The induction of cytochrome P450 by *Bacopa monnieri* Standardized extract and its constituents on HepG2 cells

Marisa Khumyat[1], Worasak Kaewkong[2], and Dumrongsak Pekthong[1,*]

[1] Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand
[2] Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand
*Corresponding author, Email: marisak62@nu.ac.th

Abstract

Bacopa monnieri (L) Wettst. (*B. monnieri*) and five major phytoconstituents have been beneficial for neuropharmacological disorders treatment. Herb-drugs interaction (HDIs) could occur due to inhibition or induction of drug metabolism enzymes. It could alter the pharmacokinetics and resulting therapeutic failure. Currently, there have been reported that *B. monnieri* extract (BME) could inhibit drug-metabolizing enzymes using recombinant Cytochrome P450 (rCYPs). However, no study related to the induction effect of the BME using HepG2 cells has been conducted. To achieve this purpose, the induction effect of the BME, as well as five major constituents on six major CYP isoforms, were analyzed using HepG2 cells. The non-cytotoxicity assay at 120 µg/mL of BME was selected to test the potential of BME to interact with the hepatic cytochrome P450 system. As the results of qRT-PCR, BME, bacoside A_3, and bacopaside II could significantly increase the CYP1A2, 2B6, and 3A4 activities and mRNA expression. Besides, bacopaside A_3 and bacopaside II might be the major phytoconstituents that drive the induction effect on CYP1A2, 2B6, and 3A4 in HepG2 cells. Until more data are available for BME, people taking simultaneously medications and BME are advised to exercise with caution since it cannot conclude from the present study whether BME could cause herb-drug or herb-herb interactions in humans, especially through lower CYP1A2, 2B6, and 3A4 activity.

Keywords**:** *Bacopa monnieri, Cytochrome P450, Induction, Herb-drug interaction (HDIs)*

1. Introduction

According to the World Health Organization (WHO), millions of people have concomitantly used herbs with prescribed and non-prescribed medicine for a synergistic effect. Besides, herbal uses in the global market tend to increase from three trillion US dollars in 2006 to five trillion US dollars in 2050 (Pan et al., 2014). However, the combination uses between the herbs and medicine may produce the herb-drug interactions (HDIs), which involve 1) alterations of drug bioavailability or efficacy and 2) chronic medical illness individuality (Eisenberg et al., 1998).

Bacopa monnieri (L) Wettst. or Brahmi is an herb belonging to the family Plantaginaceae. Brahmi is an aquatic-perennial plant found in marshy areas. It has been used in Ayurvedic medicine for the treatment of neuropharmacological disorders including cognitive enhancement and memory improvement (Saesong et al., 2019; Stough C, 2001). The major compounds, which are included bacoside A_3, bacopaside I, bacopaside II, bacopaside X, and bacopasaponin C, are responsible for memory and cognition enhancement (Saroya & Singh, 2018). Besides, *B.monnieri* extracts are found against β-amyloid toxicity (Limpeanchob, Jaipan, Rattanakaruna, Phrompittayarat, & Ingkaninan, 2008) and rich in pharmacological properties such as anti-oxidant (Kapoor, Srivastava, & Kakkar, 2009) and anti-inflammatory effects (Nemetchek, Stierle, Stierle, & Lurie, 2016). At present, this plant has been developed as dietary supplements or herbal medicine due to its good efficacy (Roodenrys S, 2002; Stough C, 2001) and less toxicity (Pravina K, 2007).

Cytochrome P450 (CYPs) is a superfamily of enzymes that play an important role in Phase I metabolism. The expression of individual P450s is regulated by two factors including endogenous factors and foreign compounds that refer to drugs and herbs or natural compounds. Also, co-administration of

herbs and drugs can lead to a pharmacokinetic interaction by induction or inhibition of specific CYP enzymes. For instance, consuming grapefruit juice has shown the inhibition effect considering drug metabolism or changing the plasma concentrations of many drug substrates for CYP3A4 (Nebert DW, 1991; Pekthong et al., 2008). Besides, co-administration of *B. monnieri* extract and anti-platelet drugs (e.g. warfarin, clopidogrel, or aspirin) and other chemical plant groups (e.g. coumarins, quinones, and vitamin K) have been increased the risk of bleeding (Leite PM, 2016). Besides, HDIs caused by consuming *B. monnieri* extracts and another drug concomitantly also occurred *via* drug-metabolizing enzymes reaction. No study related to the induction effect of *B. monnieri* extracts and their phytochemicals on drug-metabolizing enzymes using HepG2 cells has been reported yet.

