may inhibit cancer formation and/or progression and epidemiological studies have reported that diets rich in antioxidants are associated with decreased incidences of some cancers (3).

High antioxidant fruits, vegetables and herbs contain many types of phytochemicals, of which polyphenolic compounds are prevalent. Polyphenolic compounds are generally strong antioxidants (7). Their primary action involves the protection of cell constituents against oxidative damage through the scavenging of free radicals, thereby averting their deleterious effects on nucleic acids, proteins and lipids in cells (7). Therefore, consumption of plant material or extracts with high antioxidant capacities may contribute to reducing oxidative damage via non-enzymatic mechanisms and the scavenging of free radicals. However, studies into the medicinal effects of antioxidants have proved confusing, with some studies showing therapeutic effects (8, 9), whilst other studies indicate that these antioxidants may themselves be cytotoxic (10, 11). Furthermore, some polyphenolic antioxidants have been reported to induce apoptosis and may therefore also be useful as therapies for established cancers. Quercetin induces apoptosis in HT-29 (human colorectal) and

HepG2 (human liver carcinoma) cancer cell lines (12). Similarly, epigallocatechin-3-gallate (EGCG) induces apoptosis in LNCaP (human prostate) (13) and Hep2 (human laryngeal epidermoid) carcinoma cells (14). Thus, some high antioxidant plant extracts may be useful for both preventing and treating cancer.

Plants of the genus *Terminalia* (family Combretaceae) are characterised by their high antioxidant contents, which may provide them with therapeutic properties (15). Several individual *Terminalia* spp., (and traditional medicine combinations containing *Terminalia* spp.) have been reported to have potent anticancer properties. The Ayurvedic medicine Triphala (which contains *Terminalia belliricia* (Gawrt.) Roxb. and *Terminalia chebula* (Retz.) Lyons) has cytotoxic effects against thymic lymphoma cells (16, 17), human breast cancer cell lines (17, 18), human prostate cancer cell lines and human pancreatic cancer cell lines (19). Conversely, the cytotoxic effects of Triphala are negligible in normal cell lines (16, 17). *T. belliricia* extracts have also demonstrated growth inhibitory effects towards human A549 lung cancer cell lines and HepG2 hepatocarcinoma cell lines without the presence of the other plant components present in Triphala (20). *T. belliricia* extracts also have synergistic effects with conventional anticancer chemotherapeutics, enhancing the cytotoxic activity of cisplatin and doxorubicin towards the cancer cell lines, indicating their potential as synergistic cancer chemotherapeutic agents. Similarly, extracts prepared from the leaves of two Australian *Terminalia* spp. (*T. carpentariae* and *T. grandiflora*) were recently reported to be good inhibitors of Caco2 and HeLa cell proliferation (21). That study identified 3 lanostane and 2 pentacyclic triterpenoids in the extracts and postulated that they may contribute to this activity.

Another recent study reported substantially decreased cellular viability for HT29 (human colorectal adenocarcinoma) and HL60 (polymyelocytic leukemia)

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cancer cancer cell lines exposed to a *T. ferdinandiana* hydro-alcohol extract (22). The same study reported that proliferation of normal cell lines was also inhibited by the extract. In contrast, exposure of AGS (human gastric adenocarcinoma) to the *T. ferdinandiana* extract did not significantly affect cell viability. That study also reported that the *T. ferdinandiana* extract activated caspase-7 and caspase-9, as well as poly (ADP-ribose) polymerase (PARP), indicating that apoptosis induction is via an intrinsic pathway. However, whilst highlighting the potential of *T. ferdinandiana* extract for the prevention and treatment of some cancers, the previous study was limited with regards to cancer tissue and the types of cell tested. Both carcinoma cell types which were sensitive to the *T. ferdinandiana* extract in that study also have increased susceptibilities to other therapies. HT29 colorectal carcinoma cells over express p53 and therefore are highly responsive to other agents which induce apoptosis (23). Thus, susceptibility of the HT29 cell line to the *T. ferdinandiana* extract may not be indicative of the effect of the extract towards many other colorectal cancers. Similarly, the HL60 polymyelolytic cells examined in the Tan et al. study (22), are particularly responsive to compounds which interact with the retinoic acid receptor (RAR α) e.g. retinoic acid (24).

Our study was designed to extend the Tan et al. study (22) by testing *T. ferdinandiana* extracts against several different cancer cell types. Caco2 colorectal carcinoma cells were selected as a comparison to the HT29 colorectal and AGS gastric carcinoma cells examined in the earlier study. Caco2 cells are extremely well characterised and have been used in numerous anticancer studies, allowing for direct comparison of efficacies between studies. HeLa (human cervical carcinoma cell line), which are perhaps the most widely used cells for scientific research and are extremely well characterised, were included to extend the types of cancer the extracts were

tested against. The other cell lines tested in our study were selected for their differential production/secretion of proteins associated with cellular redox state and apoptotic mechanisms, allowing for further insight into the antiproliferative pathways involved. Whilst being similar in most ways, the Jeg-3 and JAR choriocarcinoma cell pair varies in the relative levels of thioredoxin expressed. The Jeg-3 cell line secretes a substantially higher level of thioredoxin than JAR cells (25). In addition, actively proliferating JAR cells produce an intracellular macrophage-like tumour necrosis factor (TNF). In contrast, Jeg-3 cells do not increase their secretion of TNF during proliferation (26). These differences may provide mechanistic details of the pathways affected by the extracts tested. The MG63 (human osteosarcoma) cell line and MC3T3-E1 (mouse primary osteoblast cell line) carcinoma pair were chosen because MC3T3-E1 cells over express collagen and mineralise in the presence of ascorbic acid (27), whereas MG63 cells express normal collagen levels. Hence, the increased levels of ascorbic acid present in the *T. ferdinandiana* fruit would assist in increased proliferation and formation of collagen and calcium matrix by MC3T3-E1 cells compared to MG63 cells. Furthermore, to the best of our knowledge, all previous studies examining the anticancer properties of *T. ferdinandiana* have concentrated on the fruit. Our study also reports on the anticancer properties of *T. ferdinandiana* leaf extracts for the first time.

Materials and methods

Plant source and extraction

The *Terminalia ferdinandiana* Exell. fruit and leaves used in this study were supplied and verified by David Boehme of Wild Harvest, Northern Territory (Australia). The fruit was pulped and frozen prior to transport and kept at -10 °C until processing.

