Indonesian Journal of Pharmacy

VOL 35 (4) 2024: 629–640 | RESEARCH ARTICLE

Thymoquinone Increased Warfarin 7-hydroxylation in Human Liver Microsomes and Induced the Expression of CYP2C9 in HepG2 Cells

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Article Info

Submitted: 27-11-2023
Revised: 06-05-2024
Accepted: 15-05-2024

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ABSTRACT

Herb-drug interaction is an interesting phenomenon that can induce therapeutic complications in patients. Warfarin is widely used as an anticoagulant, which has a narrow therapeutic index. The combination of herbal and warfarin has consequences in the therapy outcome on the attenuation of drug efficacy or increased toxicity. This study aimed to investigate the effect of thymoquinone on warfarin 7-hydroxylation activity in the human liver microsome (HLM) and CYP2C9 expression in HepG2 cells. The co-administration of thymoquinone on warfarin 7-hydroxylation was investigated using HLM and HepG2 cells. The study was divided into three groups: control, warfarin, and combination of warfarin-thymoquinone. The metabolite of 7-hydroxywarfarin (7-OH warfarin) in HLM was determined using HPLC-MS/MS. Furthermore, the effect of thymoquinone in terms of the induction of CYP2C9 expression in HepG2 cells was determined by RT-PCR. The results of the validated method used were selective for HLM 7-hydroxywarfarin, with an LLOQ of 0.62 μM, so it met the criteria for accuracy and precision for metabolite analysis. The results showed that the co-incubation of thymoquinone at 0.37mM significantly increased warfarin 7-hydroxylation activity ($P<0.05$). In addition, after 72-hour incubation, thymoquinone also significantly induced CYP2C9 expression in HepG2 cells ($P<0.05$). These findings provide valuable insights that the combination of thymoquinone with warfarin significantly increased the warfarin 7-hydroxylation activity in human liver microsomes and CYP2C9 expression in HepG2 cells, which may have an impact on the clinical outcomes of warfarin in patients.

Keywords: thymoquinone, HPLC-MS/MS method validation, 7-hydroxywarfarin, HepG2 cells, CYP2C9

INTRODUCTION

Drug-herb interactions are one type of interaction that can induce therapeutic complications in clinical outcomes. Herbal medicines can sometimes have adverse drug interactions with regular medicines. The herb-drug interaction can reduce the drug efficacy and lead to harmful side effects for the patient (Borse *et al*., 2019; Koziolek *et al*., 2019). The interaction between herbal medicines and drugs can alter their pharmacokinetics and actions, leading to additive, synergistic, or antagonist outcomes. This is mainly due to the interaction of the cytochrome P450 complex (Kucharczuk, *et al*., 2018; Zhao *et al*., 2020). CYP2C9 is a member of the P450 2C subfamily of enzymes, and CYP P450 enzymes

contribute substantially to metabolizing several medicines, including oral antidiabetic agents, NSAIDs, anticoagulants, and antihypertensives (Boyce *et al*., 2018; Daly *et al*., 2017; Green *et al*., 2016). Metabolic processes activate two receptors, namely CAR and PXR, which are orphan nuclear receptor subfamilies. PXR targets CYP target genes, including UGT1A, CYP3A4, and CYP3A1, while CAR-induced genes encode CYP2B6, CYP2C9, and CYP3A4 (Hogle *et al*., 2018; Yan & Xie, 2016).

