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***In vitro* anticancer properties of selected *Eucalyptus* species**

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Abstract

In spite of the recent advancements in oncology, the overall survival rate for pancreatic cancer has not improved over the last five decades. Eucalypts have been linked with cytotoxic and anticancer properties in various studies, however, there is very little scientific evidence that supports the direct role of eucalypts in the treatment of pancreatic cancer. This study assessed the anticancer properties of aqueous and ethanolic extracts of four *Eucalyptus* species using an MTT assay. The most promising extracts were further evaluated using a CCK-8 assay. Apoptotic studies were performed using a caspase 3/7 assay in MIA PaCa-2 cells. The aqueous extract of *E. microcorys* leaf and ethanolic extract of *E. microcorys* fruit inhibited the growth of glioblastoma, neuroblastoma, lung and pancreatic cancer cells by more than 80% at 100 µg/mL. The *E. microcorys* and *E. saligna* extracts showed lower GI₅₀ values than the ethanolic *E. robusta* extract in MIA PaCa-2 cells. Aqueous *E. microcorys* leaf and fruit extracts at 100 µg/mL exerted significantly higher cell growth inhibition in MIA PaCa-2 cells than other extracts ($p < 0.05$). Statistically similar IC₅₀ values ($p > 0.05$) were observed in aqueous *E. microcorys* leaf ($86.05 \pm 4.75 \mu\text{g/mL}$) and fruit ($64.66 \pm 15.97 \mu\text{g/mL}$), and ethanolic *E. microcorys* leaf ($79.30 \pm 29.45 \mu\text{g/mL}$) extracts in MIA PaCa-2 cells using the CCK-8 assay. Caspase 3/7 mediated apoptosis and morphological changes of cells were also witnessed in MIA PaCa-2 cells after 24 h of treatment with the extracts. This study highlighted the significance of *E. microcorys* as an important source of phytochemicals with efficacy against pancreatic cancer cells. Further studies are warranted to purify and structurally identify individual compounds and elucidate their mechanisms of action for the development of more potent and specific chemotherapeutic agents for pancreatic cancer.

Keywords: *Eucalyptus*, anticancer, pancreatic cancer, MTT, CCK-8, caspase 3/7

Introduction

Pancreatic adenocarcinoma, otherwise known as pancreatic cancer comes under the category of gastrointestinal cancers and is one of the most devastating forms of human cancers in the world (Bailey *et al.* 2016; de Sousa Cavalcante and Monteiro 2014; Pourhoseingholi *et al.* 2015). It is the 13th most common type of cancer worldwide (Jiao and Li 2010) with a median survival timeline of less than 5 months after diagnosis and a five-year survival rate of less than 5%, even after treatment (de Sousa Cavalcante and Monteiro 2014). Poor prognosis and few effective therapies make it the 8th major form of cancer-related death, resulting in more than 227,000 deaths annually worldwide (Biankin *et al.* 2012; Ghaneh *et al.* 2010; Jiao and Li 2010). Concerted attempts over the past 15 years to improve the survival rate of patients diagnosed with pancreatic cancer have, in large parts been unsuccessful, and have lagged well behind advancements in the treatment of other cancers (Okines *et al.* 2010; Vaccaro *et al.* 2012).

Natural sources such as plants, marine invertebrates and microorganisms have been extensively studied and utilised for their anticancer properties in traditional as well as modern medicine. Bioactive compounds derived from natural sources comprise more than 60% of the anticancer drugs commercially available today (Jimeno *et al.* 2004). Approximately, 50-60% of cancer patients in the United States use products isolated from different plant components, either alone or in conjunction with mainstream chemotherapy and/or radiation therapies (Wang *et al.* 2012). In respect to the management of pancreatic cancer, various natural and semi-synthetic compounds, including gemcitabine, the most commonly used pancreatic cancer drug, have been identified and characterized.

The antitumour and cytotoxic properties of *Eucalyptus* extracts and isolated individual phytochemicals contained therein have been reported in various studies (Al-Fatimi *et al.*

2005; Benyahia *et al.* 2005; Ito *et al.* 2000; Mota *et al.* 2012; Soliman *et al.* 2014; Takasaki *et al.* 2000; Takasaki *et al.* 1995; Vuong *et al.* 2015a). While, a number of reports suggested the anticancer properties of eucalypt extracts and essential oils, there is very little scientific evidence that emphasizes a direct role of eucalypts in the management of pancreatic cancer. Phenolic compounds such as gallic acid, quercetin, narigenin, myricetin, apigenin and epigallocatechin-3-gallate derived from other natural sources have been shown to exhibit anticancer activity against pancreatic cancer both *in vitro* and *in vivo* (Liu *et al.* 2012; Lou *et al.* 2012; Lyn-Cook *et al.* 1999; Melstrom *et al.* 2011; Phillips *et al.* 2011; Qanungo *et al.* 2005; Shankar *et al.* 2008; Zhou *et al.* 2010). These particular compounds are also found extensively in eucalypts, highlighting the potential of *Eucalyptus* as a source of novel therapeutic agents for the treatment of pancreatic cancer. The present study was designed to assess the anticancer activity of crude aqueous and ethanolic extracts of four different *Eucalyptus* species: *E. robusta*, *E. microcorys*, *E. saligna*, and *E. globulus*. This study was divided into two parts; with, the extracts initially evaluated for cell growth inhibition activity across a panel of cancer cell lines. The most effective crude extracts were then subsequently evaluated for their efficacy specifically against pancreatic cancer cells using both primary and secondary pancreatic cancer cell lines.

