

Antioxidant Activity of Various Fractions of Non-Tannin Phenolics of Canola Hulls

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Cyclone canola hulls were extracted with 70% (v/v) acetone. The dried crude extract was dissolved in ethanol and fractionated on a Sephadex LH-20 column using 95% (v/v) ethanol as the mobile phase. Five major fractions were isolated according to the UV absorption. All fractions exhibited marked antioxidant activity in a β -carotene–linoleate model system. Fractions I and II showed the best preventive effect against the bleaching of β -carotene. The scavenging effect of fractions I, III, and V, at 1 mg, on α, α -diphenyl- β -picrylhydrazyl (DPPH) radical was 67.4%, 80.7%, and 63.3%, respectively. Fractions II and IV showed weak DPPH scavenging effects. The reducing power of phenolics present in fractions IV and V was greater than that of fractions I–III, and the observed data correlated well ($r^2 = 0.937$; $P = 0.007$) with the total content of phenolics present in each fraction.

Keywords: *Non-tannin phenolics; canola; hulls; antioxidative properties; reducing power; scavenging activity*

INTRODUCTION

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) are commonly used as synthetic antioxidants in lipid-containing foods (Sherwin, 1990). Over the last 2 decades, there has been an increasing interest in replacing these synthetic antioxidants with natural alternatives (Howell, 1986), because of their possible role as promoters of carcinogenesis (Ito et al., 1986). Fruits, vegetables, spices, nuts, seeds, leaves, roots, and barks have thus been exploited as potential sources of natural antioxidants (Pratt and Hudson, 1990).

Advances in dehulling of canola/rapeseed (Sosulski and Zadernowski, 1981; Greilsamer, 1983; Diosady et al., 1986) may soon bring about the introduction of dehulling to the canola/rapeseed industry. The subsequent use of hulls as a component of feedstuffs may be one way of their utilization. Canola hulls have been reported to contain 6% of phenolic compounds (Naczki and Shahidi, 1998). These compounds include phenolic acids (Krygier et al., 1982), flavonoids (Durkee and Harborne, 1973), and soluble and insoluble condensed tannins (Naczki et al., 1994; Naczki and Shahidi, 1998).

Wanasundara and Shahidi (1994) reported that the antioxidant activity of crude ethanolic extracts of canola meal on canola oil was equivalent to that of TBHQ and stronger than that of BHA, BHT, and BHA/BHT/monoacylglyceride citrate (MGC). Later Wanasundara et al. (1994) isolated the most active component of these

extracts and identified it as 1-O- β -D-glucopyranosyl sinapate. Furthermore, strong antioxidative activity of ethanolic and methanolic extracts of rapeseed phenolics was reported by Amarowicz and Fornal (1995) and Nowak et al. (1992). Moreover, Shahidi et al. (1995) observed that the addition of 0.5–5% canola flour to meat resulted in 73–97% inhibition of fat oxidation as determined by the 2-thiobarbituric acid (TBA) assay. Recently, we used a β -carotene–linoleate model system to evaluate the antioxidative activity of crude extracts of phenolics isolated from low- and high-tannin rapeseed/canola hulls via extraction with 70% (v/v) aqueous acetone. It was found that low-tannin hull extracts exhibit greater antioxidative activity than high-tannin hull extracts (unpublished data). This paper reports the results of our studies on antioxidative activities of non-tannin fractions of phenolic compounds isolated from canola hulls.

MATERIALS AND METHODS

Cyclone canola hulls were prepared according to the procedure described by Sosulski and Zadernowski (1981). Hulls were extracted with hexane for 12 h using a Soxhlet apparatus and then dried at room temperature.

Soluble polyphenols were extracted from hulls twice at room temperature into 70% (v/v) aqueous acetone using a Waring blender (Waring Products Division, Dynamics Corporation of America, New Hartford, CT) for 2 min at maximum speed. The extracts were combined, evaporated to near dryness under vacuum at 40 °C, and lyophilized. The extraction yielded 5.8 g of lyophilized extract of crude tannins from 100 g of hulls.

