Bioassay Guided Fractionation of *Pittosporum angustifolium* and *Terminalia ferdinandiana*: A Lc-Ms/Ms and Gc-Ms Exploratory Study

Janice Mani * , Joel B. Johnson , Holly Hosking , Ryan Batley , Ryan Du Preez , Daniel A Broszczak , Kerry Brian Walsh , Paul Neilsen , Mani Naiker

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Article

Bioassay Guided Fractionation of *Pittosporum angustifolium* and *Terminalia ferdinandiana*: A LC-MS/MS and GC-MS Exploratory Study

Janice Mani [1,2,*], Joel Johnson [1,2,3], Holly Hosking [1], Ryan Batley [1], Ryan du Preez [1], Daniel Broszczak [4], Kerry Walsh [1,2], Paul Neilsen [1] and Mani Naiker [1,2,5]

[1] College of Science and Sustainability, CQUniversity, Bruce Hwy, North Rockhampton, QLD 4701, Australia
[2] Institute for Future Farming Systems, CQUniversity, Bundaberg, QLD 4670, Australia
[3] Present address: Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Brisbane, QLD 4072, Australia
[4] School of Biomedical Sciences, Queensland University of Technology, Brisbane, QLD 4000, Australia
[5] Jawun Research Centre, Cairns, QLD 4870, Australia
* Correspondence: Janice.mani@cqumail.com or janice.mani@gmail.com

Abstract Bioprospecting native Australian plants offers potential discovery of latent and novel bioactive compounds. Promising cytotoxic and antibacterial activity of methanolic extracts of *Pittosporum angustifolium* and *Terminalia ferdinandiana* led to further fractionation and isolation using our laboratory's bioassay guided fractionation protocol. Hence, the aim of this study was to further evaluate the bioactivity of the fractions and subfractions and characterize bioactive compounds using liquid chromatography mass spectroscopy (LC-MS/MS) and gas chromatography MS (GC-MS). Compounds tentatively identified in *P. angustifolium* Fraction 1 using LC-ESI-QTOF-MS/MS were chlorogenic acid and/or neochlorogenic acid, bergapten, berberine, 8'-epitanegool and rosmarinic acid. GC-MS analysis data showed the presence of around 100 compounds, mainly comprising of carboxylic acids, sugars, sugar alcohols, amino acids, and monoalkylglycerols. Furthermore, the fractions obtained from *T. ferdinandiana* flesh extracts showed no cytotoxicity, except against HT29 cell lines, and only Fraction 2 exhibited some antibacterial activity. The reduced bioactivity observed in the *T. ferdinandiana* fractions could be attributed to the potential loss of synergy as compounds become separated within the fractions. As a result, further fractionation, and separation of compounds in these samples were not pursued. However, additional dose-dependent studies are warranted to validate the bioactivity of *T. ferdinandiana* flesh fractions, particularly since this is an understudied species. Moreover, LC-MS/GC-MS studies confirm the presence of bioactive compounds in *P. angustifolium* Fraction 1/subfractions which helps to explain the significant acute anti-cancer activity of this plant. The screening process designed in this study has the potential to pave the way for developing scientifically validated phytochemical/bioactivity information on ethnomedicinal plants, thereby facilitating further bioprospecting efforts, and supporting the discovery of novel drugs in modern medicine.

Keywords: bioassay guided fractionation; bioprospecting; LC-MS/MS; GC-MS; Australian plants; cytotoxicity; antibacterial

1. Introduction

Bioprospecting involves a multidisciplinary approach that entails the systematic discovery, isolation, and identification of new bioactive molecules from natural biological reserves, such as plants. Whilst medicinal plants have been used to treat various health conditions for centuries, conventional bioprospecting methods are often time consuming and expensive (Abdallah et al., 2021). Moreover, the complexity of plant matrices and the occurrence of numerous phytochemicals can make the separation and analysis process quite challenging.

The process of separating phytochemicals involves isolating the constituents in the plant extracts or effective parts and purifying them into monomer compounds using physical and chemical

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methods (Feng et al., 2019). Conventional isolation methods include solvent extraction, precipitation, crystallisation, fractional distillation, salting out, and dialysis. More modern techniques include column chromatography, high performance liquid chromatography, ultrafiltration, and high-performance liquid droplet counter current chromatography. There are several chromatographic techniques available for the identification and quantification of phenolic compounds in plants, including thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography alone or coupled with mass spectrometry (MS), capillary electrophoresis (CE) (2, 26–29) and micellar electro- kinetic chromatography (MEKC) (Nour et al., 2013).

Phenolic compounds are a highly complex class of naturally occurring molecules that possess a range of therapeutic properties. As a result, significant interest has been devoted to their analysis in medicinal plants and food samples. High performance liquid chromatography (HPLC) is the most commonly utilized separation technique for this purpose (Mekky et al., 2019; Naiker et al., 2020; Sochor et al., 2010; Zanatta et al., 2021).

High performance liquid chromatography equipped with a fraction collector was the analytical tool of choice for the separation and isolation of fractions/subfractions of extracts in this study due to its characteristic features of high efficiency, speed, and automation. The separation of compounds is based on the principles of the adsorptive capacity of column stationary phase to different compounds, molecular size of the compounds, difference of dissociation degrees of the chemical constituents, and different partition coefficients between stationary and mobile phase (Feng et al., 2019).

Based on the findings from our previous study (Mani, et al., 2022), the objective of this study was to further fractionate Fraction 1 of *P. angustifolium* methanolic crude extracts into subfractions, utilizing phase 3 of the proposed bioassay guided fractionation protocol design (Figure 1), and to perform LC-MS and GC-MS analysis for compound characterisation. Additionally, since *T. ferdinandiana* flesh crude extracts had also shown some therapeutic potential, fractionation and bioassay testing on the fractions were also included.

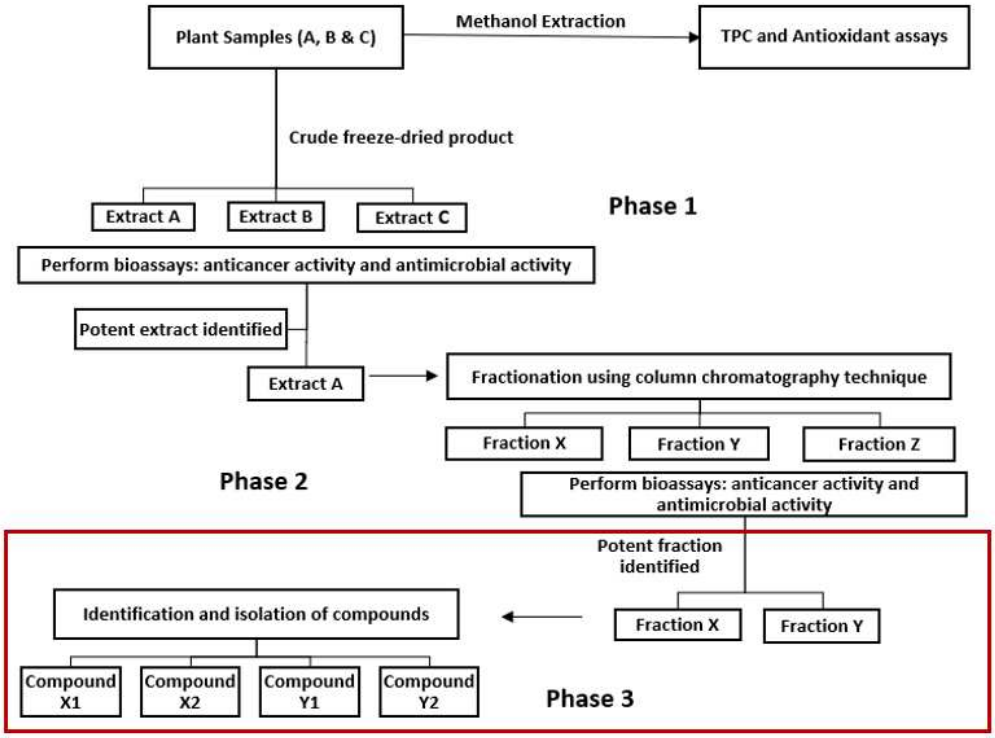

Figure 1. Bioassay guided fractionation protocol design (Mani, et al., 2022).

