ARTICLES

Flavonol Glycosides from the Seed Coat of a New Manteca-Type Dry Bean (*Phaseolus vulgaris* L.)

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Two flavonol glycosides were isolated from the methanol extracts of "Prim", a variety of *Phaseolus vulgaris* L. (Manteca-type), with a yellow seed coat color. High-performance liquid chromatography was used to isolate and identify compound **1**, kaempferol (3,4,5,4′-tetrahydroxyflavone)-3-*O*-*β*-D-glucopyranoside, and compound **2**, kaempferol 3-*O*-*β*-D-glucopyranoside-(2→1)-*O*-*β*-D-xylopyranoside. Concentrations of these compounds were 49.9 ± 0.78 mg/100 g and 58.5 ± 1.67 mg/100 g of fresh whole bean weight, respectively. These two flavonols were the only flavonoids found in Prim and our data indicate that they are responsible for imparting the yellow color to the seed coat. We were not able to indicate the biochemical effects of the eight genes responsible for seed coat color, but we show that *G* is probably not responsible for producing a flavonol 3,5-diglycoside as has been hypothesized in the literature. No proanthocyanidins (condensed tannins) were found in Prim dry bean seed coat.

Keywords: *Phaseolus vulgaris L.; flavonol glycosides; seed coat color genotype; proanthocyanidins*

INTRODUCTION

Considerable variability exists in common bean (*Phaseolus vulgaris* L.) for seed characteristics, and consumers have acquired specific preferences for various combinations of size, shape, and color of the dry seeds (Adams and Bedford 1973). In many countries these preferences formalize the commercial (market) classes, which must meet specific consumer expectations and industry standards. Seed coat color and seed size are the two main criteria that identify the numerous market classes recognized throughout the world.

Manteca is a dry bean market class with a pale-yellow seed coat and preferred by consumers in Chile. Manteca beans are favored because of their reported ease of digestibility. Beans with pale-yellow seed coats are also a popular market commodity in Mexico because of their reputation of being highly palatable and easy to digest.

Our knowledge of the seed coat color and patterns in *P. vulgaris* is mainly due to the work of Kooiman (1931), Lamprecht (1932), Feenstra (1960), and Prakken (1970, 1972). Prakken (1970,1972) reviewed the literature and in the synthesis he developed for the inheritance of seed coat color showed that eight Mendelian loci contribute: *P*, *C*, *D*, *J*, *G*, *B*, *V*, and *Rk*. According to Prakken's interpretation of the literature, *C*, *D*, and *J* are the color genes, *G*, *B*, and *Rk* are modifying genes (have an intensifying effect or darkening influence upon pale colors formed by the action of the color genes), and *V* is called the violet factor. When *V* is dominant it causes bluish or violet to black colors to develop in the seed coat.

Although the genetics of seed coat color in common bean is well established, the nature of the pigments giving rise to color is less well understood. Feenstra (1960) published a treatise on some biochemical aspects of common bean seed coat inheritance. He isolated and identified a number of compounds consisting of anthocyanins, flavonols, and leuco-anthocyanidins, all of which are broadly classed as flavonoids. Today researchers accept the fact that the pigments responsible for seed coat in *P. vulgaris* color are flavonoids. Many of the flavonoid pigments that give rise to seed coat color in beans may also impart positive health benefits as antioxidants (Hertog et al. 1993). On the other hand, some flavonoids may cause beans to darken in color upon aging and become hard to cook and digest.

Leakey (1988) proposed a hypothesis for the correspondence between genes imparting the various colors to seed coats and the pathways that might be involved in the biosynthetic interconversion of chalcone, flavones, flavonols, and anthocyanins. Leakey's scheme starts with the *P* gene called the "ground gene", which was identified by Kooiman (1931) and is necessary for the plant to produce color in the seed coat. Hence, *P* can be considered as the controlling factor for the presence

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or absence of flavonoids in seed coats. When *P* is recessive all plants produce seed with white testa regardless of what the genetic constitution is at the other seed coat color loci.

"Prim" is a new cultivar of Manteca that has been bred in Great Britain from parents not of Latin American origin but having the same pale-yellow genotype and qualities of taste, texture, and digestibility for which the Latin American Manteca market class is noted (Leakey, 1994). Prim is of the genetic constitution: *PP*, *CC*, *dd*, *jj*, *GG*, *bb*, v^{lae}, v^{lae}, and *Rk Rk* (Prakken 1972; Bassett unpublished test cross data, 1998).

