Antioxidant Activity of Crude Tannins of Canola and Rapeseed Hulls

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ABSTRACT: The antioxidant activity of crude tannins of canola and rapeseed hulls was evaluated by β-carotene-linoleate, α,α-diphenyl-β-picrylhydrazyl (DPPH) radical, and reducing power assays. Crude tannins were extracted from three samples of Cyclone canola (high-tannin) hulls and Kolner, Ligaret, and Leo Polish rapeseed (low-tannin) hulls with 70% (vol/vol) acetone. The total phenolic content in crude tannin extracts ranged between 128 and 296 mg of sinapic acid equivalents per 1 g of extract. The ultraviolet spectra of methanolic solution of canola extracts showed two absorption maxima (282 and 309 nm), whereas those of rapeseed extracts exhibited a single maximum (326 nm). Crude tannins isolated from canola hulls exerted significantly ($P \leq 0.025$) greater antioxidant activity than those from rapeseed in all three assays. The scavenging effect of all crude tannins, at a dose of 1 mg, on the DPPH radical ranged from 35.2 to 50.5%. The reducing power of Cyclone canola hull extracts on potassium ferricyanide was significantly ($P \leq 0.0025$) greater than that of rapeseed hull extracts, and the observed data correlated well ($r = 0.966$; $P = 0.002$) with the total content of phenolics present.

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KEY WORDS: Antioxidative properties, canola, condensed tannins, hulls, rapeseed, reducing power, scavenging effects.

There is a keen interest in replacing synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) with natural alternatives in food systems (1). Synthetic antioxidants have been implicated in the promotion of carcinogenesis (2). Currently fruits, vegetables, spices, nuts, seeds, leaves, roots, and barks are being investigated as potential sources of natural antioxidants (3).

Rapeseed species include *Brassica napus, B. campestris,* and *B. juncea* which are commonly known as rape, turnip rape, and leaf mustard, respectively. In Canada and Europe the seed of rape and turnip rape are regarded as rapeseed. Traditional rapeseed varieties contain from 22 to 60% of erucic acid in their oil. The name canola was adapted in 1979 in Canada for any genetically modified rapeseed variety that contained less than 2% erucic acid in its oil and less than 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 18 μmol/g whole seeds and less than 1% erucic acid in the oil. In 1985, rapeseed and canola were recognized by the United States Food and Drug Administration as different species (4).

Phenolic acids and their derivatives, as well as soluble and insoluble tannins, are the predominant phenolic compounds found in canola and rapeseed (5–7). Canola and rapeseed hulls have been reported to contain up to 6% tannins (5). Therefore, use of hulls, after dehulling, as a source of natural antioxidants may provide a means for their utilization.

Alcoholic extracts of rapeseed meal (8), rapeseed cakes (9), and legume (pea, lentil, faba bean, and broad bean) hulls (10) exhibited strong antioxidant activity in a β-carotene-linoleate model system. Wanasundara and Shahidi (11) reported that the antioxidant activity of ethanolic extracts of canola meal in canola oil was equivalent to that of TBHQ and stronger than that of BHA, BHT, and BHA/BHT/monoacylglyceride citrate (MGC). The most active component of these extracts was 1-*O*-β-D-glucopyranosyl sinapate (12). The objective of the current research was to investigate the antioxidative activities of crude extracts of tannins isolated from selected high- and low-tannin hulls of canola and rapeseed varieties.

MATERIALS AND METHODS

Seeds of Cyclone canola, grown at three different locations in western Canada, and Ligaret, Kolner, and Leo Polish rapeseed varieties were dehulled as described by Sosulski and Zadernowski (13) and Naczk *et al*. (7). Hulls were extracted with hexane for 12 h using a Soxhlet apparatus, and the hulls were dried at room temperature.

Soluble tannins were extracted twice from a sample of 60 g of hulls at room temperature into 70% (vol/vol) aqueous acetone (at a ratio of 1:10, wt/vol) using a Waring Blender (Waring Products Division, Dynamics Corporation of America, New Hartford, CT) for 2 min at maximal speed. The extracts were combined, evaporated to near dryness under vacuum at 40°C and lyophilized.