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2. Materials and Methods

2.1. Reagents

Hydrochloric acid and sodium carbonate were purchased from Chem-Supply (Australia). All other reagents including the HPLC grade methanol, were purchased from Sigma-Aldrich (Australia). Some reagents used in the cytotoxicity analysis which included the CellTiter 96® AQueous Assay (composed of solutions of tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine methosulfate; PMS), commonly known as MTS reagent, and foetal bovine serum (FBS) were obtained from Promega (United States of America) and Scientifix (Australia), respectively. The Dulbecco's Modified Eagle's Medium - high glucose (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS) solution was kept in the dark at 4°C, while the other reagents used in the bioassays were frozen until required for use. All dilutions and assay preparations used Milli-Q water. All reagents used were of analytical grade or higher purity.

2.1. Sample extraction

Approximately 2.5 g of powdered plant material was extracted in 75 mL of 90 % methanol as previously detailed (Mani, et al., 2022). The supernatant obtained was filtered using 0.45 µm Advantec filter paper and evaporated under reduced pressure at 27°C to a semi-solid consistency using a rotary evaporator. The semi-solid product was redissolved in approximately 25 mL Milli-Q water and freeze-dried under vacuum (Flexi-Dry Freeze-dryer, -47°C, 277 mTorr) for 72 h to obtain a fine lyophilized product, which was stored at 4°C in the dark until required.

2.2. HPLC fractionation and sub-fractionation of P. angustifolium extract

The HPLC conditions mentioned in our previous publication (Mani, et al., 2022) were followed with slight modifications. Briefly, a reversed-phase C18 column (Agilent Eclipse XDB-C18; 150 × 4.6 mm; 5 µm pore size) and guard cartridge (Gemini C18 4 × 2 mm) with an injection volume of 30 µL and a run time of mins with post run time of 5 mins was allowed for column flushing. The time slicing feature of the Agilent fraction collector was used to collect only Fraction 1 (0 to 12 min) from 20 mg mL^{-1} of *P. angustifolium* lyophilized product. The volume collected after multiple runs was then rotary evaporated to a semi-solid consistency and reconstituted in 30 mL Milli-Q water. This was then placed at -80 °C overnight and then freeze-dried for 72 hours. A crystalline product of a mass of 87.9 mg was obtained. The HPLC chromatogram of *P. angustifolium* Fraction 1 is depicted in Figure 2.

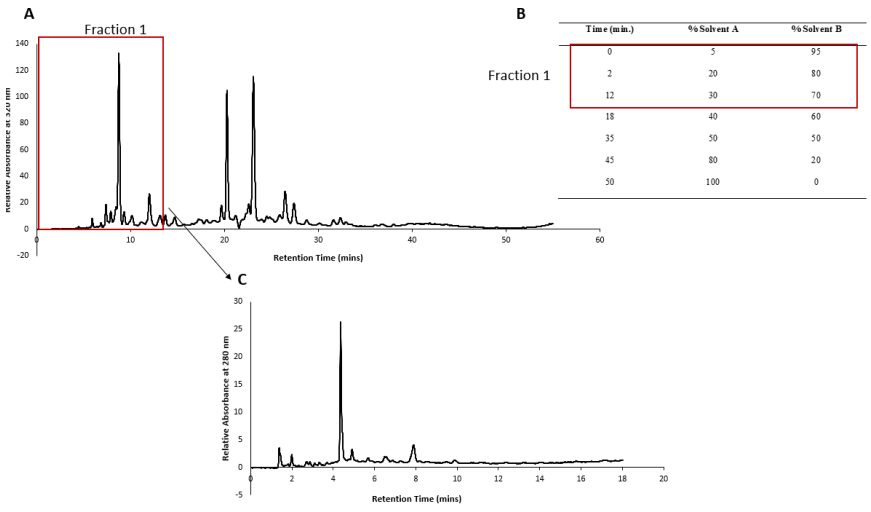

Figure 2. (A) Chromatogram of Pittosporum angustifolium extract showing retention times at which the five fractions were collected. (B) Elution gradient of P. angustifolium extract fractionation. (C) Chromatogram of P. angustifolium Fraction 1.

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The crystalline product obtained from Fraction 1 was redissolved in Milli-Q water at a concentration of 43.95 mg mL^{-1} and subjected to HPLC fractionation using gradient elution, as described in Figure 2 (B), and an injection volume of 30 µL. Retention time zones showing predominant peaks were selected for time slicing, and fractions were collected from 0-3 mins (Sub-fraction 1), 3-6 mins (Sub-fraction 2), and 6-11 mins (Sub-fraction 3), as depicted in Figure 3.

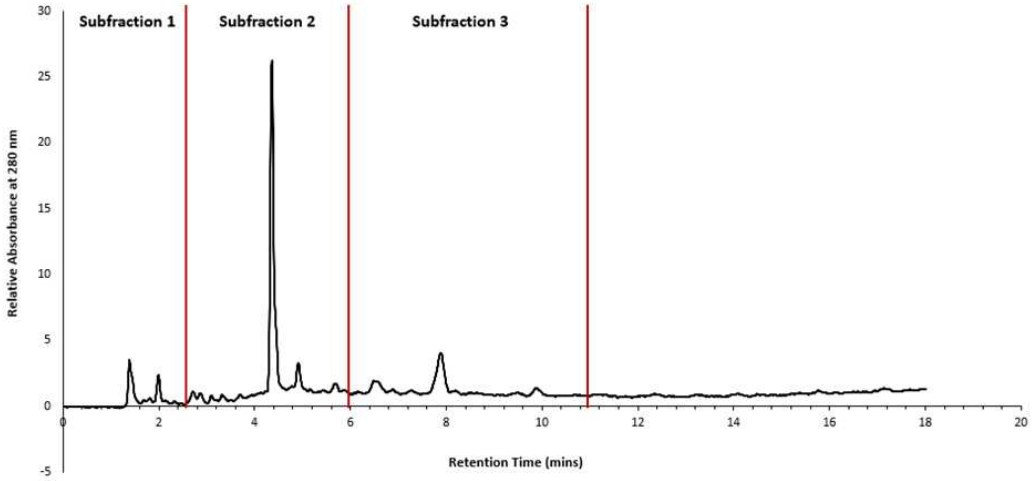

Figure 3. HPLC chromatogram of Pittosporum angustifolium Fraction 1 and the retention times (0-3 mins (Sub-fraction 1), 3-6 mins (Sub-fraction 2), and 6-11 mins (Sub-fraction 3)) at which the three subfractions fractions were collected.

2.3. HPLC fractionation of T. ferdinandiana flesh extract

The same HPLC conditions as described above (Section 2.2) and our previous publication (Mani, et al., 2022), was followed with slight modifications to the gradient elution and injection volume. The gradient elution described in Figure 2 (B) was used, and a sample injection volume of 30 µL was applied. A total run time of 50 mins was allowed to ensure that all eluents were captured in the chromatogram, and a post run time of 10 mins was allowed for column flushing.

Retention times showing predominant peaks were selected for time slicing and collection of fractions from 0-6 mins (Fraction 1), 6-16 mins (Fraction 2), 16-30 mins (Fraction 3) and 30-40 mins (Fraction 4), as depicted in Figure 4.

