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ORIGINAL ARTICLE

Alteration of Murine Cytochrome P450 Profiles in Fatty Liver Disease by Hesperidin and Myricetin

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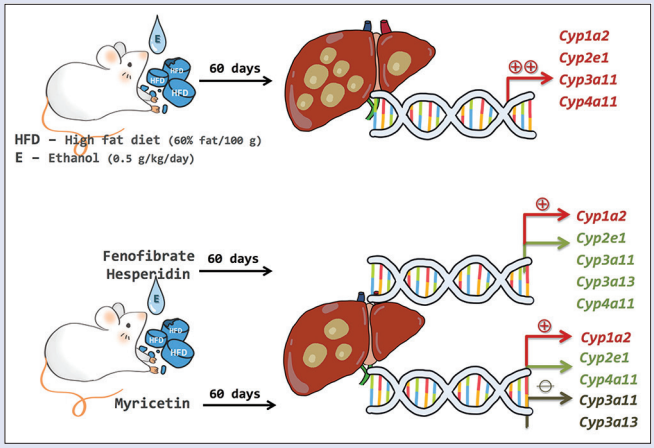
ABSTRACT

Background: Hesperidin and myricetin are anti-inflammatory and anti-oxidant flavonoids that have beneficial effects in fatty liver disease (FLD), but information regarding their effects on cytochrome P450 (CYP450) enzymes in FLD is limited. **Objectives:** This study determined the impacts of hesperidin and myricetin on CYP450 profiles in FLD mice. **Materials and Methods:** Adult female mice were fed a high fat diet (HFD, 60 kcal % fat of total food) with daily intragastrically administered ethanol (0.5 g/kg/day) in combination with either fenofibrate (40 mg/kg/day), hesperidin (50 and 200 mg/kg/day), or myricetin (50 and 200 mg/kg/day) for 60 consecutive days. Liver histomorphology was examined by oil red O staining. Hepatic enzyme activity and mRNA expression of CYP1A2, CYP2E1, CYP3A11, CYP3A13, and CYP4A11 were assessed. **Results:** HFD-induced hepatocellular damage was prevented by low dose hesperidin and myricetin. Expression of *Cyp1a2*, *Cyp2e1*, and *Cyp4a11* mRNAs as well as CYP2E1 and CYP3A activities was significantly induced by HFD plus ethanol (HE) while *Cyp3a13* expression was slightly increased. Fenofibrate and low dose hesperidin prevented HE-induced *Cyp1a2* and *Cyp2e1* expression while HE-induced *Cyp4a11* expression was prevented by all treatments. The expression of *Cyp3a13* was extensively suppressed by high-dose hesperidin and myricetin, and CYP2E1 and CYP3A activities were significantly decreased by all treatments. **Conclusion:** Alteration of CYP450 profiles by high doses of hesperidin and myricetin could lead to drug interactions. Nevertheless, low dose hesperidin prevented dysregulation of CYP450 expression in FLD and is a promising candidate for FLD treatment.

Key words: Drug interaction, ethanol, flavonoids, high fat diet, lipid metabolism

SUMMARY

- The most common mechanism of herb-drug interactions is induction/inhibition of cytochrome P450 (CYP450s), which is also a factor involved in fatty liver disease (FLD) progression. This study determined the impact of hesperidin and myricetin on CYP450 profiles in FLD mice. Female Institute for Cancer Research mice were fed a high fat diet (HFD) with daily intragastrically administered ethanol (0.5 g/kg/day) in combination with fenofibrate (40 mg/kg/day), hesperidin or myricetin (50 and 200 mg/kg/day) for 60 days. The results showed that HFD-induced hepatocellular damage was prevented by low dose hesperidin and myricetin. Expression of *Cyp1a2, Cyp2e1,* and *Cyp4a11* mRNAs as well as CYP2E1 and CYP3A activities was significantly induced by HFD plus ethanol (HE) and *Cyp3a13* expression was slightly increased. Fenofibrate and low dose hesperidin prevented HE-induced increases in *Cyp1a2* and *Cyp2e1* expression and all treatments prevented HE-induced *Cyp4a11* expression. Expression of *Cyp3a13* was extensively suppressed by high dose hesperidin and myricetin, and CYP2E1 and CYP3A activities were significantly decreased by all treatments. These alterations of CYP450 profiles by high dose hesperidin and myricetin could lead to drug interactions. Nevertheless, low dose hesperidin improved CYP450 profiles to control FLD progression. Therefore, low dose hesperidin is a promising candidate for FLD treatment.



