Inhibition studies of Cytochrome P450 2A6 by *Vernonia cinerea* Less and *Carthamas tinctorius* L. extracts

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ABSTRACT

The liver-specific cytochrome P450 2A6 (CYP2A6) enzyme play an essential role in metabolism of various endogenous and exogenous compound especially the tobacco-additive substance, nicotine, and coumarin probe substrate. The nicotine metabolism by liver-specific human CYP2A6 enzyme has been reported as an important route in nicotine detoxification and associate with smoking addiction behavior. In addition, genetic polymorphism of CYP2A6 enzyme is associated with smoking behavior, low CYP2A6 activity smoke fewer cigarettes per day. Thus, compounds that could inhibit the CYP2A6 mediated nicotine metabolism might be a good candidate for smoking cessation therapy. We had previously reported that the whole plant extract of *Vernonia cinerea* L. and. the flower extract of *Carthamus tinctorius* L are strongly inhibit the CYP2A6 enzymatic activity *in vitro*. Therefore, characterization of *V cinerea* and *C tinctorius* extracts on CYP2A6 enzyme were further investigated in this study. The *V cinerea* and *C tinctorius* were collected, macerated, extracted and fractionated into Hexane, Ethyl Acetate and Water fractions. The CYP2A6 enzyme was bacterially expressed and purified. The CYP2A6-mediated coumarin 7-hydroxylase activity was measured in the presence and absence of tested fractions. Interestingly, the F5 fractionation of Hexane fraction of *V cinerea* and the F4 and F5 fractionation of Hexane fraction (Hexane: Ethyl Acetate; 70:30) at 50 µg/ml could potently inhibit the CYP2A6 activity by 77.71%, 100% and 96.01% respectively. The inhibitory activities of this fractionation were comparable to the specific CYP2A6 inhibitor (8-methoxypsolaren: 8-MOP). These natural–derived compounds from *V cinerea* and *C tinctorius* could possibly useful for safety smoking cessation treatment.

Keywords: Cytochrome P450 2A6, Nicotine metabolism, 7-Hydroxylase activity, Inhibition, *Vernonia cinerea* L, *Carthamus tinctorius* L

1. INTRODUCTION

Cigarette smoking is widely prevalent due to the highly addictive properties caused by nicotine, a major constituent of tobacco. Because of addiction, continuing tobacco smoking leads to exposure to a diverse array of carcinogens in tobacco, causing tobacco-related diseases. Nicotine is metabolized mainly by the cytochromes P450 2A6 (CYP2A6) enzyme to cotinine, followed by conjugation with glucuronic acid and excreted in urine. However, addictive properties of nicotine lead to an exposure to various carcinogens, and as a result could contribute to the cause of lung diseases and cancers. In humans, inter-individual differences in nicotine metabolism have been shown in association with genetic polymorphisms of CYP2A6. Individuals with gene deletion variant have impaired nicotine metabolism, accordingly have reduced smoking behavior and are likely to stop smoking. Thus inhibition of CYP2A6 activity, to mimic genetic defect, could decrease nicotine metabolism and so maintain plasma nicotine level for longer periods of time and as a result may affect smoking behavior by smoking fewer cigarettes per day. Moreover reducing nicotine intake in the smokers concomitantly reduces the exposure to tobacco smoke contaminants and carcinogenic metabolites, thus decreases adverse health effects of tobacco smoking. In addition, the 8-methoxypsolaren (8-MOP), a specific inhibitor of CYP2A6 enzyme, shown to reduce number of cigarette smoke per day. However, 8-MOP causes various side effects and has been removed for smoking cessation [1-3].

Recently, commonly used Thai herbs were selected and screened for their CYP2A6 inhibitory activity by using fluorescence-based method. Interestingly, at 10% v/v, *Vernonia cinerea* tea (*VC* tea) and *Carthamas tinctorius* tea (*CT* tea) could inhibit CYP2A6-mediated coumarin 7-hydroxylase activity by 72% and 50%, respectively [4]. In this study, we aim to further investigate the inhibition activity of the *V. cinerea* and *C. tinctorius* extracts on bacterially expressed and purified CYP2A6 enzyme *in vitro*. The candidate compounds that could potential inhibited CYP2A6 activity could beneficial as an alternative safety-smoking cessation in Thailand.

2. MATERIALS AND METHODS

Preparation of herb extract and trial purification procedure

V. cinerea (whole plant) was collected in Bangsean and *C. tinctorius* (flower) was purchased from local traditional Chinese pharmacy in Chonburi province. Both plants were dry, macerate, extract and fractionate in to hexane, ethyl acetate and water as shown in Figure 1. The activity-guide assay was performed and the hexane fractions were further purified by column chromatography (CC) at various ratio of hexane:ethyl acetate and preliminary determined the purity by thin layer chromatography (TLC).

