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Mulberry (*Morus alba* L.): Risk of herb–drug interactions via alteration of phase I and II metabolizing enzymes and transporters in HepG2 cells

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ABSTRACT

Mulberry (*Morus alba* Linn; MA) is a food supplement that may cause herb–drug interactions (HDIs). Potential interactions of MA, its constituents cyanidin 3-glucoside (C3G) and rutin, and four common pharmacologically active agents were examined in HepG2 cells. Cells were incubated with 1–10 µM C3G, 1–10 µM rutin, and 125–500 µg/ml MA alone and in combination with 5 mM acetaminophen (APAP), 5 mM aspirin (ASA), 10 µM simvastatin (SV), or 50 µM caffeine (CF) for 72 hours. The expressions of phase I and II metabolizing enzymes and transporters were determined by RT/qPCR. When tested alone, MA significantly upregulated the expression of *CYP1A2* and UDP-glucuronosyltransferase 1A6 (*UGT1A6*). Cotreatment of HepG2 cells with APAP, ASA, SV, or CF, and MA resulted in upregulation of *CYP1A2* and *N*-acetyltransferase 1 expression and downregulation of ATP-binding cassette B1 and solute carrier organic anion 1B1 expression. Combining MA with APAP, ASA, or SV elevated *CYP2C19* expression, and MA and ASA coinduced the expression of *CYP2D6*. Coadministration of MA with APAP or ASA increased *UGT1A6* expression. C3G and rutin did not affect the expression of any tested genes. Consequently, MA is recommended to be taken at a low dosage due to the feasibility of MA HDIs arising from concomitant use with APAP, ASA, SV, or caffeine.

INTRODUCTION

White mulberry (*Morus alba* Linn.; MA) from the Moraceae family has been used in traditional medicine to treat ailments such as blurred vision, fever, high blood pressure, joint stiffness, and urinary tract obstruction (Bae and Suh, 2007). MA contains many bioactive components, including the anthocyanin cyanidin 3-glucoside (C3G) and the flavonoid rutin. C3G has been shown to prohibit invasion and migration of A549 human lung cancer cells by inhibiting the expression of matrix metalloproteinase-2 and the urokinase-plasminogen activator and increasing the expression of the tissue inhibitor of matrix metalloproteinase-2 and the plasminogen activator inhibitor (Chen *et al*., 2006). In addition, C3G was shown to protect against oxygen–glucose deprivation and glutamate-induced cell death in DIV-7 rat primary cortical neurons (Bhuiyan *et al*., 2011). Rutin and MA have also been shown to possess potential pharmacological activity; rutin lowered plasma glucose levels in a type II diabetes rat model (Hunyadi *et al*., 2012), and MA exhibited antihypertensive activity via the inhibition of the angiotensin I-converting enzyme in male Wistar–Kyoto rats (Yang *et al*., 2012).

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The metabolism of xenobiotics primarily occurs in the liver through the combined action of phase I cytochrome P450 (CYP) enzymes such as *CYP1A2*, *CYP2C19*, *CYP2D6*, and *CYP3A4*; phase II transferases such as sulfotransferase 1A1 (*SULT1A1*), *N*-acetyltransferase 1 (*NAT1*), and UDP-glucuronosyltransferase 1A6 (*UGT1A6*); and transporters such as ATP-binding cassette B1 (*ABCB1*) and solute carrier organic anion 1B1 (*SLCO1B1*) (Estudante *et al*., 2013; Meyer, 1996). Hence, herbal supplements that interfere with the normal activity of these enzymes and transporters could cause herb–drug interactions (HDIs).

Acetaminophen (APAP) is a common antipyretic and analgesic drug. APAP overdose causes hepatotoxicity due to the production of a toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), during *CYP1A2*, *CYP2E1*, and *CYP3A4*-mediated metabolism (Toes et al., 2005). Aspirin, or acetylsalicylic acid (ASA), is an antiplatelet agent prescribed for the prevention of cardiovascular events (Ittaman *et al*., 2014). ASA is hydrolyzed to salicylic acid by carboxylesterases in the liver and intestine (Miners, 1989). Simvastatin (SV) is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor used as an antihypercholesterolemic agent. It is metabolized in the liver by *CYP3A4*, *CYP2C8*, and *CYP2D6* (Prueksaritanont *et al*., 2003). A cup of coffee contains approximately 120 mg of the psychoactive agent caffeine (CF; methylxanthine), and at these levels, it is principally metabolized by *CYP1A2*, but at high concentrations, it is also metabolized by *CYP2D6* (Belayneh and Molla, 2020). Concomitant use of any of these agents with herbal supplements that affect CYP metabolism may therefore initiate or increase the risk of an HDI. This study aimed to investigate the impact of MA combined with APAP, ASA, SV, and CF on the expression of the pharmacologically important enzymes and transporters *CYP1A2*, *CYP2C19*, *CYP2D6*, *CYP3A4*, *SULT1A1*, *NAT1*, *UGT1A6*, *ABCB1*, and *SLCO1B1* in HepG2 cells.

