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Biophenols and Antioxidant Properties of Australian Canola Meal

Hassan K. Obied,*[,†] Yi Song,[†] Sonia Foley,[†] Michael Loughlin,[‡] Ata-ur Rehman,[§] Rodney Mailer,[§] Tariq Masud,[∥] and Samson Agboola[‡]

[†]Graham Centre for Agricultural Innovation, School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia 2678

[‡]Graham Centre for Agricultural Innovation, School of Agriculture and Wine Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia 2678

[§]Graham Centre for Agricultural Innovation, Industry and Investment NSW, Wagga Wagga, NSW, Australia

[∥]Department of Food Technology, University of Arid Agriculture, Rawalpindi, Pakistan

ABSTRACT: During the extraction of canola oil, large quantities of meal are produced. Extracting biophenols from Australian canola meal (ACM) adds value to an otherwise low-value agro-industrial byproduct. This study examined the biophenol content and the antioxidant activity of ACM, the impact of extraction conditions, and varietal differences. Sinapine was the principal biophenol in ACM. In crude and hydrolyzed extracts, 31 compounds were identified: 2 dihexosides, 2 organic acids, 4 glucosinolates, 17 sinapic acid derivatives, 2 cyclic spermidine alkaloids, caffeic acid and its dihexoside, kaempferol, and its C-glucoside. ACM showed significant free radical scavenging activity in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays. Sinapine was the chief contributor to ACM antioxidant activity, whereas kaempferol sinapoyl triglucoside isomer was the most potent antioxidant. Biophenol content ranged between 12.8 and 15.4 mg GAE/g DW. Differences among studied cultivars were generally quantitative. The Tarcoola cultivar showed the highest biophenol content and antioxidant activity.

KEYWORDS: *online ABTS, polyphenols, rapeseed, canola cake, Brassica napus, sinapine, HPLC, free radical*

INTRODUCTION

Oil from rapeseed (*Brassica napus* L.) has been used for centuries as fuel and lubricant. The cardiotoxic properties of rapeseed oil restricted its culinary use due to high erucic acid content.[1] Similarly, an abundance of antinutrient components, mainly glucosinolates and sinapine, limited the use of rapeseed cake as animal feed.[2,3] In the 1970s, canola was introduced through hybridization to overcome rapeseed toxicities and present high-quality edible rapeseed varieties. In 1985, rapeseed and canola were recognized by the U.S. Food and Drug Administration as different species.[3] The name "canola" is derived from "Canadian oil, low acid" and was registered in Canada.[4] It is used afterward to denote any cross-bred rapeseed variety that contained <2% erucic acid in its oil and <30 μmol/g of any one or any combination of the four aliphatic glucosinolates (gluconapin, progoitrin, glucobrassicanapin, and napoleiferin) in its defatted meal. This definition was revised in 1997 to a glucosinolates level of <18 μmol/g whole seeds and <1% erucic acid in the oil.[5]

From a minor crop in the late 1980s, canola is now the largest oilseed crop and third largest broad-acre crop (after wheat and barley) in Australia,[6] which makes up to 91% of total production. Australia produces around 3.2 million tonnes of canola.[6] On average, Australian canola seeds yield around 42% oil.[7] Thus, about 1.9 million tonnes of canola meal is produced annually. Canola meal, produced as a byproduct during the extraction of oil from canola seed, is mainly used as a high protein source animal feed in Australia.[8]

Studies of the biophenol composition of rapeseed meal can be traced back to the early 1970s.[9,10] The importance of phenolic compounds in canola seeds has since been continuously recognized. During the past two decades canola biophenols attracted more attention than ever before in part due to the fact that canola production has grown more rapidly than any other source of vegetable oil. Most of the available literature on canola biophenols is derived from Canadian canola.[11−13] Health benefits (e.g., anticancer, antiaging, etc.) of the canola oil/meal have been recognized, with particular interest based on the antioxidant activities of the phenolic compounds.[14,15]

No data have been reported on the phenolic composition and antioxidant activities of Australian canola meal (ACM) to date. The unique environmental and ecological properties of Australia are known to induce detectable changes in the phenolic composition of plants.[16,17] Therefore, it is crucial to determine the phenolic content and antioxidant activity of ACM for any future high-technology utilization.

MATERIALS AND METHODS

Chemicals and Reagents. The following reagents were used without further purification: Folin−Ciocalteu reagent, potassium phosphate monobasic, sodium phosphate dibasic dihydrate, formic acid, sodium nitrite, ethylenediaminetetraacetic acid (EDTA), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) from Sigma-Aldrich (Sydney, Australia); hydrochloric acid (32%) and HPLC grade methanol were obtained from Merck (Darmstadt, Germany); *n*-hexane was purchased from J. T. Baker (USA);

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anhydrous acetonitrile was from RCI Labscan (Bangkok, Thailand); sodium chloride, glacial acetic acid, and absolute ethanol were from Merck (Kilsyth, Australia); sodium carbonate was from Biolab (Clayton, Australia); sodium molybdate dihydrate, aluminum chloride, and potassium persulfate were obtained from Univar (Seven Hills, Australia); sodium hydroxide was from Rowe Scientific (Lonsdale, Australia). Water used in all analytical work was purified by a Modulab Analytical model water system (Continental Water Systems Corp., Australia). More than 30 phenolic standards were purchased from Sigma-Aldrich (Australia) and Extrasynthese (France) and used without further purification including sinapic acid, gallic acid, caffeic acid, ferulic acid, apigenin, kaempferol, quercetin, and rutin.

Sample Collection and Preparation. Seed samples from four different cultivars of canola (Tarcoola, Rainbow, Warrior, and Lantern) were obtained from Canola Breeding Program, DTIRIS (NSW Department of Trade and Investment, Regional Infrastructure and Services), at Wagga Wagga Agriculture Institute, Wagga Wagga, NSW, Australia. Portions of the canola seeds (50 g) were ground to fine powder in a coffee mill for 2 min. The powder was then defatted with petroleum ether in a Soxhlet apparatus and air-dried overnight to produce the canola meal samples.

