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In vitro inhibitory mechanisms and molecular docking of 1'-S-1'-acetoxychavicol acetate on human cytochrome P450 enzymes.

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ABSTRACT

Background: The compound, 1'-S-1'-acetoxychavicol acetate (ACA), isolated from the rhizomes of a Malaysian ethno-medicinal plant, *Alpinia conchigera* Griff. (Zingiberaceae), was previously shown to have potential in vivo antitumour activities. In the development of a new drug entity, potential interactions of the compound with the cytochrome P450 superfamily metabolizing enzymes need to be ascertain.

Purpose: The concomitant use of therapeutic drugs may cause potential drug-drug interactions by decreasing or increasing plasma levels of the administered drugs, leading to a suboptimal clinical efficacy or a higher risk of toxicity. Thus, evaluating the inhibitory potential of a new chemical entity, and to clarify the mechanism of inhibition and kinetics in the various CYP enzymes is an important step to predict drug-drug interactions.

Study design: This study was designed to assess the potential inhibitory effects of *Alpinia conchigera* Griff. rhizomes extract and its active constituent, ACA, on nine c-DNA expressed human cytochrome P450s (CYPs) enzymes using fluorescent CYP inhibition assay.

Methods/Results: The half-maximal inhibitory concentration (IC\textsubscript{50}) of *Alpinia conchigera* Griff. rhizomes extract and ACA was determined for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. *A. conchigera* extract only moderately inhibits on CYP3A4 (IC\textsubscript{50} = 6.76 ± 1.88 µg/ml) whereas ACA moderately inhibits the activities of CYP1A2 (IC\textsubscript{50} = 4.50 ± 0.10 µM), CYP2D6 (IC\textsubscript{50} = 7.50 ± 0.17 µM) and CYP3A4 (IC\textsubscript{50} = 9.50 ± 0.57 µM) while other isoenzymes are weakly inhibited. In addition, mechanism-based inhibition studies reveal that CYP1A2 and CYP3A4 exhibited non-mechanism based inhibition whereas CYP2D6 showed mechanism-based inhibition. Lineweaver-Burk plots depict that ACA competitively inhibited both CYP1A2 and CYP3A4, with a K\textsubscript{i} values of 2.36 ± 0.03 µM and 5.55 ± 0.06 µM, respectively, and mixed inhibition
towards CYP2D6 with a $K_i$ value of 4.50 ± 0.08 µM. Further, molecular docking studies show that ACA is bound to a few key amino acid residues in the active sites of CYP1A2 and CYP3A4, while one amino residue of CYP2D6 through predominantly Pi-Pi interactions.

Conclusion: Overall, ACA may demonstrate drug-drug interactions when co-administered with other therapeutic drugs that are metabolized by CYP1A2, CYP2D6 or CYP3A4 enzymes. Further in vivo studies, however, are needed to evaluate the clinical significance of these interactions.

Keywords

1'-S-1'-acetoxychavicol acetate; Drug interaction; Cytochrome P450; Inhibition; Enzyme kinetics; Molecular docking

Abbreviations

ACA, 1'-S-1'-acetoxychavicol acetate; BOMCC, 7-benzyloxymethyloxy-3-cyanocoumarin; CYPs, cytochrome P450s; DBOMF, dibenzyloxymethyl-fluorescein; DDIs, drug-drug interactions; EOMCC, 7-ethoxy-methyloxy-3-cyanocoumarin; IC$_{50}$, half maximal inhibitory concentration; $K_i$, inhibition constant; $K_m$, Michaelis constant; MOBFC, 7-(4-methoxybenzyloxy)-4-trifluoromethylcoumarin; NADP*, nicotinamide adenine dinucleotide phosphate; NCE, new chemical entity; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SD, standard deviation; $V_{max}$, the maximum velocity of the reaction.
1. Introduction

_Alpinia conchigera_ Griff. is a slender perennial herb widely distributed in eastern Bengal and southwards to Peninsular Malaysia and Sumatera. The rhizomes are used as food condiment in the northern states of Peninsular Malaysia and occasionally in folk medicine in the east coast for the treatment of skin diseases due to fungal infections (Ibrahim et al., 2000, Aziz et al., 2013). Moreover, in some states of Peninsular Malaysia, the rhizomes are used as a post-partum medicine and the young shoots are prepared into a vegetable dish (Ibrahim et al., 2009).

