

Limitations of *In Vitro* Assessments of the Drug Interaction Potential of Botanical Supplements

Authors

John S. Markowitz^{1,2,3}, Hao-Jie Zhu^{1,2}

Affiliations

¹ Department of Pharmacotherapy and Translational Research, University of Florida, Gainesville, Florida, USA

² Center for Pharmacogenomics, University of Florida, Gainesville, Florida, USA

³ Center for Drug Interaction Research and Education, University of Florida, Gainesville, Florida, USA

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Correspondence

John S. Markowitz, PharmD
Department of Pharmacotherapy and Translational Research
University of Florida College of Pharmacy
1600 SW Archer Road,
RM PG-23
Gainesville, FL 32610-0486
USA
Phone: + 1 352 273 6262
Fax: + 1 352 273 61 21
jmarkowitz@cop.ufl.edu

Abstract

Although there are inherent and recognized limitations of *in vitro* screening methodologies to assess conventional drug-drug interactions (DDIs) per industry guidelines and those adopted by independent laboratories, further limitations are being appreciated which are unique to the evaluation of botanical products and potential DDIs in which they may participate. Among the larger issues faced are the uncertainty in assigning hepatic concentrations of multiple constituents and their potential metabolites, accounting for oral bioavailability, distribution, first-pass metabolism and active metabolites. Furthermore, the wide variability in the chemical composition of commercially available botanical supplement formulations continues to be a major concern, and manufacturing standards or enforcement thereof is essentially nonexistent in most countries. Differing formulations, unspecified product excipients, administration and absorption of the therapeutic ingredient(s) of a standardized dosage

Introduction and Background

Approximately 50% of all licensed drugs that have been registered worldwide in the 25 year time period prior to 2007 were either natural products or synthetic derivatives [1]. It is now well recognized that the metabolism and disposition of conventional medications may be significantly influenced by the co-ingestion of various natural products and dietary supplements. These substances, widely used in both a complementary and alternative fashion, are inclusive of vitamins, minerals, herbal or other botanicals, amino acids or metabolic precursor substances, or combinations thereof. The present review will focus primarily on botanical dietary supplements and the limitations of widely utilized *in vitro* methods in the as-

essment of botanical-drug interactions. Increasing interest in evaluating botanical-drug interactions is largely the result of the steady growth of dietary supplement use by consumers worldwide in the last 20 years – particularly in Western countries such as the US where up to 20% of the public reports regular usage [2]. Numerous products containing biologically active phytochemicals, so-called “secondary metabolites” are widely available for use worldwide. They are often termed “herbal” or “botanical” supplements and are derived from an enormous variety of plant sources. They can be purchased in numerous forms, the most common of which are encapsulated extracts. Extracts containing natural phytochemicals are also available as beverages and tinctures and can be ingredients in sports/nutri-

form, the very presence and/or concentration of one or more phytoconstituents within a supplement are typically unknown and nontarget entities. A further issue is the absence of authentic analytical standards, and the inability to accurately screen the entities as mixtures to even approximate typical scenarios, which may occur following the ingestion of dietary supplements, adds additional layers of complexity to experimental design and difficulty in interpreting experimental results. Multiple challenges exist in experimental methodologies employed in performing *in vitro* research with conventional pharmaceuticals and those unique to botanical extracts. These obstacles prevent the investigators from effectively utilizing high-throughput models to accomplish more than essentially “flag” suspected sources of drug interactions which must be further evaluated *in vivo*, at present, in order to confirm clinical significance. This review is intended to discuss the problems and challenges in evaluating botanical-drug interactions using *in vitro* methodologies.

