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Bioassay Guided Fractionation and Determination of Total Phenolic, Flavonoid Contents and *in vitro* Radical Scavenging Activity of Fractions From *A. Macrosperma L*, Kiwi Fruit

Sujeewa Krishanthi Hettihewa[1*], Yasine Hemar[1] and HP Vasantha Rupasinghe[2]

[1]School of Chemical Sciences, University of Auckland, 23 Symonds Street, Auckland 1142, New Zealand

[2]Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS B2N 5E3, Canada

[*]**Corresponding author:** Dr.Sujeewa Krishanthi Hettihewa, School of Chemical Sciences, University of Auckland, 23 Symonds Street, Auckland 1142, New Zealand, E-mail: krishanthi2001@yahoo.com

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Abstract

This study was designed to successfully use bioassay guided fractionation to purify phenolic antioxidants rich fractions from *Actinidia macrosperma* fruit. Phenolic compounds were extracted using 70% acetone by steeping technique, and the vacuum manifold solid phase extraction (SPE) using Strata C18 cartridges was used for the preliminary purification to remove more polar compounds such as sugars and proteins from the defatted crude extract resulting in an acidified aqueous fraction (F1), while moderately polar compounds were obtained in an acidified methanol fraction (F2). The total phenolic, total flavonoid contents were determined by using Folin Ciocalteu method and aluminiumchloride method respectively. In vitro radical scavenging activity was evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Phenolics of the extracts were identified using high performance liquid chromatography coupled to diode array detector (HPLC-DAD). Total phenolic content, total flavonoid content and radical scavenging activity determined for the acidified methanol fraction (F2) (476.3 ± 12.7 mg GAE/100 g DW, 106.7 ± 7.5 mg CAE/100 g DW and 2.9 ± 0.1 mmolTrolox equivalents/100 g DW respectively) were significantly (p=0.05) higher than those for the acidified aqueous fraction (F1) (363.2 ± 18.9 mg GAE/100 g DW, 46.2 ± 0.7 mg CAE/100 g DW and 2.0 ± 1.0 mmol Trolox equivalents/100 g DW respectively). The phenolic profile, radical scavenging activity and the HPLC-DAD fingerprints of the SPE–purified extracts showed that the acidified methanol fraction (F2) was rich in flavonoids and was the most active fraction which was subjected to further purification to identify the antioxidants. The presented result along with the qualitative analysis on high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) revealed that the bioassay guided fractionation was successfully employed to purify phenolics rich fractions from *A. macrosperma* kiwi fruit.

Keywords:* *Bioassay guided fractionation; Separation; Antioxidants; Kiwi fruit; Polyphenols

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Introduction

Many plants and herbs are considered to have antioxidant properties and are extensively studied for their beneficial effects on the health of animals and humans, based on their scavenging ability of free radicals generated by environmental and metabolic factors [1,2]. Many of these antioxidant compounds, such as polyphenols, vitamin C, E and A, are present in fruits and vegetables that contribute most significantly to their total antioxidant capacity [3,4].

Kiwi fruit is the common name of the edible berry of cultivars of the woody vine of several Actinidia species. Among several Actinidia species, *Aactinidia macrosperma* is a non-commercial type kiwi fruit with orange-colored flesh, small size fruit with large seeds, and relatively thick, hairless skin. This is a well-known medicinal plant which has been extensively employed in Chinese traditional medicine [5-7].

Research on the therapeutic potential of medicinal plants validates ethnopharmacological usage of plant materials, but also isolates and focuses on the characterization of the active components [8-12]. Therefore, interest in the role of phenolic antioxidants in human health has prompted studies on the separation, isolation and characterization of active phenolic components in various plant-derived foods [13]. Various chromatographic methods available for separation (liquid-liquid chromatography, solid phase chromatography), isolation {semi preparative high-performance liquid chromatography, open column chromatography, flash column chromatography, high speed counter current chromatography (HSCCC)}, are reported in the literature [14-19].

