Inhibitory Effects of Commonly Used Herbal Extracts on UGT1A4, 1A6, and 1A9 Enzyme Activities

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ABBREVIATIONS: UGT, (UDP-glucuronosyltransferase); HLM, human liver microsomes; HIM, human intestinal microsomes; VDI, volume per dose index; RDI, recommended daily intake; Km, Michaelis-Menten constant; LC, liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; EGCG, epigallocatechin gallate; TFP, trifluoperazine; TFPG, TFP glucuronide; MPA,
mycophenolic acid; MPAG, mycophenolic acid-7-O-glucuronide; MS, mass spectrometry; SRM, single reaction monitoring; ESI, electrospray ionization.
Abstract

The aim of this study was to investigate the effect of commonly used botanicals on UGT1A4, UGT1A6, and UGT1A9 activities in human liver microsomes. The extracts screened were black cohosh, cranberry, echinacea, garlic, ginkgo, ginseng, milk thistle, saw palmetto, and valerian in addition to the green tea catechin epigallocatechin gallate (EGCG). Formation of trifluoperazine glucuronide, serotonin glucuronide, and mycophenolic acid phenolic glucuronide were used as index reactions for UGT1A4, UGT1A6, and UGT1A9 activities in human liver microsomes, respectively. Inhibition potency was expressed as the concentration of the inhibitor at 50% activity (IC$_{50}$) and the volume in which the dose could be diluted to generate IC$_{50}$-equivalent concentration (Volume/Dose index; VDI). Potential inhibitors were EGCG for UGT1A4, milk thistle for both UGT1A6 and UGT1A9, saw palmetto for UGT1A6, and cranberry for UGT1A9. EGCG inhibited UGT1A4 with an IC$_{50}$ value of (mean ± SE) 33.8 ± 3.1 µg/mL. Milk thistle inhibited both UGT1A6 and UGT1A9 with an IC$_{50}$ values of 59.5 ± 3.6 and 33.6 ± 3.1 µg/mL, respectively. Saw palmetto and cranberry weakly inhibited UGT1A6 and UGT1A9 respectively with IC$_{50}$ values > 100 µg/mL. For each inhibition, VDI was calculated to determine the potential of achieving IC$_{50}$-equivalent concentrations in vivo. VDI values for inhibitors indicate a potential for inhibition of first-pass glucuronidation of UGT1A4, UGT1A6, and UGT1A9 substrates. These results highlight the possibility of herb-drug interactions through modulation of UGT enzyme activities. Further clinical studies are warranted to investigate the in vivo extent of the observed interactions.
Conjugation of compounds with glucuronic acid represents a major disposition pathway for endogenous and exogenous compounds, including drugs and phytochemicals. Human glucuronidation enzymes (UDP-glucuronosyltransferases; UGT) are divided into two families, UGT1 and UGT2, which encompass more than 18 enzymes (Tukey and Strassburg, 2000). UGT1A4, UGT1A6, and UGT1A9 enzymes belong to the UGT1 family and conjugate a wide spectrum of drugs and phytochemicals. UGT enzymes are differentially expressed in tissues, with liver and intestine being the main sites for drug glucuronidation (Tukey and Strassburg, 2000). Substrates for UGT enzymes include many drugs (e.g. mycophenolic acid, trifluoperazine, tamoxifen, lamotrigine, and acetaminophen) and phytochemicals (e.g. quercetin, kaempferol, epigallocatechin gallate) (Oliveira and Watson, 2000; Lu et al., 2003; Kiang et al., 2005). Because many phytochemicals are glucuronidated primarily by UGT1A enzymes, there is a potential for herb-drug interaction through competing with drug substrates for this pathway (Mohamed and Frye, 2011). We previously reported the inhibitory effects of several commonly used herbal supplements on UGT1A1 and of Ginkgo biloba extract and its polyphenolic compounds quercetin and kaempferol on UGT1A9 (Mohamed and Frye, 2010; Mohamed et al., 2010). The aim of this study was to identify other potential herb-UGT interactions through screening commonly used herbal extracts for inhibitory effects on the activities of UGT1A4, UGT1A6, and UGT1A9.

Recent surveys estimate that 38% of Americans use complementary and alternative medicine, which includes herbal supplements (Barnes et al., 2008). However, the physiologic and metabolic effects of herbals and phytochemicals are often poorly
understood. One of the issues of concern to clinicians is the potential for herb-drug interactions, which may lead to poor clinical outcomes (Gardiner et al., 2008). Several case studies have described deleterious herb-drug interactions that can lead to morbidity or even mortality (Ruschitzka et al., 2000; Kupiec and Raj, 2005). Consequently, much attention has been given to investigating the effects of herbal supplements on cytochrome P-450 enzymes, the primary metabolic route for the majority of marketed drugs (Izzo and Ernst, 2009). In contrast, research is lacking regarding the potential of herbals to alter other metabolic routes including glucuronidation (Mohamed and Frye, 2011).

