Effects of herbal products and their constituents on human cytochrome P4502E1 activity

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Abstract:

Ethanolic extracts from fresh *Echinacea purpurea* and *Spilanthes acmella* and dried *Hydrastis canadensis* were examined with regard to their ability to inhibit cytochrome $P450_{2E1}$ mediated oxidation of p-nitrophenol in vitro. In addition, individual constituents of these extracts, including alkylamides from *E. purpurea* and *S. acmella*, caffeic acid derivatives from *E. purpurea*, and several of the major alkaloids from *H. canadensis*, were tested for inhibition using the same assay. *H. canadensis* (goldenseal) was a strong inhibitor of the $P450_{2E1}$, and the inhibition appeared to be related to the presence of the alkaloids berberine, hydrastine and canadine in the extract. These compounds inhibited 2E1 with K_I values ranging from 2.8 μM for hydrastine to 18 μM for berberine. The alkylamides present in *E. purpurea* and *S. acmella* also showed significant inhibition at concentrations as low as 25 μM, whereas the caffeic acid derivatives had no effect. Commercial green tea preparations, along with four of the individual tea catechins, were also examined and were found to have no effect on the activity of $P450_{2E1}$.

Keywords:

Echinacea; Cytochrome P4502E1; Hydrastis; Goldenseal; Spilanthes; Green tea

Article:

1. INTRODUCTION

Cytochrome $P450_{2E1}$ is an alcohol inducible P450 isoform that has been implicated in the generation of reactive oxygen species such as superoxide and hydrogen peroxide and may mediate the toxic effects of a variety of xenobiotic compounds (Gorsky et al.,1984; Guengerich et al., 1991; Castillo et al., 1992). This isoform has been identified in a wide range of tissue types outside the liver, including, intestines (Subramanian and Ahmed, 1995), tongue (Yang et al., 2003), kidney, and nasal mucosa (Ding et al., 1986). The widespread distribution of this isoform and its potential role in activating toxins, along with the observed induction by exposure to alcohol, suggest that compounds with the ability to inhibit $P450_{2E1}$ may be useful in the prevention of deleterious effects of various chemical toxins that are activated by this enzyme. For example, Xu et al. (2003) have shown that exposure of human hepatoma cells (HepG2) to arachidonic acid results in the generation of hydrogen peroxide, and that cells overexpressing cytochrome $P450_{2E1}$ produce elevated levels of hydrogen peroxide, implicating this P450 isoform in its formation. The hydrogen peroxide produced in these cells is genotoxic due to resulting lipid peroxidation. Moreover, treatment of the cells with the retinoid lycopene protected the cells from the $P450_{2E1}$-derived peroxide. Others have also demonstrated that inhibition of cytochrome $P450_{2E1}$ can lead to reduction in the genotoxicity of other xenobiotics whose toxicity is mediated by this enzyme (Hammond and Fry, 1997).

Herbal extracts hold promise for use in strategies for inhibiting the metabolic activation of toxic xenobiotics. Such extracts contain a diverse array of chemical species, each with the potential to act on specific cytochrome P450 enzymes in an inhibitory manner. Many examples of herbs or food products that interact with cytochrome P450 enzymes exist in the literature (Brady et al., 1988; Guengerich and Kim, 1990; Subehan et al., 2006; Koul et al., 2000), and in some instances, drug interactions have been observed (Bailey et al., 1998; Wentworth et al., 2000). However, the complex nature of the extracts is a problem from a regulatory standpoint and in understanding the biochemical affects they exert on xenobiotic metabolizing systems. Thus, it is important not only to recognize the inhibitory effect of a complete extract, but to also identify the individual components of the extract that contribute to the observed inhibitory effect.

The herbal products *Echinacea purpurea*, *Hydrastis canadensis*, green tea, and *Spilanthes acmella* are popular products that are widely used for medicinal purposes. The effects of some of these products on drug metabolism in various tissue types have been studied (Budzinski et al., 2000; Yale and Glurich, 2005; Yang and Raner, 2005; Gorski et al., 2004; Chatterjee and Franklin, 2003). However, the ethanol inducible cytochrome $P450_{2E1}$ enzyme is often neglected in such pharmacologically driven studies. Consequently, little is known regarding the potential interaction of cytochrome $P450_{2E1}$ with these natural products. Given the possible role of $P450_{2E1}$ in the generation of reactive oxygen species in the liver, and the resulting oxidative stress associated with these species, interactions that could reduce $P450_{2E1}$ activity under certain conditions may prove beneficial.

