JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Phytochemical, Antioxidant, and Antiproliferative Properties of Seed Oil and Flour Extracts of Maryland-Grown Tobacco Cultivars

Zhuohong Xie,[†] Monica Whent,[†] Herman Lutterodt,[†] Yuge Niu,[‡] Margaret Slavin,[†] Robert Kratochvil,[§] and Liangli (Lucy) Yu[†,*]

[†]Department of Nutrition and Food Science, University of Maryland, College Park, Maryland 20742, United States

[‡]Institute of Food and Nutraceutical Science, Key Laboratory of Urban Agriculture (South), School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China

[§]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland 20742, United States

ABSTRACT: To determine the possible alternative use of tobacco, the seeds representing seven Maryland tobacco cultivars were investigated for their phytochemical, antioxidant, and antiproliferative properties. Tobacco seed oils were extracted by the Soxhlet method, and analyzed for their yield, density, refractive index, fatty acid profiles, and tocopherol profile. The defatted flours were extracted in 50% acetone and 80% ethanol. The tobacco seed oil and flour extracts were analyzed for total phenolic contents (TPC) and scavenging capacities against peroxyl, hydroxyl and 2,2-diphenyl-1-picryhydrazyl (DPPH) radicals. The fatty acid compositions of phospholipids and the protein content of the flours were also analyzed. In addition, oil and flour extracts of varieties MD609 and MD609LA were evaluated for their antiproliferative effects on HT-29 human colon cancer cells. All of the tested extracts significantly inhibited HT-29 cell proliferation except that from MD609 oil. The data from this study suggest the potential alternative use of tobacco seeds in developing natural antioxidants and antiproliferative agents for improving human health.

KEYWORDS: antioxidant, radical, antiproliferation, tobacco, HT-29 cancer cell line

INTRODUCTION

The state of Maryland has about 300 years of history growing tobacco as a cash crop. Tobacco was once the currency of the state and supported southern Maryland's economy. Recently, health concerns over smoking and chewing tobacco have reduced the overall demand for the crop.[1] Between 2001 and 2005, the Southern Maryland Agricultural Development Commission enacted a voluntary buyout for tobacco farmers to decrease dependency on tobacco farming, improve the diversity of farm economy, and preserve the region's farmland.[2] Farmers in southern Maryland are now turning to grow alternative crops or raise livestock in place of tobacco.[2] However, new farm products are in need of large investment in facilities, training of staff, management, and marketing. Maryland has a suitable climate for tobacco and farmers have experience and full range of facilities for farming tobacco. Therefore, the best option for farmers may be the development of a profitable alternative use of tobacco.

There has already been research into the industrial, pharmaceutical, and food use of the tobacco plant. Some researchers have studied tobacco seed oil as a source of biodiesel, a modifier in alkyl resins, or in the production of soap and paint.[3−5] Other studies have focused on extraction of nicotine, solanesol, malic acid, and citric acid for food and pharmaceutical use.[6] As an example of tobacco use for nutrition, Chari [6] demonstrated that leaf proteins were composed of essential amino acids, and could be a source of edible proteins.[6] Several research groups found significant antioxidant capacities of leaf phenolic compounds and proteins.[7−9] Thus, the tobacco plant has demonstrated its potential value beyond traditional uses.

Tobacco seed is a byproduct from tobacco whose yield is around 617−759 kg per hectare area.[5] It is free of nicotine and its oil has a low proportion of saturated fatty acids and contains health-beneficial compounds such as tocopherols and sterols.[5,10,11] No previous study has investigated the antioxidant or antiproliferative capacities of tobacco seed extracts.

The present research was conducted to investigate the feasibility of alternative uses of tobacco seeds for nutraceutical or functional food ingredients, by determining phytochemical, antioxidant and antiproliferation activities of Maryland tobacco cultivars. Seven varieties of seed oils and defatted flours were evaluated in this study. The results from this work will be used to promote the alternative use of Maryland tobacco cultivars.

MATERIALS AND METHODS

Materials. Tobacco seeds for seven cultivars which were developed and released for public use by the University of Maryland tobacco breeding improvement program during the past 50 years were provided by Dr. Robert J. Kratochvil, Department of Plant Science & Landscape Architecture, University of Maryland, College Park. The seven cultivars tested were MD201, MD341, MD40, MD402, MD601, MD 609, and MD609LA. The majority of these cultivars were selected for release because they had improved disease resistance, yield potential, and/or quality characteristics deemed important. MD609LA is of particular interest since it was specifically developed using backcross breeding techniques to have a low nicotine concentration (approximately 20−25% of standard

Received: May 24, 2011
Accepted: August 9, 2011
Revised: July 31, 2011
Published: August 09, 2011

tobacco cultivars). The tobacco seed oils and flours were sampled and extracted on arrival.

Iron(III) chloride, fluorescein (FL), 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^{\bullet}$), 2,2′azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO) and 2,2′-azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Thirty percent ACS-grade H_2O_2 was purchased from Fisher Scientific (Fair Lawn, NJ). α,γ,δ-Tocopherols were purchased from EMD chemicals, Inc. (San Diego, CA, USA). Ultrapure water used in all experiments was prepared by an ELGA Purelab ultra Genetic polishing system with <5 ppb TOC and resistivity of 18.2 mΩ (Lowell, MA) and was used for all experiments. All components of the cell culture media were purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Oil Extraction. Tobacco seeds were ground in a standard household coffee grinder for 8 min. Ten grams of the ground tobacco seeds from each variety were extracted in approximately 100 mL petroleum ether for 3.5 h via a Soxhlet apparatus. The petroleum ether was evaporated using a Buchi Rotovapor R-200 (Flawil, Switzerland) under a reduced pressure and the remaining oils were weighed and yield of oil was calculated. The oils were stored at ambient temperature under nitrogen in the dark until further testing.

Determination of Refractive Index and Density. The refractive index values of the tobacco seed oils were determined at 25 °C according to the AOCS Official and Tentative Methods procedure *Cc* 7−25 (AOCS Cc7−25) using an ABBE Refractometer (American Optical Corporation, Buffalo, NY). The specific density was determined at 25 °C against pure water according to the AOCS Official and Tentative Methods procedure To 1b-64 (AOCS Cc7−25).

Antioxidant Extraction of Oil. Oils were extracted with methanol and the methanolic extracts were evaluated for antioxidant and antiproliferation capacities. Briefly, one gram of oil was measured into a test tube and 1 mL of methanol was added. The test tube was vortexed and then rested for one hour. The supernatant was collected. This procedure was repeated two more times. All three extracts were combined. The extraction was then kept under nitrogen in the dark until further analysis.

Antioxidant Extraction of Flour. The defatted tobacco seed flour remaining after Soxhlet extraction was dried in air overnight at ambient temperature. Approximately 1 g of the flour was extracted with 10 mL of either 80% ethanol or 50% acetone at ambient temperature overnight. The mixtures were filtered through a 0.45 μm syringe filter (Fisher Brand, Pittsburgh, PA). The extracts were kept under nitrogen in the dark until further analysis.

