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Original Article

Natural Product Communications
July 2019: 1–6
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DOI: 10.1177/1934578X19863510
journals.sagepub.com/home/npx

Effects of Anthocyanins and Melatonin From Purple Waxy Corn By-Products on Collagen Production by Cultured Human Fibroblasts

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Abstract

Cob and silk of purple waxy corn (*Zea mays* L. Ceratina Kulesh) are underutilized sources of anthocyanins, which could be extracted by various solvents: water, ethanol, methanol, 50% ethanol, and 50% methanol. Anthocyanin and melatonin levels were investigated. The quantifications of anthocyanins as cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside were determined by high-performance liquid chromatography combined with mass spectrometry (HPLC-MS/MS), and melatonin by HPLC-fluorescence. The ability of collagen production upon exposure to human skin fibroblasts from the different macerates of the cob and silk was also studied. All cob extracts showed higher level of anthocyanins than silk extracts. The 50% ethanol extract of cob showed the highest cyanidin-3-glucoside level at 2.42 ± 0.03 mg/g dried weight, whereas that of silk showed the highest content of cyanidin-3-glucoside at 1.95 ± 0.04 mg/g dried weight. Although cob extracts contained more anthocyanins than silk extracts, silk extracts could stimulate collagen production more than cob extracts. Therefore, the collagen production was likely due to the amount of melatonin in the silk extracts, which contained greater quantities than those of the cob, and the lipophilicity of melatonin or the hydrophilicity of anthocyanins also affected the ability of cell permeation. Thus, anthocyanins and melatonin levels should be considered for the biological activity study.

Keywords

anthocyanins, melatonin, purple waxy corn, cob, silk, human skin fibroblast, collagen production

Received: March 6th, 2019; Accepted: May 6th, 2019.

Anthocyanins are natural colorants with antioxidant,[1] anti-inflammatory,[2,3] wound-healing[4] and anti-candidiasis[5,6] properties, with therapeutic potentials for topical wound healing. Topical applications of low-dose anthocyanins from purple sweet potato prevent UV-induced skin damage by decreasing the amount of UVB radiation reaching the epidermis.[7] Anthocyanins from blueberries inhibited photoaging in UV-B-irradiated human dermal fibroblasts via reduction of collagenolytic matrix metalloproteinases.[8] Several by-products of purple corn (*Zea mays* L.), eg, silk, cob, and husk, are increasingly investigated for potential since they are rich in anthocyanins.[9-11] Three major anthocyanins, cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (Pg3G), and peonidin-3-glucoside (Pn3G), were found in purple corn extracts determined by high-performance liquid chromatography (HPLC) coupled with diode array spectrophotometry and mass spectrometry (MS).[12] The by-products of purple waxy corn (*Zea mays* L. var. Ceratina Kulesh) developed by Plant Breeding Research Center for Sustainable Agriculture, Faculty of Agriculture, Khon Kaen University were extracted by different solvents, eg, water, ethanol, and hydroalcoholic solvents, and it was observed that the hydroalcoholic extracts showed high contents of total phenolic compounds and anthocyanins.[13]

Skin fibroblasts play roles in regeneration and wound healing, which involves production of collagen and other extracellular matrix proteins.[14] Previous research reported that anthocyanins from blackcurrant extract increased the collagen level of a normal human female skin fibroblast cell line.[15] The

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anthocyanin complex from aqueous extracts of purple waxy corn cob and blue butterfly pea flowers (*Clitoria ternatea* Linn.) was shown to increase collagen production in cultured human gingival fibroblasts.[16]

Melatonin has been detected in plants and animals.[17,18] It influences different phases of wound repair, eg, inflammation, by regulating the release of inflammatory mediators, cell proliferation, and migration.[19,20] Melatonin increased the collagen content in cultured cells by activating membrane receptors of fibroblasts and myofibroblasts,[21] as well as increased procollagen in HaCaT keratinocytes.[22]

In this research, we investigated the effects of different solvents for extraction of some by-products from purple waxy corn: cob and silk. The active compounds, the 3 anthocyanins (C3G, Pn3G, Pg3G) and melatonin, were determined. To our best knowledge, there is no previous report on the melatonin level in purple waxy corn.