Identification of selective substrates for UGT enzymes allows screening of herb-UGT interactions using human liver microsomes. Trifluoperazine, serotonin, and mycophenolic acid were reported to be selective in vitro probe substrates for UGT1A4, UGT1A6, and UGT1A9, respectively (Court, 2005). In this study, formation of trifluoperazine glucuronide, serotonin glucuronide, and mycophenolic acid phenolic glucuronide were used as index reactions for UGT1A4, 1A6, and 1A9 enzymatic activities, respectively.
Materials and Methods

Chemicals and Reagents. Trifluoperazine (TFP; ≥ 99%), serotonin (≥ 98%), potassium phosphate dibasic, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), uridine diphosphate glucuronic acid (UDPGA), β-glucuronidase, magnesium chloride, bovine serum albumin (BSA), alamethicin, niflumic acid, and epigallocatechin gallate (EGCG; ≥ 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethanol, methanol, acetone, hecogenin acetate (93%), and 1-naphthol (> 99%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Serotonin-O-β-D-glucuronide was provided by RTI International (Research Triangle Park, NC) through the NIMH Chemical Synthesis Program. Mycophenolic acid (MPA; 98%), mycophenolic acid β-D-glucuronide (MPAG; 98%), and mycophenolic Acid-d3-β-D-glucuronide (MPA-d3-G; 98%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Herbal extracts (black cohosh, Cimicifuga racemosa; cranberry, Vaccinium marocarpon, echinacea, Echinacea purpurea; garlic, Allium sativum; ginkgo, Ginkgo biloba; ginseng, Panax ginseng; milk thistle, Silybum marianum; saw palmetto, Serenoa repens; and valerian, Valeriana officinalis) were generously provided by Finzelberg & Co. KG (Andernach, Germany) as dry powder. Table 1 summarizes the properties of the extracts screened. UltraPool® human liver microsomes (HLM), which are microsomes pooled from 150 donors to minimize lot-to-lot variability, were purchased from BD Biosciences Discovery Labware (Woburn, MA, USA).

Preparation of Herbal Working Solutions. Herbal extracts were reconstituted with the solvents originally used for extraction and standardization by the vendor (Table 1). In order to remove any insoluble contents, the mixture was centrifuged at 20,000 x g for
5 minutes and the liquid supernatant was removed. Working solutions were freshly prepared so that final herbal concentrations in screening incubations would represent the recommended daily intake of each extract in 53 L, 5.3 L, and 0.53 L. These volumes roughly represent total body fluids, and two extremes of a range of concentrations that could appear in the small intestine, assuming 100% bioavailability as previously described by Hellum et al (2007). For confirmation experiments, a range of concentrations around the rough IC\textsubscript{50} of herbal extracts was used in incubations. Concentration of organic solvents in incubations was the same in all incubations including controls and was limited to 1%. For EGCG, working solutions were freshly prepared in 10% methanol and 1.5 mM ascorbic acid, which was added to ensure EGCG stability during the experiment (Lu et al., 2003). Ginseng and ginkgo were tested as both unhydrolyzed and acid-hydrolyzed extracts due to the high content of glycosides, which are extensively hydrolyzed in vivo by stomach acids and intestinal bacteria. Acid-hydrolyzed ginseng extract was prepared by dissolving 60 mg of the powder extract in 1 mL of 60% ethanol/40% 0.5 M HCl (Sloley et al., 2006). After 90 minutes at 37°, the extract was neutralized with 0.1 M KOH and was serially diluted to prepare working solutions containing 10% ethanol. Acid-hydrolyzed ginkgo extract was prepared by dissolving 30 mg of the powder extract in 1 mL of 60% acetone/40% 5 M HCl. The acid treated extract was heated at 90°C for one hour and neutralized with 2 M KOH. Working solutions were prepared so that their concentrations were 10-fold higher than the final concentrations in incubations.

**Incubations of Herbal Extracts with TFP.** TFP was used as a probe substrate for UGT1A4 in HLM. Incubations with TFP were performed as described previously by
Uchaipichat and coworkers (2006). Briefly, the incubation mixture (final volume, 250 µL) consisted of TFP, 5 mM MgCl₂, 50 mM Tris-HCl buffer (pH 7.4), 0.1 mg/mL microsomal proteins, and alamethicin (100 µg/mg protein). Concentration of TFP in incubations was 60 µM, which corresponds to the $K_m$ in HLM (Uchaipichat et al., 2006). The mixture was pre-incubated on ice for 15 minutes. The reaction was started by adding UDPGA (final concentration, 5 mM). After the mixture was incubated for 20 min at 37°C, the reaction was stopped by adding 250 µL (4% Acetic acid/96% Methanol), vortex-mixing, and placing tubes on ice. Tubes were centrifuged for 10 min at 20,000 x g and the supernatant was transferred to HPLC tubes. Screening experiments were performed by adding herbal extracts at three different concentrations to the incubation mixture. Incubations with and without hecogenin (50 µM) were performed to serve as positive and negative controls, respectively.

