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Effect of an anxiolytic botanical containing *Souroubea sympetala* and *Platanus occidentalis* on *in-vitro* diazepam human cytochrome P450-mediated metabolism

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Keywords
anxiolytic; herbal medicine; herb–drug interactions; *Plantanus occidentalis*; *Souroubea sympetala*

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Abstract

Objectives A novel anxiolytic natural health product (NHP) containing *Souroubea sympetala* and *Platanus occidentalis* is available for the companion animal market and is currently being developed for clinical evaluation. Addressing the risk of potential NHP–drug interactions, this study investigated *S. sympetala* and *P. occidentalis* plant extracts, and their identified bioactive compounds, for effects on the activity of cytochrome P450 (CYP) isozymes and the metabolism of the conventional anti-anxiety medication diazepam.
Methods *Souroubea sympetala* and *P. occidentalis* extracts, a 1 : 1 blend of the two extracts, and five triterpenes were tested for inhibitory effects on human recombinant CYP3A4, CYP2D6, CYP2C9 and CYP2C19 activity using a fluorometric plate assay. Direct effects on the metabolism of diazepam were evaluated using human liver microsomes with drug and metabolite quantification by ultra-high-pressure liquid chromatography and mass spectroscopy.
Key findings The active substances betulinic acid (BA) and ursolic acid (UA) strongly inhibited CYP3A4 activity while UA and lupeol moderately inhibited CYP2C19. All extracts exhibited strong activity against the tested isozymes at 50–100 μg/ml. BA and all plant extracts blocked the formation of major diazepam metabolites.
Conclusions Betulinic acid, UA and both the extracts and blended product are expected to affect the metabolism of diazepam when given in high dose.

Introduction

Generalized anxiety disorder (GAD) affects health and well-being and is common across North America and abroad. According to the National Institute of Mental Health, approximately 2.7% of American adults suffer from GAD every year with an estimated 5.7% experiencing GAD at some point in their lives.[1] Due to concerns over side effects, tolerance, compliance, patient preference and the cost of conventional medications, alternative or complementary therapies have received increasing attention from both researchers and consumers in recent years.

A novel anxiolytic herbal medicine containing the dried extracts of *Souroubea sympetala* and *Platanus occidentalis* has been developed for the companion animal market and is currently being developed for clinical evaluation as a treatment for GAD for human.[2–4] *Souroubea sympetala* is a vine from the Marcgraviaceae family found in Central and South America that was selected for investigation by Costa Rican botanists based on dereplication strategy for poorly studied botanicals.[5] *Souroubea sympetala* extract was found to have significant dose-dependent anxiolytic effects in animal models including the elevated plus maze and fear-potentiated startle. Further analyses identified betulinic acid (BA) as the main active principal in the plant extract, as well as the presence of other pentacyclic triterpenes.[5] The pharmacological mode of action was shown to be attributed to agonistic action at the $GABA_A$ receptor which could be abolished with co-administration of the antagonist flumazenil.[6]

Platanus occidentalis has high concentrations of BA in the bark, which was added to the formulation to increase potency of the product.[7] Although it has only a weak anxiolytic effect in animal tests, *P. occidentalis* has a synergistic anxiolytic effect when mixed with *S. sympetala*.[8] The blended NHP mixture (Zentrol™) is marketed as an anxiolytic for canine noise aversion. Preliminary safety studies determined that there were no negative effects in canines at ten times the suggested dose.[9] These results were confirmed in larger trials with beagles.[2] However, there are still knowledge gaps in the overall safety of this product.

Metabolic interactions are one of the most common causes of adverse effects when multiple health products are taken concomitantly. Cytochrome P450 (CYP450) is the major Phase I drug-metabolizing enzyme system.[10,11] Many phytochemicals have been identified as substrates, inducers or inhibitors of drug metabolism enzymes, including several CYP isoforms.[12] Altered metabolic activity may have significant impact on the plasma concentration and half-life of a medicinal or nonmedicinal substance, which can lead to adverse reactions such as overdose or therapeutic failure.[13] St. John's wort is a well-documented example.[14] Clinical studies have also observed the increased CYP3A4 drug metabolism and the reduced absorption of drugs through P-glycoprotein (Pgp) efflux.

Our objective was to evaluate the potential risk of NHP–drug interactions related to the concomitant use of this herbal product with conventional medical products, such as the anxiolytic diazepam (DA). Plant extracts of *S. sympetala* and *P. occidentalis* alone and as a 1 : 1 w/w mixture, along with their isolated active triterpenes, were tested *in vitro* for inhibitory effects on the activity of various CYP enzymes as well as the production of DA metabolites.

Materials and Methods

Plant extracts and sample preparation

Souroubea sympetala leaves and stem were harvested from a plantation in Costa Rica managed by the Universidad Nacional (UNA) in Sarapiqui (Heredia province). Plants were authenticated to species by UNA botanists (Sanchez, Poveda and Otarola), and the voucher specimens were deposited at the Herbarium Juvenal Rodriguez, UNA and University of Ottawa herbarium (OTT19994). Bark of *P. occidentalis* (Plantanaceae) was collected in Guelph Ontario, identified by University of Ottawa botanist Dr. Julian Starr, and prepared as a voucher now deposited at the University of Ottawa herbarium (OTT19995). Dried plant material was ground through 1 mm mesh. Ground material (1 g samples) of both species was mixed (55 : 45, w : w) prior to extraction as this method produces a dry powder. The mixed plant material with extracted with 20 ml 95% ethanol by shaking at 250 rpm overnight. The supernatant was collected by vacuum filtration, and residues were re-extracted under the same conditions. A third extraction did not significantly increase yield and was not undertaken. The supernatants were then combined and dried by rotatory evaporation at 45 °C followed by lyophilization. Dried extracts were stored in −20 °C conditions until use.