MATERIALS AND METHODS

Reagents

Cyanidin-3-O-glucoside (C3G) and rutin were supplied by Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China). Dulbecco's modified Eagle medium (DMEM) (+) phenol red, DMEM/F12 phenol red-free medium, fetal bovine serum, 1× penicillin, streptomycin, and neomycin antibiotics, and 1× GlutaMAX® were supplied by Gibco® (Thermo Fisher Scientific, MA). ReverTra Ace® and random primers were products of Toyobo Co., Ltd. (Osaka, Japan). Primers were synthesized by Bio Basic, Inc. (Markham, Ontario, CA). Taq polymerase and RNase inhibitor were products of Vivantis Technologies Sdn. Bhd. (Selangor, Malaysia). Other chemicals were obtained from commercial suppliers.

Preparation of mulberry fruit (MA) powder

Mulberry fruits (Panya, T. 8 KKU No. 25978) were washed in cold water 3× and dried in a hot air oven at 50°C until the moisture content was 10%. The dried fruits were ground and sieved (60 mesh) before storage in a vacuum aluminum package. The contents of C3G and rutin were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC) using a Hypersil octadecyl-silica (ODS) column (4.0 × 250 mm, 5 µm, Agilent Technologies Inc., Santa Clara, CA) with gradient elution of 5%–80% acetonitrile in 0.1% phosphoric acid for 25 minutes at a flow rate of 1 ml/minute coupled with a dual wavelength-UV detector at 517 (for C3G) and 365 nm (for rutin). The MA contained 7.85% C3G and 7.65% rutin dry weight, respectively.

Cell culture

HepG2 cells (ATCC® HB-8065, Manassas, VI) were seeded in 24-well plates (2.5×10^5 cells/well) at sterile conditions of 37°C, 5% CO_2, and 95% relative humidity for 48 hours. The cells were incubated with 0.2% dimethyl sulfoxide (control), ketoconazole or rifampicin (10 µM), C3G or rutin (1 and 10 µM), MA (125–500 µg/ml), APAP or ASA (5 mM), 10 µM SV, and 50 µM CF for 72 hours. The cells were harvested for total RNA extraction, and mRNA expression was determined using reverse transcription-quantitative polymerase chain reaction (RT/qPCR).

Determination of mRNA expression by RT/qPCR

Total RNA was extracted from the cells as previously described (Chatuphonprasert *et al*., 2015). Concentration and purity of total RNA were assessed using a NanoDrop 2000c UV-Vis Spectrophotometer (NanoDrop Technologies, Inc., Thermo Fisher Scientific, MA) at 260/280 and 260/230 nm, respectively. Total RNA integrity was examined by 1.25% agarose gel electrophoresis and then converted to cDNA using ReverTra Ace® (Toyobo Co. Ltd., Osaka, Japan) at 25°C for 10 minutes, 42°C for 60 minutes, and 95°C for 5 minutes. cDNA was multiplied to analyze the expression of phase I, namely *CYP1A2*, *CYP2C19*, *CYP2D6*, and *CYP3A4*, phase II, namely *SULT1A1*, *NAT1*, and *UGT1A6*, transporters, namely, *ABCB1* and *SLCO1B1*, and the reference *GAPDH* with specific primers for each gene (Table 1) under conditions recommended by the supplier. The mRNA level was normalized to that of *GAPDH* and expressed as relative fold expression using the delta–delta Ct method as previously described (Chatuphonprasert *et al*., 2020).

Statistical analysis

The results are expressed as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance with Tukey's statistical *post-hoc* test. SPSS version 28.0 (Armonk, NY) was applied for the statistical analysis. Statistical difference was set at $p < 0.05$.

RESULTS

Modification of phase I metabolizing genes by mulberry fruit (MA) in HepG2 cells

Expression of *CYP1A2* mRNA was upregulated 4.2- and 13.7-fold by 250 and 500 µg/ml MA, respectively, in HepG2 cells after 72 hours treatment ($p < 0.05$), whereas C3G and rutin did not affect *CYP1A2* expression. *CYP1A2* expression was also upregulated in a dose-dependent manner after cotreatment of HepG2 cells with APAP and 500 µg/ml MA (5.8-fold), ASA and 250 or 500 µg/ml MA (6.0- and 8.5-fold, respectively), SV and 250 or 500 µg/ml MA (4.5- and 6.7-fold, respectively), and CF and 125, 250, or 500 µg/ml MA (3.8-, 6.1-, and 9.3-fold, respectively) ($p < 0.05$) (Fig. 1A). The expression levels of *CYP2C19*, *CYP2D6*,

Table 1. Primer sequences used for RT/qPCR.