Abbreviations used: CYP450: Cytochrome P450; ER: Endoplasmic reticulum; FLD: Fatty liver disease; HFD: High fat diet; ORO: Oil red O.

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INTRODUCTION

Recent evidence showed that the use of herbal plants as alternative medicines has become increasingly common leading to an increased awareness in herb-drug interactions.[1] The most related mechanism of herb-drug interactions is an inducer or inhibitor of cytochrome P450 (CYP450), a major superfamily of hepatic metabolizing enzymes for endogenous and exogenous substances, which located in endoplasmic reticulum (ER).[2,3]

Modulation of CYP450 was considered as the factor that involved in the progression of fatty liver disease (FLD) despite sex, gender, hepatic lipid deposition, and dysfunction of mitochondrial or ER.[4] Induction or inhibition in CYP450 enzymes causes inflammation and produce excess reactive oxidative species (ROS). An imbalance of this

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oxidant-antioxidant system leads to an increase of the oxidant portion and might generate FLD.[5]

Natural compounds have been found to possess significant therapeutic benefits against FLD, which flavonoids have been frequently been study in the model of FLD and seem to approach beneficial effects is required to be revealed.[6] The consumption of flavonoids associated with several health benefits through antioxidant activity, anti-inflammation, anticancer, and antiglycation.[7] Hesperidin and myricetin are anti-inflammatory and anti-oxidant flavonoids that may attenuate the progression of FLD. This study determined the effects of hesperidin and myricetin on the activity and expression of CYP1A2, CYP2E1, CYP3A11, CYP3A13, and CYP4A11 in FLD-induced mice.

MATERIALS AND METHODS

Chemicals and reagents

High fat diet (HFD) with 60 kcal % fat of total food (cas no. D12492) was a product of Research Diets (New Brunswick, USA). Ethanol was purchased from Merck (Frankfurt, Germany). Myricetin (cas no. 529-44-2) and hesperidin (cas no. 520-26-3) with purity > 95% were procured from Chengdu Biopurify (Chengdu, China). Oil red O (ORO) was purchased from Sigma-Aldrich (Missouri, USA). ReverTra Ace® was a product of Toyobo Co., Ltd. (Osaka, Japan). Taq DNA polymerase was from Vivantis® (Selangor, Malaysia). All other laboratory chemicals were of the highest purity from commercial suppliers.

Animal treatments

Five-week-old female Institute for Cancer Research (ICR) mice (17.30 ± 3.15 g) were obtained from the Northeast Laboratory Animal Center (Khon Kaen University, Khon Kaen, Thailand) and housed in the Animal Unit of Faculty of Pharmaceutical Sciences, Khon Kaen University under controlled conditions at the temperature of 23°C ± 2°C and humidity of 45% ± 2%. The Institutional Animal Care and Use Committee of Khon Kaen University approved the protocol for handling and treatment of the animals (Approval no. IACUC-KKU-12/63). At all times, the mice were housed on corncob bedding in stainless steel cages with *ad libitum* access to water and food. The mice (n = 5 for each group) were randomly divided into control, HFD plus ethanol (0.5 g/kg/day, i.g.,) (HE), HE with fenofibrate 40 mg/kg/day, i.g., (F), HE with hesperidin 50 or 200 mg/kg/day, i.g., (H50/200), HE with myricetin 50 or 200 mg/kg/day, i.g., (M50/200) and treated for 60 consecutive days. At 1 day after the last treatment, the mice were euthanized with 100 mg/kg intraperitonial Zoletil® (250 mg tiletamine HCl and 250 mg zolazepam HCl, Virbac, New Zealand). Plasma and organs were collected and immediately stored at −80°C for further analysis.