One medicinal herb, *Nigella sativa* L. (*Ranunculaceae*), also called black cumin or black seeds, is famous for spicy culinary uses and is historically precious in traditional medicine. Consuming *N. sativa* as a traditional medicine can decrease the activity of CYP2C11 and CYP2D genes in rat liver in vivo. Thymoquinone (TQ) is the primary metabolite found in *Nigella sativa* (Gougis *et al*., 2021; Hannan *et al*., 2021; Korashy *et al*., 2014). TQ possesses pharmacological properties such as hepatoprotective, immunomodulatory, anticoagulant, antidiabetic, diuretic, antidepressant, anti-inflammatory, neuroprotective, antibacterial, antioxidant, and anticonvulsant effects (Butt *et al*., 2019; Hamdan *et al*., 2019; Hannan *et al*., 2021; Hosseinzadeh *et al*., 2017; Muralidharan-Chari *et al*., 2016). TQ is believed to decrease the expression of several genes, such as CYP2C11, CYP2D7, and CYP2D8. It has been demonstrated to interact with chlorzoxazone by inhibiting CYP3A4 and CYP2C19 (Elbarbry *et al*., 2017). Previous research using hydrogen peroxide induction may boost the inhibitory impact of thymoquinone on HepG2 cell proliferation. Thymoquinone shows potential as a phytochemical in cancer treatment due to its anti-inflammatory, antioxidant, and anti-angiogenic properties (Alhmied *et al*., 2021; Ghelichkhani *et al*., 2023). TQ exhibited selective cytotoxicity against hepatocellular carcinoma HepG2 and SMMC-7721 cells (Jehan *et al*., 2020). Warfarin is an anticoagulant drug, that inhibits vitamin K-dependent coagulation factors. In vitro and in vivo studies indicate that administering warfarin with ginsenosides, a component of ginseng, can weaken its therapeutic effect, and may increase bleeding risks when combined with NSAIDs (Lin *et al*., 2020; Penner *et al*., 2022). Warfarin is metabolized by the enzyme CYP2C9 to produce its primary metabolite 7-hydroxywarfarin (Daly *et al*., 2017; Flora *et al*., 2017). A clinical case study found that using anticoagulation to manage atrial fibrillation in individuals with cirrhosis reduces the risk of stroke without significantly raising the risk of haemorrhage (Chokesuwattanaskul *et al*., 2019).

Interactions between drugs and medicinal herbs can occur in the phases of pharmacokinetics, including absorption, distribution, metabolism, and excretion, as well as the phases of pharmacodynamics, including antagonists, additives, and synergists (Gupta *et al*., 2017). Pharmacokinetic interactions between drugs in therapeutic class one do not apply to other drugs due to differences in physicochemical properties. These interactions involve key metabolic enzymes such as CYP450, resulting in clinically significant interactions. However, pharmacodynamic interactions can also increase or decrease the effectiveness of antagonist drugs without changing their pharmacokinetic profile (Niu *et al*., 2019). Drug interactions in the pharmacokinetic and pharmacodynamic phase can occur at one receptor site, multiple receptors, and non-specifically through nonspecific mechanisms of action (Ahad *et al*., 2020; Rombolà *et al*., 2020).

TQ, the primary chemical ingredient of the volatile oil of *N. sativa*, significantly inhibits the metabolic activity of four major drug-metabolizing enzymes in HLM– CYP1A2, CYP2C9, and CYP3A4 (Albassam *et al*., 2018). A previous study also reported that TQ could competitively inhibit warfarin 7-hydroxylation activity in HLM (Wang *et al*., 2022). The study suggested that the coadministration of thymoquinone or dietary supplements containing thymoquinone with warfarin might result in an unexpected potential drug interaction. Many of the reports above have shown that TQ showed interactions with warfarin but have not explained the molecular nature of the interaction at the level of CYP2C9 expression. Based on the previous studies, it is interesting to examine the interaction between TQ and warfarin by evaluating the impact on the CYP2C9-metabolizing enzyme activity in Human Liver Microsomes (HLM) and CYP2C9 expression in HepG2.

MATERIALS AND METHODS

Chemical and reagents

Thymoquinone (TQ), warfarin, 7-Hydroxy-warfarin, Chloramphenicol, NADPH, and pooled human liver microsomes were purchased from Sigma-Aldrich Pte. Ltd., Singapore. Methanol HPLC grades for liquid chromatography were purchased from PT. Merck Millipore, Indonesia. A set of HPLC-MS/MS instruments (SCIEX-Q3 4500, ESI method) and HPLC-MS/MS column (100 mm x 4.6 mm; 5µm)

were purchased from Phenomenex. Real Time PCR instrument ABi7500fast. Favorgen, FATRK-001-1 for RNA isolation and SMOBIO, RP-1400 for cDNA synthesis. The media for HepG2 cells consisted of Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% v/v fetal bovine serum (FBS) (Gibco), 1% v/v Penicillin-Streptomycin (Gibco), and 0.5% v/v Fungizone (Gibco). Trypsin-EDTA 0.25% (Gibco) The cytotoxic experiments employed the MTT Reagent [3-(4,5-dimethylthiazol-2-yl)-2,5 dimethyltetrazoliumbromide] obtained from Sigma-Aldrich Pte. Ltd. The assays were conducted in a phosphate-buffered saline solution with a pH of 7.4. RNA isolation protocol kit (Favorgen, FATRK-001-1) and cDNA synthesis (SMOBIO, RP-1400), Primary use of forward-reverse CYP2C9 (GAPDH, F: 5′-CCTTCATTGACCTCAACTA-3′, R: 5′-GGAAGGCCATGCCAGTGAGC-3′; CYP2C9, F: 5′-CTTGACACCACTCCAGTTGTC-3′, R: 5′-AGATGGATAATGCCCCAGAG-3′).