tion drinks, powders, and “energy” bars. Functional food products containing specific phytochemical constituents are also being used by the public for the treatment of some common ailments [3]. Natural products do not have to be consumed as “medicines” to be involved in a drug interaction. A notable example is grapefruit juice, components of which (i.e., furanocoumarins) have been demonstrated in both *in vitro* investigations and in numerous clinical studies to increase the bioavailability of a variety of cytochrome P450 (CYP) 3A substrates [4]. Additionally, grapefruit juice as well as other natural health products may also inhibit other major phase I enzymes such as carboxylesterase (CES) [5–7]. Consumption of cruciferous vegetables (i.e., isothiocyanates) has been implicated in inducing the metabolism of a number of CYP1A2 substrates [8,9]. The potential influence of various natural products upon the metabolism and disposition of medications has been recognized for decades, well before the recent and drastic increase in use of these agents by the general public and subsequent reaction to the discovery that one of the most popular dietary supplements, St. John’s wort (SJW), was firmly identified as a perpetrator in clinically significant botanical-drug interactions [10–12]. Thus, a generalization by the lay public that “natural means safe” soon gave over to the likewise over-generalization, that the SJW example must surely represent “only the tip of the iceberg” as an untold number of available botanical supplements were being combined with conventional drug therapies in an ever growing number of patients. Hence, a public health concern and awareness were born.

As a consequence, efforts to screen numerous botanical products for potential DDIs were soon underway with some sense of urgency given the widespread use among the general population – often in those already prone to polypharmacy, e.g., elderly, HIV positive. These initial studies generally involved the more commonly used products and assessed their influence on major metabolic enzyme systems. As data accumulated, extensive lists of suspected botanical-drug interactions began to be generated based on emerging studies and specific phytoconstituents present in these products, the majority of which remain to be documented in a convincing manner [10, 11]. There are numerous *in vitro* and *in vivo* methods available to screen botanical products for drug interaction potential, most being essentially a direct adaptation of methodologies applied to conventional medications in the assessment of DDI potential. Although many *in vivo* normal volunteer botanical-drug interaction studies have been and continue to be performed, the number of *in vitro* studies assessing the potential for drug interactions with natural products far exceeds the number of *in vivo* studies, and they remain the most cost effective and predominant type of research investigations performed to date. The use of animal models and human subjects in research on DDIs is generally limited due to a variety of factors including the associated costs of such investigations, interspecies differences that preclude the adequate predictive value of experiments, the availability of test subjects, feasibility of testing procedures, and ethical concerns about discomfort or pain caused to live subjects.

Although there are inherent and recognized limitations of *in vitro* screening methodologies to assess conventional drug interactions per industry guidelines and those adopted by independent laboratories, further limitations are being appreciated which are unique to the evaluation of botanical products and potential DDIs in which they may participate. Among the larger issues faced are the uncertainty in assigning hepatic concentrations of multiple constituents and their potential metabolites, accounting for oral

bioavailability, and distribution including first-pass metabolism and active metabolites. Furthermore, the wide variability in the chemical composition of commercially available botanical supplement formulations continues to be a major concern and manufacturing standards or enforcement thereof are essentially nonexistent in most countries. Differing formulations, unspecified product excipients, typically compounds serving the primary purpose of aiding in the manufacture, administration and absorption of the therapeutic ingredient(s) of a standardized dosage form, and the very presence and/or concentration of one or more phytoconstituents within a supplement are typically unknown. Often supplements are standardized to a single phytoconstituent in the extract which is the putative active component, and in some cases that standardization “marker” may continue in spite of evidence emerging that it is not the component responsible for exerting therapeutic effects. Further issues are the absence of authentic analytical standards, and the inability to accurately screen the entities as mixtures to even approximate typical scenarios, which may occur following the ingestion of dietary supplements, and adds additional layers of complexity to experimental design and difficulty in interpreting experimental results.

Although many normal volunteer botanical-drug interaction studies have been performed and published, it is *in vitro* investigations assessing both potential for interaction as well as mechanisms by which various natural products may interact with metabolic enzymes and transporters which account for the majority of research performed and published to date. There are many commonly used methods to assess DDIs *in vitro*. These methods have strengths as well as limitations, and their use in screening conventional drugs has been more extensively reviewed elsewhere [13–15]. This limited review is intended to focus more on the particular problems and challenges in evaluating DDIs involving botanical supplements using *in vitro* methodology, and what can and cannot be inferred from the results of such investigations.