Genes		Primers (5′→3′)	Annealing temp. (°C)	Product size (bp)
CYP1A2	F	ACAAGGGACACAACGCTGAA	60	160
	R	AGGGCTTGTTAATGGCAGTG		
CYP2C19	F	GGATTGTAAGCACCCCCTG	60	174
	R	TAAAGTCCCGAGGGTTGTTG		
CYP2D6	F	AGCTTTCTGGTGACCCCATC	61.1	135
	R	GGACCCGAGTTGGAACTACC		
CYP3A4	F	CTTCATCCAATGGACTGCATAAA	55	87
	R	TCCCAAGTATAACACTCTACACACACA		
SULT1A1	F	GTCACCGAGCTCCCATCTTC	60	76
	R	GTCTCCATCCCTGAGGGAATC		
NAT1	F	GAATTCAAGCCAGGAAGAAGCA	60	151
	R	TCCAAGTCCAATTTGTTCCTAGACT		
UGT1A6	F	AGCCCAGACCCTGTGTCCTA	58.2	76
	R	CCACTCGTTGGGAAAAAGTCA		
ABCB1/ P-glycoprotein	F	GGGATGGTCAGTGTTGATGGA	60	110
	R	GCTATCGTGGTGGCAAACAATA		
SLCO1B1/ OATP1B1	F	GAATGCCCAAGAGATGATGCTT	60	154
	R	AACCCAGTGCAAGTGATTTCAAT		
GAPDH	F	CACCATCTTCCAGGAGCGAG	61.1	72
	R	GACTCCACGACGTACTCAGC		

and *CYP3A4* mRNA were not modified by C3G, rutin, or MA alone (Fig. 1B, C, and D), and APAP, ASA, SV, and CF alone did not alter the expression of *CYP2C19*. However, cotreatment of HepG2 cells with MA and either APAP (5.8–8.0-fold), ASA (6.0–6.8-fold), or SV (6.0–7.8-fold) did upregulate the expression of *CYP2C19* (Fig. 1B). The expression of *CYP2D6* mRNA was upregulated 5.7-fold by ASA alone and 2.3–5.1-fold after cotreatment with MA and ASA ($p < 0.05$) (Fig. 1C). Cotreatment of MA and either APAP, CF, or SV did not significantly change *CYP2D6* expression. The expression of *CYP3A4* was not modified either by MA alone or by cotreatment with all of the tested agents (Fig. 1D). The known CYP modifiers ketoconazole and rifampicin differently affected CYP expression in HepG2 cells. Ketoconazole elevated *CYP1A2* (Fig. 1A) and *CYP2D6* (Fig. 1C) expression by 8.7- and 2.5-fold, respectively, while rifampicin induced *CYP3A4* expression by 2.6-fold (Fig. 1D). *CYP2C19* expression was not changed by either ketoconazole or rifampicin (Fig. 1B).

Modification of phase II metabolizing genes by mulberry fruit (MA) in HepG2 cells

After 72 hours, expression of *SULT1A1* mRNA was unchanged in HepG2 cells by treatment with either MA alone or in combination with any of the tested agents, while the known CYP modifiers ketoconazole and rifampicin elevated *SULT1A1* expression by 3.0- and 1.6-fold, respectively ($p < 0.05$) (Fig. 2A). The expression of *NAT1* mRNA was not altered by treatment with either of the known CYP modifiers, by C3G, rutin, and MA, or by all the tested agents. However, *NAT1* expression was upregulated in HepG2 cells by cotreatment with APAP and 500 µg/ml MA (11.5-fold), ASA and 250 or 500 µg/ml MA (5.9- and 19.2-fold, respectively), SV and 125–500 µg/ml MA (10.4-, 13.7-, and 12.8-fold, respectively), and CF and 125–500 µg/ml MA (9.1-, 11.4-, and 5.4-fold, respectively) ($p < 0.05$) (Fig. 2B). The expression of *UGT1A6* mRNA was not altered by rifampicin, C3G, or rutin (Fig. 2C), but MA dose-dependently upregulated *UGT1A6* expression resulting in a significant 5.1-fold induction with 500 ug/ml MA ($p < 0.05$). In addition, *UGT1A6* expression was induced by ketoconazole (2.0-fold), by APAP alone (3.9-fold) and with MA (2.6–8.4-fold), and by ASA with MA (1.8–3.2-fold). On the contrary, SV and CF suppressed the expression of *UGT1A6* mRNA approximately 0.1-fold ($p < 0.05$).