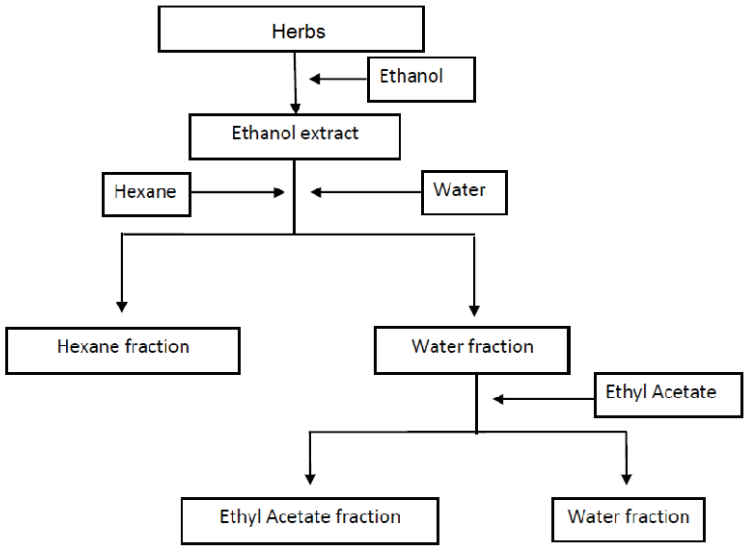

Figure 1. Extraction and fractionation flowchart of *Vernonia cinerea* less and *Carthamas tinctorius* less

Cytochrome P450 CYP2A6 activity assay

The human CYP2A6 and rat CPR, a P450s redox partner, proteins were expressed and purified as previously described [5-7], the purified enzymes were then used for P450-reconstitution enzymatic assay. Enzymatic activity of CYP2A6 to metabolize fluorescence coumarin substrate was determined as previously described [5, 7-8], with some modification. The purified human CYP2A6 was pre-incubated with rat CPR in 50 mM Tris-Cl buffer for 10 min at room temperature, followed by incubation with coumarin substrate. To determine inhibition activity, the various concentrations of extract or fraction and coumarin substrate (~ the K_m value) were incubated with enzyme mixture for 5 min before starting reaction by addition of NADPH. Production of 7-hydroxycoumarin metabolite will be measured in real time at excitation 355 nm and emission 460 nm. The P450 enzymatic activity in the presence of extract or fraction was compared with the control incubations in which DMSO solvent was added instead of extract or fraction. The actual IC_{50} values (concentration causing 50% reduction of control activity) for inhibition were calculated using GraphPad Prism5 software, version 5 (La Jolla, CA). Since 8-methoxypsolaren (8-MOP) is the known inhibitor of CYP2A6, it was used as control in the inhibition assays.

Further study of the inhibitory activity of extract and fraction on electron transfer of rat CPR was determined. The purified rat CPR was incubated with cytochrome c in 50 mM Tris-CL buffer pH 7.5, 50 µM NADPH was add to start reaction, increasing of cytochrome c (reduced form) was detected at 550 nm [6]. Specific activity was analyzed by SPSS and GraphPad Prism5 to verify remaining activity of enzyme compared between with or without *V. cinerea* and *C. tinctorius* extracts and fractions.

3. RESULTS AND DISCUSSIONS

The human CYP2A6 and rat CPR enzymes were successfully expressed and purified from bacteria-expression system into homogeneity, as determined by SDS-PAGE. The molecular mass of CYP2A6 and rat CPR were approximately 57.99 and 78.7 kDa, respectively. The cytochrome c reduction activity of the purified rat CPR is 58.69 ± 1.58 µmol of cytochrome c reduction/minute/mg protein which is comparable to previously report [6]. The *in vitro* reconstitution of rat CPR and human CYP2A6 enzyme was performed and the CYP2A6-mediated coumarin 7-hydroxylase activity was determined. The specific activity of the purified CYP2A6 enzyme is 0. 2951 ± 0.0007 µmol coumarin/minute/mg protein with a coumarin substrate binding affinity (coumarin K_m) about 3.25 ± 0.13 µM which is also comparable to previously report by our group [4].