This study focuses on evaluating the induction effect of *B. monnieri* extracts and five bioactive compounds on drug-metabolizing enzymes to assess drug safety follow the *in vitro* experiment guidelines from the US FDA Guidance for industry 2020. This finding will be useful to evaluate the HDIs of the extracts and their bioactive compounds when co-administration with other drugs as well as to predict further clinical interaction to ensure the patient's medication use for effective with maximum safety.

2. Objectives

To study the induction effect of Cytochrome P450 by *B. monnieri* standardized extract and constituents on HeG2 cells

3. Materials and Methods

3.1. Chemicals and authentic metabolite standards of induction

Bacopa monnieri standardized extract (batch No.: R63001) was purchased from GPO Ltd. (BKK, Thailand). Bacoside A_3, Bacopaside I, II, X, and Bacopasaponin C were obtained from GPO Ltd. (BKK, Thailand) and Pharmaceutical Sciences, Naresuan University (PHT, Thailand). Phenacetin, Acetaminophen, Bupropion, Hydroxybupropion, Tolbutamide, 4-Hydroxy tolbutamide, S-Mephenytoin, 4-Hydroxy mephenytoin, Bufuralol, 1'-Hydroxy bufuralol, Midazolam, and 1'-hydroxy-midazolam were purchased from Sigma–Aldrich (Thailand). All other laboratory chemicals were used as the highest purity and from commercial suppliers. HepG2-cells were purchased from GIBCO Ltd. (BKK, Thailand) and used with the permission of The Research Ethics Committee.

3.2. Preparation of *B. monnieri* extract

B. monnieri extracts prepared by the spray-dried method that received courtesy of Natural Products Research Group Research and Development Institute Government Pharmaceutical Organization (GPOs). The extract powder, containing the amount of total saponin approximately is 18% (by weight), which is a mixture of bacoside A_3, bacopaside II, bacopasoponin X, bacopasaponin C and bacopaside I. Five saponin standards and each molecular weight including bacoside A_3 (M.W.= 929.1), bacopasaponin C (M.W.= 899.07), bacopasides I (M.W.= 979.1), bacopaside II (M.W.= 929.1), and bacopaside X (M.W.= 899.1) were obtained from Pharmaceutical Sciences, Naresuan University (PHT, Thailand). (purity > 96% by HPLC).

The determination of activities in *B. monnieri* extracts and compounds according to the method of Nuengchamnong *et al.* (Nuengchamnong N, 2016) to selective the concentration of the standard substance for the ability test effect of inhibiting or inducing comprehensively. While it corresponded with the amount in the extract and its constituent standardization should be collected at a temperature of -20ºC in the light-resistant bottle, until used for experimentation.

3.3. HepG2-cell culture

Human hepatocellular carcinoma cell line HepG2(JCRB1054) purchased from JCRB Cell Bank in Japan was cultured based on a previous study by Phokrai *et al.* (Phokrai *et al.*, 2018). Initially, 70% of cell viability or more with a density equal to 0.08 x 10^6 and 0.06 x 10^6 cells/well of HepG2 cells were added to 12 and 24-well plates for determination of CYP mRNA expression, respectively. To determine CYP

activity, the 12 and 24-well biocoat and multi-well culture plates with the density were determined as 0.06 x 10^6 and 0.04 x 10^6 cells/well was incubated in DMEM low glucose (GIBCO, cat. no 11885084), which contained 10% FCS, 1% Penicillin/Streptomycin at 37°C and 5% CO_2 for 24 hours. After that the medium was changed, the systems will be treated with 3 concentrations of the reference inducers, which included 25 µM β-naphthoflavone (βNF), and 500 µM Phenobarbital (PB) or 10 µM Rifampicin (RIF), *Bacopa monnieri* extract (BME), Bacoside A_3, Bacopaside I, Bacopaside II, Bacopasaside X and Bacopasaponin C for 72 hours. The medium including a treatment compound will be changed every 24 hours. The DMSO 0.1% v/v will be used as a control group.