Mechanisms of Drug Interactions

▼ Drug interactions are generally classified as pharmacodynamic or pharmacokinetic. This review focuses mainly on pharmacokinetic interactions which occur when absorption, distribution, metabolism, or elimination are altered by another drug to produce the interaction. The primary route of drug elimination from the body is via biotransformation of the agent catalyzed by one or more enzymes [15]. Oxidative processes mediated by CYP450 enzymes are typically viewed as “functionalization” reactions said to represent phase I metabolism. In humans, approximately 70 active genes encode for various CYP450s of which CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5 are considered the most relevant to the majority of conventional pharmaceuticals. Other phase I processes include metabolic reduction and hydrolysis. Conjugation reactions (phase II metabolism) typically follow phase I metabolism and involve the introduction of a hydrophilic endogenous species to the drug molecule, ultimately producing a more polar molecule which is more readily excreted in bile, feces, or urine. Glucuronidation is one such major pathway catalyzed by one of many uridine diphosphoglucuronosyltransferases [UGTs]. Other major metabolic pathways falling under the general phase II category include sulfation, methylation, acetylation, and glutathione conjugation. There are a significant number of drug-metabolizing enzyme (DME) families and superfamilies which may exhibit

overlapping substrate specificities, and these properties are extensively reviewed elsewhere and well beyond the scope of the present review.

Phase I DMEs such as those comprising the CYP450 and serine hydrolase systems are found in essentially all tissues including the liver, intestine, lung, kidney, brain, adrenal gland, testes, heart, skin, and nasal mucosa. In the case of CYP3A4, the isoform is expressed in mucosal gut cells more abundantly than in the liver. In the liver [16], DMEs are primarily located in the endoplasmic reticulum of hepatic cells.

Enzymes involved in phase II reactions are mainly located in the cell cytosol, except UGTs, which are located in the endoplasmic reticulum. Furthermore, drug transporters such as P-glycoprotein (P-gp, *ABCB1*), multidrug-resistance-associated proteins (MRPs, *ABCC*) and breast cancer resistant protein (BCRP, *ABCG2*) are increasingly appreciated with regard to their important roles in the disposition of many drugs, DDIs, and botanical-drug interactions [17,18]. There is clear *in vitro* evidence that numerous phytochemicals are potent inhibitors of ABC transporters, although the exploitation of this property for the purposes of enhancing the therapeutic efficacy has yet to be realized in the clinical arena. Since many of the phytochemicals are charged [18], it stands to reason that both organic anion transporters (OATs), MRP2, organic cation transporters (OCTs), and P-gp are transporters of some of these compounds, and potentially may be inhibited by them as well.

The two major mechanisms of drug interactions involving enzymes and/or transporters are metabolic inhibition and induction. Metabolic inhibition can occur with almost all enzymes and transporters. Inhibitory effects on a specific drug metabolizing system can lead to toxicity as a result of increased plasma concentrations of the substrate compound and may occur as early as the first dosages administered depending on the agents involved. Toxicity may also occur if a previously minor metabolic pathway becomes favored, resulting in a toxic metabolite. Enzyme inhibition can also result in reduced clinical efficacy if the substrate is a prodrug [3,7]. Current opinion and regulatory guidance generally holds that studying inhibition of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 enzymes is of most importance from a clinical perspective. Conversely, sufficient induction CYP 450 increases drug clearance which may lead to therapeutic failure [11,19,20]. Metabolic induction does not occur with all enzymes and transporters and is presently viewed as most significant for CYP3A, CYP1A2, as well as P-gp substrates. Metabolic induction of expression of DMEs by xenobiotics, including clinical therapeutics, is mediated by activation of the nuclear receptors constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR). Primary human hepatocytes are considered to be a preferred *in vitro* model for the study of drug metabolism, especially metabolic induction relative to the models based on immortalized cells lines, such as HepG2 cells. However, primary hepatocyte models do exhibit some unique limitations. For example, the expression and activity of specific DMEs in primary hepatocytes could vary significantly between different donors due to genetic variation, pathological state, and varied techniques involved in tissue collection, storage, and transfer. Additionally, the expression of DMEs in primary hepatocytes may change over time in culture depending on *in vitro* culture conditions. All these factors may affect the predictability and reproducibility of primary human hepatocytes models. There are, however, recognized problems in detecting CAR-mediated enzyme induction as it is constantly activated in immortalized cells even

before xenobiotic activation. This situation makes *in vitro* assessment of hCAR activation extremely challenging.