HPLC-MS/MS condition assay

The analysis of 7-hydroxywarfarin in HLM used HPLC-MS/MS using a C18 column (100 mm x 4.6 mm; 5 µm) 80 Å. The mobile phase (A) consisted of 100% (v/v) water with 5 mM ammonium acetate, while (B) consisted of 100% (v/v) methanol. The flow rate used was 0.6 mL/min with a retention time of 0.91 minutes and an injection volume of 3 µL. The specific MRM transitions used for qualification were (in negative mode) m/z 307.1 → 161.0 for warfarin, m/z 323.1 → 177.0 for 7-OH-warfarin and m/z 320.8 → 152.0 for Chloramphenicol (Radko *et al*., 2019; Śniegocki *et al*., 2017).

Assay method validation

Validation of the bioanalytical methods based on guidelines from the European Medicines Agency (2011) was used to measure concentrations of 7-OH-warfarin in HLM. Guidelines for validation include selectivity, lower limit quantization (LLOQ), accuracy and precision, dilution integrity, and stability. The selectivity was initiated by injecting HLM blanks for analysis and interference at the 7-OH-warfarin peak. 7-OH-warfarin was stated with no peak interference if HLM blanks ≤ 20% of LLOQ 7-OH-warfarin. The lower limit of quantization (LLOQ) was performed to determine the lowest analyte concentration in the sample that can be measured with acceptable accuracy and precision. The LLOQ assay was determined by injecting a standard solution of 7-OH-warfarin through the HLM at concentrations of 0.25 and 0.62 µM (5 replications). LLOQ was measured at a concentration in which the percentage of differentiation value was 20%. The standard solution of 7-OH-warfarin was generated at four concentration levels within the range of the calibration curve, including LLOQ, low QC (3 x LLOQ), medium QC (30-50% calibration curve range, high QC (75% of the upper calibration curve range). The criteria of accuracy were no higher than ± 15% for QC samples and no higher than ± 20% for LLOQ. The precision criteria were met if the standard deviation (SD) was not greater than ± 15% for the QC sample in five repetitions, while that for the LLOQ was not greater than ± 20%. The integrity of the dilution must meet the established precision and accuracy criteria. The dilution integrity was determined by adding analytes of which the concentration was above ULOQ in HLM blanks. The HLM of such blanks was diluted with the HLM of the blanks with a dilution factor (2x, 5x, 10x) in 5 replications.

The 7-OH-warfarin stability test was determined using low QC (1.85µM) and high QC (4.93µM) samples, which were analyzed immediately after sample preparation. In addition, stability tests were also carried out after the low QC and high QC samples were stored at predetermined conditions (temperature) and time. The stability test consisted of room temperature (T0); the samples were stored in the freezer (-80°C) for 4 hours (T4) and 24 hours (T24); freeze-thaw (FT) stability was performed using the QC 7-OH-warfarin samples stored at temperature (-80°C) and liquidated at room temperature (3x freeze-thaw cycles); The 24-hour autosampler stability of the injection-ready QC 7-OH-warfarin sample was stored in the autosampler for 24 hours.

7-Hydroxywarfarin formation assay

The series of TQ concentrations (0.24 mM, 0.30 mM, and 0.37 mM) and warfarin (0.16 mM) were added to reaction mixture in microtubes. In addition, phosphate buffer 0.1 M, pH 7.4 (275 µL), and 25 µL HLM (FC = 0.25 mg/ml) were added, mixed thoroughly, and placed in a 37°C water shaker bath for 5 minutes. The reaction was initiated by adding 50 µL of 20 mM NADPH (FC = 1.0 mM) to a final volume of 0.5 mL, followed by 30-minute incubation at 37°C. The reaction was stopped by vigorously shaking in 500 L of cold methanol. Chloramphenicol (50µL) as IS was added into each tube. The tubes were centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was

extracted with a pipette and injected into an HPLC-MS/MS to detect 7-hydroxywarfarin.

