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Molecular Docking of *Nigella Sativa* L. with PXR Receptors and the Effect of Thymoquinone on PXR Expression in HepG2 Cells

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ABSTRACT. The interaction between herbal remedies and drugs is a fascinating phenomenon that might cause therapeutic complications in patients. Warfarin is an anticoagulant drug that provides anti-blood-clotting effects by inhibiting the formation of vitamin K-dependent coagulation factors, and warfarin interacts with the nuclear receptor PXR, subsequently modulating cytochrome P450 during the metabolism process. St. John's wort, an herbal medicine with antidepressant effects, can alter the pharmacokinetics of warfarin. Thymoquinone is one of the active compounds in *N. sativa* and has pharmacological activities such as anticoagulant, antidiabetic, diuretic, antidepressant, and anti-inflammatory effects. In this study, we investigated the compounds in *Nigella sativa* (*N. sativa*) against pregnane X receptor (PXR) and evaluated the impact of thymoquinone (TQ) administration on PXR expression in HepG2 cells. The molecular docking analysis was conducted utilizing the Molecular Operating Environment (MOE) with the PXR (PDB ID: 7AXJ). At the same time, the PXR gene expression was measured using RT-PCR instruments. The RMSD value in docking represents the deviation criteria between the native ligand position and the redocking position result, indicating the capability of MOE and PDB qualification for performing molecular docking. The docking analysis showed that warfarin had the strongest binding energy (7AXJ -6.0507) by forming hydrogen binding type on Arg410. Despite TQ being the major component, it also displayed a high affinity for the two PDB IDs (7AXJ -4.5962). Furthermore, the concurrent administration of warfarin and TQ (19.27 µM) in HepG2 cells showed a significant reduction in the relative mRNA expression of the PXR gene. Given the above-mentioned findings, our study adequately enables us to predict the mechanism behind herb-drug interactions (HDIs) implicating *N. sativa*, specifically the TQ compound to warfarin metabolism via the activation of the PXR receptor.

Keywords: Thymoquinone, expression mRNA, PXR, molecular docking, HepG2 cells.

INTRODUCTION

Drug-herbal medication interactions can induce therapeutic complications in clinical outcomes. Herbal medicines can sometimes have adverse drug interactions with regular medicines. Herbal-drug interactions can reduce drug efficacy and lead to harmful side effects for the patient (Borse 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 primarily due to cytochrome P450 complex interactions (Kucharczuk, et al., 2018; Zhao et al., 2020).

Warfarin is an anticoagulant drug that provides anti-blood clotting effects by inhibiting the formation of vitamin K-dependent coagulation factors. Warfarin is a drug that undergoes metabolism by the enzyme CYP2C9 which produces the main metabolite in 7-hydroxywarfarin (Daly et al., 2017; Flora et al., 2017). Warfarin interacts with the PXR nuclear receptor and significantly induces CYP3A4 and CYP2C9 mRNAs in cultures of primary human hepatocytes or in LS174T intestinal cells (Rulcova et al., 2010). Clinically relevant reports indicate that St. John's wort preparations, used as an herbal antidepressant, can alter the pharmacokinetics of warfarin. This interaction is due to the activation of the pregnane X receptor (PXR), making it an inducer of cytochrome P450 enzymes (most notably CYP3A4) and P-glycoprotein (Nicolussi et al., 2020). The results of in vitro and in vivo studies show that administering warfarin simultaneously with ginsenosides, the main component of ginseng, may weaken the therapeutic effect of warfarin. Conversely, combining

anticoagulants with NSAIDs can increase the risk of bleeding (Lin et al., 2020; Penner et al., 2022). According to a case clinical study, the use of anticoagulation to treat atrial fibrillation in patients with cirrhosis is associated with a decreased risk of stroke without a significant increase in the risk of haemorrhage (Chokesuwattanaskul et al., 2019).

The cytochrome P450 enzyme, enzyme conjugation, and protein transport influence the metabolism of a drug. The metabolic process involves the activation of two receptors, CAR and PXR. The constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are subgroups of orphan nuclear receptors that regulate the activity of certain metabolizing enzymes. The DNA-binding domains of CAR and PXR has a similar array of amino acids that cause the receptor to bind to similar DNA (Buchman et al., 2018). Nuclear receptor PXR has been identified for its capacity to activate genes without ligands. PXR exhibits specificity towards multiple CYP genes, including CYP3A1, CYP3A4, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP3A5, CYP3A7, CYP4F12, UGT1A1, UGT1A3, UGT1A4, and UGT1A6. In addition to its significant role in inducing drug degradation, CAR can control bilirubin degradation, a process of heme decomposition by the liver. As in xenobiotic-metabolizing enzymes, specific differences are also present in the specificity of these nuclear receptor ligands; rifampin activates human PXR but not in rats or mice. Ursolic acid efficiently reduces the activity of CYP3A4 and CYP2B6 on the hepatocellular carcinoma cell line (HepG2), which are induced by rifampin (RIF, a human PXR agonist) (Chang et al., 2017; Daujat-Chavanieu & Gerbal-Chaloin, 2020; Hogle et al., 2018). CYP2C9, a member of the P450 2C subfamily (CYP2C), is the most prominently expressed enzyme involved in the metabolism of various drugs catalyzed by CYP P450 enzymes. These drugs include oral antidiabetic drugs of the sulfonylurea group, anticoagulants, NSAIDs, and several groups of antihypertensives (Boyce et al., 2018; Daly et al., 2017; Green et al., 2016). The interaction of drugs that are considered in therapy and their activation will be significantly affected by CAR and PXR receptors. CAR and PXR are also receptors that play a role in energy metabolism. PXR has a significant role in protecting against bile acid toxicity by maintaining homeostatic bile acid (Hogle et al., 2018; Yan & Xie, 2016).

Nigella sativa L. (Ranunculaceae), also known as black cumin or black seeds, is a medicinal herb renowned for its spicy culinary usage and has a rich historical significance in traditional medicine. Consumption of *N. sativa* as a traditional medicine can reduce the expression of the CYP2C11 gene in rat liver in vivo. Additionally. the results indicate that *N. sativa* has inhibitory effects on CYP3A4 and CYP2D6 in healthy humans and in vitro at HLM, with modified AUC > 20% (Gougis et al., 2021; Korashy et al., 2014). Thymoquinone (TQ) is one of the active compounds in *N. sativa* (Hannan et al., 2021). The previous study showed that using hydrogen peroxide induction could potentially enhance the inhibitory effect of thymoquinone on the proliferation of HepG2 cells. Thymoquinone has excellent potential as a phytochemical for treating cancer because of its ability to reduce inflammation, combat oxidative stress, and inhibit the growth of new blood vessels (Alhmied et al., 2021; Ghelichkhani et al., 2023). TQ shows preferential cytotoxicity towards hepatocellular carcinoma HepG2 and SMMC-7721 cells (Jehan et al., 2020). TQ has pharmacological activities, such as anticoagulant, antidiabetic, diuretic, antidepressant, anti-inflammatory, antimicrobial, antioxidant, anticonvulsant, nephroprotective, hepatoprotective, and immunomodulatory effects (Butt et al., 2019; Hamdan et al., 2019; Hannan et al., 2021; Hosseinzadeh et al., 2017; Muralidharan-Chari et al., 2016). TQ has also been found to decrease the expression of several genes, including CYP2C11 and CYP2D7,8, and has been shown to interact with chlorzoxazone through the inhibition of CYP3A4 and CYP2C19 (Elbarbry et al., 2017).