Standards of BA, ursolic acid (UA), lupeol, β-amyrin and α-amyrin were purchased from Extrasynthese Lyon, France, and DA solution (1 mg/ml in dissolved in methanol) was obtained from Sigma-Aldrich (Oakville, ON, Canada). All standard compounds (1 mg/ml) and plant extracts were solubilized in 100% methanol at defined concentrations as a stock solution for experimental use.

Quantification of triterpenes in plant extracts

Phytochemical separation and characterization of the extracts were achieved by a Shimadzu UPLC-MS system (Mandel Scientific Company Inc., Guelph, ON, Canada) consisting of LC30AD pumps, a CTO20A column oven, a SIL-30AC autosampler and a LCMS-2020 mass spectrometer. Separation of the marker compounds was performed on a Kinetex 1.7 μm, 150 × 2.1 mm i.d. column (Phenomenex Inc., Torrance, CA, USA). The injection volume was 1 μl, column oven temperature was 55 °C and flow rate was 0.4 ml/min. The mobile phases LC-MS grade were (A) water, (B) acetonitrile with gradient elution: 65–100% B in 7 min, hold 100% B for 8 min, the system was re-equilibrated for 5 min before the next injection. Detection was achieved by using atmospheric pressure chemical ionization (APCI) operated in positive and negative selective ion monitoring mode (SIM): *m/z* at 409 ($[\mathrm{M{-}H_2O}]^+$) was used to monitor lupeol, α-amyrin and β-amyrin; *m/z* 455 ($[\mathrm{M{-}H}]^-$) was used to monitor betulinic acid and ursolic acid. Nebulizing gas flow was set at 1.5 l/min, and drying gas flow was at 10 l/min. The interface, desolvation line and heat block temperature was set at 350, 300 and 400 °C, respectively. The scan speed was set at 938 μ/s.

Calibration curves were generated by injecting dilutions (0.5–50 μg/ml) of reference standards spanning the compound concentration in samples. Each sample (0.1–1 mg/ml) was injected in triplicate, and peak area was then used to quantify the amount of each marker compound.

Cytochrome P450 assays

A microtitre fluorometric plate assay was used to assess the inhibitory capacities of terpenes and plant extracts towards different CYP450 isoforms. The assays were performed in 96-well plates under red light to minimize the exposure of fluorescent light to photosensitive material. The fluorescence was measured using a Cytation 3 Cell Imaging Multi-Mode Reader BioTek Instruments Inc., Winooski, VT, USA as outlined in Table 1.

Samples were incubated in the presence of CYP450 enzymes with fluorometric substrate and 1.08 mM NADPH in a 0.2 mM phosphate (CYP2C19, CYP2C9 and CYP3A4) or Tris buffer (CYP2C9) with a pH at 7.5 at 37 °C for 20–60 min (refer to Table 1 for enzyme-specific assay parameters). Ketoconazole 1.9 μM, quinidine 2 μM, sulfaphenazole 100 μM and tranylcypromine 100 μM were used as positive controls for CYP3A4, CYP2D6, CYP2C9 and CYP2C19, respectively. Corning® Supersomes™ Insect Cell Microsomes were used as negative control. These assays were optimized previously.[15]

IC_{50} values were obtained by using Prism GraphPad version 7.0; log [Inhibitor] vs normalized response – variable slope analysis module. Samples were tested in triplicate at a minimum of five concentrations.

Diazepam metabolism assay with human liver microsome

Human liver microsome (HLM; Corning Inc.-Life Science, Oneonta, NY, USA) was thawed in a 37 °C water bath then placed on ice until required. A 5-μl aliquot of the test extract was incubated with 2 mM NADPH, 2 mg/ml HLM and 3.5 mM DA in 100 mM PBS buffer for 90 min in a shaking incubator set at 37 °C and 200 rpm. The reaction was stopped by adding 300 μl of cold methanol. The mixture was then vortexed for 1 min and sonicated for 5 min to neutralize all protein content, and centrifuged at 13 400 *g* for 10 min. The supernatant fluid was filtered through 0.2-μ PTFE filters (Chromatographic Specialties Inc., Brockville, ON, Canada) and stored at −20 °C until analysis. Ketoconazole (10 nM) and kava–kava extract (0.05 mg/ml) were used as positive control, and 10% methanol was used as vehicle control. All samples were tested with/without HLM in triplicate.

After HLM metabolism, DA and its metabolites (Figure 1) were identified by selected ion monitoring (SIM: nordazepam, *m*/*z*: 271, oxazepam *m*/*z*: 287, and temazepam *m*/*z*: 301) using a Shimadzu UPLC-MS system (Mandel Scientific Company Inc.) consisting of LC30AD pumps, a CTO20A column oven, a SIL-30AC autosampler and a LCMS-2020 mass spectrometer. An Acquity BEH C18 column 100 × 2.1 mm, 1.7 μm particle size (Waters, Mississauga, ON, Canada), with an Acquity BEH C18 VanGuard precolumn 5 × 2.1 mm was used for separation. Mobile phase consisted of H_2O (A) and acetonitrile (B) with 0.1% formic acid in both. The gradient elution method was initiated at 10% B and then increased to 50% B over 8 min. The column was then washed with 100% B for 4 min and returned to the initial conditions in 2 min. The flow rate was set to 0.5 ml/min with the column temperature set at 50 °C. The mass spectrometer with electrospray ionization ESI interface was operated in positive scan mode. The nebulizing gas flow was set at 1.5 l/min, and drying gas flow was at 10 l/min. The desolvation line temperature and heat block temperature were set at 250 and 400 °C, respectively. The *m*/*z* range of both positive and negative scan is from 150 to 600 with 1500 μm/s scan speed.