Although there is one research study reported in the literature on the separation, isolation and identification of the phenolics in the fruit of A. macrosperma, phenolic compounds such as the flavonoids and their glycosides present in this fruit were not characterized due to the co-elution of multiple compounds. Therefore, the objective of this research study was to use bioassay guided fractionation for the separation of possible potent antioxidants, undiscovered phenolic compounds in the previous study carried out by in A.macrosperma fruit grown in New Zealand.

Materials and Methods

Sampling plant materials

The fruits of *A. macrosperma* reaching physiological maturity were collected at the Plant and Food research orchard in Te Puke Bay, New Zealand. The fruits with defects were discarded, and the remaining fruits were cut into small parts, freeze-dried and stored at -80°C. The samples were prepared by grinding the lyophilized fruit samples in the mortar using a pestle prior to the extraction.

Chemicals

Folin-Ciocalteu phenol reagent, hydrochloric acid, ferulic acid, caffeic acid, chlorogenic acid, ρ-coumaric acid, syringic acid, catechin, epicatechin, rutin, quercetin-3-O-glucoside, quercetin, luteolin, 2,2-diphenyl-2-picylhydrazyl (DPPH), and Troloxwere

purchased from Sigma, St Louis, USA. Sodium carbonate, sodium hydroxide, sodium nitrite, formic acid, and aluminum hexahydrate, were from Scharlau, Spain. gallic acid (ACROS, USA), HPLC grade methanol, HPLC grade acetonitrile, ethanol, methanol, hexane, and all other chemicals were purchased from ECP Ltd, Auckland, New Zealand.

Preparation of defatted crude extract from *A.macrosperma*

The lyophilized ground fruit sample was subjected to extraction by performing the method published [1]. Phenolic compounds were extracted by steeping lyophilized ground fruit sample (500 g) in 70% aq. acetone (500 mL × 5) in a Scott Duran bottle for 6 h in the dark with nitrogen gas purging at room temperature (23 ± 2°C) to prevent phenolic oxidation during extraction.

Bioassay guided fractionation by vacuum manifold solid phase extraction (VSPE)–Strata® SPE C_{18}

Since the defatted crude extract could contain sugars, sugar derivatives, phenolic compounds and any other hydrophilic compounds, these compounds were separated into different fractions depending on their polarity and the acidity using Strata® C_{18} SPE cartridge (12 mL, SPE; Phenomenex, New Zealand) on a vacuum manifold according to the method described by [20] with some modifications of the solvents selected for the separation (FIG.1).

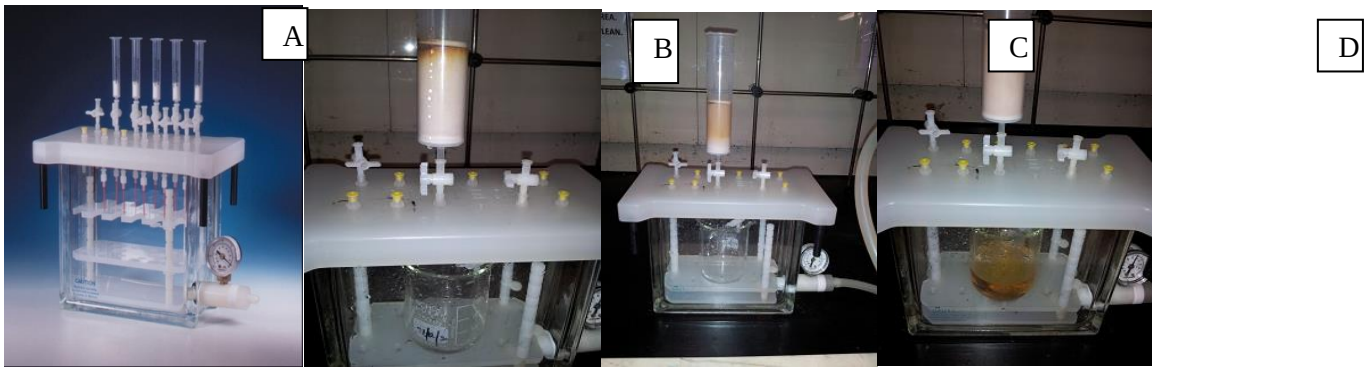


FIG.1. **Fractionation process of defatted crude extract on vacuum manifold strata solid phase C18 (A) multiple cartridges (B) collection of F1 fraction (C) collection of F2 fraction (D) after collection of F2 fraction.**