Voucher specimens are stored in the School of Natural Sciences, Griffith University, Australia. Voucher numbers of the *T. ferdinandiana* fruit and leaf specimens are GUTF1F-2016-1A and GUTF1L-2016-1A respectively. All plant materials were washed in deionised water and thoroughly dried using a Sunbeam food dehydrator. The dried materials were stored at -30 °C until required. Prior to use, the plant materials were ground into a coarse powder. Individual 1 g quantities of the powdered *T. ferdinandiana* materials were weighed into separate tubes and 50 mL of methanol, sterile deionised water, ethyl acetate, chloroform or hexane were added. All solvents were obtained from Ajax Chemicals, Australia and were AR grade. The ground *T. ferdinandiana* material was extracted in each solvent for 24 hours at 4 °C with gentle shaking. The extracts were subsequently filtered through Whatman No. 54 filter paper under vacuum. The solvent extracts were air dried at room temperature. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10 mL deionised water (containing 1% DMSO) and stored at 4 °C until use.

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays (28, 29).

Antioxidant capacity

The antioxidant capacity of each extract was assessed using the DPPH free radical scavenging method (30) with modifications. Briefly, DPPH solution was prepared

fresh each day as a 400 μ M solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). A 2 mL aliquot of each extract was evaporated and the residue resuspended in 2 mL of methanol. Each extract was added to a 96 well plate in 5, 10, 25, 50, 75 μ L volumes in triplicate. Methanol was added to each well to give a volume of 225 μ L. A volume of 75 μ L of the fresh DPPH solution was added to each well to give a total reaction volume of 300 μ L. Ascorbic acid was prepared fresh and examined across the range 0-25 μ g per well as a reference and the absorbances were recorded at 515 nm. All tests and controls were performed in triplicate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as μ g ascorbic acid equivalents per gram of original plant material extracted.

Screen for anticancer bioactivity

Cancer cell lines

The Caco2 and HeLa carcinoma cell lines used in this study were purchased from American Type Culture Collection (Rockville, USA). The MC3T3-E1 and MG63 cell lines were a gift from Professor Ann McDonnell, Griffith University, Australia. The Jeg-3 and JAR cells were donated by Dr Jenny DiTrapanni, Griffith University Australia. Caco2, HeLa, Jeg-3 and JAR cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen). The MC3T3-E1 and MG63 cell lines were grown in Dulbecco/Vogt modified Eagle's minimal essential medium: nutrient media (DMEM). Both the DMEM and RPMI medias were supplemented with 20 mM HEPES, 10 mM sodium bicarbonate, 50 μ g/mL streptomycin, 50 IU/mL penicillin, 2 mM glutamine and 10 % foetal calf serum (Invitrogen). The cells were maintained as

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monolayers in 75 mL flasks at 37 °C, 5 % CO₂ in a humidified atmosphere until approximately 80 % confluent.

Evaluation of cancer cell antiproliferative activity

Antiproliferative activity of the extracts was assessed as previously described (31). Briefly, 1 mL of trypsin (Sigma) was added to the culture flasks and incubated at 37 °C, 5 % CO₂ for 15 min to dislodge the cancer cells. The cell suspensions were then transferred to a 10 mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 mL of fresh media. Aliquots of the resuspended cells (70 µL, containing approximately 5000 cells) were added to the wells of a 96 well plate. A 30 µL volume of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37 °C, 5 % CO₂ for 12 hours in a humidified atmosphere. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. Absorbances were recorded at 490 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in triplicate, each with 3 internal replicates (i.e. 9 replicates in total for each test). The antiproliferative activity of each test was calculated as a percentage of the negative control using the following 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

Cell morphological changes were measured using bright field microscopic imaging without staining. Caco2 cells were seeded into individual wells of a 96 well plate at a density of 5×10^3 cells/100 μ L. Cells were allowed to adhere overnight. Selected *T. ferdinandiana* extracts were added to the wells at half the concentration of the IC₅₀. Cells were incubated for 24 hours at 37 °C in a humidified 5 % CO₂, 95 % air atmosphere. Each assay was performed in triplicate and the cell morphology was qualitatively appraised after a 24h incubation using an inverted phase microscope coupled with an Olympus IX70 camera with an optical zoom of 20X.

Determination of caspase 3 activity

Cellular caspase 3 activity of cells exposed to the *T. ferdinandiana* fruit and leaf extracts was quantified using a colourimetric caspase 3 assay kit (Sigma Aldrich, Australia) as per the manufacturer's instructions. This assay is based on the amount of p-nitroaniline (p-NA) molecule cleaved from the acetyl-Asp-Glu-Val-Asp (Ac-DEVD-p-NA) substrate. Test extracts was added to the Caco2 cells at a concentration equal to the IC₅₀ concentration. Untreated cells were included in the assay as a control. The cells were incubated for 3 h at 37 °C, 5 % CO₂ in a humidified atmosphere. The supernatants were aspirated and the cells were washed with 100 μ L PBS. The cells were resuspended in lysis buffer (50mM HEPES pH 7.4, 5 mM CHAPS, 5 mM DTT) and lysed on ice for 20 min. The cell debris was removed by centrifugation at 16000 g for 15 min at 4 °C. The supernatant was collected and the caspase activity was determined in triplicate at 405 nm using a Molecular Devices, Spectra Max M3 plate reader. The amount of caspase Ac-DEVD-p-NA lysed was

calculated by comparison with a p-NA standard curve. All tests were performed in duplicate and were repeated twice.

Toxicity screening

Two assay methods were used to assess the toxicity of the individual samples. The *Artemia* lethality assay (ALA) was utilised for rapid preliminary toxicity screening, whereas an MTS cellular viability assay was used as a cellular evaluation of toxicity.

***Artemia franciscana* nauplii toxicity screening**

Potassium dichromate (K₂Cr₂O₇) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay. Toxicity was tested using a modified *A. franciscana* nauplii lethality assay (32, 33). Briefly, 400 µL of seawater containing approximately 38 (mean 38.1, n = 125, SD 12.6) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µL seawater) was included on each plate. All treatments and controls were performed three times in triplicate. The wells were checked at regular intervals and the number of dead were counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC₅₀ with 95% confidence limits for each treatment was calculated using probit analysis.