A sample (550 mg) of the crude polyphenol extract was suspended in 5 mL of 95% (v/v) ethanol and applied onto a chromatographic column (2.3 \times 40 cm) packed with Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO) and equilibrated with 95% (v/v) ethanol. The column was exhaustively washed with 95% (v/v) ethanol at a flow rate of 60 mL/h. Six-milliliter fractions were collected and their absorbance values read at both 280 and 326 nm. Eluates (non-tannin phenolics) (Strum-

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eyer and Malin, 1975) were then pooled into five major fractions based on the elution profiles. After evaporation of ethanol the samples were lyophilized and residues weighed. The total phenolic compounds in each major fraction were estimated using the Folin–Denis reagent (Swain and Hillis, 1959) and the contents expressed as *trans*-sinapic acid equivalents. UV spectra of major fractions were recorded using a Hewlett-Packard 8452A diode array spectrophotometer (Palo Alto, CA).

Major fractions so separated were examined by a thin layer chromatographic (TLC) methodology on silica gel plates (Sigma Chemical Co., St. Louis, MO; 60-Å mean pore diameter, 2–25- μ m particle size, 250- μ m thickness) using diethyl ether–petroleum ether–acetic acid (80:20:1, v/v/v) (A) or *n*-butanol–acetic acid–water (3:1:1, v/v/v) (B) as a mobile phase (Amarowicz et al., 1995a,b). The phenolic bands were visualized by spraying the plates with a 1% solution of FeCl₃ in 1 M HCl (Reio, 1958). Sinapine was isolated from rapeseed meal according to the procedure described by Clandinin (1961). Caffeic, cinnamic, *p*- and *o*-coumaric, gentisic, protocatechuic, syringic, sinapic, and vanillic acids were obtained from Sigma.

The antioxidant activity of the non-tannin fraction of canola hull phenolics was determined using a β -carotene–linoleate model system (Miller, 1971). Methanolic solutions (0.2 mL) containing 2 mg of crude extract or non-tannin fraction of canola hull phenolics were added to a series of tubes containing 5 mL of a prepared emulsion of linoleate and β -carotene. Similar experiments were carried out using a known amount (0.02 or 0.2 mg) of ferulic or sinapic acid or 0.5 mg of BHA. Immediately after the addition of emulsion to tubes, the zero-time absorbance at 470 nm was recorded. Samples were kept in a water bath at 50 °C and their absorbances recorded over a 120-min period at 15-min intervals.

The scavenging effect of extracts or their fractions for α , α -diphenyl- β -picrylhydrazyl (DPPH) radical was monitored according to the method of Hatano et al. (1988) as described by Yen and Chen (1995); 0.1 mL of methanolic solution containing 200–1000 μ g of the crude extract or non-tannin fraction of canola hull phenolics as well as sinapic or ferulic acid was mixed with 2 mL of distilled water and then added to a methanolic solution of DPPH (1 mM, 0.25 mL). The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min, and its absorbance was read at 517 nm.

The reducing power of the crude extract and non-tannin canola hull phenolic fractions, as well as sinapic and ferulic acids, was determined as described by Oyaizu (1986). Phenolic fractions as well as standards (20–100 μ g) in 1 mL of distilled water were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% solution of potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Following this, 2.5 mL of trichloroacetic acid (TCA) was added and the mixture centrifuged at 1750g for 10 min. A 2.5-mL aliquot of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of a 0.1% solution of FeCl₃, and the absorbance of the reaction mixture was read at 700 nm.

Statistical analysis of data was carried out using the SigmaStat v.2.03 (ANOVA test, linear correlation) (SPSS Science, Chicago, IL). Each fraction of non-tannin phenolics as well as pure phenolic acids, for the purpose of statistical analysis, was referred to as treatment. The statistical analysis of all treatments was carried out using the ANOVA test. In addition, the Tukey's test was employed among the treatments when a statistically significant difference ($P \leq 0.05$) was found using the ANOVA test.

