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# *Eucalyptus microcorys* leaf extract derived HPLC-fraction reduces the viability of MIA PaCa-2 cells by inducing apoptosis and arresting cell cycle

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ABSTRACT

New therapeutic strategies such as the development of novel drugs and combinatorial therapies with existing chemotherapeutic agents are urgently needed to improve the clinical prognosis of pancreatic cancer. We have previously reported the antiproliferative properties of aqueous crude *Eucalyptus microcorys* extract against pancreatic cancer cell lines. In this study, bioassay-guided fractionation of the aqueous crude *E. microcorys* extract using RP-HPLC and subsequent assessment of the resultant fractions (F1–F5) for their antioxidant activity and cytotoxicity against pancreatic cancer cell lines were performed. The molecular mechanisms associated with the cytotoxicity was characterised by studying the effects of the most potent fraction-1 (F1) on apoptosis and cell cycle profiles as well as its phytochemical constituents by LC-ESI/MS/MS. F1 displayed significantly greater antioxidant activity in three different assays ($p < 0.05$). Moreover, F1 exhibited significantly greater antiproliferative activity ($IC_{50}$ = 93.11 ± 3.43 μg/mL) against MIA PaCa-2 cells compared to the other four fractions ($p < 0.05$). F1 induced apoptosis by regulating key apoptotic proteins- Bcl-2, Bak, Bax, cleaved PARP, procaspase-3 and cleaved caspase-3 in MIA PaCa-2 cells, suggesting the involvement of intrinsic mitochondrial apoptotic pathway and arrested cells at G2/M phase. A combination of gemcitabine and F1 exerted a greater effect on apoptosis and cell cycle arrest than F1 or gemcitabine alone ($p < 0.05$). LC-ESI/MS/MS revealed the tentative identities of phytochemicals present in F1 and their similarities with the phenolic compounds previously reported in *Eucalyptus* with antipancreatic cancer activity. Our study shows that the polyphenol and antioxidant-rich fraction of *E. microcorys* extract is a promising candidate for developing mono or combination therapies against pancreatic cancer.

## 1. Introduction

Molecular heterogeneity, poor prognosis and few effective therapies make pancreatic cancer one of the most lethal malignancies. It is the seventh leading cause of cancer-related death worldwide [1]. According to GLOBOCAN 2012 estimates, 338,000 new cases were diagnosed in 2012, with the highest incidence in North America and Europe and the lowest in Africa and Asia [1,2]. Pancreatic cancer has the highest mortality rate of any cancer with 3364 new diagnoses were estimated in Australia in 2018 and its incidence and mortality statistics are similar throughout the world [3–5]. Only minor advances in the treatment of pancreatic cancer have been made over the last 15 years compared to other cancers [6,7]. Pancreatic cancer patients experience an average five-year survival rate of about 7%, post-treatment due to its complex mutational landscape, few effective therapies with the emergence of resistance to chemotherapy and radiotherapy as well as poor prognosis

*Abbreviations:* 7-AAD, 7-aminoactinomycin D; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid; ANOVA, analysis of variance; ATM, ataxia telangiectasia mutated; Bak, Bcl-2 homologous antagonist killer; Bax, Bcl-2-associated protein; Bcl-2, B-cell lymphoma 2; CCK-8, cell counting kit-8; cdc2, cell division cycle 2; cdc25, cell division cycle 25; cdc25c, cell division cycle 25 homolog c; CDKN2A, cyclin-dependent kinase Inhibitor 2A; Chk-1, checkpoint kinase-1; Chk-2, checkpoint kinase-2; CUPRAC, cupric reducing antioxidant capacity; DMSO, dimethyl sulfoxide; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; HPDE, human pancreatic ductal epithelial cell; HPLC, high performance/pressure liquid chromatography; HPLC-ESI/MS/MS, high performance/pressure liquid chromatography, electrospray ionisation, mass spectroscopy, mass spectroscopy; IC50, the half-maximal inhibitory concentration; JNK, c-Jun N-terminal kinase; KRAS, Kirsten rat sarcoma; p17, protein 17; p53, protein 53; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PS, phosphatidylserine; ROS, reactive oxygen species; RP-HPLC, reversed-phase high performance/pressure liquid chromatography; TAC, total antioxidant capacity; TBS, tris-buffered saline; TBST, tris-buffered saline + 0.1% tween 20; TE, trolox equivalent; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

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[5]. It is also estimated that within a decade it will become the second cause of cancer death in the Western societies [5]. The Food and Drug Administration approved compound gemcitabine has been the drug of choice for pancreatic cancer over others such as 5-fluorouracil because of its overall clinical benefit and survival rate [6,8]. In spite of this, gemcitabine is only minimally effective, as it improves patient survival by only a matter of weeks [9]. Interestingly, gemcitabine in conjunction with other drugs such as erlotinib and capecitabine has been shown to be more efficacious than gemcitabine monotherapy [10,11]. As drug discovery is a complex process that involves a large amount of time and resources, testing existing drugs in combinatorial therapies is a logical approach for improving overall efficacy.