Fatty Acid Composition of Oil. Fatty acid methyl esters (FAME) were prepared according to a previously described laboratory protocol and subjected to gas chromatography (GC) analysis.[12] The fatty acid analysis was conducted using a Shimadzu GC-2010 with an FID and a Shimadzu AOC-20i autosampler (Shimadzu, Columbia, MD). A fused silica capillary column SPTM-2380 (30 m × 0.25 mm with a 0.25 μm film thickness) from Supelco (Bellefonte, PA) was used and helium was used as the carrier gas at a flow rate of 2.2 mL/min. Injection volume was 1 μL at a split ratio of 10/1. Oven temperature was initially 136 °C, increased by 6 °C/min until 184 °C where it was held for 3 min, then increased by 6 °C/min to a final temperature of 226 °C. Individual fatty acids were identified through comparison of GC retention time with those of fatty acid methyl ester standards. Quantification was based on the area under each fatty acid peak as compared to the total area of all fatty acid peaks. Measurements were taken in triplicate.

Fatty Acid Composition of Phospholipid. For phospholipid extraction, 1.5 g of the flour were extracted in 10 mL of a mixture of $CHCl_3$/MeOH (2:1, v:v) according to the Folch's procedure.[13] Fatty acid methyl esters (FAME) were prepared from the extract. The gas chromatography (GC) analysis was conducted in the same way as described previously for oil samples.

Tocopherol Profile. Tocopherols were determined according to a reported protocol with minor modification.[14] The tobacco oil was dissolved in methyl-*tert*-butyl ether and filtered through a 0.45 μm filter. The stationary phase was a Waters C-30 column (250 × 4.6 mm, 5 μm). The mobile phase consisted of methanol/MTBE/water, (81:15:4, v/v/v) (solvent A) and MTBE/methanol (91:9, v/v) (solvent B). The mobile phase was run from 0 to 16.7% solvent B in 15 min, 100% solvent B from 15 to 25 min and re-equilibrated with 100% solvent A from 25 to 30 min. The flow rate was 1 mL/min and injection volume was 40 μL. Tocopherol profile of each sample was detected at wavelength of 295 nm and compared to known standards for quantification. Each sample and standard was run in duplicate.

Protein Content of Tobacco Seed. The nitrogen content was determined by a Perkin-136 Elmer PE 2400-Series II, CHNS/O analyzer (PerkinElmer, USA) in a commercial analytical lab (NuMega Resonance Laboratories, San Diego, CA). The protein content was calculated by nitrogen content multiplied by 6.25.

Total Phenolic Content. The TPC of antioxidant extract was measured according to a laboratory procedure described previously.[15] Briefly, 100 μL of tobacco seed oil/flour extract, 500 μL of the Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate and 1.5 mL ultrapure water comprised the reaction mixture. Gallic acid was used as the standard. Absorbance was read at 765 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 2 h of reaction at ambient temperature in the dark. Reactions were conducted in triplicate and results were reported as gallic acid equivalents (GAE) per gram of tobacco seed oil/flour.

Relative DPPH Radical Scavenging Capacity (RDSC). The RDSC of each extract was determined following a recently reported procedure by Cheng and others.[16] Briefly, 100 μL of sample extract, standard solution of Trolox, or solvent control was added to 100 μL of freshly prepared DPPH$^{\bullet}$ solution to initiate antioxidant-radical reaction. The absorbance of the reaction mixture was measured at 515 nm during 40 min of reaction. An initial DPPH$^{\bullet}$ concentration of 100 μM was used for all reaction mixtures. RDSC values were calculated using areas under the curve relative to Trolox standard. Results were expressed as micromoles of Trolox equivalents (TE) per gram of sample (oil or flour) on a dry weight basis.

Radical Cation ABTS$^{\bullet+}$ Scavenging Capacity. The free radical scavenging capacity of sample extracts were evaluated against ABTS$^{\bullet+}$ generated according to a previously reported protocol.[17] The radical cation of ABTS is frequently used to measure the free radical scavenging capacity of foods compared to the standard trolox. Standard solutions of Trolox were prepared in Methanol for the oil and 50% acetone for the flour. ABTS cation radicals were generated by reacting a 5 mM aqueous

Table 1. Physical and Chemical Properties of Tobacco Seed and Its Seed Oil

variety	oil content (g/100 g)	protein[a] (mg/g)	refractive index[b]	density[c] (g/mL)	phospholipid (g/100 g)
MD201	43.17	368.8 ± 3.5ab	1.4708 ± 0.0003e	0.8900 ± 0.0021bc	1.11
MD341	44.09	391.3 ± 3.5d	1.4695 ± 0.0001c	0.8946 ± 0.0014 cd	1.48
MD40	43.58	376.3 ± 3.5abc	1.4703 ± 0.0001de	0.8885 ± 0.0007b	1.85
MD402	42.28	386.9 ± 0.9cd	1.4697 ± 0.0004 cd	0.8952 ± 0.0016d	1.74
MD601	45.28	365.6 ± 2.7a	1.4668 ± 0.0002b	0.8836 ± 0.0014a	1.47
MD609	44.30	378.4 ± 2.2bc	1.4651 ± 0.0002a	0.8930 ± 0.0046bcd	1.20
MD609LA	40.87	368.4 ± 4.0ab	1.4703 ± 0.0002e	0.8916 ± 0.0027bcd	1.70

[a] Data were expressed as mean ± SD ($n = 2$). Different letters represent significant differences ($P < 0.05$). [b] Data were expressed as mean ± SD ($n = 3$). Data were adjusted to that at 25 °C. Different letters represent significant differences ($P < 0.05$). [c] Data were expressed as mean ± SD ($n = 5$). Measurement was taken at 25 °C. Different letters represent significant differences ($P < 0.05$).

solution of ABTS with MnO_2 for 20 min at ambient temperature. Initial assay reaction mixtures contained 80 μL of sample extract or solvent for control and 1.0 mL of $ABTS^{\bullet+}$ solution with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured after 1 min of reaction. Results were expressed as micrograms of Trolox equivalents (TE) per gram of sample (oil or flour) on a dry weight basis.

Oxygen Radical Absorbing Capacity (ORAC) Assay. ORAC assay was conducted with fluorescein (FL) as the fluorescent probe using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland) according to a previously described laboratory protocol.[18] Standard solutions of Trolox were prepared in MeOH for oil and 50% acetone for flour. Initial reaction mixtures contained 225 μL of 8.16×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL sample extract, standard, or corresponding solvent for the blank and 25 μL of 0.36 M AAPH. FL and antioxidant extracts were mixed in a 96-well plate and preheated in plate reader for 20 min at 37 °C after which the AAPH solution was added to initiate the antioxidant-radical reactions. The fluorescence of the assay mixture was recorded every minute for 80 min at 37 °C. Excitation and emission wavelengths were 485 and 535 nm, respectively. Results were expressed as micromoles of Trolox equivalents (TE) per gram of sample (oil or flour) on a dry weight basis.