In the current study, we have examined preparations of four different medicinal plants, cone flower (*E. purpurea*), toothache plant (*S. acmella*), goldenseal (*H. canadensis*) and green tea (*Camellia sinensis*). Their abilities to inhibit cytochrome $P450_{2E1}$ from human liver microsomes and from an in vitro expression system have been evaluated. In addition, we have selected several major classes of chemical species found in each of the extracts and tested the individual compounds with respect to their inhibitory properties on the human $P450_{2E1}$ isoform.

2. MATERIALS AND METHODS

2.1. Chemicals and enzymes

All of the chemicals used in this study except for the alkylamides from *E. purpurea* and *S. acmella* were purchased from commercial suppliers Sigma Aldrich or Acros Chemical Companies. *E. purpurea* fresh roots were purchased from Pacific Botanicals (Williams, OR) and *S. acmella* fresh plants were purchased from Horizon Herbs (Williams, OR). Ethanolic extracts (95% and 33%) were prepared from this plant material according to published procedures (Cech et al., 2006a). Dried roots of *H. canadensis* were also obtained from Horizon Herbs and were extracted at a ratio of 1:5 (1 mL solvent:5 g powdered roots) in 50% ethanol:50% water. A commercial green tea extract (standardized to contain 50% polyphenols) manufactured by Spring Valley (Bohemia, NY) was used.

2.2. Purification of alkylamides from S. acmella and E. purpurea

Alkylamides were separated from the 95% ethanol extracts of *S. acmella* and *E. purpurea*. Concentrated extract (100 mL) was diluted to 200 mL with de-ionized water. A 35 g C18 reverse phase extraction cartridge was equilibrated with 100 mL of a 50% ethanol: 50% H_2O solution. The diluted *E. purpurea* solution was loaded onto the cartridge at a flow rate of 7 mL/min. After the entire 200 mL had passed through, the cartridge was washed with 100 mL of 50% ethanol:50% H_2O. The eluent was collected in 25 mL fractions and stored for later analysis. Three subsequent elutions were carried out in the same manner. The mobile phase for these three elutions consisted of 55% ethanol:45% H_2O, 60% ethanol:40% H_2O and 70% ethanol:30% H_2O, respectively. A total of four- 25 mL fractions were collected from each of the washing steps for a total of 16 fractions. At this point, each of the fractions was analyzed by HPLC to identify those containing alkylamides. Fractions containing significant amounts of the alkylamides were then submitted to HPLC purification using a semi-preparative C18 column (250 x 10 mm) with an injection volume of up to 5 mL. The column was first equilibrated in 50% acetonitrile containing 0.1% trifluoroacetic acid and the sample was analyzed using the same mobile phase with a flow rate of 1.5 mL/min and a detection wavelength of 200 nm. Individual peaks

from the chromatogram were collected and the mobile phase was evaporated. Identity of the alkylamides in the fractions was verified using an LC/MS assay described previously (Cech et al., 2006a). An identical procedure was used to purify several *S. acmella* alkylamides.

2.3. Quantification of alkylamides in E. purpurea and S. acmella extracts

Since standards for each of the alkylamides are not commercially available, a single compound (dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutlyamide) was purchased from Chromadex (Santa Anna, CA) and used as a standard for quantification of all of the isobutylamides isolated. It was assumed that the spectrophotometric properties of all of the alkylamides would be similar enough for this approach to provide a reasonable estimate of the concentrations of each in the complex extracts.