Natural products research forms an integral part of cancer therapy drug development, with 74.9% of new anticancer drugs marketed between 1981 and 2010 directly derived from natural sources [12,13]. A number of *in vitro* and *in vivo* studies have demonstrated the antipancreatic cancer properties of different plant-derived phenolic compounds such as quercetin, myricetin, apigenin, naringenin, epigallocatechin-3-gallate, to name a few [5]. Vuong et al. highlighted the potential of bioactive compounds particularly from Australian native fruits in the treatment of pancreatic cancer [14]. Over 800 species of eucalypts are found in Australia and only a few have been exploited by the pharmaceutical industry thus far [5]. Studies to date have illustrated the anticancer activity of only a limited number of eucalypts. Therefore, there remains an opportunity for natural product chemists to explore the chemical diversity of Australian eucalypts for the development of new anticancer agents. We first reported the antipancreatic cancer properties of various species of Australian eucalypts [15–17]. *Eucalyptus microcorys,* commonly known as tallowwood, is one of the least investigated *Eucalyptus* species in terms of its phytochemical composition and bioactivity. We have recently demonstrated the phytochemical, antibacterial and antifungal properties of crude aqueous extract of *E. microcorys* leaves [18]. In addition, we have illustrated the antiproliferative activity of crude aqueous *E. microcorys* extract against MIA PaCa-2 pancreatic cancer cells and highlighted its significance as an important source of phytochemicals for pancreatic cancer therapy [17]. This study aimed to advance our previous research by establishing the antioxidant activity and antiproliferative effect of RP-HPLC fractions from the aqueous crude *E. microcorys* extract against both primary and secondary pancreatic cancer cell lines. Examinations to achieve a greater understanding of the phytochemical constituents and the molecular mechanisms of action of the most potent fraction were also undertaken.

## 2. Materials and methods

### 2.1. Plant materials

*E. microcorys* fresh leaves were collected from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (33.4 °S 151.4 °E) on 2nd April 2014. The plant was identified by one of the authors (A.C.C.) and a voucher specimen deposited at the Don McNair Herbarium, The University of Newcastle (Accession number - 10499). After collection, the leaves were immediately transferred to the laboratory and stored at −20 °C to limit potential degradation of the phytochemicals. Using a dry air oven (50 °C), the leaves were dried for 5 h to constant weight, then ground to a fine powder in a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach), sieved using a 1 mm EFL 2000 stainless steel mesh sieve (Endecotts Ltd., London, England), packed in a sealed, airtight container and stored at −20 °C until tested.

### 2.2. Extraction and preparation of crude extract powder

Conventional aqueous extraction was carried out using the following method. Powdered leaf sample (5 g) was suspended in distilled water (100 mL) and heated at 85 °C for 15 min using a shaking water bath (Ratek instruments Pty Ltd., Boronia, VIC, Australia). The extract was then cooled on ice to room temperature and filtered through Whatman® No.1 filter paper. The filtrate was concentrated to one-third of its initial volume under reduced pressure (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) at a temperature of 45 °C. The concentrated extract was then freeze-dried (Thomas Australia Pvt., Ltd., Seven Hills, NSW, Australia) for 48 h at a chamber pressure of $2 \times 10^{-1}$ mbar and cryo-temperature of −50 °C.

### 2.3. RP-HPLC fractionation of E. microcorys extract

Fractionation of *E. microcorys* extract was performed using a Shimadzu LC-20AD HPLC system (Shimadzu, Rydalmere, NSW, Australia). The extract was dissolved in 70% ethanol and filtered through 0.45 µm Phenex syringe filters (Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). An autoinjector (SIL-10AP, Shimadzu, Rydalmere, NSW, Australia) was used to inject 500 µL of the extract onto a RP semi-prep Phenomenex Synergi 4U Polar-RP 80 A column (250 mm x 10 mm) (Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). The column temperature was maintained at 35 °C using a temperature controller (Phenomenex Therma Sphere TS 130, Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). The mobile phase (flow rate = 3 mL/min) comprised two solvents: distilled water + 0.1% orthophosphoric acid (mobile phase A) and 100% acetonitrile (mobile phase B). A gradient elution schedule was implemented as follows: 0–10 min, 0% B; 10–30 min, 30% B; 30–45 min, 60% B; 45–50 min, 60% B; 50–60 min, 0% B. Phytochemicals were detected at wavelengths of 190 and 254 nm using a photodiode array detector (SPD-M20 A, Shimadzu, Rydalmere, NSW, Australia). Based on retention time of the phytochemical profile, five major fractions (F1, F2, F3, F4 and F5) were obtained from the *E. microcorys* extract and collected using an auto fraction collector (FRC-10 A, Shimadzu, Rydalmere, NSW, Australia) as follows: F1: 5.25 − 8.60 min, F2: 29.70 − 37.70 min, F3: 41.50–43.00 min, F4: 50.30–52.70 min and F5: 54.00–58.40 min (Fig. 1). The collected fractions were evaporated using a rotary evaporator (40 °C), freeze-dried for 24 h ($2 \times 10^{-1}$ mbar, −50 °C), then stored at −20 °C until required. The yields of the fractions were also calculated (n = 10 runs, Fig. 1).

### 2.4. Determination of antioxidant capacity of E. microcorys fractions

#### 2.4.1. TAC assay

The TAC of the fractions was determined using the ABTS assay as described by Bhuyan et al. [19]. Trolox was used as standard and the result was expressed in mg TE per g of dry weight (mg TE/g).

#### 2.4.2. Free radical scavenging capacity

The free radical scavenging activity of the fractions was determined by the DPPH assay as described by Bhuyan et al. [20] and the result was expressed in mg TE per g of dry weight (mg TE/g).

#### 2.4.3. CUPRAC assay

To determine the cupric ion chelating capacity of the fractions, the CUPRAC assay as described by Apak et al. [21] was implemented, with the result expressed as mg of TE per g of dry weight (mg TE/g), with trolox as the calibration standard.

### 2.5. Determination of pancreatic cancer cell viability of the fractions

*E. microcorys* fractions were evaluated against two primary pancreatic cancer cell lines (MIA PaCa-2 and BxPC-3), a secondary pancreatic cancer cell line (CFPAC-1) derived from a liver metastasis site and an immortalised normal HPDE using the CCK-8 assay as per the method described by Bhuyan et al. [16]. Pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). The HPDE cells were originally from the lab of