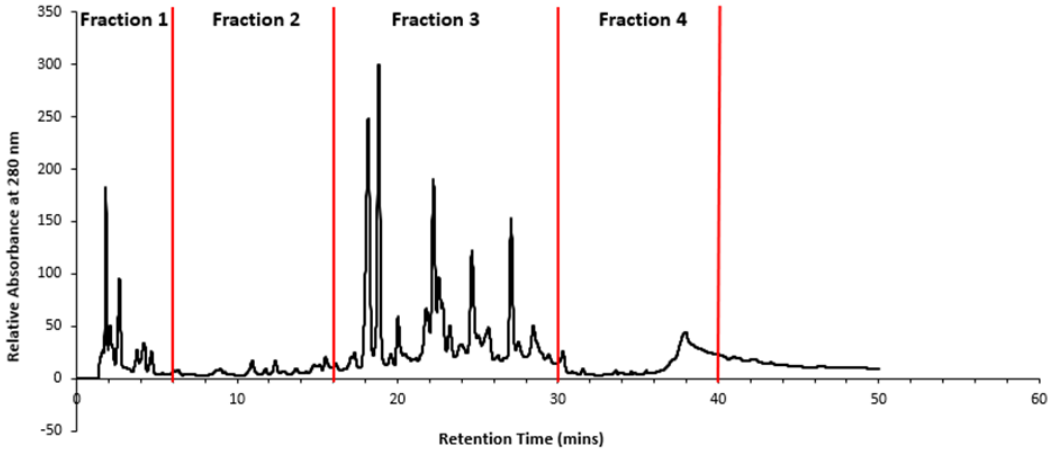

Figure 4. HPLC chromatogram of T. ferdinandiana extract and the retention times (0-6 mins (Fraction 1), 6-16 mins (Fraction 2), 16-30 mins (Fraction 3) and 30-40 mins (Fraction 4)) at which the four fractions were collected.

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2.4. Cytotoxicity Assay

The cytotoxicity of the subfractions of *P. angustifolium* Fraction 1 and fractions of *T. ferdinandiana* were assessed against HeLa (human cervical carcinoma), HT29 (human colorectal carcinoma), HuH7 (human liver carcinoma) and PH5CH8 (human epithelial cell), obtained from the University of Adelaide, using MTS assay previously described (Mani, et al., 2022). However, it was observed that the proliferation and general health of the HuH7 cells were compromised, and they were nonviable for use in the cell culture assay of *T. ferdinandiana* fractions in the later trials.

2.5. Antimicrobial Activity

The antimicrobial activity of *T. ferdinandiana* Fractions (1–4) were tested against the four bacterial strains (Gram positive -*Staphylococcus aureus* and Gram negative -*Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*) following the disk diffusion method (Mostafa et al., 2018) with slight modification as described previously (Mani, et al., 2022).

2.6. LC-MS/MS Analysis of P. angustifolium fraction

The methanolic extract of *P. angustifolium* was analyzed for targeted phenolic compounds using liquid chromatography tandem mass spectroscopy (LC-MS/MS). The analysis was performed using a Nexera X2 chromatography system, which was coupled with a Shimadzu LCMS-8040 system comprising of a CBM-20A communications bus module, DGU-20A5R degassing unit, LC-30AD pumps, SIL-30AC autosampler, and CTO-20AC column oven. The analytical method used a Raptor biphenyl column (100 mm X 2.1 mm, 2.7 µm), 5 µL injection volume, 40˚ C column temperature and flow rate of 0.6 mL min^{-1}. The mobile phase comprised water (phase A) and methanol (phase B), each containing 5 mM ammonium formate and 0.1% formic acid. The eluent was directed to the electrospray ionization (ESI) module.

Shimadzu LCMS-8040 model triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source was used to perform targeted tandem mass spectrometry on the eluting compounds. Both positive and negative ionization modes were used depending on the ionization characteristics of each analyte. The ESI conditions used were interface temperature of 350 °C, DL (Dissolution line) temperature 250 °C, and 400 °C in ESI source. Nitrogen was used as the nebulizing gas and drying gas, at flow rates of 3 L min^{-1} and 15 L min^{-1}, respectively and the interface voltage used was 4.50 kV. The LC-MS/MS data were collected and analysed in the LabSolutions software (Shimadzu, Kyoto, Japan). The PubChem database and offline version (accessed 21 March 2023) of the National Institute of Standards and Technology (NIST) Library was used to match the MS/MS spectra of phenolic compounds in the *P. angustifolium* extract.

2.7. GC-MS/MS Analysis of P. angustifolium fraction 1 and subfractions

All four samples were dissolved in 1 mL chilled MS grade water and mixed via vortex and kept on ice throughout processing where possible. Twelve aliquots were created for each sample. Three aliquots each of 1 µL, 10 µL, 100 µL and 200 µL were generated by transferring the sample into glass vial inserts. The aliquots were then frozen prior to lyophilisation at 1 mbar (room temperature) for 3.5 hrs until dry. The sample aliquots were then sealed in auto sampler vials and stored at -20 °C until analysis.

Samples were analysed on a Shimadzu TQ8050 NX system following automated trimethylsilyl (TMS) derivatisation using an AOC 6000 plus auto-sampler. The derivatisation was accomplished by adding 25 µL of 30 mg mL^{-1} methoxyamine hydrochloride in pyridine to 10 µL of dried metabolite extract. The samples were then incubated at 37 °C for 2 hrs with continuous agitation. Following this, 25 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added, with further incubation at 37 °C for 1 hr with continuous agitation. Derivatised sample was then incubated at room temperature for 1 hr prior to injection. One microliter of the derivatised samples were injected onto the GC-MS/MS by the auto-sampler at a split ratio of 5:1 and a constant flow of 1.10 mL min^{-1} and the oven temperature was maintained at 100˚C. The samples were analysed in Multiple Reaction Monitoring

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(MRM) mode using the Shimadzu Smart Metabolite Database containing 521 MRM metabolite targets. Prior to each sample being analysed, four hexane blanks were analysed to ensure there was no carry over between samples.

Data produced from the analysis of samples was extracted using LabSolutions Insight software. Metabolites that were within ± 0.1 min of the predicted retention time and within ± 25% of the relative ion ratio had their area under the curve reported for further statistical analysis. Where metabolites did not need these criteria, the area under the curve was not reported.

2.8. Statistical Analysis

All data were presented as means ± SEM for triplicate samples. Statistical analyses were performed using RStudio version 2022.12.0-353 software, with a one-way analysis of variance to determine the significance between the control and treated groups. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Pittosporum angustifolium sub fractions

3.1.1. Cytotoxic Activity and HPLC Profiling

The sub-fractionated products obtained from *P. angustifolium* Fraction 1 (GGLX F1 S1-3) were subjected to an anticancer bioassay, and the results are presented in Figure 6. There was no significant difference ($p > 0.05$) in the percentage cell viability between of the treated cells and negative control for all the tested cell lines, except for the HT29 cells ($p < 0.05$), where all three subfractions demonstrated some cytotoxic activity.

The low bioactivity observed in the subfractions may be attributed to the low doses of compounds present in these subfractions, as indicated by low peak signals in the HPLC chromatograms shown in Figure 7. Additionally, the cytotoxic effect observed in the methanolic extracts of *P. angustifolium* and its fractions may be due to the synergistic action of different compounds. On the other hand, the subfractions contain perhaps fewer isolated compounds, which may explain the low or no cytotoxicity observed.

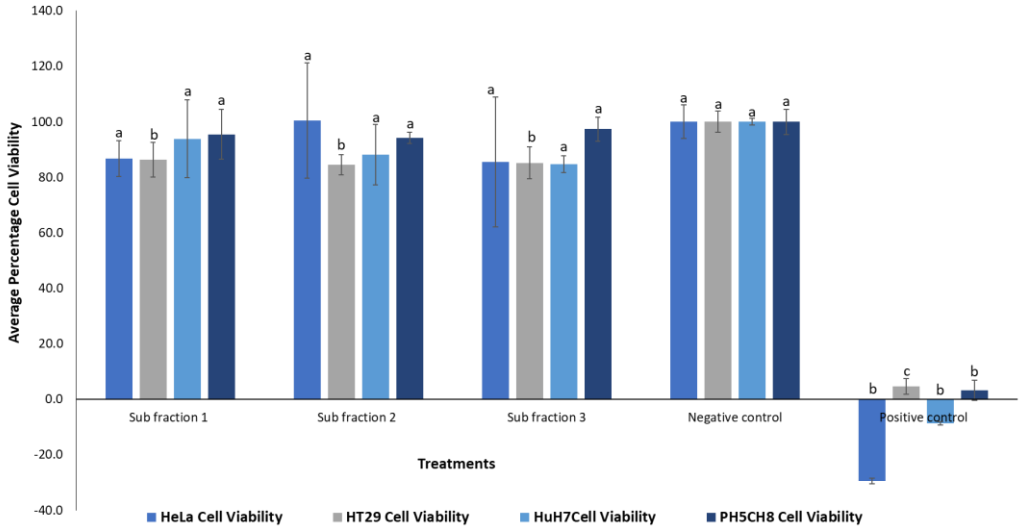

Figure 6. Percentage cell viability of cell lines treated with Pittosporum. angustifolium Fraction 1 subfractions 1, 2 and 3 of concentrations 16.55, 12.15 and 16.15 µg mL^{-1}, respectively. One-way ANOVA test indicated no significant difference (p-value > 0.05) in cytotoxicity between the different subfractions for the same cell line, denoted by same letters on the respective bars, except in the case

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of HT29 cells. Negative control: cells without treatment, positive control: cell treated with 50 ug mL-1 cisplatin (chemotherapy drug).