Optimization of Biophenol Extraction. Two different solvents (aqueous mixtures of 80% methanol v/v and 70% acetone v/v) and two extraction conditions (stirring with a magnetic stirrer and sonication in an ultrasonic bath) were investigated for extraction of canola phenols using simple solid–liquid extraction as previously described.[18]

Optimized Extraction Method. All extractions were performed at ambient temperature (21 ± 2 °C). Two grams of canola meal samples was extracted with 10 mL of solvent (aqueous methanol 80% v/v) for 30 min with continuous stirring using a magnetic stirrer at low speed. The extract was then filtered through Advantec grade no. 1 filter paper. The raffinate was re-extracted with 10 mL of solvent for 15 min and filtered over the first filtrate. The combined filtrate was defatted twice with *n*-hexane (2 × 10 mL), filtered through GF/F filter paper, and refiltered using a 0.45 μm nylon syringe filter to produce canola meal crude extract (CE). CE was stored at −20 °C until further analysis.

Acid and Base Hydrolyses of CE. Acid and base hydrolyses of CE were performed as described earlier [17] with some modification.

Acid Hydrolysis. Five milliliters of 12.0 N HCl was added to 5 mL of CE. The mixture was refluxed at 80 °C for 30 min under a condenser. The hydrolyzed solution was allowed to cool, and 1 g of NaCl was added. The mixture was extracted with ethyl acetate (30 mL × 3). The ethyl acetate combined extract was dried over anhydrous sodium sulfate, and ethyl acetate was evaporated under vacuum at 35 °C. The residue was reconstituted in methanol (10 mL) and filtered through a 0.45 μm nylon syringe filter. Acid-hydrolyzed crude extract (AHCE) was stored at −20 °C as above.

Base Hydrolysis. Five milliliters of 2.0 N NaOH was added to 5 mL of CE. The mixture was refluxed under condenser for 30 min at 80 °C. The hydrolyzed extract was allowed to cool and adjusted to pH 2 with 4.0 N HCl. NaCl (1 g) was added to the solution, and the solution was extracted with ethyl acetate (30 mL × 3). The ethyl acetate combined fraction was dried over anhydrous sodium sulfate before ethyl acetate was removed under vacuum at 35 °C. The residue was reconstituted in methanol (10 mL) and filtered through a 0.45 μm nylon syringe filter. Base-hydrolyzed crude extract (BHCE) was stored at −20 °C as above.

Acidic and Alkaline Extraction. Two grams of the canola meal samples were extracted with 16 mL of 80% aqueous methanol and 4 mL of concentrated HCl or 4.0 N NaOH, respectively, with refluxing under condenser at 80 °C for 30 min, with or without 15 mL protection solution (10 mM EDTA and 1% ascorbic acid or 10 mM EDTA and 1% sodium metabisulfite). The extracts were filtered through Advantec grade no. 1 filter paper. Extracts were adjusted to pH 2 by 4.0 N HCl and volumes made up to 20 mL using ultrapure water. The extracts were then filtered through GF/F filter paper. The extracts were divided into two equal portions. The first portion, 10 mL of the filtrate, was refiltered using a 0.45 μm nylon syringe filter and stored at −20 °C till analyzed. The second portion of the extracts, the remaining 10 mL, was further extracted with ethyl acetate as described above.

Spectrophotometric Assays. All spectrophotometric measurements were performed on a Cary 50 spectrophotometer, using Cary WinUV version 3 software (Varian, Australia).

UV–Vis Spectrum. Spectra of diluted CE (1:500 v/v in water) were recorded between 200 and 800 nm.

Folin–Ciocalteu (FC) Total Phenols. Total phenol content was determined as described earlier.[18] CE was diluted (1:5) with 80% aqueous methanol. Results were expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE/g DW).

Total Flavonoids. Total flavonoid content was determined as described previously.[17] Results were expressed as milligrams of quercetin equivalent per gram dry weight (mg QE/g DW).

Total Phenols, Total Hydroxycinnamic Acids, and Total Flavonols. Analysis was performed as described earlier.[18] The crude extract was diluted (1:20 v/v) with 80% aqueous methanol. The absorbance was measured at 280 nm to determine total phenols using gallic acid as a standard, at 320 nm to determine hydroxycinnamic acid derivatives using caffeic acid as a standard, and at 360 nm to estimate total flavonols using quercetin as a standard.

o-Diphenol Content. The *o*-diphenol content was determined as described earlier.[18] The crude extract was diluted (1:20) with 80% aqueous methanol. Results were expressed as milligrams of caffeic acid equivalents per gram dry weight of freeze-dried material (mg CAE/g).

Antioxidant Activity. CE was diluted (1:5) with 80% aqueous methanolic solution.

ABTS Assay. The ABTS radical scavenging activity of canola meal extracts was determined as described earlier.[17] Results were expressed as Trolox equivalent antioxidant capacity (TEAC) value. TEAC is defined as milligrams of canola meal dry weight giving the same percentage of $ABTS^{\bullet+}$ scavenging activity as 1 mM Trolox.

DPPH Assay. The DPPH radical scavenging activity of canola meal extracts was determined as described previously.[17] Results were expressed as TEAC value. TEAC is defined as milligrams of canola meal dry weight giving the same percentage of $DPPH^{\bullet}$ scavenging activity as 1 mM Trolox.

Chromatographic Characterization of Extracts. *High-Performance Liquid Chromatography–Diode Array Detection with Online $ABTS^{\bullet+}$ Scavenging (HPLC-DAD-ABTS).* Analysis was performed as described earlier.[17,19] Sample analysis was performed by gradient elution on a 250 mm × 4.6 mm i.d., 5 μm, Gemini C-18 column with a SecurityGuard guard cartridge (Phenomenex, Australia). Solvent A was a 0.2% formic acid solution in water, and solvent B was a 0.2% formic acid solution in methanol. Initial condition was 5% solvent B, and then solvent B increased to 80% over 65 min. The system was allowed to equilibrate at initial conditions for 15 min between runs.