Materials and methods

Plant materials

Fresh leaves of *E. robusta*, *E. microcorys* and *E. saligna* were collected on 2nd April, 2014 from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude of 33.4° S, longitude of 151.4° E). *E. globulus* fresh leaves were collected from Tinderbox, Tasmania, Australia (latitude 43° S, longitude 147.2° E) on 6th May, 2014. The collected *Eucalyptus*

species are shown in Figure 1. The plants were authenticated by the author (A.C.C.) and voucher specimens deposited at the Don McNair Herbarium (Accession numbers- *E. robusta*: 10492, *E. saligna*: 10504, *E. microcorys*: 10499, *E. globulus*: 10505). The leaves were then immediately transferred to the laboratory and stored at -20° C to avoid potential degradation of the phytochemical profile. Using a dry air oven, the leaves were dried at 70° C for 5 h to constant weight. The leaves were then ground to a fine powder using a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach), then sieved (≤ 1 mm) using a 1 mm EFL 2000 stainless steel mesh sieve (Endecotts Ltd., London, England) and packed in a sealed container and stored at -20° C until required.

Extraction of plant materials for preparation of crude extract powder

Conventional extraction was carried out using water and 70% ethanol (Bhuyan *et al* 2016; Bhuyan *et al* 2017). For aqueous extracts, 5 g of powdered leaf sample was mixed with 100 mL of distilled water and heated at 85° C for 15 min. In the case of ethanolic extracts, 5 g of powdered sample was mixed with 100 mL of 70% ethanol and macerated at room temperature for 72 h. Conical flasks (250 mL) sealed with a stopper and covered with aluminium foil were used for extraction in a shaking water bath (Ratek instruments Pty Ltd., Boronia, VIC, Australia). Aqueous extracts were immediately cooled on ice to room temperature (RT). The extracts were then filtered using Whatman® No.1 filter paper. The extraction yields were determined based on the extract (g) obtained from dried leaves (100 g). The filtrate was then concentrated using a rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) at 55° C with reduced pressure to one third of the initial volume. Concentrated extracts were then transferred to 15 mL Corning centrifuge tubes, sealed and immersed in liquid nitrogen and then freeze-dried for 48 h using a freeze

dryer (Thomas Australia Pvt., Ltd., Seven Hills, NSW, Australia) with drying chamber pressure of 2×10^{-1} mbar and cryo-temperature of -50° C.

Assessment of growth inhibition of crude Eucalyptus extracts on cancer cell lines

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cytotoxicity of the crude *Eucalyptus* extracts was screened using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Deane *et al.* 2013) to assess cell growth inhibition across a panel of cancer cell lines. The cell line panel consisted of HT29 (colon); U87, SJ-G2, SMA (glioblastoma); MCF-7 (breast); A2780 (ovarian); H460 (lung); A431 (skin); Du145 (prostate); BE2-C (neuroblastoma); and MIA PaCa-2 (pancreas) together with one non-tumour derived normal breast cell line (MCF10A). Briefly, all cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum, 50IU/mL penicillin, 50 μ g/mL streptomycin and 2mM L-glutamine. The MCF10A cells were cultured in DMEM:F12 (1:1) cell culture media, 5% heat inactivated horse serum, supplemented with penicillin (50 IU/mL), streptomycin (50 μ g/mL), 20mM Hepes, L-glutamine (2mM), epidermal growth factor (20 ng/mL), hydrocortisone (500 ng/mL), cholera toxin (100 ng/mL), and insulin (10 μ g/mL). Cells were plated in triplicate in DMEM (100 μ L) in a 96 well plate, at densities of 2500 – 4000 cells per well optimized to achieve logarithmic growth after 24 hours. The following day, 100 μ L of media with or without (control) the crude extracts was added to each well to give the final concentration of 100 μ g/mL. The cytotoxicity of the crude extracts was measured after 72 h of incubation using the MTT assay (Deane *et al.* 2013). The absorbance was read at 540 nm to determine growth inhibition based on the difference in the optical density values on day 0 versus those at the end of extract treatment. Cell growth inhibition as a percentage was determined, where a value of 100% is indicative of total

growth inhibition. A dose response curve (100 – 0.05 µg/mL) was also produced for extracts that showed promising activity, from which a GI₅₀ value was obtained representing the extract concentrations that inhibited 50% cell growth.

Determination of pancreatic cancer cell viability

To validate the anticancer activity of the selected *Eucalyptus* extracts that showed promising activity against the MIA PaCa-2 cell line in the MTT assay, they were further evaluated against two primary pancreatic cancer cell lines (MIA PaCa-2 and BxPC-3), and one secondary pancreatic cancer cell line (CFPAC-1), derived from a liver metastasis site using the Dojindo Cell Counting Kit-8 assay (CKK-8; Dojindo Molecular Technologies, INC., MD, USA). Additionally, extracts were assessed on an immortalised normal human pancreatic ductal epithelial cell line (HPDE). Pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). HPDE cells were originally from lab of Dr. M. Tsao (MD, FRCPC, University Health Network, Toronto, ON, Canada) (Furukawa *et al.* 1996).

Cell culture

MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2.5% horse serum and L-Glutamine (100 µg/mL). BxPC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS and L-Glutamine (100µg/mL). CFPAC-1 cells were cultured in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% FBS and L-Glutamine (100 µg/mL). HPDE cells were cultured in Keratinocyte Serum-free Media (KSFM) supplemented with human Recombinant Epidermal Growth Factor (rEGF) and Bovine Pituitary Extract (BPE).