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The total content of phenolic compounds in the extract was estimated using the Folin-Denis reagent (14) and expressed as *trans*-sinapic acid equivalents. The content of tannins in extracts was measured using the modified vanillin assay (7) and the proanthocyanidin assay (7) and expressed as absorbance (A) units per 1 g of hulls (A/g). Ultraviolet (UV) spectra of methanolic solutions of extracts were recorded using a Beckman 7400 diode array spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

The antioxidant activity of crude extracts of canola and rapeseed hull tannins was evaluated using a β-carotene-linoleate model system (15). Methanolic solutions (0.2 mL) containing 2 mg of crude extracts were added to a series of tubes containing 5 mL of an emulsion of linoleate and β-carotene stabilized by Tween 40, prepared as described by Wanasundara *et al*. (12). A controlled experiment was carried out using 0.5 mg of BHA. Immediately after the addition of the 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 read over 120 min at 15-min intervals.

The scavenging effect of crude tannin extracts on the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical was monitored according to the method of Hatano *et al*. (16). An aliquot (0.1 mL) of methanolic solution containing 20–100 μg of crude extract of canola or rapeseed hull tannins 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 10 s, then left to stand at room temperature for 30 min, and the absorbance of this solution was measured at 517 nm.

The reducing power of crude extracts of canola and rapeseed hull tannins was determined as described by Oyaizu (17). Crude extracts (20–100 μg), dissolved in 1 mL of distilled water, were mixed with 2.5 mL of a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 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 was centrifuged at 1750 × *g* 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 0.1% solution of $FeCl_3$, and the absorbance of the reaction mixture was read at 700 nm.

Statistical analysis of the data [analysis of variance (ANOVA) test, *t*-test, linear correlation] was carried out using SigmaStat v.2.03 (SSPS Science Inc., Chicago, IL). Each hull sample, for the purpose of statistical analysis, was referred to as a treatment. The treatments were classified into two groups; a low-tannin and a high-tannin. The statistical analysis of all treatments was carried out using the ANOVA test. In addition, the contrast test (18) was employed among the groups of treatments when a statistically significant difference ($P \leq 0.05$) among all treatments was found by the ANOVA test. Furthermore, the statistical analysis of the treatments within each group was carried out using the *t*-test.

The results presented in graphs are mean values of at least three experiments (with three replicates per experiment). No statistically significant difference (ANOVA test; $P > 0.05$) was found among the experiments for each treatment. Treatments, within each group of treatments, followed by the same superscript letter are not significantly different ($P > 0.05$; *t*-test).

RESULTS AND DISCUSSION

In previous studies (5,7) we investigated the effectiveness of various solvent systems and extraction conditions on the yield of extraction of soluble tannins from canola meals and hulls. A 70% (vol/vol) acetone was found to be the most efficient solvent. Futhermore, a two-stage extraction of hulls with this solvent was sufficient for the removal of soluble tannins. We also found that the differences in the amount of tannins extracted from the same batch of hulls on different days were not statistically significant (one-way ANOVA test). The aim of this study was to evaluate the antioxidant activity of crude tannins; therefore, 70% (vol/vol) acetone was selected for their extraction from hulls.

Canola and rapeseed hulls contained 14–2,131 mg tannins per 100 g of hulls, and the variability within the canola varieties was found to be 57–1,556 mg tannins per 100 g of hulls and 994–2,131 mg tannins per 100 g of hulls for Westar and Cyclone canola, respectively (5,7). The differences in tannin content within the canola varieties may be due to the growing location as well as the stage of seed development. More detailed studies on the effect of these variables are still necessary.

Naczk *et al*. (7) proposed to classify hulls, based on the precipitation index (PI) expressed as milligrams of dye-labeled bovine serum albumin (BSA) precipitated per milligram of soluble tannins, into low- (PI > 17.0) and high-tannin (PI ≤ 5.0) hulls. These authors suggested that more polymerized tannins were isolated from low- than from high-tannin hulls. Therefore, in this study, three samples of high-tannin hulls (Cyclone canola; PI < 5.0) and three samples of low-tannin hulls (Kolner, Leo, and Ligaret Polish rapeseed varieties; PI > 20.0) were selected to obtain information on the variability in the antioxidant properties within and among the groups of high- and low-tannin hulls.