High-Performance Liquid Chromatography–Diode Array Detection–Tandem Mass (HPLC-DAD-MS/MS). Samples were analyzed on an Agilent 1200 series liquid chromatograph (Agilent technologies, Germany) by gradient elution. The flow rate was 0.7 mL/min, and the injection volume was 5 μL. The mobile phase used was as described above. A three-step gradient elution for a total run time of 70 min was used as follows: initial conditions, 5% solvent B; solvent B increased to 80% over 65 min; then solvent B increased to 100% over 2 min; and finally back to initial conditions in 3 min. The effluent from the DAD was directed to a 6410 triple-quadrupole LC-MS (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) interface. MS analysis was performed in the negative and positive ion mode (m/z 100–1200) under the following conditions: nitrogen gas; gas temperature, 350 °C; gas flow rate, 9 L/min; nebulizer pressure, 40 psi; capillary voltage, 4 kV; cone voltage, 100 V. Data analysis was performed using Agilent MassHunter workstation version B.01.04 2008 (Agilent Technologies, Germany).

Quantitative Determination of Sinapic Acid and Sinapine. Sinapine and sinapic acid concentrations in our samples were determined by quantitative HPLC-DAD analysis. Peak areas from triplicate extracts were integrated at 280 nm and averaged. Sinapic acid

reference compound (Sigma-Aldrich) was used to construct a seven-point calibration curve for concentrations between 0.1 and 1000 μg/mL; regression coefficient R^2 = 0.993. Recoveries were determined as sinapic acid equivalent.

Statistical Analyses. All measurements were done in triplicates at least, and results were presented as means ± standard deviations. Statistical comparisons were made using one-way ANOVA and post hoc LSD test. Data analyses were performed by Microsoft Excel and PASW Statistics package version 17.0 (SPSS Inc., Chicago, IL, USA). Results were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

UV−Vis Spectrum of ACM Extract. UV−vis spectra of extracts were investigated to provide a preliminary idea about the chemical nature of canola meal biophenols and help in selecting suitable detection wavelengths for HPLC monitoring. Scanning the UV−vis spectrum within the range 200−800 nm, two peaks of equal intensities were observed at 225 and 325 nm with a shoulder at 235 (data not shown). This is indicative of the presence of hydroxycinnamic acids;[20] the absence of prominent peaks at 280 and 360 nm suggests simple phenols and flavonols are not major constituents of ACM. The findings are consistent with previous literature in which sinapic acid and its derivatives were identified as the main constituents in canola meal.[12,13,21] Sinapic acid has a λ_{max} = 322 nm, whereas sinapine has a λ_{max} = 329 nm.[12]

Recovery of Biophenols from Canola Meal. *Optimization of Extraction Conditions.* In a preliminary pilot study, we found that methanol and acetone-based solvents were superior to ethanol and acetonitrile-based solvents, and dynamic extraction conditions were more efficient than static extraction. Hence, an optimization study was conducted to compare the phenol recovery efficiencies between 80% aqueous methanol and 70% aqueous acetone with stirring or sonication. Results are given in Table 1.

Table 1. Optimization of the Extraction Conditions[a]

	TP (FC) (mg GAE/g DW)	ODP (mg CAE/g DW)	TF (mg QE/g DW)
80% aqueous methanol with stirring	20.8 ± 0.5 a	13.9 ± 0.5 a	1.2 ± 0.1 a
80% aqueous methanol with sonication	22.1 ± 4.2 a	13.5 ± 2.2 a	1.3 ± 0.3 a
70% aqueous acetone with stirring	39.2 ± 2.3 b	28.5 ± 1.1 b	4.5 ± 0.1 b
70% aqueous acetone with sonication	39.0 ± 1.6 b	26.1 ± 0.6 c	4.4 ± 0.3 b

[a]Different letters in the same column indicate significant difference at $p < 0.05$. TP (FC), total phenols measured by Folin−Ciocalteu reagent; ODP, *o*-diphenol content; TF, total flavonoids.

Results show that 70% aqueous acetone is a more efficient extraction solvent compared with 80% aqueous methanol. Whereas the total phenol content showed a 70% increased recovery, *o*-diphenol content was doubled and total flavonoid recovery was more than tripled. Acetone superiority in extraction of biophenols from plant material has been frequently reported. However, the compatibility of methanolic extracts with HPLC and the high UV cutoff of acetone at 330 nm[18] make it more appealing to use methanolic extracts for further assays to simplify sample preparation procedures. Furthermore, comparison of the fingerprint chromatograms of methanol and acetone extractions showed no qualitative differences (data not shown).

In the comparison between stirring and sonication methods, overall stirring was slightly more efficient than sonication, yet there was no statistically significant difference apart from *o*-diphenols recovery. On the other hand, it was observed that sonication promoted microemulsion formation. Hence, stirring was considered in preference to sonication for recovery of ACM biophenols.

Biophenolic Composition of ACM. The use of hyphenated liquid chromatography, especially tandem mass spectroscopy, has increased the number of biophenols reported in canola meal and facilitated the detection of complex biophenol derivatives.[12,22] By means of LC-DAD-MS/MS, we could distinguish 29 peaks in ACM extracts (Table 2; Figure 1). We could tentatively identify 27 compounds. Two peaks, 14 and 20, remained unidentified. Both compounds **14** and **20** have absorption maxima around 325 nm and ABTS scavenging activity suggesting that they may be sinapic or caffeic acid derivatives. Compound **20** has an odd molecular weight, 727 Da, most likely to be a monocholine ester.

Identification of peaks was carried out via comparison of their UV−vis and mass spectra with biophenol standards and literature values as described earlier.[23] Furthermore, the study of ABTS scavenging chromatograms (Figure 3) provided valuable insight into not only the antioxidant activity but also the chemical structures of ACM biomolecules. We reported earlier that in online ABTS scavenging assay, the *o*-diphenols entity is essential for $ABTS^{\bullet+}$ scavenging activity and compounds devoid of this structural feature, such as monophenols, failed to scavenge $ABTS^{\bullet+}$.[17] Acid and base hydrolyses of the extracts were also performed to assist in identifying aglycones and unconjugated structures (Table 2; Figure 2).