Modification of transporter genes by mulberry fruit (MA) in HepG2 cells

MA, C3G, and rutin did not modify the expression of *ABCB1* and *SLCO1B1* mRNA in HepG2 cells after 72 hours treatment (Fig. 3A and B). The expression of *ABCB1* was suppressed in HepG2 cells by cotreatment with MA and APAP (0.22–0.33-fold) or ASA (0.26–0.38-fold). *ABCB1* expression was suppressed by SV itself (0.26-fold), SV with MA (0.30–0.43-fold), CF itself (0.16-fold), and CF with MA (0.22–0.23-fold) ($p < 0.05$) (Fig. 3A). The expression of *SLCO1B1* was suppressed by cotreatment with MA and APAP (0.21–0.30-fold) and by treatment with ASA alone (0.28-fold), SV alone (0.24-fold), and CF alone (0.18-fold). Cotreatment of HepG2 cells with MA and ASA (0.24–0.36-fold), MA and SV (0.26–0.29-fold), and MA and CF (0.11–0.22-fold) also suppressed *SLCO1B1* expression ($p<0.05$) (Fig. 3B). Ketoconazole elevated *ABCB1* expression 3.7-fold and *SLCO1B1* expression 2.0-fold, and rifampicin induced *SLCO1B1* expression 1.8-fold ($p < 0.05$).