3.4. Cytotoxicity

Initially, the HepG2 cells and primary human hepatocytes cultures (10,000 cells/well) were cultured in 96-multiwell culture plates. The concentrations ranged from 0 to 480 µg/ml and were increased by 10-fold to measure cellular viability using the MTT assay (Carmichael J, 1987)**.**

3.5. Induction effect of *B. monnieri* extract and its bioactive compound on CYPs

HepG2 cells (24-well plates), treated with 2 concentrations (non-cytotoxic concentration) reference compound or *B. monnieri* extract for 72 hours, were incubated with the substrates of each CYPs. The medium contained gentamycin (50 mg/l), salicylamide (1 mM) with 5% CO_2, and 95% air. The concentrations of substrates are Phenacetin (26µM, CYP1A2) bupropion (50 µM, CYP2B6), tolbutamide (100µM, CYP2C9), S-Mephenytoin (10 µM, CYP2C19), Bufuralol (1 µM, CYP2D6) and Midazolam (100 µM, CYP3A4). The biotransformation was terminated by add 1% formic acid in acetonitrile for a stop reaction ratio of 1:1 of the sample. The samples were centrifuged at 7,000 g for 10 minutes, and then the resulting supernatant was kept at -80ºC before use. The resulting supernatant was transferred into an Eppendorf tube and then aliquoted as 20 µL to be further analyzed by LC-ESI-QTOF-MS/MS. Results were expressed as pmol of the metabolite were produced including Acetaminophen (CYP1A2), Hydroxybupropion (CYP2B6), 4'-Hydroxytolbutamide (CYP2C9), 4'-Hydroxymephenytoin (CYP2C19), 1'-Hydroxybufuralol (CYP2D6) and 1'-Hydroxymidazolam (CYP3A4) formed /million cells in well (10^6)/ incubation time (minutes).

3.6. LC-ESI-QTOF-MS/MS condition

In brief, the P450 isoform-specific metabolite was analyzed using LC-ESI-QTOF-MS/MS analysis following the previous study by Nitra *et al*. (Nuengchamnong, Sookying, & Ingkaninan, 2016). An Agilent 6540 Q-TOF-MS spectrometer (Agilent Technologies, Singapore) coupled with an Agilent 1260 Infinity Series HPLC system (Agilent, Waldbronn, Germany) was used to perform in this study. The HPLC was coupled to an electrospray ionization (ESI) source and proprietary Agilent dual nebulizer. The injection volume of the sampler and standards were adjusted to 20 µL. 0.1% formic acid in water v/v (A) and 50% Methanol in acetonitrile v/v (B) were used as the mobile phase. It was employed in the gradient mode with 95% solvent A for 0-10 minutes, after that change solvent composition to 95% B at 10-13 minutes, then stop time and post-run for 5 minutes. The ZORBAX Eclipse Plus C-18 column (4.6 mm x 100 mm, 3.5 µm) was purchased from Agilent Technologies, USA to separate compounds. The operating parameters in MS detection were described as follows: drying gas (N2) flow rate, 10.0 L/min; drying gas temperature, 350°C; nebulizer pressure, 30 PSIG; capillary, 3500 V; skimmer, 65 V; octupole RFV, 750 V; and Fragmentor voltage of 250 V in negative mode and 100 V in positive mode. The mass was set at m/z 100-1200 with a 250 ms/spectrum. Agilent LCMS-QTOF Mass Hunter Data Acquisition Software Version B.05.01 and Agilent Mass Hunter Qualitative Analysis Software B 06.0 (Agilent Technologies, USA) were used for collecting all of the acquisition and analysis of the data respectively. Each sample had been identified the structure using in both positive and negative mode including targeted MS/MS analysis. In this study, a dual-nebulizer ESI source (calibrant solution A, Agilent Technologies, USA) was used to provide accurate mass measurements (error less than 5 ppm for analysis).

