Determination of health-promoting bioactives in Australian avocados

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Food Science Australia

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FINAL REPORT

HAL PROJECT AV07003 (30/10/2008)

Determination of health-promoting compounds in Australian avocadoes

by

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15 January 2009

HAL PROJECT: AV07003

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MEDIA SUMMARY

Avocadoes are known to contain high amounts of health-benefiting phytonutrients including monounsaturated fatty acids, carotenoids, and vitamins B, C and E. Recent studies carried out using Californian-grown 'Hass' avocadoes have reported that extracts from the fruit also possess *in–vitro* (i.e. in test-tube) anti-cancer properties against prostate cancer cells.

The aim of this work was to obtain scientifically creditable information relating to the health-promoting benefits of substances found in Australian avocadoes (Hass variety). This was achieved by quantifying the major lipid-soluble phytonutrients found in avocadoes and assessing their antioxidant and *in-vitro* cancer-inhibiting properties.

Lipid-soluble extracts from Australian-grown 'Hass' avocadoes were found to contain Vitamin E (α, γ and δ forms) (1398-2643 µg/100 g fresh weight), chlorophylls (a and b forms) (2304-3680 µg/100 g fresh weight) and lutein (160-273 µg/100 g fresh weight) as their major components.

The same extracts (rich in Vitamin E, chlorophylls, and carotenoids) were found to have high *in-vitro* antioxidant activity. A sub-fraction of these extracts, containing several unidentified components, was found to significantly inhibit the growth of colon and gastric cancer cells in test-tube assays, but was less effective against leukaemia cells.

This information may now be used to further promote the use of Australian avocadoes as part of an everyday diet and strengthen their position in the mind of the national and international public as a natural, health-benefiting fruit.

TECHNICAL SUMMARY

Avocadoes are known to contain high amounts of health-benefiting phytonutrients including monounsaturated fatty acids, carotenoids, and vitamins B, C and E. Recent studies carried out using Californian-grown 'Hass' avocadoes have reported that extracts from the fruit also possess *in–vitro* (i.e. in test-tube) antiproliferative properties against prostate cancer cells.

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Lipophilic extracts (from Australian-grown fruit) rich in vitamin E, chlorophylls, carotenoids, and were found to have high antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH). A sub-fraction of these extracts was found to suppress proliferation/growth of malignant cell lines *in-vitro* particularly in relation to gastric (AGS) and colon (HT-29) cells. Several of the components likely to be responsible for this activity remain unidentified and future work should focus on their structural elucidation and quantification.

INTRODUCTION

There is ample scientific evidence that suggests a diet rich in fruits and vegetables is associated with reduced risk of many diseases. As a result, public health recommendations that aim to increase fruit and vegetable consumption can be found in most countries. For example the United States Department of Agriculture recommends the consumption of 7-9 servings of fruits and vegetables per day (1).

Avocado (Persea Americana Mil.) fruit is an oleaginous fruit with a lipid content of about 25% of its edible portion (2). The fruit is a rich source of monounsaturated fatty acids (MUFAs) especially oleic acid (3). In addition to MUFAs, avocadoes are known to contain high amounts of other bioactive substances such as carotenoids (4), vitamins B, C and E (5), phenolics (6) and phytosterols (7).

Whilst the beneficial effects of MUFAs on cardiovascular human health have been well documented (8, 9) there is limited evidence related to the potential chemopreventative properties of avocadoes. The use of phytochemicals to suppress, stop, or reverse carcinogenesis has attracted significant attention over the last few years. A recent study using lipid-soluble extracts, rich in lutein and tocopherols, from Californian 'Hass' avocadoes has reported inhibition of prostate cancer cell lines *in vitro* (10). Although lutein has been shown to possess antiproliferative properties against mammary and colon cancer cells (11, 12) it was found to be inactive against the prostate cancer lines. This led the investigators to conclude that phytochemicals other than lutein were responsible for the antiproliferative properties of the avocado extract (10). Similarly, the active components behind the growth inhibition of malignant and pre-malignant human oral cells by a chloroform avocado extract, reported in another study, were not identified (13).

All the studies described above were carried out overseas using extracts from foreign-grown fruit. Therefore our aim was to determine the levels of major lipid-soluble bioactives in Australian-grown 'Hass' fruit and assess the antioxidant and antiproliferative activity of extracts against three cancer-cell lines not previously investigated (in relation to avocado extracts). The generated information could then be used to further promote/market Australian avocadoes and strengthen their position in the mind of the national and international public as a natural, health-benefiting fruit.

MATERIALS and METHODS

Samples

Mature 'Hass' avocadoes were obtained from the Sydney Markets. The fruits originated from 3 farms located in Queensland (Sunnyspot Farm, Tinaroo Falls and Avocado Estate) and their maturity varied from ripe to very ripe, based on skin colour and firmness, at the time of analysis.

