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Purple Waxy Corn Modifies the Expression of *CYP3A4*, *N*-acetyltransferase 2, and *UGT1A6* in HepG2 and Caco-2 Cells

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ABSTRACT

Purple waxy corn (*Zea mays* L. var. ceritina Kulesh.), or black purple sticky corn, is an edible plant with antioxidant properties according to its rich anthocyanin, phenolic and flavonoid content. This study aimed to determine how purple waxy corn modified drug metabolizing genes (*CYP1A2*, *CYP2C9*, *CYP3A4*, *UGT1A6*, and *NAT2*) and a drug transporter (*OATP1B1*) in human hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cells. The cells were incubated with purple waxy corn (125 to 1,000 µg/mL) for 48 h. Cell viability, reactive oxygen species (ROS), aspartate transaminase (AST), and alanine aminotransferase (ALT) levels were investigated. The mRNA expression of target genes was determined by RT/qPCR. Cell viability remained above 80% even at the maximum tested concentration of purple waxy corn (1,000 µg/mL). Expression of *CYP1A2* and *CYP2C9* was not modified by purple waxy corn in either HepG2 or Caco-2 cells, but *CYP3A4* expression was significantly suppressed in Caco-2 cells. At the highest concentration (1,000 µg/mL), purple waxy corn markedly induced expression of *UGT1A6* and *NAT2* mRNA in Caco-2 cells. Conversely, purple waxy corn suppressed expression of *NAT2* at the highest concentration in HepG2 cells. The expression of *OATP1B1* was not affected by purple waxy corn in either cell type. Therefore, consumption of large amounts purple waxy corn could cause food-drug interaction via differential modification of *CYP3A4*, *UGT1A6*, and *NAT2*.

***Keywords*:** *Zea mays*, *CYP3A4*, *NAT2*, *UGT1A6*, Food Drug Interaction.

Introduction

Purple waxy corn (*Zea mays* L. ceritina Kulesh.), known in Thai as *Kao Kum* which means black purple sticky corn, is a popular edible plant containing numerous nutrients and natural phytochemicals, including anthocyanins, phenolics, and flavonoids.[1] The high amounts of these phytochemicals provide purple waxy corn with antioxidant, neuroprotective, and memory-enhancing activities.[2,3] Moreover, purple waxy corn has been demonstrated to have preventive properties against cataractogenesis and retinopathy in diabetic rats.[4,5] Therefore, purple waxy corn is an excellent candidate to develop as food supplement to prevent or delay chronic diseases. However, to ensure the safe consumption of food and herbal supplements over the long term, food and herb drug interactions cannot be overlooked.[6]

Cytochrome P450 (CYP) is a superfamily of monooxygenase enzymes responsible for phase I metabolism in the liver. Human CYP1A2, CYP2C9, and CYP3A4 are responsible for the biotransformation of more than 50% of clinical drugs.[7] Uridine diphosphate (UDP)-glucuronosyl transferase 1A6 (*UGT1A6*) is a conjugation enzyme in phase II metabolism that metabolizes clinical drugs including carvedilol, naproxen, valproic acid, and zidovudine.[8]

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N-acetyltransferase 2 (*NAT2*) is a phase II metabolizing enzyme that biotransforms arylamines, aromatic amines, hydrazine drugs, and procarcinogens.[9] Together, CYPs, UGT, and NAT are involved in the biotransformation and metabolism of more than 90% of market available drugs, while many phytochemicals are also substrates for CYPs and UGT.[8,10,11] In addition to phase I and II metabolizing enzymes, the organic anion transporting polypeptide 1B1 (*OATP1B1*) is an important determinant of transporter mediated drug interaction.[12,13]

Food and herbal supplements can interfere with the metabolism of clinical drugs via alteration of phase I and/or phase II metabolizing enzymes. For example, flavonoids have been shown to decrease the metabolism of felodipine and nifedipine by inhibiting CYPs. These food-drug interactions can reduce the efficacy or increase the toxicity of drugs. Citrus fruits or fruit juices are commonly reported as causes of food drug interactions[14] and many natural foods, herbs, or plant dyes can modulate expression of *CYP2A6*, *UGT1A6*, *UGT2B7*, and *OATP1B1*.[12,13,15]

