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Full Length Article

In vitro Cytotoxic Properties of Crude Polar Extracts of Plants Sourced from Australia

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ABSTRACT

Background: Numerous commercial pharmaceuticals – including anticancer, antiviral, and antidiabetic drugs have been developed from traditional plant-derived medicines. There are approximately 25,000 species of flora occurring in Australia, which are adapted to a range of harsh environments, and hence a plethora of novel compounds are still awaiting research in the context of their medicinal properties.
Objective: The current study therefore aimed to develop a systematic protocol for screening plants with potential cytotoxicity. Many studies have found polar compounds such as caffeic acid, coumaric acid, chlorogenic acid, quercetin, anthocyanins, hesperidin, kaempferol, catechin, ellagic acid, and saponins to be the bioactive components responsible for the therapeutic effects.
Methods: The total phenolic content (TPC) and antioxidant capacity (FRAP) of methanolic extracts of selected plants was first determined. A high correlation between the TPC values and FRAP values of the plant polar extracts were evident. Cell viability of the cancer cell lines were assessed using the MTS assay.
Results: Cells subjected to *Pittosporum angustifolium* Lodd. extracts at concentration of 250 $\mu g \cdot mL^{-1}$ showed no viable cells, comparable to cisplatin (a chemotherapy medication) used as a positive control (10 $\mu g \cdot mL^{-1}$). Some promising inhibitory effects were also seen with *Murrya koenigii* flower and leaves at concentration of 250 $\mu g \cdot mL^{-1}$, with only 43.46% and 63.88% cell viability, respectively. In contrast, extracts of *Citrus hystrix* (leaves) and *Sygyzium australe* (stamen) showed higher percentage cell viabilities (around 82–93%) at same concentrations. The phenolic profile of *P. angustifolium* B extracts demonstrating greater peak intensity and dominant peaks were tentatively identified as chlorogenic acid, p-coumaric acid, caffeic acid, t-ferulic acid and rutin.
Conclusion: The Australian species *Pittosporum angustifolium* was the most cytotoxic against HeLa and HT29 cells comparing to the other plant extracts tested. A HPLC profile of the *P. angustifolium* extract also showed an array of promising therapeutic phenolic compounds. Future work will aim to fractionate and isolate novel compounds from this species and test their bioactivity.

1. Introduction

Cancer is a leading cause of mortality globally accounting for nearly 10 million deaths in 2020 (Ferlay et al., 2020), despite technological advancement in conventional treatments (Dutt et al., 2019). To date, there are no anticancer drugs or treatments which are 100% safe and effective (Shukla and Mehta, 2015). Additionally, conventional treatments and synthetic based cytotoxicity drugs are often expensive and beyond the scope of low-income population. Medicinal plants and plants-based remedies therefore play a significant role in primary healthcare system in the prevention and treatments of many diseases including cancer, particularly for the rural populace (Koche et al., 2016). Several plant derived products and their analogues that have been identified as potent cytotoxicity agent include taxol (*Taxus brevifolia*), vinblastine (*Catharanthus roseus*), vincristine (*Catharanthus roseus*), topotecan (*Camptotheca acuminata)*, irinotecan (*Catharanthus roseus*), and camptothecin (*Camptotheca acuminata*) derivatives, as well as epipodophyllotoxins (Ediriweera et al., 2019; Shukla and Mehta, 2015).

Globally, herbal plants have been found to have a profound scope of phytochemicals with antioxidative properties and their use in cancer treatment have been reported to reduce adverse side effects and toxicity (Bhuyan et al., 2017; Dutt et al., 2019). Plants produce a diverse range of phytochemicals as defensive mechanisms to cope with environmental stressors (Mani et al., 2021). Hence the inexhaustible source of bioactive compounds provided by medicinal plants vary in terms of the plant species, varietal types, geographical locations and mechanism of action. Considering that Australia has approximately 25,000 species of

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Table 1
Plant samples investigated in this study.

Scientific name	Common name	Part of plant analysed
Murrya koenigii (L.) Spreng.	Curry leaf	Leaves, flowers
Syzygium australe (J.C.Wendl. ex Link) B.Hyland	Bush cherry	Stamen
Citrus hystrix DC.	Kaffir lime	Leaves
Pittosporum angustifolium Lodd. (Sample A - variety unknown)	Gumbi gumbi	Leaves
Pittosporum angustifolium Lodd. (Sample B - variety unknown)	Gumbi gumbi	Leaves

flora that have adapted to the harsh environment, there is a plethora of bioactive compounds still waiting to be characterised and investigated (Mani et al., 2021).

Moreover, Australian Aboriginal and Torres Strait Islander people have depended on traditional plant-based medicines for centuries, which has contributed to their long standing existence, as one of the world's oldest surviving cultures (Deo et al., 2016; Mani et al., 2021; Tan et al., 2015). The traditional preparatory methods of herbal remedies usually involves extraction of water soluble compounds (Biva et al., 2016; Ota and Ulrih, 2017; Sadgrove and Jones, 2014.). Many studies have also found polar compounds such as caffeic acid, coumaric acid, chlorogenic acid, quercetin, anthocyanins, hesperidin, kaempferol, catechin, ellagic acid and saponins to be the bioactive components responsible for the therapeutic effects of plants (Sakulnarmrat et al., 2013; Sakulnarmrat and Konczak, 2012; Sanches et al., 2016; Vuong et al., 2014).

Reports of cytotoxicity of several Australian plants with high antioxidant capacity are still limited or lacking (Jamieson et al., 2014), although anecdotal and empirically informed evidence shows promising cytotoxicity of Australian grown plants (Sadgrove and Jones, 2016). Our previously published study showed high total phenolic content and antioxidant capacity in a number of Australian sourced plants (Johnson et al., 2020). Subsequently, plant samples showing high phenolic and antioxidant capacity were selected for the current study to investigate their bioactivity against the HeLa and HT29 cancer cell lines a robust protocol was developed in cytotoxicity screening of crude plant extracts. HeLa and HT29 cell lines were selected as they are established cancer cell lines utilized extensively within in vitro tumor models for cervical and colorectal cancers. Moreover, HeLa cells are generally used as the first test for anticancer activity before moving to other cell lines, which can be harder to maintain. Both the HeLa and HT29 cell lines were also selected due to their quick proliferation and ease of use. This study aims to report the phytochemical quantitation and chemical profile of the selected samples and assimilate the correlation between antioxidant capacity and their cytotoxicity properties.

2. Materials and Methods

2.1. Sample collection and preparation

All plant samples were collected during the spring season of 2018 from various locations around Rockhampton and Bundaberg, in the Central Queensland, except for *Pittosporum angustifolium* unknown variety B. Dried samples of *P.angustifolium* unknown variety B was obtained from Westwood, Rockhampton Region. The plants were identified and confirmed by Professor Nanjappa Ashwath. Sample name and parts of plants collected/used are described in Table 1 (Johnson et al., 2020). Images of the plants used in this study are given in Fig. 1.

Approximately 10–20 g of healthy and viable fresh plant material (leaves, stamen, or flower) were sampled. The plant samples were washed with demineralized water, frozen at −80°C and then freeze-dried (−70°C, 700 mmTorr) for 2–6 days. The dried samples were ground into a coarse powder and sieved (<1.0 mm) (Johnson et al., 2020).

2.2. Reagents

Analytical and HPLC grade was purchased from ThermoFisher Scientific (United States of America). Hydrochloric acid and sodium carbonate were purchased from ChemSupply Australia (Adelaide, Australia). Folin-Ciocalteu reagent, gallic acid, sodium acetate, glacial acetic acid, TPTZ (2,4,6, -tris(2-pyridyl)-s-triazine), ferric chloride, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and phosphoric acid were purchased from Sigma-Aldrich (United States of America). All dilutions and assays for chemical analysis were prepared using Milli-Q water unless stated otherwise. The reagents were stored in dark at 4°C until use.