Sample extraction

The extraction of the avocado samples was based on the method described by Lu *et al.* (10) with modifications. An arc segment from each avocado (~ 30g) was cut from the side of the fruit, homogenised in acetone (30 mL, containing 0.1% butylated hydroxytoluene (BHT) as

an antioxidant) and extracted by sonication (15 min.) using the same solvent (3 x 80 mL). The extracts were combined, filtered and dried with $MgSO_4$ before reduced to about 6 mL using a rotary evaporator (40 °C). Further extraction with hexane (containing 0.1% BHT) followed (5 x 10 mL) before the extract was reduced to dryness on a rotary evaporator (40 °C) and was finally reconstituted with hexane (10 mL). The last step (i.e hexane extraction) was carried out to ensure that only the lipid-soluble components remained in the extract (referred to as **crude extract** in this report). The crude extract was subjected to HPLC analysis, antioxidant and anticancer assays before and after further purification by solid phase extraction. Saponification, using 9M KOH in 50 % ethanol (20 mL), was initially performed (on a few samples used for method development) but was not used for the remainder of the project as it was found to be detrimental for some extract components particularly oxygenated carotenoids and tocopherols. This is in agreement with recent published literature (14).

Solid phase extraction (SPE)

Solid phase extraction (SPE) was used to obtain a sub-fraction from the crude hexane extract. An aliquot (1 mL) of the crude hexane extract was loaded on a pre-conditioned Supelclean^{TM} LC-Diol cartridge (500 mg capacity, 3 mL volume, Supelco, Castle Hill, NSW, Australia). The cartridge was washed with 1 mL of 0.1% BHT in hexane. The retained components were eluted with 5 mL of acetonitrile/methanol (80/20 v/v), evaporated to dryness and reconstituted with 2 mL of an appropriate solvent before being subjected to HPLC analysis, antioxidant and anticancer assays. The SPE fraction generated in this way retained the more polar lipophilic compounds found in the crude extract but contained less chlorophylls, carotenoids and tocopherols.

High performance liquid chromatography (HPLC)

Lipophilic compounds were chromatographed using a Shimadzu HPLC system equipped with two high-pressure LC-10ADVP pumps, a SIL-10ADVP auto sampler (250 µL sampling loop), a CTO-1-ADVP column oven and a SPD-M10ADVP photodiode array detector (Shimadzu Inc., Rydalmere, NSW, Australia). A YMC Carotenoid column (C30) was used for the separation of the carotenoids: 4.6 mm i.d x 250 mm length, 5µm particle size (Waters Associates, Chippendale, NSW, Australia). The mobile phases used were methanol, MTBE, water (81:15:4) (A) and methanol, MTBE, water (6:90:4) (B) at a flow rate of 1 $mLmin^{-1}$. Analytes were eluted using a linear gradient: 0 to 100% B over 10 min followed by 100% B for another 6 min. Detection was achieved using fluorescence and visible detection (wavelengths used are shown in Table 1). Analytes were identified by comparison of their elution time with those of authentic standards (Sigma-Aldrich, Castle-Hill, NSW, Australia). Quantification was achieved by the use of external calibration curves. Values for carotenoids were expressed against the curve obtained from all-*trans*-lutein, values for tocopherols against the curve obtained from α-tocopherol whilst values for chlorophylls a and b were expressed against their own calibration curves. All reagents used in this study were HPLC grade (Merck Serono Ltd, Frenchs Forest, NSW Australia) and authentic reference compounds were purchased from Sigma-Aldrich Ltd (Castle Hill, NSW Australia).

Antioxidant analysis using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

This method was adapted from Thaipong, *et al.* (15). A series of dilutions of the avocado samples and standards were made in a solvent mixture of acetone:methanol (1:1). An aliquot (30 µL) of the sample or standard dilution was added to a solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Castle Hill, NSW) in methanol (101 µM). The mixture was vortexed briefly and left to stand in the dark. The absorbance was read after 1 h at 570 nm using a Multiskan RC Microplate Reader. The standard curve using Trolox (Sigma-Aldrich, Castle Hill, NSW) was found to be linear between 25 – 800 µM. Additional dilutions were made if the values measured were over the linear range of the standard curve. The IC_{50} values were obtained using GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, California USA). As expected from previous studies (16), the DPPH assay was found to be suitable for the analysis of the avocado lipophilic extracts in contrast to the ferric reducing antioxidant power (FRAP) assay which was initially used but deemed unsuitable due to the poor solubility of the extracts in the assay reagents.

Cell culture

All cell lines, obtained from the American Type Culture Collection, were cultured at 37°C under a humidified atmosphere containing 5% CO_2. Gastric adenocarcinoma (AGS) cells were cultured in F12-K medium containing 10% foetal bovine calf serum (FBS) (Invitrogen, Australia) and 1% Penicillin-Streptomycin (P-S) (Trace, Australia), colon adenocarcinoma (HT-29) cells were cultured in McCoy's 5A modified medium containing 10% FBS and 1% P-S and leukaemia (HL-60) cells were cultured in Iscove's modified Dulbecco's medium containing 20% FBS and 1% P-S.