The quantitative analysis of DA metabolites was performed on an Agilent 1200 series HPLC system with quaternary pump G1311A, solvent degasser G1322A, column oven G1316A and photodiode array detector G1315A. Separation of DA and its metabolites was carried out with a Synergi MAX-RP C12 column 250 × 2 mm, 4 μm particle size (Phenomenex, Mississauga, ON, Canada). Mobile phase was H_2O (A) and acetonitrile (B) +0.1% formic acid in both. The isocratic elution method was initialized with 40% B for 10 min. The column was then washed with 100% B for 5 min, and then returned to the initial condition for 1 min. The system was then re-equilibrated for 5 min before the next injection. Flow rate was set to 0.5 ml/min with oven at 55 °C. DAD was set to monitoring wavelength 240 nm.

Statistical analysis

Chemstation software Version B 3.02 was used to calculate the peak area of the major metabolites of DA. Percentage inhibition was calculated by comparing the metabolite to the appropriate vehicle control. A two-way ANOVA

Table 1 Description of the experimental conditions for the microtitre fluorometric cytochrome P450 (CYP) inhibition studies

CYP isoform	CYP (nM)	Substrate	Substrate (μM)	Excitation/emission λ (nm)	Incubation time (min)
CYP3A4 + b5	5	DBF	1	490/530	20
CYP2D6	10	AMMC	0.12	410/460	60
CYP2C9 + b5	50	7-MFC	100	410/530	60
CYP2C19 + b5	20	CEC	25	410/460	60

AMMC, 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin; b5, cytochrome b5; CEC, 3-cyano-7-ethoxycoumarin; DBF, dibenzylfluorescein; MFC, 7-methoxy-4-trifluoromethyl-coumarin.

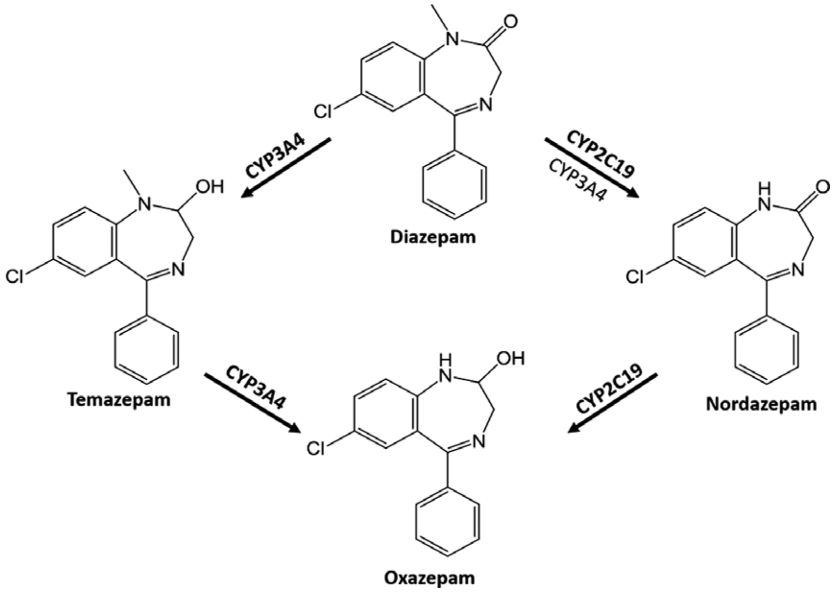

Figure 1 Phase I metabolic pathways of diazepam.

followed by both Dunnett and Sidak's multiple comparison tests was used to analyse the percentage inhibition of different DA metabolites.

Results

The percentage yield of *P. occidentalis*, *S. sympetala* and mixture extract is 3.34%, 5.23% and 4.32% dry weight of plant material, respectively. All major triterpenes in the plant extract were identified and quantified using UPLC-MS (Figure 2, Table 2). The *P. occidentalis* extract contained nearly 30% triterpenes by weight, consisting only of betulinic acid (297.9 mg/g) and ursolic acid (20.7 mg/g). The *S. sympetala* extract contained five marker compounds constituting about 15% of the extract. Amyrins were the most abundant terpenes in the extract with 55.2 and 45.6 mg/g for α-amyrin and β-amyrin, respectively. The triterpene content of the mixture was as predicted based on the observed concentrations of these compounds in *P. occidentalis* and *S. sympetala* extract, with the exception of lupeol, which was higher than expected.

All extracts exhibited strong inhibitory effects >70% on CYP3A4, 2C9 and 2C19 at a concentration at 50 μg/ml (Figure 3). The inhibition of CYP2D6 activity was weakest, as only moderate inhibition 40–70% was observed with higher concentrations of 100 μg/ml. Accordingly, the IC_{50} of all plant extracts towards CYP3A4, 2C9 and 2C19 (but not 2D6) were then obtained to further evaluate the inhibition potency (Table 3). Similar patterns were observed among all 3 CYP450 enzymes: the inhibition potency of *S. sympetala* extract was twofold to fourfold lower than both *P. occidentalis* extract and the mixture, which produced similar IC_{50} values. The high inhibition of CYP 2D6 with the positive control may be due to the fluorescence quenching of quinidine.