Cell viability

Cell viability was determined using the CCK-8 assay. CCK-8 assay is a sensitive colorimetric method for the determination of the number of viable cells using WST-8 salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium). WST-8 is reduced by dehydrogenase activities in viable cells and forms a water soluble yellow-color formazan dye. The amount of formazan dye produced is directly proportional to the number of viable cells. This method is more sensitive than other tetrazolium salts such as MTT, XTT, MTS or WST-1.

200 μ L of suspended cells were seeded into a 96 well plate at 3×10^3 cells per well for MIA PaCa-2 and 7×10^3 cells per well for BxPC-3, CFPAC-1 and HPDE and allowed to adhere for 24 h. The cells were then treated with 100 μ g/mL of crude extracts, gemcitabine (50 nM), or vehicle control (0.5% DMSO). After 72 h, 10 μ L of CCK-8 solution was added and incubated at 37 °C for 90 min. The absorbance was measured at 450 nm using a microplate reader (BIORAD Benchmark Plus™, Bio-Rad Laboratories Pty Ltd., Gladesville, NSW, Australia) and cell viability was determined as a percentage of control. To eliminate the background that occurs due to turbidity, the absorbance of the wells was also measured at 650 nm as a reference and this value was subtracted from the absorbance of the same well measured at 450 nm. All experiments were performed in replicates of 6. The IC₅₀ (concentration that inhibits cell growth by 50%) values were calculated by curve fitting the absorbance (viability) vs. log [concentration of treatment], using GraphPad Prism software version 6.0b (San Diego, CA, USA).

Muse cytofluorimetric analysis for evaluation of apoptosis

The MIA PaCa-2 cells were treated with 100 μ g/mL of the selected extracts for 24 h. Gemcitabine (1 μ M/mL) and DMSO (0.5%) were used as positive and negative controls, respectively. Using the Muse caspase 3/7 (cat #MCH100108, Merck Millipore, Billerica,

MA, USA) assay, cells were analysed for the presence of early and late apoptosis and cell death as per the manufacturer's instructions. The Muse Cell Analyzer (Merck Millipore, Billerica, MA, USA) was used to analyse the cell counts. For detection of dead cells, a marker 7-aminoactinomycin D (7-AAD) was also included in this assay as an indicator of cell membrane structural integrity. This assay distinguishes four populations of cells: Live cells: Caspase-3/7(-) and 7-AAD(-), Apoptotic cells exhibiting Caspase-3/7 activity: Caspase-3/7 (+) and 7-AAD(-), Late Apoptotic/Dead cells: Caspase-3/7(+) and 7-AAD(+) and Necrotic cells: Caspase-3/7(-) and 7-AAD(+).

Statistical analysis

All analyses were carried out at least in triplicate. Independent samples *t*-test, one-way ANOVA, and the LSD post-hoc test were performed using SPSS statistical software (version 16.0, Chicago, IL, USA) to compare the means. The results were expressed as means $\pm$ standard deviations. At $p < 0.05$, the differences between the mean values in the experiments were taken to be statistically significant.

Results

Extraction yield of selected Eucalyptus species

Extraction yields (g/100 g of DW) of the different species of *Eucalyptus* for each solvent system are shown in the Table 1. The results indicated that the extraction yields with water were significantly higher in the case of *E. robusta*, *E. saligna* and *E. globulus* in comparison to that of 70% ethanol ($p < 0.05$). In the case of *E. microcorys* leaf and fruit extracts, the difference between the extraction yields with water and 70% ethanol was not statistically significant ($p > 0.05$).

Growth inhibition of crude Eucalyptus extracts on cancer cell lines using MTT assay

Growth inhibition values of the aqueous and ethanolic extracts of *Eucalyptus* species on cancer cell lines were calculated using the MTT assay (Table 2). Overall, the aqueous and ethanolic extracts from both leaf and fruit of *E. microcorys* and ethanolic extract of *E. robusta* exhibited more than 80% cell growth inhibition in glioblastoma, neuroblastoma and lung cancer cell lines at 100 µg/mL. In addition, aqueous *E. microcorys* leaf and 70% ethanolic extracts of *E. microcorys* fruit and *E. saligna* inhibited the growth of the pancreatic cancer cell line (MIA PaCa-2) by at least 80% at 100 µg/mL. In the case of skin cancer and normal breast cell lines, both the aqueous and ethanolic extracts of *E. microcorys* fruit at 100 µg/mL concentration showed more than 80% growth inhibition. The ethanolic extract of *E. saligna* resulted in more than 80% inhibition in pancreatic, colon, ovarian and skin cancer cell lines (Table 2). The aqueous and 70% ethanolic extracts of *E. globulus* displayed low to moderate activity (11 ± 5 - $77 \pm 4\%$ growth inhibition respectively) against the tested cancer cell lines. *E. robusta* (70% ethanol), *E. microcorys* fruit and leaf (water and 70% ethanol) and *E. saligna* (70% ethanol) extracts were selected for further investigation to assess the GI₅₀ values against the cancer cell lines. Table 3 displays the GI₅₀ values obtained from the selected *Eucalyptus* extracts and gemcitabine. The aqueous and ethanolic extracts of both *E. microcorys* leaf and fruit and the 70% ethanolic extract of *E. saligna* exhibited GI₅₀ values of 68 ± 2.3 , 76 ± 3 , 63 ± 3.8 , 73 ± 4 and 53 ± 3 µg/mL, respectively, in the MIA PaCa-2 pancreatic cancer cell line. In the case of the ovarian cancer cell line, all the extracts showed low to moderate activity with GI₅₀ values ranging from 33 µg/mL (*E. saligna* ethanolic extract) to 80 ± 5 µg/mL (*E. robusta* ethanolic extract). Additionally, it was found that GI₅₀ values were particularly higher (> 100 µg/mL) in the prostate cancer cell line for all the extracts. Similarly, in the breast cancer cell line (MCF-7), ethanolic extracts of *E. robusta*,