Cellular viability assay

The *T. ferdinandiana* extracts were also screened against human primary dermal fibroblasts (HDF). The HDF cells were obtained from American Type Culture Collection (ATCC PCS-201-012) and were cultured and maintained in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific, Australia), supplemented with 10 % foetal calf serum (Invitrogen), 50 µg/mL streptomycin (Sigma-Aldrich, Australia) and 50 IU/mL penicillin (Sigma Aldrich, Australia). The cells were maintained as monolayers in 75 mL flasks at 37°C, 5 % CO₂ in a humidified atmosphere until approximately 80% confluent. Once confluency was achieved, 1 mL of trypsin (Sigma, Australia) was added to the culture flasks and incubated at 37 °C, 5 % CO₂ for 15 min to dislodge the HDF cells. The cell suspensions were then transferred to a 10 mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 mL of fresh media. Aliquots of the resuspended cells (70 µL, containing approximately 5000 cells) were added to individual wells of a 96 well plate. A volume of 30 µL of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO₂ for 24 hours in a humidified atmosphere. All extracts were screened at 200 µg/mL. The cells were then washed in PBS (pH 7.2) to remove interference due to sample colour. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. Absorbances were recorded at a test wavelength of 540 nm and a blank wavelength of 690nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in at least triplicate and triplicate controls were 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 $\leq 50\%$ of the untreated control indicated toxicity, whereas extracts or controls with $>50\%$ untreated control viability were deemed to be nontoxic.

HPLC-MS QTOF analysis

Chromatographic separations were performed as previously described (34). Briefly, 2 μL of sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1 x 100 mm, 1.8 μm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilised for the study consisted of a 5 min isocratic phase at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by a 3 min isocratic phase at 100% B. Mass spectrometry analysis was performed on an Agilent 6530 quadrupole time-of-flight spectrometer fitted with a Jetstream electrospray ionisation source in both positive and negative mode. Data was analysed using the Masshunter Qualitative analysis software package (Agilent Technologies). Each extract was analysed using the Find by Molecular Feature function to generate a putative list of compounds which was then 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). Empirical formula for unidentified compounds was determined using the Find Formula function in the software package.

Statistical analysis

Data are expressed as the mean $\pm$ SEM of at least three independent experiments. One way ANOVA was used to calculate statistical significance between control and treated groups with a P value < 0.01 considered to be statistically significant.

Results

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the dried *T. ferdinandiana* fruit and leaf powders with solvents of varying polarity yielded dried plant extracts ranging from 11 mg (*T. ferdinandiana* fruit hexane extract) to 590 mg (aqueous *T. ferdinandiana* fruit extract) (Table 1). The higher polarity methanol and water solvents extracted substantially higher yields compared with the mid to lower polarity solvents. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies showed that methanol and water extracted the widest range and the greatest relative abundance of phytochemicals (Table 1). Methanol and aqueous fruit and leaf extracts contained moderate to high levels of polyphenolics, flavonoids and tannins, as well as low to moderate levels of saponins. The ethyl acetate fruit and leaf extracts generally contained similar classes of compounds, albeit at lower levels. The chloroform and hexane extracts were much less complex, with only low abundances of polyphenolic compounds.

Antioxidant content

The DPPH assay was used to determine the antioxidant capacity of each plant extract and compared to an ascorbic acid standard curve to quantify the ascorbic acid equivalents in each extract (Table 1). The antioxidant capacity ranged from a low of 1

mg ascorbic acid equivalents per gram of dried plant material extracted (*T. ferdinandiana* fruit and leaf hexane extracts) to a high of 660 mg ascorbic acid equivalents per gram of dried plant material (methanolic *T. ferdinandiana* fruit extract). The aqueous *T. ferdinandiana* fruit extract (264 mg ascorbic acid equivalents) as well as the methanolic (150 mg ascorbic acid equivalents) and aqueous leaf extracts (340 mg ascorbic acid equivalents) also had high antioxidant capacities. The fruit and leaf ethyl acetate extracts had moderate to high antioxidant capacities of 39 and 22 mg ascorbic acid equivalents respectively.

Antiproliferative activity

Caco2 and HeLa cell lines

Aliquots of each extract were tested for the ability to inhibit proliferation of Caco2 and HeLa cell lines (Figure 1). All of the *T. ferdinandiana* fruit extracts displayed significant ($p<0.01$) antiproliferative effects against the Caco2 cells. The methanolic and aqueous *T. ferdinandiana* fruit extracts were particularly potent inhibitors of HeLa cell proliferation. Indeed, negative HeLa proliferation was noted for some extracts, suggesting that the antiproliferative activity was not only cytostatic in nature, but was possibly cytotoxic (Figure 1a). The mid to lower polarity *T. ferdinandiana* ethyl acetate, chloroform and hexane fruit extracts inhibited HeLa cell proliferation by 58%, 34% and 22.4% respectively. A different trend was apparent with *T. ferdinandiana* leaf extracts. All leaf extracts except the chloroform extract were effective inhibitors of HeLa cell proliferation (Figure 1b). The higher polarity methanolic and aqueous *T. ferdinandiana* leaf extracts strongly inhibited HeLa cell proliferation by 73% and the 69% respectively compared to the untreated control. The mid-polarity *T. ferdinandiana* leaf ethyl acetate extract produced the strongest

inhibition against HeLa cell proliferation, with a 92% decrease in proliferation. The lower polarity *T. ferdinandiana* hexane leaf extract also inhibited HeLa cell proliferation, albeit with lower efficacy (34% inhibition compared to the negative control). The *T. ferdinandiana* chloroform leaf extract did not significantly affect HeLa cell proliferation.

The extracts were similarly effective at inhibiting Caco2 cancer cell proliferation. Notably, negative proliferation was noted for the *T. ferdinandiana* methanolic and ethyl acetate leaf extracts (Figure 1b), suggesting that cytotoxic mechanisms may be involved. The *T. ferdinandiana* fruit extracts were also strong inhibitors of cell proliferation, albeit with lower potency. The methanolic, aqueous and ethyl acetate fruit extracts inhibited Caco2 proliferation by 93%, 97% and 74% respectively. The chloroform and hexane extracts prepared from both the fruit and the leaves also inhibited Caco2 cell proliferation, albeit with lower potency (40-60% inhibition compared to the untreated control).

Jeg-3 and JAR cell lines

Methanolic and aqueous *T. ferdinandiana* fruit extracts were particularly potent inhibitors of Jeg-3 and JAR cell proliferation (Figure 2). Indeed, a decrease in Jeg-3 cell proliferation was noted for the *T. ferdinandiana* methanolic fruit extract, suggesting that the antiproliferative activity was at least in part due to cytotoxic mechanisms (Figure 2a). The *T. ferdinandiana* methanolic fruit extract was also a potent inhibitor of JAR cell proliferation, inhibiting proliferation by 95% compared with the untreated control. The *T. ferdinandiana* aqueous fruit extract inhibited Jeg-3 proliferation by 99% compared to the negative control. A similar but more potent inhibition was noted with the *T. ferdinandiana* aqueous fruit extract which induced

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negative proliferation with JAR cells, indicating that the extract may be cytotoxic to JAR cells. The *T. ferdinandiana* ethyl acetate, chloroform and hexane fruit extracts inhibited Jeg-3 cell proliferation by 63%, 42% and 15% respectively. A similar trend was observed for the ethyl acetate, chloroform and hexane fruit extracts inhibition of JAR cell proliferation, with inhibitions of 60%, 39% and 22% determined respectively.