Human cell lines are useful *in vitro* models for human organs.[16] Hepatocarcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cell lines are widely employed for metabolic studies representing the liver and intestine, respectively.[16] The HepG2 cell line presents genotypic features that are similar to primary hepatocytes. The Caco-2 cell line is preferred as a model small intestinal cell line as it can be spontaneously differentiated to express either enterocytic or colonocytic characteristics.[16,17] HepG2 and Caco-2 are the most suitable *in vitro* models for understanding regulatory processes of absorption and metabolism of compounds in the liver and small intestine.[17] Moreover, the characteristics of HepG2 and Caco-2 monolayers make them excellent models to study the bioactivity of food supplements.[18,19] Hence, this study aimed to determine the effects of purple waxy corn on the mRNA expression of phase I and II

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metabolizing enzymes and a drug transporter in HepG2 and Caco-2 cells.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (+) phenol red (DMEM with phenol red, Cat. No. 11885-084), DMEM/F12 (1:1) phenol red-free medium (Cat. No. 2104-025), fetal bovine serum (FBS), 1× Glutamax®, DPBS, and 1× penicillin, streptomycin, and neomycin antibiotics (PSN) were purchased from Gibco® (New York, USA). ReverTraAce®, Thunderbird™ Probe qPCR Mix, and other reagents for RT/qPCR were products of Toyobo Co., Ltd. (Osaka, Japan). TaqMan™ gene expression assays were products of Applied Biosystem™ (Waltham, Massachusetts, USA). Other laboratory chemicals and materials were provided by commercial suppliers with analytical or molecular grade.

Purple waxy corn preparation

Purple waxy corn (*Zea mays* L.) was harvested from the Field Crop Research Station at Nong-Bau Village (16°27'45.2"N 102°37'10.7"E), Khon Kaen University, Khon Kaen, Thailand between November 2020 and January 2021. A plant specimen (PANPB-ZM 2021-001) is lodged at the Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology, Khon Kaen University. Milk powder of purple waxy corn was provided by the Research Institute for Human High Performance and Health Promotion, Khon Kaen University. The powder was precisely weighed and dissolved in deionized water to determine total phenolic, total flavonoid, and anthocyanin content, as well as the percent tannin contribution according to standard protocols.[20] The phenolic, flavonoid, and anthocyanin contents were 1.08 ± 0.31, 1.17 ± 0.28, and 1,033.3 ± 0.011 mg/g dry weight, respectively. The percent tannin contribution was 84.94 ± 4.41 %. Defined weights of the milk powder were suspended in cell culture medium and filtered through a 0.22 µm-sterile membrane filter before adding to cells.

Cell culture and treatments

HepG2 (ATCC® HB-8065, Manassas, USA) and Caco-2 (RBRC-RBC0988, RIKEN cell bank, Saitama, Japan) cells were maintained in standard DMEM supplemented with 1× Glutamax®, 10% or 20% FBS, and 1× PSN at 37°C with 5% CO_2 concentration and 95% humidity. The cells were cultured in 24 well-plates (2.5×10^5 cells/well in 0.5 mL) for 48 h before incubation with either 0.1% dimethyl sulfoxide (DMSO, control), 10 µM ketoconazole (Keto) or rifampicin (Rif), or 125, 250, 500, 1000 µg/mL of the purple waxy corn milk powder for 48 h. The medium was collected for determination of cell viability, reactive oxygen species (ROS), aspartate transaminase (AST), and alanine aminotransferase (ALT). HepG2 and Caco-2 cells (n=4-5) were harvested for total RNA preparation.

Determination of cell viability

Cell viability was determined after 48 h of treatment using the resazurin assay. Briefly, a final concentration of rezasurin at 100 µM was added into phenol red-free medium and incubated with the cells at 37°C in 5% CO_2 for 30 min. The percentage of cell viability was calculated from the amount of fluorescence due to the presence of the fluorescent dye resorufin, which was measured by a spectrofluorometric plate reader at excitation/emission of 530/580 nm.[21]

Determination of reactive oxygen species (ROS)