E.microcorys leaf and fruit and aqueous extract of *E.microcorys* leaf displayed a higher ($> 100 \mu\text{g/mL}$) GI_{50} values. Gemcitabine exhibited GI_{50} values ranging from of $1.4 \pm 1.0 \text{ nM}$ (ovarian cancer cell line) to $14 \pm 5.0 \text{ nM}$ (pancreatic cancer cell line) (Table 3).

Cell viability of primary and secondary pancreatic cancer cell lines using CCK-8 assay

The relatively lower GI_{50} values obtained from the aqueous and ethanolic extracts of *E. microcorys* leaf and fruit and ethanolic extract of *E. saligna* using the MTT assay might indicate the presence of solitary or more than one compound in the extracts with specific activity against the pancreatic cancer cell line (MIA PaCa-2). Hence, the aqueous *E. microcorys* leaf and fruit, and ethanolic *E. microcorys* leaf, and *E. saligna* extracts were further examined for their activity against other primary and secondary pancreatic cancer cell lines using the more sensitive CCK-8 assay. Between the two *E. microcorys* fruit extracts, only the aqueous extract *E. microcorys* fruit was considered for further examination due to limited availability of the fruit. Table 4 shows the cell growth inhibition (%) values in response to $100 \mu\text{g/mL}$ and $50 \mu\text{g/mL}$ of selected extracts across three different pancreatic cancer, and a normal pancreatic cell lines. Values obtained from extracts at a particular concentration were compared among each other (Table 4). In MIA PaCa-2 cells, statistically significant differences among the *E. microcorys* leaf (aqueous and ethanolic) and *E. microcorys* fruit (aqueous) extracts was observed at $100 \mu\text{g/mL}$ ($p < 0.05$) as indicated by the one-way ANOVA. Both the aqueous extracts of *E. microcorys* leaf and fruit inhibited the growth of MIA PaCa-2 cells by more than 80%. All three *E. microcorys* extracts ($50 \mu\text{g/mL}$) low to moderately inhibited ($13.65 \pm 5.43 - 53.82 \pm 7.80\%$) the cell growth of MIA PaCa-2 cells. The aqueous extract of the *E. microcorys* fruit exhibited significantly greater cell growth inhibition value than the other extracts at both 100 and $50 \mu\text{g/mL}$ ($p < 0.05$). *E.*

saligna (70% ethanol) extract displayed moderate to low activity with < 50% growth inhibition at both 100 and 50 µg/mL in MIA PaCa-2 cells (Table 4).

In the BxPC-3 cell line, the selected extracts showed < 30 % cell growth inhibition mean values both at 100 and 50 µg/mL (Table 4). Aqueous and ethanolic extracts of *E. microcorys* leaf and ethanolic extract of *E. saligna* showed statistically similar cell growth inhibition values at 100 µg/mL concentration in BxPC-3 cells, as revealed by one-way ANOVA ($p > 0.05$). Gemcitabine (50 nM) inhibited the growth of BxPC-3 cells by $71.30 \pm 2.40\%$ (Table 4). All the tested extracts at both 100 and 50 µg/mL displayed < 15% growth inhibition in CFPAC-1 cells (Table 4). Moreover, there was no significant difference among the extracts in regards to their CFPAC-1 cell growth inhibition activity at 100 µg/mL ($p > 0.05$). Growth inhibition of > 90% was observed in the normal HPDE cells when they were treated with gemcitabine (50nM) and the four *Eucalyptus* extracts (100 and 50 µg/mL) (Table 4).

As the four *Eucalyptus* extracts exhibited < 50% growth inhibition in comparison to the vehicle control in BxPC-3 and CFPAC-1 cell lines, even at the higher concentration of 100 µg/mL, only MIA PaCa-2 and HPDE cell lines were further investigated for the determination of IC₅₀ values. Table 5 represents the IC₅₀ values of the selected *Eucalyptus* extracts. One-way ANOVA suggested that there was no statistical difference among the IC₅₀ values obtained for aqueous extracts of *E. microcorys* leaf and fruit, and ethanolic *E. microcorys* leaf extracts in the MIA PaCa-2 cell line ($p > 0.05$, Table 5). The ethanolic extract of *E. saligna* displayed a significantly greater IC₅₀ value (115.52 ± 5.36 µg/mL) in comparison to the other tested extracts in the MIA PaCa-2 cell line ($p < 0.05$, Table 5). Interestingly, the IC₅₀ value of ethanolic *E. microcorys* leaf extract as ambiguous with wide standard deviations (Table 5). In the case of the normal HPDE cells, IC₅₀ values < 20 µg/mL were obtained for all four extracts with ethanolic *E. microcorys* leaf extract being the lowest at 9.06 ± 2.12 µg/mL (Table 5).