Computational studies employing targets of the PXR receptor have been identified and extensively utilized to predict interactions between drugs or herbs and medicines. An in silico docking molecular analysis has demonstrated that felodipine is a potent PXR agonist and induces CYP3A4 expression in HepG2 cells (Reddy & Nyunoya, 2021). The use of molecular docking studies in the activation pathways of PXR receptors allows for predicting metabolic mechanisms in animal investigations (Borse et al., 2019; Daujat-Chavanieu & Gerbal-Chaloin, 2020; Küblbeck et al., 2020).

Several reports indicate that thymoquinone (TQ) interacts with drugs, yet the molecular nature of this interaction at the PXR expression level remains unexplained. To address this gap, we used molecular docking results from previous studies to understand how thymoquinone administration impacts PXR gene expression in HepG2 cells. This study aims to predict the mechanism of herb-drug interaction by examining the activation pathway of the PXR receptor.

EXPERIMENTAL SECTION

Materials

In the docking method validation process, the PDB IDs for the target PXR (pregnane X receptor) receptors are 7AXJ, 1SKX, and 4XAO. The PDB IDs were acquired from the protein data bank website (https://www.rcsb.org/). The ligands used in this study were 4 chemical constituents from *Nigella sativa* (Table 1), agonist ligand 7AXJ (hyperforin) collected from Pubchem, and then drawn using Marvin Js.

The human hepatocellular carcinoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.).

Thymoquinone (TQ) and warfarin were purchased from Sigma-Aldrich Pte. Ltd., Singapore, The media for HepG2 cells consists 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-dimethyltetrazolium bromide] 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 PXR (GAPDH, F: 5′-CCTTCATTGACCTCAACTA-3′, R: 5′-GGAAGGCCATGCCAGTGAGC-3′; PXR, F: 5′-GAAGATCATGGCTGTCCTCAC-3′, R: 5′-CGTCCGT GCTGCTGAATAA-3′).

Instrumentation

Molecular docking investigations were performed using a computer system equipped with the necessary hardware for molecular modeling and computational chemistry calculations. The system was powered by Intel® Core™ i5 processor, 8GB RAM, and a 64GB flash drive. The required software included Microsoft operating system and Molecular Operating Environment (MOE) software version 2015, licensed under the Faculty of Pharmacy, UGM. The expression of PXR studies was assessed using a Multimode Microplate Reader (Spark Tecan) and Real-Time PCR instrument ABi7500fast.

Quantitative Structure-Activity Relationship (QSAR) analysis

A QSAR analysis was conducted to screen the bioactivity of the ligands using the Way2Drug/PASS server (http://www.way2drug.com/passonline/). The assessment values ranged from 0.000 to 1.000, and only molecules whose activity was Pa> Pi (Pa = Probability to be active; Pi = Probability to be inactive) were considered for further evaluation. The ligands are expected to exhibit PXR activity with a Pa > Pi value (Daniel et al., 2023; Sonkamble et al., 2018).

Molecular Docking of PXR Receptors

Preparation of PXR receptor

The 3D structure of the PXR Receptor was downloaded from the RCSB database available at https://www.rcsb.org/ in PDB format. The protein target was then opened using the MOE software. The structure of the protein chain of the PXR receptor was prepared using the Structure Preparation application in MOE. The application identified and automatically corrected issues in the protein's 3D structure. The QuickPrep panel in MOE was used to perform automatic corrections, protonation, tethering, and structure minimization for the ligand-receptor complex of PXR. Hydrogen atoms and atomic charges were added, and water molecules were removed through SEQ (sequence) tools. The prepared protein structure was saved in MOE format (.moe) (Halim et al., 2021; Marbun et al., 2023).

Design and preparation of test compounds

The designed compounds of *Nigella Sativa*, hyperforin and warfarin were inputted into the MOE software for preparation. The position of the structures with the lowest energy was determined using the Energy Minimize feature in MOE, followed by the search for the most stable conformations of the ligands. A database was created containing the test compounds with the most stable conformations for docking purposes. The files were saved in MOE format (.moe) (Halim et al., 2021; Marbun et al., 2023).

The known ligand dataset from PubChem (https://pubchem.ncbi.nlm.nih.gov/) was prepared, including the ligand structures available in SMILES code format. The 3D structures of each ligand were generated using the MOE-Builder module. Subsequently, conformational searches were performed to obtain the lowest conformational energy for each ligand, which was then minimized using the semiempirical (PM3). The optimized structures were stored in a database with the *.mdb format (Halim et al., 2021; Marbun et al., 2023).

Docking method validation

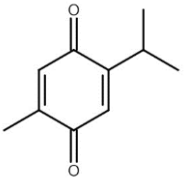
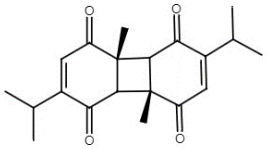
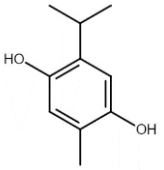
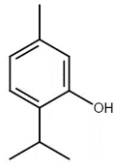
The docking method was validated through pose validation and scoring validation. Pose validation was performed by redocking the native ligand to the target protein, and the obtained RMSD values were evaluated by comparing the re-docked ligand poses with the ligand poses obtained from crystallography. The method is considered valid if the RMSD value is less than 2 (Halim et al., 2021; Marbun et al., 2023; Ramírez & Caballero, 2018).

Ligand docking and docking result analysis

The docking process was performed using MOE between compounds of *Nigella Sativa* (Table 1), hyperforin and warfarin, and the prepared isoform PXR receptor complex. A docking protocol was executed according to previously validated scoring function and pose validation results.