Chromatographic Analysis of TFP glucuronide (TFPG). HPLC analysis was performed with a Shimadzu LC-10AD VP pump (Shimadzu Scientific Instruments, Columbia, MD, USA) connected to a Waters 717 autosampler and Waters 2475 florescence detector (Waters Corporation, Milford, MA, USA). 50 µL of the incubation supernatant was injected on a reversed-phase Phenomenex Luna Phenyl-Hexyl column (2 x 100 mm, 3 µm). Isocratic chromatography was carried out at ambient temperature using a mobile phase consisting of 0.1% tri-fluoroacetic acid in acetonitrile: deionized water (30:70) at a flow-rate of 0.2 mL/min. The total run time was 15 min. TFPG was detected at an excitation wavelength of 310 nm and emission wavelength of 475 nm (Rele et al., 2004).
The identity of the TFPG peak was verified through enzymatic hydrolysis using β-glucuronidase. 60 µM TFP was incubated with HLMs as described above for 1 hour at 37°C. Then, 25 µL of 100 mM potassium phosphate buffer (pH 4.0), and 2,500 units of β-glucuronidase were added. Tubes were incubated for 16 hours at 37°C. The reaction was stopped by adding 10 µL 70% HClO₄, vortex-mixing and centrifugation at 20,000 x g for 10 minutes. The supernatant was transferred to HPLC tubes for injection. Control incubations were performed in the same way but did not contain β-glucuronidase enzyme. The TFPG peak was detected in the control incubation but not in the hydrolyzed one.

**Incubations of Serotonin with Herbal Extracts.** To investigate the effect of herbals on UGT1A6 activity, incubations of herbal extracts with HLM were performed using serotonin as a probe substrate as described by Krishnaswamy and coworkers with modifications (2003). Briefly, the incubation mixture (final volume, 100 µL) consisted of serotonin at a concentration around $K_m$ value in HLM (8 mM), 5 mM MgCl₂, 50 mM Tris-HCl buffer (pH 7.4), 0.5 mg/mL microsomal proteins, and alamethicin (100 µg/mg protein). The mixture was pre-incubated on ice for 15 minutes. The reaction was started by adding UDPGA (final concentration, 5 mM). After the mixture was incubated for 60 min at 37°C, the reaction was stopped by adding 10 µL 24% perchloric acid: acetonitrile (1:1, v/v), vortex-mixing, and placing tubes on ice. Tubes were centrifuged for 10 min at 20,000 x g and the supernatant was transferred to HPLC tubes. 1-naphthol (50 µM) was used as a positive control inhibitor in the screening assays (Fujiwara et al., 2008).
Chromatographic Analysis of Serotonin Glucuronide. Isocratic chromatography was carried out at ambient temperature on a reversed-phase Waters C18 Symmetry column (3.9 x 150 mm, 5 µm). The mobile phase consisted of 5% acetonitrile / 95% 2 mM ammonium acetate (pH 2.7). Isocratic elution at flow-rate of 1.0 mL/min was employed. The total run time was 10 min and the injection volume was 30 µL. The HPLC system consisted of a Shimadzu LC-10AD VP pump (Shimadzu Scientific Instruments, Columbia, MD, USA) connected to a Waters 717 autosampler and Waters 2475 florescence detector (Waters Corporation, Milford, MA, USA). Serotonin glucuronide was detected at an excitation wavelength of 225 nm and emission wavelength of 330 nm. To confirm the identity of serotonin-glucuronide peak, retention time was compared to serotonin glucuronide standard. In addition, serotonin glucuronide peak was collected from the HPLC eluate and analyzed using MS/MS. The isolated fraction showed abundant ions with \( m/z \) 353, which matches the \( m/z \) of serotonin-glucuronide ions in the positive mode. Upon fragmentation of the parent ion, a product ion with \( m/z \) 177 was produced, which matches the expected breakdown of the conjugate into glucuronic acid and free serotonin.

Incubations of MPA with Herbal Extracts. Incubations with MPA were performed as described previously with modifications (Mohamed et al., 2008). Briefly, the incubation mixture (100 µL) contained HLM (protein concentration, 0.16 mg/mL), alamethicin (100 µg/mg protein), MgCl₂ (5 mM), 2% BSA, and 100 mM phosphate buffer, pH 7.4. MPA was used at a concentration equivalent to the \( K_m \) value in HLM (240 µM). Microsomes were pre-incubated on ice with alamethicin for 15 minutes. The reaction was started by adding UDPGA (1 mM) and placing incubation tubes in a water
bath at 37°C for 30 minutes. The reaction was stopped by adding 300 µL of ice-cold acetonitrile and 20 µL of internal standard (20 µg/mL MPA-d3-G). Tubes were vortex-mixed for two minutes and centrifuged for 10 min at 20,000 x g. The supernatant was diluted 12-fold with purified water and 5 µL was injected into the HPLC system. Incubations of MPA with niflumic acid (70 µM) were used as positive controls (Vietri et al., 2000).