Reagents used in cytotoxicity testing bioassays were Dulbecco's Modified Eagle's Medium - high glucose (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS), L-Glutamine solution (L-glute), Penicillin-Streptomycin solution (Pen Strep) and Trypsin-EDTA solution, purchased from Sigma-Aldrich Australia. 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 Fetal bovine serum (FBS) were obtained from Promega (United States of America) and Cytiva (United States of America) respectively. The DMEM and DPBS solution were kept in the dark at 4°C, while the other reagents were frozen until required for use. All reagents used were of analytical grade purity.

2.3. Extraction protocol and measurement of TPC and antioxidant capacity

The extraction protocol for polar compounds, previously developed in our laboratory was followed (Johnson et al., 2020). Approximately 0.5 g of powdered sample was combined with 7 mL of 90% aqueous methanol (v/v). The sample was vortexed for 10 s, mixed in an end-over-end mixer for 60 min and centrifuged at 1000 × g for 10 min (Heraeus Multifuge Thermo Fisher Scientific (United States of America)). The supernatant was collected, and the extraction step was repeated with the addition of another 7 mL of 90% aqueous methanol (v/v) to the pellet. Total phenolics content (TPC) was measured using the Folin-Ciocalteu assay and the antioxidant capacity were measured using ferric reducing antioxidant capacity (FRAP) and cupric-reducing antioxidant capacity (CUPRAC) chemical assays (Johnson et al., 2021).

2.4. Extraction for cytotoxicity bioassay

The extraction protocol for cytotoxicity bioassay was similar to the method stated earlier (Section 2.3) with slight modifications. Approximately 2.5 g of the powdered sample was weighed and combined with 45 mL of 90% aqueous methanol (v/v), vortexed for 10 s and mixed in end-over-end mixer for 60 min. The sample was then centrifuged for 10 min at 1000 × g, and the supernatant was collected. The extraction step was repeated with another 45 mL of 90% aqueous methanol (v/v). The combined supernatant was filtered using 0.45 μm Advantec filter paper and rotary evaporated down to semi-solid consistency. The semi-solid product was re-dissolved in 25 mL Milli-Q water and frozen at −80°C overnight, followed by freeze drying at (−54°C,500 mTorr) for 72 h. The freeze-dried product was stored at 4°C in the dark until required. Stock solutions of the extracts were prepared by weighing the

Fig. 1. Plants used in this study. (**A**) *Syzygium australe*; (**B**) *Citrus hystrix*; (**C**) *Murrya koenigii*; (**D**) *Pittosporum angustifolium*.

appropriate amount of dried crystals and dissolving in sterilized Milli-Q water. The solution was then sterile filtered and serially diluted in DMEM media to obtain required concentrations. Stock solutions of the plant extracts were prepared fresh daily as required.

2.5. Cytotoxicity bioactivity

The HeLa (human cervical carcinoma) and HT29 (human colorectal carcinoma) cells were obtained from the University of Adelaide and cultured in DMEM, supplemented with 10% FBS, 1% Pen Step and 1% L-glute. The cells were maintained as mono layers in T-25 flask at 37°C, 5% CO_2 in a humidified condition until approximately 80% confluent.

The cytotoxicity properties of the freeze-dried plant extracts were assessed as previously described (Jamieson et al., 2014) with slight modifications. Briefly, the culture flasks were rinsed with DPBS twice, followed by an addition of 0.5 mL of trypsin and incubation at 37°C, 5% CO_2 for 10 min to dislodge the cells. The cell suspension was transferred to a 10 mL centrifuge tube, from which 50 μL aliquot was taken out and counted using trypan blue. Appropriate dilution of the cell suspension was made to obtain a final concentration of 5×10^4 cells·mL^{-1}. Aliquots of 100 μL diluted cell suspension were added to the wells of the 96 well plate (100 μL cells/well) in triplicates. The plate was incubated at 37°C, 5% CO_2, for 24 h prior to the addition of 100 μL of plant extracts or cell media (for the negative control) to individual wells. The final concentration of the extracts in the wells were 250 μg·mL^{-1}. Cisplatin, a chemotherapeutic drug against cervical cancer was used as the positive control. After the addition of the treatment, the plates were incubated for a period of 48 h, after which 10 μL of MTS reagent was added to each well, followed by a further incubation for a period of 1 h. At the end of the incubation period the absorbance was measured at 490 and 630 nm using a 96-well BIO-RAD iMark plate reader. This bioassay was repeated three times to test for reproducibility.

2.6. Phenolic profiling by high performance liquid chromatography (HPLC)

The polyphenolic content in the freeze-dried extract used in the cytotoxicity screening was evaluated using Agilent 1100 HPLC system (Agilent Technologies, Germany) with C-18 reversed phase column (Agilent Eclipse XDB-C18; 150 × 4.6 mm; 5-μm pore size), guard cartridge (Gemini C18 4 × 2 mm) and multiwavelength detector module. The analysis followed a previously developed protocol (Johnson et al., 2020b) with slight modifications. The mobile phase comprised 0.01 M phosphoric acid and methanol at a flow rate of 1 mL/min with a gradient beginning at 5% methanol (0 mins), ramping to reach 25% at 15 mins, 40% at 25 mins, 50% at 30 mins and 100% at 35 mins. The total run time was 40 mins, with a post-run flush time of 10 mins. The sample injection volume used was 5 μL. Based on retention time, the polyphenols present in the extracts were classified as hydroxybenzoic acid < hydroxycinnamic acids < flavonoids and were quantified at wavelengths 250 nm, 280 nm, and 325 nm, respectively.

2.7. Statistical analysis and data validation

All the parameters studied were subjected to statistical treatment using RStudio running version R-4.1.2 statistical software. The data were expressed as mean ± SD of duplicate chemical assays and triplicate cytotoxicity testing. One-way ANOVA followed was adopted to all the parameters under study to test the level of statistical significance. The difference was considered significant if P-value < 0.05. In the cytotoxicity testing, One-way ANOVA was used to calculate statistical significance between control and treated groups with a P-value < 0.05 considered to be statistically significant.

3. Results and Discussion

The current approach to screen plant extracts with potential cytotoxicity was achieved using the proposed bioassay guided fractionation protocol designed as shown in Fig. 2. This study demonstrates results obtained from phase 1 of the proposed protocol (Fig. 2).

3.1. The total phenolic content and antioxidant capacity

Quantitative phytochemical analysis of crude methanolic extracts of plants as reported in our earlier study (Johnson et al., 2020) are given in Table 2 and Fig. 3. It was found that the *M. koenigii* (leaves and flowers) had highest levels of TPC (5014 ± 100 and 7449 ± 107 mg GAE/100 g DW, respectively). The Australian species *P. angustifolium* of two unknown varieties A and B also showed relatively high TPC, with variety B (4296 ± 59 mg GAE/100 g DW) having almost twice the value of variety A (2723 ± 25 mg GAE/100 g DW). Moderate amounts of TPC were found in *S. australe* and *C. hystrix* (2664 ± 33 and 3086 ± 124 mg GAE/100 g DW, respectively).