The analysis was conducted on molecular docking results between the test ligands and the native ligands with their respective target proteins. The affinity of the ligands to the target could be assessed by the binding energy indicated by the docking score values obtained after the docking process. Smaller binding energy values may indicate stronger binding between the test compounds and the target, indicating more stable interactions. The docking scores of the best poses for each compound in the test ligands dataset were interpolated to the validated correlation equation to obtain predicted affinity/activity values. The docking results were analyzed for the interactions between the test compounds and the protein's amino acid residues in the pocket to identify the binding interactions that may contribute to PXR receptor (Halim et al., 2021; Marbun et al., 2023; Shivanika C et al., 2022).

Table 1. Chemical constituents of *N. sativa*

No	Compound	Structure
1	Thymoquinone (PubChem ID 10281)	
2	Dithymoquinone (PubChem ID 398941)	
3	Thymohydroquinone (PubChem ID 95779)	
4	Thymol (PubChem ID 6989)	

The Expression of PXR on HepG2 Cells

HepG2 cell culture

The HepG2 cells, derived from human hepatocellular carcinoma, were acquired from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). In DMEM, a growth medium, HepG2 cells were cultivated at 37°C with 5% CO_2. Passaging of cells occurred every 5–7 days. From confluent cultures, suspensions of HepG2 cells were generated utilizing a trypsin/EDTA solution, and the concentration of each cell was assessed using a hemocytometer. As needed, the growth medium was replaced every 3-4 days.

Cytotoxic assay of the warfarin and thymoquinone on HepG2 cells

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was employed to perform the cytotoxic assay. The cells were cultivated in a flask under controlled conditions of 37°C and a 5% CO_2 atmosphere with humidity until they reached 80% confluence. A solution containing 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid disodium salt (EDTA) was used on the cells in a flask (Sigma-Aldrich Pte. Ltd.). Cell culture was prepared and grown with DMEM medium. Cell incubation was performed for 72 hours with control warfarin, a combination of warfarin and thymoquinone at t he same concentration of 38.54, 19.27, and 9.62 μM, respectively. The cell cultures were used in this experiment when the compound did not have a cytotoxic effect rate of more than 20%, maintaining a HepG2 cell viability of over 80%.

The PXR expression in HeG2 cells by qRT-PCR

Cell incubation was performed for 72 hours with warfarin or co-incubation of warfarin and thymoquinone at the same concentration of 19.27μM. The PXR 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 final volume of 20 μL. The amplification procedure commenced with an initial denaturation phase at 95 °C for 2 minutes. This was followed by denaturation at the same temperature for 15 seconds and subsequent annealing at 64 °C for 1 minute. This cycle was repeated 40 times. The threshold value (CT) was automatically calculated by qRT-PCR.

Statistical Analysis

The data was displayed as the mean ± SD. Following a one-way ANOVA, the statistical significance between different groups was determined using a post hoc Tukey test. The statistical tests were conducted using SPSS ver.22 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined when a (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

RESULTS AND DISCUSSION

Quantitative Structure-Activity Relationship (QSAR) Analysis

The QSAR analysis results using the PASS online server indicated that the compounds exhibited bioactivity against PXR with Pa > Pi value and were selected for molecular docking analysis **(Table 2)**.

Molecular Docking of PXR Receptors

The docking method validation results **(Table 3)** of some PDB IDs for PXR receptors (7AXJ, 1SKX, and 4XAO) indicated that 7AXJ and 4XAO met the criteria of having an RSMD value less than 2Å (Ramírez & Caballero, 2018). The RMSD value in docking serves as the deviation criteria between the native ligand position and the redocking position result, indicating the capability of MOE and the qualification of PDB to perform molecular docking (Marbun et al., 2023; Tong et al., 2020). According to the docking score, 7AXJ had the highest score. The negative value indicated a high binding energy level and signified a stronger attraction between the ligand and protein **(Table 3).** Therefore, the molecule 7AXJ was chosen for molecular docking investigations with the chemical components in *Nigella sativa*.

Estradiol functions as the ID 7AXJ protein's native ligand, serving as an agonist of PXR receptors, and is responsible for activating phases I and II metabolizing enzymes. Estradiol has been found to occupy a specific position within the expansive ligand-binding pocket of PXR. It acts as an intermediary, connecting the critical polar residues Ser-247 and Arg-410 within PXR (Banerjee et al., 2013; Xue et al., 2007). Estradiol is a potent inhibitor of CYP3A4 and CYP2B6, necessitating proper documentation to ensure accurate modeling of xenobiotic and endobiotic effects on drug metabolism and potential adverse reactions (Olack et al., 2022). The docking analysis utilized to validate estradiol as a native ligand bound to the amino acids Asp205 and Arg410 from PXR is compatible with these amino acids.

The results of docking of molecules analysis of chemical compounds from *Nigella sativa* and warfarin at PXR proteins **(Table 4)** demonstrated that all compounds exhibited an affinity towards either one or both proteins, as indicated by the increasing negative values of binding energy.

The binding energy, often known as the docking score, is a quantitative measure that indicates the magnitude of the ligand-protein or macromolecule-protein interaction. A negative binding energy value indicates that the interaction between the macromolecule's receptor and ligand is stable and solid (Azmi et al., 2021; Damayanti et al., 2020; Durga & Julius, 2020).

Each compound possesses specific binding sites on the amino acid protein target on Arg410, Asp205, and Ser247 (7AXJ). Thymoquinone and dithymoquinone, including the native ligand in 7AXJ at the O atom, form bonds with Arg410 (H-acceptor). The presence of these hydrogen bonds creates a strong ligand-receptor interaction. Conversely, the agonist ligand (hyperforin) forms an explicit binding with O-Arg410 (Ionic) **(Figures 1 and 2).** The compounds in *Nigella sativa* have demonstrated an affinity for the PXR receptor, particularly the 7AXJ protein, as evidenced by its high binding energy value and the fact that it bound to Arg410 and Asp205 in the native ligand. Additionally, dithymoquinone was observed to possess the most intense binding energy, 7AXJ, in the Arg410 amino acid. Thymoquinone, the main compound found in *Nigella sativa,* was found to bind to the identical amino acid Arg410 with the native ligand.

