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Antioxidant Activity of Phenolic Compounds from Whole Cottonseed By-product

Diana Yamile Gallego Villa[1], Anna Lisa Piccinelli[2], Luigi Russo[2], Maddalena Landi[2], Luca Rastrelli[2]*

[1]Facultad de Medicina Veterinaria y de Zootecnia, Universidad Nacional de Colombia, Cra 30 N° 45-03, Bogotá, Colombia.
[2]Dipartimento di Farmacia, University of Salerno, Via Ponte don Melillo, 84084, Fisciano (SA), Italy.

*rastrelli@unisa.it

Abstract

Cottonseeds are fed to high-producing dairy cows as a source of fat and highly-digestible fiber. Previously we reported the isolation and structure characterization of different phenolic compounds, quercetin, kaemferol and benzoic acid derivatives (1-9) from whole cottonseed by-product. In this paper the antioxidant activity of all isolated compounds was determined by measuring free radical scavenging effects using the Trolox equivalent antioxidant capacity assay and the coupled oxidation of β-carotene and linoleic acid (autoxidation assay). The range of phenolic compounds present in WCS (1.90 g/kg), assayed by the Folin-Ciocalteu method, and their antioxidant properties provides a valuable database for possible nutritional studies on the impact of diet on animal health and productivity.

Key words: Whole cottonseed; by-products; flavonol glycosides; benzoic acid derivatives; antioxidant activity; feedstuff.

Introduction

Agricultural processing yields many by-products that have significant feeding value for livestock. Because of their increasing market value as feed ingredients, many producers of such materials now consider them to be "co-products" rather than "by-products." Agricultural co-products that are sometimes available at attractive prices include soy hulls, wheat midds, whole cottonseed, wet and dry corn gluten feed, wet and dried brewers grains, dried distillers grains, poultry litter and many others.

One of these is the whole cottonseed (WCS), a by-product of cotton industry, is the unprocessed and unadulterated oilseed which has been separated from the cotton fiber. WCS has been used as a supplemental feedstuff for cattle, sheep and other ruminants for over 100 years, because it can be used as a practical source of supplemental protein, forage or grain replacement in ruminant diets to reduce cost of production (Solaiman, 2007; Holffman, 1998).

The digestion of whole cottonseed in the rumen causes a slow release of nutrients. With the slow release of nutrients, a component of protein is bypass protein. This bypass protein will be available for direct absorption by the animal.

Each pound of cottonseed will provide 0.2 pounds of crude protein on a dry matter basis. Include up to 7 pounds per day in mature cow diets to provide 1.5 pounds of supplemental crude protein. Whole cottonseed provides sufficient supplements for lactating cows fed a diet containing hay that is at least 9 percent crude protein. If supplementation is required beyond 7 pounds per day, then other feeds must be blended with the cottonseed. Whole cottonseed's high fat content is the primary limiting factor for inclusion in beef cattle diets. Supplementing cottonseed at the maximum recommended feeding rate (7 pounds per day) will provide 1.1 pounds of supplemental fat, which is the maximum amount that should be fed to prevent significant reduction in fiber digestion. Furthermore, WCS is an excellent supplement to poor quality grass hay for dry and lactating cows because it supplies both energy and protein in a single feed ingredient. Use cottonseed in the diets of cows and stocker calves, but do not feed it to young, pre-ruminant calves.

Effects of lamb diets containing increasing levels of WCS on feed intake, liveweight gain, feed conversion, feeding margin and carcass characteristics were studied by Kandylis. (Kandylis et al. 1998) and it was concluded that WCS was satisfactory as a feed ingredient for growing sheep. Moreover, WCS at 15% of the total diet can be safely used for meat goat production; however, it should not be fed or fed very sparingly to young bucks raised for breeding purposes (Solaiman, 2007).

Secondary constituents in whole cottonseed has been studied because some components, including terpenoid phytoalexins have been blamed for anti-nutritive or toxic effects when the seeds were tested as animal feed (Piccinelli et al., 2007). Information about other phenolic compounds such as flavonoids in WCS is scarce and only in 2001 Zhang et al. (Zhang et al., 2001) found that glandless cotton seed contains five flavonol glycosides, including a new apiosyl derivative, recently, flavonol glycosides and benzoic acid derivatives have been reported (Piccinelli et al., 2007)

The effects of antioxidants are attracting considerable interest today in connection with the quality of processed animal products, as proved by recent studies on the effect of antioxidative activities in the living organism (Gramzow, et.al, 2002).

In contrast, studies in cattle have demonstrated the importance of antioxidant substances during the peripartal period, ultimately confirming the significance of antioxidants at times of physiological performance peaks (Allen, et.al, 2001; Gramzow, et.al, 2002), And, although not very significant to ruminants due to their restricted life spans, it would be possible to expert that ruminants fed on fodders rich in polyphenols may retain some of these desirable polyphenols in their milk or flesh which could benefit consumers (Vinson et al., 1998). In this regard, the antioxidant activity of phenolic compounds in products like WCS should be an increasingly tool in contributing to animal health and productivity and in this paper the antioxidant activity of previously isolated phenolic compounds was determined.