Arjun et al. reported comparable or slightly lower TPC values (7012 mg GAE/100 g) in *M. koenigii* (leaves), which were sourced from Tamil Nadu in India (Arjun et al., 2017). Previously, Netzel

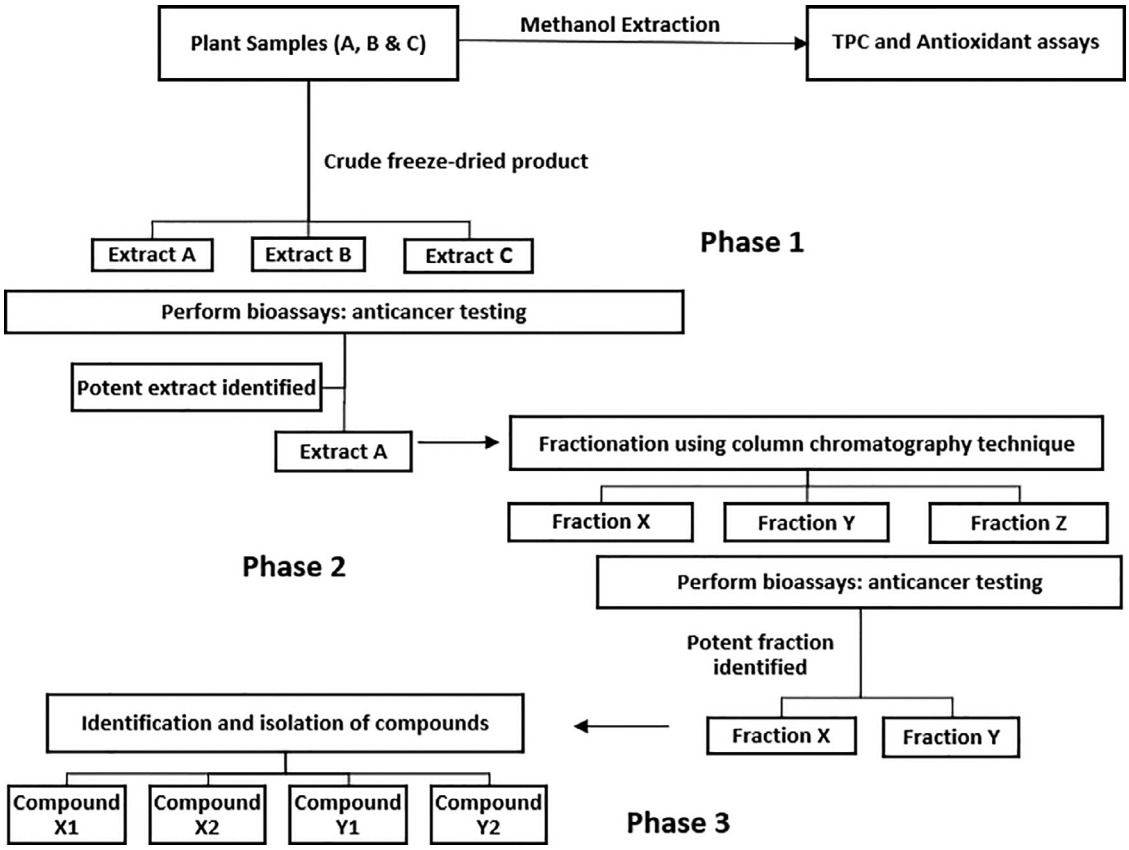

Fig. 2. Bioassay guided fractionation protocol.

Table 2
Average TP, FRAP and CUPRAC values of the plant extracts.

Samples	Avg TP (mg GAE/100 g DW)	FRAP (mg TXE/100 g DW)	CUPRAC (mg TXE/ 100 g DW
M. koenigii (leaves)	5014 ± 100	6038 ± 365	12,601 ± 519
M. koenigii (flowers)	7449 ± 107	7707 ± 348	18,055 ± 1748
S. australe (stamen)	3086 ± 124	3086 ± 124	5436 ± 123
C. hystrix	2664 ± 33	2202 ± 21	6119 ± 96
P. angustifolium (A)	2723 ± 25	2819 ± 170	7198.52 ± 594
P. angustifolium (B)	4296 ± 59	6677 ± 916	15,025 ± 1496

Values are mean ± SD ($n = 2$).

et al.reported *S. australe* TPC of 2140 ± 10 mg GAE/100 g (Netzel et al., 2007), which were lower than values reported here. Moreover, our earlier review of Australian native plants have found TPC values in the range of 760–66,050 mg GAE/100 g (Mani et al., 2021). The key role of phenolics and polyphenols, which constitute a wide variety and complex array of phytochemicals, as antioxidant, anti-inflammatory, antimicrobial, anticancer and antiviral activities, have been confirmed by earlier work (Arjun et al., 2017; Cock et al., 2015; Netzel et al., 2007; Phan et al., 2020). The ability to chelate metal ions and prevent lipid oxidation and free radical scavenging capacity makes phenolic compounds such as phenolic acids, flavonoids and tannins the most effective and salient antioxidants (Mani et al., 2021). Therefore, the estimation of the TPC of the selected plants is a vital part of this study.

Antioxidant capacity was the highest in *M. koenigii* flowers (FRAP: 7449 ± 107 mg TEX/ 100 g DW, CUPRAC: 18,055 ± 1748 mg TEX/ 100 g DW), followed by *P. angustifolium* B (FRAP: 4296 ± 59 mg TEX/ 100 g DW, CUPRAC: 15,025 ± 1496 mg TEX/ 100 g DW) (Table 2). Whereas antioxidant capacity was the lowest in *C. hystrix* (FRAP: 2202 ± 21 mg TEX/ 100 g DW, CUPRAC: 6119 ± 96 mg TEX/ 100 g DW) (Table 2). A major discrepancy was only noted for *S. australe*, whereby the FRAP values reported in this study (FRAP: 3086 ± 124 mg TEX/ 100 g DW) deemed to be higher than that reported earlier (458 ± 11 mg TEX/ 100 g DW) (Netzel et al., 2007).

The values reported in this study generally were comparable to previous values obtained from literature (Deo et al., 2016). Moreover, variations in data within literature for same plants depend on extraction solvent and protocol used (Johnson et al., 2020) and the growing conditions of the plants. Hence abiotic and biotic stressors such as climate or geographical location and evolution also influence phytochemical content and activity levels (Mani et al., 2021).

Similar trends in the antioxidant capacity methods of the plant extracts, measured using both FRAP and CUPRAC were also observed (Fig. 3) and a strong correlation between TPC and antioxidant capacity was also evident (Fig. 4). A R^2 value closer to 1 (R^2= 0.9637) suggested that there was stronger correlation between TPC, and antioxidant capacity determined by CUPRAC as opposed to FRAP (R^2= 0.8214). On the contrary, numerous studies have found that extracts with higher TPC did not always show higher antioxidant capacities (Bhardwaj et al., 2014; Fidrianny et al., 2018; Johnson et al., 2020). This may be due to differences in interaction of polyphenols, availability and nature (polar or non-polar) of compounds with antioxidant capacities (Haslaniza et al., 2015). However, it may be assumed that higher antioxidant capacity