CYP2C9 expression in HepG2 cells
HepG2 cell culture

The human hepatocellular carcinoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The HepG2 cells were cultured at 37°C in 5% CO2 in a growth medium DMEM. The cells were passaged every 5–7 days. Suspensions of HepG2 cells were produced from confluent cultures using trypsin/EDTA solution, and cell concentration was determined using a hemocytometer. The growth medium was changed every 3–4 days or as required.

Cytotoxic assay of the warfarin and thymoquinone in HepG2 cells

The cytotoxic assay was conducted using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The cells were grown at 37°C in a humidified 5% CO2 atmosphere in a flask to reach 80% confluence and passaged using a 0.25% trypsin/0.02% ethylenediaminetetra-acetic acid disodium salt (EDTA) solution (Sigma-Aldrich Pte. Ltd.). Cell culture was prepared and grown with DMEM medium. The cells were incubated for 72 hours with control warfarin, a combination of warfarin and thymoquinone at the same concentration of 38.54, 19.27, and 9.62 µM, respectively. The cell cultures were used in this experiment when the compound used did not have a cytotoxic effect rate of greater than 20%, hence maintaining a HepG2 cell viability of over 80%.

CYP2C9 expression in HeG2 cells by qRT-PCR

The cells were incubated for 72 hours with warfarin or with warfarin and thymoquinone at the same concentration of 19.27µM. The CYP2C9 expression measurements were initiated by isolating RNA from HepG2 cells using a commercially available RNA isolation procedure kit (Favorgen, FATRK-001-1). Subsequently, the concentration and purity of the collected RNA were assessed at a wavelength of 260/280 nm using a nanodrop spectrophotometer, after which the samples were stored at a temperature of -80°C. The process of cDNA synthesis was conducted by introducing RNA into the master mix in accordance with the cDNA synthesis protocol provided by SMOBIO (RP-1400).

The qRT-PCR analysis was conducted by combining 1 µl of cDNA with a PCR mixture consisting of master mix, forward primer, reverse primer, and PCR water, resulting in a total volume of 20 µl. The amplification process involved an initial denaturation step at 95°C for 2 minutes, followed by denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute, repeated for 40 cycles. The threshold value (CT) was calculated automatically by qRT-PCR.

Statistical analysis

Data were presented as mean ± SD. The statistical significance between various groups was analyzed by one-way ANOVA and a post hoc Tukey test. The statistical tests were conducted using SPSS ver.22 (SPSS Inc., Chicago, IL, USA). A (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

The condition of warfarin 7-hydroxylation activity assay

The 7-OH-warfarin concentrations were validated by bioanalysis methodologies using an acceptable, constant, and effective quantitative analytical strategy for plasma matrix and HLM analysis. HPLC-MS/MS was selected to analyze 7-OH-warfarin concentrations in HLM due to its accuracy, selectivity, and sensitivity to bioanalytic studies (Committee for Medicinal Products for Human Use, 2011; W Ju *et al*., 2014). Based on the optimization results, methanol was chosen because it produces a matrix at the best HLM without any disruptive chemicals. Methanol is a mobile phase in the HPLC-MS/MS system and can attract proteins contained in HLM. The composition of the mobile phase was determined by the optimal drug resolution value of the endogenous biological substance, the best peak form, and the retention period.

Selectivity and lower limit of quantification (LLOQ), the validation stage requires a selective method to analyze 7-OH-warfarin HLM using HPLC-MS/MS. The validation method on the selectivity test stated no peak interference at the retention time of 7-OH-warfarin on the blank HLM. The lower limit of quantification (LLOQ) was measured at concentrations of 0.25 and 0.62 µM. The data showed that LLOQ at a concentration of 0.62 µM met the criteria with a % differentiation value of ≤ 20%; there was no peak interference if the HLM was blank.

Table I. The accuracy and precision analyses of 7-OH-warfarin HLM by HPLC-MS/MS.

7-OH-warfarin Concentration (µM)	% Error Within (n=5)			% CV Within (n=5)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
0.62	-4.02	0.24	1.9	11.31	7.64	8.72
1.85	0.43	1.79	2.78	2.82	4.79	6.33
3.08	13.64	6.24	8.23	2.61	10.36	2.66
4.93	11.22	6.24	2.35	2.6	1.47	3.13

Table II. The dilution integrity 7-OH-warfarin in HLM by HPLC-MS/MS.