2.4. Cytochrome $P450_{2E1}$ assay

Cytochrome $P450_{2E1}$ activity was monitored using the substrate p-nitrophenol in human liver microsomes and expressed cytochrome $P450_{2E1}$. The $P450_{2E1}$ assay used in this laboratory has been reported previously, and was used without modification for control reactions (Larson et al., 1991). The inhibitory properties of the extracts and individual components were determined by including a known quantity of the inhibitor in the $P450_{2E1}$ assay and monitoring the effects on V_{max} and K_m for the reaction. In all cases, if ethanol or methanol was present in the stock solution of inhibitor, the solvent was evaporated, and the residue was re-dissolved in the assay buffer prior to adding the enzyme and remaining components, since ethanol is a known inhibitor of $P450_{2E1}$. This also ensured that the inhibitor was soluble in the reaction mixture at the concentrations used.

2.5. Quantification of individual components in various extracts

The four major catechins in green tea extract, EGCG, EC, EGC and ECG were quantified in a sample of the extract using a three point calibration curve as described previously (Lee et al., 2000). Caffeic acid derivatives, caftaric acid, chlorogenic acid and cichoric acid were analyzed in the 33% ethanolic *E. purpurea* extract using HPLC separation followed by ESI-MS for detection and quantification (Sasagawa et al., 2006). The HPLC system used was an HP1100 by Agilent with a C18 (50x 2.1, 3.0 µm particle size) column. Injection volume was 10 µL and flow rate set at 0.2 mL/min. Calibration curves were generated using a mixture of standard compounds purchased from Chromadex, Inc. (Santa Anna, CA, USA). The alkaloids berberine, hydrastine and canadine were quantified in the goldenseal extract using HPLC and ESI-MS with a similar method to that previously published for *Echinacea* (Sasagawa et al., 2006). Standards of the alkaloids were purchased from Sigma Aldrich in Saint Louis, MO (hydrastine and berberine) and from Sequoia Research in the UK (canadine). The method of analysis for goldenseal differed from that for *Echinacea* only by the use of a different gradient for separation as follows (where A = 20 mM ammonium acetate in 90:10 water:acetonitrile adjusted to pH 4.5 with acetic acid, and B = acetonitrile): t = 0–5 min, 15% B; t = 5 to 20 min, 15–25%B; t = 20–23 min, 25% B, t = 23–25 min, 15% B.

3. RESULTS AND DISCUSSION

3.1. Interaction of E. purpurea and S. acmella extracts with P4502E1 in vitro

Table 1 lists the inhibitory properties of *E. purpurea* and *S. acmella* extracts on human cytochrome $P450_{2E1}$ in both liver microsomes and baculovirus expressed enzyme. Extracts of *E. purpurea* root in 95% ethanol significantly inhibited the activity of cytochrome $P450_{2E1}$ in human liver microsomes when present at 0.4% full strength (2.0 µL of extract in 500 µL reaction). Inhibition was approximately 30% using a p-nitrophenol concentration of 0.020 mM. Likewise, p-nitrophenol oxidation in baculovirus expressed human $P450_{2E1}$ and P450 reductase was inhibited 30% under the same conditions. The 95% ethanol extract from whole flowering *S. acmella* plants was much less potent in its inhibition of $P450_{2E1}$. For example, 8% extract (40 µL in a 500 µL reaction) was required in human liver microsomes to achieve 40% inhibition under identical conditions. Interestingly, greater than 50% inhibition was observed in the expressed $P450_{2E1}$ using the same conditions. Based on the more potent inhibition seen with the *E. purpurea* extract, it may be concluded that either components in the *E purpurea* extract had a higher binding affinity to the $P450_{2E1}$ enzyme, or the *Echinacea* extract had a higher content of components with the ability to interact with $P450_{2E1}$. Additional experiments

were carried out using a 33% ethanolic extract of the *E. purpurea* roots. Inhibition of $P450_{2E1}$ by the 33% extract was much less potent, where no inhibition was seen when 2 μL of the extract was used in a 500 μL reaction using human liver microsomes or expressed P450.