At 50 μg/ml, individual triterpenes showed selective inhibitory effects on the tested CYP450 enzymes (Figure 3). BA strongly inhibited CYP3A4 activity but had very limited effect on CYP2D6, CYP2C9 and CYP2C19. UA exhibited the strongest inhibitory effects on all 4 CYP450 isoforms, particularly CYP3A4. Lupeol showed only moderate inhibition of CYP2C19 activity, as did β-amyrin, whereas α-amyrin had very limited inhibitory effect on any of the four isoforms. β-amyrin also showed highest inhibition on CYP2D6. BA and UA were subsequently tested to obtain IC_{50} for CYP3A4 (Table 3).

In the HLM incubation mixture, all three metabolites of DA reported for CYP450-mediated biotransformation were detected by UPLC-MS (Figure 4) although oxazepam was present below the limit of quantification. All plant extracts inhibited the formation of DA metabolites, especially at the highest tested concentration, 0.2 mg/ml, which reduced metabolite formation by nearly 50% (Figure 5). Of the triterpenes examined, only BA altered the HLM metabolism of DA. In the presence of 25 μg/ml BA, the formation of temazepam and nordazepam was inhibited by 43% and 45%, respectively. LP, AAM and BAM slightly increased the catalytic activity with respect to the vehicle control, possibly due to hormesis, which is frequently seen in natural product assays.

Two-way ANOVA and Dunnett's multiple comparison tests showed that ketoconazole, betulinic acid and all plant

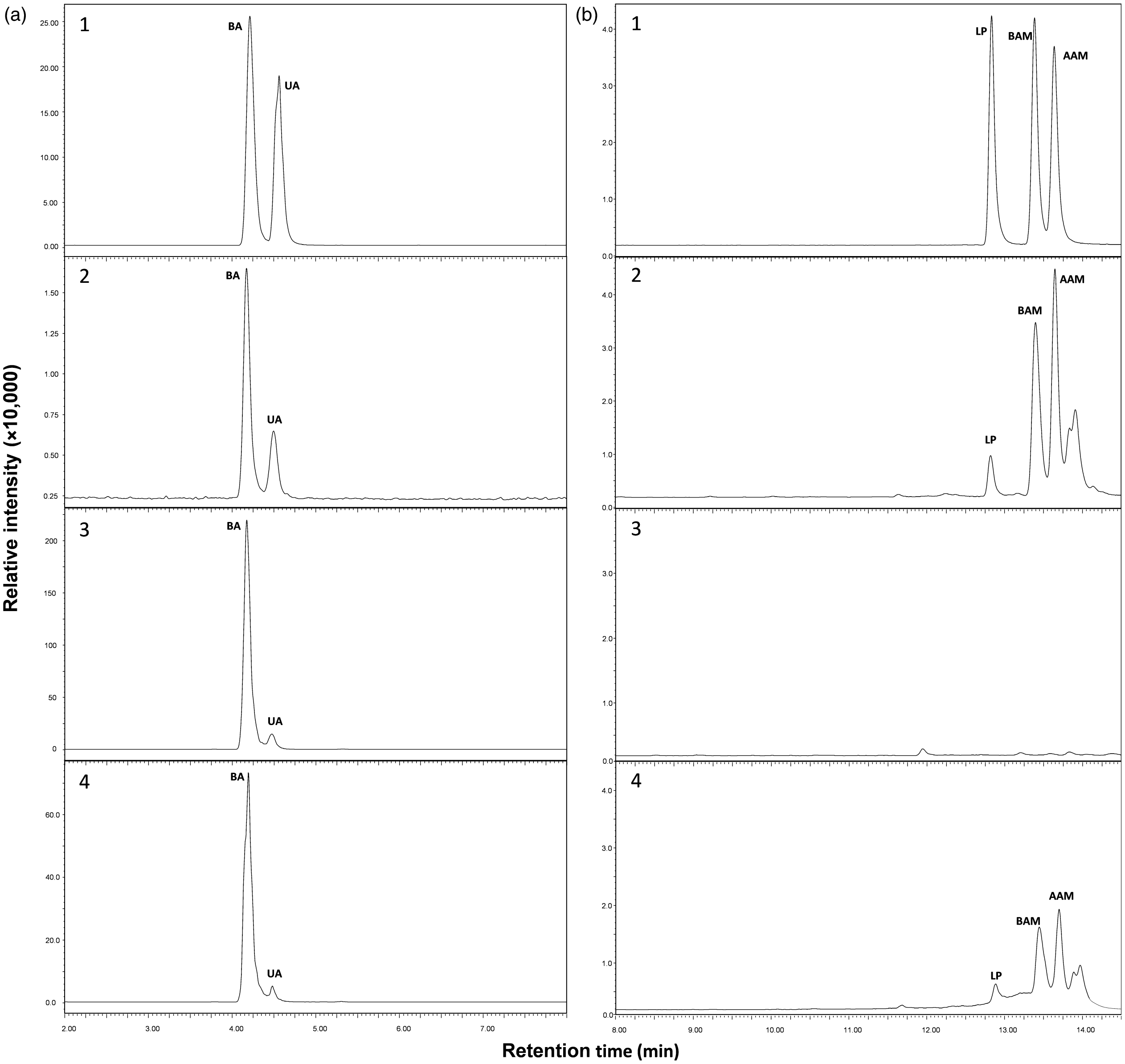

Figure 2 UPLC-MS selected ion monitoring (SIM) chromatogram of triterpenes in plant extracts. (a) Negative SIM monitoring $m/z = 455$ ($[M-H]^-$); (b) positive SIM monitoring $m/z = 409$ ($[M-H_2O]^+$). 1, Standard compounds mixture; 2, *Souroubea sympetala* extracts; 3, *Platanus occidentalis* extract; 4, extracts of a combined mixture of the two herbals. AAM, α-amyrin; BA, betulinic acid; BAM, β-amyrin; LP, lupeol; UA, ursolic acid.