Phenolic acids and their derivatives, as well as condensed tannins, are the predominant phenolics of crude tannin extracts (6). The total content of phenolics in crude tannin extracts was determined by the Folin-Denis assay and expressed as sinapic acid equivalent per gram of extract, as this reagent is sensitive to both classes of phenolics. On the other hand, the content of condensed tannins was estimated by the proanthocyanidin and vanillin assays. These two assays are specific for condensed tannins and are commonly used for their quantitation (19). Tannins isolated from plant materials are mixtures of polymeric compounds that differ in their sensitivity toward the reagents used for their determinations (19). Therefore, the tannin contents shown in Table 1 are expressed in absorbance (A) equivalents per gram of extract (A/g) as it is difficult to find an appropriate standard. The contents of total phenolics and condensed tannins in crude extracts are shown in Table 1. The crude tannins of Cyclone canola hulls contained twice as much total phenolics as those of the Polish

TABLE 1
Total Phenolic and Tannin Content in Crude Extracts of Canola and Rapeseed Hull Tannins[a]

Cultivar	Total phenolics (mg/g)[b]	Tannin content	
		A_{500}/g[c]	A_{550}/g[d]
Cyclone #1	270[a] ± 8	283[a] ± 10	656[a] ± 14
Cyclone #2	296[a] ± 6	278[a] ± 8	653[a] ± 23
Cyclone #3	224[b] ± 10	174[b] ± 8	223[b] ± 8
Kolner	128[c] ± 7	7[c] ± 4	14[c] ± 5
Leo	136[c] ± 6	7[c] ± 4	14[c] ± 6
Ligaret	148[c] ± 10	15[d] ± 7	37[d] ± 9

[a]Total phenolics and tannin content (within a column) followed by the same roman superscript letter are not significantly different (*t*-test; $P > 0.05$).
[b]Expressed as sinapic acid equivalents.
[c]Determined using the vanillin assay.
[d]Determined using the proanthocyanidin assay.

rapeseed varieties. On the other hand, the crude tannins of canola hulls contained 10–40 times more soluble condensed tannins than rapeseed hulls.

The UV spectra of methanolic solutions of crude tannins of Cyclone canola hulls exhibited two absorption maxima (282 and 309 nm), whereas those of rapeseed hulls displayed one maximum at a longer wavelength (326 nm) (Fig. 1). In a preliminary study, the crude tannins of Cyclone canola were fractionated on a Sephadex LH-20 column according to the procedure described by Strumeyer and Malin (20). The methanolic solution of condensed tannin fraction exhibited an absorption maximum at 282 nm whereas the nontannin fraction of hull phenolics dissolved in methanol displayed absorption maxima at 282 nm (minor peak) and 309 nm (major peak). The absorption maximum at the longer wavelength (309 or 326 nm) may be due to the presence of phenolic acids, notably hydroxycinnamic acid derivatives (21), and the absorption maximum at the shorter wavelength (282 nm) may be due to the presence of *p*-hydroxybenzoic acid and flavone/flavonol derivatives (11,22). The analysis of crude tannin

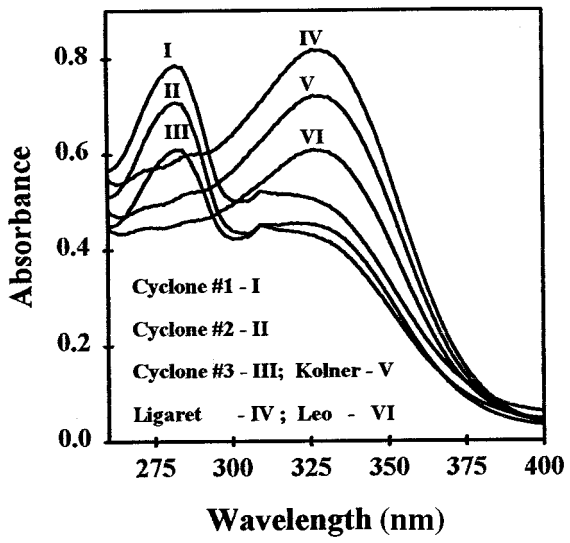

FIG. 1. Ultraviolet spectra of crude tannins of canola (Cyclone #1–3) and rapeseed hulls in methanol.

spectra also indicates that extracts obtained from rapeseed hulls are low in flavonoids.

The extracts of potential antioxidants from plant materials are a complex mixture of phenolics with a different number and arrangement of hydroxyl and methoxy groups, and differing degrees of polymerization. It is well known that the antioxidant activities of phenolics are dictated by their molecular structure (23). Polyphenols are known to exhibit stronger antioxidant activity than monophenols, and the antioxidant activity of a phenol is also affected by its degree of polymerization. In addition, phenols with a second hydroxyl group in the *ortho* and *para* positions possess stronger antioxidant activity than those with it in the *meta* position (23,24). Ariga and Hamano (25) correlated the ability of flavonoid oligomers to scavenge free radicals with their degree of polymerization. Recently, Brand-Williams *et al*. (26) and Bondet *et al*. (27) showed that the structural characteristics of antioxidants affected their reaction rate with the DPPH radical. They found that the reaction rate of a majority of phenolics tested slowed as a steady state was reached after 1 to 6 h of reaction. Because of this the evaluation of antioxidant activity of complex mixtures of phenolics is difficult and it has to be limited to the estimation of the total antioxidant activity of the system. This also complicates the interpretation of experimental results. Numerous methods and their modifications have been proposed for the evaluation of antioxidant activity of potential antioxidants (28). Of these, the β-carotene-linoleate assay, the DPPH, and the reducing power assays are most commonly used for the evaluation of antioxidant activity of biological extracts.