The natural 1’-S-1’-acetoxychavicol acetate (ACA) (Fig. 1) isolated from the rhizomes of _Alpinia conchigera_ Griff. is one of the most medicinally active phytochemical substances provokes apoptosis-mediated cell death towards several human tumour cell lines _in vitro_ and _in vivo_ (Awang et al., 2010; In et al., 2012; Arshad et al., 2015). Moreover, a combination of ACA and cisplatin (CDDP) potentiates the cytotoxic effects of CDDP _in vitro_, and improve the chemotherapeutic regime with increased efficacy at a lower concentration of CDDP in an animal model. ACA was found to down-regulate NF-κB activation, and reduce the expression of NF-κB regulated genes such as proinflammatory (_COX-2_) and proliferative (_cyclin D1_) in tumour tissues (In et al., 2012). Nonetheless, ACA exhibits some clinical development limitations such as poor aqueous solubility and non-specific targeting of tumour cells, making the compound less favourable for cancer treatment. Further development of ACA to address these drawbacks was carried out by using a novel drug complex formulation comprising of ACA and a recombinant human alpha fetoprotein (rhAFP). Conjugation of ACA-rhAFP treatments show a higher reduction in tumour volume of human lung and prostate xenografted tumour models as compared to the standalone ACA, with less systemic toxicities, for instance, body weight loss and inflammation of vital organs (Arshad et al., 2015).
As a potential antitumour agent, ACA might be used in combination with other drugs, which may have a narrow therapeutic index and a sharp dose toxicity curve (Beijnen and Schellens, 2004). Clinically, the use of combinations therapy can augment the potential for drug-drug interactions (DDIs) which can lead to severe drug adverse events or decreased drug efficacy. Therefore, the United States Food and Drug Administration (US FDA) guidelines clearly define the need to investigate possible interactions of a new chemical entity (NCE) with the family of cytochrome P450 metabolizing enzymes during the preclinical drug development phase. Hence, it is prudent to assess the potential of ACA to induce DDIs, before proceeding into clinical studies. Drug interactions may arise through the inhibition and/or induction of drug-metabolizing enzymes such as cytochrome P450s (CYPs), which are primarily found in the liver, and transporter proteins (e.g., p-glycoprotein) (Zhang et al., 2010). CYP-mediated drug interactions is a prime concern because CYP enzymes are involved in the oxidative metabolism of more than 70% of prescription drugs (Tyagi et al., 2010). The major CYPs involved in the hepatic metabolism of most drugs include CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP2E1, that contribute to the metabolism of more than 90% of endogenous and exogenous compounds (Lin, 2006).

To the best of our knowledge, this is the first report on the modulatory effects of A. conchigera extract and ACA on the activities of recombinant human CYP isoforms. The present work aims at investigating the in vitro inhibitory effects of A. conchigera extract and ACA on recombinant c-DNA expressed human CYPs using a fluorogenic assay. Those CYP isoforms found to be inhibited by ACA were further assessed to identify the mode of inhibition, which was determined as mechanism-based (time-dependent) and reversible inhibition (competitive, non-competitive or uncompetitive). Finally, two-dimensional (2-D) and three-dimensional (3-D) visualization to understand how ACA interact with particular CYP isoforms was investigated using computational molecular docking analysis.
2. Material and methods

2.1 Compounds and reagents

Rhizomes of *Alpinia conchigera* Griff. were collected from Jeli province of Kelantan, an east-coastal state of Peninsular Malaysia. The sample was extracted and identified by Professor Dr. Halijah Ibrahim from the Institute of Biological Science, Division of Ecology and Biodiversity, Faculty of Science, University of Malaya (Arshad et al., 2015).