CE contained biophenols and nonbiophenolic components (Table 2). Early eluting peaks, 1−4, in CE chromatogram (Figure 1) showed no UV absorbance around 280 nm and did not have $ABTS^{\bullet+}$ scavenging activity. On the basis of their mass spectrum, peaks 1 and 2 were assigned as dihexoses, most probably gentiobiose and sophorose.[22,24] We previously reported the presence of peaks 3 and 4 in other plants.[17] On the basis of their molecular ions $[M - H]^-$ at m/z 133 and 191, UV spectra, and literature data,[25] peaks 3 and 4 were identified as malic and citric acids, respectively. Farag et al. detected malic and citric acids in all canola tissues apart from seeds.[25] This can be ascribed to their extraction under liquid nitrogen of ground seeds versus hexane refluxing prior to extraction in our procedures.

Four major glucosinolates (Table 2; Figure 1) were tentatively identified in ACM, namely, progoitrin **5**, gluconapin **8**, 4-hydroxyglucobrassicin **17**, and glucobrassicanapin **18**, on the basis of matching their UV and mass spectra with the literature.[26,27] Detection of glucosinolates in biophenolic canola extracts has been encountered before.[28] Using ion trap mass spectroscopy in the negative ionization mode, Millán et al. could identify 8 glucosinolates,[26] whereas Fang et al. reported 11 glucosinolates by applying positive ionization mode.[29] Hence it is not our aim to profile canola glucosinolates; only glucosinolates showing prominent peaks in our chromatograms are reported.

In agreement with the UV−vis spectra of the extracts, sinapic acid derivatives were the most prevalent biophenols (Figures 1 and 3). Sinapic acid derivatives are estimated to constitute 99%

Table 2. Major Peaks Identified in ACM Extracts[a]

peak	RT	λ_{max} (nm)	ESI$^-$	assigned structure	MW	ABTS	ref
1	4.2		**387**, 341	dihexose	342	−	
2	4.9		**387**, 341	dihexose	342	−	
3	6.3	218	**133**	malic acid	134	−	25
4	9.1	219	**191**	citric acid	192	−	25
5	15.0	220	**388**	progoitrin	389	−	38
6a	17.6	328, 237	663, 422, **354**, 294	*trans*-sinapine 1	310	++	39
6b	17.8	331, 239	663, 422, **354**, 294	*trans*-sinapine 2	310	++	
7	21.6	327, 297s	**503**	caffeoyl dihexoside	504	++	24
8	24.0	220	**372**	gluconapin	373	−	38
9	24.7	326, 250s	453, **385**	4′-glucosylsinapic acid	386	−	24
10	25.6	303b, 232	**530**, 494	feruloyl cyclic spermidine alkaloid	495	−	22
11	26.1	311b	**530**, 494	cyclic spermidine alkaloid	495	−	22
12	27.0	326, 275	**729**	unknown	730	++	
13	27.7	327, 265	**385**	1-*O*-*β*-D-glucopyranosyl sinapate	386	++	
14	28.2	330, 290	**547**	unknown	548	++	
15	28.9	333, 275, 240s	977, **385**	kaempferol sinapoyl triglucoside isomer	978	+++	22
16	30.5	338, 268	1045, 977	kaempferol sinapoyl triglucoside isomer	978	+	22
17	30.6	220, 295	**463**	4-hydroxyglucobrassicin	464	−	26
18	34.1	220	**386**	glucobrassicanapin	387		29
19	35.9	323, 231	291, **223**	*cis*-sinapic acid	224	−	32
20	37.0	325, 300s	726	unknown	727	+	
21	37.5	323, 230	291, **223**	*trans*-sinapic acid	224	++	33
22	38.6	333, 268	**977**, 487	kaempferol sinapoyl triglucoside isomer	978	++	22
23	40.3	331, 231	407, **339**, 223	sinapoyl malate isomer	340	++	40
24	40.9	331, 231	407, **339**, 223	sinapoyl malate isomer	340	++	40
25	42.2	327, 230	**753**	disinapoyl dihexoside	754	++	41
26	43.2	327, 229	**959**	trisinapoyl dihexoside	960	++	22
27	44.2	331, 232	**591**	disinapoyl hexoside	592	++	42
28	46.2	326, 230	**1183**, 591	tetrasinapoyl dihexoside	1184	++	
29	48.6	330, 229	**591**	disinapoyl hexoside	592	++	22
30	14.2	285, 230	NI (ESI$^+$ 149)	degradation product of sinapic acid	148	−	
31	30.5	323, 300s, 241	**179**	caffeic acid	180	++	43
32	38.8	311, 323s, 270, 280s	**487**	unknown	488	−	
33	44.7	364, 264	**447**	kaempferol *C*-hexoside	448	++	
34	47.1	326, 234	305, **237**	methyl sinapate	238	++	35
35	56.1	364, 265	**285**	kaempferol	286	++	33
36	42.4	307, 229	**419**	unknown	420	−	

[a]Peak number from Figure 1; RT, retention time; ESI$^-$, electrospray ionization major peaks; MW, molecular weight; ABTS, ABTS$^{\bullet+}$ scavenging activity in online assay; NI, did not ionize under negative ESI conditions; b, broad peak; s, peak shoulder; values in bold represent base peak; +, low ABTS scavenging activity; ++, good ABTS scavenging activity; +++, potent ABTS scavenging activity.

of the canola meal biophenols.[4] Upon base hydrolysis, *trans*-sinapic acid peak **21** constituted 90% of the total detected peak area at 280 nm (Figure 2C). In accord with most available literature, sinapoyl choline, sinapine **6**, was the principal sinapic acid derivative in ACM (Figure 1). *cis*- and *trans*-sinapic acids were the only free phenolic acid detected in ACM (Table 2). The *cis*-isomer, **19**, is more polar and elutes earlier than the *trans*-isomer, **21**. The literature on the presence of free phenolic acids in canola meal is contradictory.[11,25,30] By means of biophenol standards, we confirmed the absence of vanillic, *p*-hydroxybenzoic, gentisic, protocatechuic, syringic, *p*-coumaric, and ferulic acids in our samples. After acid hydrolysis, we could detect minute amounts of caffeic acid **31** (Figure 2).