The effect of crude tannin extracts on the coupled oxidation of linoleic acid and β-carotene was compared to that of BHA (Fig. 2). Crude extracts of canola hull tannins exhibited the greatest antioxidant activity, but the values were lower than that of BHA in the β-carotene-linoleate model system. The antioxidant activity of crude extracts of rapeseed hull tannins was lower than that of crude extracts of canola tannins.

The scavenging activity of crude tannins of canola and rapeseed hulls was determined by the DPPH assay. The absorption maximum of a stable DPPH radical in methanolic solution was previously reported to be at 515 nm (26), but in our study it was found at 517 nm. The absorption at this characteristic wavelength disappears as the reaction between the antioxidant molecules and radicals progresses. The decrease in the absorbance depends on the concentrations of the antioxidant and the radical, the molecular structure of the antioxidant, and its kinetic behavior (26,27). Therefore, the decrease in absorbance at the characteristic wavelength is a measure of the radical scavenging activity of the antioxidant employed. The original DPPH assay (16), used in this study, measures the change in the absorbance after 30 min of reaction at room temperature. Recently Brand-Williams *et al*. (26) modified this procedure to take into account the different kinetic behavior of antioxidants. However, the use of this modified procedure is limited to the evaluation of scavenging activity of pure antioxidants since a knowledge of molecular

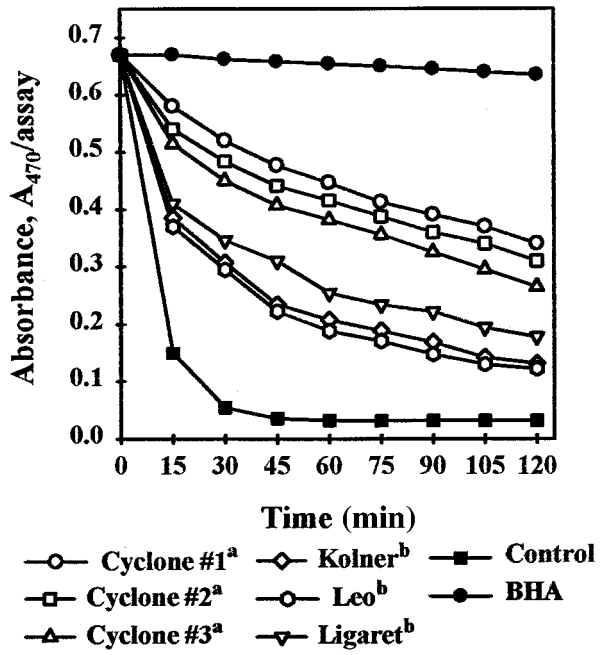

FIG. 2. Antioxidant activity of crude tannins of canola (Cyclone #1–3) and rapeseed hulls in a β-carotene-linoleate model system, as measured by changes in absorbance values at 470 nm. The same roman superscript letter indicates no significant difference (*t*-test; $P > 0.05$) within the group. BHA, butylated hydroxyanisole.

structures of the compounds is essential. Figure 3 shows that crude tannins of canola hulls exhibit a somewhat greater scavenging activity than those of their rapeseed counterparts. The scavenging effects of the canola and rapeseed hull tannins, expressed as the ratio of the decrease in the absorbance at 517 nm to the absorbance of DPPH solution in the absence of phenolics, ranged from 44.5 to 50.5% and from 35.2 to 40.7% at a dose of 1 mg of extract for canola and rapeseed crude tannins, respectively. These results were similar to those reported by Yen and Chan (29) for black tea extracts at a dose of 2 mg of extract.