Due to their significantly lower IC₅₀ values compared to the *E. saligna* extract (70% ethanol) in the MIA PaCa-2 cell line, the aqueous extracts of *E. microcorys* leaf and fruit, and ethanolic extract of *E. microcorys* leaf were further studied for the induction of caspase 3/7 mediated apoptosis by using Muse caspase 3/7 assay. After 24 h of treatment, all extracts activated caspase 3/7 mediated apoptosis in the MIA PaCa-2 cell line (Figure 2). Treatment stimulated a net increase in the percentage of total apoptotic cells in comparison to the negative control (0.5% DMSO). The percentages of total apoptotic and live cells displayed by aqueous extracts of *E. microcorys* leaf and fruit were comparable to that of the positive control (gemcitabine). More than 12% of cells undergoing caspase 3/7 mediated apoptosis were detected after 24 h of treatment with the aqueous extracts of *E. microcorys* leaf and fruit, and gemcitabine (Figure 2). Morphological changes of the MIA PaCa-2 cells after 24 h of treatment with the selected extracts were also observed (Figure 3). Treated cells lost their characteristic elongated shape and became more circular. Membrane blebbing as a result of apoptosis was also observed (Figure 3).

Discussion

A greater extraction yield is often desired from a species or extraction method for further testing and utilisation of the extracts. The difference in the extraction yields can be attributed to the difference between the availability of extractable components, sample type, extraction solvent and technique implemented (Sultana *et al.* 2009). Our observations were in agreement with the previous findings of Sultana *et al.* (2009) who reported that a greater extraction yield can be achieved with extraction under reflux regardless of plant material and solvent used. We found that three out of four *Eucalyptus* species tested in our study showed significantly

higher extraction yield under reflux with water in comparison to the maceration with 70% ethanol.

The MTT assay was implemented to assess cell growth inhibition by the extracts across a panel of cancer cells as it is routinely used for high-throughput drug screening, and the correlation between the MTT assay and cell counting ($r > 0.98$) is well established (Hussain *et al.*, 1993). Our findings suggested that the anticancer activity of aqueous *E. robusta* freeze-dried extract against glioblastoma, neuroblastoma, breast, ovarian, lung and skin cancer cell lines is not different to that of aqueous *E. robusta* spray-dried extract previously demonstrated by Vuong *et al.* (2015b). The authors reported an 86% reduction in cell viability of MIA PaCa-2 cell line using 100 $\mu\text{g/mL}$ of aqueous *E. robusta* spray-dried extract. Our results revealed a somewhat comparable value of $79 \pm 2\%$ growth inhibition in MIA PaCa-2 cells for a 100 $\mu\text{g/mL}$ of aqueous *E. robusta* freeze-dried extract. Mota *et al.* (2012) reported the significant antiproliferative effects of ethanolic *E. globulus* bark extract in MDA-MB-231 human breast adenocarcinoma cells. In our study, aqueous and ethanolic extracts of *E. globulus* leaf inhibited the MCF-7 breast cancer cells only by $41 \pm 3\%$ and $22 \pm 2\%$, respectively. The relatively lower activity of the extracts against breast cancer cells in contrast to the previous study can be explained by the differences in breast cancer cell lines and plant materials used in both the studies. Vuong *et al.* (2015b) reported the GI_{50} values of aqueous *E. robusta* spray-dried extract in glioblastoma, neuroblastoma, breast, ovarian, lung and skin cancer cell lines which are similar to the GI_{50} values of ethanolic *E. robusta* extract obtained from MTT assay in our study.

E. microcorys and *E. saligna* extracts exhibited $< 30\%$ growth inhibition in BxPC-3 and CFPAC-1 cell lines even at high concentration in the present study. The lower efficacy of the same extracts in BxPC-3 and CFPAC-1 cells than MIA PaCa-2 can be attributed to the variations in genetic profiles of the cell lines. For instance, *KRAS* (Kirsten rat sarcoma viral

oncogene homolog), *TP53* (encoding the p53 protein), *CDKN2A*, and *SMAD4* are the four most common types of mutations found in these pancreatic cancer cell lines, however, their mechanisms of occurrence vary among the cell lines (Deer *et al.* 2010).

The GI_{50} values obtained from MTT assay were compared with the IC_{50} values from CCK-8 assay for the aqueous *E. microcorys* leaf and fruit extracts and ethanolic *E. microcorys* leaf and *E. saligna* extracts against MIA PaCa-2 and HPDE cell lines. The IC_{50} value from a typical dose response curve is similar to the GI_{50} value provided the drug inhibits the growth of the cells completely (Paraiso *et al.* 2012). IC_{50} values between 10 - 50 and 50 - 100 $\mu\text{g/mL}$ represent strong and moderate cytotoxicity, respectively (Doll-Boscardin *et al.*, 2012). We have previously established that aqueous *E. microcorys* leaf and fruit extracts possessed significantly higher total phenolic contents than aqueous extract of *E. saligna* (Bhuyan *et al.* 2016). It was also demonstrated that aqueous *E. microcorys* leaf extract had significantly higher saponin content compared to the aqueous *E. saligna* extract (Bhuyan *et al.* 2016). Plant phenolics and saponins have been reported to possess cytotoxicity and anticancer properties in a number of studies (Man *et al.* 2010; Podolak *et al.* 2010; Wahle *et al.* 2010). In addition, the synergistic cytotoxic and anti-inflammatory effects of flavonoids and saponins have also been reported (Puangraphant and de Mejia 2009; Chávez-Santoscoy *et al.* 2016). The significantly lower IC_{50} value attained from the tested *E. microcorys* extracts in MIA PaCa-2 cells is potentially due to the presence of specific phenolic compounds and saponins. However, the IC_{50} value of 70% ethanolic extract of *E. microcorys* against MIA PaCa-2 cells was calculated with wide standard deviations. It could be attributed to the significantly lower efficacy ($< 30\%$, $p < 0.05$) of ethanolic *E. microcorys* extract against MIA PaCa-2 cells even at a higher concentration (100 $\mu\text{g/mL}$).