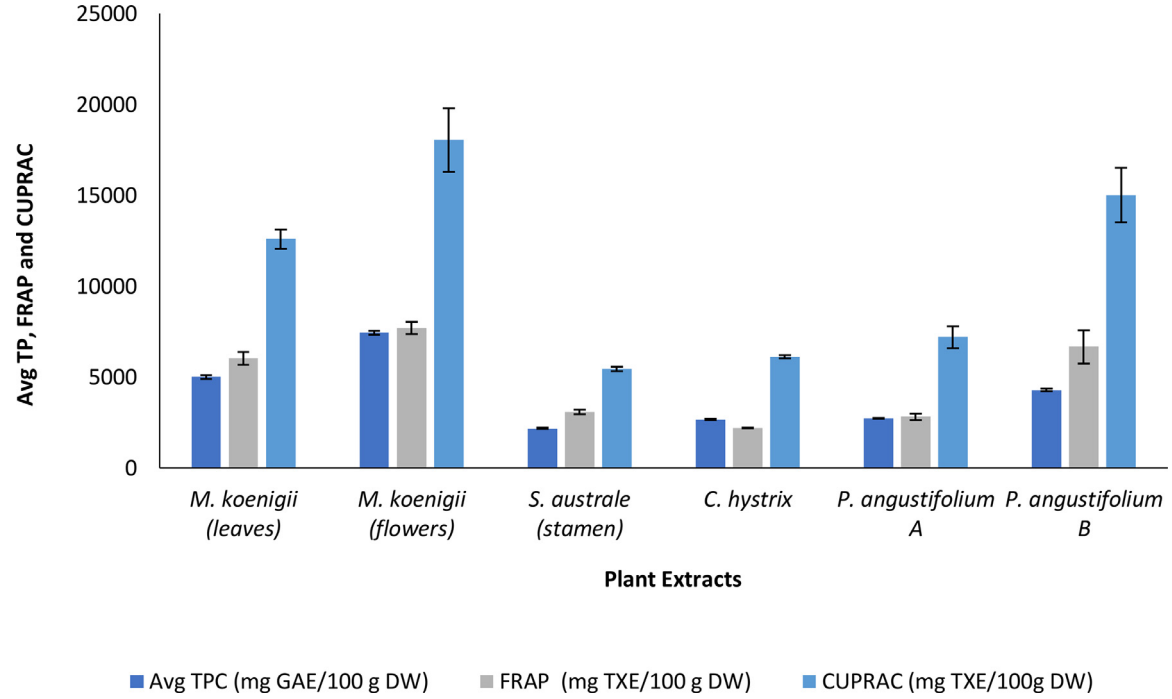

Fig. 3. Graph of total phenolic content and antioxidant capacity.

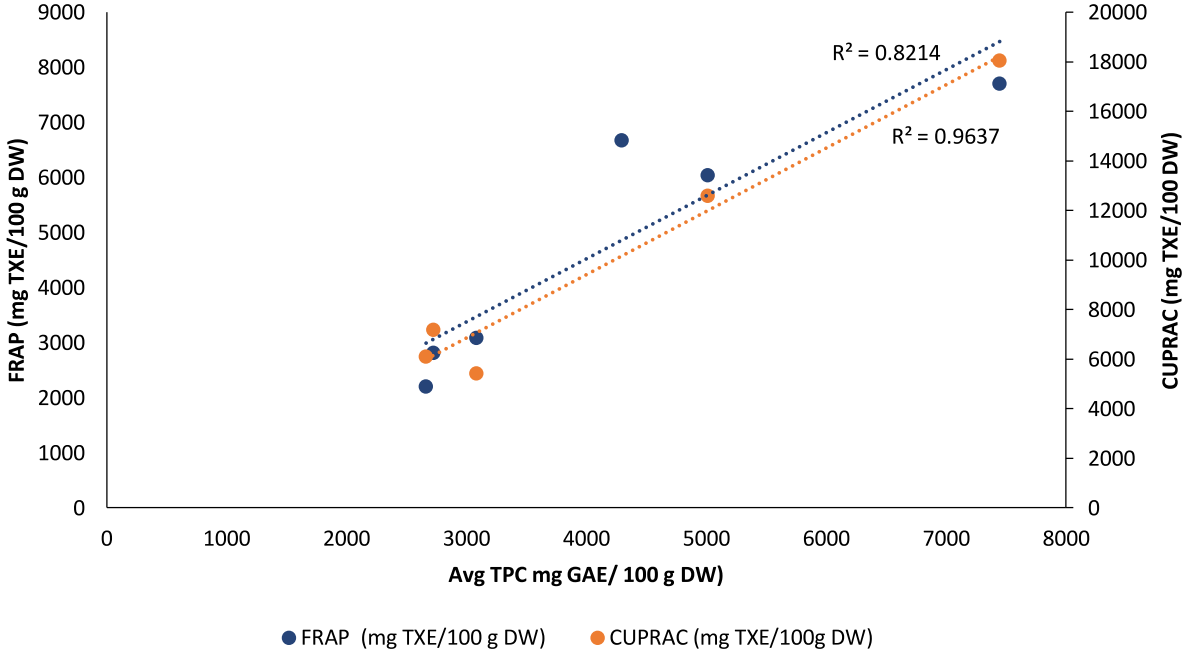

Fig. 4. Correlation between TPC and antioxidant capacity plant samples.

would potentially result in higher cytotoxicity activities (Vuong et al., 2014).

3.2. Cytotoxicity bioactivity

The results of percentage cell viability of HeLa cells upon treatment with the crude plant polar extracts are given in Fig. 5A. Qualitatively, the extract treated cells which the most potent effect (no cellular respiration) appeared light pink in color in the presence of MTS reagent, whereas wells with viable cells with cellular respiration occurring showed dark maroon color. The *P. angustifolium* extracts showed the highest potency with no viable cells, comparable to the positive control (cisplatin, a chemotherapeutic drug), followed by the *M. koenigii* flowers and leaves extract (43 ± 10% and 64 ± 13%, respectively). Extracts of *S. australe* and *C. hystrix* were the least potent with% cell viability of 86–87%, which were not statistically different to the negative control (100% cell viability).

To further investigate the cytotoxic activity, colon cancer cell lines (HT29 cells) were treated with *M. koenigii* (Flower and leaves) and *P.angustifolium* (A and B) extracts and the findings are as shown in