Examination of hepatic morphological and histological features by oil red o staining

ORO staining was modified from a previous study.[5] Liver tissue was collected and histomorphology was examined under a light microscope. The microscopic slide with embedded liver tissue was stained with ORO and further evaluated for hepatic histological features at ×20 magnification using a Motic AE2000 inverted microscope (Kowloon, Hong Kong). The image was analyzed and displayed on screen using Motic image plus 3.0 software (Kowloon, Hong Kong).

Determination of CYP3A activity using erythromycin N-demethylase activity

The reaction mixture of erythromycin and microsomes was incubated with 1 mM NADPH to initiate the reaction. Trichloroacetic acid (12.5%) was added to stop the reaction before centrifugation at 1,900 rpm for 15 min. The supernatant was incubated with 30% ammonium acetate for 30 min. The absorbance was measured using a microplate reader (EnSight™ multimode plate reader, Perkin Elmer, California, USA) at a wavelength of 405 nm and compared with the standard formaldehyde.[8]

Determination of CYP2E1 activity using aniline hydroxylase activity

The reaction mixture of 100 mM aniline HCl, 100 mM nicotinamide and 10 mg/mL microsomes was generated. Trichloroacetic acid (20%) was added to stop the reaction before centrifugation at 1900 rpm for 15 min. The supernatant was mixed with 20% Na_2CO_3 and 4% phenol solution. The absorbance was measured using a microplate reader (EnSight™ multimode plate reader) at a wavelength of 630 nm compared with the standard 4-aminophenol.[8]

Determination of methoxyresorufin *O*-demethylase activity

The assessment of methoxyresorufin *O*-demethylase (MROD) was modified from a previous study.[8] The reaction mixture of 10 mg/mL microsomes and 5 mM methoxyresorufin was made and the formation of resorufin was immediately measured by spectrofluorometry using a microplate reader (EnSight™ multimode plate reader) with an excitation wavelength of 520 nm and an emission wavelength of 590 nm compared with the standard resorufin.

Quantitative determination of CYP450 mRNA expressions using RT/real-time polymerase chain reaction

Total RNA was reverse transcribed to cDNA using ReverTra Ace® at 25°C for 10 min, 42°C for 60 min, and 95°C for 5 min. mRNA expression of CYP450 genes, i.e., *Cyp1a2*, *Cyp2e1*, *Cyp3a11*, *Cyp3a13*, *Cyp4a11,* and a reference gene, *Gapdh*, were subjected to qPCR with specific primers (Bio Basic Inc., Toronto, Canada) [Table 1] under conditions recommended by the supplier (Applied Biosystems, Branchburg, NJ, USA) and calculated by $2^{-(\Delta\Delta Ct)}$ to determine a change of fold difference.[5,9,10]

Statistical analysis

The data were analyzed using the one-way analysis of variance followed by Tukey *post hoc* test (IBM SPSS statistics version 23, IBM, Armonk, New York, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Histological and morphological features of hesperidin or myricetin-treated fatty liver disease mouse livers

Histological and morphological features of the mouse liver carcass and ORO-stained sections are shown in Figure 1. Control group demonstrated normal hepatic histomorphology. HE greatly enlarged and discolored the whole liver compared to the control. ORO staining (red staining) demonstrated that HE caused liver injury with cells appearing swollen and containing balloon gaps with the accumulation of disseminated TG. Treatment with Either F, H, or M prevented the HE-induced FLD morphological changes. Low dose H and M attenuated TG accumulation by more than high doses, as shown in the ORO-stained liver histomorphology.