Chemicals such as α-naphthoflavone, tranylcypromine, miconazole, sulfaphenazole, quinidine, terfenadine, ketoconazole, anhydrous acetonitrile and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Missouri, USA), and montelukast was obtained from Cayman Chemicals (Michigan, USA). Vivid® P450 screening kits were purchased from Life Technologies™ (California, USA). Each kit contains P450 reaction buffer, P450 BACULOSOME® Plus Reagent, fluorescent substrates (7-benzylxoxymethyloxy-3-cyanocoumarin (BOMCC), 7-ethoxy-methyloxy-3-cyanocoumarin (EOMCC), dibenzyloxymethyl-fluorescein (DBOMF), and 7-(4-methoxybenzyloxy)-4-trifluoromethylcoumarin (MOBFC)), fluorescent standard, the regeneration system (333 mM glucose-6-phosphate and 30 U/ml glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate pH 8.0), and 10 mM NADP+ in 100 mM potassium phosphate, pH 8.0.

2.2 Isolation of ACA from *Alpinia conchigera* Griff. rhizomes extract

Air-dried and powdered rhizomes of *Alpinia conchigera* (2.1 kg) were extracted with hexane (7.0 L) at room temperature (72 hrs). The solvent was evaporated in vacuum to give the hexane extract, which was subjected to column chromatography (CC) on silica gel (Merck Kiesegel 60) eluting with a stepwise gradient of hexane-ethyl acetate (100:0 to 50:50). The
fractions were collected separately and concentrated in vacuum at 40 °C. The fractions with similar TLC profiles were pooled together to give six sub-fractions, which were then subjected to further chromatographic analysis, which yielded 1’-(S)-1’-acetoxychavicol acetate as the major constituent. The structure of this compound was determined based on comparison of its spectral data with those reported in the literatures (Mitsui et al., 1976; Barik et al., 1987; Yang et al., 1999).

The isolated compound was injected into Waters HPLC system equipped with Eclipse Plus C18 column (3.5 µm, 4.6 mm X 150 mm I.D., Agilent Technologies). The HPLC profile of the gradient eluent for ACA was; (A) deionized water and 0.1% formic acid and (B) acetonitrile; 0-10 min (5- 0% A); 10-12 min (0-10% A); 12-14 min (10-20% A) at a flow rate of 0.8 ml/min and the injection volume was 10 µL. The absorbance was recorded at 254 nm. The strong peak was detected at retention time (RT) = 2.44 min with 98% of purity (Supp. Figs. 1A and 1B)

2.3 In vitro CYP450 inhibition study

The inhibitory effects of Alpinia conchigera Griff. rhizomes extract and ACA on the catalytic activities of cDNA-expressed human P450 enzymes were carried out according to the manufacturer’s instructions of Vivid® P450 screening kit. To determine the IC$_{50}$, working concentrations (3.125- 200 µg/ml) of Alpinia conchigera Griff. rhizomes extract was prepared from 2 mg/ml stock solution, whereas the working concentrations of ACA (1.56- 100 µM) was prepared from 0.1 M stock solution. The extract and compound were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in the incubation mixture was kept ≤ 0.1%. The assays were performed using Costar 96-well black, non-treated polystyrene assay plates with flat bottoms (Corning Inc, New York, USA) in the kinetic assay mode.
Into each well, a 40 μl of various concentrations of the extract and test compound solution or positive inhibitor control was incubated with 50 μl of pre-mixture of CYP 450 BACULOSOME® Plus Reagents, the regeneration system in Vivid® reaction buffer or 50 μl of reaction buffer alone as a background control at room temperature (25 °C) for 20 min. Then, the reaction was initiated by an addition of 10 μl/well of a mixture of reconstituted substrate and NADP⁺ in Vivid® reaction buffer. The plate was read immediately for fluorescence changes every 1 min at room temperature for 60 min using an Infinite® 200 Microplate reader (Tecan, Männedorf, Switzerland) with appropriate excitation and emission wavelengths for each P450 enzyme. The readings were displayed as relative fluorescence units (RFU). The final inhibitor concentrations of the inhibitors were chosen according to the Vivid® P450 screening kit protocol. All measurements were performed in triplicate. The percentages of inhibition for each concentration of the test compound or the inhibitor control was calculated using the following formula.