3.7. CYPs isoform mRNA expression

HepG2 cells (12-well plates), treated with 2 concentrations (non-cytotoxic concentration;60 and 120 µg/ml) reference compound or bacoside A_3, bacopaside II, bacopaside X, bacopasaponin C and bacopaside I for 48 h have analyzed the activity of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 mRNA expression. The total RNA obtained from hepatocytes was extracted using TriZol® reagent (Invitrogen, France). The RNA was measured using spectrophotometry. The cDNA was analyzed by beginning from 500 ng of total RNA using an iScript kit from Biorad company (France) at 42°C for 30 minutes. Then the diluted RT reaction (1:10) as 10 µl will be used for real-time PCR amplification using SYBR Green kit and denaturation step software at 95°C for 3 minutes with 40 cycles of PCR (denaturation, 95°C, 10 secs; annealing, 58°C, 1 minute) and one cycle at 55°C for 1 minute. The measuring of PCR product infusion step (final phase of the reaction) using human sense and reverse primers are described as follows Table 1. These results will be shown as fold change and compare to the control group (i.e. DMSO treated cultures). The over 2-fold change will be the ability as CYPs inducing (> 2-fold mRNA expression) or repressing (< 2-fold mRNA expression). The Cq value are calculated from qRT-PCR product occurred by equation following as step, (i) ΔCq = Cq (Target gene) – Cq (Reference gene), (ii) Exponential expression transform: ΔCq , (iii) Expression = $2^{-\Delta Cq}$ respectively

Table 1 RNA sequencing of CYPS and Actin for identification

CYPs	RNA sequencing
CYP1A2	5'-GGGCACTTCGACCCTTACAA-3'and 5'-GCACATGGCACCAATGACG- 3'
CYP2B6	5'-AGGGCCCCTTGGATTTCCG-3' and 5'-GGCCATACGGGAGGCCCTTG - 3'
CYP2C9	5'-CCTATCATTGATTACTTCCCG- 3' and 5'-AACTGCAGTGTTTTCCAAGC-3'
CYP3A4	5'-CACAAACCGGAGGCCTTTTG-3' and 5'-ATCCATGCTGTAGGCCCCAA- 3'
Actin	5'-GGGCACGAAGGCTCATCATT- 3' and 5'-AGTCGGTTGGAGCGAGCATC-3'

3.8. Statistics

The one-way analysis of variance and Dunnett's test was used to assess the significance of the difference between the groups. The level of significance was set at $P < 0.05$.

4. Results and Discussion

4.1. Cytotoxicity

At 0.1% DMSO showed more than 75% cell viability that confirmed the solvent effect did not present. The cytotoxicity of *B. monnieri* extract, as well as its five bioactive compounds, was evaluated on cell lines using an MTT assay. As the results, the CC_{50} value of *B. monnieri* extract was 788.8 µg/mL while the CC50 values of bioactive compounds were greater than 100 µg/mL (100 µM). These results might suggest that other compounds contribute to the cytotoxicity of *B. monnieri* extract. At 120 µg/mL of *B. monnieri* extract containing less than 2.5 % of bioactive compounds, thus, the bioactive compounds were accounted for less than 3.0 µM. Hence, the concentration of the bioactive compounds at 2.5 µM was selected to further perform the induction effect experiment. 50% cytotoxic concentration (CC_{50}) values of *B. monnieri* extract and bioactive compounds were summarized in Table 2.

Table 2 Cytotoxicity test of *B. monnieri* and five bioactive compounds in HepG2-cell showing CC_{50} values

	BME (µg/mL)	Ba A_3 (µM)	Ba I (µM)	Ba II (µM)	Ba X (µM)	Ba C (µM)
CC_{50}	788.8	965.6	460.6	3493	1892	149.7

BME is *B. monnieri* extract, Ba A_3 is bacopaside A_3, Ba I is bacopaside I, Ba II is bacopaside II, Ba C is bacopasaponin C, Ba X is bacopaside X

As the MTT assay results, despite alone 0.5% DMSO, showed the cytotoxic to cell line with CC_{50} value of 2.817 µM and the percentage of cell viability was still 75%. This result suggested that alone the CC_{50} value did not reflect the cytotoxicity. The graphs explaining the concentration of *B. monnieri* extract and bioactive compounds versus the percentage of cell viability are shown in Figure 1. This result suggested that consumption of 300 and 600 mg/day *B. monnieri* extract (dose in mg / human plasma volume in mL) might not produce the hepatotoxic.