At 48 h after treatment, ROS was determined by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method. Briefly, the medium was mixed with 60 nM of DCFH-DA and incubated under light protection for 40 min at room temperature. Fluorescence intensity of dichlorofluorescein was measured by a spectrofluorometric plate reader at excitation/emission of 484/530 nm. The level of ROS was calculated by comparison with a standard curve of hydrogen peroxide.[19]

Determination of AST and ALT levels

At 48 h after treatment, the medium was incubated with either AST (10 mM L-aspartate and 1.7 mM α-ketoglutarate) or ALT substrate (300 mM L-alanine and 0.7 mM α-ketoglutarate) at 37°C for 30 or 20 min, respectively. Then 2,4-dinitrophenylhydrazine was added and left for 20 min, followed by the addition of sodium hydroxide. Absorbance was measured at 505 nm. The levels of AST and ALT were calculated by comparison with a standard curve of sodium pyruvate and are expressed as international units per liter (IU/L).[19]

Quantitative analysis of mRNA expression by RT/qPCR

At 48 h after treatment, total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method.[21] Total RNA was reverse transcribed using ReverTraAce® kit. Expression of *CYP1A2* (Hs00167927_m1), *CYP2C9* (Hs02383631_s1), *CYP3A4* (Hs00604506_m1), *UGT1A6* (Hs01592477_m1), *NAT2* (Hs01854954_s1), and *OATP1B1* (Hs00272374_m1) mRNA was determined using reverse transcriptase-quantitative polymerase chain reaction (RT/qPCR) and normalized to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs02786624_g1) using the probe-primers of TaqMan™ gene expression assays with Thunderbird™ reagents. The relative fold expression was calculated using the delta-delta Ct method.[21]

Statistical analysis

The results are expressed as mean ± S.D. (n = 4-5 per group) and were statistically analyzed using analysis of variance (ANOVA) with Tukey's honest significant difference test (SPSS version 23, Chicago, IL, USA). $p \leq 0.05$ or 0.001 was considered as statistically significant.

Results and Discussion

Effects of purple waxy corn on cell viability, ROS, AST, and ALT

The percentage cell viability was fluorometrically determined by resazurin assay to measure the ability of living cells to enzymatically reduce resazurin (a non-fluorescence dye) to resorufin, a pink fluorescence dye.[22] Keto strongly reduced the viability of HepG2 and Caco-2 cells ($p < 0.001$, Figures 1A and 1B). Rif and purple waxy corn both slightly reduced cell viability to around 80%, but cell morphology remained normal. There was no significant difference in cell viability among the tested purple waxy corn concentrations (125 to 1,000 µg/mL), which is consistent with a previous study that reported that purple waxy corn at 3,000 µg/mL reduced Hep G2 cell viability to 60%.[23] Hence, the study continued using these concentrations. For ROS analysis, the DCFH-DA method was employed based on the reaction of ROS with DCFH-DA to create highly fluorescent dichlorofluorescein.[19] Keto and Rif generated significant amounts of ROS in both cell types (Figures 1C and 1D), whereas purple waxy corn did not. AST and ALT assays were employed to indicate cell injury (Figure 2).[19] Keto and Rif significantly increased AST and ALT levels in HepG2 and Caco-2 cells. Nonetheless, we continued to employ Keto and Rif at 10 µM due to their modulatory effects on CYP expression.[24,25] All tested concentrations of purple waxy corn did not significantly affect AST and ALT levels. These observations suggested that purple waxy corn was not hazardous to the cells at concentrations ranging from 125 to 1,000 µg/mL.

Effects of purple waxy corn on the mRNA expression of CYPs

Keto induces *CYP1A2* and *CYP3A4* expression via activation of the aryl hydrocarbon receptor and pregnane X receptor, respectively.[24] Keto has been reported as an inhibitor for *CYP2C9* and a drug transporter,[26] while Rif is a typical *CYP3A4* inducer.[25] Therefore, Keto and Rif were chosen as positive controls in this study.

Keto significantly induced expression of *CYP1A2* mRNA in both HepG2 and Caco-2 cells ($p < 0.001$, Figure 3A and 3B), while Rif extensively suppressed *CYP1A2* expression in HepG2 cells. Purple waxy corn did not modify *CYP1A2* expression in either cell type. Human CYP1A2 is abundantly expressed in the liver[7] and fundamentally metabolizes almost all anti-depressant, anti-psychotic, anti-inflammatory, anesthetic, and analgesic drugs.[27] Hence, purple

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waxy corn exhibits a low risk of drug interaction with CYP1A2 substrates in the liver and intestine.