Since dry bean seeds are the plant part of economic importance and because consumers are becoming more concerned about the chemistry of the food they eat, we find it a worthwhile research study to examine the relationship between seed coat colors and the chemistry defining the colors. Because there is little knowledge of the flavonoid pigments associated with yellow or orange colored bean seeds (Leakey, 1988), we undertook the present investigation to identify the flavonoid compounds in Prim dry bean. Specific objectives were to (1) identify and quantify the flavonoids found in Prim seed coats and (2) establish the relationship between the Mendelian genes controlling the pale-yellow color in Prim and the seed coat flavonoid pigments present.

EXPERIMENTAL PROCEDURES

Materials. Prim beans were bred by Dr. C. L. A. Leakey (Personal communication) to chemically resemble the "Manteca", or butter (from the Spanish mantequilla), bean which is grown primarily in Chile. Approximately 2 kg of Prim beans were obtained from Mr. Eric Klassen, Contract Production Manager, Johnson Seeds, Arborg, Manitoba, Canada.

Seed Coat Removal. Ten, 100-g (fresh weight) measures of beans were washed and then placed in enough distilled water to soak the beans. After 15 min of soaking the seed coats were separated from the cotyledons and then freeze-dried. The water exudate from the soaked beans was also freeze-dried and stored. The seed coats were lyophilized, placed in a small blender, and ground to a fine powder about H_2O extracts.

Instrumentation. A recycling preparative HPLC system (LC20, Japan Analytical Co. Tokyo) was used for separation of the extracts. Quantification of flavonol compounds **1** [kaempferol (3,4,5,4′-tetrahydroxyflavone)-3-*O*-*β*-D-glucoside] and **2** [kaempferol 3-*O*-*β*-D-glucopyranoside-(2→1)-*O*-*β*-D-xylopyranoside] was achieved on a Shiseido Capcell Pak reverse-phase C18 column (4.6 × 250 mm, 5 μM), using a Waters 600 multisolvent pump, a 996 photodiode array detector, and a 717 autosampler. All chromatograms were analyzed with Waters Millenium 2010 software. The gas chromatography system used was a Hewlett-Packard 5890 series II (J&W Scientific, Folsom, CA) equipped with a DB 17 column (30 m × 0.25 mm with 0.25-μm film) fitted with flame ionization detector (FID) and a 7673 autosampler. The oven temperature was programmed from 50 °C (5 min isothermal) to 210 °C at 3 °C /min and then to 270 °C at 5.0 °C/min for a total run time of 51.7 min. The injector temperature was kept at 130 °C. The carrier gas was helium with a flow rate of 160 mL/min and detector temperature of 275 °C. 1D and 2D NMR spectra were obtained on a VXR 500 MHz with Varian software at the Max T. Rogers NMR facility in the Department of Chemistry, Michigan State University. Pure flavonoids **1** (13.0 mg) and **2** (17.0 mg) were dissolved in DMSO-d_6 solution and the following analyses performed: ^{1}H and ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), gradient heteronuclear multiple quantum correlation (GHMQC), double quantum filtered correlated spectroscopy (DQF−COSY), through orbital total correlation spectroscopy (TOCSY), as well as gradient heteronuclear multiple bond correlation spectroscopy (GHMBC).

Extraction and Isolation of Compounds 1 and 2. Dried, ground bean seed coat (100 g) was packed into a glass column (5 × 30 cm) and then extracted sequentially with hexane (500 mL), EtOAc (500 mL), MeOH 100% (1.5 L), MeOH:H_2O 1:1 (2 × 1 L). Extracts were dried under reduced pressure in a rotary evaporator, weighed, and transferred into vials. Yields obtained were 0.113, 0.092, 1.83, 2.30, and 10.11 g, respectively.

TLC Analysis. The crude MeOH extract was subjected to chromatography on Analtech silica gel HLF plates (250 μ) with solvent systems of $CHCl_3$:MeOH 4:1 and 1:1 and $CHCl_3$:MeOH:H_2O 4:1:0.5. Plates were visualized under UV light and then sprayed with 20% H_2SO_4, charred with a heat gun, and again visualized under 254 and 366 nm. To test for the presence of proanthocyanidins, the MeOH, MeOH:H_2O, and H_2O extracts were chromatographed on avicell cellulose plates (250 μm) with a solvent system of butanol:acetic acid:water 4:1:5 (Markham 1982). Plates were then sprayed with 5% vanillin in EtOH and concentrated HCl 4:1.