Cell proliferation assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Castle Hill, NSW Australia) colorimetric assay was used to assess the antiproliferative activity of the crude and SPE-treated avocado extracts following desolvation and reconstitution into tetrahydrofuran (THF). Cells ($5x10^3$ cells in 100 µL medium) were plated into each well of the 96-well microplates. After they were incubated for 24 h, the cells were treated with various concentrations of the extracts for another 24 h. Then MTT solution was added to each well and incubated for another 4 h. The resulting MTT formazan product, produced by the mitochondrial enzymes of living cells, was dissolved by the addition of 100 µL of DMSO. The amount of formazan was determined by measuring the absorbance at 595 nm with a microplate reader (Bio-Rad, model 550). The reduction of MTT to formazan takes place only when mitochondrial reductase enzymes are active, and therefore this conversion can be used as a measure of living cells.

Liquid chromatography-mass spectrometry (LC-MS)

Atmospheric pressure chemical ionisation (APCI)

Atmospheric pressure chemical ionisation (APCI) coupled to LC-MS was carried out to obtain additional information on the identity of some (i.e. those for which reference standards were not available) of the carotenoids present in the extracts. Analysis was performed on a Quantum triple stage quadrupole (TSQ) mass spectrometer (ThermoFinnigan, NSW, Australia) equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector and an autosampler. The column, mobile phase, flow rate and gradient were identical to those described above. Ions were generated using an APCI source in the positive mode under conditions set following optimisation using lutein. Data was acquired in the full scan mode (200-800 m/z).

Electrospray ionisation (ESI)

Electrospray ionisation (ESI) coupled to LC-MS was carried out to obtain additional information on the identity of the constituents of the SPE-treated extracts. The TSQ system this time was fitted with an ESI source. Ions were generated in the positive mode under conditions set following optimisation using quercetin. A Luna-(2) (C_{18}, 2.1 mm i.d x 150 mm length, 5µm particle size) (Phenomenex, Lane Cove, NSW, Australia) column was used for separation. The mobile phases used were water (A) and acetonitrile (B), both containing 0.5% formic acid, at a flow rate of 1 $mLmin^{-1}$. Analytes were eluted using a linear gradient: 80 to 100% B over 10 min. Data was acquired in the full scan mode (200-400 m/z).

RESULTS

Chemical composition of lipid-soluble avocado extracts

Lutein was the major carotenoid detected in the lipid-soluble avocado extracts followed by several other minor oxygenated and non-oxygenated carotenoids (violaxanthin isomers, antheraxanthin, α- and β-carotene). α-Tocopherol was the major form of vitamin E found in the extract together with lesser amounts of γ- and δ-tocopherol. The extracts were also found to be rich in chlorophylls a and b (Figure A1 in Appendix). The quantitative values for the major lipophilic components found across the fruits investigated are shown below (Table 1). The concentration of these components was found to vary quite widely between fruits as can be seen from the concentration ranges shown in Table 1. This was not unexpected as a previous study carried out on Californian 'Hass' avocadoes has also highlighted inter-fruit variation in concentration of phytochemicals (e.g. lutein 213-361 µg/100 g FW, α-tocopherol 2537-3278 µg/100 g FW) (10).

Table 1: Major lipid-soluble phytochemicals identified in Australian-grown 'Hass' avocadoes

Compound	Concentration range (µg/100g fruit FW[a])	Quantitation[b]
α-Tocopherol	1197-2151	α-Tocopherol (fluorescence)
γ-Tocopherol	118-232	α-Tocopherol (fluorescence)
δ-Tocopherol	83-260	α-Tocopherol (fluorescence)
Chlorophyll a	1073-1465	Chlorophyll a (Vis[a] at 432 nm)
Chlorophyll b	1231-2215	Chlorophyll b (Vis at 470 nm)
Lutein (all-*trans*-)	160-273	Lutein (Vis at 445 nm)
Violaxanthin (all-*trans*-)[c]	25-58	Lutein (Vis at 445 nm)
Violaxanthin (*cis*-)[c]	18-42	Lutein (Vis at 445 nm)
Antheraxanthin (all-*trans*-)[c]	18-48	Lutein (Vis at 445 nm)
α-carotene (all-*trans*-)	6-13	Lutein (Vis at 445 nm)
β-carotene (all-*trans*-)	15-52	Lutein (Vis at 445 nm)

[a] FW: fresh weight; Vis: visible absorbance
[b] Denotes the calibration curves and detection mode used to obtain the quantitative values.
[c] Tentative identification based on UV-Visible spectral information, pseudomolecular ion $[M+H]^+$ from APCI-LC-MS and elution time on C_{30} column only.

Antioxidant capacity of lipid-soluble avocado extracts

The antioxidant capacity of the 'Hass' lipid-soluble extracts was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing antioxidant power (FRAP) assays. The FRAP assay was found not to be suitable for the analysis of the 'Hass' extracts as the physical properties of components in the extracts interfered with the assay. The presence of coloured components in the extracts or the generation of secondary reaction products between the reagent (i.e. chromogen) and the samples analysed are possibly responsible for such interferences (17). The DPPH assay however produced meaningful results that indicate that avocadoes can be a very good source of natural lipophilic antioxidants. The antioxidant activity of the lipophilic crude avocado extracts, expressed as the concentration of sample in solution able to reduce the initial DPPH concentration by 50% (IC50), ranged on average from 62.2 to 129.0 mg/mL (Table 2). The antioxidant activity values for the SPE-treated extracts were much higher (i.e they possessed less antioxidant activity) than those obtained from the crude extracts (Figure A2). Chemical analysis of the SPE-treated extracts showed that they contained no tocopherols and much less carotenoids and chlorophylls compared to the crude hexane extracts. This suggests that components in the extracts such as tocopherols, chlorophylls and carotenoids are responsible for the antioxidant activity seen in the lipophilic avocado extracts. Indeed, when plotted, the antioxidant capacity of 'Hass' avocado lipophilic extracts was found to correlate well with the sum of the concentration of six components (α-,γ- and δ-tocopherols, chlorophylls a and b, and lutein) (Figure A3).