Percentage inhibition

\[
\text{Percentage inhibition} = \left[1 - \frac{\text{RFU of test or inhibitor} - \text{Blank}}{\text{Solvent control} - \text{Blank}}\right] \times 100 \quad (\text{Cheng and Guengerich, 2013}).
\]

2.4 Mechanism-based inhibition studies of ACA

The determination of mechanism-based inhibition of CYP1A2, CYP2D6 and CYP3A4 was carried out by a modified method of Thomford et al., 2016. To determine whether ACA is a time-dependent inhibitor of CYP enzymes, multiple concentrations of ACA were preincubated in CYP450 BACULOSOME® Plus Reagents and in the absence or presence of NADPH in Vivid® reaction buffer. After 30 min preincubation, a mixture of substrate and NADP⁺ were added to initiate the reaction. The fluorescent intensities of the product were
measured every min for 30 min at an excitation and emission wavelength of 415/460 nm using an Infinite® 200 Microplate reader (Tecan, Männedorf, Switzerland). The percentage of inhibition, and subsequently the IC$_{50}$ values of ACA in the reaction with or without NADPH preincubation were measured.

2.5 Enzyme kinetics measurement

In order to determine the type of reversible inhibition and apparent K$_i$ values of ACA in CYP1A2 enzyme, four concentrations of substrate EOMCC (2, 3, 4, 6 µM) were incubated with various concentrations of ACA (0, 2.5, 5, 7.5, 10 µM). For the assessment of inhibitory effect of ACA on CYP2D6 and CYP3A4 enzymes, four concentrations of substrate BOMCC (4, 8, 10, 15 µM) were used in the reaction with various concentrations of ACA (0, 3, 6, 8, 12 µM). Enzyme kinetics were evaluated for ACA with IC$_{50}$ values less than 10 µM according to the In vitro CYP450 inhibition study.

2.6 Molecular docking study

Based on the enzyme inhibition results, a molecular docking analysis on the interaction of ACA with CYP1A2, CYP2D6 and CYP3A4 was performed using the AutoDock Vina version 1.1.2 software to visualize the binding conformations of the compound in the enzymes (Trott and Olson, 2010). The X-ray crystal structures of human CYP1A2 (PDB code: 2HIF), CYP2D6 (PDB code: 3QM4) and CYP3A4 (PDB code: 4D6Z) were obtained from the RCSB Protein Databank (http://www.rcsb.org/). The ligand (ACA) was prepared using ChemBio3D Ultra version 12.0 (PerkinElmer, Massachusetts, USA) and the energy minimization was carried out using the General Atomic and Molecular Electronic Structure System (GAMESS) (PerkinElmer, Massachusetts, USA). The binding results were
visualized as 3-D and 2-D diagrams using Discovery Studio Visualization version 4.5 (Accelrys, Inc., CA).

2.7 Statistical analysis

The quantitative data were expressed as mean ± SD results obtained from at least three independent experiments. GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, USA) was used to calculate the IC$_{50}$ values and enzyme kinetic parameters (K$_m$, V$_{max}$) by regression analysis. The mechanism of inhibition was determined graphically from the Lineweaver-Burk plots. The K$_i$ values were determined using the secondary plots constructed based on the slope of Lineweaver-Burk plots.
3. Results and discussion

3.1 Inhibitory effect of A. conchigera Griff. rhizomes extract and ACA on recombinant human CYPs

The inhibitory effects of A. conchigera Griff. rhizomes extract and ACA on nine human recombinant CYP enzymes were investigated to predict potential drug-drug interactions. The mean IC$_{50}$ values and the standard deviation (SD) of plant extract, ACA or the positive controls are shown in Table 1. A. conchigera exhibits moderate inhibition against CYP3A4 (IC$_{50}$ = 6.76 ± 1.88 µg/ml) whereas other CYPs, CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6, CYP2E1 and CYP3A5, show weak or no inhibition when treated with the extract (Table 1 and Supp. Figs. 2A - 2H). Again, ACA was screened on nine CYP isoforms and DDI is predicted based on the following criteria: IC$_{50}$ < 1 µM suggesting a strong inhibition, 1 µM < IC$_{50}$ < 10 µM suggesting a moderate inhibition, and IC$_{50}$ > 10 µM suggesting a weak inhibitory potency (Bell et al., 2008). We found that ACA show moderate inhibition on the enzymatic activities of CYP1A2, CYP2D6 and CYP3A4, with IC$_{50}$ values of 4.55 ± 0.11 µM, 7.43 ± 1.15 µM and 9.17 ± 1.87 µM, respectively (Figs. 2A, 2C and 2E); whereas all positive controls for the respective CYP isoforms display strong inhibition with IC$_{50}$ values of 0.12 ± 0.03 µM, 0.01 ± 0.003 µM and 0.13 ± 0.02 µM (Figs. 2B, 2D and 2F). ACA however, demonstrates weak inhibition on other CYP isoforms (Supp. Figs. 3A, 3C, 3E, 3G, 3I and 3K); whereas all positive controls for respective CYP isoforms exhibit strong to moderate inhibition (Supp. Figs. 3B, 3D, 3F, 3H, 3J and 3L). These results indicate that both plant extract and ACA could inhibit the activity of CYP3A4, which is the most abundant drug metabolizing CYP isoform. Together with ACA, other active compounds present in the extract may be involve in the inhibition of CYP3A4 activity, which explains the higher inhibition of the plant extract compared to ACA.
3.2 Mechanism-based and non-mechanism-based inhibitory potencies studies of ACA on CYPs