The results presented in graphs are mean values of at least three determinations. Treatments followed by the same superscript letter in a graph legend are not significantly different ($P > 0.05$; Tukey's test).

RESULTS AND DISCUSSION

Figure 1 shows the elution profiles at 280 and 326 nm of the 70% (v/v) acetone extract of canola hull

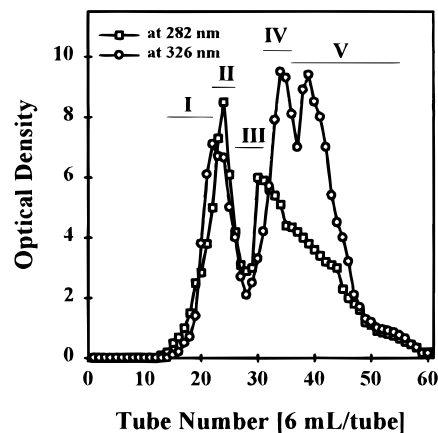


Figure 1. Sephadex LH-20 chromatography of Cyclone canola hull non-tannin phenolics using ethanol.

Table 1. Percentage of Recovery of Non-Tannin Canola Hull Phenolic Extracts, Their Total Phenolic Content, and Spectral Data^a

fraction no.	rel content (%) ^b	total phenolic content (mg/g) as sinapic acid equiv	spectral data (nm)	
			maximum	shoulder
I	23.7	14	284, 328	
II	35.2	21	282, 324	
III	22.6	26	280	326
IV	8.4	106	324	
V	10.1	112	270, 328	

^a Separated on a Sephadex LH-20 column. ^b Percent (%) of total non-tannin phenolic fraction.

phenolics using a Sephadex LH-20 column and 95% (v/v) ethanol as the mobile phase. The eluate was comprised of approximately 52% crude polyphenols or 3.02% the weight of the hulls. The phenolics eluted with ethanol were subsequently separated into five major fractions. The use of a Sephadex LH-20 column for fractionation of phenolics is commonplace. Amarowicz et al. (1992), using Sephadex LH-20 and methanol as a mobile phase, fractionated ethanolic extracts of rapeseed meal into five fractions, and Wanasundara et al. (1994), using Sephadex LH-20 and 95% (v/v) ethanol as a mobile phase, separated ethanolic extracts of canola meal into seven major fractions containing phenolics.

The mass balance of the separation of phenolic acids on the Sephadex LH-20 column is shown in Table 1. The relative contents of fractions I–III were higher than those of fractions IV and V. However, fractions IV and V contained more phenolics, expressed as sinapic acid equivalents, than fractions I–III. Wanasundara et al. (1994) reported a similar distribution pattern of relative content of canola meal phenolic fractions and their total contents of phenolics.

The UV spectra for fractions I, II, and V showed two maxima, while fraction III exhibited one maximum and one shoulder and fraction IV displayed one maximum only (Table 1). The absorption peaks at the longer wavelengths (324–328 nm) may be due to the presence of phenolic acids, notably hydroxycinnamic acid derivatives (Kozłowska et al., 1983; Naczka et al., 1992), and those at the shorter wavelengths (270–284 nm) may be due to the presence of *p*-hydroxybenzoic acid and flavone/flavonol derivatives (Mabry, 1970; Macheix et al., 1990; Wanasundara et al., 1994; Amarowicz et al., 1996).

Each fraction of phenolic compounds, separated on Sephadex LH-20, was examined by the TLC methodol-