The discrepancies in the percentage of cell growth inhibition, GI_{50} and IC_{50} values of tested *Eucalyptus* extracts obtained from MTT and CCK-8 assays can be explained by the technical

differences in the calculation of the values and the mechanisms of the assays. The IC₅₀ value does not take into account the initial cell population unlike GI₅₀ (Tong 2010). Moreover, the MTT assay primarily measures only the mitochondrial dehydrogenase activity of the cells (Ishiyama *et al.* 1996; Maioli *et al.* 2009), whereas, CCK-8 involves most of the dehydrogenase in a cell. Also, the formazan produced in CCK-8 assay is water soluble in nature and does not form crystals like MTT (Tominaga *et al.* 1999). The formazan crystals formed in MTT assay damage cells by puncturing membranes during exocytosis (Riss *et al.* 2004; Tominaga *et al.* 1999). Previous reports also suggested the unreliability of MTT assay when working with plant extracts, phenolic compounds and antioxidants as they can interfere with the assay by reducing the tetrazolium salt even in the absence of cells (Bruggisser *et al.* 2002; Maioli *et al.* 2009; Peng *et al.* 2005; Shoemaker *et al.* 2004; Wisman *et al.* 2008). Factors such as pH and glucose supply in the cell culture media can also affect the reduction of tetrazolium salt in MTT assay (Marshall *et al.* 1995; Sims and Plattner 2009). Altered cell metabolism due to some drugs can lead to changes in mitochondrial activity, which in turn influences the MTT assay by decreasing the reduction of tetrazolium salt (Hayon *et al.* 2003; Sims and Plattner 2009). Another important factor that can explain the discrepancies between the results obtained from MTT and CCK-8 assays in the present study is the difference in cell passage numbers used. Gene expression, morphology and cell development can be affected by the long-term subculturing (Deer *et al.* 2010) and can result in drug resistance and reduction in apoptosis (Ajabnoor *et al.* 2012; Pronsato *et al.* 2013).

The process of programmed cell death, apoptosis is downregulated in cancer (Shoja *et al.* 2015), therefore, induction of apoptosis by a drug or an extract is essential for reducing the viability of cancer cells. Activation of caspase 3/7 is considered a hallmark of apoptosis as these two effector caspases act downstream in the apoptotic pathway (Cullen and Martin 2009; Shoja *et al.* 2015). Activation of caspase 3/7 was observed in MIA PaCa-2 cell line by

the *E. microcorys* extracts after 24 h of treatment. Morphological changes such as cell shrinkage, rounding and plasma membrane blebbing due to caspase mediated apoptosis have also been reported in numerous studies (Coleman *et al.* 2001; Sebbagh *et al.* 2001; Ebrahimi Nigjeh *et al.* 2013). We also observed cell shrinkage, rounding and membrane blebbing of MIA PaCa-2 cells when treated with *E. microcorys* extracts for 24 h.

A number of studies in the literature have reported the IC₅₀ values of extracts, essential oils and compounds isolated from other *Eucalyptus* species such as *E. camaldulensis*, *E. torquate*, *E. sideroxylon*, *E. cladocalyx* and *E. benthamii* against various cancer cell lines including human breast adenocarcinoma, human bladder carcinoma, human promyelocytic leukemia cells, Jurkat, HeLa and ovarian cancer cells (Al-Fatimi *et al.* 2005; Ashour 2008; Benyahia *et al.* 2005; Doll-Boscardin *et al.* 2012; Hrubik *et al.* 2012; Singab *et al.* 2011; Topçu *et al.* 2011). However, there is very little information in the literature about the activity of *E. microcorys* extract against pancreatic cancer cells.

Conclusion

Three out of four *Eucalyptus* species showed significantly higher extraction yields under reflux with water compared to the maceration with 70% ethanol at RT. The MTT assay revealed that the aqueous extract of *E. microcorys* leaf and ethanolic extract of *E. microcorys* fruit inhibited the cell growth of glioblastoma, neuroblastoma, lung and pancreatic cancer cells by more than 80% at 100 µg/mL. The selected *E. microcorys* leaf and fruit and *E. saligna* (70% ethanol) extracts displayed lower GI₅₀ values than *E. robusta* (70% ethanol) extract in MIA PaCa-2 pancreatic cancer cell line in MTT assay. The CCK-8 assay further revealed that the aqueous *E. microcorys* leaf and fruit extracts at 100 µg/mL concentration exerted significantly higher cell growth inhibition (89.96 ± 0.91 and $93.75 \pm 2.01\%$,

respectively) in MIA PaCa-2 cells compared to other tested extracts ($p < 0.05$). By contrast, < 30% growth inhibition was observed in BxPC-3 and CFPAC-1 cells by four tested *Eucalyptus* extracts at both 100 and 50 $\mu\text{g/mL}$ concentrations. The IC_{50} values of aqueous *E. microcorys* leaf ($86.05 \pm 4.75 \mu\text{g/mL}$) and fruit ($64.66 \pm 15.97 \mu\text{g/mL}$) and ethanolic *E. microcorys* leaf ($\sim 79.30 \pm 29.45$) extracts were found to be statistically similar ($p > 0.05$) in MIA PaCa-2 cells using CCK-8 assay. Our findings in regards to the GI_{50} and IC_{50} values of the crude extracts are particularly promising as crude extracts are mixtures of multiple components comprising both active and inactive compounds and hence higher doses are often required to observe an activity in comparison to standard chemotherapeutic drugs. Nevertheless, further investigations are warranted to isolate and identify more refined and solitary compounds from these extracts for achieving effective anticancer activity against pancreatic cancer cells, even with lower doses. Future studies to elucidate the mechanisms of action of the purified compounds from the *Eucalyptus* species are also crucial to develop therapeutic agents with greater potency and specificity against pancreatic cancer.