Table 2 Concentration of triterpenes mg/g in *Souroubea sympetala* and *Platanus occidentalis* extracts alone and in a combined mixture (MIX)

Extract	Betulinic acid	Ursolic acid	Lupeol	α-amyrin	β-amyrin
P. occidentalis	279.87 ± 18.20	20.67 ± 1.82	N/A	N/A	N/A
S. sympetala	22.29 ± 0.08	0.78 ± 0.02	8.87 ± 0.14	55.19 ± 1.25	45.56 ± 0.95
MIX	159.27 ± 21.14	9.20 ± 1.58	7.21 ± 0.63	22.01 ± 0.82	23.27 ± 0.66

Quantification results presented as mean ± SEM, $n = 3$. N/A, not applicable.

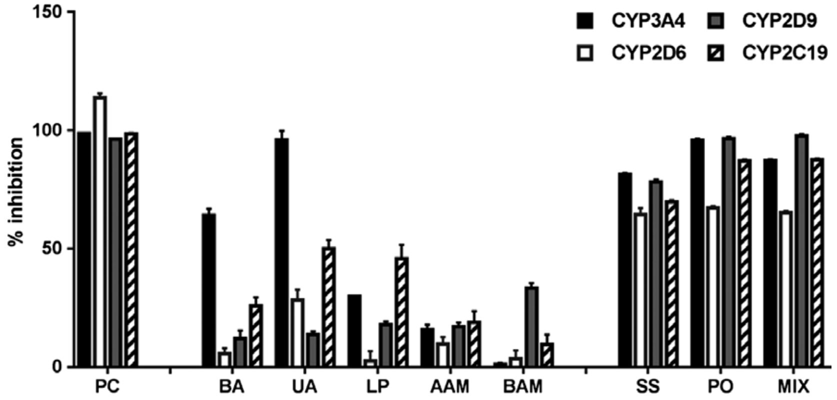

Figure 3 Mean per cent inhibition of cytochrome P450 3A4, 2D6, 2C9 and 2C19 isoforms by positive controls (left), triterpenes (centre) and herbal extracts (right) relative to vehicle control. The triterpenes and *Souroubea sympetala* and *Platanus occidentalis* extracts were tested at 50 μg/ml except with CYP2D6 assay (100 μg/ml). Data presented as mean ± SEM of at least three independent experiments. PC, positive control. AAM, α-amyrin; BA, betulinic acid; BAM, β-amyrin; LP, lupeol; MIX, a combined mixture of the two herbal extracts; PO, *P. occidentalis*; SS, *S. sympetala*; UA, ursolic acid. Positive control used in the assay was ketoconazole 1.9 μM, quinidine 2 μM, sulfaphenazole 100 μM and tranylcypromine 100 μM for CYP3A4, CYP2D6, CYP2C9 and CYP2C19, respectively. 20% Methanol was used as vehicle.

Table 3 Relative potency of cytochrome P450 inhibition elicited by *Souroubea sympetala* and *Platanus occidentalis* plant extracts alone and as a combined mixture (MIX) and five major triterpenes as determined by IC50 concentrations μg/ml (mean ± SEM, *n* ≥ 3)

	CYP3A4	CYP2C9	CYP2C19
Betulinic acid	16.98 ± 0.75	>50	>50
Ursolic acid	6.03 ± 0.24	>50	>50
Lupeol	>50	>50	>50
α-amyrin	>50	>50	>50
β-amyrin	NA	>50	>50
S. sympetala	9.92 ± 0.38	13.48 ± 0.94	26.06 ± 0.91
P. accidentalis	3.08 ± 0.14	3.56 ± 0.14	10.19 ± 0.37
MIX	3.98 ± 0.14	3.72 ± 0.13	9.17 ± 0.41

extracts at high concentration (0.2 mg/ml) significantly block the DA metabolism *in vitro*. The two-way ANOVA and Sidak multiple comparison tests indicated percentage inhibition on two major DA metabolites formation was not significantly different ($P > 0.05$) than each other in all tested groups.

Discussion

Many common anxiolytic medications may cause serious adverse effects such as benzodiazepine overdose.[16,17] Accordingly, the administration of these medications is often based on the risk to benefit ratio, which can be narrow in certain if not many populations. Co-administration of other health or dietary products that may inhibit normal CYP-mediated metabolism may increase the rate and potency of adverse effects. Such events may completely alter the risk to benefit ratio of these products.

The inhibitory potential of several active substances found in *S. sympetala* and *P. occidentalis* extracts towards CYP450 isoforms had been previously reported,[18–20] but the plant extracts had not been examined. Our results suggest that both plant extracts and the mixture have potential to inhibit CYP450 enzyme activity. *P. occidentalis* showed threefold to fourfold stronger CYP450 inhibition relative to the traditionally used *S. sympetala*. The potency of the mixed extract was similar to that of *P. occidentalis*, suggesting that its addition to *S. sympetala* not only boosts the anxiolytic effect[4] but also increases the potential risk of herb–drug interaction when co-administrated with other therapeutic products.