With transporters in general, inhibition and induction can lead to both an increase or decrease in plasma/active site concentrations depending upon substrate specificity, the direction of transport with respect to the active site of the drug and the specific type of transporter affected. Thus prediction of drug interactions through changes in transport interaction must take into consideration the direction of transport with respect to the individual drug and active site(s). Further, the interplay between metabolism and transport is only now beginning to be investigated.

***In Vitro* Methods of Predicting Drug Interactions: Strengths vs. Weaknesses**



The investigation into the DDI potential of a new chemical entity which may represent a promising candidate for clinical development has been a long-standing area of interest and investigation. Withdrawal of medications such as terfenadine, astemizole, cisapride, and mibefradil from the market demonstrate the risk of DDIs which had gone largely unrecognized prior to their approval by the US Food and Drug Administration (FDA). Drug interaction studies for new drug applications (NDAs) filed between 1987 and 1991 were largely *in vivo* studies with potential coadministered drugs, whereas for NDAs submitted between 1992 and 1997, the majority of studies involved metabolic mechanisms and *in vitro* methodology [21]. Despite current limitations in the extrapolation of *in vitro* drug metabolism data to the *in vivo* environment, *in vitro* studies remain the mainstay of initial evaluations, largely due to the high throughput nature of these investigations and the substantially reduced costs relative to *in vivo* studies [21,22].

Studies are typically carried out utilizing high-throughput *in vitro* study paradigms to assess compounds for DDI potential. The FDA has recently reported an increase in the inclusion of *in vitro* data in NDAs and acknowledges the importance of these data in evaluating these applications and eventual product labeling [21,22]. Thus, the use of *in vitro* studies is clearly a valuable tool in drug development continuing to evolve in both methodology and interpretation. Although *in vitro* screening methods are both standard and accepted procedures in research and development efforts within the scientific community and pharmaceutical industry, a number of limitations are recognized in both the manner in which data are generated and how the extrapolation of such data to the *in vivo* situation is attempted on the compounds studied, metabolic pathway assessed, and system utilized [3]. Preclinical evaluation of drug metabolism and disposition as well as drug interaction assessments utilize a variety of *in vitro* enzyme and drug transporter sources and methodologies including hepatic and intestinal microsomes, liver homogenates, S9 fractions, cDNA expressed individual CYPs, or more purified enzyme preparations, primary hepatocytes, liver slices, and immortalized cell lines. Often cell lines that are deficient in, or overexpress a specific transporter such as P-gp may be utilized [22].

Although these methods are clearly more rapid and less expensive than animal and clinical studies, inherent limitations exist in both the methodology used and the utilization of generated *in vitro* data. Chief among these include the arbitrary assignment of drug concentration at the enzymatic and/or transporter site, the difficulty accounting for or even estimating presystemic metabolism, and the contribution of both known and unknown metabolites [3,14,18,23].

Limitations	<ul style="list-style-type: none"> ▶ Limited or unknown absorption or bioavailability of the botanical constituents to their proposed site(s) of action ▶ Uncertainty regarding clinically relevant concentrations of free compound versus conjugates or other metabolites formed <i>in vivo</i> ▶ Metabolites of botanical extracts are poorly characterized for most extracts and may contribute to the net inhibitory or inductive effects observed ▶ Single constituents often used in testing which are not reflective of typical multi-constituent extracts which may contribute of the net inhibitory or inductive effects observed ▶ There is a known large product to product variability and known difficulties in the characterization and standardization of products with complex phytochemical profiles. This may lead to difficulties in reproducibility of experiments
Advantages	<ul style="list-style-type: none"> ▶ Relatively easy to perform in most laboratories investigating drug interactions ▶ Inexpensive in comparison to animal studies and clinical trials ▶ Specific mechanisms can be evaluated under carefully controlled conditions

Table 1 General limitations and advantages of *in vitro* botanical-drug interaction studies.