Few researchers have reported a broad/skewed peak for sinapine on reversed phase HPLC chromatograms.[13,31] The sinapine peak 6 detected by Agilent HPLC-DAD-MS (Figure 1) split into two peaks [6a and 6b] (Figure 3A) on the Varian HPLC-DAD-ABTS chromatograph, although the same column and mobile phases were used. We could rule out the possibility of being an artifact (double peak) via injecting standards, catechuic acid and catechin, with similar retention times to sinapine. Both compounds showed a single sharp peak. In addition, two peaks [6a and 6b] were observed in the ABTS chromatogram (Figure 3B), although a different UV detector was used. Peaks 6a and 6b both have the same molecular weight yet they have slightly different UV−vis spectra (Table 2). Hence, **6a** and **6b** can be two sinapine stereoisomers. Examining sinapine structural formula (Figure 3), the two peaks can be either *cis*/*trans* isomers or sterically hindered conformers. *cis*-Sinapine has never been reported so far, although *cis*-sinapic acid was frequently reported in canola meal.[32,33]

Furthermore, sinapic acid esters with hexoses, dihexoses, kaempferol, malic acid, and methanol were found (Table 2). In agreement with Liu et al., two sinapic acid hexoses, **9** and **13**, were detected in ACM.[32] The literature shows that the second most abundant sinapic acid derivative after sinapine is sinapoyl glucoside, 1-*O*-*β*-D-glucopyranosyl sinapate.[5,12] Hence, com-

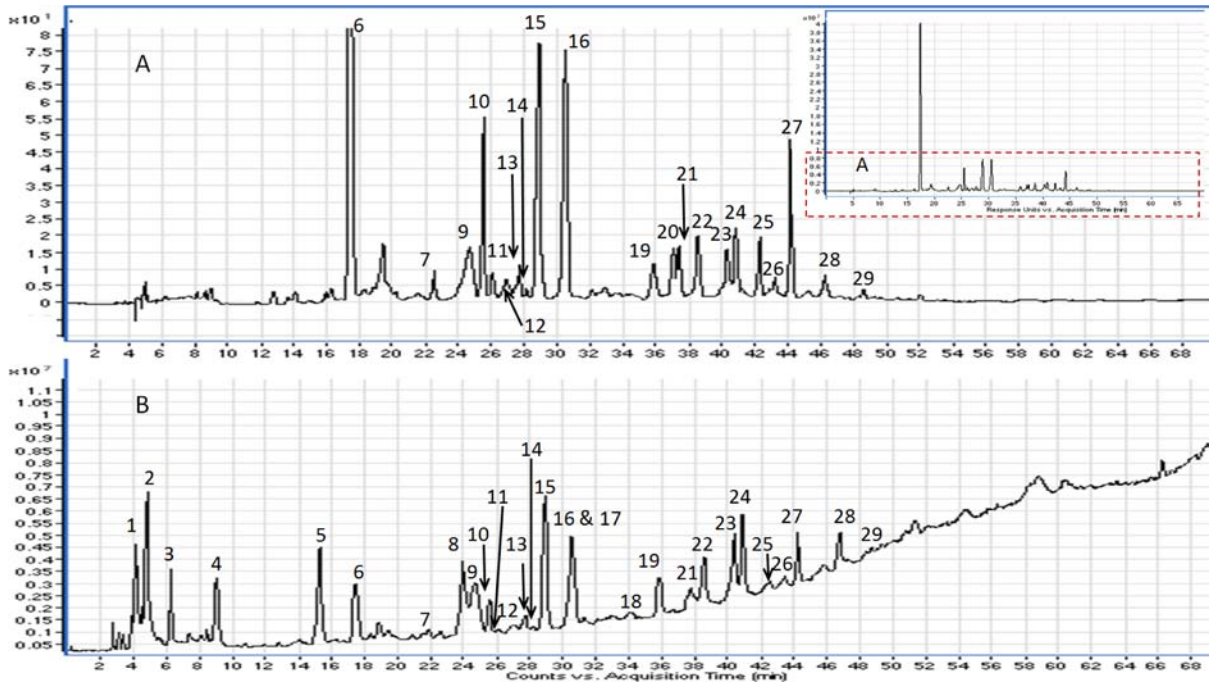

Figure 1. HPLC-DAD-MS/MS chromatograms of ACM crude extract (Tarcoola cv.): (A) chromatogram at 280 nm; (B) total ion chromatogram.

pounds **9** and **13** are most likely glucoside derivatives.[32] Peak 13 showed ABTS scavenging activity as expected from an ester of sinapic acid with glucose. Thus, we assigned compound **13** as 1-*O*-*β*-D-glucopyranosyl sinapate. The absence of an ABTS peak for peak 9 (Figure 3B) can be attributed to blocking of the *p*-hydroxy group of sinapic acid via ether/glycoside linkage with glucose; thus, compound**9** was tentatively assigned as 4′-glucosylsinapic acid. Two isomers of sinapoyl glucoside were discovered in Chinese canola,[32] but no earlier reports of 4′-glucosylsinapic acid were found. Disinapoyl hexoside **27** was detected in ACM. In accordance with Baumert et al., we identified caffeoyl dihexoside **7** but did not detect any monosinapoyl dihexoside derivatives.[22] Meanwhile, disinapoyl **25** and trisinapoyl **26** dihexosides were found in ACM. For the first time, we report the presence of tetrasinapoyl dihexoside **28** (Table 2). More complex sinapoyl glucoside derivatives were also reported in canola meal, in which the glucoside is further linked to a flavonoid, most commonly kaempferol.[22,29] Three isomers of kaempferol sinapoyl trihexose, **15**, **16**, and **22**, were detected in our sample, similar to the earlier findings of Baumert et al.[22] Quercetin sinapoyl trihexoside was previously identified in canola inflorescence but not in canola seed.[25] Peaks 23 and 24 were tentatively assigned as sinapoyl malate isomers. Although many reports were found for *trans*-sinapoyl malate in Brassica,[25] only a few studies have documented the presence of *cis*-sinapoyl malate.[34] Methyl sinapate **34** was detectable only in acid-hydrolyzed extracts (Figure 2A,B), supporting earlier findings[35] about being an artifact formed via esterification with methanol in the extraction solvent under acidic conditions.