Expression of *CYP2C9* is inducible by Rif,[28] which is consistent with the significant induction of *CYP2C9* mRNA seen in both cell types in the present study (Figure 3C, $p < 0.001$). Conversely, Keto strongly inhibited *CYP2C9* expression (Figures 3D; $p < 0.001$). Corresponding to the *CYP1A2* expression results, purple waxy corn did not alter expression of *CYP2C9* in either cell type. CYP2C9 is responsible for the metabolism of clinical drugs like fluoxetine, losartan, phenytoin, tolbutamide, torsemide, warfarin, and non-steroidal anti-inflammatory drugs (NSAIDs).[26] Therefore, as purple waxy corn did not affect the expression of *CYP2C9*, co-administration of purple waxy corn with CYP2C9 drug substrates should be acceptable.

CYP3A4 metabolizes a large and diverse range of molecules and is the main metabolizer of over 50% of clinical drugs, including bronchodilators, and antiviral, antibacterial, antifungal, lipid-lowering, and anti-hypertensive drugs.[26] Keto and Rif significantly induced expression of *CYP3A4* mRNA in HepG2 and Caco-2 cells ($p < 0.001$; Figures 3E and 3F). Purple waxy corn significantly suppressed *CYP3A4* expression in Caco-2 cells, but not in HepG2 cells. Differences in liver and intestine tissue could explain this divergence.[29,30] Therefore, care should be taken for consumption of purple waxy corn with CYP3A4 substrates.

Effects of purple waxy corn on UGT1A6, NAT2, and OATP1B1 expression

After phase I metabolism by CYPs, phase II conjugation reactions are responsible for the biotransformation of xenobiotics. UGT1A6, a member of the UDP-glucuronosyl transferase family, is responsible for the conjugation of many drugs, such as aspirin, naproxen, carvedilol, zidovudine, and valproic acid.[8] CYP and UGT enzymes together are responsible for the metabolism of more than 90% of market drugs and many phytochemicals are also substrates for CYPs and UGTs. For example, prenylflavonoids have been shown to up-regulate expression of *UGT1A6* mRNA in Caco2 cells[10] and quercetin, a well-known bioactive flavonoid, was reported to induce *UGT1A6* expression in a Caco-2 model.[31] For the present study, expression of *UGT1A6* was not affected by any treatments in HepG2 cells, compared to the control (Figure 4A). In contrast, both Keto and Rif significantly induced *UGT1A6* expression in Caco-2 cells ($p<0.001$; Figure 4B). In addition, the highest concentration of purple waxy corn showed a significant induction of *UGT1A6* expression in Caco-2 cells ($p<0.05$). Therefore, purple waxy corn might up-regulate *UGT1A6* expression according to its flavonoid content.

A number of hydrazine drugs (i.e. anti-depressant and anti-diabetic agents) and procarcinogens (e.g. arylamines and aromatic amines) are metabolized by *N*-acetyltransferase 2 (NAT2).[9] *NAT2* mRNA is constitutively expressed in several tissues, but it is abundantly expressed in the liver, small intestine, and colon.[32,33] Keto and the highest concentration of purple waxy corn significantly suppressed expression of *NAT2* in HepG2 cells ($p<0.001$; Figure 4C), while Keto, Rif, and the highest concentration of purple waxy corn extensively up-regulated *NAT2* expression in Caco-2 cells (Figure 4D). A previous study demonstrated that the flavonoid quercetin inhibited NAT2 activity in healthy volunteers.[34] Hence, it is likely that purple waxy corn at high concentration suppresses *NAT2* expression in HepG2 cells due to its flavonoid content. The divergence of liver and intestine tissue characteristics could explain these differences in *NAT2* regulatory expression.