Material and Methods

Plant Material

Whole cottonseed is a by-product of cotton production and acreage is expanding in the North Italy. Whole cottonseed (WCS) was furnished by Cereal Comm Feed Company (Brescia, Italy).

Extraction and Isolation Procedure of Compounds 1-9

The dried and powdered WCS (405 g) was defatted with hexane and $CHCl_3$ and then extracted with MeOH to give 19 g of residue. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH soluble portion (5.8 g) which was chromatographed twice on a Sephadex LH 20 CC (Pharmacia, Uppsala, Sweden) (1 m x 3 cm i.d.) with flow rate of 0.5 mL/min; 90 fractions of 8 mL were collected. After TLC analysis (Si-gel, *n*-BuOH-AcOH-H_2O 65:15:25, $CHCl_3$-MeOH-H_2O 70:30:3) fractions with similar R_f were combined giving four major fractions (I-IV) which were further purified by HPLC on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a Waters m-Bondapak C-18, 10 mm (7.8 x 300 mm, flow rate 2.5 mL/min) column and a U6K injector. Fractions were purified using MeOH-H_2O as the eluent as reported previously (FOCH)

Total Polyphenols Assay

Estimation of the global polyphenol content in the extracts was performed according to the Folin-Ciocalteu method. Whole Cotton Seed was extracted with acetone/water (7:3) employing a Dionex Accelerated Solvent Extractor ASE-200. A portion (1.15 mg) of this extract was dissolved in MeOH (2 mL), and the extract was diluted 10-fold with water. Folin-Ciocalteu reagent (0.5 mL; Merck) was added to the diluted solutions (0.5 mL), followed by 0.5 mL of a 100 g/L solution of Na_2CO_3. The absorbance was measured at 720 nm (Shimadzu UV-2101 spectrophotometer) with a blank sample (water plus reagents) in the reference cell. Quantification was obtained by reporting the absorbances relative to the calibration curve of quercetin used as standard phenol.

TEAC Assay.

Pure compounds were tested by using the TEAC assay. The TEAC value is based on the ability of the antioxidant to scavenge the radical cation 2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonate) ($ABTS^{\bullet+}$) measured by spectrophotometric analysis. $ABTS^{\bullet+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The $ABTS^{\bullet+}$ solution was then diluted with phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Samples were diluted with methanol to produce solutions of 0.3, 0.5, 1, 1.5, and 2 mM concentration. The reaction was initiated by the addition of 1 mL of diluted ABTS to 10 µL of each sample solution. Determinations were repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol) and was plotted as a function of concentration compound or standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical Co., Gillingham, Dorset, U.K.). The antioxidant activities of compounds **1–9** are expressed as TEAC values in comparison with TEAC activity of reported reference compound quercetin (Re, et.al, 1999). The TEAC value is defined as the concentration of standard Trolox with the same antioxidant capacity as a 1 mM concentration of the antioxidant compound under investigation.

Autoxidation of β-Carotene Assay

Oxidation of linoleic acid was measured according to the method described by Pratt (Pratt, et.al, 1992). Quantities of linoleic acid (20 mg) and Tween 20 (200 mg) were placed in a flask, and a solution of 2 mg of *β*-carotene in 10 mL of $CHCl_3$ was added. After removal of $CHCl_3$, 50 mL of distilled water saturated with oxygen for 30 min was added. Aliquots (200 mL) of each compound, dissolved in ethanol to a 15 mg/mL solution, were added to each flask with shaking. Samples without test compounds were used as blanks, and a sample with 2,6-di-*tert*-butyl-4-methoxyphenol (BHT, Aldrich Chemical Co.) was used as a control substance. Samples were subjected to oxidation by placing in an oven at 50 °C for 3 h. The absorbance was read at 470 nm at regular intervals. The antioxidant activity (AA) was calculated with the equation

$$AA = [1 - (A0 - At)]/(A00 - A0t) \times 100$$

where A_o = absorbance at the beginning of the incubation, with test compound; A_t = absorbance at the time *t*, with test compound; A_{oo} = absorbance at the beginning of the incubation, without test compound; and and A_{ot} = absorbance at the time *t*, without test compound.

Results and Discussion

Whole Cotton Seed was extracted successively with hexane, $CHCl_3$, and MeOH. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH soluble portion which was subjected to chromatography on Sephadex LH-20 and HPLC.

Nine phenolic compounds were isolated (**Fig. 1**): quercetin 3-O-{β-D-apiofuranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside} (**1**), kaempferol 3-O-{β-D-apiofuranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside} (**2**), quercetin 3-O-[β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside] (**3**), quercetin 3-O-β-D-glucopyranoside (**4**), kaempferol 3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**5**), quercetin 3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**6**), kaempferol 3-O-α-L-rhamnopyranoside (**7**), gallic acid (**8**) and 3,4-dihydroxybenzoic acid (**9**). The structures and molecular formule of compounds **1-9** were determined from their ESI-MS spectra, as well as from 1D and 2D ^1H and ^{13}C NMR data (FOCH).