Table 2. QSAR analysis of the selected ligands for PXR bioactivity using the PASS Online server

No	Compound	Pa	Pi	Activity
1	Thymoquinone	0.231	0.006	PXR Agonist
2	Dithymoquinone	-	-	-
3	Thymohydroquinone	0.231	0.006	PXR Agonist
4	Thymol	0.251	0.005	PXR Agonist
5	Hyperforin	0.045	0,032	PXR Agonist
6	Warfarin	-	-	-

Table 3. Validation of the outcome of docking procedures for multiple PDB IDs of the PXR receptor with its native ligand

Receptor	ID PDB	Co-crystallized Ligand (native ligand)	Docking score	RMSD (Å)	Ligand atom	Amino acid	Binding type	Distance (Å)
PXR	7AXJ	Estradiol	-5.6610	1.6458	O	Asp205	H-donor	2.79
					O	Arg410	H-acceptor	2.96
	1SKX	Rifampicin	-8.7558	4.4065	O	Arg410	Ionic	3.59
	4XAO	(4S)-2-methyl-2.4-pentanediol	-3.4603	1.6973	-	-	-	-

Table 4. Results of molecular docking interactions between compounds and the protein 7AXJ

No	Compound	Docking score	RMSD-Refine (Å)	Ligand atom	Amino acid	Binding type	Distance (Å)
1	Thymoquinone	-4.5962	1.7507	O	Arg410	H-acceptor	3.06
2	Dithymoquinone	-6.1963	1.3883	O	Arg410	H-acceptor	3.33
3	Thymohydroquinone	-4.7082	0.5603	O	Asp205	H-donor	3.05
4	Thymol	-4.5774	1.2027	O	Ser247	H-donor	3.04
5	Agonist ligand (Hyperforin)	-8.4957	1.4682	O	Arg410	Ionic	3.99
6	Warfarin	-6.0507	1.5018	O	Arg410	H-acceptor	3.53
				O	Arg410	Ionic	3.09

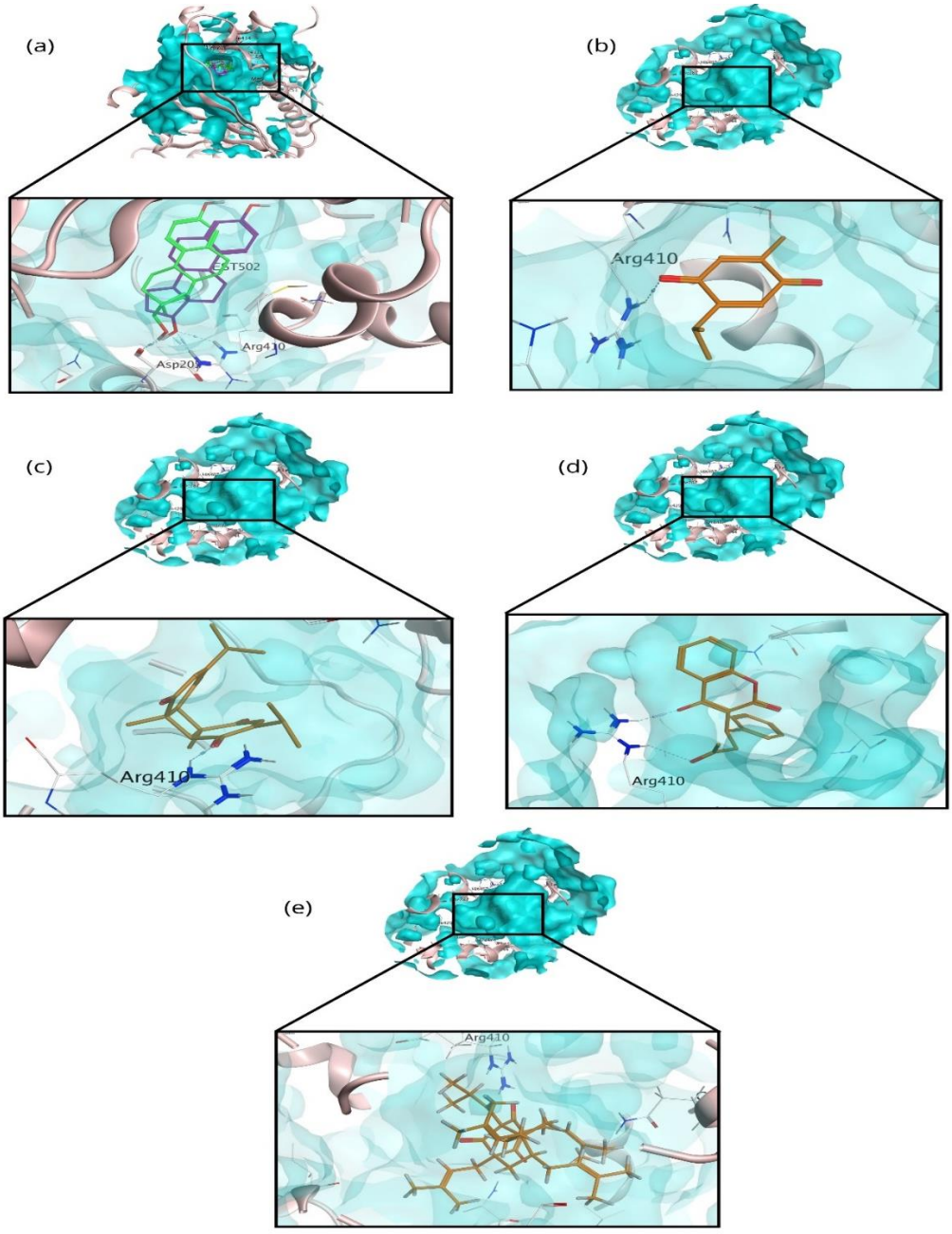


Figure 1. Redocking the position of a 3D-conformation ligand using the proposed method: (a) 7AXJ to Estradiol (RMSD:1.6458 Å), the green structure represents the native ligand, and the purple structure represents Estradiol; ligand interaction *Nigella sativa* chemical constituent and Warfarin to 7AXJ using MOE. 7AXJ-Thymoquinone (b); 7AXJ-Dithymoquinone (c); 7AXJ-Warfarin (d); 7AXJ-Agonist (Hyperforin) (e)