Limitations of *In Vitro* Studies Specific to Botanical Supplement Assessment

All of the aforementioned limitations must be monitored and carefully considered for appropriate interpretation of *in vitro* studies in extrapolating to interactions in humans. Nonetheless, *in vitro* studies still provide much value in identifying specific botanical products or constituents that may potentially pose an interaction risk and thus serve as a “signal” that *in vivo* studies may be warranted to confirm clinical relevance. In addition to the general limitations of *in vitro* studies which are noted above, due to the nature of these multi-constituent, nonstandardized, and generally not fully characterized products which make up botanical extracts and dietary supplements, unique challenges and caveats exist in the methods utilized in their evaluation as well as the interpretation of published reports. An overview of these limitations as well as advantages of *in vitro* study of botanical supplements is presented in **Table 1**. Unlike uncooked fruits and vegetables wherein the degree of phytochemical exposure is dependent on the quantity ingested, exposure in botanical supplements is dependent on the dose, frequency, and formulation and is in theory, quantifiable [24]. In the case of the dietary supplement industry in which little manufacturing oversight is required, this could prove to be yet another source of variability leading to difficulties in interpretation of data. Botanical products contain a wealth of naturally occurring phytochemicals including alkaloids, xanthines, coumarins, terpenes, steroids, lipids, carotenoids, flavonoids, isoflavones, isothiocyanates, phenolic, cinnamic and amino acids, as well as di- and tripeptides [23,24]. This list is not to be construed as exhaustive as each class encompasses a wide variety of derivatives and stereoisomers, which expands further upon being subject to presystemic metabolism. In fact the differential absorption and bioavailability of stereoisomers has scarcely been investigated although there is precedent for drastic differences in the inhibitory activity of naturally derived stereoisomers quinine and quinidine relative to CYP2D6 and drug transporters [25,26]. It would be virtually impossible to perform meaningful clinical studies with most individual constituents. Thus, screening with *in vitro* methodologies has been a feasible method of predicting which botanical/dietary supplements may be involved in interactions with conventional drugs and warrant further “confirmatory” normal volunteer studies to assess true clinical relevance [3].

The selection of relevant concentrations of botanical supplements to assess in *in vitro* study systems is far more difficult than that of assessing conventional compounds as there is often little to no data published on what systemic concentrations are

reached for phytoconstituents, and almost none that have been replicated. Indeed, the assignment of physiologically relevant concentrations for even the most thoroughly characterized conventional medication is a well-known limitation of *in vitro* studies; for almost all botanical products, there is little to no adequate pharmacokinetic data available on any but the most studied product. Furthermore, even these products which have undergone some formal study are limited to dose and formulation specific investigations, typically measuring only a few “marker” constituents that are characteristic of the product. These compounds may or may not represent the current prevailing thought on the purported active constituents. However, a given supplement may continue to be standardized based upon the content of an irrelevant compound while contents of “nonstandardized” constituents vary widely between proprietary products and perhaps within manufactured lots [27]. An accurate assessment of pharmacokinetic data is particularly problematic at present given the lack of comprehensive human disposition studies using well characterized botanical supplements. Of the small number of studies that are available for review, overall conclusions are limited for a number of reasons including use of different formulations, different or unknown dosages, and differences in what is measured analytically [3]. A problem frequently encountered in the existing *in vitro* literature is the use of inappropriately high concentrations of single isolated constituents obtained from commercial sources when only a small fraction of the compound may actually be bioavailable. Most natural products are generally subject to first-pass metabolism and to a much larger extent than conventional pharmaceutical agents which are in most cases specifically developed to be substantially bioavailable or otherwise formulated as prodrugs [3]. In addition, many are less bioavailable due to their hydrophilic nature or large molecular size. A number of recent studies assessing the biological activities of many phytochemical constituents of botanical products *in vitro* have been criticized due to the failure of investigators to acknowledge that these constituents do not exist when the native compounds are given *in vivo* but rather are present as conjugated metabolites that are formed primarily in the small intestine [3, 28]. Since unconjugated compounds do not ever reach the liver in many cases in any appreciable quantity, *in vitro* investigations to assess and predict the effects of these compounds on hepatic metabolism and transport are inaccurate [29].

