Cytotoxicity of Methanol and Aqueous Olive Pomace Extracts Towards Pancreatic Cancer Cells In Vitro

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Abstract
Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 8%, and as such the search for novel treatments is vital. Olive pomace contains phenolic compounds which have displayed anti-cancer properties, however, the anti-pancreatic cancer potential of crude olive pomace extracts has yet to be determined. Therefore, the phenolic compound content and the cytotoxic activity of olive pomace extracts was determined in vitro. Moreover, the efficacy of water as an extraction solvent for the recovery of phenolic compounds from olive pomace was determined. Incubation with methanol pomace extract (100 µg/mL) for 72 hours caused growth inhibition in all cells tested; HT29 (colon) 35%, A2780 (ovarian) 53%, H460 (lung) 22%, A431 (skin) 38%, Du145 (prostate) 32%, BE2-C (neuroblastoma) 31%, MCF-7 (breast) 57%, and U87, SJ-G2, SMA (glioblastoma) 49%, 43% and 46% respectively, and one non-cancer derived cell line MCF-10A (normal breast) 36% growth inhibition and reduced the viability of pancreatic cancer cells in a dose-dependent manner. Low concentrations (50 µg/mL) of methanol pomace extract increased the viability of non-tumorigenic cells (HPDE) compared to vehicle controls (20% increase). Additionally, the methanol extract contained a higher concentration of total phenolic compounds compared to the water extract (63.34 ± 4.82 and 51.55 ± 4.23 µg tyrosol equivalents/mL, respectively). Further work on the cytotoxicity of olive pomace extracts towards pancreatic cancer cells, and the protective effect on non-tumorigenic cells is warranted.

Keywords
Olea europaea, Olive oil, Phytochemicals, Polyphenols, Oleuropein, HPLC, Waste utilisation

Introduction
Epidemiological data has shown that consumption of a Mediterranean-style diet is associated with a reduced risk for most cancers [1-4]. One characteristic difference between Mediterranean diets and other healthy diets is the high consumption of olive oil [5]. Accumulating evidence suggests that microconstituents found in olive oil, including oleic acid and phenolic compounds, provide health benefits [6-8]. Interestingly, only approximately 2% of phenolic compounds originally present in the pre-processed olive actually end up in the olive oil [9, 10]. As such, olive waste products constitute a wealth of available phenolic compounds with known health benefits.

Phenolic compounds in olive oil and olive waste arise from the enzymatic...
conversion of secoiridoids glycosides (oleuropein, demethylleuropein and ligstroside) that are found in olive fruits by β-glycosidase during the mechanical extraction process [11-13]. The concentration and type of phenolic compounds in olive waste extracts will vary widely due to factors such as cultivar, growing conditions, location, time of harvest as well as extraction conditions [14-16]. Moreover, the different olive waste products (olive leaf, pomace and wastewater) will have different profiles due to the partitioning behavior of the phenolic compounds [17].

Olive waste extracts have displayed numerous health benefiting properties including reducing hyperlipidemia [7], antioxidant activity and reduction of oxidative stress and hypoglycemia [18], protective effects against cancers of the brain [19], colon [20] and breast [21]. Although the in vitro anti-cancer properties of olive pomace extracts have been confirmed in certain cell lines, investigations on pancreatic cancer cells has yet to be demonstrated.

Pancreatic cancer has a 5-year survival rate of less than 8%. Resistance to conventional treatment and its complex molecular heterogeneity contributes significantly to these dismal statistics. Current treatment options only increase survival by approximately 6 months. Moreover, chemotherapeutic drugs (such as gemcitabine) are highly toxic to the patient’s healthy tissue. Therefore, the search for novel drugs which selectively induce apoptosis in cancer cells without being toxic to healthy tissue is a key strategy. Considering the link between the consumption of a Mediterranean diet and reduced cancer tissue is a key strategy. Considering the link between the consumption of a Mediterranean diet and reduced cancer incidence and mortality [4], as well as the protective effects of olive phenolic compounds observed previously, olive pomace extracts warrant detailed investigation.