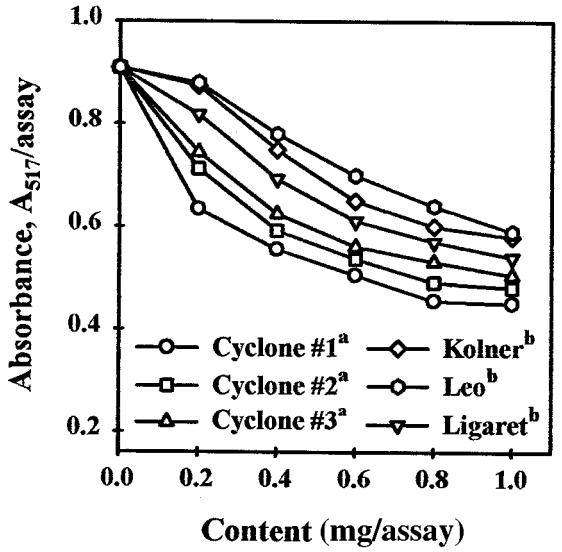

FIG. 3. Scavenging effect of crude tannins of canola (Cclone #1–3) and rapeseed hulls on the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical, as measured by changes in absorbance values at 517 nm. The same roman superscript letter indicates no significant difference (*t*-test; $P > 0.05$) within the group.

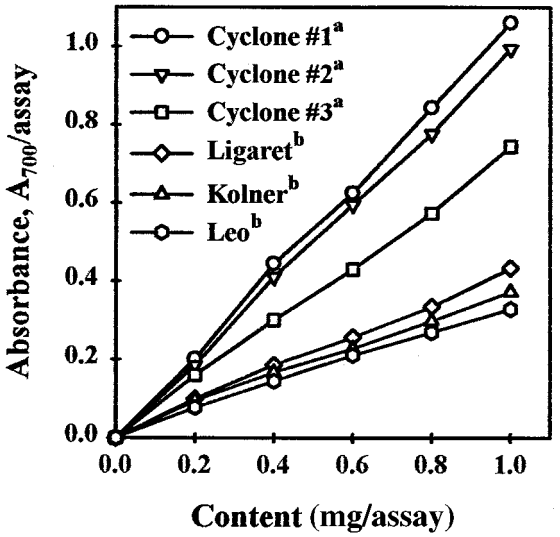

FIG. 4. Reducing power of crude tannins of canola (Cyclone #1–3) and rapeseed hulls as measured by changes in the absorbance values at 700 nm. The same roman superscript letter indicates no significant difference (*t*-test; $P > 0.05$) within the group.

Figure 4 shows the reducing power of crude tannins of canola and rapeseed hulls as a function of the total phenolic concentrations. The results indicate that canola hull extracts exhibited a greater reducing power than rapeseed hull extracts. Thus, phenolics present in canola hull extracts are good electron donors and could terminate the radical chain reaction by converting free radicals to more stable products. The observed reducing power of crude tannin extracts, expressed as the absorbance of samples at 700 nm per 1 mg of extract, correlated well ($r = 0.966$; $P = 0.002$) with the total content of phenolics present in them (Table 1). The reducing powers of canola and rapeseed hulls were similar to those reported by Amarowicz *et al.* (30) for ethanolic extracts of evening primrose seeds.

The observed differences among the antioxidant activities of treatments (hulls) (Figs. 2–4) were found to be statistically significant (one-way ANOVA test; $P = 0.01$). Subsequently, the contrast method (18) was employed for the evaluation of differences between the means of the group of treatments, i.e., low- and high-tannin hulls. The results of this analysis showed that a statistically significant difference was found ($P = 0.005$ for the DPPH assay; $P = 0.025$ for the reducing power assay; $P = 0.0025$ for the β-carotene-linoleate assay) in antioxidant activities between the group treatments for all assays used in this study. The results of the statistical analysis for treatments within each group of treatments are shown in Figures 2–4.

The significantly ($P \leq 0.025$) stronger antioxidant activity of canola hull crude tannins compared to those of rapeseed

hulls may be due to (i) the difference in total phenolics and tannins contents (Table 1), (ii) the diversity in structural characteristics of potential phenolic antioxidants present in crude tannin extracts, (iii) a synergism of phenolics with one another and/or other components present in each extract (11), and (iv) different kinetic behaviors of potential antioxidants as reported by Brand-Williams *et al*. (26). Crude tannin extracts of canola hulls contained 10–40 times more condensed tannins (Table 1) than those of rapeseed hulls. Therefore, the tannin fraction of canola extracts may also contribute to their greater antioxidant activity. Further work is required to evaluate the antioxidant activity of tannin and nontannin fractions of canola and rapeseed hulls as well as to isolate and identify the active components of canola and rapeseed hull phenolics.

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