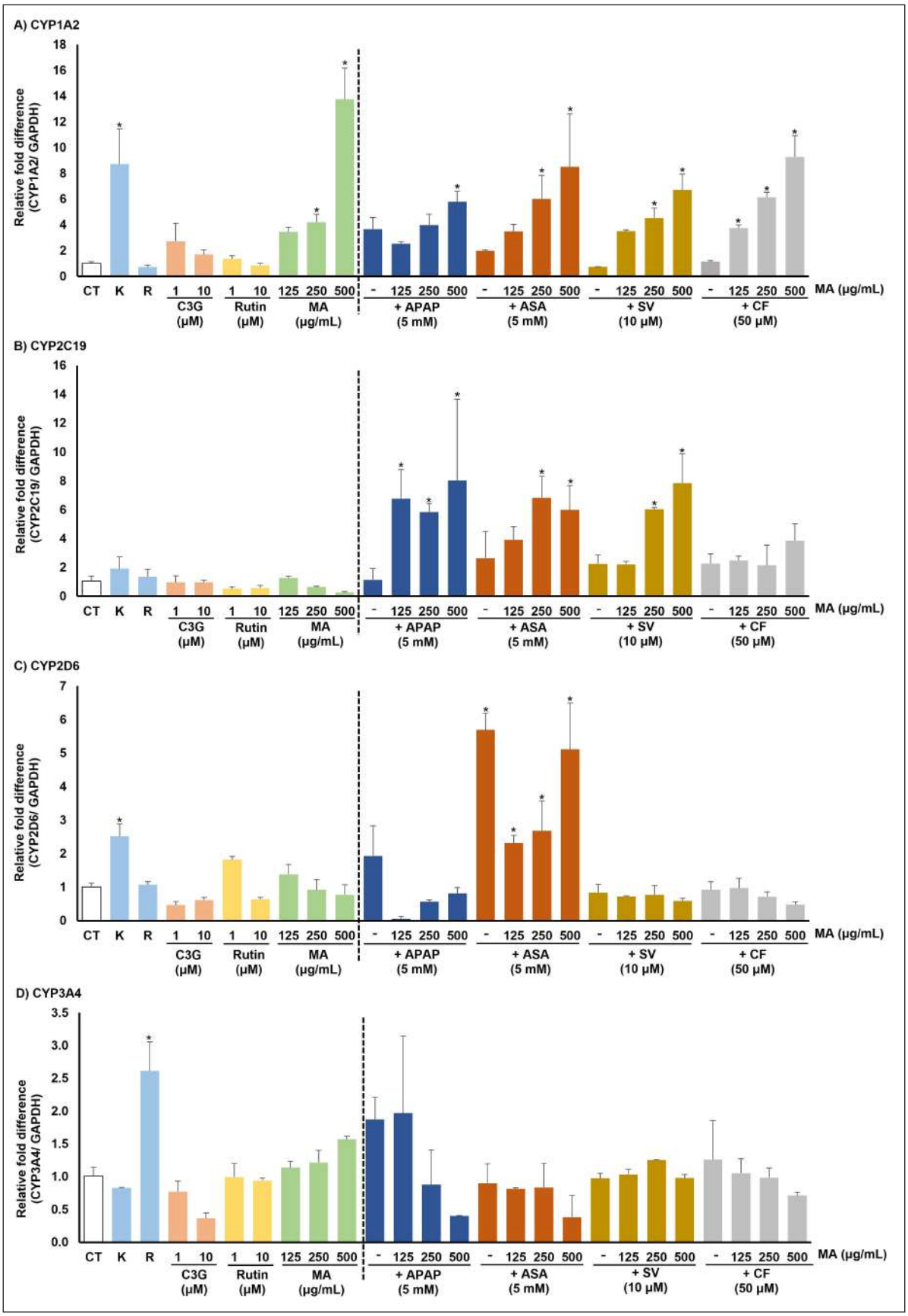

Figure 1. Modification of phase I metabolizing genes by mulberry fruit (MA) in HepG2 cells. CT, 0.2% DMSO (control); K, 10 μM ketoconazole; R, 10 μM rifampicin; C3G, cyanidin 3-glucoside; APAP, acetaminophen; SV, simvastatin; CF, caffeine. *$p < 0.05$ versus CT. $n = 4–5$.