We previously identified an optimal method for the extraction of phenolic compounds from olive pomace using water as the extraction solvent. While water is a cheaper and green alternative to organic solvents, the selectivity of water for phenolic compounds is low in comparison. Methanol (50-80%) has been identified as the most selective solvent for the preparation of olive extracts high in desirable compounds (50-80%) has been identified as the most selective solvent for the preparation of olive extracts high in desirable compounds including oleocanthal, oleuropein as well as the flavonoids luteolin and apigenin. Therefore, the aims of this study were including oleocanthal, oleuropein as well as the flavonoids luteolin and apigenin. Therefore, the aims of this study were to determine the anti-pancreatic cancer activity of methanol pomace extracts and compare the phenolic compound content of aqueous pomace extracts to methanol pomace extracts.

Methods

Materials

HPLC methanol, HPLC ethanol, orthophosphoric acid, syringic acid and tyrosol were purchase from Sigma-Aldrich (Temecula, MS, USA). CCK-8 reagent was purchased from Dojindo Molecular Technologies Inc., Rockville, MD, USA. Dulbecco’s Modified Eagle Medium (DMEM), Keratinocyte Serum-Free Media (K-SFM), Roswell Park Memorial Institute medium (RPMI), Iscove's Modified Dulbecco's Medium (IMDM), trypsin-EDTA, L-glutamine and Phosphate Buffered Saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA). Foetal Bovine Serum (FBS) and Horse Serum (HS) was purchased from Interpath (Heidelberg West VIC, Australia). Luminata Classic western Horse Radish Peroxidase substrate was purchased from MERCK-Millipore (Temecula, MA, USA). Pancreatic cancer cells; MIA PaCa-2 (ATCC® CRL-1420™), BxPC-3 (ATCC® CRL-1687™) and CFPAC-1 (ATCC® CRL-1918™), were purchased from ATCC (Manassas, VA, USA) and immortalised normal pancreatic ductal epithelial cells (HPDE) were a gift from the lab of Dr. M. Tsao (MD, FRCP, University of Health Network, Toronto, ON, Canada). All cells have been authenticated by CellBank Australia (Westmead, NSW, Australia).

Sample preparation and extraction

Olive pomace was collected in April 2015 from Houndsfleld Estate, Lochinvar, the Hunter Valley, NSW. The olive paste came from fruit of the Frantoio cultivar which was picked on Friday 17th April, stored overnight at 14 °C before milling on Saturday 18th April using a semi-continuous Enrossi 150 traditional olive oil pressing system (Enoagricola Rossi, Calzolari di Umbieride, Perugia, Italy). The paste temperature during the malaxing phase was 22-23 °C. Olive pomace was stored for 12 h at -20 °C before freeze drying. Phenolic compound extracts were prepared according to Goldsmith et al [22]. Briefly, 5 g of dried pomace was added to 250 mL of water or 50% methanol and placed in ultrasound bath (Soniclean, 220 V, 50 Hz and 250 W model 250HD, Soniclean, Pty Ltd, Thebarton, SA, Australia) for 75 min at 250 W power. Extracts were concentrated (their volume reduced) using a rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, Australia), freeze dried in a FD3 freeze drier with the temperature at ~40 °C for 48 h (Thomas Australia Pty. Ltd., Seven Hills, NSW, Australia) and then stored at ~20 °C until further analysis.

Determination of total phenolic compounds

The extracts were analyzed according to [23] with minor modifications. Briefly, a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW Australia) and a 250 ± 4.6 mm Synergi 4 µm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW, Australia) with detection at 320 nm [24] was used. The column was maintained at 30 °C, the flow rate was 1 mL/min and three solvents were used for the mobile phase Solvent A: 0.1% orthophosphoric acid, Solvent B: 100% Methanol, Solvent C: 100% Ethanol. A gradient elution schedule was used according to the following: 0 min A 96%, B 2%, C 2%; 0-40 min A 40%, B 30%, C 30%; 40-60 min A 40%, B 30%, C 30%; 60-62 min A 96%, B 2%, C 2%. Peaks were identified at 320 nm. Syringic acid was used as an internal standard. A tyrosol standard curve was developed which was linear between 0-500 µg/mL (peaks with normalised area less than the intercept (0 µg/mL) of the tyrosol standard curve (y = mx + b) were excluded) and total peak areas of chromatograms expressed as µg tyrosol equivalents/mL.