Hydroxyl Radical Scavenging Capacity (HOSC). HOSC assay was conducted with 50% acetone solutions according to a previously published protocol [19] using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Standard solutions of Trolox were prepared in 50% acetone for flour. Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL of standard, sample extract, or blank, 40 μL of 0.1990 M H_2O_2 and 60 μL of 3.43 mM $FeCl_3$. Fluorescence was measured every minute for 3 h with an excitation wavelength of 485 nm and emission wavelength of 535 nm. HOSC values were expressed as micromoles of Trolox equivalents (TE) per gram of sample (oil or flour) on a dry weight basis.

HT-29 Colon Cancer Cell Proliferation Inhibition. Extracts were prepared for cell treatment by redissolving antioxidant extracts into DMSO solution and the final concentrations of oil or flour in cell media were equal to 0, 1, 5, 10 mg/mL. HT-29 human colorectal adenocarcinoma cell proliferation inhibition was investigated according to a previously reported laboratory protocol.[20] The cells were grown in a humidified atmosphere at 37 °C and 5% carbon dioxide in a culture media of McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. The cells (5×10^3 cells/well) were seeded on a 96-well culture plates. After 24 h incubation, the media were replaced by 100 μL appropriate treatment media containing 0, 1, 5, 10 mg/mL sample and 0.3% DMSO. The treatment media were filtered through a 0.2 μm pore, retrograde cellulose filter prior to treatment of cells. ATP-Lite 1 step kit (Perkin-Elmer Life and Analytical Sciences, Shelton, CT) was used to determine cell proliferation. During short reaction between ATP-Lite and ATP in cells, the emitted luminescence, which was directly proportional to the number of living cells, was determined by a Victor^3 multiwell plate reader (Perkin-Elmer, Turku, Finland) immediately prior to treatment and at 4, 24, 48, 72, and 96 h after initial treatment. A separate plate was used for each reading. Treatment media were replaced every 24 h until a reading was taken on that plate.

Statistical Analysis. Tocopherol and nitrogen determination were conducted in duplicate while other tests were conducted in triplicate. Data were reported as mean ± SD. One-way ANOVA and Tukey's test were employed to identify differences in means. Correlation was analyzed using a two-tailed Pearson's correlation test. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Physical and Chemical Properties of Seed and Seed Oil. Tobacco seed oils appeared in a yellow to light yellow color. Oil content, refractive index, density, color and phospholipid content of oil are shown in Table 1. The tobacco seeds exhibited an oil content ranging from 41 to 45 g/100 g seed. MD601 seed had the highest oil content of 45 g/100 g seeds. These data were compatible with those (30−49 g/100 g seed) in seeds of the Bulgaria tobacco varieties,[11] twice more than those in soybean (17−24%),[21] and comparable to those in rapeseed (35−39%).[22] Phospholipid is a group of lipid which is the major component of cell membrane, a precursor of a neural transmitter, and can be used as an emulsifier in food processing. In tobacco seeds, the extracted phospholipid was within a range of 1.11−1.85 g/100 g seed. The results suggested that tobacco seeds are rich in oil and have the potential to provide a dietary source of phospholipid.

The total protein content is also shown in Table 1. Nitrogen content was determined by CHNS/O analyzer and the protein content was calculated from the nitrogen content. The total protein content ranged from 366 to 391 mg/g seed. Among the

Table 2. Fatty Acid Compositions of the Studied Tobacco Seed Oils[a]

	fatty acid (g/100 g total fatty acids)						
	MD201	MD341	MD40	MD402	MD601	MD609	MD609LA
16:0	10.00 ± 0.01e	9.90 ± 0.01d	8.57 ± 0.01b	8.49 ± 0.03a	8.73 ± 0.00c	8.74 ± 0.03c	8.45 ± 0.01a
16:1	0.18 ± 0.00bc	0.19 ± 0.01c	0.16 ± 0.02abc	0.11 ± 0.02ab	0.12 ± 0.07abc	0.09 ± 0.01a	0.12 ± 0.00abc
18:0	2.69 ± 0.01b	2.86 ± 0.01 g	2.73 ± 0.00 cd	2.75 ± 0.01e	2.75 ± 0.01de	2.83 ± 0.01f	2.64 ± 0.00a
18:1	10.62 ± 0.00e	11.45 ± 0.01f	10.47 ± 0.01d	10.45 ± 0.01d	9.61 ± 0.02b	9.47 ± 0.01a	10.21 ± 0.00c
18:2	75.46 ± 0.03b	74.66 ± 0.03a	77.03 ± 0.02c	77.17 ± 0.04d	77.74 ± 0.06f	77.78 ± 0.04f	77.53 ± 0.01e
18:3	0.79 ± 0.00bc	0.67 ± 0.02a	0.76 ± 0.02bc	0.77 ± 0.01bc	0.80 ± 0.01 cd	0.84 ± 0.03de	0.77 ± 0.00bc
20:0	0.17 ± 0.01a	0.17 ± 0.00a	0.17 ± 0.00a	0.17 ± 0.00a	0.16 ± 0.01a	0.16 ± 0.01a	0.17 ± 0.00a
20:1	0.09 ± 0.01a	0.10 ± 0.01a	0.11 ± 0.01a	0.10 ± 0.01a	0.09 ± 0.01a	0.10 ± 0.01a	0.10 ± 0.01a
MUFA	10.89 ± 0.01f	11.74 ± 0.02 g	10.74 ± 0.02e	10.66 ± 0.03d	9.82 ± 0.06b	9.65 ± 0.03a	10.43 ± 0.01c
PUFA	76.25 ± 0.04b	75.33 ± 0.01a	77.79 ± 0.01c	77.93 ± 0.04d	78.54 ± 0.05f	78.62 ± 0.01f	78.30 ± 0.01e
SFA	12.86 ± 0.03f	12.92 ± 0.01 g	11.47 ± 0.02c	11.41 ± 0.03b	11.63 ± 0.02d	11.73 ± 0.02e	11.26 ± 0.01a

[a] Data were expressed as mean ± SD (n = 3). Different letters within each column represent significant differences ($P < 0.05$). SFA: saturated fatty acids. MUFA: total monounsaturated fatty acids. PUFA: total polyunsaturated fatty acids.