The absence of free flavonoids such as apigenin, catechin, luteolin, naringenin, and quercetin was confirmed in ACM. Upon hydrolysis, the only flavonoid aglycone detected in ACM was kaempferol **35** (Table 2; Figure 2). Metabolic profiling of Egyptian canola revealed the presence of isorhamnetin, isoquercetrin, quercetin, naringenin, kaempferol, and their glycosides, whereas only kaempferol glycosides were detected in canola seeds.[25]

A cyclic spermidine alkaloid was identified in German canola seeds,[22,29] and a hexoside derivative was identified in transgenic canola.[24] In ACM, two isomers of the cyclic spermidine alkaloid were identified, **10** and **11**. This is the first report of two isomers for this alkaloid in canola meal.

Upon acid and base hydrolyses, seven more peaks, 30−36, were observed (Table 2), in addition to the main peak of *trans*-sinapic acid **21** (Figure 2). Whereas base hydrolysis produced principally *trans*-sinapic acid, acid hydrolysis resulted in generation of a new major peak, 30, which was present neither in the unhydrolyzed nor in the base-hydrolyzed extracts. Peak 30 was the most abundant peak in the acid hydrolyzed extract followed by *trans*-sinapic acid **21** (Figure 2A). Therefore, compound **30** is either a degradation product of sinapic acid or an artifact of the acid hydrolysis process. The compound failed to ionize under negative ESI conditions, whereas a peak was generated under positive ionization conditions with an m/z of 149. This compound is highly polar with a retention time, 14.2 min, less than that of sinapine, 17.6 min, and after that of gallic acid, 13 min. The intense UV absorption at 285 nm suggests a hydroxybenzoic acid derivative. Lack of ABTS scavenging activity (Table 2) negates the presence of dihydroxy or trihydroxy phenolic groups. Thus, the high polarity is most probably due to a charged molecule.

Peak 33 was assigned as kaempferol-*C*-hexoside. *C*-Glycosides are known for their resistance to hydrolysis under acidic conditions. Although peak 32 was detected under acidic conditions, both 32 and 36 were prominent under base hydrolysis conditions. Autoxidation of alkaline solution of sinapic acid results in the formation of lignan derivatives such as thomasidioic acid, molecular weight 446 Da.[36] Seven lignan derivatives were reported in *Brassica napus*.[29]

Antioxidant Activity of ACM. 1-*O*-*β*-D-Glucopyranosyl sinapate was reported as the most active antioxidant in canola meal in a lipid model system.[37] Meanwhile, in the DPPH

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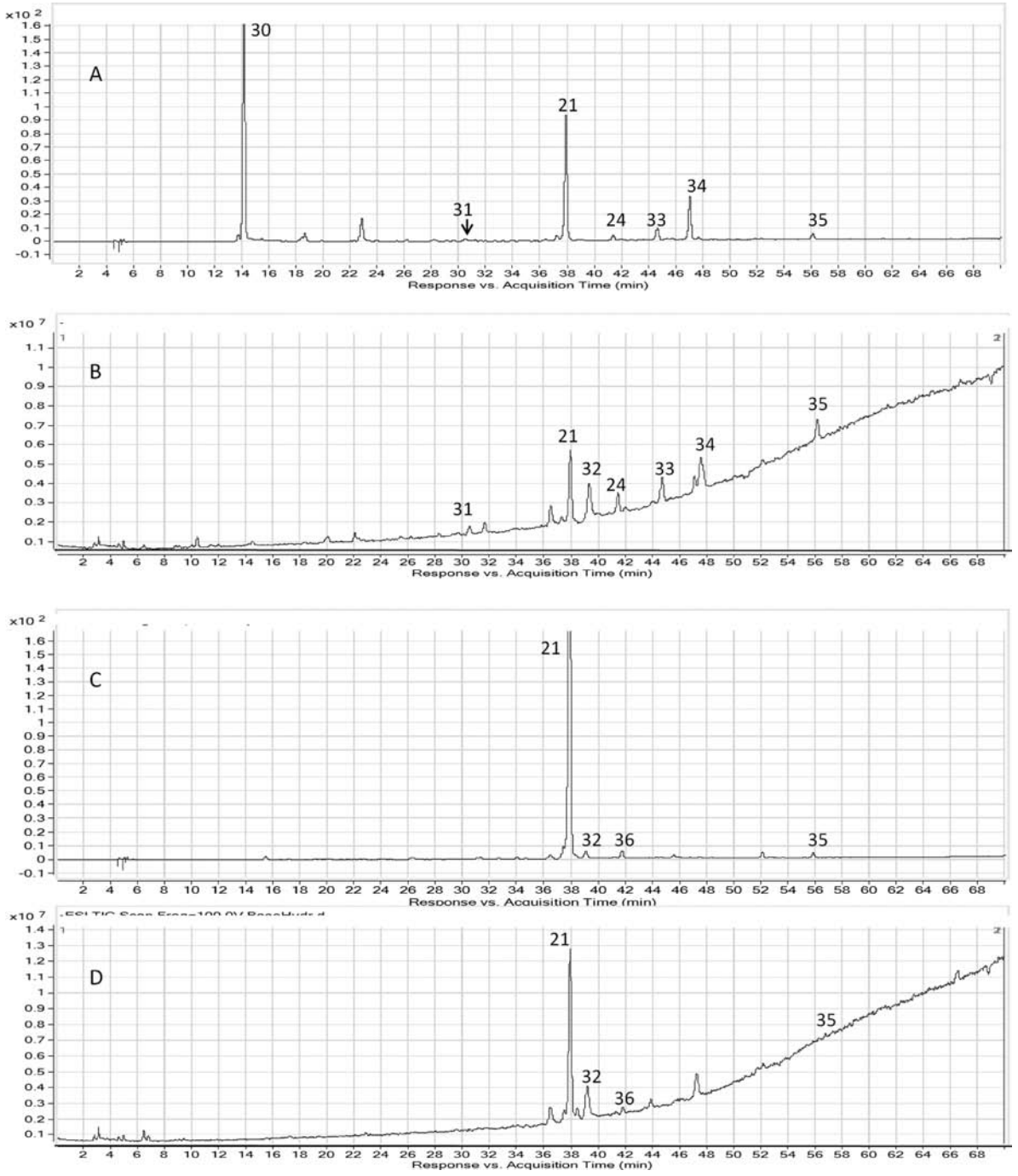