**MPAG LC-MS/MS Assay.** MPAG was determined by LC-MS/MS on a ThermoFinnigan Surveyor series HPLC system connected to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Corp., San Jose, CA, USA) using electrospray ionization (ESI), as described previously (Mohamed et al., 2008). Briefly, 5 µL of each sample was injected on a reversed-phase Phenomenex (Torrance, CA, USA) Synergi Fusion-RP18 column (100 × 2 mm, 4 µm). The mobile phase consisted of (A) 1 mM acetic acid in deionized water and (B) 1 mM acetic acid in acetonitrile. Gradient elution at a flow-rate of 0.22 mL/min was employed with the following steps: at start of the run, 30% B for one min, then increased to 90% B in 0.75 min, held at 90% B between 1.75 and 3.1 min, and from 3.6 to 6.5 min, the column was re-equilibrated at 30% B. Analysis was carried out in the single reaction monitoring (SRM), negative ion mode using the mass transitions of \( m/z \) 495 → 319 and \( m/z \) 498 → 322 for MPAG and MPA-d3-G, respectively.

**Data Analysis.** Remaining enzyme activity was calculated from the peak area of the glucuronide metabolites formed in herbal extract incubations expressed as a percent of control. Remaining enzyme activity and herbal extract concentration data were fitted to
equation 4-1 using Prism 5.02 (GraphPad Software, San Diego, CA, USA) to estimate IC$_{50}$ values.

Volume per dose index (VDI) was calculated using equation 6-1 and was used as a measure of the potential of IC$_{50}$ concentrations to be reached in vivo as described by Strandell et al. (2004). The VDI is defined as the volume in which one dose should be dissolved in order to obtain the corresponding IC$_{50}$ concentration.

$$\text{Volume/Dose index (L) } = \frac{RDI}{IC_{50}} \quad (6-1)$$

($RDI$: recommended daily intake)
Results

A total of 35 herb-UGT enzyme pairs were evaluated, each at three different concentrations. Results from the screening experiments are summarized in Table 2. Rough IC$_{50}$ and VDI values were estimated based on remaining enzyme activity data at the three concentrations of each herbal extract. VDI was used to select the herb-UGT interactions to investigate further. A VDI cutoff value was considered to be 5 L for UGT1A4 interactions and 2 L for UGT1A6 and 1A9 interactions. This was based on an expression study that showed that UGT1A6 and 1A9 are expressed in the intestine and the liver while UGT1A4 is mainly expressed in the liver (Ohno and Nakajin, 2009).

Herbal extracts that showed inhibition of a UGT enzyme with VDI values exceeding the specific cutoff value in the screening experiments were studied further in confirmatory assays to estimate accurate IC$_{50}$ and VDI values. For all reported IC$_{50}$ values, goodness of fit ($r^2$) of the nonlinear regression curve was greater than 0.9.

Effect of Herbal Extracts on TFPG Formation...Effect of 10 herbal extracts on UGT1A4 activity was achieved through incubations of pooled HLM with TFP and monitoring formation of TFPG as an index of UGT1A4 activity. For milk thistle and acid-hydrolyzed ginkgo extracts, evaluation of their effects on UGT1A4 activity was not possible due to interference of the herbal extracts with TFPG florescence. All the tested extracts inhibited TFPG formation with different potencies (Figure 1). Herbal extracts showing rough IC$_{50}$ values less than 100 µg/mL were (mean ± SE) EGCG (34.4 ± 4.1 µg/mL), black cohosh (69.7 ± 4.8 µg/mL), and saw palmetto (70.6 ± 9.3 µg/mL) (Table 2). Only EGCG inhibited UGT1A4 with VDI value exceeding 5 L. This finding was confirmed by incubating TFP with increasing concentrations of EGCG. Best-fit IC$_{50}$ was
(mean ± SE) 33.8 ± 3.1 µg/mL and VDI value was 7.4 L based on daily dose of 250 mg (Table 3, Figure 2).

**Effect of Herbal Extracts on Serotonin Glucuronide Formation.** Milk thistle, saw palmetto, EGCG, and echinacea inhibited serotonin glucuronide formation with IC$_{50}$ values of (mean ± SE) 66.9 ± 3.5, 131.8 ± 21.5, and 183.6 ± 29.8 µg/mL, respectively (Table 2, Figure 1). A VDI cutoff value of 2 L was applied to select which extracts to study further. Only saw palmetto and milk thistle exceeded the VDI cutoff with values of 2.4 L and 9.0, respectively (Table 2).

Precise IC$_{50}$ and VDI values were determined for inhibition of serotonin glucuronide formation by milk thistle and saw palmetto (Figure 3). Best-fit IC$_{50}$ values were 59.5 ± 3.6 and 103.5 ± 10.7 for milk thistle and saw palmetto, respectively. VDI values were 6.3 and 3.1 L for milk thistle, and saw palmetto, respectively (Table 3).