Table 3. Fatty Acid Compositions of the Phospholipid[a]

	fatty acid (g/100 g total fatty acids)						
	MD201	MD341	MD40	MD402	MD601	MD609	MD609LA
16:0	8.91 ± 0.02bc	8.03 ± 0.01ab	7.80 ± 0.03a	7.56 ± 0.01a	9.11 ± 0.03c	9.14 ± 0.01c	9.27 ± 0.91c
18:0	2.92 ± 0.04ab	3.01 ± 0.00b	3.02 ± 0.03b	2.80 ± 0.01a	3.34 ± 0.06c	3.46 ± 0.02c	3.80 ± 0.14d
18:1	11.61 ± 0.01b	13.11 ± 0.00e	11.92 ± 0.03c	11.64 ± 0.01b	10.64 ± 0.04a	10.52 ± 0.01a	12.25 ± 0.18d
18:2	75.49 ± 0.05bc	74.85 ± 0.05b	76.28 ± 0.12 cd	76.90 ± 0.02d	75.95 ± 0.11 cd	75.65 ± 0.01bc	72.72 ± 0.98a
18:3	0.76 ± 0.02a	0.69 ± 0.02a	0.72 ± 0.03a	0.81 ± 0.02a	0.74 ± 0.04a	0.86 ± 0.01a	1.52 ± 0.34b
20:0	0.12 ± 0.03a	0.18 ± 0.01a	0.15 ± 0.01a	0.17 ± 0.00a	0.18 ± 0.03a	0.27 ± 0.01b	0.36 ± 0.03c
20:1	0.12 ± 0.03a	0.14 ± 0.04a	0.11 ± 0.04a	0.13 ± 0.01a	0.04 ± 0.08a	0.11 ± 0.01a	0.07 ± 0.01a
MUFA	11.73 ± 0.04b	13.25 ± 0.04e	12.03 ± 0.03c	11.77 ± 0.01b	10.68 ± 0.06a	10.63 ± 0.01a	12.32 ± 0.19d
PUFA	76.25 ± 0.06c	75.54 ± 0.03b	77.00 ± 0.09de	77.71 ± 0.01e	76.69 ± 0.14 cd	76.51 ± 0.01 cd	74.24 ± 0.65a
SFA	12.02 ± 0.07bc	11.21 ± 0.01ab	10.97 ± 0.06a	10.78 ± 0.30a	12.63 ± 0.09 cd	12.86 ± 0.00 cd	13.43 ± 0.83d

[a] Data were expressed as mean ± SD ($n = 3$). Different letters within each column represent significant differences ($P < 0.05$). SFA: saturated fatty acids. MUFA: total monounsaturated fatty acids. PUFA: total polyunsaturated fatty acids.

samples, MD341 had the highest protein content (391 mg/g seed), which was followed by MD402 (387 mg/g seed).

Fatty Acid Composition of Oil. Palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1, n-9), linoleic (18:2, n-6), α-linolenic (18:3, n-3), arachidic (20:0) and eicosenoic (20:1, n-9) acids were found in all tobacco seed oils, with linoleic acid as the primary fatty acid (74.7−77.8%) and followed by oleic and palmitic acids (Table 2). Linoleic acid is an essential fatty acid which cannot be synthesized in human body. It was the most prevalent PUFA in all of the tested oils, with the highest level observed in MD609 (78%). The linoleic acid in tobacco seed oil is about 1.5 times higher than that in soybean oil (42−53%),[20] while olive oil has less than 5% of linoleic acid.[23] The very high level of linoleic acid showed the potential for tobacco oil to be a potential dietary source of essential fatty acid. Oleic acid, which is the major fatty acid in olive oil (>80%), is believed to lower the incidence of cardiovascular disease.[23,24] The tobacco seed oils contained 9−12% oleic acid, with the highest level detected in MD341.

The oils contained high levels of unsaturated fatty acids. They had a polyunsaturated fatty acid (PUFA) level of 75−79%, with MD609 demonstrating the highest level of 79%. The monounsaturated fatty acid (MUFA) level ranged from 10 to 12%, in which MD341 had the highest value of 12%. The PUFA content was higher than that in Bulgarian tobacco varieties (8−52%) and higher than that in soybean oil (42−63%),[11,25] whereas MUFA content was comparable to that in cotton seed (15−22%) and grape seed oils (12−28%).[26]

Fatty Acid Composition of Phospholipid. The fatty acid composition of phospholipid is shown in Table 3. The phospholipid samples had similar composition to the neutral oil samples. The MUFA ranged from 11 to 13%, the PUFA ranged from 74 to 78% and the SFA ranged from 11 to 13%. Unlike the neutral oils, palmitoleic acid (16:1) was not detected in any of the phospholipid samples.

Tocopherol Profile. The α-, γ- and total tocopherol profiles of the seven varieties of tobacco seed oils are shown in Table 4. The highest level for α-tocopherol was recorded in MD601 seed oil (4.8 mg/kg oil), and followed by MD609LA (4.3 mg/kg oil). MD201 contained the highest level of γ-tocopherol (88.3 mg/kg oil), followed by MD402 (83.7 mg/kg oil). MD201 also had the

Table 4. Tocopherol Profile[a]

variety	α-tocopherol (mg/kg)	γ-tocopherol (mg/kg)	total tocopherol (μmol/kg)
MD201	3.678 ± 0.377c	88.325 ± 1.571e	217.730
MD341	1.219 ± 0.113a	28.262 ± 0.093a	70.636
MD40	2.621 ± 0.027b	47.223 ± 0.329b	119.224
MD402	3.462 ± 0.310bc	83.725 ± 2.004e	208.116
MD601	4.789 ± 0.319d	77.523 ± 1.247d	196.259
MD609	4.132 ± 0.065 cd	69.243 ± 1.157c	175.303
MD609LA	4.342 ± 0.359 cd	75.119 ± 0.464d	187.727

[a] Data were expressed as mean ± SD (n = 2). Different letters within each column represent significant differences (P < 0.05).

highest total tocopherol content (217.7 μmol/kg oil), followed by MD402 (208.1 μmol/kg oil). Total tocopherol is reported in μmol/kg due to the different molecular weight of α- and γ-tocopherol isomers. Previous research on different Bulgarian tobacco varieties showed a wide range of total tocopherol content (11.2−166.9 μmol/kg).[10] The tocopherol values of Maryland tobacco varieties in the present work were comparable to those of Bulgarian tobacco varieties, grapeseed (37.1−263.4 μmol/kg) and palm (9.3−1478.2 μmol/kg) oils, but was lower than that of soybean oil (234.5−6353.9 μmol/kg) and some varieties of the olive oil (95−755 μmol/kg).[26,27] Although different in total tocopherols, the tobacco seed oils had a similar ratio between their α- and γ-tocopherols.

Protein Content of Tobacco Seed. The nitrogen content was determined by the CHNS/O analyzer and the protein content was calculated from the nitrogen content. The total protein content ranged from 366 to 391 mg/g seed. Among the samples, MD341 had the highest protein content (391 mg/g seed), which was followed by MD402 (387 mg/g seed).