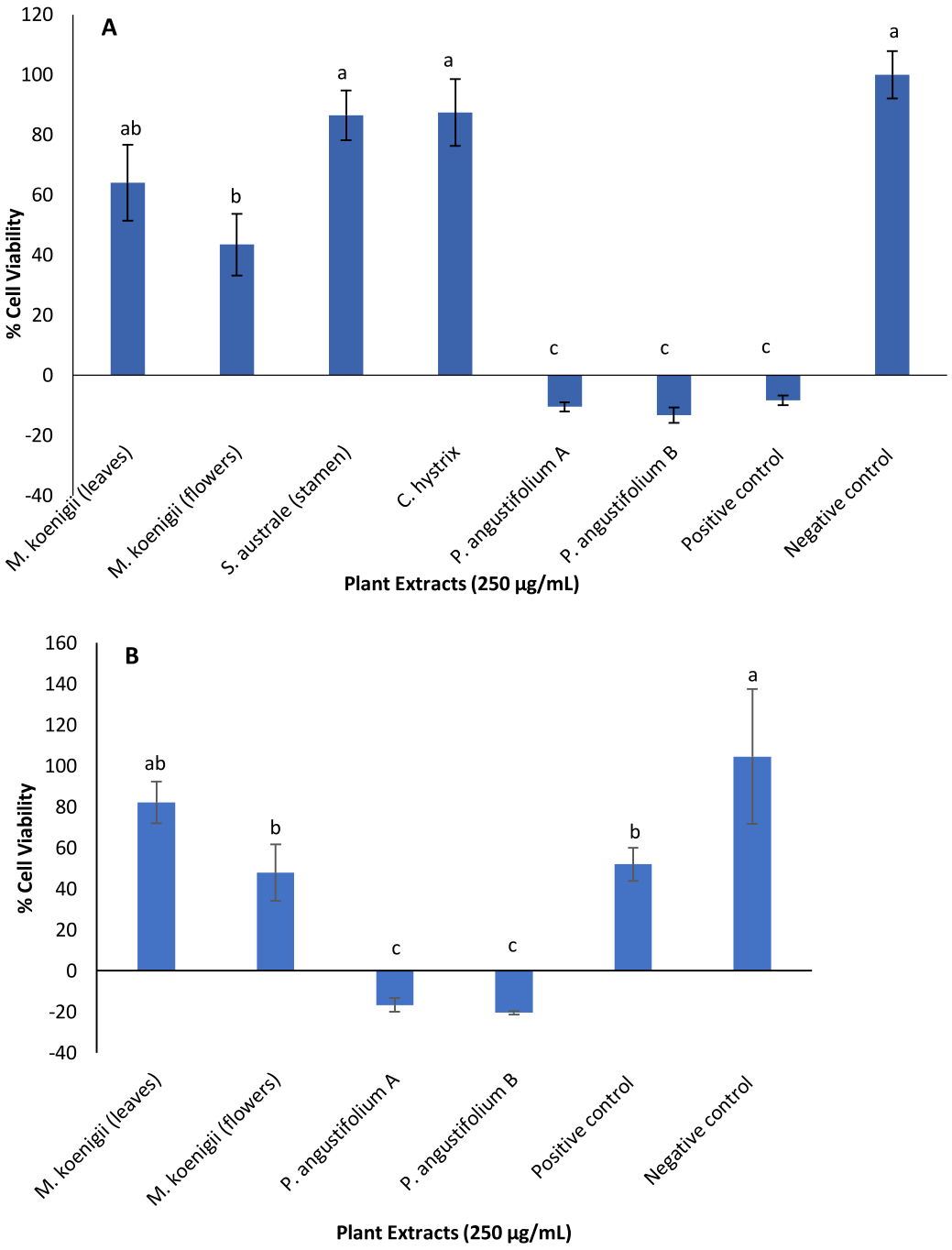

Fig. 5. The percentage cell viability of **(A)** HeLa cells and **(B)** HT29 treated with plant extracts at 250 µg·mL^{-1} concentration. One-way ANOVA test indicated a statistically significant difference (*P*-value < 0.05) between the different plant extracts, denoted by different letters on the bar, 'a' indicates significant difference as compared with positive; 'b' indicates significant difference as compared with positive/negative control; 'c' indicates significant difference as compared with negative control; 'ab' indicates significant difference as compared with positive/negative control. Negative control: cells without treatment; positive control: cells treated with 10 µg·mL^{-1} cisplatin (chemotherapy drug).

Fig. 5B. The cytotoxic effect of the extracts against HT29 was similar to the activity against HeLa, whereby the *P.angustifolium* extracts showed the highest potency, with no viable cells. These extracts were deemed more potent in comparison to the positive control (52 ± 8% cell viability). The *M. Koenigii* flower extracts also showed comparatively similar levels of potency (48 ± 14% cell viability) to the positive control. Whereas no significant difference was found in the percentage cell viabilities between the negative control and the *M. koenigii* leaf extracts. The cellular morphology of the cells before and after the addition of the *P. angustifolium* B extracts are shown in Fig. 6.

Although the mechanism of action via which this cytotoxic effect occurs was not investigated in the current study, previous literature have found phytochemicals to inhibit growth of HeLa cells via apoptosis (Palasap et al., 2014). Palasap et al. used caspase activity assay to demonstrate the apoptosis pathway of fractions obtained from *Caesalpinia mimosoides* Lamk where fraction F21, comprising of Gallic acid (68.49%), caffeine (3.44%) vanillic acid (5.59%), ferulic acid (20.63%) and resveratrol (1.85%), was found to increase the caspase activity of HeLa cells (Palasap et al., 2014).

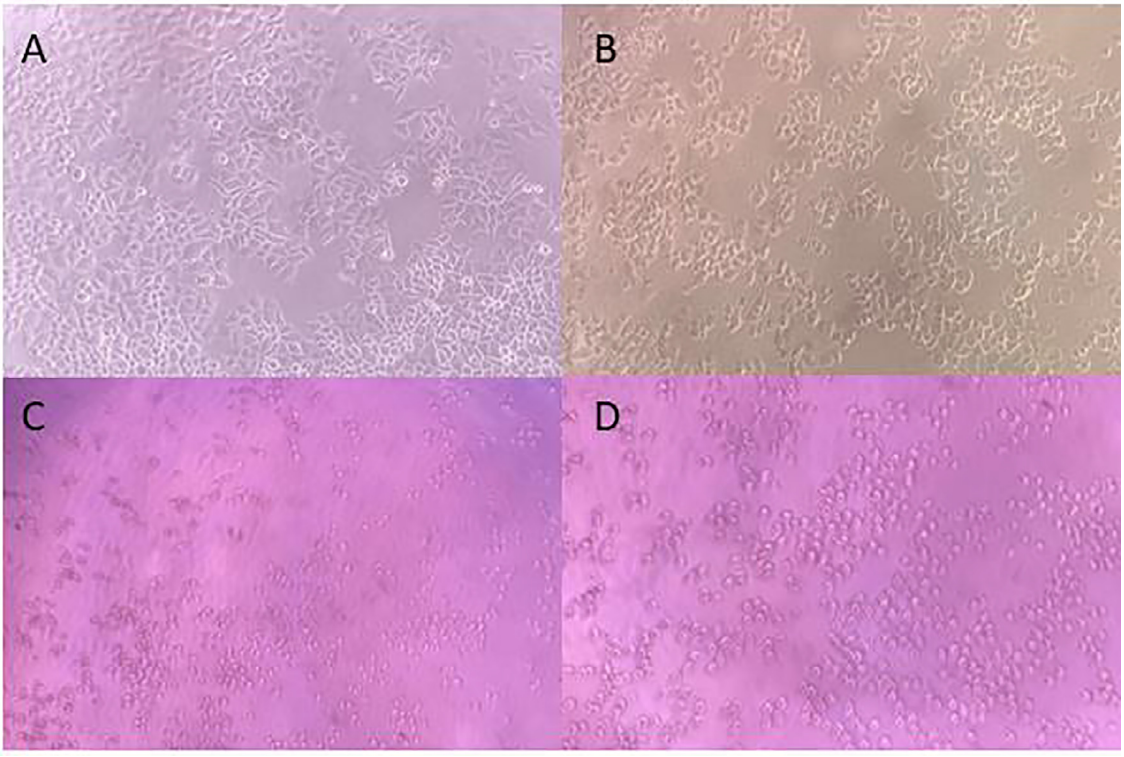

Fig. 6. Microscopic observation of cellular morphology of **(A)** HeLa, **(B)** HT29 cell before the addition of the plant extracts, **(C)** HeLa and **(D)** HT29 cells after addition of *P. angustifolium* B.