Dilution Factor	Mean ± SD Recovery (µM)	Accuracy (% Error)	Precision (% CV)
2x	100.26 ± 3.81	0.26	3.80
5x	98.18 ± 7.31	-1.82	7.45
10x	84.68 ± 6.23	-15.32	7.36

Data are represented as mean ± SD from 5 replications.

Table III. The stability test of 7-OH-warfarin in HLM by HPLC-MS/MS.

Stability Study		Mean ± SD Recovery	Accuracy (% Error)	Precision (% CV)
Short-Term Stability				
T0		84.59 ± 3.35	-15.41	3.96
		106.94 ± 2.93	6.94	2.74
T4		105.64 ± 5.76	5.64	5.45
		99.18 ± 6.69	-0.82	6.74
T24	Low QC 1.85 µM	106.17 ± 7.69	6.17	7.25
	High QC 4.93 µM	97.31 ± 12.18	-2.69	12.52
Freeze-thaw		108.15 ± 4.85	8.15	4.48
		99.69 ± 12.85	-0.31	12.89
Autosampler		107.87 ± 9.00	7.87	8.35
		97.58 ± 15.78	-2.42	16.18

The data are represented as the mean ± SD from 3 replications. The stability of a freeze-thaw (-80°C) sample was performed by 3 cycles.

The calibration curve was based on between the area (Y axis) and the 7-OH-warfarin concentration (X axis), resulting in a linear regression equation y = 0.01534 x + 0.04997 (r = 0.99500, r^2 = 0.99003). Linearity results showed that analytical methods had a proportional relationship between detector response and analyte concentration variations.

Accuracy and precision from four concentration levels within the calibration curve's range, accuracy, and precision data were acquired, including LLOQ concentration (0.62 µM), low QC (1.85 µM), medium QC (3.08 µM), and high QC (4.93 µM). The accuracy criterion was no higher than ± 15% for QC samples and no greater than ± 20% for LLOQ. The precision criterion was met if the standard deviation (SD) was not higher than ± 15% for the QC sample in 5 repetitions, not greater than ± 20% for LLOQ (Table I). Therefore, the proposed bioanalytical approach can determine the concentration of analytes in Human Liver Microsomes with accuracy and precision in LLOQ (Bruin *et al*., 2020; Committee for Medicinal Products for Human Use, 2011).

Dilution Integrity. The results of the dilution integrity test that have met the criteria showed that the bioanalysis method used can examine the diluted sample with precision and accuracy. Based on (Table II), the integrity test of dilution has met the criteria according to the parameters of accuracy and precision (% error ≤ 15%, % CV ≤ 15%).

Stability tests were performed to verify whether the analyses in plasma remained stable and did not degrade during bioanalysis and storage. Stability of the sample at room temperature (T0),

freezer (-80°C) at specified times (T4), 24 hours (T24), and freeze-thaw (FT); and in autosampler (24 hours), shows good stability. No degradation occurs at 7-OH-warfarin during storage, and various conditions could be seen from the % error value of ≤ 15% and % CV ≤ 15%. (Table III). The stability test conducted during the validation method demonstrated that the levels of 7-hydroxywarfarin did not exhibit any major variations and remained within the stipulated requirements outlined in the recommendations provided by the European Medicines Agency (Committee for Medicinal Products for Human Use, 2011).

The present study aims to validate the bioanalysis method for 7-hydroxywarfarin, which has previously demonstrated selectivity, accuracy, precision, dilution Integrity, and stability when applied to spiked plasma samples. This validated approach can now be used to analyze 7-hydroxywarfarin levels combined with TQ in HLM. Internal standards are commonly employed in drug bioanalysis testing to reduce discrepancies, particularly in the HPLC-MS/MS method. The utilization of internal standards has the potential to enhance the values of accuracy and precision (Imre *et al*., 2019; Wright *et al*., 2019).