A mechanism-based inhibition is also known as an irreversible inhibition. In an irreversible inhibition, when an inhibitor is metabolized by a CYP enzyme, the reactive metabolite can still tightly bind to the active site of the enzyme resulting in a long inhibition period. Alternatively, an irreversible inhibition can also occur when a metabolite-intermediate complex is formed. The complex is then binds tightly to the heme of the CYP, and causes a catalytic position to become inactive (Fontana et al., 2005).

An IC$_{50}$ value shift assay was carried out to evaluate the mechanism-based and non-mechanism-based inhibitory potencies of ACA on CYP1A2, CYP2D6 and CYP3A4 (Table 2). The IC$_{50}$ ratio was calculated by dividing the IC$_{50}$ value of the test compound without NADPH preincubation divided by the IC$_{50}$ of the test compound with NADPH 30 min preincubation. According to Berry and Zhao, 2008, a test compound is considered a mechanism-based inhibitor when the IC$_{50}$ ratio the test compound in the reaction without or with NADPH preincubation is ≥ 1.5. Our study results show that, the IC$_{50}$ ratios of ACA for CYP1A2 and CYP3A4 are 0.83 ± 0.03 and 0.78 ± 0.03 respectively, which are less than 1.5 indicating that ACA is a non-mechanism-based inhibitor for these CYP isoforms. In contrast, with an IC$_{50}$ ratio = 3.23 ± 0.17 for CYP2D6, ACA is possibly a mechanism-based inhibitor of this enzyme. The study was verified using α-naphthoflavone, quinidine and ketoconazole, which are non-mechanism based inhibitors of CYP1A2, CYP2D6 and CYP3A3, respectively (Pirotta et al., 2014; Bertelsen et al., 2003; Winitthana et al., 2011) (Figs 3A-3F).

3.3 Enzyme kinetic studies

The modes of inhibition of ACA on CYP1A2, CYP2D6 and CYP3A4 by ACA were characterized by enzyme kinetic analysis. The Lineweaver-Burk plots and the corresponding
secondary plot for the inhibition of the three CYP isoenzymes by different concentrations of ACA are shown in Fig. 4. These plots also provided the $K_m$ and $V_{max}$ values of the enzymatic reactions. The derived $K_m$ and $V_{max}$ are used to create a secondary plot of $K_m/V_{max}$ vs. concentrations of the test compound. The $K_i$ value is obtained at the point where the lines of the graph intersect at the y-axis. This y-axis interception provides information on the affinity inhibition of the compound (Tracy et al., 2008).

As presented in Figs. 4A and 4E, the Lineweaver-Burk plots intersect a common point on the y-axis, indicating ACA is a competitive inhibitor of CYP1A2 and CYP3A4. The $K_i$ (mean ± S.D.) values for these enzymes were 2.36 ± 0.03 µM and 5.55 ± 0.06 µM, respectively, determined from the secondary plots shown in Figs. 4B and 4F. On the other hand, ACA shows a non-competitive (mixed-type) inhibitor of CYP2D6, where the Lineweaver-Burk plots intersected slight to the left of the y-axis (Fig. 4C). The $K_i$ (mean ± S.D.) value was 4.50 ± 0.08 µM, obtained from the secondary plot (Fig. 4D). All the inhibition constant values and modes of inhibition of CYP enzymes by ACA are summarized in Table 3.