Figure 2. HPLC-DAD-MS/MS chromatograms of hydrolyzed canola meal crude extract (Tarcoola cv.): (A) acid hydrolysis chromatogram at 280 nm; (B) acid hydrolysis total ion chromatogram; (C) base hydrolysis chromatogram at 280 nm; (D) base hydrolysis total ion chromatogram.

radical scavenging assay, sinapic acid was a more potent scavenger than sinapine followed by 1-*O*-*β*-D-glucopyranosyl sinapate. Examining the ABTS scavenging chromatogram (Figure 3B), we can ascertain that the total antioxidant activity of ACM is chiefly due to sinapines **6a** and **6b**, kaempferol sinapoyl triglucoside isomer **15**, disinapoyl glucoside **27**, 1-*O*-*β*-D-glucopyranosyl sinapate **13**, unknown sinapoyl derivative **20**, tetrasinapoyl dihexoside **28**, kaempferol sinapoyl triglucoside isomer **22**, and disinapoyl dihexoside **25**. In quantitative terms, sinapine was the most abundant antioxidant. Meanwhile, the most potent antioxidant in ACM was kaempferol sinapoyl triglucoside isomer **15**. Three different kaempferol sinapoyl triglucosides were detected in ACM, in accord with earlier findings[22] (Figure 3). The ABTS scavenging activities of the three isomers vary on the basis of the way kaempferol, sinapic acid, and glucose are bound to each other.

Biophenol Content and Antioxidant Activity: Impact of Cultivar. Total phenols, hydroxycinnamic acids, flavonols, *o*-diphenols, flavonoids, and free radical scavenging activities were significantly affected by cultivar (Table 3). Total phenol contents were 12.8−15.4 (FC) and 7.3−9.5 (280 nm) mg GAE/g DW. Total phenol content in canola byproducts in literature varied from 6.4 to 18.4 mg/g DW.[12] *o*-Diphenols were the most abundant class of biophenols, ranging from 9.2

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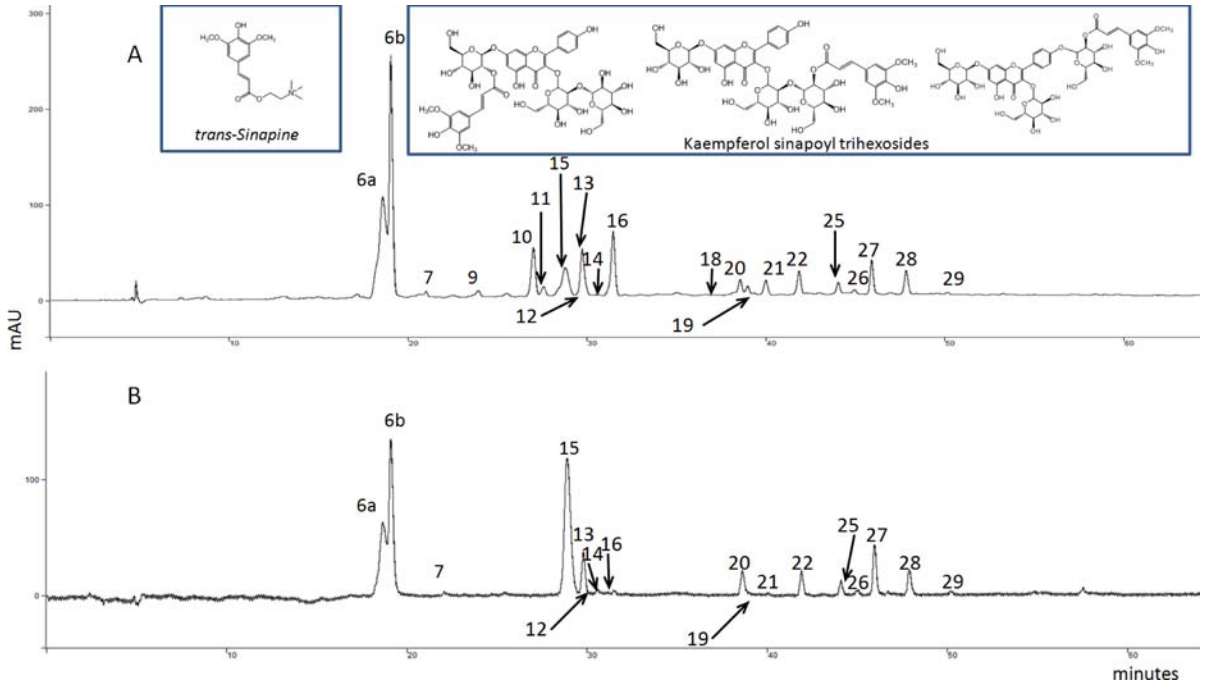

Figure 3. HPLC-DAD-ABTS chromatograms of canola meal crude extract (Tarcoola cv.): (A) at 280 nm; (B) ABTS scavenging at 414 nm.