Growth inhibition screening of cancer cell lines

The cytotoxic potential of methanol olive pomace extract
was assessed. Briefly, the growth inhibition of eleven cancer cell lines including MIA PaCa-2 (human pancreatic carcinoma), HT29 (human colorectal adenocarcinoma), A2780 (human ovarian carcinoma), H460 (human lung carcinoma), A431 (human skin epidermoid carcinoma), Du145 (human metastatic prostate carcinoma), BE2-C (malignant neuroblastoma), MCF-7 (ER+ human breast adenocarcinoma), and U87, SJ-G2, SMA (human glioblastoma) and one non-cancer derived cell line MCF-10A (non-tumorigenic breast epithelial) was determined. Briefly, all cancer cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine. The MCF-10A cells were cultured in DMEM:F12 (1:1) cell culture media, 5% heat inactivated horse serum, supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin, 20 mM Hepes, 2 mM L-glutamine, 20 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 µg/mL insulin. Cells were plated in triplicate in 100 µL DMEM on a 96 well plate, at a density of 2500–4000 cells per well. When cells were at logarithmic growth after 24 h, medium without (control), and medium with methanol olive pomace extract (100 µg/mL) was added to each well to give a final concentration of 100 µg/mL (day 0). The MTT assay was employed whereby absorbance was read at 540 nm to determine growth inhibition after 72 h of incubation based on the difference between the optical density values on day 0 and those at the end of drug exposure. Cell growth inhibition as a percentage was determined where a value of 100% is indicative of total growth inhibition.

Viability of pancreatic cell lines treated with methanol olive pomace extract

Human pancreatic cancer (MIA PaCa-2) cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 2.5% horse serum and L-glutamine (100 µg/mL), BxPC-3 cells were cultured in RPMI supplemented with 10% FBS and L-glutamine (100 µg/mL) and CFPAC-1 cells were cultured in IMDM supplemented with 10% FBS and L-glutamine (100 µg/mL). Human pancreatic ductal epithelial cells (HPDE) were cultured in KSFM supplement free media. All cells were maintained at 37 °C under 5% CO2.

Pancreas cell viability was determined using the Dojindo Cell Counting Kit-8. Human pancreatic cancer (MIA PaCa2, BxPC-3 and CFPAC-1) cells and non-tumorigenic pancreas (HPDE) cells were seeded into a 96 well plate at 3000-10000 cells per well and allowed to adhere for 24 h (in order to achieve logarithmic growth). Cells were then treated within the range of 0-200 µg/mL of crude extract, gemcitabine (0-50 nM), a combination of gemcitabine and methanol pomace extract or vehicle control. After 72 h, a 10% CCK-8 solution in media, was added before incubating at 37 °C for 180 min. Absorbance was measured at 450 nm and cell growth inhibition.

Statistical Analysis

GraphPad Prism Version 7.0 was used to conduct a student's t-test on HPLC total phenolic compounds comparing Methanol and Water total phenolic compounds. Repeated Measures two-way ANOVA and the Dunnett's multiple comparisons test were used to compare combination treatment cells to gemcitabine only controls (n = 6). Significance value was set at p < 0.05. All experiments were performed in triplicate.

Results and Discussion

Total phenolic compound content of olive pomace extracts.

There are numerous methods that can be used for the determination of phenolic compounds in crude extracts; the gold standard of which is HPLC [25, 26]. In this study, total olive phenolic compounds were evaluated using RP-HPLC by identifying the main peaks at the wavelength of 320 nm (Figure 1) Peaks were quantified using a tyrosol standard curve. The methanol extract had a higher content of phenolic compounds (63.34 ± 4.82 µg tyrosol equivalents/mL; Table 1) compared to the water extract (51.55 ± 4.23 µg tyrosol equivalents/mL; Table 1). Methanol has been confirmed as an ideal solvent for the extraction of phenolic compounds from olive materials [12, 13, 24, 27, 28], with 80% methanol the official method for the identification of phenolic compounds in olive oil [29]. However, due to the different chemical composition and the low oil content of olive pomace compared to olive oil, authors have previously argued that a lower percentage of methanol or ethanol (50%) is better able to effectively extract the phenolic compounds present [30].