3.4 Molecular Docking

Molecular docking studies for CYP1A2, CYP2D6 and CYP3A4 were further carried out to visualize the binding of ACA with each CYP isoforms. ACA binds to the active cavity of human CYP1A2 (PDB code: 2HIF) with a binding free energy of -7.0 kcal/mol (Fig. 5A). A previous study has shown that the amino acid residues, including Thr124, Phe125, Phe226, Phe260, Gly316, and Ala317, in the active site of CYP1A2 are responsible for ligand binding (Yang et al., 2012). As shown in Fig. 5B, Phe125, Phe226, Gly316, and Ala317 residues are critical for the binding of ACA to CYP1A2. Pi-Pi stacking interactions can be seen between...
aromatic rings of ACA and Phe125, Phe226 in the distance of approximately 5.52 and 4.97 Å, respectively. A Pi–amide interaction was found between the aromatic ring of ACA and Gly316 at a distance of 4.97 Å. In addition, aromatic ring and C=O group of ACA interacts with Ala317 through Pi-alkyl (4.08 Å) and alkyl (3.39 Å), respectively. It is also found that ACA does not bind to the active cavity of CYP1A2 in the presence of an inhibitor, α-naphthoflavone (data not shown). The finding demonstrates the competitive inhibitory property of ACA on this CYP isoenzyme.

The results of the molecular docking show that ACA binds to CYP2D6 (PDB code: 3QM4) with a binding energy of -7.2 kcal/mol. It has been reported that Phe120, Glu216 and Asp301 were critical amino acid residues for substrate binding of CYP2D6 active sites. It was suggested that Glu216 and Asp301 play a key role in the interaction between CYP2D6 and one of the strong inhibitors, quinidine (McLaughlin et al., 2005). Another study show that carvedilol, an CYP2D6 substrate, demonstrated Pi-Pi interaction with Phe483 (Mo et al., 2012). Figs. 5C and 5D reveal that Phe483 amino acid residue interacts with ACA through Pi-Pi stacking and Pi-alkyl interaction at a distance of 4.65 Å, while other key active site residues previously reported by others were not involved. It can also be seen that Thr54 and Thr375 residues interact with ACA via hydrogen bonding, whereas Phe481 and Leu372 interact via alkyl-terminal double bond and Pi-terminal double bond. The results suggests that ACA might be binding to the allosteric site near to the substrate binding site of the enzyme.

The molecular docking analysis also reveals that ACA binds to an active site cavity of CYP3A4 (PDB code: 4D6Z) with a binding energy of -7.3 kcal/mol. The 4D6Z was chosen for docking based on the completeness and a higher resolution (1.93 Å) of the crystal structure (Kaur et al., 2015). Previous studies show that Arg106, Phe215, Arg372 amino acid residues are keys for binding of a well-known inhibitor ketoconazole (Ekroos and Sjögren,
and Phe57, Arg112, Phe215, Arg372 are important to bind substrate such as testosterone (Zhou et al., 2013). As shown in Figs. 5E and 5F, the aromatic ring of ACA interact via Pi-Pi stacking with Phe215 at a distance of 3.93 Å whereas the cation-Pi interaction formed with Arg106 at a distance of 4.80 Å. Moreover, Arg106, Thr224 and Arg372 residues are involved in the interaction with the C=O or C–O groups of ACA via hydrogen bonding. Pi-sigma bond interaction is formed with Phe57 while Ile223 and Phe220 show Pi-terminal double bond interaction with ACA. Hence, the results suggested that ACA binds to active cavity of CYP3A4.