**Effect of Herbal Extracts on MPAG Formation.** Black cohosh, cranberry, echinacea, ginseng, acid-hydrolyzed ginseng, and milk thistle inhibited MPAG formation (Figure 1). However, only milk thistle (rough IC$_{50}$ = 35.9 ± 4.3 µg/mL, VDI = 16.7 L) and cranberry (rough IC$_{50}$ = 260.5 ± 33.0 µg/mL, VDI = 3.8 L) exceeded the VDI cutoff of 2 L and were selected for further study (Table 6-2). Precise best-fit IC$_{50}$ and VDI values for milk thistle and cranberry were 33.6 ± 3.1 µg/mL and 17.9 L, and 230.4 ± 32.9 µg/mL and 3.1 L, respectively (Table 3, Figure 4).
Discussion

In this study, 10 commonly used herbal extracts were screened for their effects on the glucuronidation activity of UGT1A4, 1A6, and 1A9 in pooled HLM. UGT enzyme activities were measured in vitro using selective substrates—TFP for UGT1A4, serotonin for UGT1A6, and MPA for UGT1A9 (Court, 2005). Based on VDI values, the most potent inhibitors were EGCG for UGT1A4, milk thistle for both UGT1A6 and UGT1A9, saw palmetto for UGT1A6, and cranberry for UGT1A9. These findings highlight the possibility of herb-drug interactions through modulation of UGT enzyme activity. The likelihood of the observed in vitro interactions to occur in vivo depends on characteristics of the herb, the drug substrate, the specific enzyme, and the potency of the inhibition.

UGT1A4 is known to be the primary enzyme that catalyzes N-glucuronidation of primary, secondary and aromatic amines, which includes TFP, lamotrigine, tamoxifen, tacrolimus, and azole antifungals (Kiang et al., 2005; Rowland et al., 2006; Bourcier et al., 2010; Zhou et al., 2010; Laverdiere et al., 2011). In addition, UGT1A4 shows O-glucuronidation activity towards steroidal compounds (Green and Tephly, 1996). Hecogenin is a known inhibitor of UGT1A4-mediated TFP glucuronidation with IC₅₀ values of 1.5 µM (Uchaipichat et al., 2006). Compared to hecogenin, EGCG is a non-selective UGT1A4 inhibitor with moderate potency. EGCG has previously been shown to inhibit estradiol-3-O-glucuronidation, an index for UGT1A1 activity, with a lower IC₅₀ value (7.8 µg/mL) (Mohamed et al., 2010). In addition, in this study EGCG showed some weak inhibitory activities toward UGT1A6 and UGT1A9 (Figure 1).

Pharmacokinetic studies show that maximum plasma concentrations of EGCG are more
than 10-fold less than the observed IC$_{50}$ values following consumption of high dose (800 mg) EGCG (Foster et al., 2007). This suggests that inhibition of UGT1A4-mediated systemic glucuronidation by EGCG is unlikely. However, based on VDI of the inhibition of 7.4 L for 250 mg dose, effect of EGCG on hepatic first pass metabolism of UGT1A4 substrates is possible and will be augmented with higher EGCG doses. EGCG has been studied at doses that reach 800 mg daily for its antioxidant and anti-cancer effects (Chow et al., 2005). Considering higher doses of EGCG (800 mg), the VDI will be 23.6 L, indicating that the 800 mg dose can be diluted in up to 23.6 L and still inhibit UGT1A4 activity by up to 50%. The effect of EGCG on glucuronidation of the UGT1A4 substrates TFP, lamotrigine, tamoxifen, and imipramine warrants further investigation.

UGT1A6 is typically a low affinity enzyme that catalyzes glucuronidation of drug substrates including acetaminophen, naproxen, and deferiprone (Bowalgaha et al., 2005; Kiang et al., 2005; Limenta et al., 2008). Milk thistle and saw palmetto inhibited serotonin glucuronidation with IC$_{50}$ concentrations attainable if the daily doses of milk thistle (600 mg) or saw palmetto (320 mg) are diluted with 6.3 and 3.1 L, respectively. The observed milk thistle IC$_{50}$ for UGT1A6 is equivalent to a total flavonolignans concentration of 22.6 µg/mL; this is about 1000-fold higher than observed plasma concentration following intake of 600 mg milk thistle extract (Schrieber et al., 2008). Taken together, milk thistle extract is more likely to inhibit UGT1A6-mediated first pass rather than systemic metabolism. On the other hand, no pharmacokinetic data are available on saw palmetto. Based on IC$_{50}$ value exceeding 100 µg/mL and VDI of 3.1 L, saw palmetto will be expected to have mild, if any, inhibition of UGT1A6-mediated metabolism in vivo (Table 2).
UGT1A9 catalyzes glucuronidation of a wide range of substrates including MPA, propofol, raloxifene, and flavopiridol (Kiang et al., 2005). In the current study, milk thistle and cranberry inhibited MPAG formation, which was used as an index reaction for UGT1A9 activity in HLM (Court, 2005). For milk thistle extract, the IC₅₀ value was 33.6 µg/mL, which is equivalent to 12.7 µg/mL flavonolignans. Again, this concentration is much higher than the expected plasma concentration of flavonolignans following milk thistle intake (Schrieber et al., 2008). Therefore, inhibition of systemic metabolism of UGT1A9 substrates by milk thistle extract is not likely. Conversely, based on the range of intestinal fluid volume of 0.5 to 5 liters, a single 600-mg dose of milk thistle may result in putative concentrations of 120 to 1200 µg/mL. Accordingly, inhibition of first pass metabolism of UGT1A9 substrates by milk thistle extract is possible.