The role of metabolites is almost entirely unknown for most supplements, not only with regard to any therapeutic contribution or participation in DDIs, but the very existence as of yet uncharacterized compounds. The potential for unknown or uninvestigated constituents or metabolites to influence a given enzyme

or transporter of interest cannot be excluded from consideration. Further, the limited commercial availability of many phytochemicals and their principal metabolites precludes their initial screening using *in vitro* systems. Even if single constituents or metabolites were available, examining them as individual entities *in vitro* would be unlikely to be representative of the *in vivo* situation following ingestion of these supplements [3]. Plant extracts contain a mixture of compounds which are often structurally related and have similar biological properties. When consumed in a mixture they may have additive or antagonistic activities, which may not be apparent when an entity is tested in isolation.

The use of high concentrations of single constituents in *in vitro* study models when in fact the botanical extract is a complex mixture can result in the *in vitro* system being exposed to an exaggerated and unrealistic concentration. Additionally, if hepatic enzymatic or transporter activity is of interest, the issue of bioavailability needs to be closely considered, independent of first pass potential. If a compound accesses the portal circulation and is thus exposed to the liver, hepatic enzymes are theoretically vulnerable to whatever activity the compound might have. Extensive metabolism might result in low amounts of the compound reaching the central compartment. If there are other issues such that the compound never gets through the intestinal wall, the only enzymes or transporters that might be affected would be intestinal [e.g., CYP3A, P-gp]. Additionally, the variable content of specific constituents, variability between what are ostensibly the same products with regard to plant sources, quality control and lot to lot variability, recommended dosage, dose frequency, and duration of dosing regimens make extrapolations of *in vitro* findings to the *in vivo* situation exceedingly difficult [3, 13]. With multiple and structurally similar components found in most botanical extracts, these extracts may be particularly susceptible to the generation of “false” positive/negative results through some laboratory assay. For example, spurious data could be generated if a fluorescent probe is used in a DDI study which involves the detection of a compound(s) with autofluorescent or fluorescence quenching properties. This may be especially problematic when the analytic assay is not capable of differentiating individual compounds (e.g., a method based on microplate reader determinations). This issue can be circumvented through careful evaluation of the methodology and the use of appropriate probes or a more selective analytical method (e.g., HPLC, LC-MS).

In Vitro Findings Versus In Vivo Studies in Humans

The discrepancies between *in vitro* studies and the *in vivo* DDI study are well illustrated by the example of milk thistle [*Silybum marianum* (L.) Gaertn. (Fam. Asteraceae)]. Milk thistle botanical supplements purportedly exert hepatoprotective effects via components found within the extract of milk thistle seeds, called silymarin, which are a variable mixture composed of the flavonolignans silybin (also termed silibinin) A & B, isosilybin, silydianin, silychristin, and taxifolin. Most milk thistle supplements are standardized to contain at least 70% silymarin. Utilizing human *in vitro* study methodologies including both microsomal and hepatocyte assays following exposure to what were believed to be physiologically relevant concentrations of silymarin and its components has been found to produce inhibitory effects on the metabolism of CYP3A and CYP2C9 substrates by a number of independent investigators [30–32], although Zuber and associates

(2002) reported that inhibition of CYP3A did not occur at relevant concentrations [33]. Additionally, there is *in vitro* evidence of inhibition of the phase II [conjugative] enzymes UGT 1A6/9/1 [31] and P-gp [34, 35]. Furthermore, following the oral feeding of silybin to mice, significant *induction* of two phase II enzymes was reported, glutathione S-transferase and quinone reductase, respectively [36].