Cytotoxicity of olive pomace extracts

Considering that the methanol pomace extract contained a higher quantity of total phenolic compounds, the cytotoxicity of the methanol extract was determined. The growth inhibition of 100 µg/mL of methanol pomace extract was determined in a panel of different cancer cells. The strongest cytotoxicity was observed in breast cancer (MCF-7) and ovarian cancer cells (A2780) followed closely by glioblastoma cells (U87 and SJ-G2) and pancreatic cancer cells (MIA PaCa-2).

![Typical HPLC chromatograms](image.png)
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(57%, 53%, 49%, 43% and 42% growth inhibition respectively; Table 2). Crude olive waste extracts have been shown to display toxicity towards colon cancer and gastric cancer cells \textit{in vitro} [31], while olive leaf extracts have been shown to reduce proliferation in glioblastoma [32, 33], colon cancer cells [34], mesothelioma [35], breast cancer [36] and pancreatic cancer cells [23]. However, olive leaf and olive pomace extracts have very different phenolic compound contents [37-39]. In fact, a number of compounds are formed during olive oil processing. Therefore, it is important to also determine the toxicity of olive pomace extracts. Moreover, the cytotoxicity of olive pomace extracts has not been investigated in pancreatic cancer previously.

Dose response of pancreatic cells treated with olive pomace extract

To further explore the activity of olive pomace extracts against pancreatic cancer cells, we tested the response of different pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1) as well as non-tumorigenic cells (HPDE) treated with different concentrations of methanol pomace extract (0-200 µg/mL). Methanol pomace extract reduced the viability of all pancreatic cancer cells at each dose within the treatment range (Figure 2). Interestingly, the viability of HPDE cells when treated with 50 µg/mL of olive pomace extract increased compared to the vehicle control (20% increase). While the protective activity of olive pomace extracts has not been observed previously, crude olive leaf extracts have displayed protective action against the DNA damage of leukocytes [40], UV induced skin cancer in mice [41] as well as renal toxicity in diabetic rats [18]. These data highlight the potential of olive pomace extracts as protective agents for pancreatic cells.

Combination treatment of olive pomace extracts and gemcitabine

Gemcitabine is a highly toxic chemotherapy drug used to treat pancreatic cancer. We analyzed the effects of the methanol pomace extract combined with gemcitabine to evaluate the utility of olive pomace extracts as a supplement to chemotherapy (Figure 3); the rationale for this is to explore whether the concentration of gemcitabine required to inhibit the proliferation of pancreatic cancer cells could be reduced, thereby decreasing toxicity and providing a protective effect from gemcitabine to non-tumorigenic cells.

Pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1), as well as non-tumorigenic pancreas cells (HPDE), were treated with 50 µg/mL of crude extract combined with 0-50 nM of gemcitabine. Interestingly, the methanol pomace extract displayed a protective effect from gemcitabine in the

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time</th>
<th>Methanol extract (µg tyrosol equivalents/mL)</th>
<th>Water extract (µg tyrosol equivalents/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.615</td>
<td>3.09 ± 0.1</td>
<td>4.86 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>18.185</td>
<td>1.54 ± 0.67</td>
<td>2.29 ± 0.32</td>
</tr>
<tr>
<td>3</td>
<td>19.233</td>
<td>2.07 ± 0.23</td>
<td>3.37 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>20.281</td>
<td>4.58 ± 0.06</td>
<td>6.11 ± 0.65</td>
</tr>
<tr>
<td>5</td>
<td>20.982</td>
<td>2.40 ± 0.36</td>
<td>3.39 ± 0.4</td>
</tr>
<tr>
<td>6</td>
<td>22.924</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>23.372</td>
<td>2.99 ± 0.34</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>23.781</td>
<td>3.44 ± 0.72</td>
<td>4.24 ± 0.87</td>
</tr>
<tr>
<td>9</td>
<td>24.559</td>
<td>6.42 ± 0.03</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>25.017</td>
<td>6.68 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>27.881</td>
<td>1.48 ± 0.66</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>28.125</td>
<td>5.46 ± 0.38</td>
<td>5.68 ± 0.4</td>
</tr>
<tr>
<td>13</td>
<td>29.219</td>
<td>9.21 ± 0.01</td>
<td>6.49 ± 0.97</td>
</tr>
<tr>
<td>14</td>
<td>29.607</td>
<td>4.84 ± 0.54</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>31.415</td>
<td>9.14 ± 0.22</td>
<td>7.77 ± 0.51</td>
</tr>
<tr>
<td>Total</td>
<td>63.34 ± 4.82</td>
<td>51.55 ± 4.23</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Total biophenols of the methanol and water pomace extracts determined by HPLC. Peak numbers correspond to chromatogram peaks identified in figure 1.