All leaf extracts also significantly inhibited Jeg-3 and JAR cell proliferation (Figure 2b) with the methanolic, aqueous and ethyl acetate *T. ferdinandiana* leaf extracts being particularly strong inhibitors of Jeg-3 cell proliferation. Indeed, negative Jeg-3 proliferation was noted for the methanolic and ethyl acetate *T. ferdinandiana* leaf extracts, suggesting that the antiproliferative activity may be due to cytotoxic pathways (Figure 2b). Similarly, negative proliferation values were observed for JAR cells exposed to the aqueous and ethyl acetate *T. ferdinandiana* leaf extracts. The aqueous *T. ferdinandiana* leaf extract also strongly inhibited Jeg-3 proliferation by 83% compared to the negative control. The *T. ferdinandiana* chloroform and hexane leaf extracts were less potent, inhibiting Jeg-3 cell proliferation by 62% and 35%. These extracts also inhibited JAR cell proliferation by 42% and 31% respectively (Figure 2b). Thus, the *T. ferdinandiana* (fruit and leaf) extracts were potent inhibitors of both Jeg-3 and JAR carcinoma cell proliferation.

MC3T3-E1 and MG63 cell lines

The *T. ferdinandiana* methanolic, aqueous and ethyl acetate fruit extracts were potent inhibitors of MC3T3-E1 cell proliferation. Indeed, negative MC3T3-E1 proliferation was noted for the *T. ferdinandiana* methanolic and aqueous fruit extracts, suggesting that cytotoxicity may contribute to the antiproliferative activity of these extracts

against this cell line (Figure 3a). The *T. ferdinandiana* fruit ethyl acetate extract was also a potent antiproliferative agent, inhibiting 80% of MC3T3-E1 proliferation compared to the untreated control. In contrast, the lower polarity chloroform and hexane fruit extracts inhibited cellular proliferation to lesser extents (46% and 33% respectively). Similar trends were apparent for the *T. ferdinandiana* leaf extracts. The methanolic and ethyl acetate leaf extracts induced negative proliferation. The aqueous, chloroform and hexane extracts were also good inhibitors of MC3T3-E1 cell proliferation, albeit with lower apparent potency. The *T. ferdinandiana* leaf extracts were similarly effective inhibitors of MC3T3-E1 cell proliferation (Figure 3b). Negative MC3T3-E1 proliferation was noted for the methanolic and ethyl acetate leaf extracts, suggesting that they may possess cytotoxic activity. The higher polarity *T. ferdinandiana* aqueous leaf extract was less potent, although significant inhibition (66%) was still achieved. In contrast, the lower polarity chloroform and hexane leaf extracts inhibited cellular proliferation at higher than expected levels, with MC3T3-E1 cell proliferation inhibited by 81% and 62% respectively (Figure 3b). The strong inhibition by the lower polarity extracts was unexpected as opposing trends were noted for all other cell types.

All *T. ferdinandiana* fruit extracts also significantly inhibited MG63 cell proliferation compared to untreated control cell proliferation (Figure 3a). The higher polarity (methanolic and aqueous) and the mid polarity (ethyl acetate) extracts were particularly strong inhibitors of MG63 cell proliferation. Indeed, negative MG63 proliferation was noted for the methanolic and ethyl acetate *T. ferdinandiana* fruit extracts, indicating that cytotoxic mechanisms may be involved in the inhibition of proliferation. The aqueous *T. ferdinandiana* fruit extract also strongly inhibited MG63 proliferation by 83% compared to the negative control. The lower polarity *T.*

ferdinandiana chloroform and hexane fruit extracts also inhibited MG-63 cell proliferation, albeit by a lesser degree (55% and 28% respectively). The *T. ferdinandiana* leaf extracts were also effective inhibitors of MG63 cell proliferation, with some notable exceptions. The methanolic and aqueous *T. ferdinandiana* leaf extracts were the strongest inhibitors of MG-63 cell proliferation, with negative MG63 proliferation noted for aqueous leaf extract (Figure 3b). The ethyl acetate and chloroform *T. ferdinandiana* leaf extracts also inhibited MG63, with cell proliferation inhibited by 69% and 37% respectively.

IC₅₀ Determination

The antiproliferative efficacy of the *T. ferdinandiana* extracts was further quantified by determining their IC₅₀ values (Table 2). The leaf extracts were generally more potent inhibitors of proliferation than the fruit extracts against all cell lines tested. The *T. ferdinandiana* leaf ethyl acetate extract was a particularly potent inhibitor of cell proliferation, with IC₅₀ values as low as 6 and 102 µg/mL against MC3T3-E1 and Caco2 carcinoma cells respectively. This extract was also a potent inhibitor of all other cell lines, with IC₅₀'s generally <500 µg/mL. The methanolic *T. ferdinandiana* leaf extract was also a potent inhibitor of proliferation against Jeg-3 and MC3T3-E1 cells, with IC₅₀ values of 147 and 40 µg/mL respectively. The fruit *T. ferdinandiana* extracts were moderate to good inhibitors of carcinoma cell proliferation. As noted for the leaf extracts, the ethyl acetate and methanolic fruit extracts were generally substantially more potent than the other *T. ferdinandiana* fruit extracts.

Cell imaging studies

The cell antiproliferative studies demonstrate that the methanolic, aqueous and ethyl acetate *T. ferdinandiana* extracts have profound effects on the proliferation of all carcinoma cell lines tested. Indeed, not only was cell growth blocked, but in some cases, negative cellular proliferation was recorded. These large decreases in cell proliferation compared to the untreated control cells indicate that these extracts may not just inhibit growth by blocking cell cycle progression, but may act via cytotoxic/apoptotic mechanisms. Cell imaging studies were undertaken to test this hypothesis. The Caco2 cell line was chosen for cell imaging studies as it is a commonly used cell line in similar studies (allowing for comparisons), has well defined cell morphology, has a reasonable doubling time compared with many other cell lines and was highly responsive to the *T. ferdinandiana* extracts.