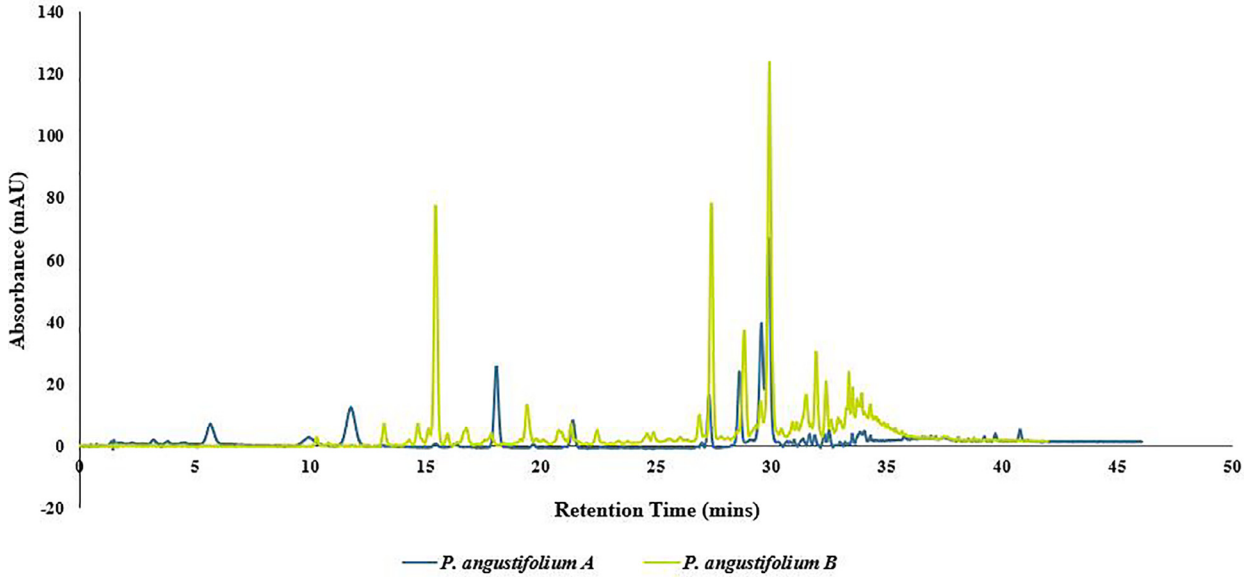

Fig. 7. HPLC profile of *P. angustifolium* A and B at detection wavelength of 320 nm.

A previous study found the fruits of *S. australe* to be more potent (IC_{50}: 134 μg·mL^{-1}) towards HeLa cells and non-cytotoxic towards normal cells (Selectivity index(SI): 14.02–45.97) compared to the leaves from these species (IC_{50}:187 μg·mL^{-1} and SI: 0.86–1.57) (Jamieson et al., 2014). Notably, whilst *M. koenigii* extracts showed higher antioxidant capacities than *P. angustifolium,* it had a lower cytotoxicity against the HeLa cells. This anomaly may have been due to variation in the phytoconstituents of the extracts and the sensitivity of the cell towards the *M. Koenigii* extracts compared to the *P.angustifolium*.

Bäcker et al. (Bäcker et al., 2016) has previously evaluated the cytotoxicity of isolated saponins from *P. angustifolium* which revealed that the predominant structural feature for a cytotoxic activity are acyl substituents at the oleanane aglycon backbone against three tumorigenic cell lines (MCF7—human breast cancer, 5637—human urinary bladder carcinoma, LN18—human glioblastoma) and one non-tumorigenic cell line (HaCaT—human keratinocyte) up to the low μM-range. However, the availability of literature aligning to the current study on *P. angustifolium* extracts against HeLa cell lines in our knowledge is nonexis-

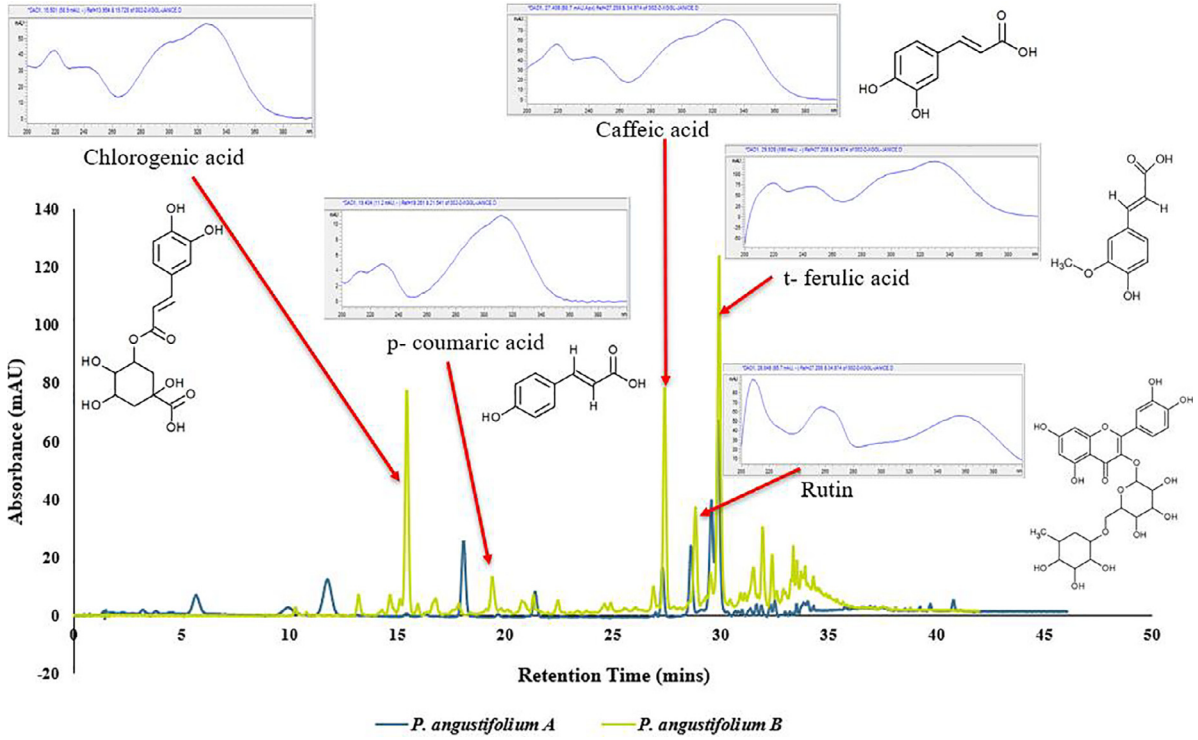

Fig. 8. HPLC profile and peak identification of *P. angustifolium* B.

tent. Additionally, whilst phytochemical screening has identified numerous triterpenes in the leaves of *P. angustifolium* of (Bäcker et al., 2013;Bäcker et al., 2015; Bäcker et al., 2016; Bäcker et al., 2014), due to the occurrence of different varieties of these species and variations in their phytochemistry (as shown in Fig. 7), there still remains a large scope for investigation. Different varieties may have different phytochemicals in varying concentrations which may lead to different biological activities.

3.3. Phenolic profiling by high performance liquid chromatography (HPLC)

HPLC analysis was performed on the two promising anticarcinogenic extracts (*P. angustifolium* A and B), with the phenolic profiles attained shown in Fig. 7. Classes of phenolic compounds obtained from literature (Ooh et al., 2014) with similar HPLC separating conditions suggests the occurrence of hydroxybenzoic acids and hydroxycinnamic acids at retention times shown in the chromatogram (Fig. 7).

Based on the phenolic profile, peaks of *P. angustifolium* B extracts were found to show peaks with greater intensity (Fig. 7). Further comparison of the main peaks identified in extract B with the UV spectrums of the standards of some common plant phenolic compounds were conducted. The dominant peaks were tentatively identified as chlorogenic acid, p-coumaric acid, caffeic acid, t-ferulic acid and rutin from comparison of their UV spectra and retention times to authentic standards as depicted in Fig. 8. Bäcker et al. (Bäcker et al., 2016) reported on the identification of polyphenolic compounds from *P. angustifolium* for the first time. They isolated quercetin glycosides rutin, isoquercitrin, 600-(3-hydroxy-3- methylglutaroyl)-isoquercitrin as well as 3,4- (4) and 4,5-dicaffeoylquinic acid, similar to the postulated findings of this study (Bäcker et al., 2014). Moreover, cytotoxicity of these compounds are also well documented (Bajpai et al., 2005; Bhuyan et al., 2017; Mani et al., 2020; Nour et al., 2013). For instance, Ekbatan et al. (Ekbatan et al., 2018) demonstrated that cell proliferation of Caco-2 cells decreased by 50% at effective concentration (EC_{50}) of 431 ± 51.84 μM with equimolar mixtures of chlorogenic acid and its major colonic microbial metabolites which included caffeic acid, 3-phenylpropionoic acid and benzoic acid. The cytotoxicity effects occurred at significantly lower concentrations of each compound (chlorogenic acid, caffeic acid, 3-phenylpropionoic acid and benzoic acid) within the mix, as opposed when cells were treated singly (one compound at a time). This indicated that the enhanced anti-colon cancer activity was due to their synergistic activity (Ekbatan et al., 2018). It can therefore be inferred that higher occurrence of these predominant species of polar phenolic compounds in extract B may be responsible for the greater anticarcinogenic effect.