These findings are important as CYP3A4 is one of the major hepatic enzymes that metabolizes many pharmaceuticals in the benzodiazepine class, as well as several selective serotonin reuptake inhibitor (SSRI)/serotonin–norepinephrine reuptake inhibitor (SNRI) medications including escitalopram and buspirone.[21,22] CYP2C19 contributes to the metabolism of many benzodiazepine class medications as an alternative pathway to CYP3A4.[23] CYP2D6 is mainly involved in the metabolism of SSRI/SNRI class medications including duloxetine, paroxetine and fluoxetine.[24,25] Although many SSRIs such as fluoxetine are metabolized by CYP2D6, they are also inhibitors of CYP2D6. Other studies suggest that, when the CYP2D6 contribution to fluoxetine metabolism diminishes over long-term treatment, CYP2C9, 2C19 and 3A4 may serve as alterative metabolic pathways.[26] Other major CYPs such as CYP1A2, CYP2B6, CYP2C8 and CYP2E1 may play a minor role in diazepam metabolism, and the inhibitory

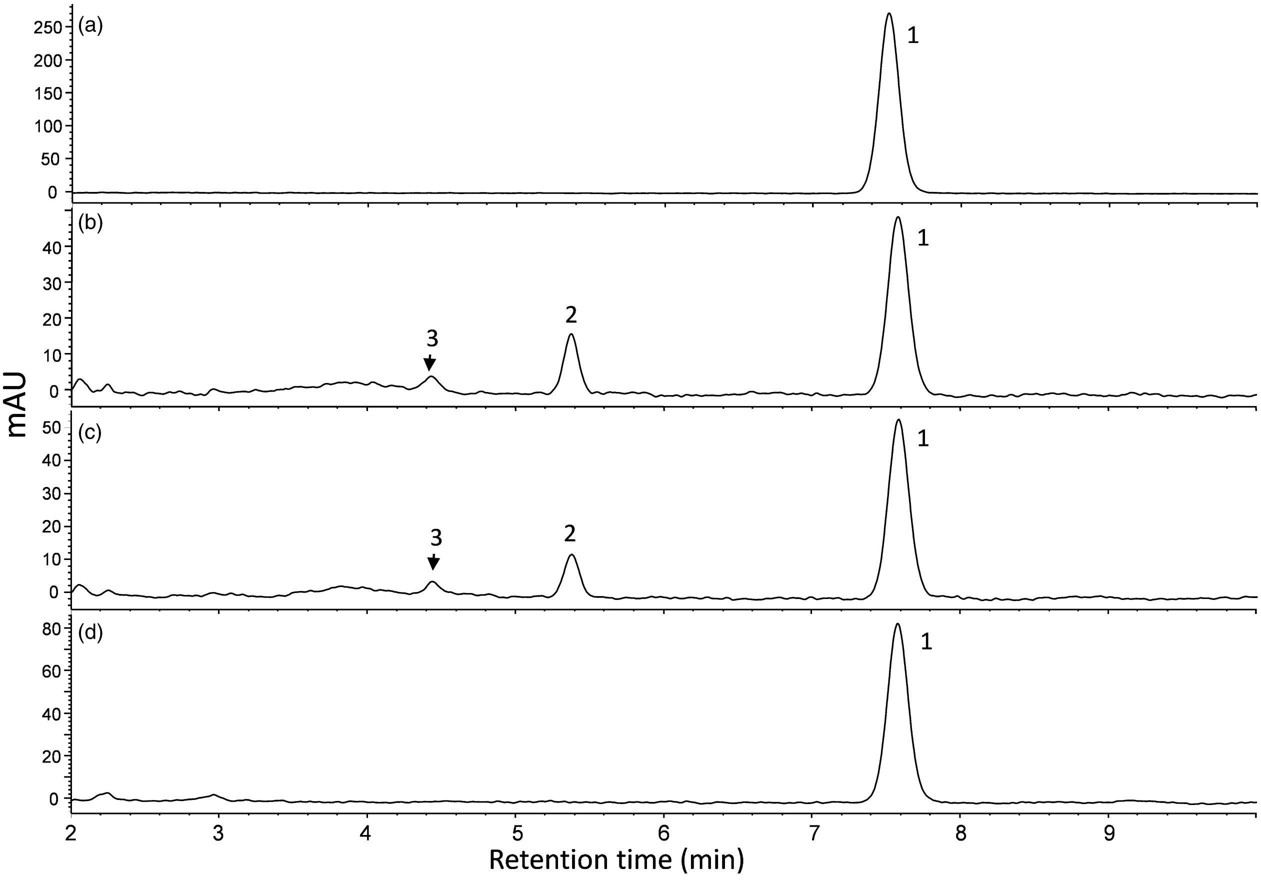

Figure 4 HPLC chromatograms of diazepam (DA) metabolite formation under different conditions across time. All chromatograms were monitored at a wavelength of 240 nm. (a) DA standard. (b) DA incubated with human liver microsomes (HLM) and NADPH. (c) DA incubated with HLM, NADPH and mixed plant extract. (d) DA incubated with HLM only. 1, Diazepam, 2, temazepam and 3, nordazepam.

effects of extract of *S. sympetala* and *P. occidentalis* could be considered in future studies.

Whereas such inhibitory effect increases the risk of NHP–drug interactions, it could also be beneficial when the herbal product is used alone. Several studies indicate that CYP450 isoforms are involved in the metabolism of the pharmacologically active pentacyclic triterpene.[18,27] CYP450 inhibition may reduce the metabolic elimination of these components, resulting in earlier onset of activity and longer half-life that possibly contribute to the synergetic effect of the 1 : 1 v/v mixed plant extract observed *in vivo*.[4]

Compared to the plant extracts, IC_{50} values for individual triterpenes were at least twofold higher (except for UA towards CYP3A4). Other minor secondary metabolites reported in *S. sympetala* include: 2-hydroxyursolic acid, taraxenyl *trans*-4-hydroxy-cinnamate, naringenin, methyl ursolate, eriodytiol, methyl 2-α-hydroxyursolate, methyl 2-α-hydroxymaslinate, methyl betulinate and condrilla sterol.[5] Naringenin has been reported to inhibit a number of CYP450 isoforms *in vitro* including CYP1A1, CYP1A2, CYP1B1, CYP2C9 and CYP3A4.[28] Several of these substances are recognized as derivatives of the major triterpenes, and they may process similar CYP450 inhibition effects. This suggests that the overall composition of the herbal drug needs to be evaluated for its potential for CYP450 inhibition.