However, when formal drug interaction study protocols were undertaken in healthy volunteers, at least three published studies appear to exonerate milk thistle extracts from participating in any significant metabolic interaction involving CYP3A or P-gp. First, Piscitelli and coworkers [37] detected no interaction between milk thistle supplements containing silymarin and the protease inhibitor and CYP3A/P-gp substrate, indinavir. Similarly, employing a different study design, DiCenzo and coworkers found no influence of milk thistle supplementation on indinavir concentrations [38]. Gurley and associates determined that milk thistle supplementation in normal volunteers reduced midazolam (CYP3A probe substrate) clearance only minimally [39]. Lastly, our group also determined there to be no significant effect upon midazolam pharmacokinetics (administered as part of a probe drug cocktail) following exposure to the proprietary milk thistle supplement Legalon® (Markowitz, unpublished data).

The reason for these conflicting results in *in vitro* and *in vivo* findings with silymarin or one or more of its components are not readily apparent. All *in vivo* studies utilized different milk thistle supplements, and it is well recognized that silymarin supplements differ substantially from one product to another [40]. Additionally, the *in vivo* microenvironment where DDIs occur differs significantly from the conditions (i.e., incubation buffer) employed in the *in vitro* studies, and consequently, biological responses of enzymes and transporters to silymarin as well as other xenobiotics could be different between *in vitro* and *in vivo* studies. Furthermore, like most *in vitro* studies, the aforementioned milk thistle experiments did not take into account the high plasma protein binding rate of silybin, which was approximately 70% according to a previous report [41]. A possible scenario is that the inhibition of isolated CYP3A *in vitro* could in fact be fully compensated for *in vivo* by alternative metabolic pathways which are unaffected by silymarin. Finally, after oral administration, silymarin is rapidly metabolized to conjugated metabolites by sulfatases and β -glucuronidases, to the extent that plasma concentrations of conjugated metabolites far exceed the concentrations of free parent compounds [42]. Thus, the discrepancies of the *in vitro* and clinical findings may be in part due to the presence of a high concentration of silymarin metabolites following biotransformation *in vivo*.

Other Considerations

Solvents utilized in *in vitro* studies may adversely influence the integrity of the assay

Researchers may arbitrarily dissolve the product in a solvent or buffer which may contain dimethyl sulfoxide (DMSO), ethanol, or other organic solvent. The solubility of the constituents is likely to further contribute to experimental variability. Some phytoconstituents are inherently difficult to solubilize. For example, lycopene, a carotenoid with potent antioxidant effects and abundant in red tomatoes, watermelon, pumpkin, plum, persimmon, and many other fruits in which it imparts a reddish color is of great research interest. However, it is essentially insoluble in

water, sparingly in ethanol, methanol, cyclohexane, and DMSO, soluble in ethyl ether and acetone, and freely soluble in chloroform and benzene. Further, like many phytoconstituents, it is unstable and may readily oxidize at room temperature. Thus, placing the compound into cell culture for metabolism or transport studies can prove extremely difficult to accomplish while still maintaining cell viability by avoidance of toxic solvents.

Derived cell lines must be carefully chosen based upon a specific assay and substrates

The cell type/line utilized in *in vitro* studies may overexpress certain enzymatic proteins that adversely affect drug metabolism in numerous ways. For example, the Caco-2 cell line derived from heterogeneous human epithelial colorectal cells. Caco-2 cells are most commonly used not as individual cells, but as a confluent monolayer on a cell culture filter (e.g., Transwell). When cultured in this manner, the cells differentiate to form a polarized epithelial cell monolayer that provides a physical and biochemical barrier to the passage of ions and small molecules. The Caco-2 monolayer is extensively utilized as an *in vitro* model of the human small intestinal mucosa in order to predict the absorption of orally administered drugs. The correlation between the *in vitro* apparent permeability across Caco-2 monolayers and the *in vivo* fraction absorbed is well established. Caco-2 cells express tight junctions, microvilli, and a number of enzymes and transporters that are characteristic of such enterocytes including P450 enzymes, peptidases, esterases, P-gp, and some uptake transporters for amino acids, bile acids, carboxylic acids, and others. However, such cell lines may also prove to be liabilities in studying some pharmacological or investigative agents due to their overexpression of specific enzymes, transporters, and other proteins. For example, Caco-2 cells were found to be largely inappropriate for studying the metabolism or transport of human carboxylesterase 1 (hCES1) dependent drug substrates (e.g., methylphenidate) or prodrugs (e.g., oseltamivir) due to the overexpression of hCES1 within this cell line [43]. A further consideration is the observed variability and heterogeneous nature of Caco-2 cells which may differ significantly between laboratories and even form different subclones during culture. For these reasons, quantitative comparisons of data generated by different laboratories have to be performed with caution and with recognition of these potential confounders.