The antioxidant activity of the avocado lipophilic extracts, expressed as IC50 values against DPPH, was found to be higher (i.e. lower IC50 values) than that obtained from lipophilic extracts from various other fruits and walnuts (Figure A4). However, it must be noted that the total antioxidant activity of a fruit/food item depends on both lipophilic and hydrophilic components.

Table 2. Antioxidant activity, expressed as IC50 values against DPPH, of crude and SPE-treated avocado lipophilic extracts (two extractions per fruit). The R^2 values indicate the fit of the theoretical line used to obtain the IC50 values.

Sample	IC50 (mg/mL)	R^2	Mean	%RSD
1A-crude	83.2	0.989	81.7	2.6
1B-crude	80.2	0.997		
2A-crude	131.2	0.993	129.0	2.4
2B-crude	126.9	0.997		
3A-crude	75.0	0.945	73.5	2.9
3B-crude	72.0	0.945		
4A-crude	67.2	0.804	65.4	4.0
4B-crude	63.5	0.877		
5A-crude	124.5	0.977	122.2	3.1
5B-crude	119.5	0.983		
6A-crude	105.6	0.991	105.6	0.1
6B-crude	105.1	0.955		
7A-crude	84.9	0.969	89.4	7.2
7B-crude	94.0	0.972		
8A-crude	85.9	0.901	83.4	4.4
8B-crude	80.7	0.949		
9A-crude	62.2	0.925	62.2	0.1
9B-crude	62.5	0.925		
10A-crude	67.5	0.958	62.5	11.4
10B-crude	57.4	0.885		
4A-SPE	650.4	0.999	633.4	0.6
4B-SPE	645.2	0.998		
6A-crude	604.5	0.999	607.4	0.7
6B-crude	610.2	0.999		

Anti-cancer properties of lipid-soluble avocado extracts

To assess the potential *in-vitro* anti-cancer properties of 'Hass' avocadoes, crude and SPE-treated lipid-soluble extracts were assayed against human promyelocytic leukaemia (HL-60), colon (HT-29) and gastric adenocarcinoma (AGS) cell lines using the MTT colourimetric assay. This assay is known to be an accurate and reliable pre-clinical tool for the evaluation of chemotherapeutic agents (18). Both, the crude and SPE-treated, avocado extracts suppressed the proliferation of cancer cells in a dose depending manner. The SPE-purified fraction was found to be much more active against all cell lines than the crude extracts or individual components (lutein, chlorophyll b, β-carotene) alone (Figure 1). The reasons for the difference in activity between the crude and SPE-treated extracts are not known but antagonistic effects between different extract components therapeutic agents are not uncommon in pharmacological studies (e.g. 19-20). Pure components were found to be inactive, when tested individually, even at concentrations much higher than their highest measured concentration in avocadoes (15 times higher for chlorophyll b, 25x for lutein, 125x for β-carotene). Although cytotoxic synergy between phytochemicals has been shown in several studies (e.g. 21), the composition of the SPE fraction (i.e. it contained no lipids, much less carotenoids, vitamin E and chlorophylls compared to the crude hexane extract) suggests that components other than carotenoids, vitamin E or chlorophylls were responsible for the *in-vitro* anti-cancer properties of the lipophilic avocado extracts. The *in-vitro* activity of the avocado extract was found to be cell-line specific as greater growth inhibition was observed in the HT-29 and AGS cells than the HL-60 cells (Figure 2).

Analysis of the active extracts by liquid chromatography-mass spectrometry (LC-MS) using positive electrospray ionisation highlighted the presence of several peaks in the *m/z* 200-400 region (Figure 3). An identical profile was obtained from a chloroform-based avocado extract (data not shown). Such an extract has been previously reported to selectively inhibit both premalignant and malignant human oral epithelial cells lines by targeting cell cycle regulatory proteins and increasing intracellular reactive oxygen (13). Persin, a known avocado-leaf toxin for lactating livestock (22) has been shown to have potent cytostatic and cytotoxic effects towards breast-cancer cells (23) and is likely to be one of the components in the active fraction. Other fatty acid-derived alkanols that have been shown in previous work to have liver injury suppressing activity (24) are likely to be part of this active fraction, based on LC-MS data, but several other components remain unidentified. The mass spectra of most peaks in the active fraction indicate the presence of hydroxylated components (Figure 3b) which explains their retention on the diol-based SPE cartridges used for fractionation.