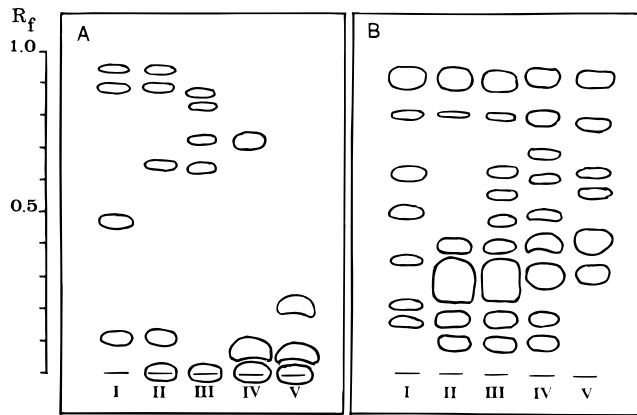


Figure 2. TLC chromatograms of Cyclone canola hull non-tannin phenolic fractions separated on a Sephadex LH-20 column: (A) mobile phase, diethyl ether–petroleum ether–acetic acid (80:20:1, v/v/v); (B) mobile phase, *n*-butanol–acetic acid–water (3:1:1, v/v/v).

ogy on silica gel. The TLC chromatograms (Figure 2) revealed the presence of a number of phenolic compounds in each fraction. On the chromatogram developed with petroleum ether–diethyl ether–acetic acid, fractions IV and V showed only three spots, while fraction I displayed four spots and fractions II and III exhibited five spots. More spots were visualized on the chromatogram developed with *n*-butanol–acetic acid–water; fractions II and V showed six spots, fraction I seven spots, and fractions III and IV nine spots. On the basis of R_f values of the spots visualized on the chromatograms developed with petroleum ether–diethyl ether–acetic acid, the presence of free ferulic acid ($R_f = 0.87$) was evident in fractions I and II. In addition, sinapine ($R_f = 0.30$) was detected in fractions IV and V, based on the chromatograms developed with *n*-butanol–acetic acid–water. However, free caffeic, cinnamic, *p*- and *o*-coumaric, gentisic, protocatechuic, syringic, sinapic, and vanillic acids were not present in any fractions of non-tannin canola hull phenolics. According to Krygier et al. (1982) these phenolic acids may be present in canola hulls as esters and/or glycosides. Further work is needed to identify the chemical structure(s) of the other non-tannin phenolic compounds of canola hulls.

The effect of various fractions of non-tannin canola hull phenolics on the coupled oxidation of linoleic acid and β -carotene was compared to that of BHA (see Figure 3A). Fractions I and III exhibited the highest antioxidant activity ($P \leq 0.05$), but these were lower than that of BHA in a β -carotene–linoleate model system. The antioxidant activity of fractions II and V was somewhat lower than that of fractions I and II. Fraction IV and the crude extract showed comparatively strong antioxidant activity during the first 40 min of determination and similar antioxidant activity to that of ferulic acid at a level of 0.2 mg (Figure 3B). Moreover, ferulic acid exhibited greater antioxidant activity than sinapic acid, but all phenolic acids were less effective than those of fractions I–V. Antioxidant activities of non-tannin fractions of canola hull phenolics were, in general, greater than those reported by Wanasundara et al. (1994) for phenolic fractions isolated from ethanolic extracts of canola meal. It should also be noted that fractions I and III with the best preventive effect against the bleaching of β -carotene contained only 14 and 26 mg of phenolics/g of sample, respectively (Table 1).

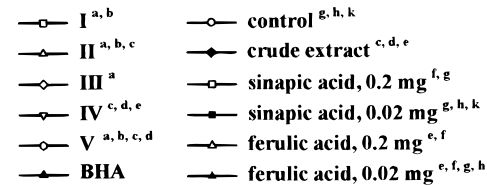
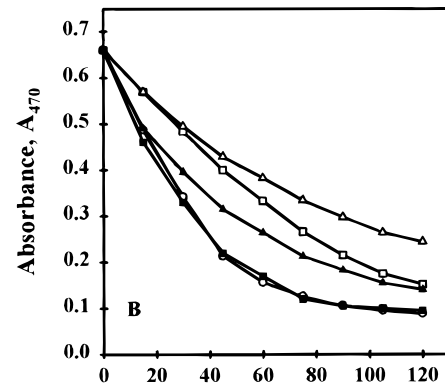
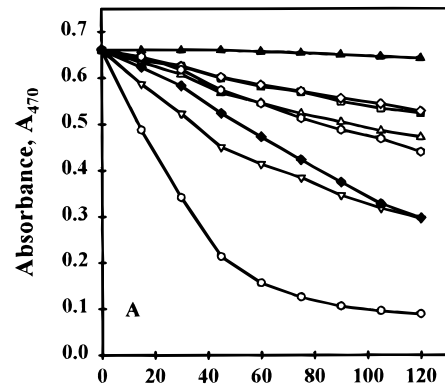