Table 1: Forward and reverse primer sequences

Genes		Forward and reverse primers (5′ → 3′)	$T_{annealing}$ (°C)	References
Cyp1a2	*F*	AAG ATC CAT GAG GAG CTG GA	50.0	Design by primer pair 1 program
	R	TCC CCA ATG CAC CGG CGC TTT CC		
Cyp2e1	*F*	TCC CTA AGT ATC CTC CGT GA	56.0	[9]
	R	GTA ATC GAA GCG TTT GTT GA		
Cyp3a11	*F*	TTT GGT AAA GTA CTT GAG G	64.0	[10]
	R	CTG GGT TGT TGA GGG AAT C		
Cyp3a13	*F*	TGT GTG GCT ATC ACA GAT	55.0	[10]
	R	AAA TAC CCA CTG GAC CAA G		
Cyp4a11	*F*	TGC CCA TGA TCA CAC AGA TGG AG	60.0	Design by primer pair 1 program
	R	TGA ATG TGT CCA CCT CAG CAC GT		
Gapdh	*F*	CCT CGT CCC GTA GAC AAA ATG	57.4	[9]
	R	TGA AGG GGT GGT TGA TGG C		

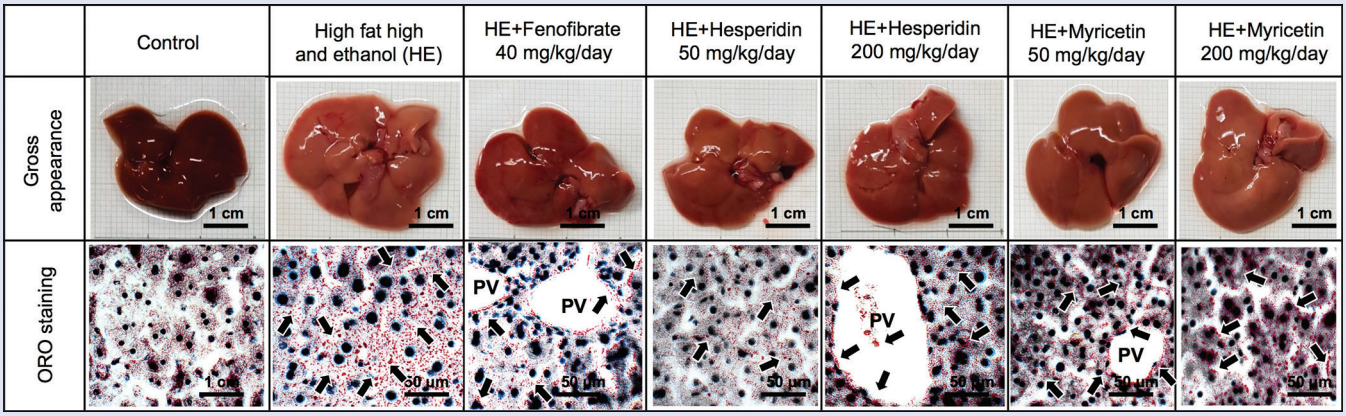

Figure 1: Effects of hesperidin and myricetin on histomorphological features of fatty liver disease induction in the mouse livers. Black arrows indicate lipid accumulation

Cytochrome P450 profiles of hesperidin or myricetin-treated fatty liver disease mouse livers