Preparative HPLC. The methanol crude extract (350 mg) was dissolved in 30 mL of 100% MeOH, and then water was added until precipitation occurred. The mixture was then centrifuged at 20000*g* for 10 min and the residue recovered in $CHCl_3$ (50 mg). The supernatant was dried, resuspended in H_2O:ACN 70:30 (30 mL), purified by recycling preparative HPLC using a H_2O:ACN 70:30 mobile phase at a flow rate of 4.0 mL/min, and detected at 265 nm. Each injection volume was 3 mL, containing 35 mg of the extract, and a total of 10 injections were made. Extracts were purified on two Gaigel ODS A-343-10 columns (20 × 250 mm, 10 μM) connected in series. After separation of the crude MeOH extract by recycling preparative liquid chromatography, compounds **1** and **2** were collected pure as single peaks.

Quantification of Compounds 1 and 2 by HPLC. An isocratic system of ACN:H_2O 30:70, $\lambda = 200-600$ nm, flow rate 1.0 mL/min over 10 min was employed with an equilibration time of 20 min for a total run time of 30 min to achieve separation of the compounds. Compounds **1** and **2** in the MeOH, MeOH:H_2O extracts and the H_2O exudate of the Manteca seed coat were quantified by using purified standards characterized by various NMR methods. Standard solutions of compounds **1** and **2** were made up to achieve concentrations of 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/mL and analyzed immediately prior to injecting three replicates of the unknowns at 1.0 mg/mL. A calibration curve was then prepared for compounds **1** and **2** with an extraction wavelength of 349 nm.

Acid Hydrolysis of Compound 2. Compound **2** (0.7 mg) was dissolved in 2.5 mL of 100% H_2O in a 50 mL round-bottom flask and refluxed with 2.5 mL of 2 N HCl in MeOH:H_2O 1:1 for 2 h. This reaction mixture was next evaporated to dryness under reduced pressure in a rotary evaporator. The residue was then redissolved in H_2O (5.0 mL) and extracted with EtOAc (5.0 mL) in a separatory funnel. The water fraction containing the sugars was then analyzed by GC.

GC Analysis of the Sugars in Compound 2. The water fraction from the hydrolysis of **2** was dried and prepared for GC analysis along with 0.1 mg each of the standards (Sigma) (+)-D-glucose, (+)-D-xylose, (+)-D-arabinose, (+)-D-galactose, (+)-D-ribose, and (+)-D-fructose. The dried samples were dissolved in 1.0 mL of dry pyridine (standing over NaOH pellets for at least 12 h) containing 30 mg/mL of hydroxylamine hydrochloride and 0.5 mg/mL *β*-phenyl-D-glucopyranoside as an internal standard. This solution was shaken overnight under anhydrous conditions. Samples were then placed on a dry heater at 75 °C and vortexed every 20 min for 1 h to complete the oxygenation reaction. After the solution cooled to room temperature, 1.0 mL of hexamethyl disylazane and 100 μL of TFA were added to the above solution and mixed thoroughly, and the supernatant was transferred to autoinjector vials.

RESULTS AND DISCUSSION

The hexane and EtOAc extracts of Prim seed coat were oily in nature with a slight yellow color. The dried