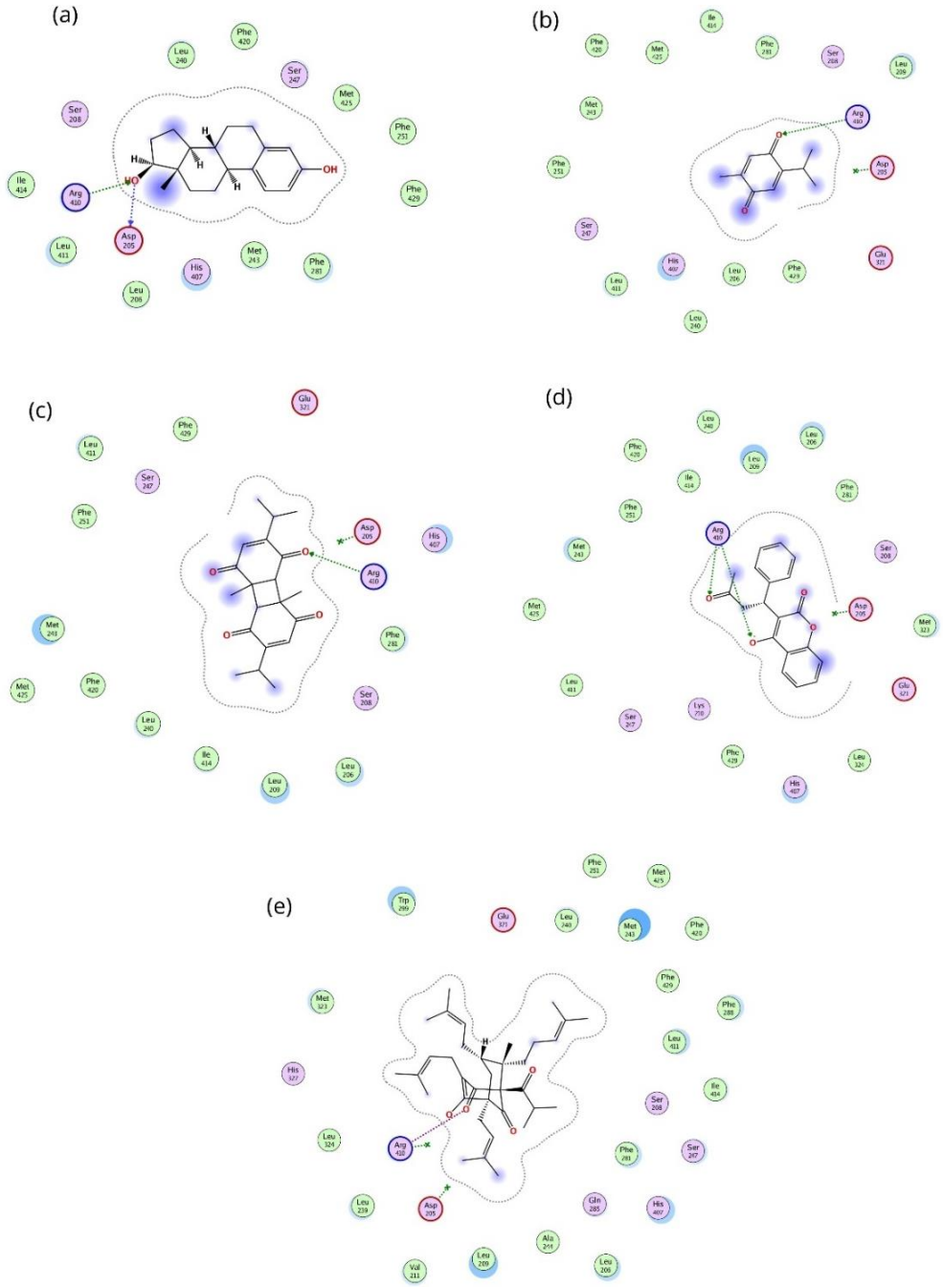


Figure 2. Redocking the position of a 2D-conformation ligand using the proposed method: (a) 7AXJ with Estradiol and ligand interaction *Nigella sativa* chemical constituent and Warfarin to 7AXJ using MOE. 7AXJ-Thymoquinone (b); 7AXJ-Dithymoquinone (c); 7AXJ-Warfarin (d); 7AXJ-Agonist (Hyperforin) (e)

The Expression of PXR on HepG2 Cells

An in vitro study was conducted to investigate the molecular mechanism of thymoquinone interaction. Specifically, it focused on examining the expression of genes involved in warfarin metabolism, particularly PXR, in the human hepatoma cell line HepG2. 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. The increase in expression of the PXR gene has profound implications for the gene products, namely mRNA and the PXR enzyme. An increase in such products will significantly impact the drug metabolism processes, potentially leading to faster drug action in the body.

The combination of warfarin and TQ at a concentration of 38.54 μM exhibits more than 20% cytotoxicity. This finding is of utmost importance as it suggests a potential risk associated with this combination. In contrast, warfarin or its combination with TQ (19.27 and 9.62 μM) exhibits a relatively minimal cytotoxic effect and demonstrated cell viability values over 80%. Similarly, the single compound of

warfarin exhibited cell viability values greater than or equal to 80% at 9.62 μM or higher concentrations (Figure. 3).

The results of initial cytotoxic screening in a single warfarin combination (warfarin-TQ) against HepG2 cells at 72-hour incubation, with viability of ≥ 80% for a concentration of 19.27 μM, showed that the concentration given could maintain HepG2 survival. Furthermore, the expression assay of PXR of the concentration of warfarin (control) and the combination was measured at a concentration of TQ at 19.27 μM (Table 5).

The determination of mRNA isolation purity, a crucial step in our experiment, is based on the 260/280 ratio. The A260/A280 absorbance ratio for each sample was precisely measured at 2.1 using a spectrophotometer, ensuring accurate results. The mRNA isolation purity test showed a 260/280 ratio within the range of 2.0, indicating a high level of purity without protein contamination (Gandhi et al., 2020).

Our study aimed to assess the impact of TQ on the CYP2C9 gene, a key player in warfarin metabolism in HepG2 cells. To do this, we utilized qRT-PCR, a method that amplifies and quantifies the DNA quantity from the target gene. The selected housekeeping gene, GAPDH, served 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. The results of this analysis would shed light on the ability of TQ to induce changes in the CYP2C9 gene.

Our experiments on HepG2 cells revealed a significant change in the expression of the PXR gene when warfarin and TQ were administered simultaneously. The mRNA expression value was notably higher than the control, indicating a significant increase in PXR expression. Specifically, the expression of PXR in HepG2 cells increased at a concentration of 19.27 μM following treatment with warfarin. However, when warfarin was combined with TQ, there was a significant reduction in the expression of PXR ($p < 0.046$) compared to warfarin alone, suggesting an inhibitory effect of the combination (Figure. 4).