The cob and silk of purple waxy corn were extracted by different solvents, eg, water (H), ethanol (E), methanol (M), 50% ethanol in water (HE), and 50% methanol (HM), to provide yields (%) of cob extracts of 7.9, 3.2, 6.7, 10.3, and 9.3, respectively, whereas those of silk extracts were 14.6, 3.4, 8.7, 17.2, and 15.1, respectively. Most of the hydroalcoholic solvents provided higher yields than ethanol and methanol extracts.[13]

The HPLC-MS/MS method was convenient for simultaneous quantitation of mixed samples of C3G, Pn3G, and Pg3G using the molecular ions ($[M + H]^+$) at m/z 499, 433, and 463 at the retention times of 2.15, 2.21, and 2.23 minutes, respectively. The results obtained, shown in Figure 1, illustrate that C3G was the predominant compound in all extracts. The highest contents of C3G, Pg3G, and Pn3G were 2.42 ± 0.03, 0.68 ± 0.02, and 0.99 ± 0.01 mg/g dried weight, detected from the HE extracts of cob, whereas 1.95 ± 0.04, 0.59 ± 0.01, and 0.16 ± 0.00 mg/g dried weight, from the HE extracts of silk, respectively (Figure 1a–c). These present results support our previous finding that HE extracts of cob of this purple waxy corn also gave the highest total anthocyanins using a pH differential method.[13] Additionally, the extraction process conditions of anthocyanins from frozen açai (*Euterpe oleracea* Mart.) reported that a mixture of ethanol/water (50:50 v/v) with no acid was the best solvent for extraction.[23]

The viability of human skin fibroblasts, relative to ascorbic acid used as the positive control, at 24 hours exposure to the H, E, M, HE, or HM extracts of either cobs or silks at 0.5 and 1 mg/mL (abbreviated as 0.5 and 1, respectively) is shown in Figure 2(a). Most of the silk and cob extracts caused no toxicity to human skin fibroblasts, except the ethanolic extract of silk at 1 mg/mL, with a very low relative cell viability (<0.5). Figure 2(b) compares the relative amount of collagen produced by human skin fibroblasts after 24-hours treatments with the extracts, relative to ascorbic acid. All of the silk extracts from H, HE, and HM, and only the cob extracts from H (both 0.5 and 1), HE1, and HM1 show collagen induction to a higher extent than ascorbic acid. Thus, with no inference on cell viability when the cells were exposed to H, HE, and HM extracts of cob and silk, collagen induction was the beneficial effect.

Overall, most of the cob and silk extracts in our study did not obviously promote cell viability, when compared with ascorbic acid. However, several silk extracts in H, HE, and HM as well as M1, and some cob extracts in H, HE1, and HM1 could induce collagen production to 1.2 to 2 times higher than ascorbic acid. Interestingly, upon exposure to E1 extract of silk, the cells with the lowest relative cell viability of less than 0.5 could produce about 80% of collagen, relative to that of ascorbic acid, suggesting an extensive promotion of cellular collagen production, but not the cell viability.

It is noted that the highest contents of C3G, P3G, and Pe3G of silk, extractable from HE, also provided the highest relative collagen production, about twice of that induced by ascorbic acid. C3G, which was previously reported to induce

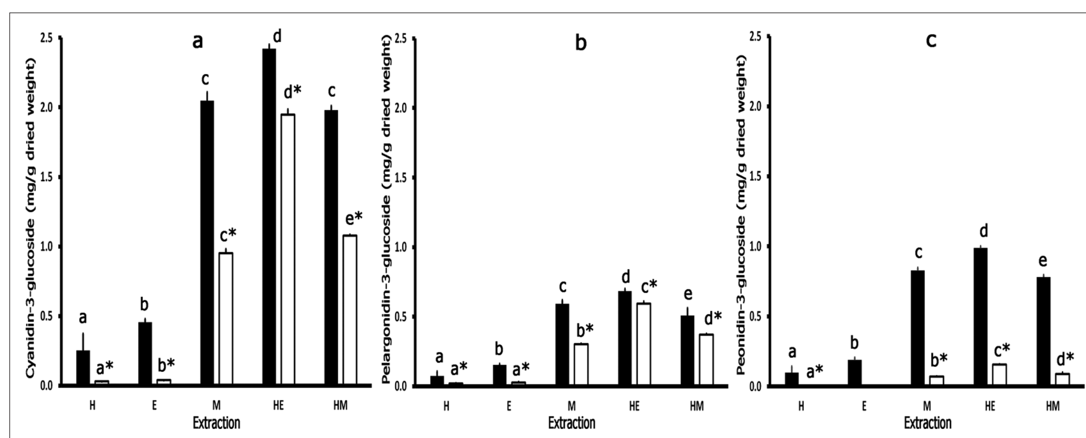

Figure 1. Average cyanidin-3-glucoside (a), pelargonidin-3-glucoside (b), and peonidin-3-glucoside (c) contents in the various extracts: water (H), ethanol (E), methanol (M), 50% ethanol in water (HE), and 50% methanol in water (HM), in comparison between cob (black columns) and silk (white columns) (n = 3). Error bars represent SDs. The same letters on each column are not significantly different (P < 0.05).