Table 3. Effect of Canola Cultivars on Biophenol Content and Antioxidant Activity of ACM Extracts[a]

cv.	TP (FC)	TP 280 nm	HCA	flavonols	ODP	TF	ABTS	DPPH
Tarcoola	15.4 ± 0.5 a	9.5 ± 0.6 a	10.4 ± 0.4 a	6.3 ± 0.01 a	12.0 ± 0.2 a	2.8 ± 0.01 a	67.9 ± 2.0 a	220.8 ± 4.9 a
Rainbow	12.8 ± 0.5 b	7.3 ± 0.2 b	7.9 ± 0.4 b	5.0 ± 0.2 b	10.1 ± 0.2 bc	2.3 ± 0.11 b	59.1 ± 1.4 b	174.6 ± 2.9 b
Warrior	14.7 ± 0.06 c	8.9 ± 0.1 a	9.3 ± 0.5 a	5.9 ± 0.5 a	10.8 ± 0.3 b	3.1 ± 0.01 c	65.9 ± 1.1 a	191.8 ± 5.8 b
Lantern	12.9 ± 0.5 b	7.5 ± 0.03 b	7.3 ± 0.3 b	4.6 ± 0.3 b	9.2 ± 0.3 c	2.6 ± 0.06 a	62.7 ± 1.2 c	175.2 ± 16.2 b

[a]Different letters in the same column indicate statistically significant difference. TP (FC), total phenols (Folin−Ciocalteu) expressed as mg GAE/g DW; TP 28, total phenols measured at 280 nm expressed as mg GAE/g DW; HCA, hydroxycinnamic acids measured at 320 nm expressed as mg CAE/g DW; flavonols, total flavonol content measured at 360 nm expressed as mg QE/g DW; ODP, *o*-diphenol content expressed as mg CAE/g DW; TF, total flavonoids expressed as mg QE/g DW; ABTS, offline $ABTS^{\bullet+}$ scavenging activity expressed as TEAC; DPPH, DPPH scavenging activity expressed as TEAC.

to 12.0 mg CAE/g DW. We found that sinapic acid gives positive reaction with the *o*-diphenol assay. Flavonoids were the least abundant in canola meal, ranging from 2.3 to 3.1 mg QE/g DW. The relative difference between the highest total phenol content, that of Tarcoola cv., and the lowest phenol content, that of Rainbow cv., was approximately 20%. This is in parallel to *o*-diphenol content, 23%, and hydroxycinnamic acids content, 30%.

Cultivars can be ranked according to their total phenol content as follows: Tarcoola > Warrior > Lantern = Rainbow. Consistently, Tarcoola showed the highest phenol content in all employed assays, whereas Rainbow was the lowest. There was no statistically significant difference between Lantern and Rainbow samples in all assays ($p < 0.05$) apart from the total flavonoids and ABTS scavenging assay. Rainbow and Lantern extracts contained less biophenols than Tarcoola in all assays. Concurrently, the biophenol content of Tarcoola was significantly higher ($p < 0.05$) than that of Warrior in all assays except total phenols (280 nm) and total flavonols content.

The antioxidant capacity of ACM was determined using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays. Both assays showed similar relative ranking of extracts, which is proportional to total phenol content: Tarcoola ≥ Warrior > Lantern = Rainbow. In both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging assays, Tarcoola cv. had the highest antioxidant activity among all samples, whereas Rainbow showed the lowest antioxidant activity. There was no significant difference between Tarcoola and Warrior in the $ABTS^{\bullet+}$scavenging assay.

To assess the presence of qualitative differences among cultivars, HPLC-DAD (280 nm), HPLC-ABTS, and total ion chromatograms (data not shown) were compared peak to peak. All cultivars showed identical phenol profiles in terms of peak identities. Thus, the impact of cultivar on phenolic content is essentially quantitative rather than qualitative.

Recovery of Sinapine and Sinapic Acid. Sinapine concentration in CE was up to 3.9 ± 0.2 mg sinapic acid equiv/g DW. Acid extraction of canola meal resulted in degradation of sinapic acid, whereas upon alkaline extraction of canola meal, sinapic acid recovery reached 10 mg/g DW. The abilities of EDTA/ascorbic acid and EDTA/sodium metabisulfite were assessed as preservatives during recovery to reduce possible oxidative degradation. Ascorbic acid solutions produced instantaneous intense brown coloration; hence, we did not continue any further with them. Paradoxically, sodium metabisulfite preserved extracts showed lower recovery than nonpreserved extracts, which suggests a prooxidant effect for sodium metabisulfite.

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In conclusion, aqueous acetone had superior extraction ability over methanol, and stirring was preferred over sonication in the recovery of canola meal biophenols. Sinapic acid derivatives were identified as the major biophenols in ACM, and sinapine was the most abundant derivative. ACM extracts showed significant antioxidant activities in both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, and sinapine was found to be the major contributor to the antioxidant activity of canola meal extract on a weight basis. Kaempferol sinapoyl triglucoside was the most potent radical scavenger in ACM extracts. The biophenol content and antioxidant activities of the studied cultivars showed up to 30% relative difference, Tacoola cv. showing the highest phenol content and antioxidant activity. The impact of cultivar on the phenol content and antioxidant activity was mainly quantitative. Australian canola cake is a rich source for the recovery of sinapine (0.4% w/w) and sinapic acid (1% w/w). In addition, there is a potential for the isolation of highly potent antioxidants.

AUTHOR INFORMATION

Corresponding Author

*(H.O.) Postal address: School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia 2678. Phone: (61) 2 6933 2161. Fax: (61) 2 6933 2587. E-mail: hobied@csu.edu.au.

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Notes

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ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ACM, Australian canola meal; CAE, caffeic acid equivalent; CE, crude extract; cv., cultivar; DPPH$^{\bullet}$, 2,2′-diphenyl-1-picrylhydrazyl radical; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FC, Folin−Ciocalteu; GAE, gallic acid equivalent; HPLC-DAD, high-performance liquid chromatography−diode array detection; MS/MS, tandem mass; QE, quercetin equivalent

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