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Conflict of interest

The authors declare no conflict of interest.

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Figure caption

Figure 1: *E. robusta* (a), *E. microcorys* (b), *E. saligna* (c) and *E. globulus* (d).

Figure 2: Effect of control (0.5% DMSO) (a), Gemcitabine-1 $\mu\text{M/mL}$ (b), *E. microcorys* leaf (water)- 100 $\mu\text{g/mL}$ (c), *E. microcorys* leaf (70% Ethanol)- 100 $\mu\text{g/mL}$ (d) and *E. microcorys* fruit (water)- 100 $\mu\text{g/mL}$ (e) on the apoptosis profile of MIA PaCa-2 cells studied by Muse Caspase 3/7 assay using Muse cytofluorimetric analysis.

Figure 3: Morphological changes of MIA PaCa-2 cells after treatment with gemcitabine (c), *E. microcorys* leaf (water) (d), *E. microcorys* leaf (70% ethanol) (e) and *E. microcorys* fruit (water) (f) for 24 h. Arrows indicate rounded cells and membrane blebbing at 40x magnification. No morphological changes between before (a) and after (b) treatment with 0.5% DMSO for 24 h were observed in the control.

a

c

b

d

a **b** **c** **d** **e**

a **b** **c** **d** **e** **f**

Table 1. Extraction yields of different species of *Eucalyptus* (n=3) using conventional extraction with water and 70% Ethanol.

	Extraction yield (g/100 g of DW)	
	Water	70% ethanol
<i>E. robusta</i>	12.75 ± 0.22 ^a	10.97 ± 0.43 ^b
<i>E. microcorys</i> (leaf)	14.75 ± 1.85 ^a	13.15 ± 0.28 ^a
<i>E. microcorys</i> (fruit)	6.26 ± 0.78 ^a	5.44 ± 0.10 ^a
<i>E. saligna</i>	7.55 ± 0.27 ^a	6.42 ± 0.03 ^b
<i>E. globulus</i>	10.08 ± 0.77 ^a	8.74 ± 0.10 ^b

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($p < 0.05$).

Table 2. Cell Growth Inhibition (%) in response to 100 µg/mL of extracts across various cancer cell lines (n = 3) using MTT assay. Higher values indicate greater growth inhibition.

		<i>E. robusta</i>		<i>E. microcorys</i>				<i>E. saligna</i>		<i>E. globulus</i>	
				Leaf		Fruit					
		Water	70% Ethanol	Water	70% Ethanol	Water	70% Ethanol	Water	70% Ethanol	Water	70% Ethanol
MIA PaCa-2	Pancreas	79 ± 2	65 ± 5	82 ± 2	67 ± 1	73 ± 2	80 ± 4	79 ± 4	84 ± 2	77 ± 4	20 ± 1
HT29	Colon	40 ± 6	65 ± 9	60 ± 6	72 ± 4	64 ± 4	78 ± 5	21 ± 3	89 ± 1	20 ± 3	26 ± 3
U87	Glioblastoma	34 ± 5	2 ± 3	36 ± 4	32 ± 5	42 ± 3	20 ± 5	35 ± 4	8 ± 4	35 ± 2	11 ± 5
SJ-G2	Glioblastoma	65 ± 9	85 ± 10	90 ± 7	95 ± 9	97 ± 4	103 ± 4	39 ± 8	47 ± 4	38 ± 7	27 ± 8
SMA	Glioblastoma (Murine)	52 ± 18	30 ± 13	56 ± 8	43 ± 11	59 ± 8	51 ± 8	45 ± 14	48 ± 9	40 ± 8	27 ± 14
MCF-7	Breast	39 ± 4	44 ± 4	43 ± 2	41 ± 6	56 ± 3	51 ± 4	53 ± 5	70 ± 3	41 ± 3	22 ± 2
A2780	Ovarian	63 ± 4	73 ± 4	75 ± 5	77 ± 4	86 ± 3	79 ± 4	64 ± 7	96 ± 0	56 ± 8	44 ± 1
H460	Lung	82 ± 14	85 ± 5	93 ± 2	95 ± 2	96 ± 2	98 ± 1	37 ± 13	64 ± 3	39 ± 12	46 ± 10
A431	Skin	57 ± 16	71 ± 9	75 ± 7	81 ± 6	87 ± 4	89 ± 4	39 ± 14	86 ± 2	48 ± 10	37 ± 8
Du145	Prostate	33 ± 3	35 ± 3	30 ± 3	22 ± 3	42 ± 4	33 ± 4	52 ± 8	49 ± 6	37 ± 13	14 ± 4
BE2-C	Neuroblastoma	68 ± 8	87 ± 9	90 ± 7	92 ± 8	97 ± 5	100 ± 4	43 ± 6	59 ± 2	44 ± 7	40 ± 4
MCF10A	Breast (Normal)	66 ± 6	67 ± 6	78 ± 4	79 ± 4	83 ± 3	84 ± 3	58 ± 7	44 ± 1	66 ± 6	53 ± 8

Table 3. Growth inhibition values (GI₅₀, concentration that inhibits cell growth by 50%) of the *Eucalyptus* (µg/ml) extracts and gemcitabine (nM) using the MTT assay. Lower value indicates greater growth inhibition efficiency.