Table 1
Inhibition of human $P450_{2E1}$ by various extracts (the total volume for each reaction was 0.500 mL)

Extract	[*p*-nitrophenol]	Enzyme	% Inhibition
Echinacea extracted in 95% ethanol (2 μL)	0.020 mM	HLM	27
		Expressed 2E1	29
Echinacea extract in 33% ethanol (2 μL)	0.020 mM	HLM	0
		Expressed 2E1	0
Spilanthes extracted in 95% ethanol (40 μL)	0.020 mM	HLM	40
		Expressed 2E1	60

E. purpurea extracts prepared using solvents with high ethanol content generally contain significant quantities of hydrophobic alkylamides, while *E. purpurea* extracts prepared in ethanol/water mixtures contain greater quantities of the more hydrophilic caffeic acid derivatives (Sasagawa et al., 2006). The higher inhibitor activity of the extract with higher ethanol content suggests that alkylamides rather than caffeic acid derivatives are likely inhibitory compounds in *E. purpurea*. This hypothesis is supported by the observation that neither of the two main caffeic acid derivatives, caftaric acid and cichoric acid, showed any inhibition of $P450_{2E1}$ at concentrations as high as 0.400 mM (data not shown). In vivo studies by Gorski et al. have shown that *E. purpurea* extracts reduced the oral clearance of CYP1A2 substrates and also modulate the activity of P4503A4 in liver and intestines (Gorski et al., 2004). The Gorski study did not, however, specifically address the 2E1 isoform.

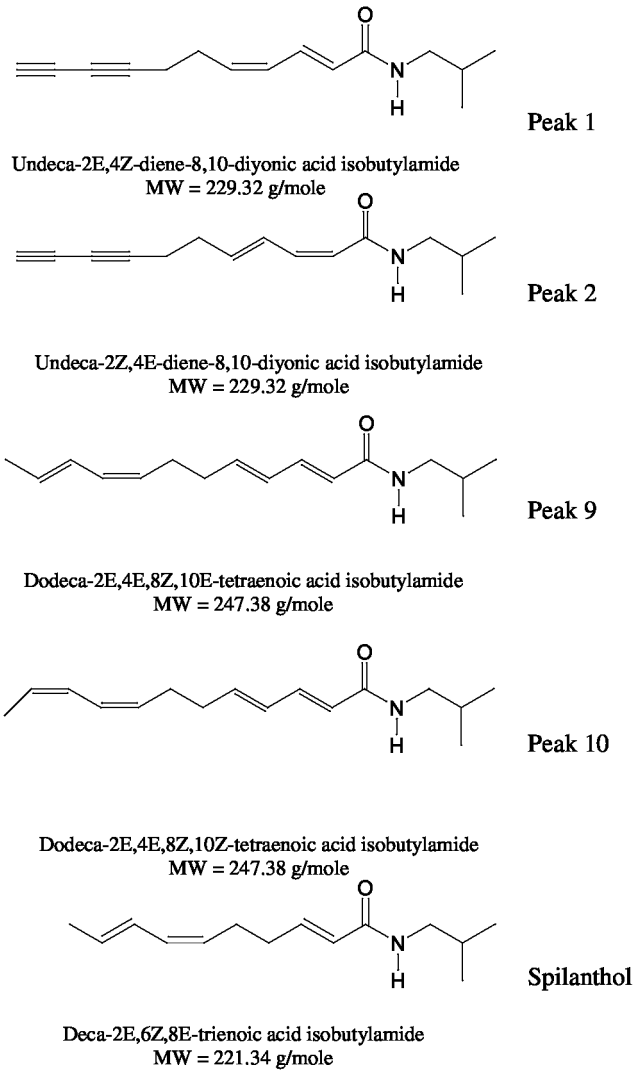

Fig. 1. Chemical structures of major alkylamide components from *Echinacea purpurea* and *Spilanthes acmella*. Compounds 1, 2, 9 and 10 were isolated from *E. purpurea* (95% ethanol extract), whereas spilanthol was isolated from an *S. acmella* extract (95% ethanol).