The key organelles for comparison between the treated and untreated cells in these studies are the nucleus, cytoplasm, vacuoles and cell membrane, all of which were clearly visible at 20X optical magnification of the cell in the untreated control cell images (Figure 3a, 3d, 3g). The outline of the viable cells are clear, distinct and compact without apparent abnormalities in size and density. Stress granules were not visible at this magnification. These are normally indicators of cellular stress. Typical apoptotic phenomena were noted in the morphology of the cells tested against the ethyl acetate (Figure 4a), methanolic (Figure 4b) and aqueous (Figure 4c) extracts when tested at sub-lethal concentrations. These images are consistent with the findings of the cell proliferative assays which showed that ethyl acetate, methanolic and aqueous *T. ferdinandiana* extracts strongly inhibited Caco2 proliferation. This correlates with the morphological characteristics noted in this study. Apoptosis is generally characterised by the presence of an intact membrane, marginalisation of the cytoplasm and chromatin condensation (35). These characteristics were clearly visible

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in the Caco2 cells treated with the *T. ferdinandiana* fruit (Figure 4b) and leaf ethyl acetate extracts (Figure 4c). Marginalisation of the cytoplasm of cells undergoing apoptosis also produces an enormously large centrally placed vacuole which is clearly visible in these figures. The presence of stress granules in the visible fields indicates that the cells are under stress. ‘Blebbing’ is the appearance of ‘spherical bubbles’ on the surface of the membrane and is a typical characteristic apparent in apoptotic cells. ‘Blebbing’ is also seen in cells tested against the ethyl acetate fruit and leaf extracts. These cells also displayed a loss in membrane integrity with no clear outline of the cell visible, extensive chromatin condensation and the presence of numerous vacuoles. Also visible for the ethyl acetate extract treatments were the ‘ghosts’ of cells where the cellular contents are no longer present within cell. The morphological changes observed with these treatments are indicative of the induction of apoptosis. Similarly, the fruit and leaf methanolic extracts (Figures 4e and 4f respectively), as well as the aqueous fruit and leaf (Figures 4 h and 4i respectively), showed similar morphological features, indicating that these cells are also undergoing apoptosis.

Determination of caspase 3 activity

As the cell imaging studies detected cell morphology changes in Caco2 cells exposed to the *T. ferdinandiana* that were consistent with apoptosis, further studies were undertaken to confirm this. Activation of caspase 3 activity was quantified following exposure to the extracts as caspase 3 as activated by both extrinsic and intrinsic apoptotic pathways. Thus, its activity relative to the untreated control is elevated, regardless of the apoptotic pathway that is activated in the cell. The ethyl acetate, methanolic and aqueous *T. ferdinandiana* fruit and leaf extracts induced significant activation of caspase 3 activity following 3 h exposure (Figure 5). In general, the leaf

extracts exhibited greater caspase 3 activity than the corresponding fruit extracts. Furthermore, the ethyl acetate extracts induced greater levels of caspase 3 activity compared to the other solvent extractions, followed by the methanolic extracts, then the aqueous extracts.

Quantification of toxicity

All extracts were initially screened at 2000 $\mu\text{g/mL}$ in the *Artemia* nauplii assay (Figure 6). For comparison, the reference toxin potassium dichromate (1000 $\mu\text{g/mL}$) was also tested in the bioassay. The lower polarity chloroform and hexane fruit and leaf extracts, as well as the fruit ethyl acetate extract, induced $< 50\%$ mortality and were thus considered to be non-toxic. In contrast, the methanolic and aqueous *T. ferdinandiana* fruit extracts, as well as the methanolic, aqueous and ethyl acetate leaf extracts, displayed $>50\%$ mortality rates at 24h and were thus deemed to have apparent toxicity. To further quantify the effect of extract concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. Table 2 shows the LC_{50} values of the extracts towards *A. franciscana*. No LC_{50} values are reported for the fruit ethyl acetate, chloroform or hexane extracts, nor for the leaf chloroform and hexane extracts, as less than 50 % mortality was seen for all concentrations tested. The methanolic and ethyl acetate *T. ferdinandiana* leaf extracts were also deemed to be non-toxic ($\text{LC}_{50} > 1000 \mu\text{g/mL}$) following 24 hours exposure. In contrast, the methanolic fruit extract and the aqueous extracts of both the fruit and leaves extracts, displayed low toxicity (LC_{50} values: 875, 983 and 928 $\mu\text{g/mL}$ respectively) following 24 hours exposure. However, it is noteworthy that the toxicity detected in our study generally correlated with the extracts with the highest antioxidant capacities. Indeed,

the fruit methanol and aqueous extracts contained 600 and 263 mg ascorbic acid equivalents respectively (Table 1). Similarly, the aqueous leaf extract had an antioxidant capacity of 340 mg ascorbic acid equivalents. Previous studies have reported that the high antioxidant content of *T. ferdinandiana* fruit is largely due to high levels of ascorbic acid (36, 37). Although the *A. franciscana* nauplii lethality assay is generally quite robust, it is noteworthy that brine shrimp are sensitive to changes in pH (38). Acidic pH can suppress the rate of mitochondrial protein synthesis and potentially be fatal to the growth and development of nauplii. Indeed, previous studies have reported that extracts high in ascorbic acid can provide fallacious toxicity determinations (39). Thus, this assay may have overestimated the toxicity of these extracts. Cellular based assays may provide a better measure of toxicity for high antioxidant extracts. In contrast to the results obtained in the *Artemia* nauplii bioassay, all *T. ferdinandiana* fruit and leaf extracts demonstrated a lack of toxicity towards normal human primary dermal fibroblasts, with cellular viability for all tests substantially >50% of the untreated control. All extracts were therefore deemed to be either nontoxic, or of only low-moderate toxicity.

HPLC-MS QTOF analysis

As the *T. ferdinandiana* leaf ethyl acetate extract generally displayed the greatest anti-proliferative potency towards all carcinoma cell lines, it was deemed the most promising extract for further phytochemical analysis. Optimised HPLC-MS parameters were developed and used to profile the *T. ferdinandiana* ethyl acetate leaf extract. In total, 18 compounds were putatively identified by comparison to the Metlin metabolomics and phytochemicals (developed in this laboratory) databases (Table 3). Interestingly, these compounds were dominated by tannins and flavonoids. Indeed, 11

of the 18 compounds identified (61%) were tannins. Furthermore, tannins accounted for approximately 28% of the total compound abundance of this extract (determined as the % of the total peak area in the negative ionisation mode chromatogram). Four flavonoids (vitexin, rutin, luteolin and quercetin) were also identified in this extract. These compounds collectively accounted for approximately 14% of the total compound abundance in this extract.