OATP1B1 is an important uptake transporter expressed on the sinusoidal side of hepatocytes and a predictor of transporter-mediated drug interactions because some herbs can modulate expression of *OATP1B1*.[12,13] Although *OATP1B1* is principally expressed in hepatic cells, it is also nominally expressed in colonic or intestinal cells.[35] Current information about food-drug interactions via OATP1B1 is limited. Dietary phenolic acids might interfere with the function of human organic anion transporters[36], but detailed information about the specific OATP1B1 isoform is lacking. Expression of *OATP1B1* was excessively suppressed by Keto in both HepG2 and Caco-2 cells (Figures 4E and 4F). In contrast, Rif markedly induced *OATP1B1* expression in Caco-2 cells (Figure 4F), while purple waxy corn did not change *OATP1B1* expression in either cell type.

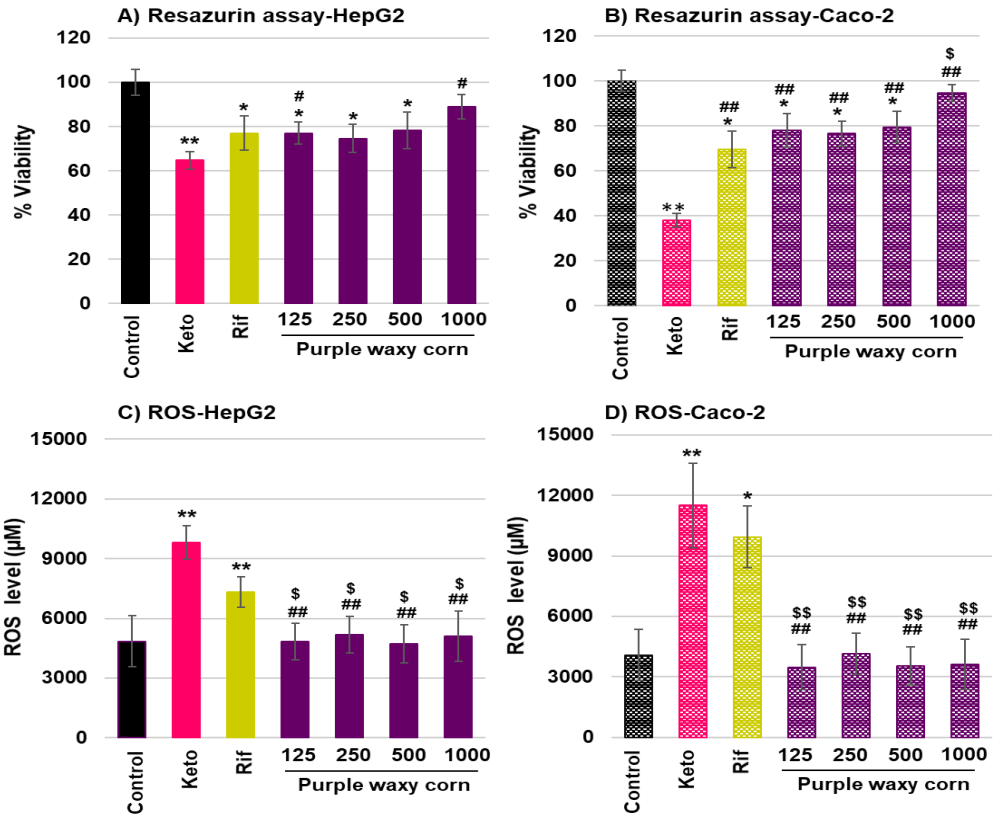

Figure 1: Effects of purple waxy corn on cell viability and reactive oxygen species (ROS).
$^{*}p < 0.05$, $^{**}p < 0.001$, VS control; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ VS Keto (10 µM ketoconazole). $^{\$}p < 0.05$, $^{\$\$}p < 0.001$ VS Rif (10 µM rifampicin); Purple waxy corn's concentrations are 125-1000 µg/mL (n = 4-5).