Individual alkylamides present in *E. purpurea* extracts did, indeed, inhibit $P450_{2E1}$. A total of 11 different alkylamides were detected in the extract used in this study and the structures of several of these are shown in Fig. 1. The components were identified based on molecular weights and fragmentation patterns observed with electrospray mass spectrometry as described previously (Cech et al., 2006a). The alkylamides were separated by HPLC (Fig. 2) and tested individually for their ability to inhibit $P450_{2E1}$. Four of these components, undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide, undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Fig. 1) appeared to be most effective at inhibiting the P450 at the concentrations isolated. The isomeric pair of dodeca-tetraenoic acid compounds (peaks 9 + 10) could not be separated, so the mixture of isomers was examined with respect to inhibition of 2E1. Inhibition data for the isolated compounds is shown in Table 2. At a total isobutylamide concentration of 0.124 mM, the isomeric mixture of compounds found in peak 9 and 10 inhibited 2E1-dependent p-nitrophenol oxidation by 40% or 50% in human liver microsomes (HLM) and expressed 2E1, respectively when 0.020 mM substrate was used. Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide (peak 1) also inhibited $P450_{2E1}$, as did undeca-2Z,4E-diene-8,10- diynoic acid isobutylamide (peak 2). These two compounds were more potent inhibitors of the P450 than those present in peaks 9 + 10, however, their concentration in the extract appeared to be somewhat lower. For example, when the 2E,4Z isomer was present in the reaction at 0.030 mM, the oxidation of p-nitrophenol was inhibited by 50 and 53% for human liver microsome and expressed 2E1, respec-

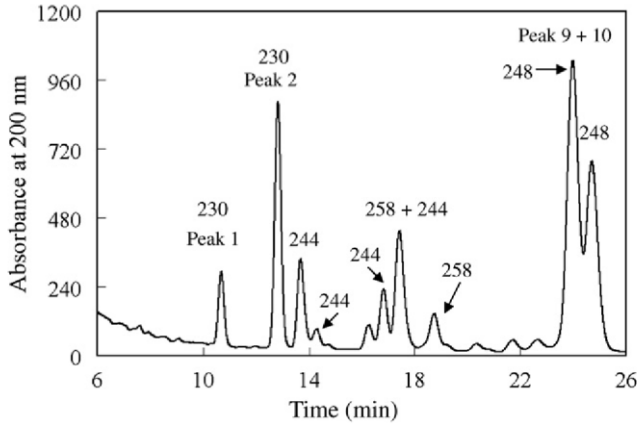

Fig. 2. HPLC chromatogram showing the relative abundance of 11 different alkylamides from a 100% ethanol extract from *Echinacea purpurea*. The molecular weight of each of the components was determined by isolating the fractions containing that peak and subjecting them to ESI-MS analysis. The molecular weights of the *protonated molecular ion* that corresponds to each peak is indicated on the chromatogram. It should be noted that these molecular weights are one larger than the weights shown in Fig. 1 because of the additional mass of the proton.

Table 2
Inhibition of human $P450_{2E1}$ by isolated isobutylamides from *E. purpurea* and *S. acmella* (the total reaction volume was 0.500 mL)

Isobutylamide from Fig. 1	[*p*-nitrophenol]	Enzyme	% Inhibition
I @ 0.030 mM	0.020 mM	HLM	50
		Expressed 2E1	53
II @ 0.025 mM	0.020 mM	HLM	40
		Expressed 2E1	62
IX + X @ 0.125 mM	0.020 mM	HLM	40
		Expressed 2E1	53
Spilanthol @ 0.067 mM	0.020 mM	HLM	55
		Expressed 2E1	75

tively, in the presence of 0.020 mM substrate. Under the same conditions, the 2Z, 4E isomer (peak 2) inhibited 2E1 by 40 and 60% in HLM and expressed P450, respectively, at an alkylamide concentration of 0.025 mM. To determine the reversibility of the inhibition by the alkylamides, a 10-fold higher concentration of the human liver microsomes were pre-incubated with the inhibitor and NADPH at 30°C for 20 min. The microsomes that were pretreated in this manner were then diluted 10-fold into a secondary reaction mixture containing the substrate p-nitrophenol and fresh NADPH. Activity of the sample was then compared with the activity of control samples prepared from microsomes pretreated with either the alkylamide alone (no NADPH), or NADPH alone (no alkylamide). The observed activities of all of the pretreated microsomes were identical, indicating a reversible type inhibition by the alkylamide. Several recent studies have shown that cytochrome P450 metabolizes *Echinacea*-derived alkylamides in vitro (Cech et al., 2006b; Matthias et al., 2005a). It has also been reported that one of the isolated alkylamides with a terminal acetylenic group appears to inhibit P450-dependent alkylamide metabolism via a mechanism-based (irreversible) process. We did not see a mechanism-based type inhibition of $P450_{2E1}$. Based on these observations, it is likely that the metabolism of alkylamides observed in the previous studies was not $P450_{2E1}$-dependent.