Effect of thymoquinone on warfarin 7-hydroxylation in HLM

The present study has employed the internal standard of chloramphenicol to achieve favorable separation outcomes in the bioanalysis of 7-OH warfarin. The presence of the 7-OH warfarin peak in HLM was observed in the chromatogram, following the administration of warfarin alone and in combination with TQ. HPLC-MS/MS was employed to verify the presence of 7-OH warfarin in the HLM sample. This confirmation was achieved through the detection of the $[M+H]^-$ ion protonation, which exhibited a mass-to-charge ratio (m/z) of 323.1 → 177.0 for 7-OH-warfarin and m/z 320.8 → 152.0 for chloramphenicol (Radko *et al*., 2019; Śniegocki *et al*., 2017; W Ju *et al*., 2014).

The effect of TQ was evaluated based on the concentration of warfarin metabolite (7-hydroxywarfarin) catalyzed by CYP2C9 in HLM. The statistical analysis showed that there was a difference among the three concentrations of thymoquinone ($P < 0.05$), using warfarin as a control (Figure 1). The concentration of TQ (0.24 and 0.30 mM) showed an increase in 7-OH warfarin concentration but was not significant compared to the control. In addition, the effect of TQ (0.37 mM) showed a significant increase in the 7-OH warfarin activity compared to the control ($P < 0.05$) (Figure 1).

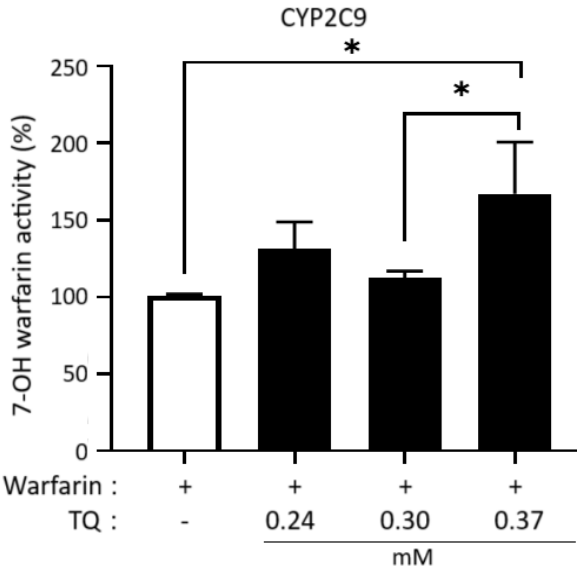

Figure 1. The percentage of 7-OH warfarin activity for each group against the control in HLM, with 3 replications. The effect of TQ on 7-hydroxywarfarin activity in HLM is expressed as mean ± SD. * $P < 0.05$ indicated significant compared to control or TQ (0.30mM).

An increase in the side effects of chemical drugs and reduced patient adherence will significantly affect the severity of the disease. This encourages people to switch to using herbs as the main therapies and drugs as the adjunct therapy. Although herbal medicine is considered natural, it contains various ingredients that can potentially modulate the activity of drug metabolism enzymes and cause changes in the pharmacokinetics and pharmacodynamics of co-administration of drugs (Elbarbry *et al*., 2017). The metabolic phase of the drug in the body is inseparable from the role of Cytochrome P450 enzymes in the liver. The role of cytochrome P450 enzymes in the liver can turn drugs into active, inactive, or toxic substances. The previous study reported that the modulation of CYP enzymes from various herbal medicines has side effects on the therapy (Rombolà *et al*., 2020). Suppression of proteins that metabolize medicines may also contribute to higher plasma levels of concurrently ingested drugs, prolonging their pharmacological effects and increasing the incidence of drug-induced toxicity and severe adverse effects (Koziolek *et al*., 2019; Li *et al*.,

2019). Warfarin and thymoquinone are commonly used as anticoagulants, while warfarin also has a narrow therapeutic index. Therefore, uncontrolled warfarin usage or in conjunction with other drugs can cause anticoagulation effects (Ageno *et al*., 2012; Muralidharan-Chari *et al*., 2016).