Total Phenolic Content. The total phenolic content was reported in the gallic acid equivalents (GAE) on a per oil or flour weight basis, since gallic acid was used as the standard chemical in the TPC assay,.[15,28] The tobacco seed oils had a total phenolic content ranging from 0.15 to 0.52 mg gallic acid equivalents/g oil (Figure 1A). The highest TPC value of 0.52 mg GAE/g oil was detected in MD402 extract. 609LA oil had the lowest level of TPC (0.15 mg GAE/g oil)—approximately 1/5 of values of other varieties. The tobacco seed oils (except 609LA variety) had about 1/4 of the previously reported TPC values of blueberry, raspberry, marionberry, and boysenberry seed oils, which ranged from 1.49 to 2.00 mg GAE/g oil.[29] These TPC values of tobacco seed oils were comparable to that of olive oil (0.13−0.32 mg GAE/g oil).[28]

The tobacco seed flours showed much higher total phenolic content than seed oils (Figure 1B). TPC values for 50% acetone extracts was 1.24−2.26 mg GAE/g flour, in which MD609LA had the highest level. TPC value for 80% ethanol extracts was 1.20−1.44 mg GAE/g flour, of which MD609 had the highest level. The TPC values of the tobacco seed flour were less than that of black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flours (15−186 mg GAE/g flour),[30] however were comparable to that of hard winter wheat bran (2.7−3.5 mg GAE/g),[17] pumpkin and cardamom seed flours (1.6−1.9 mg GAE/g flour).[31] Diets high in certain phenolics have been associated with reduced risk of chronic disease.[32] The phenolic content of tobacco seed oils and flours suggested their potential as health food ingredients.

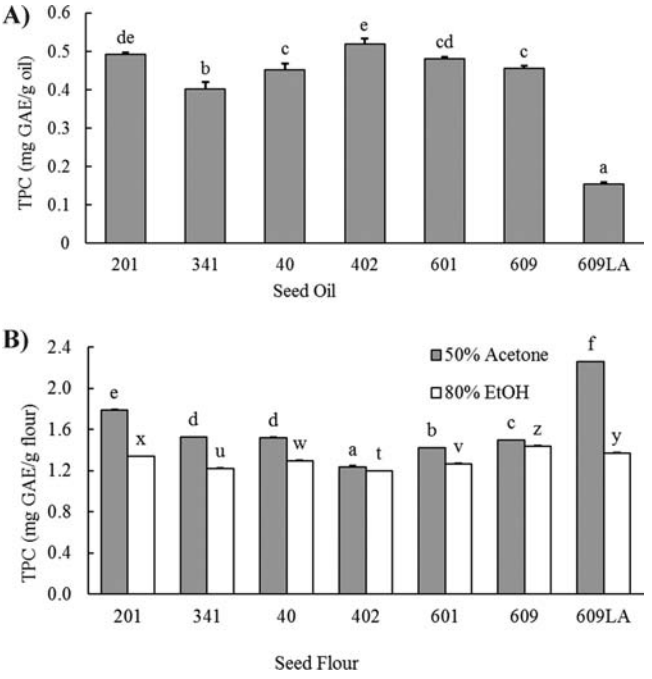

Figure 1. Total phenolic contents (TPC) s of tobacco seeds (A) oil and (B) flour expressed as gallic acid equivalents (GAE). The 201, 341, 40, 402, 601, 609 and 609LA represent Maryland tobacco cultivars MD201, MD341, MD40, MD402, MD601, MD609, and MD609LA. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences (P < 0.05).

Antioxidant Properties. Antioxidant properties were determined using the relative DPPH radical scavenging capacity (RDSC), radical cation $ABTS^{\bullet+}$ scavenging capacity, oxygen radical absorbing capacity (ORAC), and hydroxyl radical scavenging capacity (HOSC) assays. The RDSC value is standardized to Trolox equivalents (TE), and thus is possible to be compared across different laboratories. MD609 had the highest RDSC value of oils (0.58 μmol TE/g) (Figure 2A). The 50% acetone flour extracts showed an RDSC range of 2.65−4.37 μmol TE/g, whereas the 80% ethanol flour extracts showed a lower range of 2.74−3.33 μmol TE/g. (Figure 2B). The highest RDSC level was demonstrated by MD609LA flour in both solvent systems, which was comparable to the value of pumpkin seed flour (2.2 μmol TE/g).[31] These results indicated that 50% acetone was more efficient in extracting antioxidant agents. The RDSC value and TPC value of tobacco seed flour were in agreement with previous research on solvent extraction on wheat bran and soybean indicating 50% acetone was preferred for TPC and most of the antioxidant assays.[33,34] Thus all other assays for tobacco seed flours were based on 50% acetone extracts. In radical cation $ABTS^{\bullet+}$ scavenging capacity, the range for oil extract was from 0.16 to 0.28 μmol TE/g whereas the range for the flour extract was from 3.42 to 6.89 μmol TE/g (Figure 3). MD201 had the greatest value in oil extracts yet the highest level in flour extracts was observed in MD609LA. The values of tobacco seed flour were higher than that of hard winter wheat (1.1−1.9 μmol TE/g),[15] but lower than that of soft wheat (14.3−17.6 μmol TE/g).[18] The trends and values were close to that of the RDSC, as expected, possibly because these two assays belong to

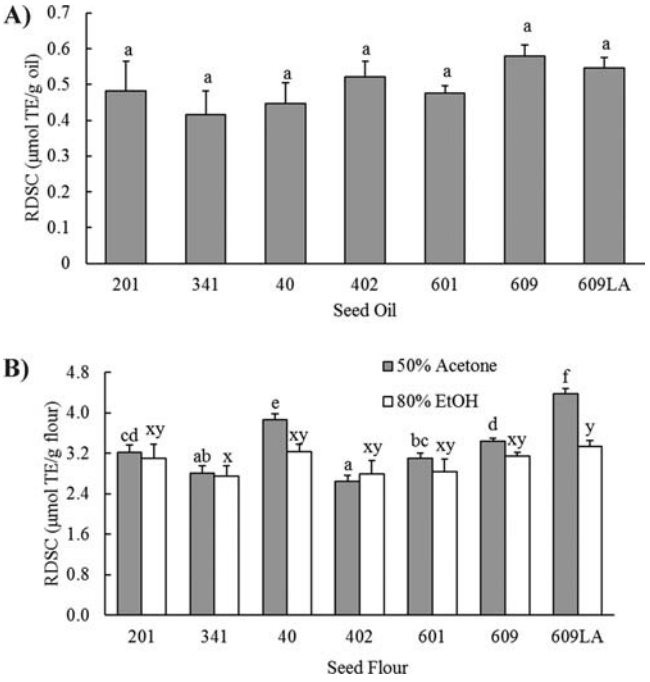

Figure 2. Relative DPPH radical scavenging capacity (RDSC) of tobacco seeds (A) oil and (B) flour expressed as trolox equivalents (TE). The 201, 341, 40, 402, 601, 609, and 609LA represent Maryland tobacco cultivars MD201, MD341, MD40, MD402, MD601, MD609, and MD609LA. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences (P < 0.05).