Since the main goal of this study was to perform an initial screening of plants with potential anticarcinogenic activity, toxicity testing and inhibitory concentrations (IC_{50}) of the extracts is not reported herein. Furthermore, the next phase of this study (Phase 2 of the Bioassay guided fractionation protocol- Fig. 2) entails “cutting” the chromatogram into fractions and screening these fractions for their bioactivity, with an aim to isolate, purify and identify a novel anticarcinogenic compound.

4. Conclusion

This study found the Australian species *P. angustifolium* to be the most potent extract comparing the other plant extracts tested against the HeLa cells. Additionally, the HPLC profile of the extract also showed an array of promising therapeutic phenolic compounds. Hence, further fractionation and isolation of novel compounds from this species is warranted. It should be noted that since the *P. angustifolium* extracts have showed high cytotoxic effect against the cancer cell line, it may also have cytotoxic effects against normal cells. Performing toxicity testing and determining the IC_{50} of *P. angustifolium* extracts against normal human cells and other cancer cell lines would further validate its effectiveness as cytotoxic agent. Moreover, it can be concluded that the proposed bioassay guided protocol is simple, robust and an effective way of screening plants with potential cytotoxicity. The next phase of the protocol will aim to identify bioactive compounds in the active fractions of the plant extracts. Finally, since bioactive triterpenoid saponins have been identified previously by numerous authors, future research on *P. angustifolium*

should also include the investigation of these compounds in addition to the therapeutic phenolic compounds.

Ethical Approval

Not applicable.

Data Availability

Nil.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Janice Mani: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Joel Johnson:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Holly Hosking:** Methodology, Writing – review & editing. **Kerry Walsh:** Writing – review & editing. **Paul Neilsen:** Conceptualization, Writing – review & editing, Supervision. **Mani Naiker:** Conceptualization, Writing – review & editing, Supervision, Project administration.

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Supplementary Materials

Nil.

References

Arjun, P., Semwal, D., Semwal, R., Malaisamy, M., Sivaraj, C., Vijayakumar, S., 2017. Total Phenolic Content, Volatile Constituents and Antioxidative Effect of Coriandrum sativum, Murraya koenigii and Mentha arvensis. Nat. Prod. J. 7 (1), 65–74. doi:10.2174/2210315506666161121104251.

Bäcker, C., Drwal, M.N., Preissner, R., Lindequist, U., 2016. Inhibition of DNA–Topoisomerase I by Acylated Triterpene Saponins from Pittosporum angustifolium Lodd. Nat. Prod. Bioprospect. 6 (2), 141–147. doi:10.1007/s13659-016-0087-5.

Bäcker, C., Jenett-Siems, K., Bodtke, A., Lindequist, U., 2014a. Polyphenolic compounds from the leaves of pittosporum angustifolium. Biochem. Syst. Ecol. 55, 101–103. doi:10.1016/j.bse.2014.02.015.

Bäcker, C., Jenett-Siems, K., Siems, K., Niedermeyer, T.H.J., Wurster, M., Bodtke, A., Lindequist, U., 2015. Taraxastane-type triterpene saponins isolated from Pittosporum angustifolium Lodd. Zeitschrift Fur Naturforschung - Section B Journal of Chemical Sciences 70 (6), 403–408. doi:10.1515/znb-2015-0005.

Bäcker, C., Jenett-Siems, K., Siems, K., Wurster, M., Bodtke, A., Chamseddin, C., Crüsemann, M., Lindequist, U., 2013. Triterpene Glycosides from the Leaves of Pittosporum angustifolium. Planta Med. 79 (15), 1461–1469.

Bäcker, C., Jenett-Siems, K., Siems, K., Wurster, M., Bodtke, A., Niedermeyer, T.H.J., Lindequist, U., 2014b. New mono- and bisdesmosidic triterpene glycosides from Pittosporum angustifolium Lodd. Zeitschrift Fur Naturforschung - Section B Journal of Chemical Sciences 69 (9–10), 1026–1044. doi:10.5560/ZNB.2014-4143.

Bajpai, M., Pande, A., Tewari, S.K., Prakash, D., 2005. Phenolic contents and antioxidant activity of some food and medicinal plants. Int J Food Sci Nutr 56 (4), 287–291. doi:10.1080/09637480500146606.

Bhardwaj, R., Yadav, A., Sharma, R., 2014. Phytochemicals and antioxidant activity in Boerhavia diffusa. Int J Pharm Pharm Sci 6 (1), 344–348.

Bhuyan, D.J., Vuong, Q.V., Bond, D.R., Chalmers, A.C., van Altena, I.A., Bowyer, M.C., Scarlett, C.J., 2017. Exploring the Least Studied Australian Eucalypt Genera: corymbia and Angophora for Phytochemicals with Anticancer Activity against Pancreatic Malignancies. Chem. Biodivers. 14 (3), 1–8. doi:10.1002/cbdv.201600291.

Biva, I.J., Ndi, C.P., Griesser, H.J., Semple, S.J., 2016. Antibacterial constituents of Eremophila alternifolia: an Australian aboriginal traditional medicinal plant. J Ethnopharmacol 182, 1–9. doi:10.1016/j.jep.2016.02.011.

Cock, I.E., Winnett, V., Sirdaarta, J., Matthews, B., 2015. The potential of selected Australian medicinal plants with anti-Proteus activity for the treatment and prevention of rheumatoid arthritis. Pharmacogn Mag 11 (42), 190. doi:10.4103/0973-1296.157734.

Deo, P., Hewawasam, E., Karakoulakis, A., Claudie, D.J., Nelson, R., Simpson, B.S., Smith, N.M., Semple, S.J., 2016. In vitro inhibitory activities of selected Australian medicinal plant extracts against protein glycation, angiotensin converting enzyme (ACE) and digestive enzymes linked to type II diabetes. BMC Complement Altern Med 16 (1), 1–11. doi:10.1186/s12906-016-1421-5.

Dutt, R., Garg, V., Khatri, N., Madan, A.K., 2019. Phytochemicals in Anticancer Drug Development. Anticancer Agents Med Chem 19 (2), 172–183.

Ediriweera, M.K., Tennekoon, K.H., Samarakoon, S.R., 2019. In vitro assays and techniques utilized in anticancer drug discovery. J. Appl. Toxicol. 39 (1), 38–71. doi:10.1002/jat.3658.

Ekbatan, S.S., Li, X.Q., Ghorbani, M., Azadi, B., Kubow, S., 2018. Chlorogenic acid and its microbial metabolites exert anti-proliferative effects, S-phase cell-cycle arrest and apoptosis in human colon cancer caco-2 cells. Int J Mol Sci 19 (3), 1–13. doi:10.3390/ijms19030723.