Discussion

Diets consisting of fruits and vegetables rich in polyphenolic compounds (flavonoids, tannins and phenolic acids), have been linked with reduced morbidity and mortality induced by chronic diseases (3). Many polyphenolic compounds are potent antioxidants which may scavenge free radicals, prevent inflammation and retard progression of carcinogenesis. Many chronic diseases are caused by the inability to regulate the redox state of the cell. Hence, it has been postulated that by altering the cellular redox state, it may shift the cell back to its normal state or induce apoptosis in aberrant carcinoma cells. Interestingly, the most potent antiproliferative activity against all cancer cells tested in our study correlated with the extracts with mid to high antioxidant capacity, indicating that the extracts may function via modulation of cellular redox status. Conversely, the chloroform and hexane extracts which had substantially lower antioxidant capacities displayed much lower antiproliferative activities. Interestingly, a previous study has reported a similar relationship between antioxidant activity and antiproliferative activity for extracts prepared from fresh apples (40). That study reported that apples with skin possessed a far superior radical scavenging activity compared to apples without skin. The greater phenolic content

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present in the skin (identified as quercetin glycosides) may contribute to the greater antiproliferative activity of these extracts via increased free radical scavenging activity. The same study also trialled apples with and without skin against Caco-2 (human colorectal carcinoma) and HepG2 (human liver tumour) cells. Interestingly, both apple extracts induced cell proliferation at low concentrations, but significantly reduced tumour cell proliferation at higher concentrations. The authors postulated that these effects may be due to the presence of both phenolic acids and flavonoids in the flesh and skin of the apple. Thus, it is likely that polyphenolic compounds can induce proliferation of carcinomas at lower concentrations, but inhibit their growth at higher concentrations as a result of their redox potential (40).

All of the *T. ferdinandiana* extracts were good inhibitors of HeLa and Caco2 proliferation. Indeed, some of the extracts were particularly potent anti-proliferative agents. Several *T. ferdinandiana* fruit and leaf extracts not only completely blocked cellular proliferation, but also induced cellular metabolic rates lower than those at the commencement of the experiment (as determined by an MTS colorimetric assay). This indicates that not only had the cell number not increased over the time-course of the experiment, but the metabolic rate (which is indicative of cellular activity and thus cell number) had decreased. This is a noteworthy result as such decreases in metabolic activity are generally indicative of cell death. Thus, it is likely that the anti-proliferative mechanism of these extracts may involve the induction of apoptosis and further studies using diagnostic cell line pairs and cell imaging were undertaken to further understand the antiproliferative activity of the *T. ferdinandiana* extracts.

The ability of the extracts to differentially inhibit the proliferation of Jeg-3 and JAR cells was examined as Jeg-3 cells produce substantially higher thioredoxin levels than JAR cells. Thus any difference in the effects of the extracts on these cells may be

indicative of redox related mechanisms. Interestingly, substantially greater inhibition of proliferation was noted against Jeg-3 than JAR cells. Indeed, the extracts potency against Jeg-3 cells was generally twice that seen for JAR cells. For example, the *T. ferdinandiana* methanolic fruit extract had an IC₅₀ value of 525 µg/mL for Jeg-3, but an IC₅₀ value of 1050 µg/mL for JAR (Table 2). This trend was apparent in all the higher polarity and mid polarity extracts of both *T. ferdinandiana* fruit and leaf. Although the increased levels of thioredoxin produced by Jeg-3 cells were anticipated to have had a protective function, this was not the case. Instead, Jeg-3 proliferation inhibited proliferation more effectively than JAR cell proliferation. The increased levels of TNF produced by JAR cells may have functioned as a growth promoter and instead served a protective role in those cells. Similar outcomes were seen in a previous study, where JAR cells proliferated more rapidly than Jeg-3 due to increased levels of intracellular TNF (26). As the *T. ferdinandiana* methanolic fruit extract had the highest antioxidant content recorded at 660 mg ascorbic acid equivalents per g, the increased levels of thioredoxin produced by Jeg-3 cells may not have played as significant a role in protection as expected as the extract itself has an inherently high antioxidant capacity. Nonetheless, ascorbic acid can function as a reducing agent and may have been instrumental in maintaining an already reduced cellular redox state, thereby lessening or negating the effects of thioredoxin. Furthermore, the possible involvement of TNF in the JAR cells as a promoter of cellular proliferation instead of cellular inhibition may indicate that an extrinsic pathway may not be involved in apoptosis. Indeed, a recent report which examined the antiproliferative activity of a hydro-alcohol *T. ferdinandiana* fruit extract against different cell lines reported that the extract activated caspase-7, caspase-9 and poly (ADP-ribose) polymerase (PARP) (22).

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The *T. ferdinandiana* leaf and fruit extracts were also good antiproliferative agents against the MC3T3-E1/MG-63 cell pair. However, the mechanisms of action may be fundamentally different to those of the Jeg-3/JAR pair. MC3T3-E1 cells are a normal murine osteoblastic cell line which has increased proliferative capacity in the presence of ascorbic acid. The increased levels of ascorbic acid present in *T. ferdinandiana* methanolic, aqueous and to a much lesser degree the ethyl acetate extracts would be expected to favour the increased proliferation of this cell line. However, the opposite trend was apparent in our study. Indeed, the *T. ferdinandiana* methanolic fruit, aqueous fruit, methanolic leaf, aqueous leaf and the ethyl acetate leaf extracts inhibited MC3T3-E1 proliferation to a much greater extent than MG-63 cells, and instead induced negative proliferation in MC3T3-E1, possibly by a cytotoxic mechanism. Indeed, past studies have documented a susceptibility of MC3T3-E1 cells to TNF (41). It is possible that extract compounds may have upregulated TNF expression, thereby inducing apoptosis, although this remains to be verified. Furthermore, the MG-63 cells have no known susceptibility to TNF, and may even follow an intrinsic mitochondrial pathway or an extrinsic Fas-activated pathway. Hence, MG-63 cell proliferation may not be inhibited by the presence of any such compounds. Thus, it may be postulated that the increased inhibition of MC3T3-E1 proliferation in comparison to MG-63 may suggest a TNF induced extrinsic apoptotic pathway.

Cell images of Caco2 cells exposed to the ethyl acetate, methanolic and aqueous extracts indicated that apoptosis had been induced. This was supported by the caspase-3 studies, which showed the *T. ferdinandiana* ethyl acetate, methanolic and aqueous extracts of both fruit and leaf induced significantly elevated caspase-3 in Caco2 cells. Elevation of caspase-3 levels is common to both extrinsic and intrinsic

pathways and does not differentiate between which of these pathways is induced. However, a recent study reported that a *T. ferdinandiana* fruit extract activated caspases consistent with activation of an intrinsic pathway. Neither that study nor our study tested the levels of caspases associated with extrinsic pathways of apoptosis so it is still possible that they are also elevated and that the extracts may induce apoptosis via multiple pathways. Also noteworthy, whilst our cell imaging studies clearly displayed all morphological characteristics associated with cytotoxicity, they were unable to reveal cytostatic activity. To the best of our knowledge, no other studies have tested *T. ferdinandiana* fruit or leaf extracts for the ability to block the cell cycle. The extracts tested against the cell lines are crude mixtures and are complex, containing a large number of compounds. They may illicit multiple mechanisms simultaneously within cells. Therefore, whilst our studies (and previous studies by Tan et al. (22)) demonstrate that *T. ferdinandiana* induce apoptosis, different compounds within the extracts may also act as cell cycle inhibitors and have cytostatic effects. Thus, further studies testing for cell cycle arrest are also warranted.