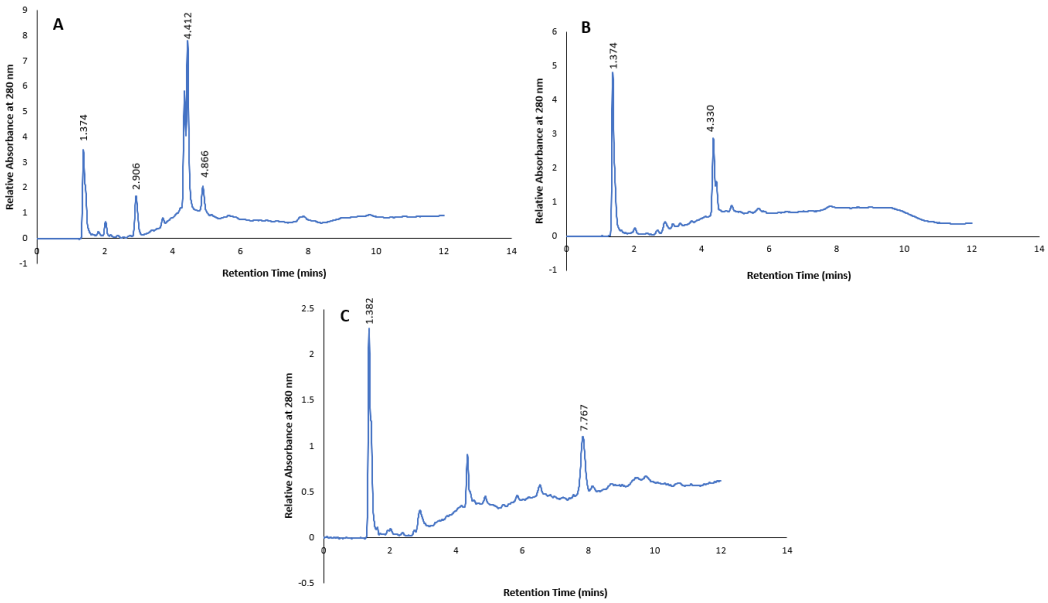

Figure 7. HPLC profiles of Pittosporum angustifolium subfractions 1 (A), subfraction 2 (B) and subfraction 3 (C).

Although numerous studies have identified triterpenoid saponins, terpenoids, phenols, and coumarin compounds isolated from *P. angustifolium* as having potential anticancer properties (Bäcker, et al., 2014a; Beh & Teoh, 2022; Blonk & Cock, 2019; Phan et al., 2020; Winnett et al., 2017), only a few have been shown to be effective *in vitro*. Backer et al (2016) screened ten acylated saponins for their ability to inhibit human DNA-topoisomerase I, an enzyme responsible in resolving torsional stress associated with DNA replication, transcription, and chromatin condensation. Inhibitors of DNA-topoisomerase I can inhibit the proliferation of cancer cells, and such agents are commonly used in chemotherapy for their antiproliferative effects. However, their effects on the metastasis of cancer cells remain unclear (Liu et al., 2019).

In previous work, Backer et al (2015) isolated two new taraxastane-type triterpene saponins, which were evaluated against four cell lines. However, no cytotoxic activity was observed up to a concentration of 130 µM. In a similar sub fractionation study of *Syzygium polyanthum* (Wight.), the crude methanol extract showed higher bioactivity in terms of hypoglycemic effect compared to the fraction, subfraction, and squalene (the major chemical compound and a triterpene isolated from *S. polyanthum* leaf extract) (Widyawati et al., 2022). Therefore, the cytotoxic properties of *P. angustifolium* could possibly be from a synergistic effect, similar to that seen in other studies.

In addition, authentic standards of selected polyphenols (4-hydroxybenzoic acid, caffeic acid, catechin, catechol, chlorogenic acid, gallic acid, isovanillic acid, neochlorogenic acid, protocatechuic acid, syringic acid, tyrosol and vanillic acid) were subjected to the same HPLC gradient elution as the subfractions, and the combined chromatograms with retention times are shown in **Figure 8**. When compared to the retention times of the predominant peaks in the chromatograms of the subfractions **(Figure 7)**, only one peak at retention time 4.412 min in subfraction 1 could tentatively be identified as gallic acid based on similar UV spectrum as the standard. However, there were some discrepancies between the retention time of the standard gallic acid (4.009 mins) and the subfraction (4.412 mins). In subfraction 2, none of the retention time peaks or UV spectra matched any of the authentic standards. On the other hand, the retention time of a predominant peak at 7.768 mins in subfraction 3 was tentatively identified as 4-hydroxybenzoic acid, as similar retention time (7.775 mins) and UV spectrum was evident (**Figure 8**).

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As the subfractions of *P. angustifolium* did not show any significant cytotoxic activity, further separation and isolation was deemed impractical and hence were not pursued.

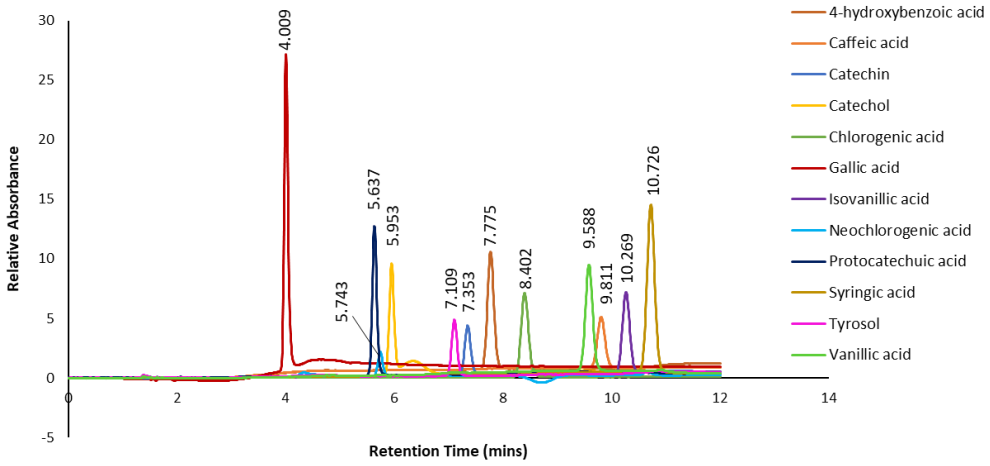

Figure 8. Chromatogram and retention times of selected phenolic standards.

3.1.2. LC- MS Analysis of P. angustifolium Fraction 1

While the subfractions of *P. angustifolium* fraction 1 did not demonstrate significant cytotoxic properties or contain compounds of interest, fraction 1 was found to be effective in reducing cell viability in the tested cancer cell lines, and it was also safer in terms of its selectivity index compared to the other fractions (Mani et al., 2022). Moreover, given the possibility of a synergistic effect at play in dictating the cytotoxic behaviour of *P. angustifolium* fraction 1, characterizing the potential phenolic metabolites in the fraction was considered a valuable pursuit.

Using LC-ESI-QTOF-MS/MS, untargeted screening, and characterization of phenolic compounds in Fraction 1 was performed. The obtained MS/MS spectra were compared with NIST and PubMed database libraries, as well as published literature, to putatively confirm the presence of phenolic metabolites (**Table 3**). Among ten prominent peaks, only peaks 1, 4, 7-9 were tentatively identified (**Figure 9)** in Fraction 1.