Antioxidant Activity

The antioxidant activity of the isolated compounds **1-9** was first tested in TEAC assay. The radical scavenger activity of the tested compounds was expressed as TEAC values; TEAC value is defined as the concentration of standard Trolox with the same antioxidant capacity as a 1 mM concentration of the antioxidant compound under investigation. TEAC results for compounds **1-9** and quercetin, used as reference compound, are summarized in **Table 1**. Quercetin derivatives (**1, 3, 4** and **6**) showed anyway a good activity with respect to the other kaempferol glycosides, probably due to the presence of the B ring *o*-dihydroxyl. All of the other metabolites yielded lower TEAC values comparable to that of the reference compound. The antioxidative effect of pure compounds **1-9** on the autoxidation of linoleic acid was also determined. The values of AA measured at t 60 and 120 min, employing bleaching of b-carotene as a model system, are reported in **Table 1**. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are the targets of lipid peroxidation. It is generally thought that the inhibition of lipid peroxidation by antioxidants may be due to their free radical scavenging activities. All tested compounds were weakly active in this test with respect to the reference compound BHT. The most active compounds were **1, 4**, and **8** even though at t 120 min their activities strongly decreased. In the literature it is reported that the flavonol C-3 hydroxyl group is responsible for the high inhibition of b-carotene oxidation in the heterogeneous system (Mora, et.al, 1990). Comparison of the antioxidant activity of flavonol aglycons with the activity of their glycosides showed that the blockage of the C-3 hydroxyl group resulted in a total loss of antioxidant activity. Our AA results are in agreement with this general consideration, all compounds having lower activity than the synthetic antioxidant BHT.

WCS contains fractions that are rich in flavonol glycosides, including the 7 compounds identified. They contain the aglycons of quercetin and kaempferol, and carbohydrate moieties, (as mono-, di- and trisaccharides), linked to the aglycons at the C-3 position. The quantitative total polyphenol content (1.90 g/kg) obtained from the Folin-Ciocalteu assays, generally considered as the method of choice to estimate total phenol contents in plant extracts (Scalbert 1992), and the structural variability are interesting due to the alimentary and taxonomic properties ascribed to flavonol glycosides. The range of phenolic compounds present in WCS could improve the quality and shelf life of farm products by their ability to inhibit lipid peroxidation. As these phenolic compounds are increasingly being regarded as contributing to animal health and productivity, it is important that considerations of the nutritive value of forages should include, not only the primary metabolites, but also the phenolic compounds which they contain.

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Table 1. Antioxidant Activities of Compounds **1-9** in the TEAC and Autoxidation Assays[a]

Compound	TEAC value (mM) ± SD[b]	Autoxidation assay	
		t) 60 min	*t*) 120 min
1	1.98 ± 0.01	32.4	14.9
2	1.31 ± 0.09	22.0	12.1
3	1.76 ± 0.03	27.3	14.2
4	1.92 ± 0.01	29.5	13.5
5	0.75 ± 0.05	26.2	4.4
6	1.76 ± 0.03	24.6	14.0
7	0.74 ± 0.02	11.3	10.0
8	1.26 ± 0.11	29.3	6.1
9	1.15 ± 0.05	24.2	5.0
Quercetin	2.77 ± 0.02		
BHT[c]		69.7	64.6

[a]For protocols used, see Materials and Methods.

[b]*n* = 3.

[c]BHT = 2,6-di-*tert*- butyl-4-methoxyphenol; standard control substance

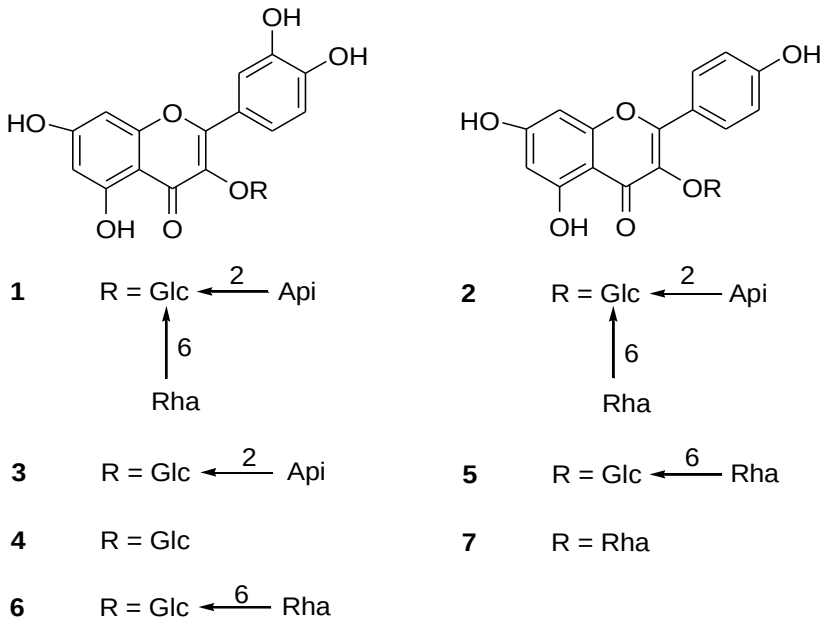

Figure 1. Flavonol glycosides **1-7** isolated from whole cotton seeds.ca