In this study, we screened specific UGT enzyme activities using HLM rather than human intestine microsomes (HIM) or expressed enzymes. The difference between expressed enzymes and HLM is that the first contain single UGT enzymes while the latter contain all the hepatic isoforms. Therefore, HLM are closer to the in vivo environment due to the availability of other UGT enzymes that may form heterodimers, which has been reported for some UGT enzymes and may affect enzyme activity (Ouzzine et al., 2003). Since our goal was to screen for interactions that may have clinical significance, the use of HLM was more appropriate. This was made feasible by the availability of selective substrates for different UGT enzymes in HLM (Court, 2005). Similarly, HIM contain all the intestinal UGT enzymes. However, no selective substrates for individual UGT enzymes have been described in HIM.
Calculation of VDI provides a helpful tool to predict the likelihood of achieving IC50-equivalent concentrations in the intestine or plasma in the absence of clinical data (Strandell et al., 2004). Although this approach is sufficient for the purpose of screening and hypothesis generation, it is limited by not considering the extent of absorption of phytochemicals through tissue and cellular barriers. Use of VDI assumes that the concentration in the gastrointestinal lumen is equivalent to that in the endoplasmic reticulum of intestinal epithelial cells where UGT enzymes are located. This assumption may lead to overestimation of the extent of the inhibition, since many phytochemicals are poorly absorbed through the intestinal wall. Therefore, the results need to be confirmed in clinical studies and, where available, IC50 values to be compared with unbound in vivo concentrations. It is worth noting that using VDI to describe inhibition potency changes the order of significance of inhibitors compared to IC50 values. For example, based on rough IC50 values, black cohosh and saw palmetto are equipotent inhibitors of UGT1A4 activity (rough IC50 = 69.7 µg/mL and 70.6 µg/mL; Table 2). However, the daily dose of saw palmetto is eight-fold higher than that of black cohosh (320 mg versus 40 mg). Thus, ingesting 320 mg of saw palmetto is expected to result in higher extent of UGT1A4 inhibition compared to ingesting 60 mg of black cohosh.

In summary, in this study, 10 herbal extracts were screened for inhibition of three UGT1A enzymes—UGT1A4, UGT1A6, and UGT1A9. We report inhibition of UGT1A4 by EGCG, UGT1A6 by milk thistle and saw palmetto, and UGT1A9 by cranberry and milk thistle extracts. Among these, EGCG inhibition of UGT1A4 and milk thistle inhibition of UGT1A6 and UGT1A9 are likely to affect first-pass glucuronidation of substrates, which will be important for drugs with high first-pass extraction ratios. The in
vivo effects of these interactions on pharmacokinetics of UGT1A4, UGT1A6, and UGT1A9 substrates remain to be determined in clinical studies.
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Authorship Contributions

Participated in research design: Mohamed and Frye.

Conducted experiments and performed data analysis: Mohamed.

Wrote or contributed to the writing of the manuscript: Mohamed and Frye.
References


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Footnotes
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the authors and does not necessarily represent the official views of the National Center
For Complementary & Alternative Medicine or the National Institutes of Health.
Legends for figures

**Figure 1.** Effect of commonly used herbal extracts on UGT1A4, UGT1A6, and UGT1A9 enzyme activities.

HLM were co-incubated with herbal extracts and A) TFP for UGT1A4 activity, B) serotonin for UGT1A6 activity, and C) mycophenolic acid for UGT1A9 activity. Three concentrations were tested for each herbal extract which represent extract daily intake in 53 L (small-dotted bars), 5.3 L (checkered bars), and 0.53 L (striped bars). Formation of TFPG, serotonin glucuronide, and MPAG were detected in respective herbal incubations. Percent of activity was calculated as the percent of glucuronide peak area in herbal incubations as compared to negative controls. Each value represents mean of duplicate incubations. Error bars represent positive standard error.

**Figure 2.** Inhibitory effect of green tea catechin EGCG on TFPG formation in HLM.

Increasing concentrations of EGCG were incubated with 60 µM TFP, 0.1 mg/mL alamethicin-activated HLM, 5 mM UDPGA, and 5 mM MgCl2 for 20 minutes at 37°C. Formation of TFPG was used as an index for UGT1A4 activity in HLM incubations. Each data point represents mean of duplicate incubations. Error bars represent two-sided standard error of the mean. Data points were fitted to IC50 equation as described under Materials and Methods. Goodness of fit r² value was 0.98.