Alterations in the hepatic CYP450 profiles of hesperidin or myricetin-treated mice following FLD induction are depicted in Figure 2. HE significantly upregulated *Cyp1a2, Cyp2e1, Cyp3a11* and *Cyp4a11* mRNAs and slightly increased *Cyp3a13* expression. Treatment with F, H or M reduced expression of *Cyp1a2, Cyp2e1* and *Cyp4a11* mRNA compared to HE-induced FLD [Figure 2a]. Treatment with F and H restored *Cyp3a11* or *Cyp3a13* expression to uninduced levels, while M suppressed expression of *Cyp3a11* or *Cyp3a13* to levels lower than control. The activities of the CYP1A2, CYP2E1, and CYP3A enzymes are shown in Figures 2b-d. HE sharply increased MROD [Figure 2b], aniline hydroxylase [Figure 2c] and erythromycin *N*-demethylase activities [Figure 2d], representing CYP1A2, CYP2E1, and CYP3A responsive enzymes, respectively, while treatment with F, H, and M prevented all of the HE-induced increases in the enzyme activity. Notably, treatment with M consistently lowered HE-induced CYP3A activity below the control.

DISCUSSION

The prevalence of nonalcoholic FLD in the Northeast of Thailand is higher in women than men at all ages.[11] Hence, female ICR mice were employed in this study. Co-consumption of HFD and ethanol significantly increased liver sizes and changed several hepatic histomorphological features, which correlated with previous study. Hepatomegaly with a discolored appearance marked by TG depositing in the liver.[12] HE induces the unfolded protein response and leads to ER expansion and proliferation, resulting in CYP450 modification.[13,14] The observations in the current study demonstrate that progression of FLD correlated with CYP450 activity and expression, namely *Cyp1a2, Cyp2e1, Cyp3a11, Cyp3a13,* and *Cyp4a11*. Alteration of CYP450s in FLD has been previously reported as induction of *Cyp2e1* and *Cyp4a11* and suppression of *Cyp3a* in humans and mice.[15] Induction of *Cyp2e1* expression in HE-induced FLD has been documented in both rodents and humans.[16] HE-induced FLD revealed the increases in *Cyp1a2* mRNA and protein expression while previous study suggested that HFD decreases in CYP1A2 protein and activity in patients.[17] The current study found that the combination of HFD and ethanol (HE) up-regulated expression of *Cyp2e1* mRNA superior to ethanol alone, which correlates to a study that showed an unsaturated fatty acid-rich diet-induced nonalcoholic steatohepatitis lessened FLD progression compared to ethanol.[16] This indicates that HFD and additional ethanol induces *Cyp2e1*. Induction of *Cyp2e1* produces toxic metabolites coupled with oxidative stress causing liver injury and worsening FLD. Hesperidin has previously demonstrated its antioxidant action in FLD by reducing MDA levels and slowing the progression of fibrosis at doses of 40–500 mg/kg/day.[18] Myricetin increased mitochondria density and decreased NO production in mice at low dose (50 mg/kg/day),[19,20] while doses of 150 and 200 mg/kg/day reduced serum glucose, leptin and MDA levels, suppressed PPAR-γ and SREBP-1c,[21] and attenuated-seizures.[22] Therefore, hesperidin and myricetin doses of 50 and 200 mg/kg/day were employed to examine FLD treatment in this study. Fenofibrate inhibited *Cyp2e1* in the current study, which correlates to a previous study.[23] Low dose hesperidin also showed an inhibitory effect against *Cyp2e1*, whilst higher doses did not. This is