A recent study showed that the use of herb-warfarin can cause clinical severity interactions, so it must be avoided or closely monitored. The mechanism of herb-warfarin interaction can influence the pharmacokinetic and pharmacodynamic mechanisms of warfarin. Pharmacokinetic interactions can influence the processes in which warfarin is adsorbed, metabolized, and distributed, with the main focus being cytochrome P450 for warfarin metabolism (Ge *et al*., 2014; Holbrook, 2005). The anticoagulant response is also influenced by several drugs that induce or inhibit warfarin metabolism and genetic polymorphisms that can modulate the expression or activity of CYP2C9, the clearance mediating isoform of S-warfarin. CYP2C9 contributes the largest proportion to the metabolism of S-warfarin, which is much more potent than the R-enantiomer clinically. Possible role of dietary factors other than vitamin K and medications or supplements as causes of anticoagulation instability in patients treated with warfarin. The use of herbs showing effects on cytochrome P450s, especially on CYP2C9, CYP1A2, CYP3A4, or CYP2C19, will affect warfarin plasma concentrations, which may be one reason for herb-warfarin interactions, as therapy using John's wort and ginseng can potentially reduce warfarin anticoagulation by inducing CYP2C9 activity (Ge *et al*., 2014; Greenblatt & Moltke, 2005; Holbrook, 2005).

The results of this study demonstrated that it is most likely that drug interactions occurred after the administration of warfarin with TQ or TQ-containing herbs characterized by an increase in the concentration of 7-OH warfarin. As we know, the most extensive metabolism of warfarin is catalyzed by CYP2C9. TQ (0.24 and 0.30 mM) showed no significant increase in 7-OH warfarin activity, whereas the activity significantly increased at a concentration of 0.37 mM. This result differs from previous reports (Albassam *et al*., 2018), which may be influenced by various factors, including temperature, incubation time, and the stability of HLM levels, which can impact the metabolic processes of compounds in HLM.

Cytotoxic assay of the warfarin and thymoquinone in HepG2 cells

To investigate the molecular mechanism of interaction with thymoquinone, an in vitro study was performed to determine the expression of genes involved in warfarin metabolism, specifically CYP2C9, in the human hepatoma HepG2 cells. HepG2 cells are commonly used in drug interaction research because they can express numerous genes involved in drug metabolism, particularly those associated with phase I metabolic processes. These cells are utilized to investigate possible inductors or inhibitors of metabolic gene expression, hence facilitating the assessment of drug interactions. Gene expression translates the information in a gene used to synthesize mRNA. The increase in CYP2C9 expression has implications for the gene products, such as mRNA and the CYP2C9 enzyme. An increase in the number of such products will impact drug metabolism and lead to a change in drug activity in the body.

The combination of warfarin and TQ at 38.54 μM exhibited higher than 20% cytotoxicity. In contrast, warfarin alone or combined with TQ (19.27 and 9.62 μM) exhibited a relatively minimal cytotoxic effect and demonstrated higher than 80% cell viability. Similarly, the single compound of warfarin exhibited cell viability greater than or equal to 80% at 9.62 μM or higher concentrations. The results of initial cytotoxic screening in a single warfarin or in combination (warfarin-TQ) against HepG2 cells at 72-hour incubation had a viability of ≥ 80% for a concentration of 19.27 μM, showing that the concentration given could maintain HepG2 survival. Furthermore, the assay of the CYP2C9 expression at the control warfarin and the combination was measured at a concentration of TQ at 19.27 μM.

CYP2C9 expression in HepG2 cells

The purity of mRNA isolation was determined based on the ratio between 260/280. The results of measuring the quality of mRNA at the A260/A280 absorbance ratio for each sample were 2.1. The samples were passed at 260 and 280 nm wavelengths in a spectrophotometer. The results of the mRNA isolation purity test on the sample ratio of 260/280 showed that it was still in the range of 2.0, which means pure mRNA isolation and not contaminated with a protein (Desjardins & Conklin, 2010; Gandhi *et al*., 2020). Subsequently, the samples were analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR), a technique capable of amplifying and quantifying the quantity of DNA from the target gene. The selected housekeeping gene, GAPDH, was

utilized as a reference gene to assess the relative expression levels of CYP2C9 mRNA in HepG2 cells following treatment with warfarin alone and in combination with TQ. This study aimed to assess the potential induction effect of TQ on the CYP2C9 gene, which is involved in the metabolism of warfarin in the human cell line (HepG2).

The results obtained from the investigation on HepG2 cells revealed that the simultaneous administration of warfarin and TQ led to a significant modification in CYP2C9 expression compared to the control group. CYP2C9 expression in HepG2 cells increased at a concentration of 19.27 μM following treatment with warfarin alone or in combination with TQ.