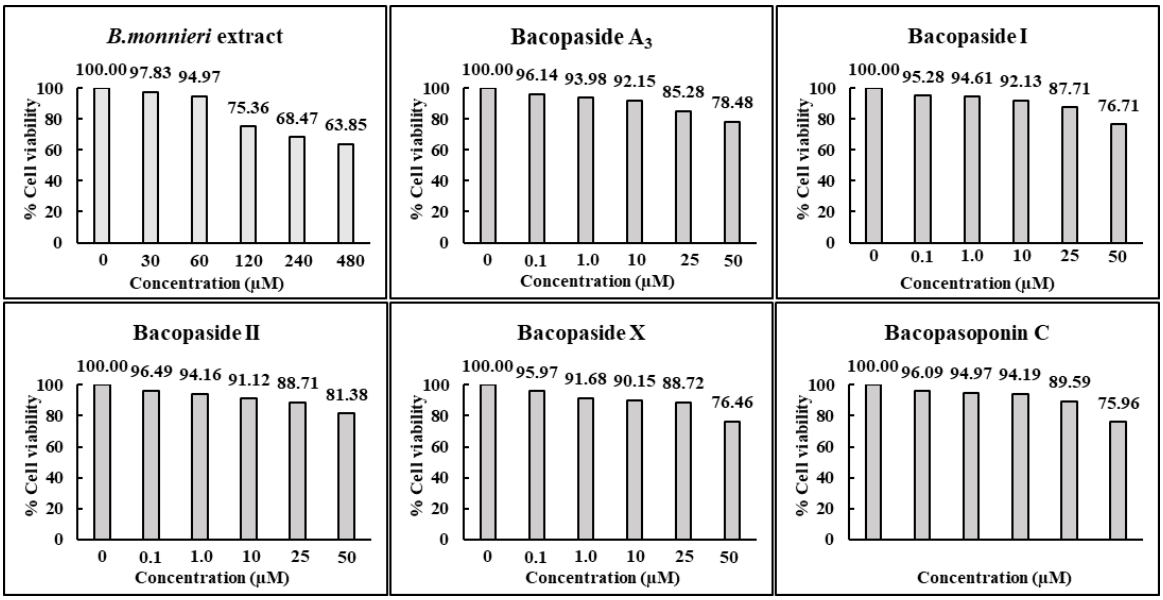

Figure 1 Illustrate the percentage of cell viability versus concentration of *B. monnieri* extract as well as its five bioactive compounds in HepG2-cell

4.2. LC-ESI-QTOF-MS/MS analysis

In vitro induction effect of *B. monnieri* extract and bacopaside I, which assumed as a major bioactive constituent drive several pharmacological effects, were analyzed in HepG2 cells using LC-ESI-QTOF-MS/MS (positive mode). The metabolites peaks including acetaminophen, hydroxybupropion, 4'-hydroxy tolbutamide, 4'-hydroxymephenytoin, 1'-hydroxybufuralol, and 1'-hydroxymidazolam were identified at 152.074, 256.111, 235.110, 278.175, and 342.079 *m/z*, respectively (data did not show). As the analysis results, the extract significantly increased CYP1A2, 2B6, 2C9, and 3A4 enzyme activity when compared to a control (DMSO). While bacopaside I significantly increased CYP1A2 and 3A4 enzyme activities as shown in Figure 2. These results suggested not only bacopaside I could induce the drugs-metabolism enzyme activity, but also other phytoconstituents the effect to the enzyme activity.