collagen production,[15] was the major component of anthocyanins extracted from cob and silk. However, significantly higher C3G contents from all extracts of cob than those of silk ($P < 0.05$) did not seem to exhibit collagen induction in the same manner as those of the silk extracts. Alcoholic or hydroalcoholic solvents could obtain several other phenolic compounds. The phytochemical screening of crude extracts from cob and silk of purple waxy corn indicated some chemicals such as carbohydrate, phenolic compounds, and flavonoids with the highest total phenolic compounds and total anthocyanins of cob and silk from HE extracts.[13] Therefore, not only anthocyanins but also some other active compounds might implicate to collagen production.

In the attempts to explain the phenomenon, melatonin was previously reported to have a role in skin function modulation involving melatonin receptors on the surface of skin cells, resulting in promotion of collagen.[24,25] With its water solubility of about 0.8 mg/mL and its effective dose in nanogram level, it is speculated that the extracts of purple waxy corn may contain melatonin, which also induces the collagen production in human skin fibroblasts. Total melatonin contents, extracted by methanol and analyzed by HPLC fluorescence, in the silk of 93.63 ± 0.18 ng/g dried weight were significantly higher than that of the cob of 74.72 ± 3.02 ng/g dried weight ($P < 0.05$). This may contribute the explanation on the effect of cob and silk extracts on collagen induction.

The hydrophilic nature of anthocyanins presents an obstacle in transport across the cell membrane as evidenced by a relatively low absorption of anthocyanins through Caco-2 cells compared to the relevant anthocyanidins, the aglycone forms.[26] On contrary, a previous cell study showed that very low-dose melatonin could promote collagen production.[24] Additionally, a physicochemical study showed its high permeation and partition coefficient.[27] Although substantially low contents of melatonin were determined, it may have profoundly influenced on cellular functions as the transport across the cell membrane could be enhanced by its hydrophobicity, high permeation characteristics and the presence of melatonin receptors on the cell surface.

A wide range of melatonin contents from 10 to 2034 ng/g dry weight could be obtained from various cultivars of corn seeds.[28] This study showed that cob and silk, 2 of several by-products of the purple waxy corn, also could be the sources of melatonin, as well as anthocyanins, depending on the optimum extraction solvents and procedures. It is essential to verify the relationship between these bioactive compounds produced during the development and growth of the plants.

Experimental

Materials and Chemicals

Cobs and silks of purple waxy corns (*Zea mays* L. var. Ceratina Kulesh) were harvested by the Plant Breeding Research Center for Sustainable Agriculture, Faculty of Agriculture, Khon Kaen University, in May 2018, dried at 50°C and ground to powder. Dulbecco's modified Eagle medium (DEMEM, Invitrogen Cooperation [Gibco], USA), 96-well plates (SLP Life Science, Korea), premium fetal bovine serum (FBS, Invitrogen Cooperation [Gibco], USA), penicillin/ streptomycin/amphotericin B (Invitrogen Cooperation, [Gibco], USA), trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen Cooperation [Gibco], USA), phosphate buffer saline (Lonza, USA), microtubes (Gibco, USA), human