The C18 SPE cartridges were preconditioned with methanol (20 mL) and conditioned with 2% formic acid in Milli-Q-water (100 mL). The freeze dried defatted crude extract (2.220 g in 3 ml of 2% formic acid Milli-Q-water) was applied to the SPE cartridge and 2% formic acid in Milli-Q-water (100 mL) was passed through the SPE cartridge to elute sugars and highly polar compounds such as phenolic acids into fraction F1. The rest of the compounds remaining on the cartridge were eluted with 2% formic acid in methanol (100 mL), giving fraction F2. The solvents from the fractions F1 and F2 were removed on a rotary evaporator below 35°C under vacuum and subjected to freeze drying and then were stored at -80°C. This process was repeated several times on two SPE cartridges to attain enough material for the liquid-liquid extraction.

Liquid-liquid extraction

The acidified methanol fraction (F2) was selected for further fractionation using liquid-liquid extraction as described by [1].

Liquid-liquid extraction of the sample was carried out between ethyl acetate and water in a separatory funnel several times until the ethyl acetate upper layer became colourless. The combined upper ethyl acetate layers (yellow colour) which was rich in moderately polar compounds and the lower aqueous layer (red) which was rich in more polar compounds were collected separately (F3 and F4 respectively) then concentrated using a rotary evaporator below 35°C under vacuum. Fractions F3 and F4 were subjected to freeze drying and stored at -80°C.

Determination of phenolic profile and radical scavenging activity

The phenolic content (TPC) by Folin-Ciocalteu assay, total flavonoid content (TFO) by aluminium chloride colorimetric method and radical scavenging activity by DPPH assay, of fractions (F1-F4) were performed according to the methods described [1].

Analytical high performance liquid chromatography coupled to diode array spectrophotometry (HPLC-DAD) analysis

The phenolic profiles in all fractions were qualitatively determined according to the procedure described [1].

Statistical analysis

All measurements obtained for phenolic profiles and radical scavenging activity were conducted in triplicate and the results are expressed as mean ± SD. The effect of the fractionation and purification steps on the total phenol content, total flavonoid content and antioxidant capacity values were analysed by analysis of variance (ANOVA) using OriginPro8 software. Pairwise multiple comparisons were evaluated by Tukeyss' significance difference test in OriginPro8. Differences at p= 0.05 were considered significant.

Results and Discussion

Sample preparation and extraction

The preparation of an extract consisted of disintegrating and homogenizing the sample followed by transferring the compounds of interest to the suitable solvent. The extraction step was especially delicate when dealing with reactive compounds like phenols. To overcome the detrimental effect of air causing oxidation, the homogenization was carried out by steeping the lyophilized fruit sample of *A. macrosperma* in the extraction solvent under a nitrogen gas purge. Polar compounds such as phenolic compounds are vacuole–bound in living plant cells [21]. During freeze drying and homogenization, the vacuoles distort or disrupt, allowing the formation of strong hydrogen bonds between phenolic compounds and other molecules such as proteins, polysaccharides and nucleic acids. Therefore, phenolic extracts of plant materials are always a mixture of different classes of chemicals that are soluble in the solvent system used [22]. Thus, an additional step of partitioning the crude extract with hexane was carried out to remove carotenoids and other lipophilic compounds that could be present in the fruit matrix. The two immiscible phases of phenolic-enriched defatted lower aqueous phase and carotenoids–enriched upper hexane phase were collected separately. The UV-visible spectra scanned in the range of 250 nm-700 nm showed a strong peak at 280 nm for the phenolic rich phase and several peaks between 400-600 nm were observed for the carotenoid–

enriched upper phase which is consistent with a high carotenoid content. Some of the carotenoids were identified by comparison with a xanthophyll (mixture of lutein and β-carotene) standard, ran under the same conditions on a UV-vis spectrophotometer. Recent studies have reported that the fruit of *A. macrosperma* is rich in carotenoids (lutein, β-carotenes, zeaxanthin,