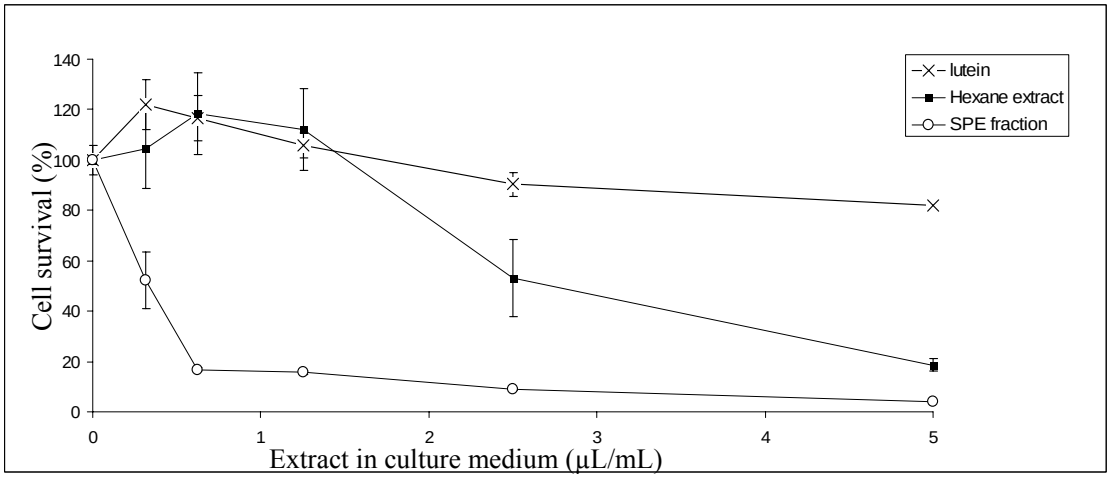

Figure 1: Proliferation of HT-29 cancer cells after exposure to lutein (authentic reference standard), the crude hexane extract and the SPE-treated fraction from an Australian-grown 'Hass' avocado using the MTT assay (n=4). Assuming complete extraction, 1 µL extract will contain equivalent lipophilic phytochemicals found in approx. 1.5 mg fruit fresh weight. The concentration of the solution used for lutein was 170µmol/L which is 25 times higher than the estimated concentration of the extract with the highest lutein content.

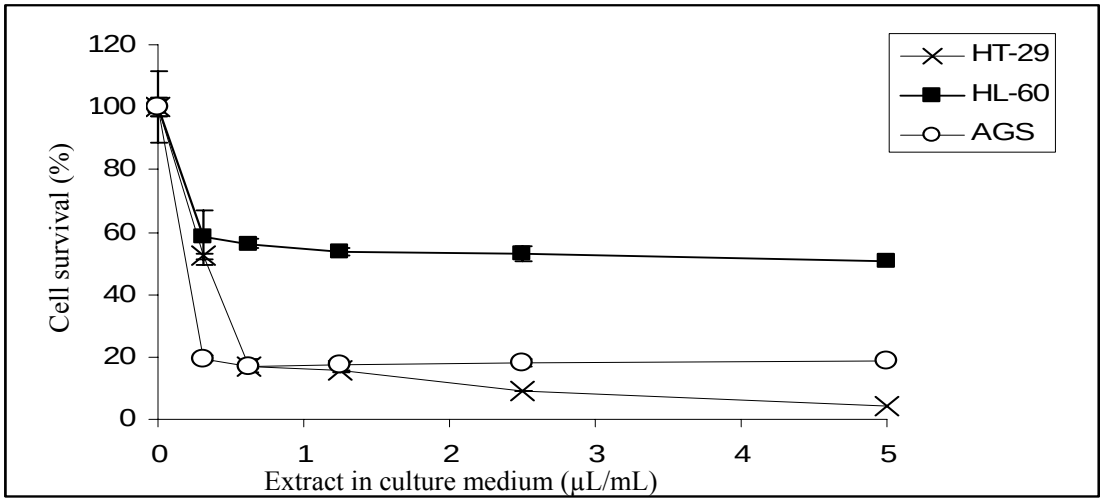

Figure 2: Proliferation of HT-29, AGS, and HL-60 cancer cells after exposure to SPE-treated lipophilic extracts from the same Australian-grown 'Hass' avocado using the MTT assay (n=4). Assuming complete extraction, 1 µL extract will contain equivalent lipophilic phytochemicals found in approx. 1.5 mg fruit fresh weight.