Pharmaceutical excipients

When performing *in vitro* or *in vivo* assessments of drug interaction potential, seldom appreciated is the potential influence of some excipients, generally regarded as inert, which are added into various oral dosage formulations. Common examples include lactose, microcrystalline cellulose, starch, stearate lubricants, sorbital, and others. Although excipients are generally believed to be devoid of any ability to disturb the PK and/or PD of “actives” they are routinely combined with, in certain cases, the concentration and disposition of certain medications may be influenced. Excipients have many functional groups that may interact with specific pharmaceutical entities [44, 45].

Conclusions

In vitro methods have been widely used in attempts to predict potential drug interactions with botanical products, but to date, few significant drug interactions have actually been discovered or ac-

curately predicted with botanical products. Instead, much of the research has led to clinical studies with negative outcomes. While the exoneration of a given botanical product is an important finding and may produce some measure of comfort to clinicians, the expense and effort to perform these studies is not trivial.

In vitro studies utilized to assess drug interaction potential with natural products have all the limitations of *in vitro* studies with conventional drugs including assignment of hepatic concentrations, accounting for first-pass metabolism and active metabolites. Additionally, the lack of commercial standards, and the inability to accurately screen the entities as mixtures remain a strategic interest [46]. These complications have contributed to the discrepancies between outcomes predicted by *in vitro* results and observed *in vivo* effects.

Cell-based models are probably the method of choice for *in vitro* investigation of transporter-mediated DDIs since intracellular substrate accumulation cannot be determined in non-cell-based models. However, non-cell-based models, such as cell membrane preparations, could prove to be very useful for rapid assessment of the *affinity* of a specific transporter to its substrates. For DMES-mediated DDI, non-cell-based systems such as hepatic and intestinal microsomes, s9 fractions, and purified individual enzymes are more rapid and also more applicable to the development of a high-throughput screening assay. However, one limitation of non-cell-based models is the inability of such models to take into account the potential interplay of drug transporters and DMSs. For example, a liver microsome incubation study might suggest a tested drug is a potent inhibitor of a specific CYP 450 enzyme. However, *in vivo*, such a DDI may never occur if the inhibitor is a substrate of an efflux transporter (e.g., P-gp, BCRP) that prevents the inhibitor from reaching the requisite concentration to cause enzyme inhibition.

Other high-throughput approaches including the use of *in silico* models have been increasingly utilized to evaluate the potential dispositional characteristics of even theoretical compounds. Although *in vitro* approaches are clearly more rapid and less expensive than clinical studies, and may generate an early signal of drug interaction potential or provide insight into the mechanistic aspects of DDIs, inherent limitations are recognized in all available *in vitro* models. These include perhaps most prominently the difficulties in the assignment of hepatic concentrations reflective of likely *in vivo* scenarios due to a variety of sources of variability, the lack of clinical pharmacokinetic data of numerous pharmacologically active constituents of a given herbal product, and the difficulty accounting for first-pass metabolism, the potential production and contribution of metabolically active metabolites and others. Accordingly, it may be time to reevaluate the value of *in vitro* screening of individual phytoconstituents and extract mixtures and critically assess the clinical implications from *in vitro* studies that are conducted, with consideration to the many limitations.

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Conflict of Interest

▼
The authors report no conflicts of interest.

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