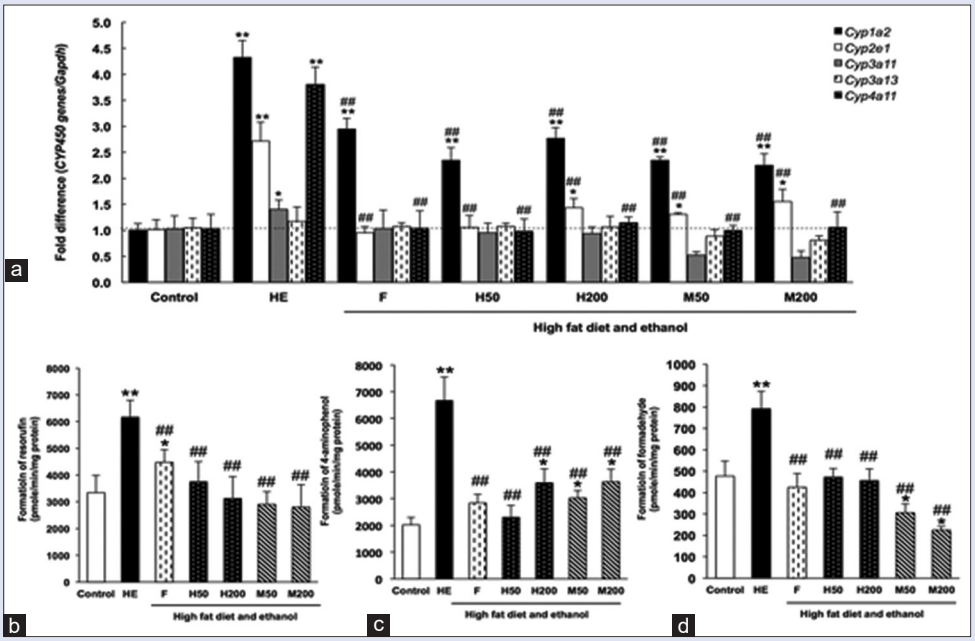

Figure 2: Effects of hesperidin and myricetin on (a) cytochrome P450 mRNA expression, (b-d) CYP1A2, CYP2E1, and CYP3A activities in fatty liver disease induction in the mouse livers. HE: High fat diet + ethanol; F: Fenofibrate; H: Hesperidin; M: Myricetin. *$P < 0.05$, **$P < 0.001$ versus Control; ##$P < 0.001$ versus HE

probably due to saturation of the hesperidin absorption and elimination mechanisms resulting in an increase in hesperetin, an active metabolite of hesperidin that might trigger oxidative stress at high doses.[23] Another CYP450 that accelerates FLD progression and promotes ROS production is *Cyp4a11*. Zhang *et al.* reported up-regulation of hepatic *CYP4A* expression in the livers of FLD patients and in three murine FLD models.[24] The elevation of *Cyp4a11* by HFD and/or ethanol stimulated an increase in inflammatory cytokines.[25] In addition, an increase in *Cyp4a11* up-regulated phosphorylated p65, which is a co-factor of the nuclear factor-κB signaling pathway.[25] Fenofibrate and flavonoids suppressed the expression of *Cyp4a11* mRNA. This suggests that the more inflammatory cytokines decrease, the more *Cyp4a11* expression is down-regulated. *Cyp3a* is responsible for the metabolism of numerous therapeutic substances. HE did not induce *Cyp3a11* and *Cyp3a13* expression, which correlates to the study of Feierman *et al.* (2003) that ethanol more likely induced CYP3A activity rather than HFD, glucose or the mixtures.[26] In addition, it was reported that down-regulation of hepatic *Cyp3a4* expression affected the severity of FLD and increased diabetes phenomena in humans.[27] The decreases in *Cyp3a11* and *Cyp3a13* expression from high dose hesperidin and myricetin suggest that these regimens could not improve FLD status in this model, which correlates to the previous study. Xu *et al.* noted that myricetin-induced ER dysfunction and apoptosis in human cells.[28] This corresponded to the finding of Choi *et al.* that myricetin inhibited CYP3A4-mediated metabolism.[29] Hence, the higher doses of hesperidin and myricetin could not improve CYP450 expression.

CONCLUSION

Low dose hesperidin improved *Cyp1a2*, *Cyp2e1*, *Cyp3a4*, and *Cyp4a11* expression profiles as well as CYP2E1 and CYP3A activities in the female FLD mice to reduce FLD progression. Since flavonoids are increasingly popular as alternative medicines, it is becoming more likely that they are being consumed with modern medicines. Therefore, it is worthwhile to confirm pharmacological functions of hesperidin-associated regulatory pathways of FLD, at least in regard to CYP450-mediated biotransformation.

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Conflicts of interest

There are no conflicts of interest.

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