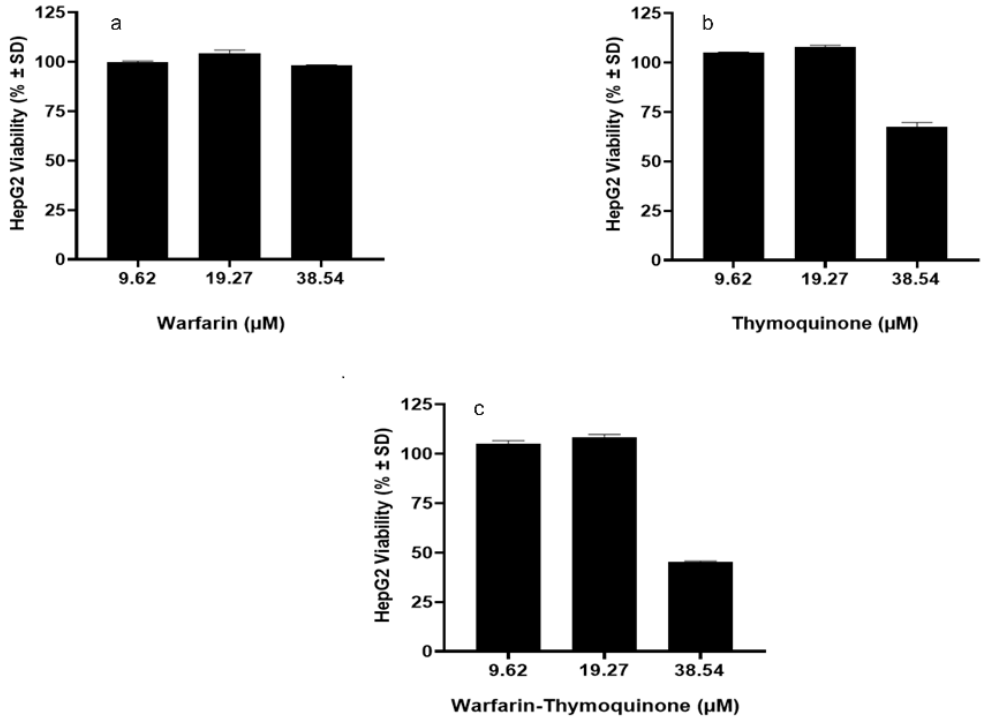


Figure 3. The figure presents the cell viability values (%) of HepG2 cells after a single treatment with different concentrations of warfarin (9.62, 19.27, and 38.54 μM) (a), thymoquinone (9.62, 19.27, and 38.54 μM) (b), and a combination of warfarin and thymoquinone (at the same concentrations) (c). The cells were incubated for 72 hours in DMEM media, and cell viability was calculated using the MTT method with 3 replications.

Table 5. HepG2 cell viability of single and combination administration during 72-hour incubation

Compound	HepG2 viability (% ± SD)
Thymoquinone (19.27 μM)	105.10 ± 0.21
Warfarin (19.27 μM)	99.84 ± 0.55
Warfarin (19.27 μM) + Thymoquinone (19.27 μM)	105.00 ± 1.60

Data are represented as mean ± SD from 3 replications.

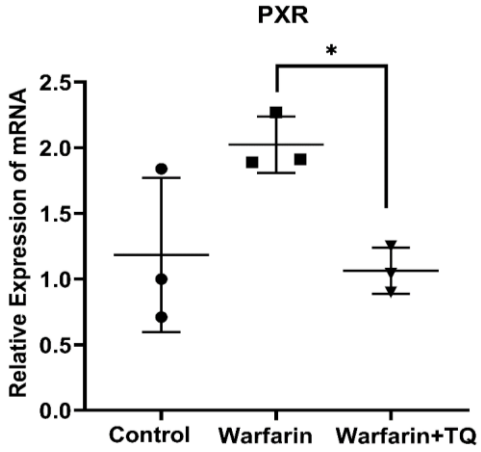


Figure 4. Relative expression of PXR mRNA in HepG2 cell cultures after treatment with warfarin alone and in combination with TQ for 72 hours. Each test group was carried out with 3 replications. * $p < 0.05$ indicated the significance compared to warfarin alone.

Drug-responsive nuclear receptors and hepatic transcriptional factors are recognized for their role in regulating the transcription of CYP2C genes by interacting with their promoters. Moreover, cross-talk between HNF4α and PXR/CAR is necessary for optimal induction in response to drugs (Hogle et al., 2018). The present study has demonstrated that molecular docking analysis of PXR shows a strong binding energy between thymoquinone and the target protein. This suggests that treatment of warfarin and TQ in HepG2 cells can lead to a significant decrease in the expression of the PXR gene. These findings highlight the potential for drug-herbal drug interactions that may result in therapeutic issues in clinical settings.

CONCLUSIONS

The compounds found in *Nigella sativa*, specifically thymoquinone, have shown to exhibit the highest binding energy for the nuclear receptors PXR, as demonstrated by in silico analyses. Moreover, the concurrent administration of warfarin and TQ in HepG2 leads to a significant decrease in the relative mRNA expression in the PXR gene. Therefore, this study suggests predicting the mechanism of herb-drug interactions (HDIs) from *Nigella sativa*, especially the thymoquinone, on warfarin metabolism through the PXR receptor activation pathway.

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CONFLICT OF INTEREST

No conflicts of interest were disclosed by the authors in this work.

AUTHOR CONTRIBUTIONS

AM, AN, AEN, EL and PM were involved in designing the study. AM conducted the experimental studies. Additionally, AM, AN, AEN, EL and PM contributed to composing the manuscript and analyzing the data. After careful review and agreement by all authors, the final version of this manuscript was submitted.

REFERENCES

Alhmied, F., Alammar, A., Alsultan, B., Alshehri, M., & Pottoo, F. H. (2021). Molecular mechanisms of thymoquinone as anticancer agent. *Combinatorial Chemistry & High Throughput Screening*, *24*(10), 1644–1653. https://doi.org/10.2174/1386207323999201027225305

Azmi, M., Syed, I. P., & Koneru, A. (2021). In silico docking analysis and admet prediction of thymoquinone derivatives against ovarian cancer. *Oriental Journal of Chemistry*, *37*(1), 221–228. https://doi.org/10.13005/ojc/370130

Banerjee, M., Robbins, D., & Chen, T. (2013). Modulation of xenobiotic receptors by steroids. *Molecules*, *18*(7), 7389–7406. https://doi.org/10.3390/molecules18077389

Borse, S. P., Singh, D. P., & Nivsarkar, M. (2019). Understanding the relevance of herb–drug interaction studies with special focus on interplays: A prerequisite for integrative medicine. *Porto Biomedical Journal*, *4*(2), e15. https://doi.org/10.1016/j.pbj.0000000000000015

Boyce, M. L., Zayac, A., Davis, A., Badrick, T., Anoopkumar-Dukie, S., & Bernaitis, N. (2018). Impact of aspirin on warfarin control as

measured by time in therapeutic range. *Basic & Clinical Pharmacology & Toxicology*, *123*(4), 504–508. https://doi.org/10.1111/bcpt.13037

Buchman, C. D., Chai, S. C., & Chen, T. (2018). A current structural perspective on PXR and CAR in drug metabolism. *Expert Opinion on Drug Metabolism & Toxicology*, *14*(6), 635–647. https://doi.org/10.1080/17425255.2018.1476488

Butt, A., Nisar, N., Mughal, T., Ghani, N., & Altaf, I. (2019). Anti-oxidative and anti-proliferative activities of extracted phytochemical compound thymoquinone. *Journal of the Pakistan Medical Association*, *69*(10), 479–1485. https://doi.org/10.5455/JPMA.302643156