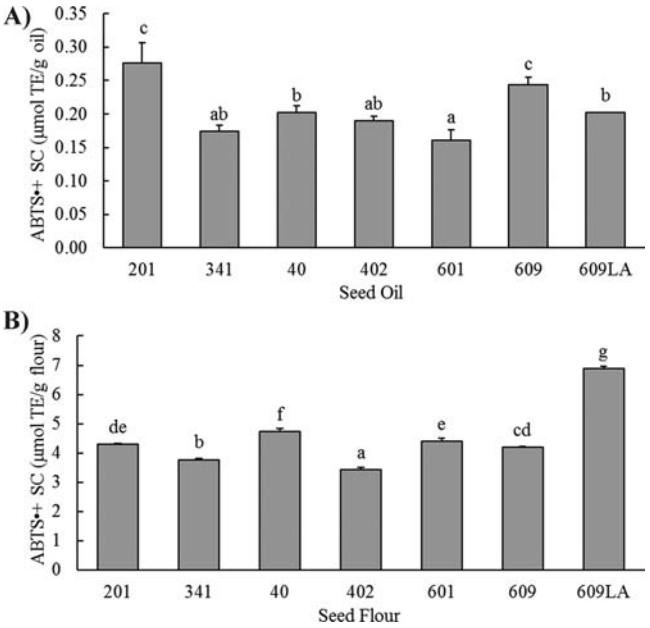

Figure 3. Radical cation $ABTS^{\bullet+}$ scavenging capacity of tobacco seeds (A) oil and (B) flour expressed as trolox equivalents (TE). The 201, 341, 40, 402, 601, 609, and 609LA represent Maryland tobacco cultivars MD201, MD341, MD40, MD402, MD601, MD609, and MD609LA. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences (P < 0.05).

electron transfer (ET) assays in which the probes (oxidants) abstract electrons from antioxidants and lead to color change.[35]

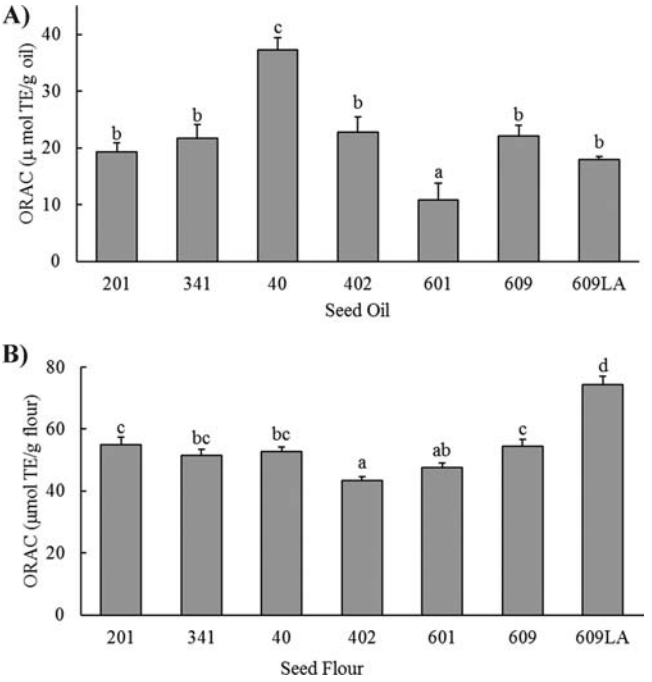

Figure 4. Oxygen radical absorbing capacity (ORAC) of tobacco seeds (A) oil and (B) flour expressed as trolox equivalents (TE). The 201, 341, 40, 402, 601, 609, and 609LA represent Maryland tobacco cultivars MD201, MD341, MD40, MD402, MD601, MD609, and MD609LA. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences (P < 0.05).

Alternatively, ORAC is a widely accepted assay for antioxidant quenching of peroxyl radicals based on hydrogen atom transfer (HAT) mechanism. ORAC values for tobacco seed oils ranged from 11 to 37 μmol TE/g, and MD40 had the highest value (Figure 4A). These values for seed oils were about one to three times greater than that of extra-virgin olive oil [36] and comparable to blueberry and cold-pressed red raspberry seed oils.[29] ORAC values for tobacco seed flours were in a range of 44−74 μmol TE/g, and MD609LA had the highest ORAC (Figure 4B). These ORAC values were comparable to those of whole soybean seed (58 μmol TE/g) [36] and cold-pressed mullein seed flour (98 μmol TE/g).[31]

The hydroxyl radical is a highly reactive radical that can damage carbohydrates, nucleic acids, lipids, and amino acids, and thus may lead to chronic disease. The hydroxyl radical scavenging capacity (HOSC) of tobacco seed flour was in a range of 25 −53 μmol TE/g, and MD609LA had the highest HOSC value (Figure 5). The HOSC values of tobacco seed flours were comparable to that of the cold-pressed pumpkin, mullein, and cardamom seed flours (22−75 μmol TE/g).[31] The antioxidant properties of oil extracts showed no significant correlation to TPC (Figure 1), nor to tocopherol content. However, TPC of flour extracts was significantly correlated with RDSC (R = 0.783, P < 0.05), radical cation $ABTS^{\bullet+}$ scavenging capacity (R = 0.901, P < 0.01), ORAC (R = 0.967, P < 0.001) and HOSC (R = 0.923, P < 0.005), which implied that phenolic compounds might be the major contributors to the antioxidant properties of the flour extracts. These results suggested that both the tobacco seed oils and defatted seed flours contain high levels of antioxidants. Further research is needed to determine the specific bioactives in tobacco seed oil and flour extracts.

HT-29 Cell Proliferation. The DMSO solution of MD609 and MD609LA seed oils and flours were evaluated for their antiproliferation capacities at dose levels of 1, 5, and 10 mg flour or oil equivalents per mL of culture media (Figure 6). These two varieties were selected in order to determine whether the breeding effort to lower the alkaloid content (MD609LA) would alter the potential anticancer activities of the seeds. The rest of the varieties were not tested due to the cost. In this cell study, the MD609 and MD609LA flour extracts eliminated all the living cells during 96 h of exposure at the 10 mg/mL treatment concentration, whereas the same extracts only decreased cell growth at 5 mg/mL. This dose-dependent effect of flour extracts indicated that they had an inhibitory effect on HT-29 cell proliferation, which was comparable to the cold-pressed black raspberry and cranberry seed flour extracts reported by Parry and others.[30]

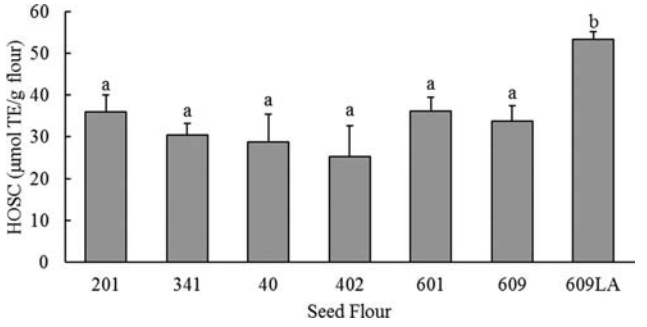

Figure 5. Hydroxyl radical scavenging capacity (HOSC) of tobacco seed flours expressed as trolox equivalents (TE). The 201, 341, 40, 402, 601, 609, and 609LA represent Maryland tobacco cultivars MD201, MD341, MD40, MD402, MD601, MD609, and MD609LA. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters represent significant differences ($P < 0.05$).