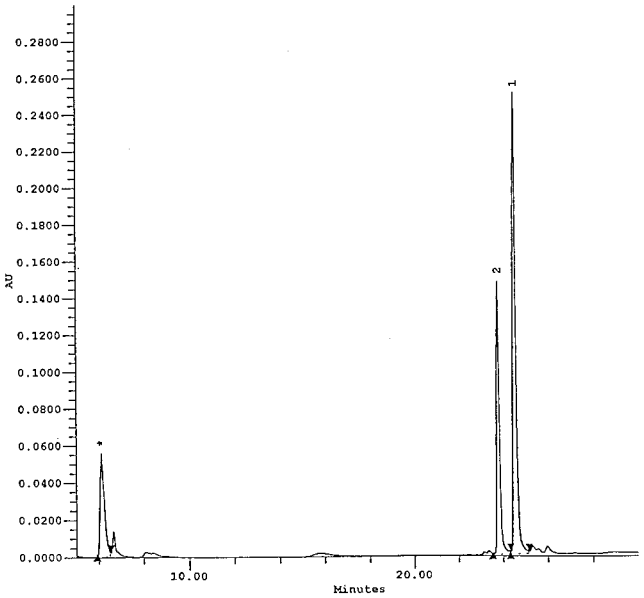

Figure 1. HPLC profile of the methanol extract from the Manteca bean seed coats. * = unidentified, 1 = kaempferol 3-*O*-*β*-D-glucoside, 2 = kaempferol 3-*O*-*β*-D-glucopyranosyl-(2→1)-*O*-*β*-D-xylopyranoside. Gradient system, 10:90 ACN:H_2O to 90:10 ACN:H_2O over 20 min, total 40 min run time, λ = 347 nm.

MeOH and MeOH:H_2O 1:1 and H_2O extracts were yellow to yellow-brown powders. Preliminary HPLC profiles of the H_2O, the MeOH, and the MeOH:H_2O extracts all showed the presence of two main compounds with absorption peaks at 266 and 347 nm, we refer to them throughout the text as compounds **1** and **2** (Figure 1). We arbitrarily chose the MeOH extract to isolate these compounds.

Kaempferol 3-*O*-*β*-glucopyranoside (1) was obtained as a yellow-brown film: ^{1}HNMR δ 12.5 (1 H, s, H-5), 6.08 (1 H, s, H-6), 6.31 (1 H, s, H-8), 8.02 (2 H, d, J = 8.88 Hz, H-2′,6′), 6.86 (2 H, d, J = 8.83 Hz, H-3′,5′), 5.41 (1 H, d, J = 7.54 Hz, H-1″), 3.32 (1 H, m, H-2″), 3.12 (1 H, m H-3″), 3.22 (1 H, m, H-4″), 3.24 (1 H, m, H-5″), 3.55 (1 H, d, J = 11.5 Hz, H-6A″), 3.30 (1 H, m, H-6B″); ^{13}C NMR (ppm) 156.07 (C-2), 133.12 (C-3), 177.31 (C-4), 161.15 (C-5), 98.82 (C-6), 164.84 (C-7), 93.67 (C-8), 156.39 (C-9), 103.69 (C-10), 120.83 (C-1′), 130.79 (C-2′,6′), 115.06 (C-3′,5′), 101.91 (C1″), 74.18 (C-2″), 77.44 (C-3″), 69.86 (C-4″), 76.40 (C-5″), 60.81 (C-6″).

Kaempferol 3-*O*-*β*-D-glucopyranoside-(2→1)-*β*-D-xylopyranoside (2) was obtained as a light yellow film: ^{1}H NMR (ppm) δ 12.5 (1 H, s, H-5), 6.10 (1 H, s, H-6), 6.31 (1 H, s, H-8), 8.04 (2 H, d, J = 9.0 Hz, H-2′,6′), 6.87 (2 H, d, J = 8.85 Hz, H-3′,5′), 5.70 (1 H, d, J = 7.52 Hz, H-1″), 3.34 (1 H, m, H-2″), 3.09 (1 H, m, H-3″), 3.24 (1 H, m, H-4″), 3.42 (1 H, m, H-5″), 3.52 (1 H, d, J = 11.5 Hz, H-6A″), 3.29 (1 H, m, H-6B″), 4.58 (1 H, d J = 7.29 Hz, H-1‴), 3.04 (1 H, m, H-2‴), 3.14 (1 H, m, H-3‴), 3.24 (1 H, m, H-4‴), 3.72 (1 H, dd, J = 16.34, 6.18 Hz, H-5A‴), 3.20 (1 H, m, H-5B‴); ^{13}C NMR (ppm) 154.66 (C-2), 132.59 (C-3), 176.94 (C-4), 161.07 (C-5), 99.31 (C-6), 165.05 (C-7), 93.85 (C-8), 156.48 (C-9), 102.78 (C-10), 120.89 (C-1′), 130.71 (C-2′,6′), 115.13 (C-3′,5′), 97.88 (C-1″), 81.73 (C-2″), 76.04 (C-3″), 69.53 (C-4″), 76.78 (C-5″), 60.48 (C-6″), 104.44 (C-1‴), 73.78 (C-2‴), 76.04 (C-3‴), 69.37 (C-4‴), 65.62 (C-5‴).