**Figure 3.** Inhibition of serotonin glucuronide formation by saw palmetto and milk thistle extracts.

Increasing concentrations of A) saw palmetto and B) milk thistle extracts were incubated with 8 mM serotonin, 5 mM MgCl2, 0.5 mg/mL alamethicin-activated HLM, and 5 mM UDPGA for 60 minutes at 37°C. Serotonin glucuronide formation was used
as an index of UGT1A6 enzyme activity in HLM incubations. Each data point represents mean of duplicate incubations. Error bars represent two-sided standard error of the mean. Data points were fitted to IC_{50} equation as described under Materials and Methods. Goodness of fit r^2 value was 0.96 and 0.99 for saw palmetto and milk thistle, respectively.

Figure 4. Inhibition of MPAG formation by cranberry and milk thistle extracts.

Increasing concentrations of A) cranberry and B) milk thistle extracts were incubated with 240 µM mycophenolic acid, 5 mM MgCl2, 2% BSA, 0.16 mg/mL alamethicin-activated HLM, and 1 mM UDPGA. Formation of MPAG was used as an index for UGT1A9 activity in HLM incubations. Each data point represents mean of duplicate incubations. Error bars represent two-sided standard error of the mean. Data points were fitted to IC50 equation as described under Materials and Methods. Goodness of fit r^2 value was 0.95 and 0.99 for cranberry and milk thistle, respectively.
Tables

Table 1. List of herbal extracts screened for UGT1A4, UGT1A6, and UGT1A9 inhibition.

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Scientific Name of Origin</th>
<th>Percent of Key Components (w/w)*</th>
<th>Solvent**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black cohosh rhizome extract</td>
<td>Cimicifuga racemosa</td>
<td>≥ 5% Total Triterpen glycosides</td>
<td>50% ethanol</td>
</tr>
<tr>
<td>Cranberry press juice</td>
<td>Vaccinium marocarpon</td>
<td>&gt; 40% Total Proanthocyanidins</td>
<td>96% Ethanol</td>
</tr>
<tr>
<td>Echinacea root extract</td>
<td>Echinacea purpurea</td>
<td>≥ 3% Cichoric acid</td>
<td>60% ethanol</td>
</tr>
<tr>
<td>Garlic bulb extract</td>
<td>Allium sativum</td>
<td>≥ 3.25% Allin</td>
<td>80% methanol</td>
</tr>
<tr>
<td>Ginkgo biloba leaf extract</td>
<td>Ginkgo biloba</td>
<td>≥ 24% Ginkgo flavoglycosides</td>
<td>60% acetone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ 6% Terpene lactones</td>
<td></td>
</tr>
<tr>
<td>Ginseng root extract</td>
<td>Panax ginseng</td>
<td>≥ 5% Total Ginsenosides</td>
<td>60% ethanol</td>
</tr>
<tr>
<td>Milk Thistle herb extract</td>
<td>Silybum marianum</td>
<td>37.9% Total silymarin flavonolignans</td>
<td>80% acetone</td>
</tr>
<tr>
<td>Saw Palmetto fruit extract</td>
<td>Serenoa repens</td>
<td>&gt;85% Total fatty acids</td>
<td>96% ethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 0.1 Sterols</td>
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</tr>
</tbody>
</table>
Table 1. Continued

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Species</th>
<th>Concentration</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valerian root extract</td>
<td><em>Valeriana officinalis</em></td>
<td>≥ 0.1 Valerenic acids</td>
<td>70% ethanol</td>
</tr>
<tr>
<td>Epigallocatechin gallate (EGCG)</td>
<td><em>Camellia sinensis</em></td>
<td>&gt; 97% EGCG</td>
<td>100% methanol</td>
</tr>
</tbody>
</table>

*Values provided by manufacturer.

**Used by manufacturer for standardization
Table 2. Effect of commonly used herbal extracts on UGT1A4, UGT1A6, and UGT1A9 activity. Each herbal extract was co-incubated at three concentrations with TFP (for UGT1A4), serotonin (for UGT1A6), and mycophenolic acid (for UGT1A9) and HLM. Formation of TFPG, serotonin-glucuronide, and MPAG were used as index reactions for activity of UGT1A4, UGT1A6, and UGT1A9 enzyme activities, respectively. Formation of glucuronides was compared in incubations with herbal extract to negative control incubations. Data represent best-fit IC50 ± standard error. Goodness of fit r² value was > 0.9 for all reported IC50 value. Volume/Dose index (VDI) was calculated by dividing the daily intake of each herb by the rough IC50 value. Recommended daily intake (RDI) values were determined based on PDR for Herbal Medicine (Gale Group., 2001) and commercially available products.