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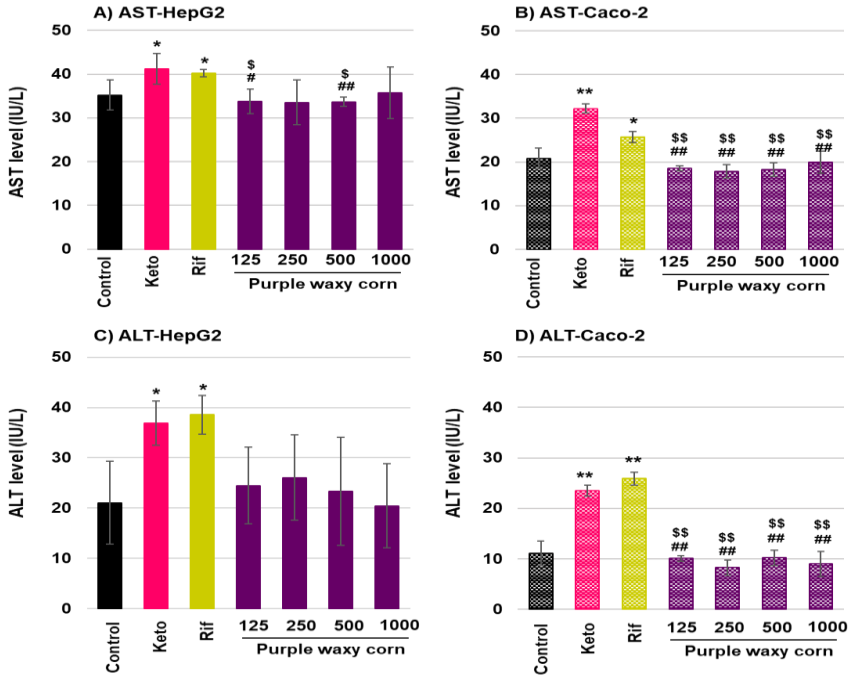

Figure 2: Effects of purple waxy corn on AST and ALT level
$^{*}p < 0.05$, $^{**}p < 0.001$, VS control; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ VS Keto (10 µM ketoconazole). $^{\$}p < 0.05$, $^{\$\$}p < 0.001$ VS Rif (10 µM rifampicin); Purple waxy corn's concentrations are 125-1000 µg/mL (n = 4-5).