The downfield portion of the 1D ^{1}H NMR spectrum of compounds **1** and **2** showed a 4 H AA ‘XX’ system at 8.02 and 6.86 ppm and a 2H AX system at 6.31 and 6.10 ppm, which is characteristic of a kaempferol nucleus (Slimestad et al., 1993). The anomeric sugar protons were found considerably downfield from the other sugar protons and the doublet at 5.41 in the spectrum of compound **1** together with the integration data revealed a kaempferol-to-sugar ratio of 1:1. The anomeric protons of **2** at 5.71 and 4.58 ppm indicated a kaempferol-to-sugar ratio of 1:2. The coupling constants of the anomeric protons in compounds **1** and **2** were both greater than 7.0 Hz, indicating a *β*-linkage of the glucose to the aglycon for both compounds (Agrawal, 1992). The rest of the sugar region for compound **1** was identified by comparison of the carbon spectrum with published ^{13}C spectral data for kaempferol glucoside (Agrawal, 1992).

To assign the proton and carbon signals for compound **2,** a variety of 2D NMR techniques were employed. The sugars from the water fraction were identified by GC as glucose (retention time 10.90 min compared to 10.88 min for the reference standard) and xylose (retention time of 6.16 min compared to 6.15 min for the reference standard). The DEPT NMR spectrum showed peaks at δ 60.81 and 65.2, respectively, which identified the CH_2 carbons of the glucose and xylose moieties in compound **2**. These, along with the anomeric protons, served as entry points in the DQF-COSY spectrum for sequential assignment of sugar protons in compound **2**. Assignment of the corresponding carbon signals of the sugars were made in the GHMQC experiment. The xylose linkage to the glucose was identified from the ^{13}C spectrum by the downfield shift of carbon 2 of the glucose from 75.50 to 81.72 ppm. This linkage was also confirmed by the TOCSY experiment which showed the direct connectivity of the anomeric proton of the xylose to H2″ of the glucose. The coupling constant for the xylose anomeric proton was greater than 7.0 ppm, which indicated a *β* linkage between the glucose and xylose. The linkage of the glucose to C3 of kaempferol was identified by comparing the literature values of kaempferol glucoside (Agrawal, 1989) to those of compound **2** and considering the fact that the C3 in compound **2** was shifted upfield by 3.1 ppm. This linkage was further confirmed by a GHMBC experiment, which showed the connectivity of the H1″ anomeric proton of the glucose to the C3 of the kaempferol. Therefore, compound **2** was identified as kaempferol 3-*O*-*β*-D-glucopyranosyl-(2→1)-*β*-D-xylopyranoside. Concentrations of compounds **1** and **2** quantified by analytical HPLC were calculated to be 49.9 ± 0.78 mg/g and 58.5 ± 1.67 mg/100 g of fresh whole bean weight. These peaks were identified by co-injection of the extracts with standards identified by NMR.

A kaempferol 3-monoglucoside and kaempferol 3-glucoxyloside were previously isolated and identified by Feenstra (1960) from a lemon yellow bean with genotype *sh sh* (now *j j*), *C_ v*^{1ae} *v* ^{lae}. Our compounds **1** (Figure 2) and **2** (Figure 3) correspond to the respective kaempferol glycosides discovered by Feenstra (1960). However, Feenstra (1960) did not identify the compounds using NMR and as a result could not determine the sites for the interglycosidic linkage of the kaempferol 3-glucoxyloside nor the quantities of either compound in fresh bean. Our detailed 2-D NMR structural data for compound **2** indicates a 2→1 linkage between the glucose and xylose.

We believe that these two kaempferol compounds we

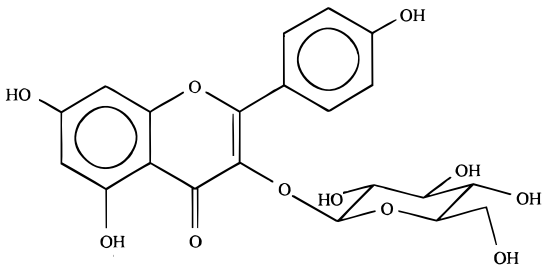

Figure 2. Structure of compound **1**, kaempferol 3-*O*-*β*-D-glucoside.