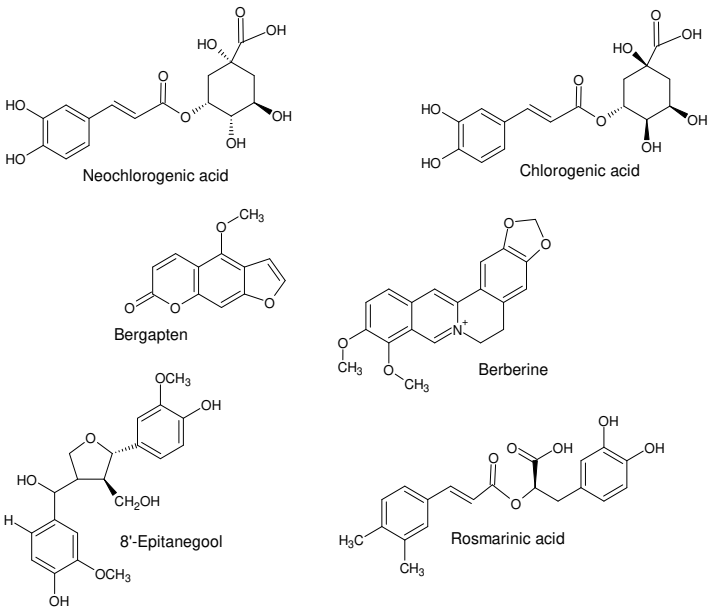

Figure 9. Proposed phenolic metabolites in Pittosporum angustifolium Fraction 1.

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Table 3. Tentative LC-MS characterization of compounds in Pittosporum angustifolium Fraction 1.

Peak no.	Proposed compound	Molecular formula	RT (min)	Mode of ionisation	Molecular Weight (g/mol)	Observed Precursor mass (m/z)	Theoretical mass (m/z)	Product ions (MS/MS)	Literature
1	Chlorogenic acid	$C_{16}H_{18}O_9$	9.13	positive	354.31	355.10	355.00*	65.0, 188.0, 219.0, 275.8	
1	Neochlorogenic acid	$C_{16}H_{18}O_9$	9.13	positive	354.31	354.31	355.00	65.0, 188.0, 219.0, 275.8	Xiao et al 2016
2	Unidentified	Unidentified	23.29	negative	Unidentified	229.10	Unidentified	157.1, 102.2	
3	Unidentified	Unidentified	29.15	positive	Unidentified	313.10	Unidentified	223.1, 158.2, 102.2	
4	Berberine	$C_{20}H_{18}NO_4^+$	30.65	positive	336.4	336.20	336.12**	287.2	Jiao et al 2018
5	Unidentified	Unidentified	33.09	positive	Unidentified	378.2	Unidentified	102.1, 249.2	
6	Unidentified	Unidentified	34.11	positive	Unidentified	326.9	Unidentified	102.3, 185.1, 228.3	
7	Bergapten	$C_{12}H_8O_4$	36.48	positive	216.042	217.10	217.05*	129.0, 202.0	
8	Rosmarinic acid	$C_{18}H_{16}O_8$	44.44	positive	360.3	361.20	361.09**	181.05, 139.04	
9	8'-epitanegool	$C_{20}H_{24}O_7Na$	45.07	positive	399.39	399.2	399.14	287.3, 304.2	Jiao et al 2018
10	Unidentified	Unidentified	46.07	positive	Unidentified	403.2	Unidentified	102.2, 329.2, 361	

* NIST Library ** PubChem .

Compound 1 (*m/z* 355.10) chromatogram and mass spectrum as shown in Figure 10 was identified as either chlorogenic acid or its isomer, neochlorogenic acid, both of which belong to the caffeoylquinic acid class of molecules. These compounds are known for their strong antioxidant, anticancer, anti-inflammatory, and antifungal properties (Jiao et al., 2018). Previous studied have also identified chlorogenic acid in *P. angustifolium* leaves (Bäcker, Jenett-Siems, Bodtke, et al., 2014; Beh & Teoh, 2022; Phan et al., 2020). Additionally, our previous work have suggested the presence of chlorogenic acid in crude MeOH extracts of *P. angustifolium* leaves (Mani, et al., 2022; Mani, et al., 2022a).

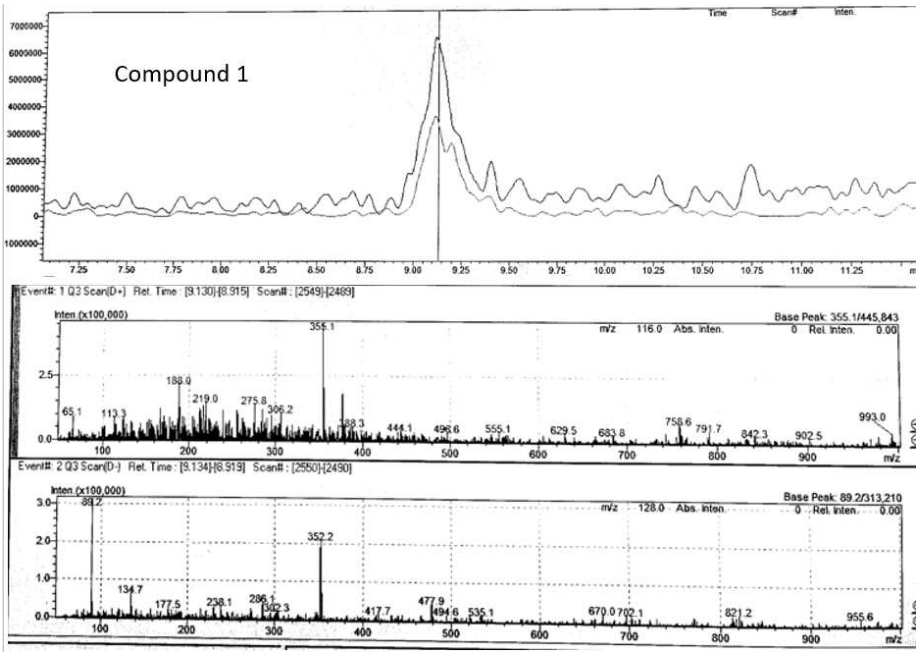

Figure 10. Chromatogram and mass spectrum of compound 1 in Pittosporum angustifolium Fraction 1.

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Compound 4 (m/z 336.20) chromatogram and mass spectrum as shown in Figure 11 was tentatively identified as berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride), a benzyl tetra isoquinoline alkaloid. Berberine been previously extracted from roots of various plants such as Berberis vulgaris, B. aristotle, B. aquifolium, Hydrastus canadensis, Pellodendron chenins, and Coptidis rhizomes (Rauf et al., 2021). Numerous authors have reported the broad spectrum therapeutic potential of berberine due to its action against diabetes, hypertension, depression, obesity, inflammation, and cancer (Jang et al., 2008; Kulkarni & Dhir, 2010; Rauf et al., 2021; Samadi et al., 2020). However, this is the first study to speculate the occurrence of berberine in P. angustifolium. Therefore, further investigation to confirm this finding is warranted.