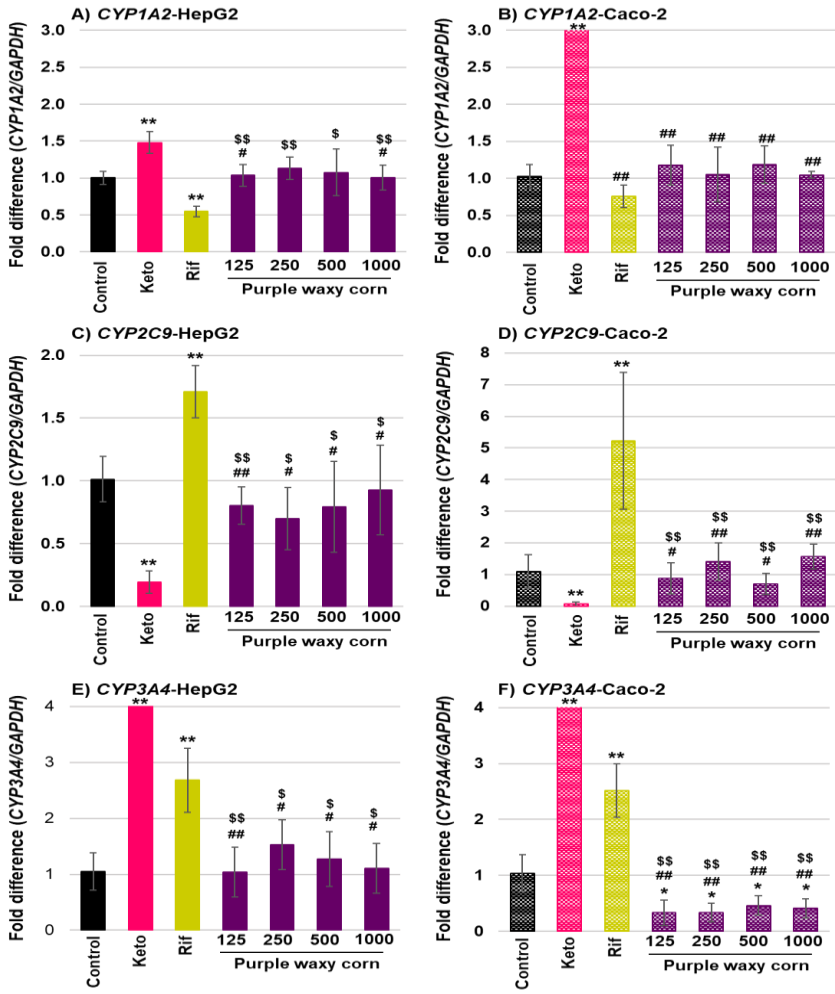

Figure 3: Effects of purple waxy corn on *CYP1A2*, *CYP2C9*, and *CYP3A4* mRNA expression.
$^{*}p < 0.05$, $^{**}p < 0.001$, VS control; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ VS Keto (10 µM ketoconazole). $^{\$}p < 0.05$, $^{\$\$}p < 0.001$ VS Rif (10 µM rifampicin); Purple waxy corn's concentrations are 125-1000 µg/mL (n = 4-5).

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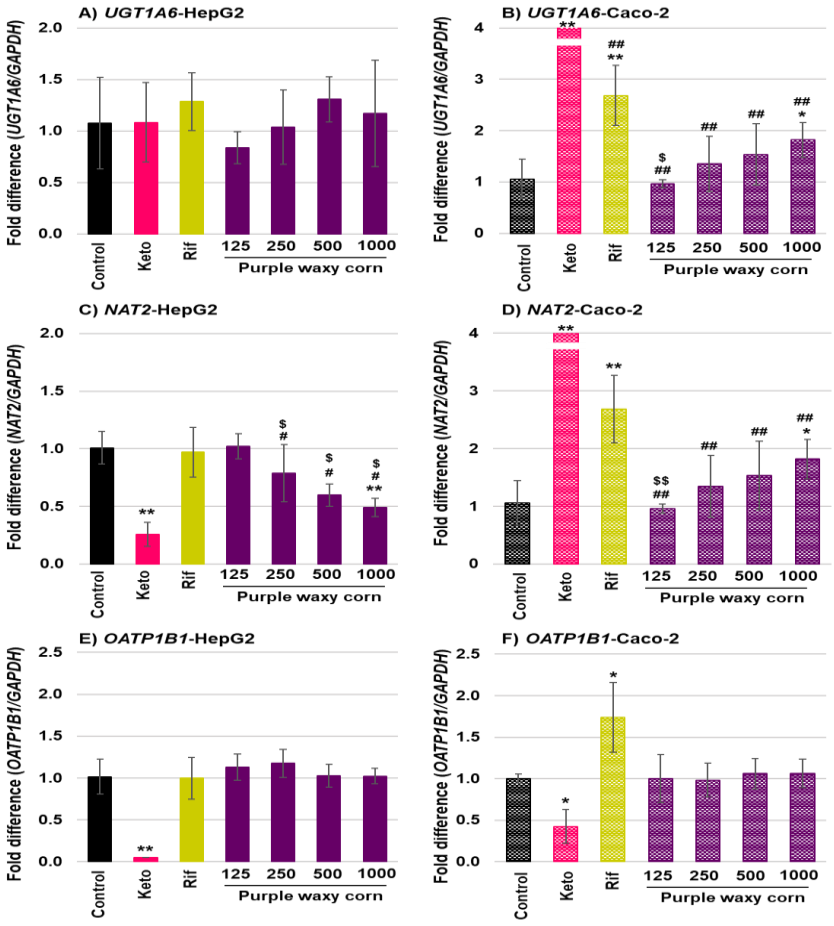

Figure 4: Effects of pineapple on *UGT1A6*, *NAT2*, and *OATP1B1* mRNA expression.
$^{*}p < 0.05$, $^{**}p < 0.001$, VS control; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ VS Keto (10 µM ketoconazole). $^{\$}p < 0.05$, $^{\$\$}p < 0.001$ VS Rif (10 µM rifampicin); Purple waxy corn's concentrations are 125-1000 µg/mL (n=4-5).

Conclusion

Purple waxy corn may cause food-drug interactions via altered *CYP3A4*, *UGT1A6*, and *NAT2* expression. Therefore, the consumption of high amounts of purple waxy corn with *CYP3A4*, *UGT1A6*, and *NAT2* substrates for long times should be a concern for the safe and effective development of purple waxy corn as a food supplement.

Conflicts of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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