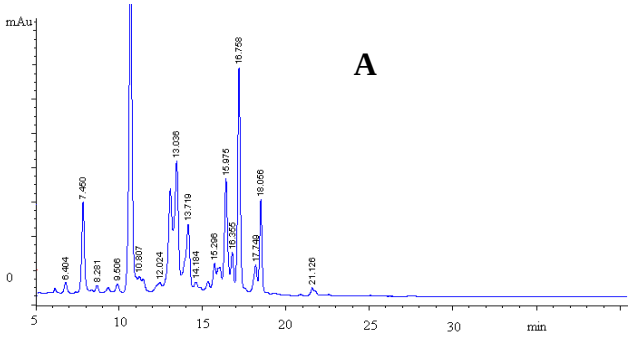
violaxanthin) and chlorophylls (chlorophyll a and b) [14].

Fractionation by solid phase extraction technique on C18 Strata cartridges

The phenolics–enriched defatted crude extract (2.22 g) was fractionated by solid phase extraction technique on reverse phase silica bonded C18 Strata cartridges on a vacuum manifold. The colourless acidified aqueous (F1, 2.01 g) and red coloured acidified methanol (F2, 0.21 g) fractions were obtained from a single cartridge in a single run. This process was repeated on two cartridges several times. The phenolic profiles and antioxidant capacities were determined for lyophilized crude extract, defatted crude extract, F1 and F2 fractions followed by HPLC-DAD analysis. Total phenolic content, total flavonoid content and radical scavenging activity determined for the acidified methanol fraction (F2) (476.3 ± 12.7 mg GAE/100 g DW, 106.7 ± 7.5 mg CAE/100 g DW and 2.9 ± 0.1 mmolTrolox equivalents/100 g DW respectively) were significantly (p=0.05) higher than those for the acidified aqueous fraction (F1) (363.2 ± 18.9 mg GAE/100 g DW, 46.2 ± 0.7 mg CAE/100 g DW and 2.0 ± 1.0 mmolTrolox equivalents/100 g DW respectively).

The most active fraction F2 obtained after repeated runs on two SPE cartridges was selected for further studies on the purification and identification of flavonoids in *A. macrosperma* fruit. Fraction F2 (3 g) was partitioned between ethyl acetate and water to obtain fractions F3 (615 mg in a single run) which showed strong yellow colour representing flavonols and F4 (2.31 g in a single run) which showed red colour representing flavan-3-ols. The total phenolic content, total flavonoid content, total flavanol content and radical scavenging activity (189.9 ± 10.7 mg GAE/100 g DW, 58.1 ± 3.8 mg CAE/100 g DW, 140.2 ± 3.5 mg CAE/100 g DW, 1.9 ± 0.1 mmolTrolox equivalents/100 g DW respectively) showed by the aqueous fraction (F4) were significantly (p=0.05) higher than those of the ethyl acetate fraction (F3) (84.1 ± 5.0 mg GAE/100 g DW, 41.2 ± 1.2 mg CAE/100 g DW, 16.1 ± 1.9 mg CAE/100 g DW, 0.5 ± 0.1 mmolTrolox equivalents/100 g DW respectively). The significantly high radical scavenging activity determined for the aqueous fraction (F4) could be due to the higher total flavanol content in F4. Monomeric (catechin, epicatechin), dimeric, trimeric and polymeric (proanthocyanidins) flavanols are all well-known antioxidants [23,24].