Figure 3. Antioxidant activity of Cyclone canola hull non-tannin phenolic fractions separated on a Sephadex LH-20 column (A) and ferulic and sinapic acids (B). Treatments followed by the same superscript letters in the graph legend are not significantly different ($P > 0.05$; Tukey's test).

Similarly, Wanasundara et al. (1994) demonstrated that the best antioxidant activity was exhibited by a fraction of canola meal phenolics which contained only 34 mg of phenolics/g of sample. On the other hand, Amarowicz et al. (1996) observed that the antioxidant activity of ethanolic extracts of mustard correlated well with the total content of phenolics in its isolated fractions. The high antioxidant activity of rapeseed/canola fractions, low in total phenolic content, may be dictated by the molecular structure of phenolics involved. According to Zadernowski et al. (1991), the molecular structure of rapeseed phenolics plays an important role in their antioxidant activity. Cort (1974) and Pokorny (1987) have also shown that substituted monophenolic compounds exhibit a greater antioxidant activity than unsubstituted monophenolics. Furthermore, Wanasundara et al. (1994) suggested that synergism of phenolics with one another and/or other components present in each fraction may contribute to the high antioxidant activity of rapeseed/canola fractions low in phenolics.

DPPH assay evaluates the ability of antioxidants to scavenge free radicals. According to Hochstein and Atallah (1988), the antimutagenic activity of antioxi-

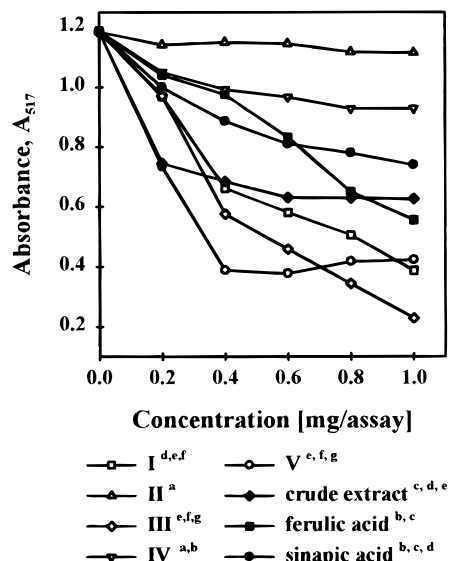


Figure 4. Scavenging effect of Cyclone canola hull extract and its non-tannin phenolic fractions and ferulic and sinapic acids on DPPH radical. Treatments followed by the same superscript letters in the graph legend are not significantly different ($P > 0.05$; Tukey's test).

dants is due to their ability to scavenge free radicals or induce antioxidative enzymes. Figure 4 shows the scavenging activity of each fraction of non-tannin canola hull phenolics as compared with ferulic and sinapic acids. The concentration of ferulic and sinapic acids used in this experiment reflected the content of phenolics in fractions IV and V (Table 1). Fractions I, III, and V exhibited a greater scavenging activity than ferulic and sinapic acids. The scavenging effect of these fractions, at a level of 1 mg, expressed as the ratio of the decrease in the absorbance at 517 nm to the absorbance of DPPH solution in the absence of phenolics at 517 nm (Yoshida et al., 1989), was 67.4%, 80.7%, and 63.3% for fractions I, III, and V, respectively. Fractions II and IV showed weak scavenging effects ranging from 6% (fraction II) to 21.7% (fraction IV). The scavenging effects of fractions I, III, and V were greater than those reported by Yen and Chan (1995) for green and black tea extracts. These authors reported that scavenging effects of tea extracts ranged from 49% to 66.1% at a 2-mg level of extract.