A different letter denotes significant difference (p < 0.05).

Table 2: Dose screen: Cells from cancer tissues of different organs of origin as well as non-cancer derived breast cells (MCF10A). Percentage (%) cell growth in response to 100 µg/mL of methanol pomace extract (the higher the value the greater the growth inhibition).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell line</th>
<th>Growth Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>HT29</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>U87</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>Breast</td>
<td>MCF-7</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>Ovarian</td>
<td>A2780</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Lung</td>
<td>H460</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>Skin</td>
<td>A431</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>Prostate</td>
<td>Du145</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>BE2-C</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>SJ-G2</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>Pancreas</td>
<td>MIA</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>SMA</td>
<td>46 ± 16</td>
</tr>
<tr>
<td>Breast</td>
<td>MCF10A</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

Figure 2: Dose response of pancreatic cancer cells (CFPAC-1, MIA PaCa-2 and BxPC-3) and non-tumorigenic cells (HPDE) when treated with 0-200 µg/mL of crude methanol pomace extract.
pancreatic cancer cells and not the non-tumorigenic pancreas cells (Figure 3). The combination of olive pomace extract and 10 nM gemcitabine caused an increase in the viability of MIA PaCa-2 (20% increase, p < 0.0001, Figure 3A) and CFPAC-1 (16% increase, p < 0.0001, Figure 3C) cells compared to 10 nM of gemcitabine alone, the combination of extract and 25 nM gemcitabine increased the viability of BxPC-3 (7% increase, p = 0.0194, Figure 3B) and MIA-PaCa-2 (14% increase, p = 0.0001, Figure 3A) compared to the 25 nM of gemcitabine alone and the combination of extract and 50 nM of gemcitabine increased the proliferation of CFPAC-1 cells (11% increase, p = 0.0066, Figure 3C) compared to the 50 nM of gemcitabine alone. Additionally, there was no difference between the combination-treatment HPDE cells and the gemcitabine only cells (Figure 3D).

Acknowledgements

The authors would like to acknowledge the University of Newcastle for the provision of funding and Houndsfield estate for the provision of pomace samples.

Conflicts of Interest

The authors declare no conflicts of interest

References

10. Klen TJ, Vodopivec BM. 2012. The fate of olive fruit phenols during oil extraction causes the formation of various compounds [10], with their activity in cancer yet to be investigated. Moreover, the activity of olive pomace extracts combined with lower doses of gemcitabine warrant investigation.

Conclusion

Olive pomace extracts are a rich source of phenolic compounds. Methanol extraction increased the yield of phenolic compounds from olive pomace compared to extraction with water. Olive pomace methanol extracts displayed cytotoxicity towards glioblastoma, breast, ovarian and pancreatic cancer cells. While olive leaf extracts have previously displayed toxicity towards breast cancer and glioblastoma cells, this study is the first to determine the cytotoxicity of olive pomace extracts in lung, ovarian, neuroblastoma and pancreatic cancer cells. Moreover, the protective effect of low doses of olive pomace extracts on non-tumorigenic pancreas cells warrants further investigation.

This has not been observed previously. In fact, olive leaf extracts have previously displayed a synergistic effect with the breast cancer chemotherapy agent doxorubicin [42] as well as increasing the efficacy of temozolomide [33] and bevacizumab [32] therapy in glioblastoma cells. These differences are likely due to the unique profile of phenolic compounds in olive pomace extract compared to olive leaves. The process of olive oil extraction causes the formation of various compounds [10], with their activity in cancer yet to be investigated. Moreover, the activity of olive pomace extracts combined with lower doses of gemcitabine warrant investigation.

Figure 3: Combination treatment of cancer cells (MIA PaCa-2, CFPAC-1 and BxPC-3) and non-tumorigenic cells (HPDE) with 50 µg/mL of methanol pomace extract and a range of gemcitabine concentrations (0-50 nM).

** denote significant difference from gemcitabine only controls (p < 0.05).
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