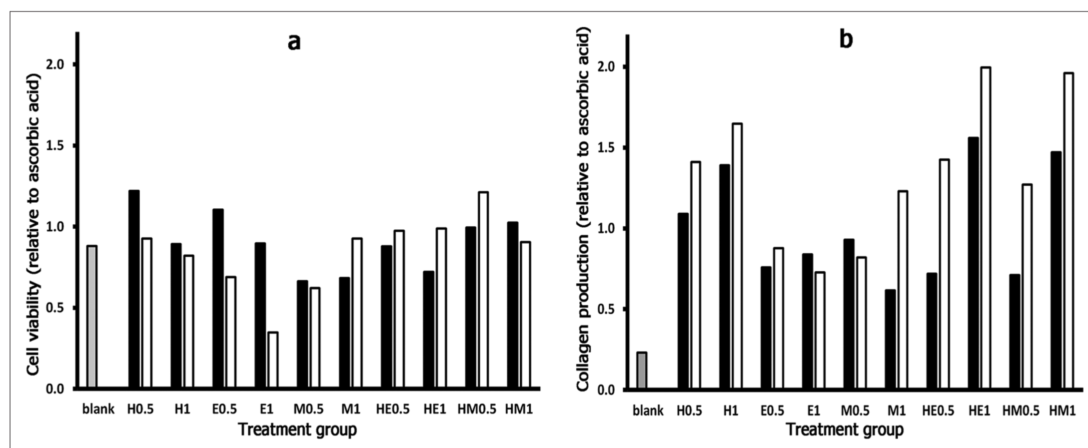

Figure 2. Effects of purple waxy corn extracts on human skin fibroblasts after incubation for 24 hours, expressed as relative to 2 mM ascorbic acid: the cell viability (a) and collagen production (b) of cob (black columns) and silk (white columns) extracts of purple waxy corn and blank (untreated sample in gray columns). The treatment groups are the extracts of cob and silk by various solvents: water (H), ethanol (E), methanol (M), 50% ethanol in water (HE), and 50% methanol in water (HM) with 0.5 and 1 mg/mL. 3-(4,5- dimethylthiazol-2-yl-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used to assay cell viability, and Sirius-picric acid to monitor collagen ($n = 8$).

collagen type I (acid-soluble, Sigma, USA), Sircol Dye reagent (Sirius Red in picric acid), acetonitrile (HPLC grade), methanol (99.9%, AR grade), and ethanol (99.5%, AR grade) from Merck (Germany) were used as received. The anthocyanin standards C3G (≥98.0%), Pn3G (≥7.0%), and Pg3G (≥97.0%, HP), and melatonin were purchased from Sigma-Aldrich (Germany).

Sample Extraction

Samples (20 g) were accurately weighed and macerated with 300 mL of water (H), ethanol (E), methanol (M), HE, or HM, and stirred with a magnetic stirrer at 25 ± 2°C for 24 hours. Each extraction was repeated 3 times, and then filtered through Whatman filter paper no.1; all filtrates were collected for freeze-drying in an EYELA Freeze dryer FDU-1200 (Tokyo Rikakikai Co., Ltd.). The extracts were stored in light-protected tightly closed containers at 4 ± 1°C until use.

Determination of C3G, Pg3G, and Pn3G

A stock of mixed standard anthocyanins containing C3G, Pg3G, and Pn3G at a weight ratio of 3:1:1, respectively, was prepared in 5% formic acid in methanol and diluted to a concentration range of C3G of 15 to 1 800 ng/mL, and Pg3G and Pn3G of 5 to 600 ng/mL. For melatonin, the standard range was 0.05 to 1.0 ng/mL. The 10 mg dried extract sample was dissolved in 5% formic acid in 10 mL methanol, sonicated for 15 minutes, centrifuged at 3000 rpm for 10 minutes and separated from the supernatant, which was diluted to a volume ratio of 1:5 with 5% formic acid in methanol. All samples and standards were filtered through a 0.2 μm syringe filter and kept in amber-glass vials before injection. The validated HPLC-MS/MS procedure was performed for anthocyanin determination using a triple quadrupole machine (API 3200 MS/MS System, ABSciex) equipped with a binary HPLC pump (Hewlett-Packard 1100, Series HPLC Value System) with Analyst software, and a column (Poroshell 120 SB-C18 [4.6 × 75 mm, 2.7 μm]) with a drop-in guard cartridge (Agilent Technologies, USA). Two mobile phases, 5% formic acid (phase A) and methanol (phase B), were subjected to a series of gradient elution, as follows: 0 to 10 minutes, 20% to 30% of phase B, 10 to 13 minutes, 30% to 100% of phase B, and 13 to 15 minutes, isocratic at 100% of phase B, and then a re-equilibration period of 3 minutes with 20% of phase B between individual runs. Operating conditions were as follows: a flow rate of 0.3 mL/min, a controlled column temperature of 30°C, the sample temperature at ambient, and an injection volume of 0.01 mL. MS parameters were as follows: ionization mode with ESI positive mode, a scan range of 400 to 500 amu, and a scan rate of 1 scan/sec. The source parameters were turbo ion spray voltage of 5 500 V, source temperature (TEM) 550°C, TEM of nebulizer gas (gas 1) 50°C, TEM of heater gas (gas 2) 60°C, curtain gas pressure 25 psi, and collision gas pressure 5 psi. For MS2 analysis, the collision energy and the collision cell exit potential were used at 5 to 130 V and 0 to 58 V, respectively, with a scan range of 400 to 500 amu. Method validation using linearity, precision, accuracy, and recovery was performed. Ten of all extracted samples were subjected to LC-MS/MS and the C3G, Pg3G, and Pn3G contents were compared with standard curves.