DA is a fast-acting benzodiazepine used to treat generalized anxiety disorder with rapid onset action after oral administration and a long half-life from 30 to 100 h.[29] All three major metabolites of DA (Figure 1) retain pharmacological activity.[30] Like DA, temazepam and oxazepam are full agonists of benzodiazepine site of the $GABA_A$ receptor complex and elicit similar anxiolytic activity. The half-life of temazepam and oxazepam is 8–20 and 8–12 h, respectively. Nordazepam is a partial agonist of the benzodiazepine site with reduced pharmacological effect relative to DA and a similar half-life (20–100 h), much longer than temazepam and oxazepam which are more active metabolites.[31] Thus, inhibiting DA metabolism or favouring the

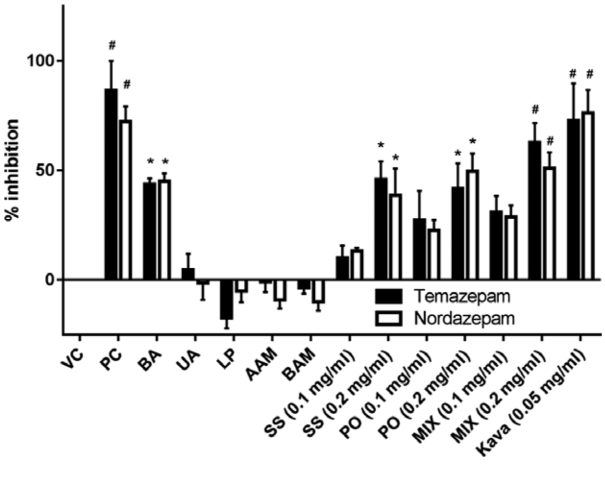

Figure 5 Per cent inhibition of major diazepam metabolite formation by *Souroubea sympetala* and *Platanus occidentalis* extracts and triterpenes (50 μg/ml) relative to vehicle controls. Mean ± SEM n = 3, *BA n = 2. 10% methanol is vehicle control (VC). Ketoconazole 10 nM was the positive control PC. AAM, α-amyrin; BA, betulinic acid; BAM, β-amyrin; LP, lupeol; MIX, a combined mixture of the two herbal extracts; PO, *P. occidentalis*; SS, *S. sympetala*; UA, ursolic acid. The *kava–kava* extract was included as a positive botanical control. A two-way ANOVA with Dunnett's multiple comparisons test was used to compare percentage inhibition to VC. $^{*}P \leq 0.05$; $^{\#}P \leq 0.01$.

formation of nordazepam may result in extended half-life. With repeated administration, accumulating plasma levels could eventually cause benzodiazepine overdose and lead to severe adverse effects including central nervous system depression, impaired balance, ataxia and slurred speech.[16] At higher concentration, all extracts inhibited the metabolism of DA in the HLM assay, significantly reducing the formation of both temazepam and nordazepam compared to vehicle control. Unlike the CYP inhibitory results obtained using probe substrates, the inhibition of DA metabolism by the extracts was similar. BA is the only triterpene that showed inhibition of DA metabolism. Even though UA showed very potent inhibitory effect on CYP3A4-mediated metabolism of the probe DBF, no inhibitory effects were observed on DA metabolism. This may due to the allosteric behaviour or selectivity of CYP3A4. Although still under debate, it is well accepted that multiple substrates can simultaneously bind to CYP3A4 inside or near the active site, affecting catalytic activity.[32] As a result, potency may vary when different substrates are used in the assay.[33]

The findings in this *in-vitro* study suggest that the extracts of *S. sympetala* and *P. occidentalis,* alone or in combination, have the potential to affect the efficacy and safety of co-administered DA products, as well as other benzodiazepines, in certain conditions. The observed inhibition of CYP-mediated metabolism, however, could also be beneficial when the extracts are not consumed with other medications since the pharmacologically active triterpenes are also metabolized though the CYP450 system, possibly increasing their bioavailability and enhancing anxiolytic effect. Finally, clinical study is warranted to investigate whether the results observed here can be applied to humans.

Declarations

Conflict of interest

John Thor Arnason, Tony Durst and Pablo Sanchez are principals in Souroubea Botanicals Inc. which has brought the research to the market in the animal healthcare field. All other authors have no conflict of interest.