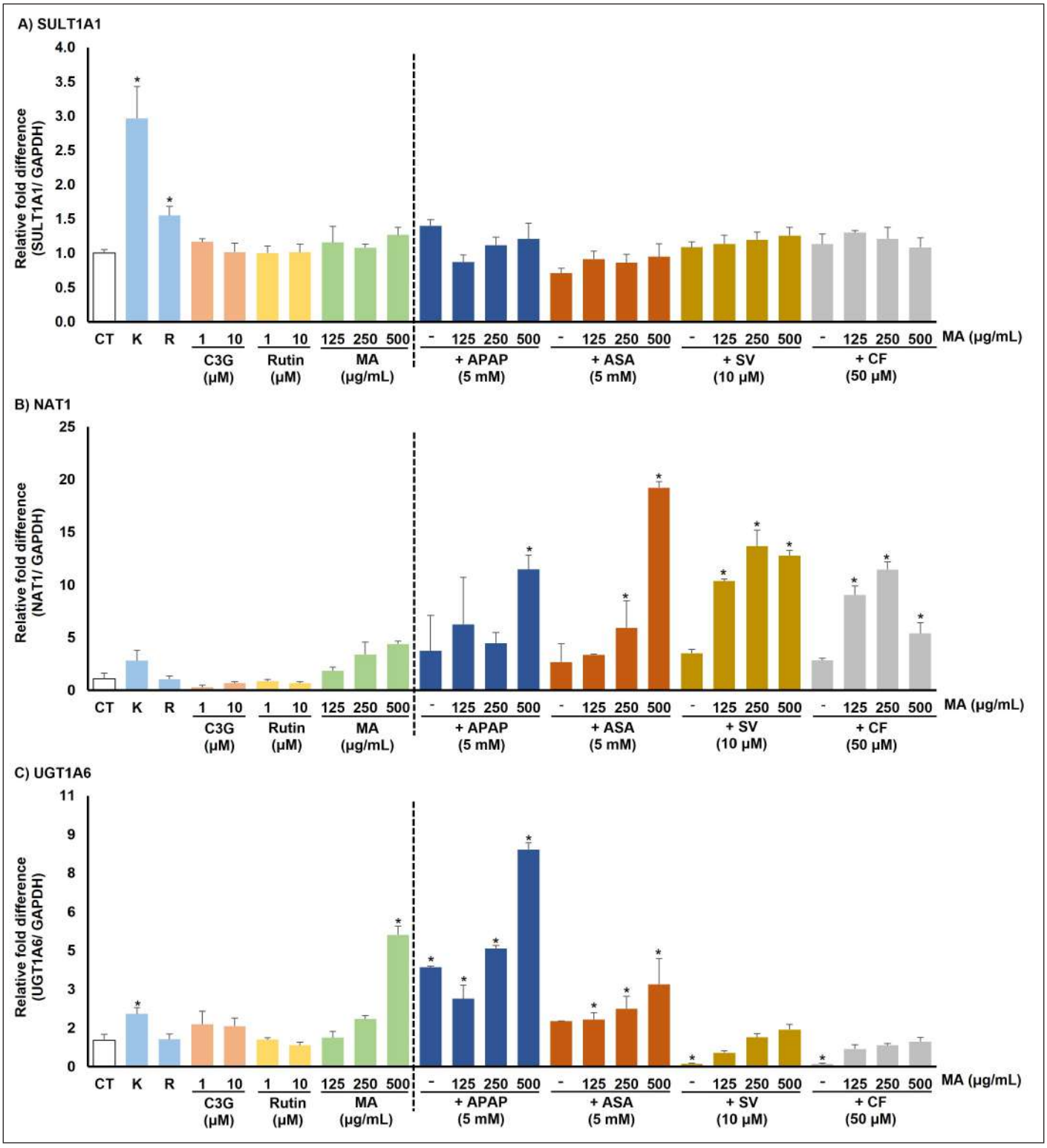

Figure 2. Modification of phase II metabolizing genes by mulberry fruit (MA) in HepG2 cells. CT, 0.2% DMSO (control); K, 10 µM ketoconazole; R, 10 µM rifampicin; C3G, cyanidin 3-glucoside; APAP, acetaminophen; SV, simvastatin; CF, caffeine. *$p < 0.05$ versus CT. $n = 4–5$.

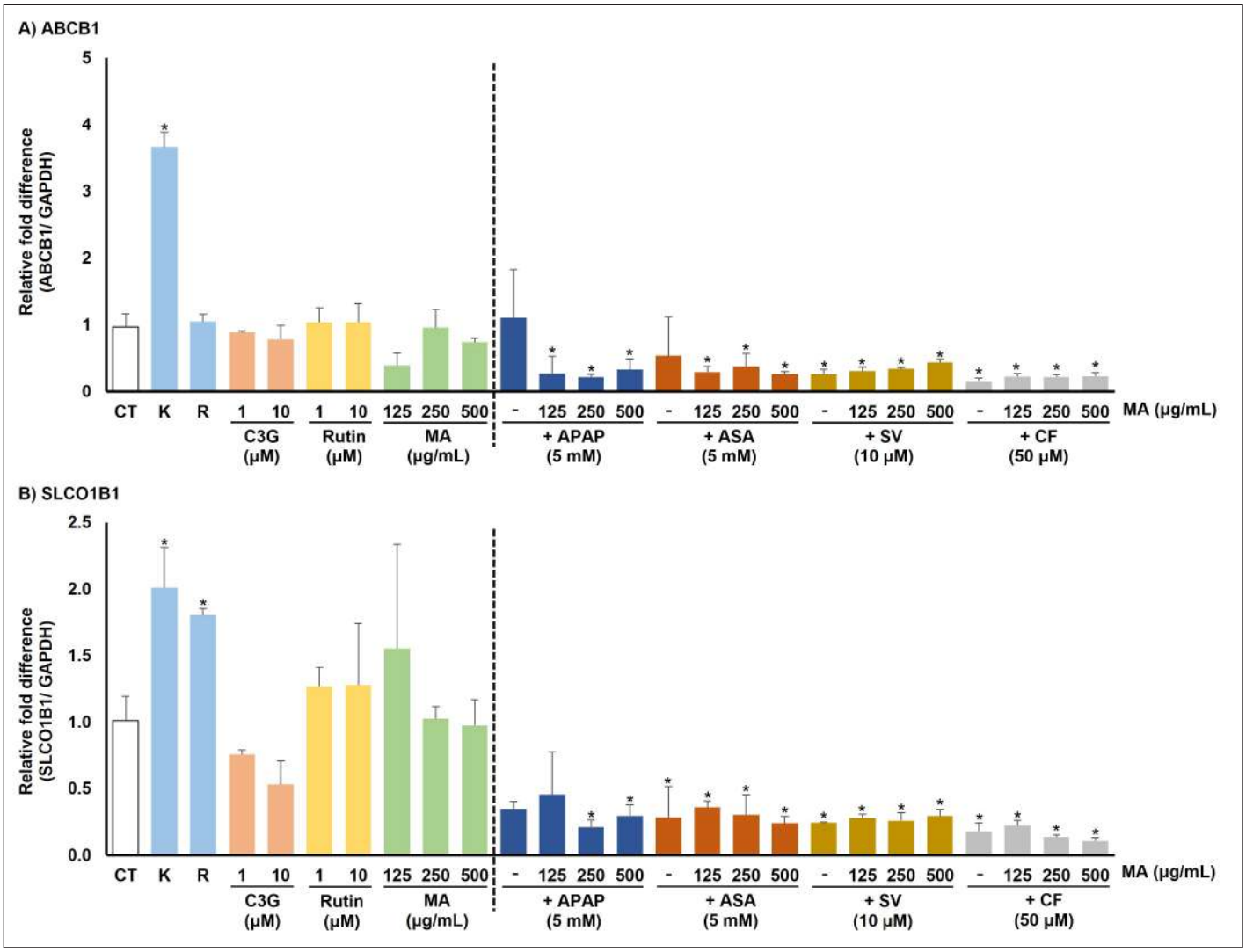

Figure 3. Modification of transporter genes by mulberry fruit (MA) in HepG2 cells. CT, 0.2% DMSO (control); K, 10 μM ketoconazole; R, 10 μM rifampicin; C3G, cyanidin 3-glucoside; APAP, acetaminophen; SV, simvastatin; CF, caffeine. *$p < 0.05$ versus CT. $n = 4–5$.