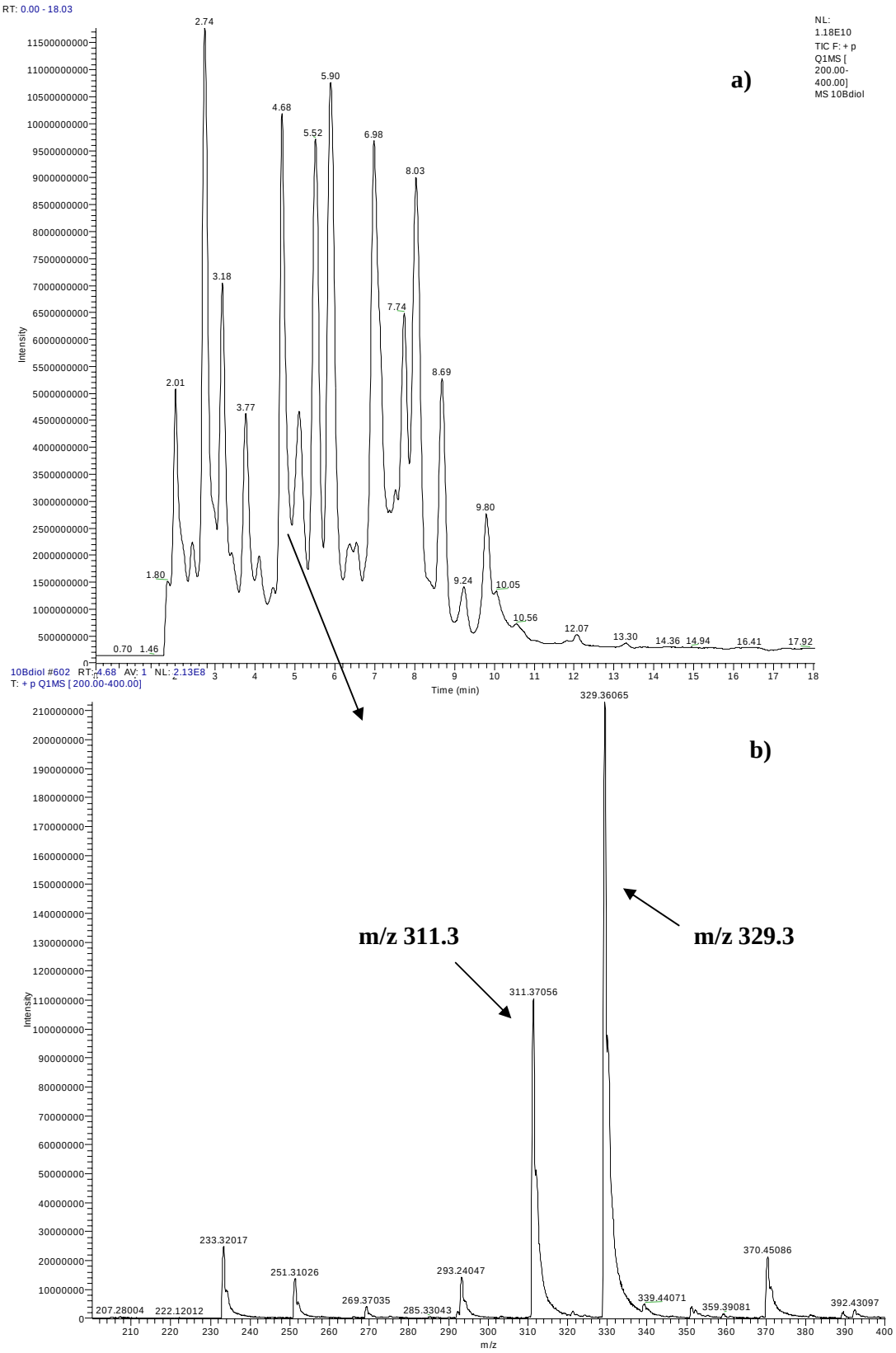

Figure 3: a) Total ion chromatogram (m/z 200-400, positive electrospray ionisation mode) of the SPE-treated fraction from a lipophilic avocado extract and b) mass spectrum of peak at retention time 4.68 minutes illustrating the presence of an OH- group on the molecule (difference of 18 amu between the two most abundant m/z).

DISCUSSION

The lipopihilic extracts from Australian-grown avocadoes were shown to possess very good *in-vitro* antioxidant and anticancer properties. Several components present in the extracts were found to be responsible for these effects. Carotenoids (primarily lutein), chlorophylls and tocopherol (i.e. Vitamin E) isomers contributed most to the antioxidant activity of the extracts whilst fatty acid derivatives (e.g persin, persenones) as well as several unidentified components are likely to be responsible for the *in-vitro* antiproliferative properties of the extracts against the three malignant cell lines (AGS, HT-29 and HL-60) tested. It is now well accepted that both antioxidant and antiproliferative activities should be considered when fruits are assessed for their health-promoting potential (25). This study has shown that avocadoes contain a suite of lipophilic compounds with antioxidant and antiproliferative properties and hence should form part of every balanced diet.

TECHNOLOGY TRANSFER

Findings from this study were presented at the 5th Pigments in Food Congress-for quality and health held in Helsinki during 14-16 August 2008 (*Lipophilic bioactives in Australian-grown 'Hass' Avocadoes,* ISBN 978-952-10-4846-3, pp. 192-194). A manuscript related to this work is currently in preparation for submission to an international peer-reviewed scientific journal.

RECOMMENDATIONS

Scientific

Further work should be carried out focusing on the elucidation/identification and subsequent quantification of the components responsible for the strong *in-vitro* antiproliferative activity exhibited by the SPE-treated lipophilic avocado extracts. These components together with the other known lipophilic antioxidants found in avocadoes (e.g. carotenoids, tocopherols) could then be used as markers for the production of fruit with enhanced health-benefiting properties leading to novel avocado-based functional foods.