Whilst further studies are necessary to identify the compounds responsible for inhibiting proliferation in the *T. ferdinandiana* extracts, the presence of high levels and a high diversity of tannins in the ethyl acetate leaf extract was particularly interesting. Tannins can influence the cellular redox state (15) and therefore may have contributed to the inhibition of proliferation reported in our study. Furthermore, tannins have direct anti-proliferative effects against a variety of carcinoma cell lines. Gallotannins have been reported to inhibit cellular growth through a variety of mechanisms including binding cell surface molecules including lipoteichoic acid and proline-rich cell surface proteins (42), and by inhibiting glucosyltransferase enzymes (43). Ellagitannins are also highly potent inhibitors of cell growth and have also been

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reported to function via several mechanisms including interaction with cytoplasmic oxidoreductases (42, 44). The ellagitannin arjunin extracted from a related *Terminalia* spp. (*T. arjuna*) has been reported to inhibit carcinoma proliferation and to stimulate apoptosis (45). Furthermore, a number of studies have reported high tannin contents in a variety of plants used in traditional medicine to treat cancer (15, 46).

Four flavonoids (vitexin, rutin, luteolin and quercetin) were also identified in this extract. Interestingly, a recent study reported good anti-proliferative properties for quercetin (47). When tested at a concentration of 100 µg/mL against human CAL-27 and KB oral carcinoma cell lines, HT29 and HCT-116 colorectal cancer cells, and LNCaP and DU145 prostate cancer cells, quercetin inhibited cell proliferation by as much as 80 % of the untreated cell proliferation rate. Unfortunately, that study did not report IC₅₀ values, making it difficult to compare the efficacy with other compounds/other studies. A different study reported that other flavonoids also induce apoptosis in human LNCaP prostate cancer and MDA-MB-231 breast cancer cells (48). That study reported a strong relationship between the induction of apoptosis and an inhibition in fatty acid synthesis. When exogenous palmitate was added to the treated cells, apoptosis was suppressed, indicating that the mechanism by which flavonoids induce apoptosis is associated with their inhibition of fatty acid synthesis. Several flavonoids including quercetin have also been reported to inhibit glutathione S-transferase P1-1 and GS-X pump activity in MCF7 cells, with IC₅₀ values between 0.8 and 8 µM, indicating that these flavonoids may be useful for potentiating apoptosis in multidrug resistant cells (49). Furthermore, similar flavonoids have been identified in many plant extracts with anti-proliferative activity (50-52).

Whilst the use of foods with high levels of antioxidants may be an attractive option in the prevention and treatment some cancers, oxidative stress is not the sole

factor in the development and progression of cancer. Genetic factors, dietary habits and environmental factors may all contribute to cancer development. Furthermore, even in oxidative stress induced cancers, dietary antioxidants are of limited value as it is difficult to supply enough of the beneficial antioxidants directly in the food to function effectively as chemoprotectants and/or chemotherapeutics. Instead, high antioxidant extracts are required to obtain a high enough dosage to be effective. Despite this, numerous studies have reported the beneficial effects of using high levels of phytochemical antioxidants in cancer prevention and treatment. Therefore, high antioxidant plant extracts such as the *T. ferdinandiana* fruit and leaf extracts examined in this report may also prove to be useful anticancer agents.

Conclusions

The results of this study demonstrate the potential of the ethyl acetate, methanolic and aqueous *T. ferdinandiana* fruit and leaf extracts to block proliferation of some cancer cells. The low toxicity of the extracts further indicates their potential in the treatment and prevention of some cancers. Purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents.

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Figure legends

Figure 1: Antiproliferative activity of the *T. ferdinandiana* (a) fruit and (b) leaf extracts against Caco2 and HeLa cancer cell lines measured as percentages of the untreated control cells. NC = untreated control; M = methanolic *T. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; Cis = cisplatin control (50 µg/mL). Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control (p<0.01).

Figure 2: Antiproliferative activity of the *T. ferdinandiana* (a) fruit 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. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana*

extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; Cis = cisplatin control (50 µg/mL). Results are expressed as mean percentages $\pm$ SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

Figure 3: Antiproliferative activity of the *T. ferdinandiana* (a) fruit 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. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; Cis = cisplatin control (50 µg/mL). Results are expressed as mean percentages $\pm$ SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

Figure 4: Morphological effects of viable Caco2 cells (a) without treatment, and cells exposed to (b) *T. ferdinandiana* fruit ethyl acetate extract, (c) *T. ferdinandiana* leaf ethyl acetate extract, (d) untreated control, (e) *T. ferdinandiana* fruit methanol extract; (f) *T. ferdinandiana* leaf methanol extract, (g) untreated control, (h) aqueous *T. ferdinandiana* fruit extract, and (i) aqueous *T. ferdinandiana* leaf extract.

Figure 5: Caspase 3 activity of Caco2 cells treated with half IC_{50} concentrations of *T. ferdinandiana* fruit and leaf ethyl acetate, methanolic and aqueous extracts. NC =

untreated control; FE = fruit ethyl acetate extract; FM = methanolic fruit extract; FW = aqueous fruit extract; LE = leaf ethyl acetate extract; LM = methanolic leaf extract; LW = aqueous leaf extract. Results are expressed as mean $\pm$ SEM of the absorbance at 405 nm. * indicates results that are significantly different to the untreated control ($p<0.01$).

Figure 6: The lethality of the *T. ferdinandiana* extracts (2000 $\mu\text{g/mL}$) and the potassium dichromate control (1000 $\mu\text{g/mL}$) towards *Artemia* nauplii following 24 hours exposure. NC = negative control; M = methanolic *T. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; PC = positive control (1000 $\mu\text{g/mL}$ potassium dichromate). Shaded bars represent fruit extracts and open bars represent leaf extracts. All tests were performed in at least triplicate and the results are expressed as mean $\pm$ SEM. * indicates results that are significantly different to the negative control ($p<0.01$).