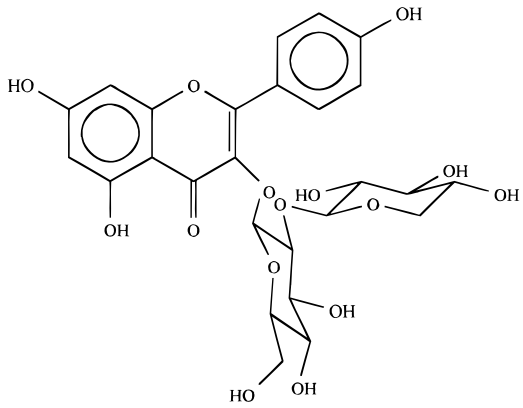

Figure 3. Structure of compound **2**, kaempferol 3-*O*-*β*-D-glucose-(2→1)-*β*-D-xyloside.

purified and identified in the extracts are the pigments imparting yellow color to Prim's seed coat. We draw this conclusion because of the yellow color of the kaempferol mono (**1**)- and di (**2**)-glycosides, the fact that other flavonoids and carotenoid pigments were not detected in the extracts, and the finding that when the purified standards were cochromatographed with the extracts the yellow spots in the extracts corresponded to the same yellow standard spots.

An objective of the current study was to establish the relationship between the Mendelian genes controlling seed coat color in Prim and the seed coat flavonoid pigments present. In the present study the only flavonoids we found in the seed coat of Prim were flavonol glycosides. Our findings agree with the observations of Feenstra (1960) who found that only flavonol glycosides were formed in the presence of *C C, v*^{lae} *v*^{lae} genotypes. Moreover, when Feenstra (1960) examined the chemistry of 12 genotypes that represented all possible homozygous combinations of *J j, Cr Cr, cu cu, V V,* and *v*^{lae} *v*^{lae}, two had yellow seed coats. In the lemon-yellow one (genotype *j j, C C, v*^{lae} *v*^{lae}) Feenstra (1960) found kaempferol 3-monoglucoside and kaempferol 3-glucoxyloside, which are the two compounds we found in Prim. For the second yellow genotype, a canary yellow seed coat and genotype *J J, C C, v*^{lae} *v*^{lae}, Feenstra (1960) found only the kaempferol 3-monoglucoside in addition to leucocyanidin and small amounts of quercitin 3-monoglucoside and leucopelargonidin.

Leakey (1988) believes and Feenstra's (1960) data suggest that the *J* gene is responsible for the production of proanthocyanidins. Prim is recessive at the *J* locus (*j j*) and no proanthocyanidins were detected. Prim beans have only four dominant color genes (Leakey, 1988): the "ground gene", *P*, on which all other genes are hypostatic, *C*, which is the complementary color gene (Prakken, 1972), *G*, which is the yellow-brown factor, and *Rk*, which is the gene imparting red color to seed coats but is not expressed in Prim. Leakey (1988) also hypothesized that *G* may control for the production of a 3,5-diglycoside on the quinonoid ring of the flavonol, but it is clear from the ^{1}H NMR data that a sugar residue at the 5-position is absent in compounds **1** and **2**; the proton of the 5-hydroxy group is clearly present at 12.5 ppm and is shifted far downfield due to deshielding by the oxygen present at the 4-position of the kaempferol. Hence, our data are at variance with Leakey's (1988) hypothesis.

With regard to the anecdotal evidence for ease of digestibility of Manteca beans, it is important to note that in Prim we did not find polyphenolics such as proanthocyanins and condensed tannins which have been strongly implicated to reduce the digestibility of protein (Lindgren, 1975; Rannenkamp, 1977; Elias et al., 1979; Reddy et al., 1985; Aw and Swanson, 1985) and starch (Griffiths and Moseley, 1980). Condensed tannins would normally be detected in the methanol and water extracts (Salunkhe, 1990), but when these extracts were spotted on cellulose TLC plates and then sprayed with 5% vanillin in EtOH, red or purplish red spots characteristic of tannins (Markham, 1982) were not observed. We did not ascertain the effect of compounds **1** and **2**, the two kaempferol glycosides, on bean seed digestibility. However, it is doubtful that these would have a negative impact on digestibility because the minimum molecular weight for a flavonoid molecule to complex with protein would require the presence of dimers (Salunkhe et al., 1990), which were not found in our extracts.

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