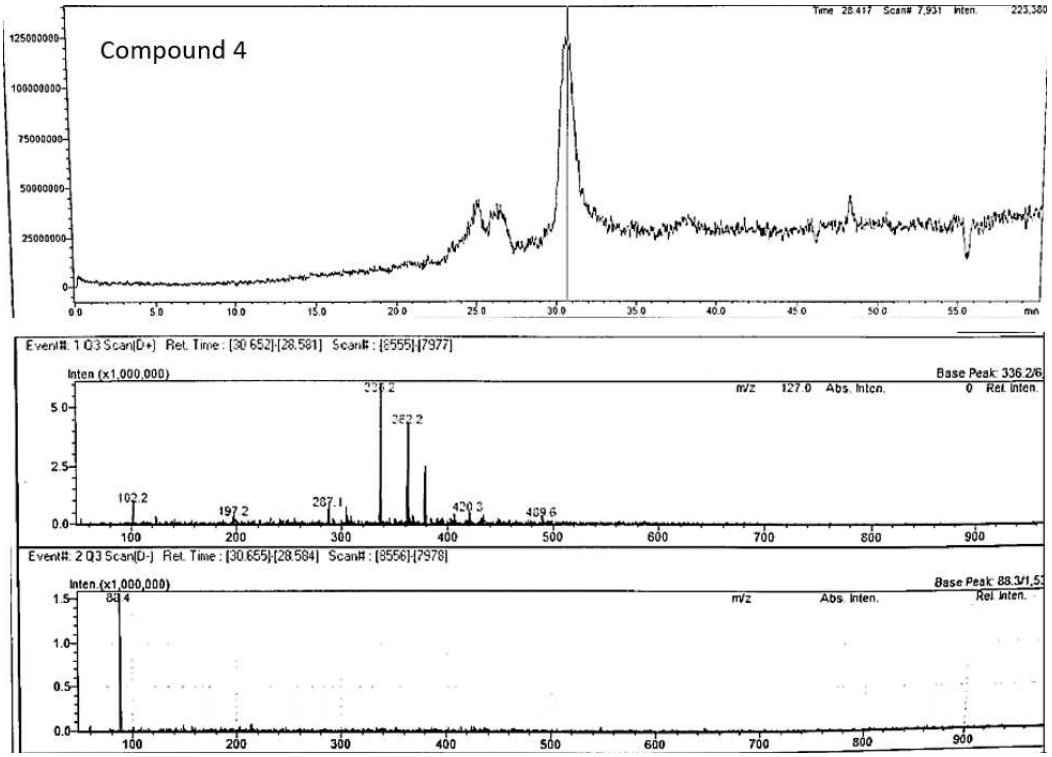

Figure 11. Chromatogram and mass spectrum of compound 4 in Pittosporum angustifolium Fraction 1.

Compound 7 (m/z 217.10) chromatogram and mass spectrum as shown in Figure 12 was identified as bergapten (5-methoxypsoralen), which belongs to the class furocoumarin. This furanocoumarin derivate is commonly found in bergamot essential oil, other citrus essential oils, and grapefruit juice, as well as in a wide variety of medicinal plants from the *Rutaceae* and *Umbelliferae* families such as figs, parsley, celery, and anise (Quetglas-Llabrés et al., 2022). Pharmacological studies have shown that bergapten has various properties, including neuroprotection, organ protection, anticancer, anti-inflammatory, antimicrobial, and antidiabetic effects (Liang et al., 2021; Quetglas-Llabrés et al., 2022). However, this is the first to report its potential occurrence in *P. angustifolium*, and further investigations are required to confirm this claim.

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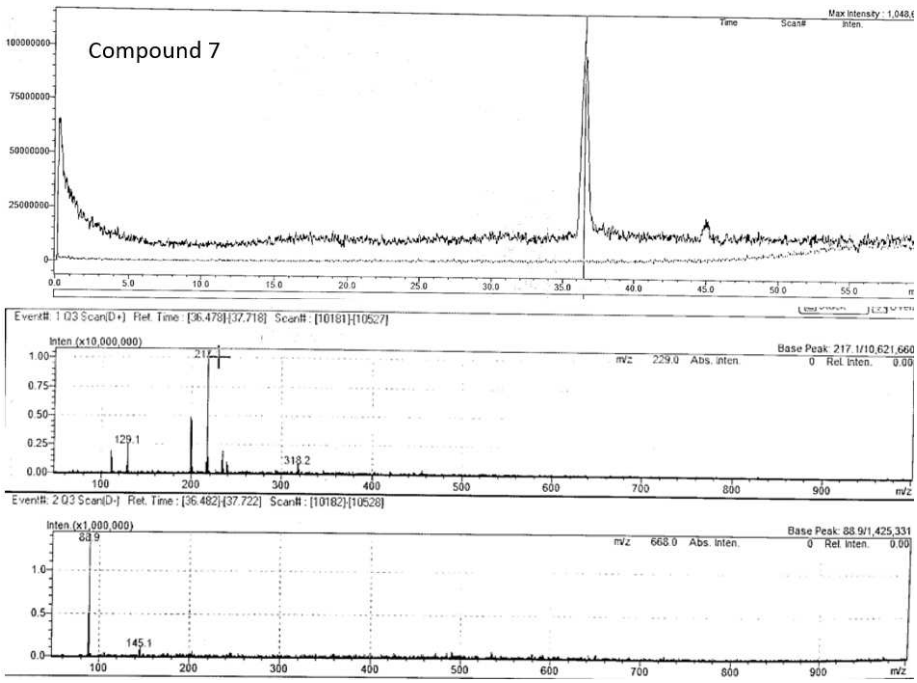

Figure 12. Chromatogram and mass spectrum of compound 7 in Pittosporum angustifolium Fraction 1.

Compound 8 (m/z 361.20) chromatogram and mass spectrum as shown in Figure 13 was tentatively identified as rosmarinic acid, which belongs to the hydroxycinnamic acid class of phenolic acids and is commonly found in fruits, herbs and medicinal plants (Ali et al., 2022). It is produced by *Boraginaeceae* and subfamily *Nepetoideae* of the *Lamiaceae* plant species. Initially, it was extracted as a pure compound from rosemary (*Rosmarinus officinalis*) (Nunes et al., 2017). This compound has shown potent biological activities in combating human diseases such as cancer, diabetes, neurodegenerative disorders, cardiovascular disease, and inflammatory disorders (Noor et al., 2022). While this is the first study to tentatively report its occurrence in *P. angustifolium*, further investigation is needed to confirm this finding.

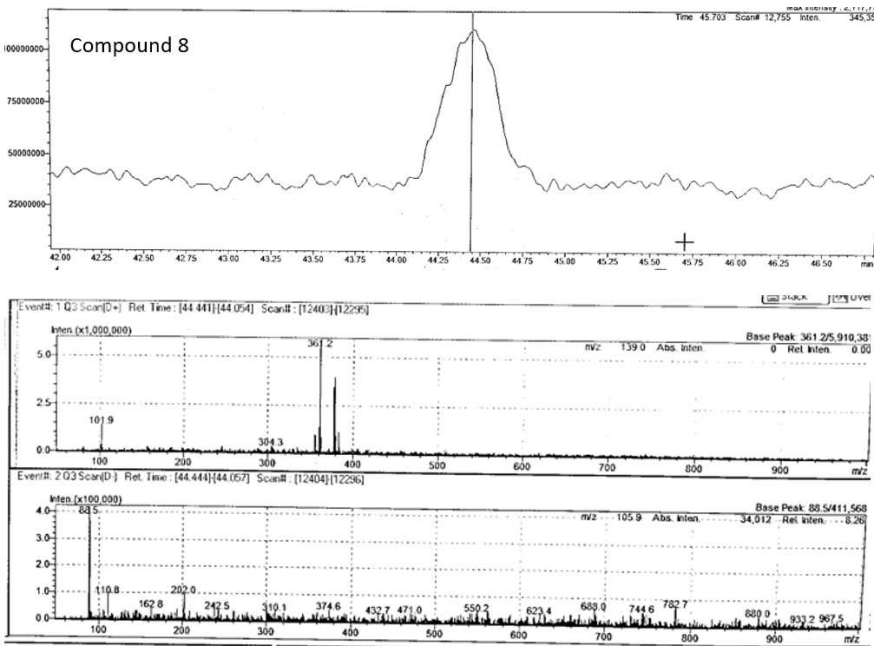

Figure 13. Chromatogram and mass spectrum of compound 8 in Pittosporum angustifolium Fraction 1.

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Compound 9 (m/z 403.2) chromatogram and mass spectrum as shown in Figure 14 was tentatively identified as 8'-epitanegool, classified as phenylpropanoids. Previous study has demonstrated promising *in silico* antiviral results similar to the main alkaloids (Omar et al., 2023). This compound has been previously identified in *Tinospora sinensis*, a type of Chinese folk medicine (Jiao et al., 2018), and literature on its therapeutic potential is limited or non-existent. Additionally, this is the first study to tentatively identify this compound in *P. angustifolium*.