MD609 oil extracts suppressed the growing of HT-29 cells only at the concentration of 10 mg/mL, whereas that of MD609LA oil had no significant effect at any concentration. The variety with lower alkaloid content (MD609LA) lost the ability to suppress growing of HT-29 cells after breeding. The trend of antiproliferative capacity against HT-29 cancer cells was correlated with that of TPC value, suggesting that phenolics may be an important contributor for their antiproliferative capacity. Other bioactive compounds such as tocopherols may also contribute to their antiproliferative capacity. The possible mechanism may include but is not limited to modulating the cellular oxidative status, suppressing inflammation reactions such as those catalyzed by COX-2, inducing apoptosis, and cellular toxic effect. Further research is required to determine the specific group of compounds contributing to anticancer capacity and to confirm antiproliferative activities on different cells and in vivo.

In summary, the present work revealed the antioxidant and antiproliferative properties of tobacco seeds for the first time. The high level of unsaturated fat and phospholipid, significant amount of antioxidants, and effect on growth inhibition of cancer cells indicated that the high-yield tobacco seed oil may be obtained with special fatty acid composition or other beneficial components and serve as edible oil. The tobacco seed flours may provide dietary sources of natural antioxidants and contain antiproliferative components. Development of health products from these flours and oils has the potential to be excellent alternative uses of Maryland grown tobacco. The results of this research may also be applicable to tobaccos growing in other states. Additional research is still required to further investigate the effects of food formulation, processing, and storage on the availability of these beneficial components and properties, as well as the phytochemical components and biochemical mechanisms involved in antiproliferative properties, in order to better utilize the oil and flour for health promotion and disease prevention.

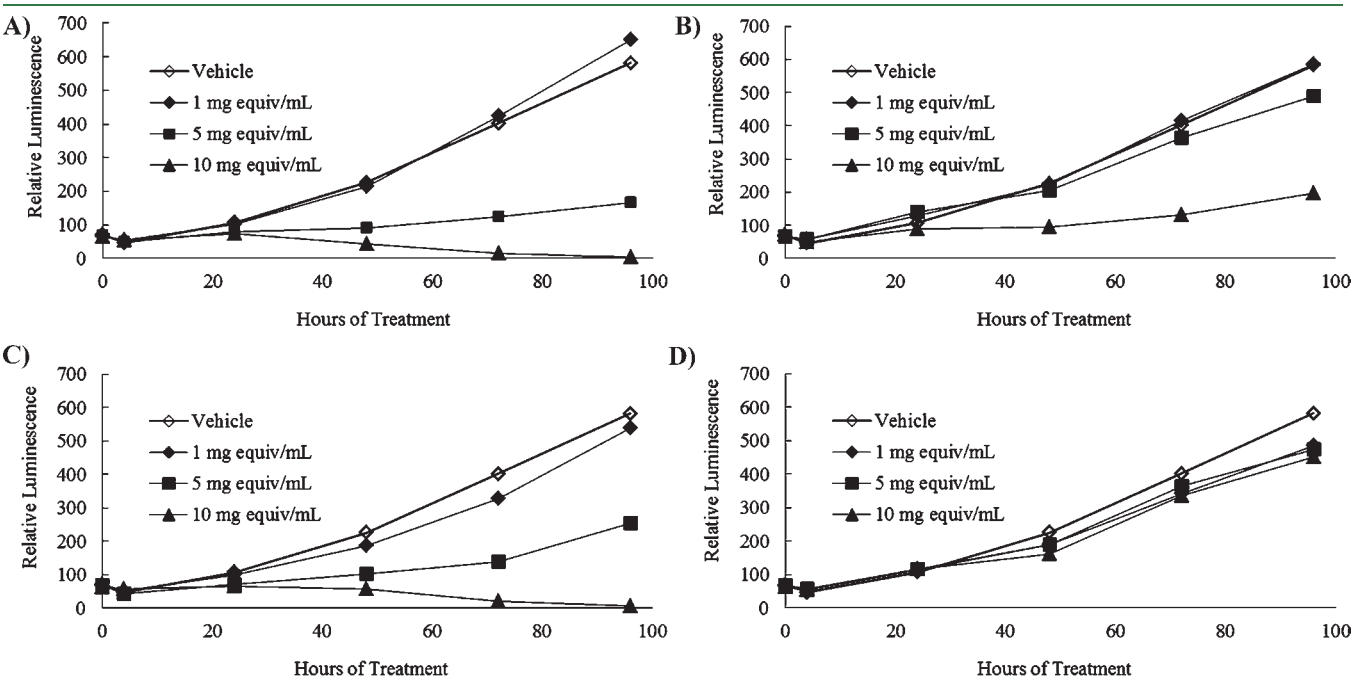

Figure 6. Dose and time effects of the tobacco seed flour and oil extracts on HT-29 cell growth. The final concentrations of the seed flour and oil extracts were 1, 5, and 10 mg flour/oil equivalents/mL in the initial culture media. (A) The effect of MD609 flour extract, (B) The effect of MD609 oil extract, (C) The effect of MD609LA flour extract, and (D) The effect of MD609LA oil extract.

AUTHOR INFORMATION

Corresponding Author

*Tel: (301) 405-0761; Fax: (301) 314-3313; E-mail: lyu5@umd.edu.

Funding Sources

This research was partially supported by a research grant on alternative use of tobacco from the USDA, a grant from the Joint Institute for Food Safety and Applied Nutrition (JIFSAN), a grant from the Maryland Grain Producers Utilization Board (MGPUB), and a grant from the Maryland Soybean Board.

REFERENCES

(1) Yancy, C. H. *Buyout brings changes to Maryland farm landscape*. Southwest Farm Press, April 2004. http://southwestfarmpress.com/news/maryland-tobacco-buyout/ (accessed July 25, 2011)

(2) Southern Maryland Agricultural Development Commission, Tri-County Council. Southern Maryland tobacco crop conversion program key accomplishments - FY 2004 (July 2003 - June 2004) [Online], 2004. http://www.somarylandsogood.com/pdf/Report.2004.pdf (accessed July 25, 2011)

(3) Giannelos, P. N.; Zannikos, F.; Stournas, S.; Lois, E.; Anastopoulos, G. Tobacco seed oil as an alternative diesel fuel: Physical and chemical properties. *Ind. Crop. Prod.* **2002**, *16*, 1–9.

(4) Ogunniyi, D. S.; Odetoye, T. E. Preparation and evaluation of tobacco seed oil-modified alkyd resins. *Bioresour. Technol.* **2008**, *99*, 1300–1304.