DISCUSSION

Coadministration of MA with *CYP1A2*-responsive drugs, such as antipsychotics (e.g., chlorpromazine, clozapine, and olanzapine), naproxen, theophylline, propranolol, and CF (Zanger and Schwab, 2013), or with UGT1A6-responsive drugs, such as valproic acid, APAP, and morphine (Kiang *et al*., 2005), could result in subtherapeutic levels of these drugs due to the upregulation of *CYP1A2* and *UGT1A6* expression by MA and subsequent increased rate of metabolism and elimination of these substrates. On the other hand, increased metabolism *CYP1A2*-responsive prodrugs, such as flutamide, melatonin, and retinoic acid, resulting from coadministration of MA might result in supratherapeutic levels of the pharmacologically active agent (Kang *et al*., 2008; Nebert and Dalton, 2006). Furthermore, concomitant use of MA with any drug substrates of phase I, *CYP1A2*, *CYP2C19 and CYP2D6*, and phase II, *NAT1* and *UGT1A6*, metabolizing genes, and transporters *ABCB1* and *SLCO1B1* presents a risk for an HDI. The upregulation of *CYP1A2* and *NAT1* expression by MA combined with APAP, ASA, SV, or CF could result in HDIs with the concomitant use of drugs that are substrates of *CYP1A2* or *NAT1*, such as *p*-aminosalicylic acid (Kawamura *et al*., 2005). Importantly, the induction of *CYP1A2* might bring about APAP hepatotoxicity. At therapeutic levels, APAP is primarily metabolized via phase II glucuronidation and sulfation with the remainder biotransformed through *CYP1A2*, *CYP2E1*, and *CYP3A4* metabolism into a toxic byproduct, NAPQI, that is eventually detoxified by the glutathione system (Toes et al., 2005). The upregulation of *CYP1A2* mRNA could therefore promote the generation of NAPQI, which would result in excessive generation of reactive oxygen species and initiate lipid peroxidation of cell membranes, resulting in necrosis and/or apoptosis of hepatocytes (Jin and Park, 2012). Notably, the upregulation of *UGT1A6* by either MA or APAP would be likely to reverse toxicity from NAPQI accumulation since glucuronidation of APAP is regulated by *UGT1A1*, *UGT1A6*, and *UGT1A9* (Walter Bock and Köhle, 2005). Not only is caffeine a specific substrate of *CYP1A2*, but also it can inhibit *CYP1A2* activity. One report demonstrated delayed metabolism of anticancer (methotrexate), antidepressant (e.g., amitriptyline, fluvoxamine, and imipramine), antipsychotic (e.g., clozapine, haloperidol, and olanzapine), and cardiovascular (e.g., propranolol, verapamil, and warfarin) drugs after concomitant use with caffeine, due to their common

metabolizing enzymes (Carrillo and Benitez, 2000). Hence, the coconsumption of caffeine and MA might boost the risk of HDIs due to their synergistic interference with *CYP1A2* metabolism. Additionally, *CYP1A2* and *NAT1* are closely involved in the biotransformation of arylamine procarcinogens into active carcinogens. High levels of *CYP1* expression in ER-independent cancers increase the formation of reactive arylamines, which are a particular cause of bladder toxicity and magnify the prospect of bladder cancer (Go *et al*., 2015). The NAT-catalyzed acetylation of arylamine is associated with carcinogenesis in various organs. For example, the overexpression of *NAT1* has been observed in estrogen receptor-positive breast cancer (Sim *et al*., 2014). Consequently, regardless of any potential HDIs, MA might also promote arylamine-induced carcinogenesis via induction of *CYP1A2* and *NAT1* expression. On the contrary, since arylamine is also a *UGT1A6* substrate (Walter Bock and Köhle, 2005) and *UGT1A6* is broadly expressed in many organs of the body, including the esophagus, intestine, kidneys, liver, skin, and urinary bladder, upregulation of *UGT1A6* by MA could also feasibly reverse carcinogenesis (Fagerberg *et al*., 2014).