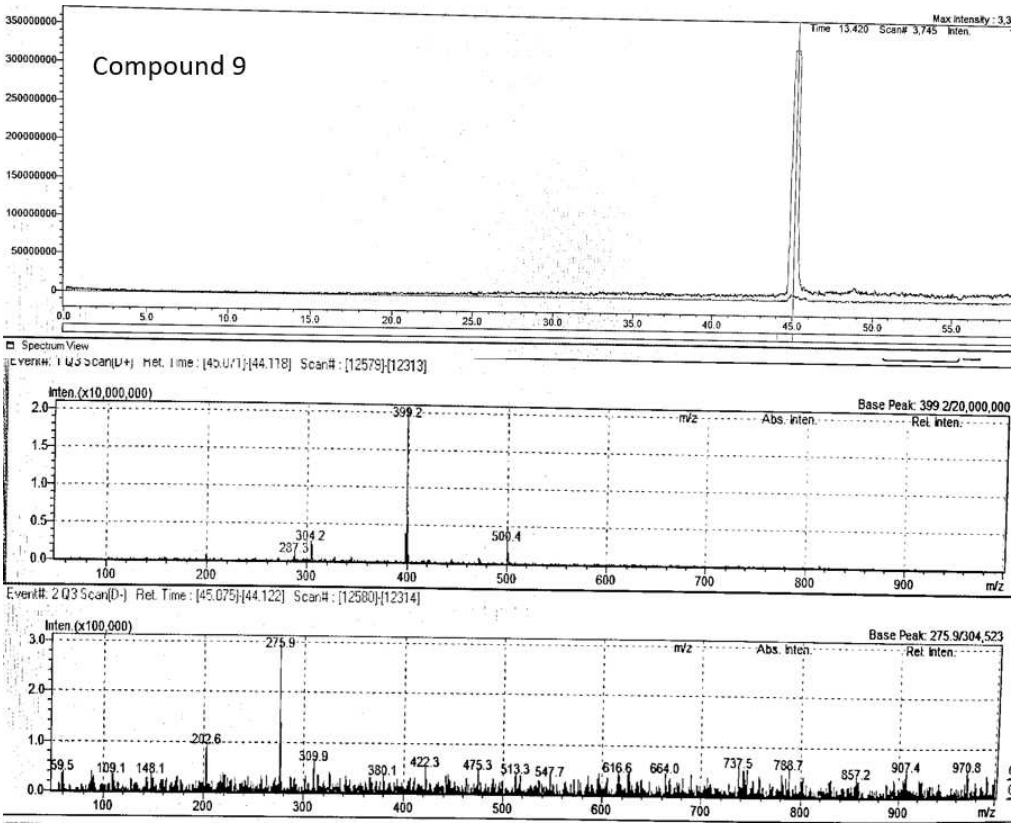

Figure 14. Chromatogram and mass spectrum of compound 9 in Pittosporum angustifolium Fraction 1.

If the identities of these compounds are confirmed in Fraction 1 of *P. angustifolium*, then it will not only strongly support its antioxidant and anticancer properties, as determined in this study, but also the anecdotal claims of Indigenous Australians (Phan et al., 2020). Furthermore, detailed studies to confirm the identity of these compounds and/or to discover other bioactive compounds in *P. angustifolium* crude, fractions and subfractions were conducted using GC-MS.

3.1.3. GC- MS Analysis of Pittosporum angustifolium Fraction 1 and sub-fractions

Targeted GC-MS/MS analysis in MRM acquisition mode identified a total of 103 compounds, belonging to various classes of primary and secondary plant metabolites. The main primary metabolites identified are classified as carbohydrates, amino acids, proteins, lipids, purines and pyrimidines of nucleic acids. On the contrary, the secondary metabolites identified were classified into three main groups; (a) nitrogen-containing compounds such as alkaloids, glucosinolates, and cyanogenic glycosides, (b) phenolic compounds such as phenylpropanoids and flavonoids, and (c) terpenes (Rabizadeh et al., 2022). However, only twenty predominantly found compounds in Fraction 1 and Fraction 1 subfractions are reported in Figure 15 and Table 4 which mainly belong to the class of carbohydrates and amino acids.

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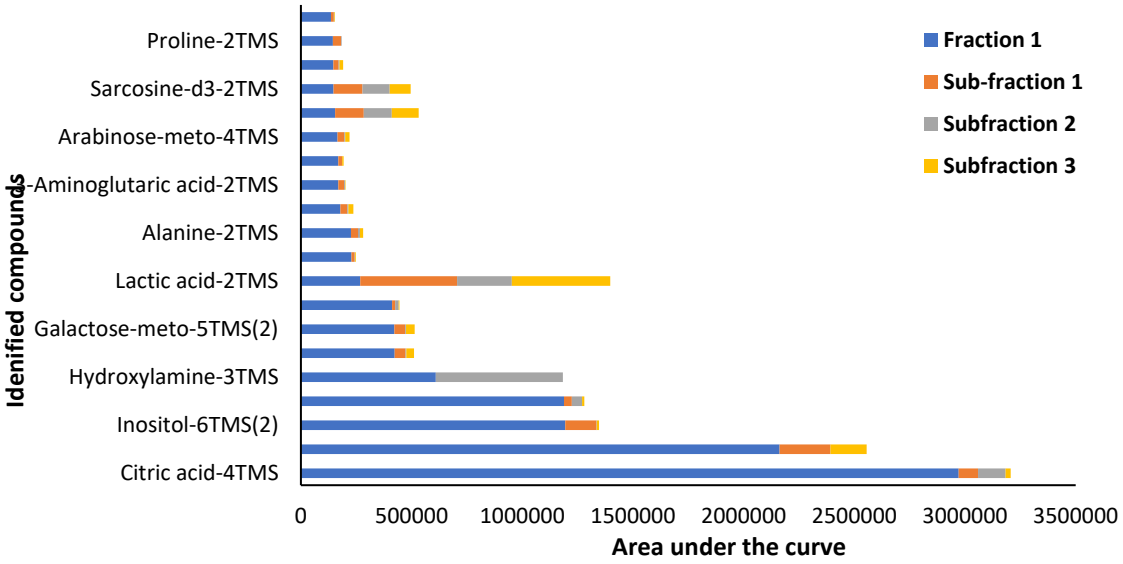

Figure 15. Targeted GC-MS/MS compounds identified in Pittosporum angustifolium Fraction 1 and Fraction 1 sub-fractions 1,2 and 3.

Table 4. Target GC-MS/MS peak area and classification of compounds identified in Pittosporum angustifolium Fraction 1 and Fraction 1 sub-fractions 1, 2 and 3.

	Target	Fraction 1	Sub-fraction 1	Subfraction 2	Subfraction 3	Compound Class
1	Citric acid-4TMS	2971610	87281	124060	23465	Carboxylic acids
2	Glucose-meto-5TMS(1)	2162365	230045	0	163277	Carbohydrate
3	Inositol-6TMS(2)	1194216	141218	1123	10389	Carbocyclic sugar
4	2-Aminopimelic acid-3TMS	1188393	35263	46722	10226	Amino acid
5	Hydroxylamine-3TMS	609044	0	574334	0	Hydroxylamine
6	Glucose-meto-5TMS(2)	423257	49736	3456	34808	Carbohydrate
7	Galactose-meto-5TMS(2)	422411	49066	3587	38804	Carbohydrate
8	1,5-13C2-Citric acid	412327	13144	16485	3281	Carboxylic acids
9	Lactic acid-2TMS	268289	437458	247071	444661	Carboxylic acids
10	1,6-Anhydroglucose-3TMS	228502	14470	1080	4143	Carbohydrate
11	Alanine-2TMS	225866	32076	8606	14237	Amino acid
12	Xylose-meto-4TMS(1)	178497	31305	4775	22749	Carbohydrates
13	3-Aminoglutaric acid-2TMS	169217	26046	4177	2165	Amino acid
14	4-Aminobutyric acid-3TMS	169048	17510	0	6009	Amino acid
15	Arabinose-meto-4TMS	165445	30249	4666	19117	Carbohydrates
16	Palmitic acid-TMS	154588	129131	126973	120981	Saturated fatty acid
17	Sarcosine-d3-2TMS	147242	130615	123520	94609	Amino acid
18	Lyxose-meto-4TMS(2)	146571	23892	3754	16430	Carbohydrates
19	Proline-2TMS	144359	37171	3022	0	Amino acid
20	Malic acid-3TMS	135932	12652	2318	3148	Carboxylic acids

Inositol (Figure 16), the third most abundant compound identified in Fraction 1 was of interest due to its previously reported anti-atherogenic, anti-oxidative, anti-inflammatory and anti-cancer properties (Siracusa & Napoli, 2022). Clinical trials using inositol in pharmacological doses have shown promising results in the management of gynaecological diseases, respiratory stress syndrome, Alzheimer's disease, metabolic syndrome, and cancer (Bizzarri et al., 2016). Inositol occurs naturally in all eukaryotes and is involved in several biological processes (Siracusa & Napoli, 2022). In mammals inositol is produced in the liver and kidney and myo-inositol (inositol isomer) and its MDPIderivatives in particular are involved in biological functions which include modulation of glucose metabolism, calcium release in cell signalling, chromatin and CSK remodelling, gene transcription, proliferation, apoptosis, and proper structural development (Bizzarri et al., 2016). In plants, inositol is well known for acting a stress ameliorator and controls multiple aspects of plant signalling and physiology (Amaral & Brown, 2022). On this premise the cytotoxic effects of Fraction 1 may likely be due to the predominant occurrence of inositol. However, in our knowledge this study

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is the first to report the occurrence of inositol in *P. angustifolium* and thus further investigation are required to confirm this finding.