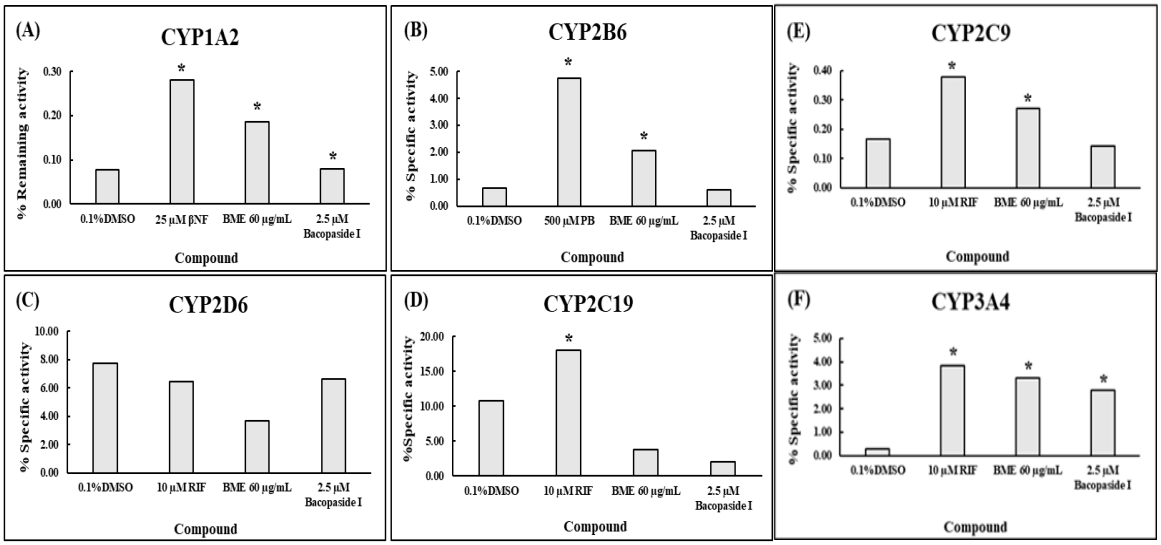

Figure 2 Illustration of the induction effect of *B. monnieri* extract, bacopaside I, and the positive control on CYP1A2 (A), CYP2B6 (B), CYP2D6 (C), CYP2C19 (D), CYP2C9 (E), and CYP3A4 (F). the results are significantly different from control by the Dunnett's test (*) $p > 0.05$ with respect to the control.

4.3. CYPs isoform mRNA expression

To investigate the induction effect of the extract and each bioactive compound in HepG2-cells, the mRNA expression was determined using RT-PCR. The expression levels of CYP1A2, 2B6, and 3A4 were showed in Figure 3. As result, 60 and 120 µg/mL *B. monnieri* extract showed 8-fold and 10-fold increasing of CYP1A2 mRNA expression respectively while a positive control, is BNF, could increase 24-fold mRNA expression. Also, at 60 and 120 µg/mL, *B. monnieri* extract showed 2-fold and 7-fold increasing of CYP2B6 mRNA expression respectively while a positive control, is PB, showed the highest mRNA expression induction. Despite 60 µg/mL did not show the induction effect on CYP3A4 mRNA expression, 120 µg/mL showed an approximately 4-fold increase of CYP3A4 mRNA expression. These results suggested the induction effect of *B. monnieri* extract might be concentration-dependent manner. In addition, *B. monnieri* extract and five bioactive compounds had no induction effect on CYP2C9 mRNA expression as shown in Figure 3. As mRNA expression results, bacopaside A_3 and bacopaside II showed the highest induction of CYP1A2, 2B6, and 3A4 expression. Besides, bacopasaponin C showed a 4.7-fold increase in CYP1A2 mRNA expression. These results suggested that bacopaside A_3 and bacopaside II might be the major bioactive compounds that drive the induction of CYP1A2, 2B6, and 3A4 mRNA expression.

Based on our results, concomitantly consuming *B. monnieri* extract and CYP1A2, 2B6, and 3A4 substrates might occur HDIs and alter the pharmacokinetics, resulting in therapeutic failure. However, the extract and bioactive compounds did not alter the CYP2C9 mRNA levels. This result suggested co-administration of the extract and CYP2C9 substrates might not produce any effect on the body. An assumption of the phenomenon might be that the *B. monnieri* extract and its bioactive compounds did not express the induction effect on CYP2C9 due to the difference in primer between HepG2 cell line and normal human hepatocyte sequencing at the position of 144 bp (Chen. J& Raymond. K, 2006). Due to marked species cells differences in the expression and regulation of CYPs, the results obtained with treated cell lines could not be directly extrapolated to normal cells in humans.

Based on the qRT-PCR results, the western blotting assay was performed to further confirm the induction effect of the extract, bacopaside A3, and its isomer compound namely bacopaside II in HepG2-cells. Our experiment results showed that the extract at a low concentration did not increase the CYP2B6

and 3A4 mRNA levels while at a high concentration could increase the CYP2B6 and 3A4 mRNA levels. This phenomenon suggested a concentration-dependent manner.