Cotreatment of MA with APAP, ASA, or SV elevated expression of *CYP2C19*, which could lead to subtherapeutic levels of co-administered *CYP2C19*-responsive drugs, such as antidepressants (e.g., amitriptyline and imipramine), voriconazole, progesterone, and omeprazole (Zanger and Schwab, 2013). On the other hand, the antiplatelet prodrug clopidogrel, which needs bioactivation by the *CYP2C19* enzyme to become active, might enhance inhibition of platelet reactivity when co-administered with MA and the tested drugs, leading to the risk of bleeding (Sibbing *et al*., 2010). Previously, *CYP2C19* activity was shown to be increased by low-dose ASA (50 mg/day) over 7 days in male nonsmoking healthy volunteers (Chen *et al*., 2003). In this study, induction of *CYP2C19* was not observed by ASA alone, but it was clearly noted after cotreatment with MA. Hence, MA might synergize with ASA to enhance *CYP2C19* activity.

The expression of *CYP2D6* was significantly induced by cotreatment with MA and ASA. Therefore, the combined use of MA and ASA with *CYP2D6* substrates, such as the antidepressant amitriptyline, antihistamines (e.g., diphenhydramine and loratadine), antipsychotics (e.g., aripiprazole, chlorpromazine, and risperidone), cardiovascular drugs (e.g., carvedilol, metoprolol, and propranolol), and opioid derivatives (e.g., codeine, dextromethorphan, and tramadol), might expedite metabolism and elimination of these drugs (Zanger and Schwab, 2013).

The expression of *ABCB1* and *SLCO1B1* was suppressed by cotreatment with MA and the tested drugs. *ABCB1*, or multidrug resistance 1 (*MDR1*) encoded P-glycoprotein, is an efflux transporter of harmful xenobiotics and drugs that is located in the intestinal epithelium. Hence, downregulation of *ABCB1* might increase the toxicity of some of its substrates, including chemotherapeutics (e.g., cyclosporine, doxorubicin, methotrexate, paclitaxel, and vinblastine), the antigout agent colchicine, the cardiac glycoside digoxin, and hydrocortisone (Estudante *et al*., 2013). Moreover, some multidrug-resistant cancers utilize *ABCB1* as a tool for chemotherapeutic transportation, resulting in resistance to chemotherapeutics such as doxorubicin, paclitaxel, topotecan, and vinblastine (Sharom, 2008). The suppression of *ABCB1* expression by cotreatment with MA and APAP, ASA, SV, or CF could be employed as a strategy against this type of MDR. *SLCO1B1* encodes the organic anion transporter polypeptide 1B1 protein, which is a liver-specific influx transporter of endogenous compounds and drugs. Coadministration of MA with the tested drugs could delay the onset of action and/or decrease the efficacy of drugs that use this transporter, such as antibiotics (e.g., cefazolin and rifampicin), the anticancer agent methotrexate, antihypertensive drugs (e.g., enalapril and valsartan), protease inhibitors (e.g., efavirenz and nevirapine), and HMG-CoA reductase inhibitors (e.g., atorvastatin, pravastatin, and rosuvastatin) (Nakanishi and Tamai, 2012).

It is important to note that HepG2 cells have been developed as a surrogate for human hepatocytes in hepatotoxicity screening to overcome the limited availability and phenotypic instability of primary human hepatocyte cultures (Gerets *et al*., 2012). Nevertheless, various transcriptional regulators in HepG2 cells are known to be expressed at lower levels, including the aryl hydrocarbon receptor, the constitutive androstane receptor, the glucocorticoid receptor, the pregnane X receptor, and the vitamin D receptor (Aninat *et al*., 2006; Tolosa et al., 2016). Therefore, the expression of metabolizing genes in HepG2 cells, including *CYP1A1*, *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, and *CYP3A4*, might deviate from primary hepatocytes, and the present findings should be confirmed in further *in vivo* and clinical studies.

CONCLUSION

Although MA has been developed as an herbal supplement with promising pharmacological properties, consumption of MA with other pharmaceuticals might give rise to HDIs and undesirable effects through its upregulation of *CYP1A2* and *UGT1A6*. Furthermore, the concomitant use of MA with APAP, ASA, SV, or caffeine extensively modified the expression of several phase I (*CYP1A2*, *CYP2C19*, and *CYP2D6*) and phase II (*NAT1* and *UGT1A6*) metabolizing enzymes and transporters (*ABCB1* and *SLCO1B1*). In order to avoid potential HDIs, the use of MA at a low dose and with low frequency is recommended, particularly when used concomitantly with APAP, ASA, SV, or caffeine.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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AUTHORS' CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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