Industry

The information generated in this study may be used as a tool to further promote/market Australian avocadoes and strengthen their position in the mind of the national and international public as a natural functional food.

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APPENDIX

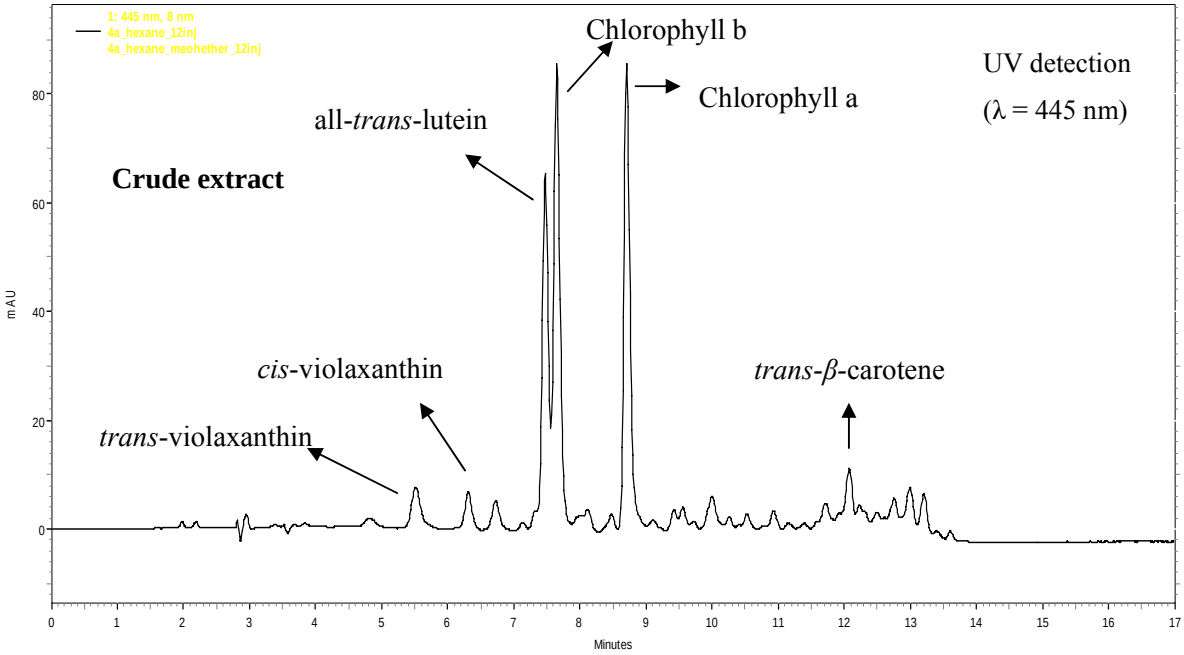

Figure A1. HPLC trace of a lipophilic crude extract from avocado illustrating the major carotenoids and chlorophylls present.

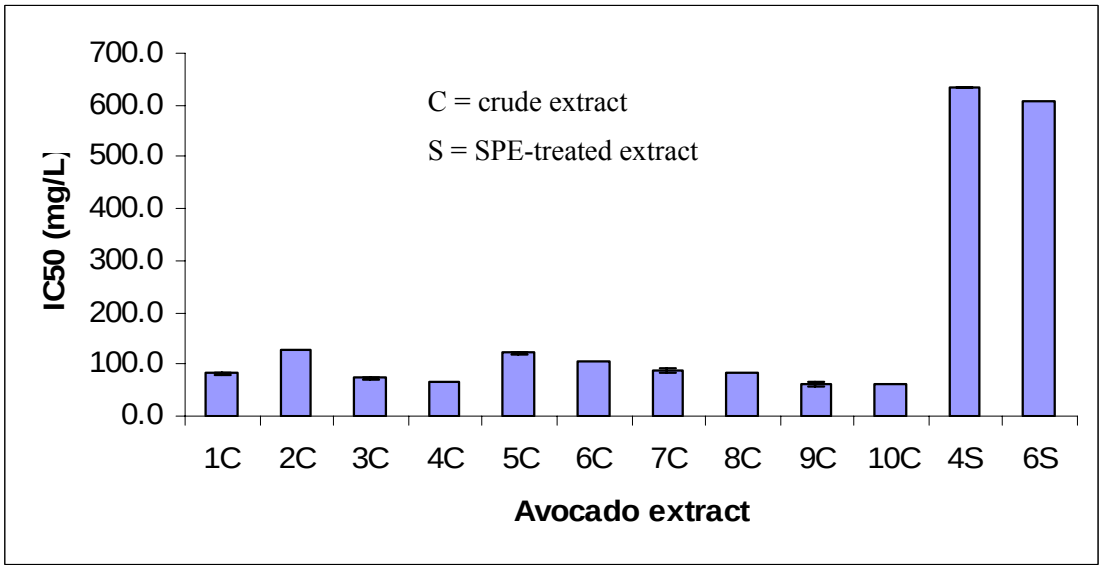

Figure A2. Antioxidant activity, expressed as IC50 values against DPPH, of crude and SPE-treated avocado lipophilic extracts. The crude extracts exhibit much higher activity than the SPE-treated extracts.