5. Conclusion

The induction effect on the *B. monnieri* extracts and their bioactive constituent was preliminary investigate using HepG2-cells. Our finding found that *B. monnieri* extract at 300-600 mg did not produce the hepatotoxic. This result might suggest a safety profile of *B. monnieri* extract. Based on *in vitro* study, *B. monnieri* extract, bacoside A_3, bacopaside I, and bacopaside II could significantly increase the CYP1A2 and 3A4 enzyme activity. Also, the *B. monnieri* extracts alone significantly increased the mRNA and activity of CYP2B6. Therefore, concomitantly consuming *B. monnieri* extract with CYP1A2 substrates (such as phenacetin, theophylline, propranolol, and caffeine), CYP2B6 substrates (such as efavirenz, sibutramine, and bupropion), and CYP3A4 substrates (such as diazepam, erythromycin, and cyclosporine) should be careful. However, the animal study should be performed before the pre-clinical study. In the future, the authors further investigate the induction effect of the extract and five bioactive compounds using primary human hepatocytes.

6. Acknowledgements

This work was financially supported by Government Pharmaceutical Organization-Merieux Biological Products Co., Ltd. Also, we would like to thank the biochemical laboratory, Faculty of Medical Science Naresuan University, for their help with developing HepG2-cells culture protocol.

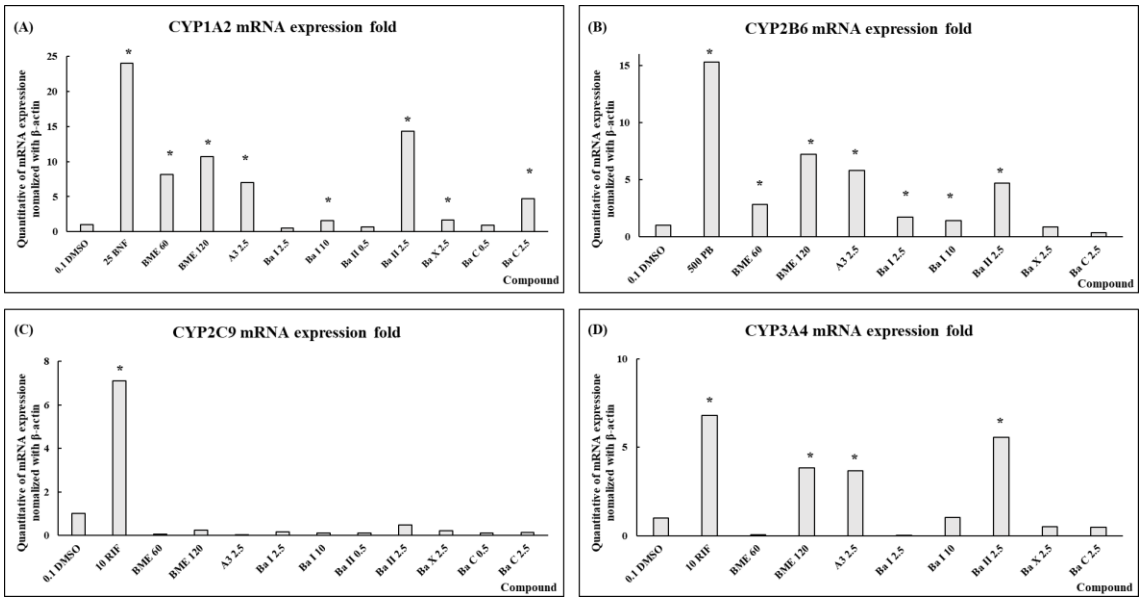

Figure 3 Illustration of the CYP1A2 (A), CYP2B6 (B), CYP2C9 (C), and CYP3A4 (D) mRNA expression fold of 60-120 µg/mL of B. *monnieri* extract (BME 60-120), 2.5 µM of bacoside A_3 (A3 2.5), 2.5 µM of bacopaside I (Ba I 2.5), 10 µM of bacopaside I (Ba I 10), 2.5 µM bacopaside II (Ba II 2.5), 2.5 µM of bacopaside X (Ba X 2.5), and 2.5 µM of bacopasaponin C (Ba C 2.5). (*) $p > 0.05$ with respect to control

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