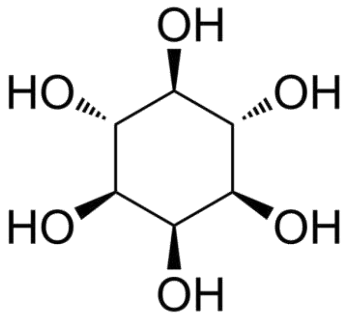

Figure 16. Chemical structure of inositol.

3.2. Terminalia ferdinandiana Fractions

3.2.1. Cytotoxic Activity

The lyophilised methanolic flesh extract of *T. ferdinandiana* was fractionated into four fractions, as listed in Table 5. The cytotoxicity of these fractions was evaluated against HeLa, HT29 and PH5CH8 cell lines using the concentrations given in Table 5. The results of this experiment are presented in Figure 17.

Table 5. Terminalia ferdinandiana flesh (KPF) lyophilised fractions.

Fractions	Crystal product obtained (mg)	Concentrations of fractions tested (mg/mL)
KPF1	38.10	0.095
KPF 2	169.00	0.423
KPF 3	77.40	0.194
KPF 4	17.60	0.044

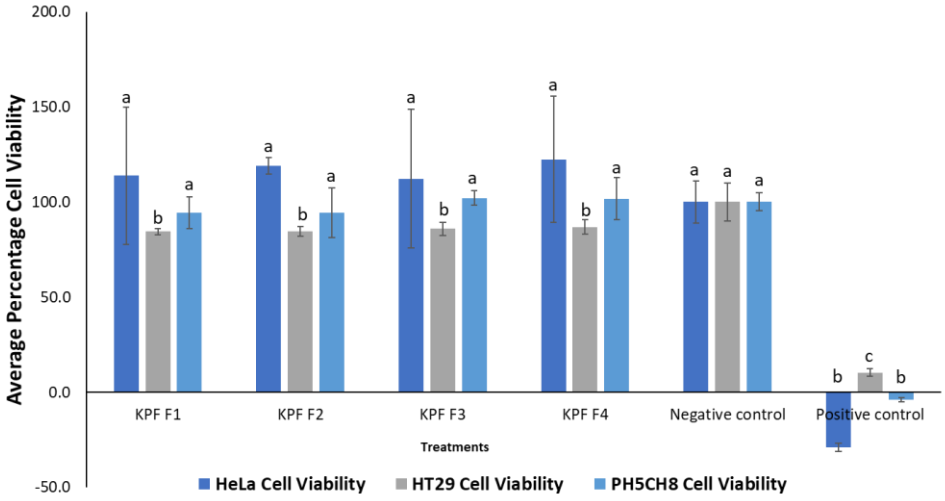

Figure 17. Percentage cell viability of cell lines treated with Terminalia ferdinandiana Fractions. One-way ANOVA test indicated no significant difference ($p > 0.05$) in cytotoxicity between the different subfractions for the same cell line, denoted by same letters on the respective bars, except in the case of HT29 cells. Negative control: cells without treatment, positive control: cell treated with 50 ug mL^{-1} cisplatin (chemotherapy drug).

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None of the fractions showed significant ($p > 0.05$) cytotoxic activity against HeLa and PH5CH8 cell lines, except for slight cytotoxicity observed against the HT29 cell line. This cytotoxicity was significantly different from the negative control ($p < 0.05$). To minimize matrix effect, lower doses of the fractions were used in the bioassay. However, in future studies, higher doses could be utilized to investigate potential higher toxicity.

While previous literature (Akter et al., 2018; Deo et al., 2016; Tan, Konczak, Ramzan, & Sze, 2011) has shown cytotoxic properties of *T. ferdinandiana* , there are limited studies on their fractions (Tan, et al., 2011). As such this study is crucial in paving the way for future fractionation studies of this species.

3.2.2. Antibacterial Activity

Considering the promising antibacterial activity demonstrated by the crude flesh extract of T. ferdinandiana (Mani et al., 2022), the fractions were also evaluated against four bacterial strains. The results are presented in Table 6.

Table 6. Average zone of inhibition (n=3) of Terminalia ferdinandiana flesh fractions.

Kakadu plum fractions	Concentrations µg/mL	Bacterial strain zone of inhibition (mm)			
		Gram positive	Gram negative		
		S. aureus	*E. coli*	*P. aeruginosa*	*S. typhimurium*
1	58.1	4.00 ± 0.71	0.00	0.00	0.00
2	169.0	4.30 ± 0.35	3.20 ± .10	2.10 ± 0.20	2.30 ± 0.40
3	77.9	0.00	0.00	0.00	0.00
4	17.6	4.20 ± 0.25	0.00	0.00	0.00
Positive control (gentamicin)	10	13.67 ± 0.58	16.33 ± 0.58	13.00± 0.10	12.67 ± 0.58

All values are given as means ± 2 SD (n = 3).

The antibacterial activity exhibited by the fractions was relatively mild, and in some cases, (Fraction 3), no activity was observed. Fraction 2 demonstrated the highest activity and was the only fraction that inhibited the growth of all tested bacterial strains compared to the other fractions, possibly because it was the most concentrated fraction. Overall, even though the flesh extracts of *T. ferdinandiana* fruit have previously demonstrated antibacterial activity in several studies (Akter et al., 2021; Gorman et al., 2019; Noé et al., 2019) including our previous studies (Mani et al., 2022), the low activity of the fractions suggests that the bioactive compounds in the lyophilised extract may work synergistically to produce its antibacterial property.

Despite the small amount of existing literature on the therapeutic potential of *T. ferdinandiana*, this study provides the first report on the bioactivity of its fractions. However, further rigorous testing in necessary to validate the data obtained, especially in terms of dose-dependent anticancer and antibacterial effects. Therefore, more elaborate investigation on this understudied native fruit is warranted.

4. Conclusion

Whilst *P. angustifolium* crude leaf extracts and fractions demonstrated strong cytotoxic activities, no significant activity was evident in the subfractions. Thereby suggesting that the bioactivity may be attributed to the synergistic effect of the phenolic compounds present. Moreover, LC-MS/GC-MS studies confirm the presence of bioactive compounds in *P. angustifolium* fraction 1/subfractions which helps to explain the significant acute anti-cancer activity of this plant. Compounds tentatively identified in *P. angustifolium* Fraction 1 using LC-ESI-QTOF-MS/MS were chlorogenic acid and/or neochlorogenic acid, bergapten, berberine, 8'-epitanegool and rosmarinic acid. GC-MS analysis indicated predominant occurrence of compound inositol in *P. angustifolium* fraction 1 which may be responsible for its anticancer properties. This is the first study to report the occurrence of the above-

mentioned compounds in *P. angustifolium*, and further investigations are required to confirm these findings. Furthermore, fractions of *T. ferdinandiana* flesh extracts showed no cytotoxicity, except against HT29 cell lines and only Fraction 2 showed some antibacterial activity against the bacterial strains tested. The reduced bioactivity in the *T. ferdinandiana* fractions may again also be due to loss of synergy as compounds become separated in the fractions. Further dose dependent studies to validate the bioactivity *T. ferdinandiana* flesh fractions is warranted for this understudied species.

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