Ferlay, J., Ervik, M., Lam, F., Colombet, M., Mery, L., M, P., Al, E, 2020. Global Cancer Observatory: Cancer Today. International Agency for Research on Cancer, Lyon https://www.who.int/health-topics/cancer#tab=tab_1.

Fidrianny, I., Octaviani, G.D., Kusmardiyani, S., 2018. Study of antioxidant profile and phytochemical content of different organs extracts of Morinda citrifolia L. Journal of Pharmaceutical Sciences and Research 10 (8), 2102–2105.

Haslaniza, H., Wan Yaacob, W.A., Osman, H., Maskat, M.Y., 2015. Interaction of antioxidants and organic acid from noni (Morinda citrifolia L.) juice with ion exchange resins during deodorization via deacidification. Der Pharma Chemica 7 (9), 9–21.

Jamieson, N., Joseph, P., Cock, I.E., 2014. The Anti-Proliferative Properties of Australian Plants with High Antioxidant Capacities Against Cancer Author Short Communication The Anti-Proliferative Properties of Australian Plants with High Antioxidant Capacities Against Cancer Cell Lines. Pharmacognosy Communications doi:10.5530/pc.2014.4.8.

Johnson, J., Collins, T., Mani, J., Naiker, M., 2021. Nutritional Quality and Bioactive Constituents of Six Australian Plum Varieties. International Journal of Fruit Science 21 (1), 115–132. doi:10.1080/15538362.2020.1860863.

Johnson, J., Collins, T., Power, A., Chandra, S., Skylas, D., Portman, D., Panozzo, J., Blanchard, C., Naiker, M., 2020a. Antioxidative properties and macrochemical composition of five commercial mungbean varieties in Australia. Legume Science 2 (1), 1–11. doi:10.1002/leg3.27.

Johnson, J., Collins, T., Walsh, K., Naiker, M., 2020. Solvent extractions and spectrophotometric protocols for measuring the total anthocyanin, phenols and antioxidant content in plums. Chem. Pap. 74 (12), 4481–4492. doi:10.1007/s11696-020-01261-8.

Johnson, J., Mani, J., Ashwath, N., Naiker, M., 2020. Potential for Fourier transform infrared (FTIR) spectroscopy toward predicting antioxidant and phenolic contents in powdered plant matrices. Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy 233, 118228. doi:10.1016/j.saa.2020.118228.

Koche, D., Shirsat, R., Kawale, M., 2016. An overview of major classes of phytochemicals: their type and role in disease prevention. Hislopia Journal 9 (1), 2016.

Mani, J., Johnson, J., Steel, J., Broszczak, D., Neilsen, P., Walsh, K., Naiker, M., 2020. Natural product-derived phytochemicals as potential agents against coronaviruses: a review. Virus Res. 284. doi:10.1016/j.virusres.2020.197989.

Mani, J.S., Johnson, J.B., Hosking, H., Ashwath, N., Walsh, K.B., Neilsen, P.M., Broszczak, D.A., Naiker, M., 2021. Antioxidative and therapeutic potential of selected Australian plants: a review. J Ethnopharmacol 268 (November), 113580. doi:10.1016/j.jep.2020.113580.

Netzel, M., Netzel, G., Tian, Q., Schwartz, S., Konczak, I., 2007. Native Australian fruits - a novel source of antioxidants for food. Innovative Food Science and Emerging Technologies 8 (3), 339–346. doi:10.1016/j.ifset.2007.03.007.

Nour, V., Trandafir, I., Cosmulescu, S., 2013. HPLC determination of phenolic acids, flavonoids and juglone in walnut leaves. J Chromatogr Sci 51 (9), 883–890. doi:10.1093/chromsci/bms180.

Ooh, K.F., Ong, H.C., Wong, F.C., Sit, N.W., Chai, T.T., 2014. High performance liquid chromatography profiling of health-promoting phytochemicals and evaluation of antioxidant, anti-lipoxygenase, iron chelating and anti-glucosidase activities of wetland macrophytes. Pharmacogn Mag 10 (39), S443–S455. doi:10.4103/0973-1296.139767.

Ota, A., Ulrih, N.P., 2017. An overview of herbal products and secondary metabolites used for management of type two diabetes. Front Pharmacol 8 (JUL), 1–14. doi:10.3389/fphar.2017.00436.

Palasap, A., Limpaiboon, T., Boonsiri, P., Thapphasaraphong, S., Daduang, S., Suwannalert, P., Daduang, J., 2014. Cytotoxic effects of phytophenolics from Caesalpinia mimosoides Lamk on cervical carcinoma cell lines through an apoptotic pathway. Asian Pac. J. Cancer Prev. 15 (1), 449–454. doi:10.7314/APJCP.2014.15.1.449.

Phan, A.D.T., Chaliha, M., Hong, H.T., Tinggi, U., Netzel, M.E., Sultanbawa, Y., 2020. Nutritional value and antimicrobial activity of Pittosporum angustifolium (gumby gumby), an australian indigenous plant. Foods 9 (7). doi:10.3390/foods9070887.

Sadgrove, N.J., Jones, G.L., 2016. Phytochemical variability of Pittosporum angustifolium lodd. (Pittosporaceae): a traditional and contemporary Aboriginal Australian medicine. Acta Hortic. 1125 (November), 303–308. doi:10.17660/ActaHortic.2016.1125.39.

Sadgrove, Nicholas J., Jones, G.L., 2014. Medicinal compounds, chemically and biologically characterised from extracts of Australian Callitris endlicheri and C. glaucophylla (Cupressaceae): used traditionally in Aboriginal and colonial pharmacopoeia. J Ethnopharmacol 153 (3), 872–883. doi:10.1016/j.jep.2014.03.054.

Sakulnarmrat, K., Fenech, M., Thomas, P., Konczak, I., 2013. Cytoprotective and pro-apoptotic activities of native Australian herbs polyphenolic-rich extracts. Food Chem 136 (1), 9–17. doi:10.1016/j.foodchem.2012.07.089.

Sakulnarmrat, K., Konczak, I., 2012. Composition of native Australian herbs polyphenolic-rich fractions and in vitro inhibitory activities against key enzymes relevant to metabolic syndrome. Food Chem 134 (2), 1011–1019. doi:10.1016/j.foodchem.2012.02.217.

Sanches, J.R., França, L.M., Chagas, V.T., Gaspar, R.S., dos Santos, K.A., Gonçalves, L.M., Sloboda, D.M., Holloway, A.C., Dutra, R.P., Carneiro, E.M., Cappelli, A.P.G., Paes, A.M., de, A., 2016. Polyphenol-rich extract of Syzygium cumini leaf dually improves peripheral insulin sensitivity and pancreatic islet function in monosodium L-glutamate-induced obese rats. Front Pharmacol 7 (MAR), 1–16. doi:10.3389/fphar.2016.00048.

Shukla, S., Mehta, A., 2015. Anticancer potential of medicinal plants and their phytochemicals: a review. Revista Brasileira de Botanica 38 (2), 199–210. doi:10.1007/s40415-015-0135-0.

Tan, A.C., Konczak, I., Li, G., Roufogalis, B.D., Sekhon, B., Sze, D.M.Y., 2015. An ethnopharmacological approach to the preliminary screening of native Australian herbal medicines for anticancer activity. Journal of Complementary and Integrative Medicine 12 (3), 245–249. doi:10.1515/jcim-2014-0017.

Vuong, Q.V., Hirun, S., Phillips, P.A., Chuen, T.L.K., Bowyer, M.C., Goldsmith, C.D., Scarlett, C.J., 2014. Fruit-derived phenolic compounds and pancreatic cancer: perspectives from Australian native fruits. J Ethnopharmacol 152 (2), 227–242. doi:10.1016/j.jep.2013.12.023.