Acknowledgements

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References

1. Harvard Medical School. National Comorbidity Survey (NCS). (2017, August 21). Retrieved from https://www.hcp.med.harvard.edu/ncs/index.php. Data Table 1: Lifetime prevalence DSM-IV/WMH-CIDI disorders by sex and cohort; Data Table 2: 12-month prevalence DSM-IV/WMH-CIDI disorders by sex and cohort.
2. Masic A *et al.* Safety evaluation of a new anxiolytic product containing botanicals *Souroubea* spp. and *Platanus* spp. in dogs. *Can J Vet Res* 2018; 82: 3–11.
3. Durst T *et al.* Anxiolytic marcgraviaceae compositions containing betulinic acid, betulinic acid derivatives, and methods. U.S. Patent 8,623,848, issued January 7, 2014.
4. Liu R *et al.* New botanical anxiolytics for use in companion animals and humans. *AAPS J* 2017; 19: 1626–1631.
5. Puniani E *et al.* Ethnopharmacology of *Souroubea sympetala* and *Souroubea gilgii* (Marcgraviaceae) and identification of betulinic acid as an anxiolytic principle. *Phytochemistry* 2015; 11: 73–78.
6. Mullally M *et al. Souroubea sympetala* (Marcgraviaceae): a medicinal plant that exerts anxiolysis through interaction with the GABA$_A$ benzodiazepine receptor. *Can J Physiol Pharmacol* 2014; 92: 758–764.
7. Carballo-Arce AF. *Phytochemical Investigations of Costa Rican Marcgraviaceae and Development of Insecticide Synergists*. Ottawa, ON: University of Ottawa; 2013 (dissertation).
8. Villalobos P *et al.* Clinical observations and safety profile of oral herbal products, *Souroubea* and *Platanus* spp; a pilot-toxicology study in dogs. *Acta Vet* 2014; 64: 269–275.

9. Bertz RJ, Granneman GR. Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* 1997; 32: 210–258.
10. Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and Inhibitors. *Drug Metab Rev* 1997; 29: 413–580.
11. Wanwimolruk S, Prachayasittikul V. Cytochrome P450 enzyme mediated herbal drug interactions (Part 1). *EXCLI J* 2014; 13: 347–391.
12. Brantley SJ *et al.* Herb-drug interactions: challenges and opportunities for improved predictions. *Drug Metab Dispos* 2014; 42: 301–317.
13. Mai I *et al.* Hyperforin content determines the magnitude of the St John's wort–cyclosporine drug interaction. *Clin Pharmacol Ther* 2004; 76: 330–340.
14. Whitten DL *et al.* The effect of St. John's wort extracts on CYP3A: a systematic review of prospective clinical trials. *Br J Clin Pharmacol* 2006; 62: 512–526.
15. Tam TW *et al.* Actions of ethnobotanically selected Cree anti-diabetic plants on human cytochrome P450 isoforms and flavin-containing monooxygenase 3. *J Ethnopharmacol* 2009; 126: 119–126.
16. Greenblatt DJ *et al.* Acute over dosage with benzodiazepine derivatives. *Clin Pharmacol Ther* 1977; 21: 497–514.
17. Sacre L *et al.* Toxicodynetics in nordiazepam and oxazepam overdoses. *Ann Pharm Fr* 2017; 75: 163–171.
18. Seervi M *et al.* Assessment of cytochrome P450 inhibition and induction potential of lupeol and betulin in rat liver microsomes. *Drug Metab Pers Ther* 2016; 31: 115–122.
19. Kim TW *et al.* Cytochrome P450-catalyzed brassinosteroid pathway activation through synthesis of castasterone and brassinolide in *Phaseolus vulgaris*. *Phytochemistry* 2014; 65: 679–689.
20. Moreira Fde L *et al.* A non-michaelian behavior of the *in vitro* metabolism of the pentacyclic triterpene alfa and beta amyrins by employing rat liver microsomes. *J Pharm Biomed Anal* 2013; 84: 14–19.
21. Rao N. The clinical pharmacokinetics of escitalopram. *Clin Pharmacokinet* 2007; 46: 281–290.
22. Mahmood I, Sahajwalla C. Clinical pharmacokinetics and pharmacodynamics of buspirone, an anxiolytic drug. *Clin Pharmacokinet* 1999; 36: 277–287.
23. Fukasawa T *et al.* Effects of genetic polymorphism of cytochrome P450 enzymes on the pharmacokinetics of benzodiazepines. *J Clin Pharm Ther* 2007; 32: 333–341.
24. Jin X *et al.* Pre-clinical evaluation of CYP 2D6 dependent drug-drug interactions between primaquine and SSRI/SNRI antidepressants. *Malar J* 2016; 15: 280.
25. Hemeryck A, Belpaire FM. Selective serotonin reuptake inhibitors and cytochrome P-450 mediated drug-drug interactions: an update. *Curr Drug Metab* 2002; 3: 13–37.
26. Mandrioli R *et al.* Fluoxetine metabolism and pharmacological interactions: the role of cytochrome p450. *Curr Drug Metab* 2006; 7: 127–133.
27. Cichewicz RH, Kouzi SA. Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Med Rev Rev* 2004; 24: 90–114.
28. Shimada T *et al.* Structure-function relationships of inhibition of human cytochromes P450 1A1, 1A2, 1B1, 2C9, and 3A4 by 33 flavonoid derivatives. *Chem Res Toxicol* 2010; 23: 1921–1935.
29. Hoffman EJ *et al.* Anxiety disorders: a comprehensive review of pharmacotherapies. *Mt Sinai J Med* 2008; 75: 248–262.
30. Hoffmann-La Roche Ltd. *Valium Product Monograph*. Basel, Switzerland: Hoffmann-La Roche Ltd, 2018.
31. Haddad LM, Winchester JF. *Clinical Management of Poisoning and Drug Overdose*, 3rd edn. Philadelphia, PA: WB. Saunders Company, 1998.
32. Sevrioukova IF, Poulos TL. Current approaches for investigating and predicting cytochrome P450 3A4-ligand interactions. In: Hrycay EG, Bandiera SM, eds. *Monooxygenase, Peroxidase and Peroxygenase Properties and Mechanisms of Cytochrome P450*. Cham: Springer, 2015: 83–105.
33. Kenworthy KE *et al.* CYP3A4 drug interactions: correlation of 10 *in vitro* probe substrates. *Br J Clin Pharmacol* 1999; 48: 716–727.