<table>
<thead>
<tr>
<th>Extract</th>
<th>RDI (mg)</th>
<th>Rough IC50 (µg/mL)</th>
<th>VDI (L/dose)</th>
<th>Rough IC50 (µg/mL)</th>
<th>VDI (L/dose)</th>
<th>Rough IC50 (µg/mL)</th>
<th>VDI (L/dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black cohosh</td>
<td>40</td>
<td>69.7 ± 4.8</td>
<td>0.6</td>
<td>NA</td>
<td>NA</td>
<td>321.6 ± 102.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Cranberry</td>
<td>1000</td>
<td>742.7 ± 118.7</td>
<td>1.3</td>
<td>&gt; 1000</td>
<td>&lt; 1.0</td>
<td>260.5 ± 33.0</td>
<td>3.8*</td>
</tr>
<tr>
<td>Echinacea</td>
<td>400</td>
<td>116.1 ± 25.1</td>
<td>3.4</td>
<td>241.0 ± 23.4</td>
<td>1.7</td>
<td>858.3 ± 158.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Garlic</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Ginkgo biloba</td>
<td>240</td>
<td>268.2 ± 48.9</td>
<td>0.9</td>
<td>NA</td>
<td>NA</td>
<td>PB</td>
<td>PB</td>
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Table 2. Continued

<table>
<thead>
<tr>
<th>Acid-hydrolyzed</th>
<th>240</th>
<th>Interf</th>
<th>-</th>
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<th>NA</th>
<th>PB</th>
<th>PB</th>
</tr>
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<tr>
<td>ginkgo biloba</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginseng</td>
<td>550</td>
<td>368.4 ±</td>
<td>1.5</td>
<td>NA</td>
<td>NA</td>
<td>298.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>66.6</td>
<td>± 29.1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Acid-hydrolyzed</td>
<td>550</td>
<td>288.0 ±</td>
<td>1.9</td>
<td>&gt; 1000</td>
<td>&lt; 0.6</td>
<td>524.3</td>
<td>1.0</td>
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<tr>
<td>ginseng</td>
<td>42.8</td>
<td>± 60.5</td>
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<td></td>
</tr>
<tr>
<td>Milk thistle</td>
<td>600</td>
<td>Interf</td>
<td>66.9 ±</td>
<td>9.0*</td>
<td>35.9 ±</td>
<td>16.7*</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>3.5</td>
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</tr>
<tr>
<td>Saw palmetto</td>
<td>320</td>
<td>70.6 ±</td>
<td>4.5</td>
<td>131.8</td>
<td>2.4*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.3</td>
<td></td>
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</tr>
<tr>
<td>Valerian</td>
<td>100</td>
<td>406.5 ±</td>
<td>2.5</td>
<td>&gt; 1000</td>
<td>&lt; 1.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>35.3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Epigallocatechin</td>
<td>250</td>
<td>34.39 ±</td>
<td>7.3*</td>
<td>183.6</td>
<td>1.4</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>gallate (EGCG)</td>
<td>4.1</td>
<td>29.8</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

NA, data points did not fit IC50 curve;

PB: IC50 values for inhibition of UGT1A9 by ginkgo and acid-hydrolyzed ginkgo extracts have been previously reported (Mohamed and Frye, 2010). Ginkgo and acid-hydrolyzed ginkgo extracts inhibited MPAG formation in HLM with IC50 values of 84.3 ± 11.6 and 20.9 ± 3.6 μg/mL, respectively. Considering dose of 240 mg, this would result in VDI of 2.9 and 11.4 L/Dose for unhydrolyzed and acid-hydrolyzed ginkgo extracts, respectively.

Interf: Addition of herb interfered with fluorescence detection of glucuronide

* indicates volume/dose index values that exceed the cutoff for further investigation.
Table 3. Determination of inhibitory potency of selected UGT1A4, UGT1A6, and UGT1A9 herbal inhibitors. Extracts were selected if their VDI based on rough IC50 values exceeded 4 L for UGT1A4, or 2 L for UGT1A6 and UGT1A9. Several concentrations of each extract were co-incubated with alamethicin-activated HLM and TFP (for UGT1A4), serotonin (for UGT1A6), or MPA (for UGT1A9). Percent of remaining activity was measured as the formation of each glucuronide in herbal incubation as a percent of negative control. IC50 values were calculated by fitting data points to IC50 equation Hill equation as described under Materials and Methods. Values reported are best-fit IC50 values ± standard error. Goodness of fit r² value was ≥ 0.95 for all reported IC50 value. Volume per dose index (VDI) was calculated by dividing the daily intake of each herb by the IC50 value. Recommended daily intake (RDI) values were determined based on PDR for Herbal Medicine (Gale Group., 2001) and commercially available products.

<table>
<thead>
<tr>
<th>UGT Enzyme</th>
<th>Extract</th>
<th>RDI (mg)</th>
<th>IC50 (µg/mL)</th>
<th>VDI (L/dose)</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A4</td>
<td>EGCG</td>
<td>250</td>
<td>33.8 ± 3.1</td>
<td>7.4</td>
<td>1.0</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>Milk thistle</td>
<td>600</td>
<td>59.5 ± 3.6</td>
<td>6.3</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Saw palmetto</td>
<td>320</td>
<td>103.5 ± 10.7</td>
<td>3.1</td>
<td>1.4</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Cranberry</td>
<td>1000</td>
<td>230.4 ± 32.9</td>
<td>3.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Milk thistle</td>
<td>600</td>
<td>33.6 ± 3.1</td>
<td>17.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 1

A.

B.

C.
Figure 3

A. Saw Palmetto Extract Concentration (µg/mL) vs. Percent of Control

B. Milk Thistle Extract Concentration (µg/mL) vs. Percent of Control