Figure 5 shows the reducing powers of non-tannin canola hull phenolics as well as ferulic and sinapic acids as a function of their concentration. Results indicate that fractions IV and V exhibited greater reducing powers than fractions I, II, and III (Figure 5A). Thus, phenolics present in fractions IV and V were good electron and hydrogen donors and could terminate the radical chain reaction by converting free radicals to more stable products. No statistically significant difference ($P > 0.05$) in reducing powers among fractions I–III was noted. The observed activity data for each fraction correlated well ($r^2 = 0.937$; $P = 0.007$) with its total content of phenolics (Table 1). Ferulic and sinapic acids showed reducing powers similar to those of fractions IV and the crude tannins extract (Figure 5A,B). Moreover, the reducing power of canola hull phenolic fractions was similar to that of ethanolic extracts of evening primrose phenolics containing 103 mg of phenolics/g of sample (Amarowicz et al., 1999).

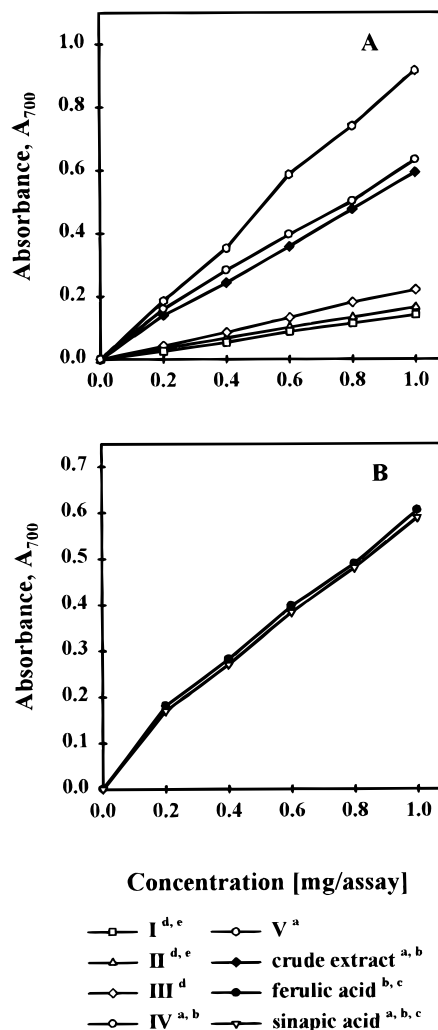


Figure 5. Reducing power of Cyclone canola hull extract and its non-tannin phenolic fractions and ferulic and sinapic acids. Treatments followed by the same superscript letters in the graph legend are not significantly different ($P > 0.05$; Tukey's test).

CONCLUSIONS

Separation of the acetone (70% v/v) extract of canola hulls afforded five fractions containing non-tannin phenolics. On the basis of thin layer chromatograms, the presence of free ferulic acid in fractions I and II and sinapine in fractions IV and V was confirmed. All fractions of canola hull non-tannin phenolics exhibited strong antioxidant activities in a β -carotene–linoleate model system. The antioxidant activity of fractions I, III, and V was greater than that of the crude extract as well as ferulic and sinapic acids. Fractions I, III, and V showed a strong scavenging effect for the DPPH radicals. This indicates that these fractions may also possess a strong antimutagenic activity. The reducing power of each fraction correlated well ($r^2 = 0.937$; $P > 0.05$) with the total content of phenolics present.

The justification as to why fractions I, III, and V displayed strong antioxidant activities is a difficult task because (i) each fraction is a complex mixture of phenolics with unknown structural characteristics; the difference in molecular structure may be in the arrangement of hydroxyl and methoxy groups, in the presence of ester and/or glycosidic bonds, and in the degree of association of the molecules involved; (ii)

synergism of phenolics with each other as well as with non-phenolic compounds may also contribute to the total antioxidative activity of each fraction; (iii) the methodologies employed estimate only the total antioxidative activity of the system. Therefore, further work is required to isolate and identify the active component(s) of canola hull phenolics present in fractions I, III, and V and to determine the kinetics and mechanism of their antioxidative activities.

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