The main alkylamide present in *S. acmella* is spilanthol (structure shown in Fig. 1). This compound was isolated using the same procedure utilized for *Echinacea* extracts, and a sample containing highly purified spilanthol was obtained (Fig. 3). The identity of this compound as spilanthol was verified by comparison of retention time and molecular weight with literature values (Nagashima and Nakatani, 1992). The concentration of alkylamide was estimated using a standard solution of the alkylamide, dodeca2-(E),4(E)-dienoic acid isobutylamide

(Chromadex) and comparing integrated peak areas of spilanthol to those of known amounts of standard. When present at 0.067 mM, spilanthol inhibited $P450_{2E1}$ activity by 55% and 75% in human liver microsome and expressed 2E1 samples, respectively.

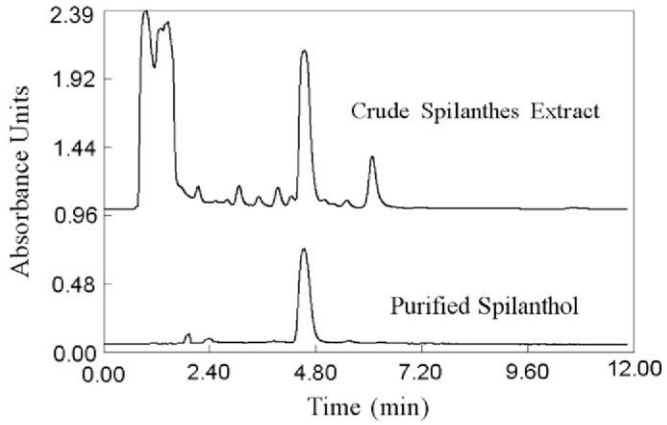

Fig. 3. HPLA chromatograms showing alkylamide content of raw (100% ethanol) *Spilanthes acmella* extract and a sample of spilanthol purified from the crude extract. Spilanthol is the primary alkylamide component of the raw extract.

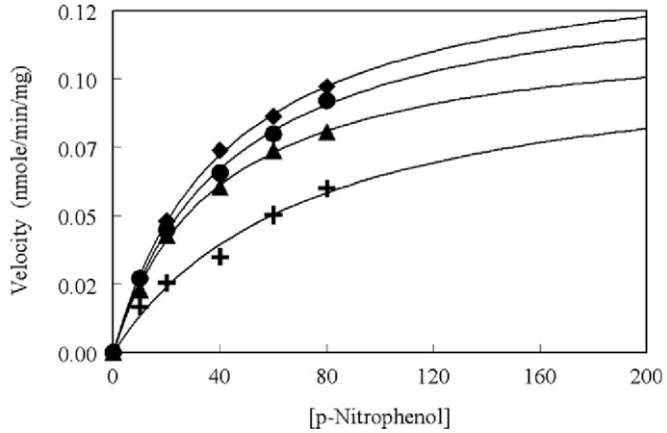

Fig. 4. Michaelis–Menton plots showing the inhibition of cytochrome $P450_{2E1}$-dependent oxidation of *p*-nitrophenol by (+) hydrastine, (●) canadine, and (▲) berberine, in pooled human liver microsomes. Activities are compared to the control reaction (◆) in which no inhibitor was present.

Based on these data, it appears that the alkylamides present in *Echinacea* and *Spilanthes* extracts (95% ethanol) are effective inhibitors of $P450_{2E1}$ at concentrations as low as 20 µM. Several recent studies have been published indicating serum alkylamide concentrations reaching the 500 nM range (Matthias et al., 2005b; Woelkart et al., 2005). Although these concentrations are roughly 40-fold lower than those used in the current study, serum concentrations may not be an accurate indication of conditions within the ER of the liver. Consequently, the physiological significance of this inhibition is still not clear, and additional research is needed with regard to the bioavailability of these compounds.