Chang, H.-Y., Chen, C.-J., Ma, W.-C., Cheng, W.-K., Lin, Y.-N., Lee, Y.-R., Chen, J.-J., & Lim, Y.-P. (2017). Modulation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) activation by ursolic acid (UA) attenuates rifampin-isoniazid cytotoxicity. *Phytomedicine*, *36*, 37–49. https://doi.org/10.1016/j.phymed.2017.09.016

Chokesuwattanaskul, R., Thongprayoon, C., Bathini, T., Torres-Ortiz, A., O'Corragain, O. A., Watthanasuntorn, K., Lertjitbanjong, P., Sharma, K., Prechawat, S., Ungprasert, P., Kröner, P. T., Wijarnpreecha, K., & Cheungpasitporn, W. (2019). Efficacy and safety of anticoagulation for atrial fibrillation in patients with cirrhosis: A systematic review and meta-analysis. *Digestive and Liver Disease*, *51*(4), 489–495. https://doi.org/10.1016/j.dld.2018.12.001

Daly, A., Rettie, A., Fowler, D., & Miners, J. (2017). Pharmacogenomics of CYP2C9: functional and clinical considerations. *Journal of Personalized Medicine*, *8*(1), 1. https://doi.org/10.3390/jpm8010001

Damayanti, S., Martak, N. A. S., Permana, B., Suwandi, A., Hartati, R., & Wibowo, I. (2020). In silico study on interaction and preliminary toxicity prediction of *Eleutherine americana* components as an antifungal and antitoxoplasmosis Candidate. *Indonesian Journal of Chemistry*, *20*(4), 899. https://doi.org/10.22146/ijc.48570

Daniel, N., Ferdinand, F., & Aditya, P. A. (2023). In silico targeting CYP51 of *Naegleria fowleri* using bioactive compounds from Indonesian plants. *Journal of Pharmacy & Pharmacognosy Research*, *11*(5), 841–862. https://doi.org/10.56499/jppres23.1693_11.5.841

Daujat-Chavanieu, M., & Gerbal-Chaloin, S. (2020). Regulation of CAR and PXR expression in health and disease. *Cells*, *9*(11), 2395. https://doi.org/10.3390/cells9112395

Durga, B., & Julius, A. (2020). In silico docking studies of thymoquinone as potential anti-cancer drug target on lung cancer cells. *Clinical Medicine*, *07*(03), 11.

Elbarbry, F., Ung, A., & Abdelkawy, K. (2017). Studying the inhibitory effect of quercetin and thymoquinone on human cytochrome P450 enzyme activities. *Pharmacognosy Magazine*, *13*(52). http://dx.doi.org/10.4103/0973-1296.224342

Flora, D. R., Rettie, A. E., Brundage, R. C., & Tracy, T. S. (2017). CYP2C9 genotype-dependent warfarin pharmacokinetics: Impact of CYP2C9 genotype on R- and S-warfarin and their oxidative metabolites. *The Journal of Clinical Pharmacology*, *57*(3), 382–393. https://doi.org/10.1002/jcph.813

Gandhi, V., O'Brien, M. H., & Yadav, S. (2020). High-quality and high-yield RNA extraction method from whole human saliva. *Biomarker Insights*, *15*, 1–9. https://doi.org/10.1177/1177271920929705

Ghelichkhani, S., Saffari-Chaleshtori, J., Ghaffari, F., & Nili-Ahmadabadi, A. (2023). The cytotoxic effect of thymoquinone enhance on HepG2 cell line due to induction of fenton reaction by hydrogen peroxide: An in vitro and in silico study. *Asian Pacific Journal of Cancer Prevention*, *24*(5), 1809–1815. https://doi.org/10.31557/APJCP.2023.24.5.1809

Gougis, P., Hilmi, M., Geraud, A., Mir, O., & Funck-Brentano, C. (2021). Potential cytochrome P450-mediated pharmacokinetic interactions between herbs, food, and dietary supplements and cancer treatments. *Critical Reviews in Oncology/Hematology*, *166*, 103342. https://doi.org/10.1016/j.critrevonc.2021.103342

Green, B., Mendes, R. A., Van Der Valk, R., & Brennan, P. A. (2016). Novel anticoagulants –an update on the latest developments and management for clinicians treating patients on these drugs. *Journal of Oral Pathology & Medicine*, *45*(8), 551–556. https://doi.org/10.1111/jop.12441

Halim, S. A., Sikandari, A. G., Khan, A., Wadood, A., Fatmi, M. Q., Csuk, R., & Al-Harrasi, A. (2021). Structure-based virtual screening of tumor necrosis factor-α inhibitors by cheminformatics approaches and bio-molecular simulation. *Biomolecules*, *11*(329), 1-18. https://doi.org/10.3390/biom11020329

Hamdan, A., Haji Idrus, R., & Mokhtar, M. H. (2019). Effects of *Nigella sativa* on type-2 diabetes mellitus: A systematic review. *International Journal of Environmental Research and Public Health*, *16*(24), 4911. https://doi.org/10.3390/ijerph16244911

Hannan, Md. A., Rahman, Md. A., Sohag, A. A. M., Uddin, Md. J., Dash, R., Sikder, M. H., Rahman,

Md. S., Timalsina, B., Munni, Y. A., Sarker, P. P., Alam, M., Mohibbullah, Md., Haque, Md. N., Jahan, I., Hossain, Md. T., Afrin, T., Rahman, Md. M., Tahjib-Ul-Arif, Md., Mitra, S., Kim, B. (2021). Black cumin (*Nigella sativa* L.): A comprehensive review on phytochemistry, health benefits, molecular pharmacology, and safety. *Nutrients*, *13*(6), 1784. https://doi.org/10.3390/nu13061784

Hogle, B. C., Guan, X., Folan, M. M., & Xie, W. (2018). PXR as a mediator of herb–drug interaction. *Journal of Food and Drug Analysis*, *26*(2), S26–S31. https://doi.org/10.1016/j.jfda.2017.11.007

Hosseinzadeh, H., Tavakkoli, A., Mahdian, V., & Razavi, B. M. (2017). Review on clinical trials of black seed (*Nigella sativa*) and its active constituent, thymoquinone. *Journal of Pharmacopuncture*, *20*(3), 179–193. https://doi.org/10.3831/KPI.2017.20.021

Jehan, S., Zhong, C., Li, G., Zulqarnain Bakhtiar, S., Li, D., & Sui, G. (2020). Thymoquinone selectively induces hepatocellular carcinoma cell apoptosis in synergism with clinical therapeutics and dependence of p53 status. *Frontiers in Pharmacology*, *11*, 555283. https://doi.org/10.3389/fphar.2020.555283