4. Conclusions

The inhibitory effects of A. conchigera Griff. rhizomes extract and ACA on human cytochrome P450 enzymes were investigated. The results of this in vitro study reveal that the plant extract can moderately inhibit CYP3A4 whereas ACA also shows similar inhibition on the three major CYP isoenzymes, namely CYP1A2, CYP2D6 and CYP3A4. The inhibitory activity on other CYP isoenzymes is however, negligibly. ACA was further investigated for mode of inhibition where it shows competitive inhibition of CYP1A2 and CYP3A4, and mixed inhibition of CYP2D6. Additionally, ACA shows mechanism-based inhibition of CYP2D6, which may potentially generate more drug-related adverse effects than reversible inhibitors. Finally, in silico molecular docking was performed to evaluate the interactions of ACA with the active cavities of these enzymes. The results suggest that ACA binds to the key amino acid residues in the active sites of CYP1A2 and CYP3A4. But there is only one residue in CYP2D6 binding site for ACA. Collectively, the results show ACA will not cause severe DDI. DDI of ACA should only be monitored if it is co-administered with CYP1A2, CYP2D6 and CYP3A4 substrates. Further in vivo investigation is warranted to evaluate the clinical relevance of these moderate inhibitory effects of ACA on the CYP isoforms.
Acknowledgments

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Conflict of interest

The authors declare no conflict of interest in this work.
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Legends for Tables, Figures and supplementary data

**Table 1** IC\textsubscript{50} values of *A. conchigera* Griff. rhizomes extract and ACA and known CYP inhibitors.

<table>
<thead>
<tr>
<th>CYP Isoforms</th>
<th><em>A. conchigera</em> extract IC\textsubscript{50} (µg/ml)\textsuperscript{a}</th>
<th>ACA IC\textsubscript{50} (µM)\textsuperscript{a}</th>
<th>Positive controls IC\textsubscript{50} (µM)\textsuperscript{a}</th>
<th>Inhibition strength of ACA \textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>13.30 ± 1.22</td>
<td>4.55 ± 0.11</td>
<td>0.12 ± 0.03 (α-naphthoflavone)</td>
<td>Moderate</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>76.38 ± 6.79</td>
<td>22.87 ± 3.59</td>
<td>1.99 ± 0.21 (Miconazole)</td>
<td>Weak</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>13.78 ± 3.08</td>
<td>20.38 ± 4.15</td>
<td>0.47 ± 0.019 (Montelukast)</td>
<td>Weak</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>15.53 ± 3.36</td>
<td>20.63 ± 1.13</td>
<td>0.02 ± 0.004 (Miconazole)</td>
<td>Weak</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>31.66 ± 2.40</td>
<td>7.43 ± 1.15</td>
<td>0.01 ± 0.003 (Quinidine)</td>
<td>Moderate</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>56.33 ± 4.14</td>
<td>28.14 ± 2.57</td>
<td>5.98 ± 0.83 (Tranylcypromine)</td>
<td>Weak</td>
</tr>
<tr>
<td>CYP2J2</td>
<td>Not detected</td>
<td>12.18 ± 0.61</td>
<td>0.17 ± 0.01 (Terfenadine)</td>
<td>Weak</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>6.76 ± 1.88</td>
<td>9.17 ± 1.87</td>
<td>0.13 ± 0.02 (Ketoconazole)</td>
<td>Moderate</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>188.40 ± 7.36</td>
<td>47.95 ± 2.37</td>
<td>0.12 ± 0.02 (Ketoconazole)</td>
<td>Weak</td>
</tr>
</tbody>
</table>

\textsuperscript{a} IC\textsubscript{50} values are expressed as mean ± S.D. from three independent experiments (n=3)
Inhibition strength is based on $IC_{50} < 1 \, \mu M$ showing strong, $1 \, \mu M < IC_{50} < 10 \, \mu M$ suggesting moderate, and $IC_{50}> 10 \, \mu M$ showing weak inhibitory potency.

**Table 2** $IC_{50}$ values and ratios of ACA and selective inhibitors of CYP1A2, CYP2D6 and CYP3A4 with or without NADPH.

<table>
<thead>
<tr>
<th>CYP isoform</th>
<th>Studied compound</th>
<th>$IC_{50}$ (without NADPH preincubation) (µM)</th>
<th>$IC_{50}$ (with NADPH preincubation) (µM)</th>
<th>$IC_{50}$ ratio $^a$ (mean ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>ACA</td>
<td>4.61</td>
<td>5.58</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>α-Naphthoflavone</td>
<td>0.08</td>
<td>0.11</td>
<td>0.73 ± 0.05</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>ACA</td>
<td>10.73</td>
<td>3.32</td>
<td>3.23 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
<td>0.002</td>
<td>0.003</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>ACA</td>
<td>5.34</td>
<td>6.94</td>
<td>0.78 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.08</td>
<td>0.06</td>
<td>1.33 ± 0.04</td>
</tr>
</tbody>
</table>

$IC_{50}$ values are expressed as mean ± S.D. from three independent experiments ($n=3$).