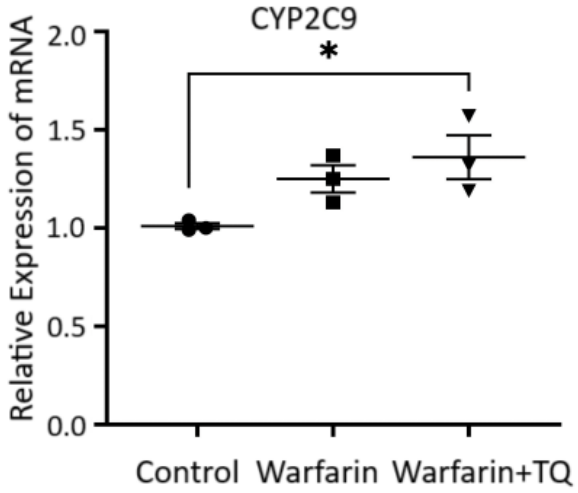

Figure 2. Relative expression of CYP2C9 mRNA in HepG2 cell cultures after treatment with warfarin alone and in combination with TQ for 72 hours. Each test group was carried out with 3 replications. *$P < 0.05$ indicated the significance compared to control.

The mRNA expression value was much higher than the control (Figure 2). The combination of warfarin with TQ demonstrated a significant increase in expression ($P < 0.035$) compared to the control cells, suggesting that they exhibited an inductive effect. In the interim, the co-administration of warfarin and TQ resulted in a marginal elevation in the CYP2C9 compared to the administration of warfarin alone. Similarly, in the case of the single administration of warfarin, there was an increase, but not significant, in CYP2C9 expression compared to the control cells.

The present study demonstrated that the concurrent administration of warfarin and TQ in HepG2 cells showed a significant increase in the relative mRNA expression of the CYP2C9 gene and may support the effect of TQ on warfarin 7-hydroxylation in HLM. Inhibition or induction of the CYP enzyme can affect the increase or decrease in the levels of drugs in plasma and alter the pharmacological action if simultaneously taken. Therefore, the influence of TQ on the increasing activity of the CYP2C9 enzyme supports the hypothesis, i.e., prolonged exposure to herbs or the chemical elements of herbs may contribute to the adverse pharmacological consequences of clinically utilized drugs.

It is well known that orphan nuclear receptor subfamilies, CAR-induced genes encode CYP2B6, CYP2C9, and CYP3A4 (Goodman *et al*., 2010). Drug-responsive nuclear receptors and hepatic transcriptional factors bind to cis elements within CYP2C gene promoters to regulate the transcription of CYP2C genes. HNF4α is probably the most important receptor for upregulating the constitutive expression of the CYP2Cs in the liver. Moreover, cross-talk between HNF4α and PXR/CAR is necessary for optimal induction in response to drugs (Chen & Goldstein, 2009). The mechanism of CYP2C9 induced by TQ may be via HNF4α or crosstalk pathway with PXR/CAR, but it is still unclear and requires further research experiments. The interaction between the administration of warfarin and TQ can increase the expression of CYP2C9 and, therefore, has implications for increasing the metabolic activity of warfarin. TQ or TQ-containing medicinal herbs as the main active compound are sometimes taken simultaneously with prescription medicines primarily metabolized by the CYP2C9 enzyme. These findings support evaluating and monitoring warfarin efficacy and safety, especially for drugs for narrow window therapeutic index.

CONCLUSION

The present study demonstrated that the administration of warfarin combined with TQ significantly increased the metabolic activity of the CYP2C9 enzyme in the human liver microsomes. In addition, the co-administration of TQ with warfarin significantly increased the CYP2C9 expression in HepG2 cells but not in control warfarin. These results indicate that TQ can potentially modify the metabolic activity of this enzyme and may change the clinical outcome of warfarin in humans.

ACKNOWLEDGMENTS

The authors thank The Indonesian Ministry of Education, Culture, Research, and Technology

and Universitas Gadjah Mada Yogyakarta for the BPPDN scholarships and support for Doctoral studies at the Faculty of Pharmacy, Universitas Gadjah Mada.

This research was funded by The Doctoral Dissertation Research Grant (No.1860/UN1/DITLIT/Dit-Lit/PT.01.03/2022), The Indonesian Ministry of Education, Culture, Research, and Technology 2022 and Universitas Gadjah Mada Final Project Recognition Program (3143/UN1.P.III/DIT-LIT/PT/2021).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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