Korashy, H., Al-Jenoobi, F., Raish, M., Ahad, A., Al-Mohizea, A., Alam, M., Alkharfy, K., & Al-Suwayeh, S. (2014). Impact of herbal medicines like *Nigella sativa*, *Trigonella foenum-graecum,* and *Ferula asafoetida*, on cytochrome P450 2C11 gene expression in rat liver. *Drug Research*, *65*(07), 366–372. https://doi.org/10.1055/s-0034-1384604

Küblbeck, J., Niskanen, J., & Honkakoski, P. (2020). Metabolism-disrupting chemicals and the constitutive androstane receptor (CAR). *Cells*, *9*(10), 2306. https://doi.org/10.3390/cells9102306

Lin, J. F., Fan, L. L., Li, B. W., Zhao, R. R., Jiang, L. G., Zhang, B. C., Lu, Y. S., & Shao, J. W. (2020). A study to evaluate herb-drug interaction underlying mechanisms: An investigation of ginsenosides attenuating the effect of warfarin on cardiovascular diseases. *European Journal of Pharmaceutical Sciences*, *142*, 105100. https://doi.org/10.1016/j.ejps.2019.105100

Marbun, P., Hakim, A. R., Ujiantari, N. S. O., Sudarmanto, B. S. A., & Nugroho, A. E. (2023). In silico pharmacokinetics study of 2,5-dibenzylidenecyclopentanone analogs as mono-ketone versions of curcumin. *BIO Web of Conferences*, *75*, 04002. https://doi.org/10.1051/bioconf/20237504002

Massadeh, A. M., Al-Safi, S. A., Momani, I. F., Al-Mahmoud, M., & Alkofahi, A. S. (2007). Analysis of cadmium and lead in mice organs: Effect of *Nigella sativa* L. (black cumin) on the distribution and immunosuppressive effect of cadmium-lead mixture in mice. *Biological Trace Element Research*, *115*(2), 157–167. https://doi.org/10.1007/BF02686027

Muralidharan-Chari, V., Kim, J., Abuawad, A., Naeem, M., Cui, H., & Mousa, S. (2016). Thymoquinone modulates blood coagulation in vitro via its effects on inflammatory and coagulation pathways. *International Journal of Molecular Sciences*, *17*(4), 474. https://doi.org/10.3390/ijms17040474

Nicolussi, S., Drewe, J., Butterweck, V., & Meyer Zu Schwabedissen, H. E. (2020). Clinical relevance of St. John's wort drug interactions revisited. *British Journal of Pharmacology*, *177*(6), 1212–1226. https://doi.org/10.1111/bph.14936

Olack, E. M., Heintz, M. M., & Baldwin, W. S. (2022). Dataset of endo- and xenobiotic inhibition of CYP2B6: Comparison to CYP3A4. *Data in Brief*, *41*, 108013. https://doi.org/10.1016/j.dib.2022.108013

Penner, L. S., Gavan, S. P., Ashcroft, D. M., Peek, N., & Elliott, R. A. (2022). Does coprescribing nonsteroidal anti-inflammatory drugs and oral anticoagulants increase the risk of major bleeding, stroke and systemic embolism? *British Journal of Clinical Pharmacology*, *88*(11), 4789–4811. https://doi.org/10.1111/bcp.15371

Kucharczuk, C. R., Ganetsky, A., & Vozniak, J. M. (2018). Drug-drug interactions, safety, and pharmacokinetics of EGFR tyrosine kinase inhibitors for the treatment of non–small cell lung cancer. *Journal of the Advanced Practitioner in Oncology*, *9*(2). https://doi.org/10.6004/jadpro.2018.9.2.5

Ramírez, D., & Caballero, J. (2018). Is it reliable to take the molecular docking top scoring position as the best solution without considering available structural data?. *Molecules*, *23*(5), Article 5. https://doi.org/10.3390/molecules23051038

Reddy, R. T., & Nyunoya, T. (2021). Identification of novel pregnane X receptor (PXR) agonists by in silico and biological activity analyses and reversal of cigarette smoke-induced PXR downregulation. *Biochemical and Biophysical Research Communications*, *555*, 1–6. https://doi.org/10.1016/j.bbrc.2021.02.145

Rulcova, A., Prokopova, I., Krausova, L., Bitman, M., Vrzal, R., Dvorak, Z., Blahos, J., & Pavek, P. (2010). Stereoselective interactions of warfarin enantiomers with the pregnane X nuclear receptor in gene regulation of major drug-metabolizing cytochrome P450 enzymes. *Journal of Thrombosis and Haemostasis*, *8*(12), 2708–2717. https://doi.org/10.1111/j.1538-7836.2010.04036.x

Shivanika C, Kumar, S. D., Ragunathan, V., Tiwari, P., Sumitha A., & Devi, P. B. (2022). Molecular docking, validation, dynamics simulations, and pharmacokinetic prediction of natural compounds against the SARS-CoV-2 main-protease. *Journal of Biomolecular Structure and Dynamics*, *40*(2), 585–611. https://doi.org/10.1080/07391102.2020.1815584

Sonkamble, V. V., Wagh, N. S., & Kamble, L. H. (2018). Inhibition of α-amylase and α-glucosidase by (6RS)-22-hydroxy-23,24,25,26,27-pentanor-vitamin-D3-6,19-sulfur dioxide-adduct, manoalide and 5β-cholestane-3α,7α,12α,24,25,26-hexol isolated from acetone extract of *Helianthus annuus* L. seeds. *International Journal of Pharmacy and Pharmaceutical Sciences*, *10*(5), 39–49. https://doi.org/10.22159/ijpps.2018v10i5.25309

Tong, X. Y., Liao, X., Gao, M., Lv, B. M., Chen, X. H., Chu, X. Y., Zhang, Q. Y., & Zhang, H.Y. (2020). Identification of NUDT5 inhibitors from approved drugs. *Frontiers in Molecular Biosciences*, *7*, 44. https://doi.org/10.3389/fmolb.2020.00044

Xue, Y., Moore, L. B., Orans, J., Peng, L., Bencharit, S., Kliewer, S. A., & Redinbo, M. R. (2007). Crystal structure of the pregnane X receptor-estradiol complex provides insights into endobiotic recognition. *Molecular Endocrinology*, *21*(5), 1028–1038. https://doi.org/10.1210/me.2006-0323

Yan, J., & Xie, W. (2016). A brief history of the discovery of PXR and CAR as xenobiotic receptors. *Acta Pharmaceutica Sinica B*, *6*(5), 450–452. https://doi.org/10.1016/j.apsb.2016.06.011

Zhao, D., Chen, J., Chu, M., Long, X., & Wang, J. (2020). Pharmacokinetic-based drug–drug interactions with anaplastic lymphoma kinase inhibitors: A review. *Drug Design, Development and Therapy*, *14*, 1663–1681. https://doi.org/10.2147/DDDT.S249098