H. canadensis (goldenseal) extracts were also examined with respect to their ability to block cytochrome $P450_{2E1}$ dependent oxidation of p-nitrophenol. Previous studies have shown that certain alkaloid compounds present in goldenseal extracts effectively inhibited several pharmacologically important cytochrome P450 isoforms, including 3A4 and 2D6 (Budzinski et al., 2000). Berberine and hydrastine, the two main alkaloids present in this extract, were especially effective, with KI values in the mid micro-molar range (25–100 µM). In vivo studies have also been performed using *H. canadensis* extracts, and inhibition of $P450_{2D6}$ and 3A4/5 were noted (Gurley et al., 2005). In our studies, goldenseal extracts inhibited the 2E1 enzyme significantly in human liver microsomes. For example, 1 µL of a 95% ethanol extract in a 0.500 mL reaction inhibited the oxidation of

p-nitrophenol in human liver microsomes by >50% using 0.020 mM substrate. On the basis of our chemical analysis of the goldenseal extract, this corresponds to a final alkaloid concentration of 1.7 μM berberine, 1.2 μM hydrastine, and 0.10 μM canadine. Inhibition of $P450_{2E1}$ by goldenseal extracts appeared to be competitive in nature, with a K_I of 0.1% extract. Several of the main alkaloid compounds from *H. canadensis* were then tested individually with regard to their inhibition of 2E1 (Fig. 4). The three compounds hydrastine, berberine and canadine were found to significantly inhibit this enzyme with low micromolar K_I values (Table 3). All three inhibitors raised K_m and lowered V_{max} values for the oxidation of p-nitrophenol, indicating a mixed type of inhibition. The K_I values reported are the lower of the two values determined for competitive and non-competitive type inhibition. In particular, berberine, which is the most abundant alkaloid in *H. canadensis*, had a K_I of 18 μM, indicating even more potent inhibition of this isoform than 3A4 and 2D6, as previously reported (Budzinski et al., 2000). Studies in rat showed that upon oral dosing with 4.38 g/kg body weight of an herbal extract (Huang-Lian-Jie-Du) containing 245 mg berberine and 60 mg palmatine, maximum plasma concentrations of these compounds were 6.2 and 3.1 ng/mL, respectively (Lu et al., 2006). These values are well below the inhibitory concentrations observed in the current study; however, the low plasma levels could be a direct result of rapid first-pass metabolism in the liver, where concentrations may be much higher.

Hydrastine was the most potent of the alkaloid compounds tested at inhibiting $P450_{2E1}$ dependent oxidation of p-nitrophenol. The K_I for this compound was determined to be 2.8 μM. Although hydrastine is present at lower concentrations than berberine in ethanolic H. canadensis extracts, the much lower inhibition constant indicates

Table 3
Inhibition of $P450_{2E1}$ activity in human liver microsomes by goldenseal extract and individual alkaloid components

Inhibitor	[*p*-nitrophenol]	% Inhibition	K_I
Goldenseal extract (1 μL of 50% ethanol extract)	0.020 mM	55%	0.1% extract
Berberine (4.0 μM)	0.020 mM	11%	18 μM
Canadine (4.0 μM)	0.020 mM	8%	17 μM
Hydrastine (4.0 μM)	0.020 mM	64%	2.8 μM

Final reaction volumes were 0.500 mL.

that it could significantly contribute to the inhibitory properties of *H. canadensis* extract toward cytochrome $P450_{2E1}$ activity.

Green tea extracts and the associated catechins were also examined for inhibition of human cytochrome $P450_{2E1}$. Although complete extracts (1.0–5.0 mg/mL) appeared to inhibit the 2E1 enzyme slightly (data not shown), none of the catechins, up to a final concentration of 100 μM, showed any inhibition toward 2E1.
In conclusion, we have shown that several alkaloid compounds from *H. canadensis* extracts are very potent cytochrome $P450_{2E1}$ inhibitors, with hydrastine being most potent with a K_I of 2.8 μM, followed by canadine and berberine with K_I values of 17 μM and 18 μM, respectively. We have also shown that the alkylamide compounds in *E. purpurea* and *S. acmella* extracts show modest inhibition of $P450_{2E1}$, with K_I values as low as 20 μM, and that commercial green tea extract has a minimal effect on $P450_{2E1}$ metabolism.

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