		<i>E. robusta</i>	<i>E. microcorys</i>				<i>E. saligna</i>	Gemcitabine
			Leaf		Fruit			
		70% Ethanol	Water	70% Ethanol	Water	70% Ethanol	70% Ethanol	nM
MIA PaCa-2	Pancreas	94 ± 6	68 ± 2.3	76 ± 3.0	63 ± 3.8	73 ± 4	53 ± 3	14 ± 5.0
HT29	Colon	103 ± 13	95 ± 9	79 ± 6	85 ± 7	78 ± 6	53 ± 2	14 ± 3.0
U87	Glioblastoma	>200	146 ± 16	156 ± 39	133 ± 25	198 ± 37	177 ± 9	2.8 ± 0.0
SJ-G2	Glioblastoma	82 ± 11	69 ± 3	72 ± 9	62 ± 1	63 ± 3	103 ± 10	1.9 ± 0.0
SMA	Glioblastoma (Murine)	123 ± 15	81 ± 18	104 ± 18	79 ± 16	100 ± 13	101 ± 16	2.9 ± 0.0
MCF-7	Breast	122 ± 3	115 ± 7	112 ± 19	98 ± 10	105 ± 10	77 ± 5	3.6 ± 1.0
A2780	Ovarian	80 ± 5	67 ± 4	67 ± 6	52 ± 2	70 ± 5	33 ± 0	1.4 ± 1.0
H460	Lung	78 ± 3	72 ± 1	73 ± 2	65 ± 3	69 ± 2	90 ± 3	10 ± 3.0
A431	Skin	92 ± 11	84 ± 7	77 ± 7	68 ± 7	74 ± 8	47 ± 7	5.8 ± 2.0
Du145	Prostate	146 ± 14	162 ± 21	175 ± 15	151 ± 13	158 ± 19	119 ± 10	2.1 ± 0.0
BE2-C	Neuroblastoma	74 ± 9	68 ± 4	73 ± 10	61 ± 4	61 ± 3	81 ± 10	6.0 ± 1.0
MCF10A	Breast (Normal)	91 ± 6	75 ± 2	77 ± 6	69 ± 5	70 ± 3	106 ± 2	3.0 ± 0.0

Table 4. Cell Growth inhibition (%) in response to 100 µg/mL and 50 µg/mL of extracts and 50nM of gemcitabine across various pancreatic cell lines (n = 6) using CCK-8 assay. Higher values indicate greater growth inhibition.

		<i>E. microcorys</i>				<i>E. saligna</i>	Gemcitabine
		Leaf		Fruit			
		µg/mL	Water	70% Ethanol	Water	70% Ethanol	50nM
MIA PaCa-2	Pancreas	100	89.96 ± 0.91 _a	28.30 ± 5.04 _b	93.75 ± 2.01 _c	45.42 ± 2.67 _d	86.34 ± 0.60
		50	21.61 ± 6.02 _a	15.48 ± 4.62 _{ab}	53.82 ± 7.80 _c	13.65 ± 5.43 _b	
BxPC-3	Pancreas	100	26.06 ± 11.60 _a	17.54 ± 12.28 _a	13.25 ± 5.50 _b	17.47 ± 5.32 _a	71.30 ± 2.40
		50	16.74 ± 11.78 _a	15.25 ± 13.00 _a	10.53 ± 6.10 _a	10.23 ± 4.54 _a	
CFPAC-1	Pancreas	100	12.68 ± 4.90 _a	11.67 ± 4.65 _a	14.66 ± 3.03 _a	10.42 ± 3.45 _a	80.13 ± 2.10
		50	8.94 ± 2.50 _a	8.59 ± 4.52 _{ab}	6.45 ± 4.48 _b	6.76 ± 3.21 _b	
HPDE	Pancreas (Normal)	100	93.91 ± 0.34 _a	94.32 ± 0.14 _a	95.76 ± 0.19 _b	93.45 ± 0.76 _c	96.06 ± 0.08
		50	92.34 ± 1.43 _{ab}	93.91 ± 0.43 _a	95.12 ± 0.18 _c	90.52 ± 1.32 _b	

All the values are means ± standard deviations.

_{a,b,c} Values in the same row not having the same subscript letter are significantly different ($p < 0.05$) from each other.

Table 5. IC₅₀ values (µg/ml) (concentration that inhibits cell growth by 50%) of the selected *Eucalyptus* extracts using CCK8 assay. Lower value indicates greater growth inhibition efficiency.

		<i>E. microcorys</i>		<i>E. saligna</i>	
		Leaf		Fruit	
		Water	70% Ethanol	Water	70% Ethanol
MIA PaCa-2	Pancreas	86.05 ± 4.75 _a	~ 79.30 ± 29.45 _a	64.66 ± 15.97 _a	115.52 ± 5.36 _b
HPDE	Pancreas (Normal)	16.25 ± 1.34 _a	9.06 ± 2.12 _b	15.96 ± 1.87 _a	12.43 ± 3.43 _{ab}

All the values are means ± standard deviations.

_{a,b,c} Values in the same row not having the same subscript letter are significantly different ($p < 0.05$) from each other.