Inhibitory herbs extract, hexane fraction, ethyl acetate fraction and water fraction on CYP2A6 enzyme was measured by fluorescence spectrometry assay, and analyzed by GraphPad Prism5 (ANOVA, p-value < 0.05). Interestingly, At 100 µg/ml of *V. cinerea,* hexane, ethyl acetate, and water fraction could inhibit CYP2A6 by 88.51% 93.43% and 45% respectively. Interestingly, at 50 µg/ml of *C. tinctorius,* ethanolic extract and all fractions could potently inhibit CYP2A6 enzymatic activity by 100%. Due to very low amount of ethyl acetate fractions, thus we aim to preliminary purify candidate compounds in hexane fractions by column chromatography. Various solvent composition were test, however, the composition of hexane:ethyl acetate at 70:30 gave high resolution of chemical compounds on TLC plate. At this separating condition, the *V. cinerea* was separated into 6 fractions while *C. tinctorius* was separated into 8 fractions. The inhibitory activities of each fraction at 50 µg/ml were determined. Interestingly, the F5 fractionation of hexane fraction of *V. cinerea* and the F4 and F5 fractionation of Hexane fraction (hexane: ethyl acetate; 70:30) at 50 µg/ml could potently inhibit the CYP2A6 activity by 77.71%, 100% and 96.01% respectively (Figure 2A and 2B). The inhibitory activities of this fractionation were comparable to the specific CYP2A6 inhibitor (8-methoxypsolaren: 8-MOP)

As CPR plays role in electrons transfer for P450-mediated metabolism *in vitro*, and the effect of plant extract on CPR activity could impair metabolic function of various P450 isoforms, resulting in diverse un-predictable side-effects of herb-drug interaction. We found that both F5 fractionation of *V. cinerea* and F4 and F5 of *C. tinctorius* (50 µg/ml) have no inhibitory activity against rat CPR enzyme (data not show), implicated that the candidate compound in Hexane fractionation could not be affected other P450 enzyme activity through inactivation of CPR enzyme. The specificity of inhibition against other P450 enzymes is waiting to determine in near future.

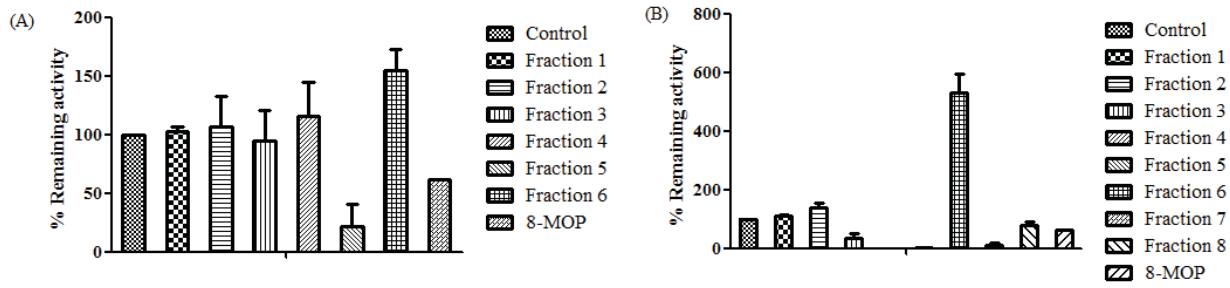

Figure 2. CYP2A6 remaining activity compared of 50 µg/ml of *V. cinerea* fractions (A), 50 µg/ml of *C. tinctorius* fractions (B) compared with 2 µM 8-MOP (specific CYP2A6 inhibitor).

Although, *V. cinerea* tea (whole plant) is widely available and well known in Thailand as an effective alternative smoking cessation strategy in Thailand [9], little is known about how *V. cinerea* tea could helps to quit smoking. Thus, according to the results, useful information that *V. cinerea* tea could strongly inhibit CYP2A6 activity may be a value added and strongly support the use of *VC* tea in smoking cessation therapy. In addition, the *C. tinctorius* (flower) is also widely available for healthcare treatment, not smoking cessation. This potent inhibition activity of *C. tinctorius* extract is value information for being used of *C. tinctorius* extract for smoking cessation. Thus, these natural-derived compounds from *V. cinerea* and *C. tinctorius* could probably suitable as a safe natural product for decreasing nicotine metabolism, an alternative safety smoking cessation therapy.

4. CONCLUSIONS

The liver-specific CYP2A6 is an important enzyme that plays an important role in nicotine metabolism in human. The CYP2A6 enzymatic activity has been reported to associate smoking behavior and number of cigarette smoked per day. Smoker who possess an impair CYP2A6 activity have reduced smoking behavior and are likely to stop smoking [1-3]. Thus inhibition of CYP2A6 activity could decrease nicotine metabolism and so maintain plasma nicotine level for longer periods of time and may affect smoking behavior by smoking fewer cigarettes per day [2, 3]. The results from this study indicated that the F5 fractionation (Hexane fraction) of *V. cinerea* and the F4 and F5 fractionation (Hexane fraction) of *C. tinctorius* (50 µg/ml) could effectively inhibit the CYP2A6 activity compared with CYP2A6 inhibitor (8-MOP). These natural-derived compounds from *V. cinerea* and *C. tinctorius* could possibly useful for safety smoking cessation treatment.

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