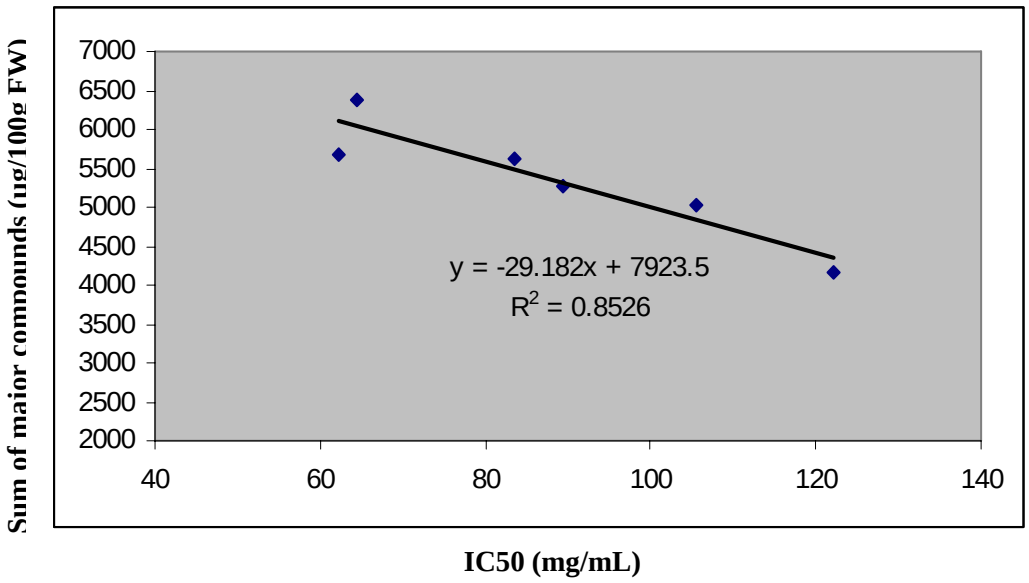

Figure A3. Correlation of the antioxidant capacity values (expressed as IC50) of lipid-soluble extracts from Australian-grown 'Hass' avocadoes with the sum of the concentration of six major compounds (α-,γ- and δ-tocopherols, chlorophylls a and b, and lutein). The smaller the IC50 value, the greater the antioxidant capacity of an extract. Higher concentrations of tocopherols, chlorophylls and lutein in avocadoes leads to higher antioxidant capacity values.

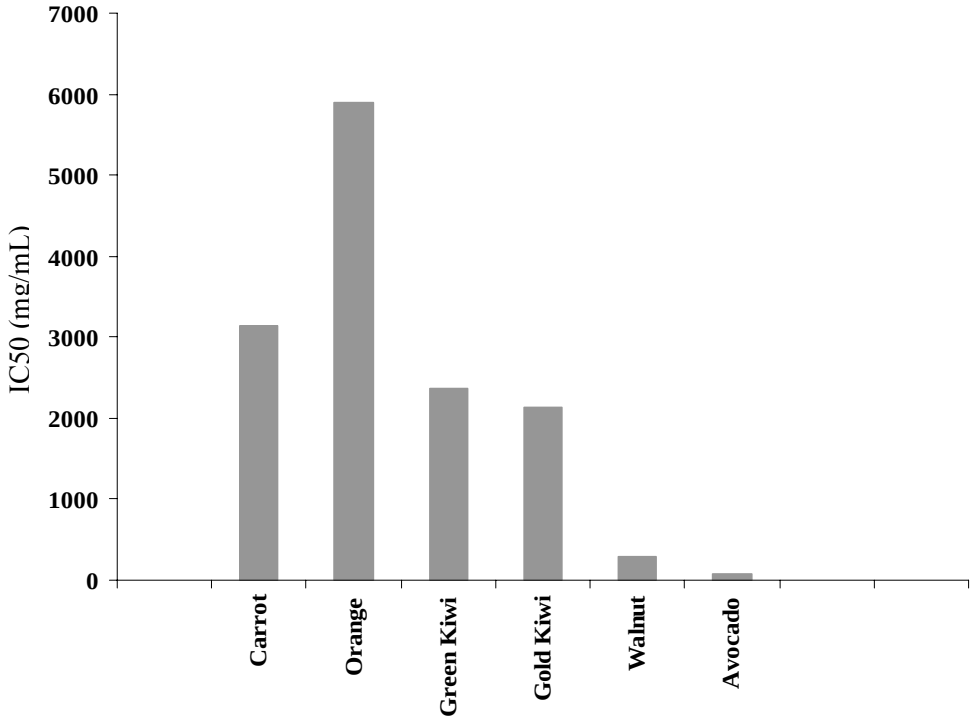

Figure A4. Antioxidant activity, expressed as IC50 values against DPPH, of lipophilic extracts from various fruits/nuts. Small columns indicate high antioxidant activity.