(5) Parlak, A.; Karabas, H.; Ozsert, I. Variables affecting the yield of oil from tobacco seed. *Asian J. Chem.* **2009**, *21*, 1917–1924.

(6) Chari, M. Tobacco, many value-added by-products. *The Hindu Survey of Indian Agriculture* **1995**, 87–91.

(7) Liu, W.; Yong, G.; Fang, L.; Wang, S.; Bai, H.; Jiang, J.; Liu, S. Free and conjugated phytosterols in cured tobacco leaves: influence of genotype, growing region, and stalk position. *J. Agric. Food Chem.* **2008**, *56*, 185–189.

(8) Matringe, M.; Ksas, B.; Rey, P.; Havaux, M. Tocotrienols, the unsaturated forms of vitamin E, can function as antioxidants and lipid protectors in tobacco leaves. *Plant Physiol.* **2008**, *147*, 764–778.

(9) Wang, H.; Zhao, M.; Yang, B.; Jiang, Y.; Rao, G. Identification of polyphenols in tobacco leaf and their antioxidant and antimicrobial activities. *Food Chem.* **2008**, *107*, 1399–1406.

(10) Zlatanov, M.; Angelova, M.; Antova, G. Composition of tobacco seed oils. *Sci. Agric. Bohem.* **2007**, *38*, 60–72.

(11) Zlatanov, M.; Angelova, M.; Antova, G. Lipid compostion of tobacco seeds. *Bulg. J. Agric. Sci.* **2007**, *13*, 539–544.

(12) Yu, L.; Adams, D.; Watkins, B. A. Comparison of commercial supplements containing conjugated linoleic acids. *J. Food Compos. Anal.* **2003**, *16*, 419–428.

(13) Folch, J.; Lees, M.; Sloane-Stanley, G. H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1956**, *226*, 497–509.

(14) Darnoko, D.; Cheryan, M.; Moros, E.; Jerral, J.; Perkins, E. G. Simultaneous HPLC analysis of palm carotenoids and tocopherols using a C-30 column and photodiode array detector. *J. Liq. Chromatogr. Relat. Technol.* **2000**, *23*, 1873–1885.

(15) Yu, L.; Haley, S.; Perret, J.; Harris, M.; Wilson, J.; Qian, M. Free radical scavenging properties of wheat extracts. *J. Agric. Food Chem.* **2002**, *50*, 1619–1624.

(16) Cheng, Z.; Moore, J.; Yu, L. High-throughput relative DPPH radical scavenging capacity assay. *J. Agric. Food Chem.* **2006**, *54*, 7429–7436.

(17) Moore, J.; Cheng, Z.; Su, L.; Yu, L. Effects of solid-state enzymatic treatments on the antioxidant properties of wheat bran. *J. Agric. Food Chem.* **2006**, *54*, 9032–9045.

(18) Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J. Agric. Food Chem.* **2005**, *53*, 6649–6657.

(19) Moore, J.; Yin, J.; Yu, L. Novel fluorometric assay for hydroxyl radical scavenging capacity (HOSC) estimation. *J. Agric. Food Chem.* **2006**, *54*, 617–626.

(20) Slavin, M.; Kenworthy, W.; Yu, L. Antioxidant properties, phytochemical composition, and anti-proliferative activity of Maryland-grown soybeans with colored seed coats. *J. Agric. Food Chem.* **2009**, *57*, 11174–11185.

(21) Muhammad, A.; Khalil, S. K.; Marwat, K. H.; Khan, A. Z.; Khalil, I. H.; Arifullah, A.; Arifullah, S. Nutritional quality and production of soybean land races and improved varieties as affected by planting dates. *Pak. J. Bot.* **2009**, *41*, 683–689.

(22) Yang, M.; Shi, L.; Xu, F.; Lu, J.; Wang, Y. Effects of B, Mo, Zn, and their interactions on seed yield of rapeseed. *Pedosphere* **2009**, *19*, 53–59.

(23) Terés, S.; Barceló-Coblijn, G.; Benet, M.; Álvarez, R.; Bressani, R.; Halver, J. E.; Escribá, P. V. Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. *Proc. Natl. Acad. Sci.* **2008**, *105* (37), 13811–13816.

(24) Aranda, F.; Gómez-Alonso, S.; Rivera del Álamo, R. M.; Salvador, M. D.; Fregapane, G. Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: Comparison with other Spanish cultivars. *Food Chem.* **2004**, *86*, 485–492.

(25) Slavin, M; Cheng, Z; Luther, M; Kenworthy, W; Yu, L. Antioxidant properties and phenolic, isoflavone, tocopherol and carotenoid composition of Maryland-grown soybean lines with altered fatty acid profiles. *Food Chem.* **2009**, *114*, 20–27.

(26) Codex Alimentarius (FAO/WHO). Codex standard for named vegetable oils. Codex-Stan 210 (Amended 2003, 2005) [Online], 2005. http://www.codexalimentarius.net/download/standards/336/CXS_210e.pdf (accessed July 25, 2011)

(27) lo Curto, S.; Dugo, G.; Mondello, L.; Errante, G.; Russo, M. T. Variation in tocopherol content in Italian virgin olive oils. *Ital. J. Food Sci.* **2001**, *13*, 221–228.

(28) Baiano, A.; Gambacorta, G.; Terracone, C.; Previtali, M. A.; Lamacchia, C.; La Notte, E. Changes in phenolic content and antioxidant activity of italian extra-virgin olive oils during storage. *J. Food Sci.* **2009**, *74*, C177–C183.

(29) Parry, J.; Su, L.; Luther, M.; Zhou, K.; Yurawecz, M. P.; Whittaker, P.; Yu, L. Fatty acid composition and antioxidant properties of cold-pressed marlonberry, boysenberry, red raspberry, and blueberry seed oils. *J. Agric. Food Chem.* **2005**, *53*, 566–573.

(30) Parry, J.; Su, L.; Moore, J.; Cheng, Z.; Luther, M.; Rao, J.; Wang, J.; Yu, L. Chemical compositions, antioxidant capacities, and anti-proliferative activities of selected fruit seed flours. *J. Agric. Food Chem.* **2006**, *54*, 3773–3778.

(31) Parry, J.; Cheng, C.; Moore, J.; Yu, L. Fatty acid composition, antioxidant properties, and anti-proliferative capacity of selected cold-pressed seed flours. *J. Am. Oil Chem. Soc.* **2008**, *85*, 457–464.

(32) Boudet, A. Evolution and current status of research in phenolic compounds. *Phytochemistry* **2007**, *68*, 2722–2735.

(33) Zhou, K.; Yu, L. Effects of extraction solvent on wheat bran antioxidant activity estimation. *Lebensm-Wiss Technol.* **2004**, *37*, 717–721.

(34) Xu, B. J.; Chang, S. K. C. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *J. Food Sci.* **2007**, *72*, 159–166.

(35) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(36) Absorbance capacity (ORAC) of selected foods – 2007 [Online], Nov 2007. http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/ORAC/ORAC07.pdf (accessed July 25, 2011).