HPLC-DAD chromatograms along with the UV-vis spectra obtained at 360 nm showed that the phenolic compounds present in defatted crude extract were successfully fractionated on C18 cartridges (FIG. 2A-2C). Compounds with shorter retention times such as phenolic acids (maximum absorption wavelength between 300-320 nm), were eluted with acidified water (fraction F1) (FIG. 2B) while compounds with longer retention times such as flavonols and their glycosides (maximum absorption wavelength between 330-360 nm) were eluted with acidified methanol (fraction F2) as shown in (FIG. **2C**)**.**



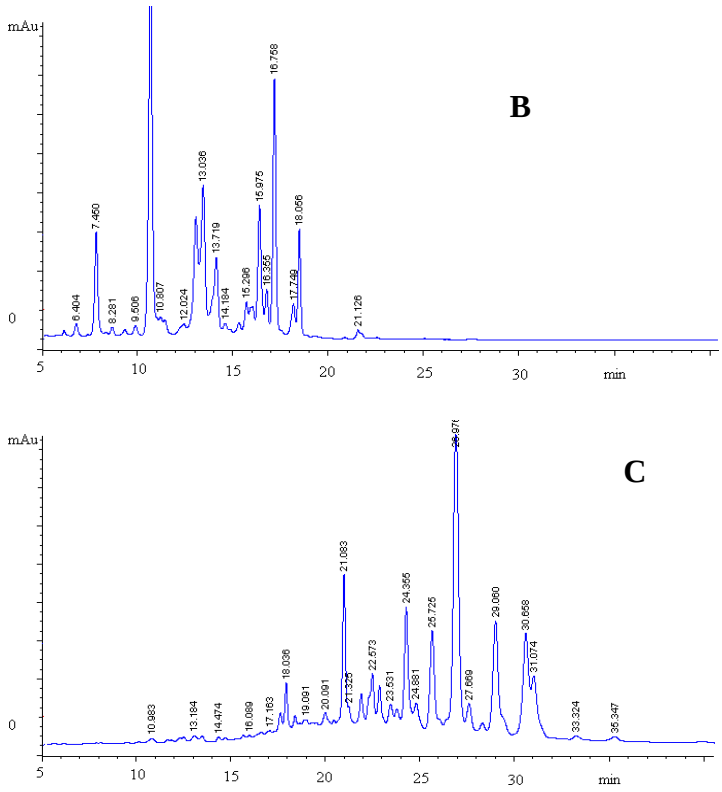


FIG. 2. **HPLC-DAD chromatograms of (A) defatted crude, (B) acidified aqueous fraction-F1 and (C) acidified methanol fraction-F2 recorded at 360 nm.**

The elution order of phenolic compounds on reverse phase mode has been described previously [25]. More polar phenolics elute at the beginning of the chromatogram, with the following order: hydroxybenzoic acids, flavan-3-ols, hydroxycinnamic acids, coumarins, flavanones, dihydrochalcones, flavonols and flavones. Within the same polyphenol class (a) the retention time decreased as more hydroxyl groups were present in the polyphenol, (b) if the polyphenol contained less polar substituents, such as methoxy groups, the retention time increased, (c) if the chemical structures of polyphenolic compounds include sugars, the polyphenols eluted before their aglycones. Our observations are in agreement with research on the phenolic composition of kiwi fruit juice extracted from A. deliciosa cv. Hayward using a similar fractionation process on SPE cartridges as [26]. Using HPLC-DAD analysis, they characterized strongly acidic phenolics such as p-coumaric acid, protocatechuic acid, syringic aldehyde, and derivatives of coumaric acid were eluted into acidified water, and weakly acidic phenolic compounds such as catechin, epicatechin, procyanidins, dimeric and tetramericflavanols, quercetinglucoside, quercetinrutinoside, quercetinrhamnoside, kaempferolrhamnoside and kaempferolrutinoside were eluted with acidified methanol [26].

HPLC-DAD chromatograms obtained from F3 and F4 are shown in FIG. 3 and 4.