Determination of Melatonin

Two grams of sample powder was suspended in 20 mL methanol and ultrasonicated for 1 hour at room temperature. After centrifugation at 4 000 rpm for 10 minutes, the supernatant was filtered through Whatman No. 1 paper. The supernatant was then evaporated under vacuum in a rotary evaporator at 40°C. The residues were redissolved in 2 mL 5% methanol-water solution for further isolation of melatonin using solid phase extraction. A Strata C18-E cartridge (3 mL, C18, 500 mg, Phenomenax) was first activated with 10 mL methanol, followed by 10 mL of pure water. Two milliliters prepared samples were added to the column. After drying, 10 mL of 5% methanol-water solution was used to elute the interference impurities. Finally, the retained melatonin was eluted at a low flow rate using 5 mL of 80% methanol-water solution. The fraction samples were dried, under vacuum in a rotary evaporator, and dissolved with 2 mL of mobile phase (30% acetonitrile in 0.01 mM phosphate buffer at pH 7.2), which was filtered through a 0.45 μm pore size membrane using a syringe filter into amber-glass vials before analysis. HPLC fluorescence analysis: Analysis of melatonin content was performed using a previously validated method, as described.[29] The HPLC (Shimadzu LC 20A, Japan) was fitted with a fluorescence detector fixed at excitation and emission wavelengths of 285 and 345 nm, respectively, and a reverse phase C18 column (5 μm, 150 mm × 4.6 mm, Luna 5 μm PFP(2) 100A, CA, USA); the mobile phase was composed of 30% acetonitrile in 0.01 mM phosphate buffer, pH 7.2 and the flow rate was 1 mL/min at 25°C. The mobile phase and all samples were filtered through a cellulose acetate membrane (0.2 μm, Ministart, Satorius) prior to use.

Cell Studies

Human skin fibroblasts, gifted by Dr Wilairat Leeanansaksiri, were grown in 75 cm^3 tissue culture flasks in complete medium (10% FBS and 90% Dulbecco's modified Eagle medium and 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B) in a humidified CO_2 incubator containing 5% CO_2 at 37°C. Reaching its plateau, the cells were trypsinized by 0.25% trypsin/EDTA solution for 5 minutes. The cells were transferred in concentrations of 10 000 cells/well into a 96-well plate (about 0.1 mL per well) and incubated for 24 hours in a humidified incubator containing 5% CO_2 controlled at 37°C prior to use. Samples were dissolved and diluted in free FBS medium. The blank or negative control was the medium without FBS, whereas the positive control was 2 mM L-ascorbic acid. Cell viability of the cultured cells in

96-well plates was estimated by 3-(4,5- dimethylthiazol-2-yl-2-yl)-2,5-diphenyltetrazolium (MTT). In brief, the prepared cells were subjected to 24 hours exposure to either the samples or the controls, then the medium was removed to mix with MTT for 1 hour, followed by absorbance reading at 550 nm using a microplate reader (SunriseTM, Tecan Group Ltd., Männedorf, Switzerland), with Magellan data analysis software (Tecan Group Ltd., Männedorf, Switzerland). Collagen was analyzed by the picrosirius red method based on selective binding of Sirius red, in acid conditions to fibrillar collagens (type I), specifically to the (Gly-X-Y)*n* helical structure.[30] The concentrations of the samples were obtained by comparing with standards at a concentration range of 0.1 to 0.7 mg/mL of human collagen type I.

Statistical Analysis

All values represent the means ± SDs and statistically compared by one-way analysis of variance. Paired *t*-test or independent *t*-test was used for comparisons of results from cell studies. The significant level was considered at 0.05.

Acknowledgments

Assoc. Prof Dr Bhalang Suriharn from the Plant Breeding Research Center for Sustainable Agriculture, Faculty of Agriculture, Khon Kaen University is thanked for providing the plant materials, while Dr Wilairat Leeanansaksiri (Suranaree University of Technology) is thanked for the supply of human skin fibroblasts. Miss Sathaporn Satsue and Miss Bunthrawan Longsri are also thanked for cell assay and melatonin determination.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was partially funded by Khon Kaen University (Incubation for Researchers Project 2013-2015 [grant number 56/01]), the Graduate School, Khon Kaen University funding for Miss Thitima Rimdusit (grant number 57/02), Faculty of Pharmaceutical Sciences funding (Startup Research Pharm KKU, 2017 (grant number 61/01) and Melatonin Research Group (grant number 60/04), Khon Kaen University, Khon Kaen, Thailand.

Supplemental Material

Supplemental material for this article is available online.

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