Figure 1: Antiproliferative activity of the *T. ferdinandiana* (a) fruit and (b) leaf extracts against Caco2 and HeLa cancer cell lines measured as percentages of the untreated control cells. NC = untreated control; M = methanolic *T. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; Cis = cisplatin control (50 $\mu\text{g/mL}$). Results are expressed as mean percentages $\pm$ SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

298x339mm (96 x 96 DPI)

Figure 2: Antiproliferative activity of the *T. ferdinandiana* (a) fruit 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. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; Cis = cisplatin control (50 $\mu\text{g/mL}$). Results are expressed as mean percentages $\pm$ SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

286x364mm (96 x 96 DPI)

Figure 3: Antiproliferative activity of the *T. ferdinandiana* (a) fruit 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. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; Cis = cisplatin control (50 $\mu\text{g/mL}$). Results are expressed as mean percentages $\pm$ SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

296x355mm (96 x 96 DPI)

Figure 4: Morphological effects of viable Caco2 cells (a) without treatment, and cells exposed to (b) *T. ferdinandiana* fruit ethyl acetate extract, (c) *T. ferdinandiana* leaf ethyl acetate extract, (d) untreated control, (e) *T. ferdinandiana* fruit methanol extract; (f) *T. ferdinandiana* leaf methanol extract, (g) untreated control, (h) aqueous *T. ferdinandiana* fruit extract, and (i) aqueous *T. ferdinandiana* leaf extract.

240x273mm (96 x 96 DPI)

Figure 5: Caspase 3 activity of Caco2 cells treated with half IC₅₀ concentrations of *T. ferdinandiana* fruit and leaf ethyl acetate, methanolic and aqueous extracts. NC = untreated control; FE = fruit ethyl acetate extract; FM = methanolic fruit extract; FW = aqueous fruit extract; LE = leaf ethyl acetate extract; LM = methanolic leaf extract; LW = aqueous leaf extract. Results are expressed as mean $\pm$ SEM of the absorbance at 405 nm. * indicates results that are significantly different to the untreated control ($p < 0.01$).

232x158mm (96 x 96 DPI)

Figure 6: The lethality of the *T. ferdinandiana* extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *Artemia nauplii* following 24 hours exposure. NC = negative control; M = methanolic *T. ferdinandiana* extract; W = aqueous *T. ferdinandiana* extract; E = ethyl acetate *T. ferdinandiana* extract; C = chloroform *T. ferdinandiana* extract; H = hexane *T. ferdinandiana* extract; PC = positive control (1000 µg/ml potassium dichromate). Shaded bars represent fruit extracts and open bars represent leaf extracts. All tests were performed in at least triplicate and the results are expressed as mean ± SEM. * indicates results that are significantly different to the negative control ($p < 0.01$).

293x158mm (96 x 96 DPI)

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

	Fruit extracts					Leaf extracts				
	M	W	E	C	H	M	W	E	C	H
Yield (mg)	364	590	25	102	11	331	471	59	59	58
Extract concentration (mg/mL)	36	59	4	10	1	33	50	60	60	60
Antioxidant capacity (mg AA equivalents/g dried plant material)	660	264	39	7	1	150	340	22	5	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 that phytochemical class; M = methanolic extract;

W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; AA = ascorbic acid.

Table 2: The IC₅₀ of the *T. ferdinandiana* fruit and leaf extracts against the cancer cell lines and detection of toxicity and quantification of LC₅₀ values (µg/mL).

		Fruit extracts					Leaf extracts				
		M	W	E	C	H	M	W	E	C	H
Carcinoma cell IC ₅₀ 's (ug/mL)	HeLa	1250	1060	800	NA	NA	2240	606	487	NA	NA
	Caco2	438	1520	600	NA	NA	1244	1230	102	NA	NA
	Jeg-3	525	1040	645	NA	NA	147	330	441	733	NA
	JAR	1050	1170	1290	NA	NA	602	752	690	NA	NA
	MC3T3-E1	443	667	780	ND	ND	40	740	6	3540	ND
	MG63	1046	780	357	3720	ND	846	1500	384	3540	ND
Toxicity	<i>Artemia nauplii</i>	875	983	NA	NA	NA	1239	928	1486	NA	NA
	LC ₅₀ 's (ug/mL)										
	HDF viability assay	-	-	-	-	-	-	-	-	-	-

Values indicate the mean IC₅₀ or LC₅₀ values of triplicate determinations. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NA = an IC₅₀ or LC₅₀ 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.

Table 3: Qualitative HPLC-MS/MS analysis of the *T. ferdinandiana* leaf ethyl acetate extract, elucidation of empirical formulas and putative identification of each compound.

Putative identification	Empirical formula	Molecular mass	Retention time	Relative abundance (%) total peak area)	
				Negative	Positive
				ionisation	ionisation
				mode	mode
Protocatechuic acid	C ₇ H ₆ O ₄	154.0272	0.522	2.34	
Chebulinic acid	C ₄₁ H ₃₂ O ₂₇	956.113	8.996	1.3	6.03
Palmitic acid	C ₁₆ H ₃₂ O ₂	256.2412	23.868	3.38	
Exifone	C ₁₃ H ₁₀ O ₇	278.0433	9.32	1.13	
Ellagic acid	C ₁₄ H ₆ O ₈	285.1943	4.277	0.83	0.56
Gingerol	C ₁₇ H ₂₆ O ₄	294.1836	13.017	1.25	
Ellagic acid dihydrate	C ₁₄ H ₁₀ O ₁₀	338.0281	1.085		0.77
Trimethyl ellagic acid	C ₁₇ H ₁₂ O ₈	344.0538	14.237	1.3	0.85
Chebolic acid	C ₁₄ H ₁₂ O ₁₁	356.0391	0.356	4.66	4.49
Vitexin	C ₂₁ H ₂₀ O ₁₀	432.1064	9.397	0.77	5.59
Rutin	C ₂₇ H ₃₀ O ₁₆	610.521	8.73	2.84	7.99
Corilagin	C ₂₇ H ₂₂ O ₁₈	634.0819	7.803	10.74	0.69
Luteolin	C ₂₁ H ₂₀ O ₁₁	448.1025	8.345	9.67	
Quercetin	C ₁₅ H ₁₀ O ₇	302.0434	11.293		1.03
Punicalin	C ₃₄ H ₂₂ O ₂₂	782.0621	9.363	2.21	5.65
Castalagin	C ₄₁ H ₂₆ O ₂₆	934.0715	8.692	1.1	

Corilagin	$C_{27}H_{22}O_{18}$	634.082	7.654	1.32	1.66
Chebulagic acid	$C_{41}H_{30}O_{27}$	954.0965	9.878	2.52	

% peak area refers to the peak area for the individual compound expressed as a % of the total area under peaks for the chromatogram in the relevant mode.

Terminalia ferdinandiana Exell. fruit and leaf extracts inhibit proliferation and induce apoptosis in selected human cancer cell lines

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