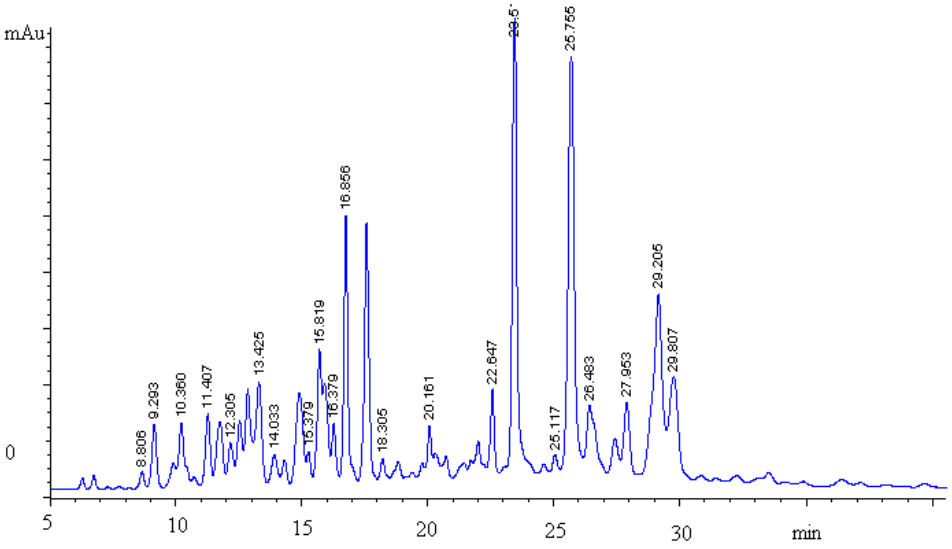


FIG. 3. **HPLC-DAD chromatogram of the ethyl acetate fraction (F3) at 360 nm.**

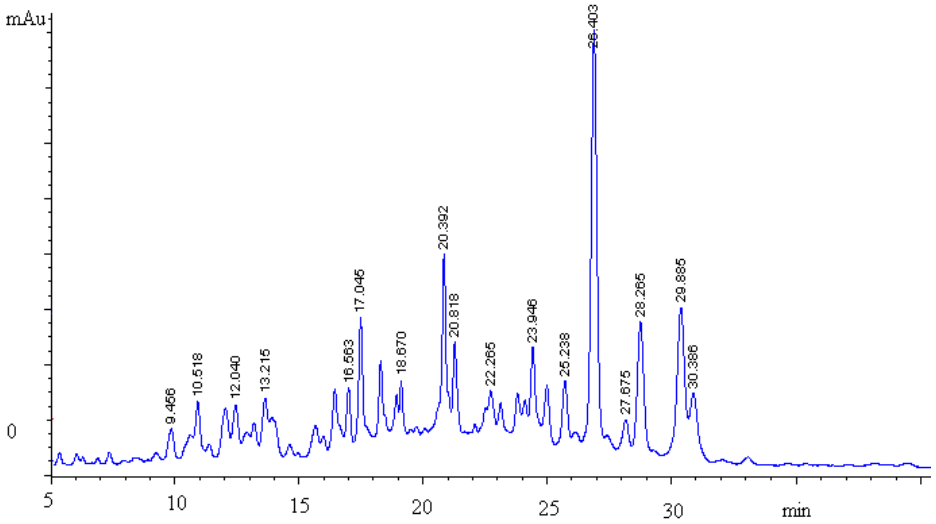


FIG. 4. **HPLC-DAD chromatogram of the aqueous fraction (F4) at 360 nm**

Conclusion

This study was designed to use bio assay guided fractionation to identify previously undiscovered phenolic antioxidant compounds in *A. macrosperma* fruit. Vacuum manifold solid phase extraction (SPE) using Strata C18 cartridges was used for the preliminary purification to remove more polar compounds such as sugars and proteins from the defatted crude extract resulting in an acidified aqueous fraction (F1), while moderately polar compounds were obtained in an acidified methanol

fraction (F2). The phenolic profile, radical scavenging activity and the HPLC-DAD fingerprints of the SPE–purified extracts showed that the acidified methanol fraction (F2) was rich in flavonoids and was the most active fraction which was subjected to further purification to identify the antioxidants.

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