$^a$ $IC_{50}$ ratios of studied compound were calculated by dividing $IC_{50}$ value of without NADPH preincubation divided by $IC_{50}$ value of with NADPH preincubation.

**Table 3** Inhibition constants and modes of inhibition of CYP isoenzymes by ACA.
<table>
<thead>
<tr>
<th>CYP isoenzymes</th>
<th>Kᵢ (µM)ᵃ</th>
<th>Mode of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>2.36 ± 0.03</td>
<td>Competitive</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>4.50 ± 0.08</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>5.55 ± 0.06</td>
<td>Competitive</td>
</tr>
</tbody>
</table>

Kᵢ values are displayed as (mean ± S.D.) from three experiments (n=3)

ᵃKᵢ values are obtained from the secondary plots of respective CYP enzyme activity using the slopes of the Lineweaver-Burk plots vs. various concentration of ACA.

**Fig. 1.** Chemical structure of ACA.

![Chemical structure of ACA](image)

**Fig. 2.** Concentration dependent inhibitory effects of ACA and positive controls on CYP1A2, CYP2D6 and CYP3A4 (A, C, E depict the inhibitory effects of ACA and B, D, F represents the inhibitory effects of positive controls). All data are expressed as mean ± S.D. of triplicates.
Fig. 3. Dose response curves shift for time-dependent inhibition determination. Effects of ACA on recombinant human CYP1A2 (A), CYP2D6 (C) and CYP3A4 (E), and positive control inhibitors on CYP1A2 (B), CYP2D6 (D) and CYP3A4 (F) enzymatic activity with and without a 30-minute preincubation with NADPH. Percentage of response activity for without preincubation (circles) and 30 min preincubation (squares) is shown. Each point represents the mean ± S.D. of triplicate determinations.
Fig. 4. Lineweaver-Burk plots and the corresponding secondary plots for the inhibition of CYPs. Primary Lineweaver-Burk plots for evaluating the types of inhibition on CYP1A2 (A), CYP2D6 (C) and CYP3A4 (E) by ACA. Secondary plots of slopes of the Lineweaver-Burk Plots versus ACA concentrations presented to identify inhibition potency of CYP1A2 (B), CYP2D6 (D) and CYP3A4 (F). Each point represents the mean ± S.D. of triplicate determinations.
Fig. 5. Molecular docking analysis demonstrates binding positions of ACA in human CYP1A2 (PDB code: 2HIF), CYP2D6 (PDB code: 3QM4) and CYP3A4 (PDB code: 4D6Z). These three-dimensional illustrations show the interactions of ACA with human CYP1A2 (A), CYP2D6 (C) and CYP3A4 (E) at the labelled amino acid residues, while the two-dimensional diagrams display the interactions of ACA in the active cavities of CYP1A2 (B), CYP2D6 (D) and CYP3A4 (F). Colours of the dotted lines describe the types of interactions as followed: Pi–Pi interaction (magenta), Pi-sigma (violet), H-bonding (green), Pi-cation (orange), alkyl and Pi-alkyl (light magenta).
Graphical abstract
Supp. Fig. 1. HPLC chromatogram (A) and $^{13}$C-NMR data (B) of 1'-S-1'-acetoxychavicol acetate.

Supp. Fig. 2. Concentration dependent inhibitory effects of *A. conchigera* Griff. rhizomes extract on CYP1A2 (A), CYP2B6 (B), CYP2C8 (C), CYP2C19 (D), CYP2D6 (E), CYP2E1 (F), CYP3A4 (G) and CYP3A5 (H). All data are expressed as the mean ± S.D. of triplicates.

Supp. Fig. 3. Concentration dependent inhibitory effects of ACA and positive control on CYP2B6, CYP2C8, CYP2C19, CYP2E1, CYP2J2 and CYP3A5 (A, C, E, G, I and K represents the inhibitory effect of ACA, and B, D, F, H, J and L represents the inhibitory effect of positive controls, respectively). All data are expressed as the mean ± S.D. of triplicates.