Physicochemical Characterization of *Mitragyna speciosa* Alkaloid Extract and Mitragynine Using *In Vitro* High Throughput Assays

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**Abstract:** *Mitragynine*, a major active alkaloid of *Mitragyna speciosa*, acts as an agonist on \(\mu\)-opioid receptors, producing effects similar to morphine and other opioids. It has been traditionally utilized to alleviate opiate withdrawal symptoms. Besides consideration about potency and selectivity, a good drug must possess a suitable pharmacokinetic profile, with suitable absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) profile, in order to have a high chance of success in clinical trials.

**Material and Method:** The purity of mitragynine in a *Mitragyna speciosa* alkaloid extract (MSAE) was determined using Ultra-Fast Liquid Chromatography (UFLC). *In vitro* high throughput ADME-Tox studies such as aqueous solubility, plasma protein binding, metabolic stability, permeability and cytotoxicity tests were carried out to analyze the physicochemical properties of MSAE and mitragynine. The UFLC quantification revealed that the purity of mitragynine in the MSAE was 40.9%.

**Results:** MSAE and mitragynine are highly soluble in aqueous solution at pH 4.0 but less soluble at pH 7.4. A parallel artificial membrane permeability assay demonstrated that it is extensively absorbed through the semi-permeable membrane at pH 7.4 but very poorly at pH 4.0. Both are relatively highly bound to plasma proteins (> 85 % bound) and are metabolically stable to liver microsomes (> 84 % remained unchanged). In comparison to MSAE, mitragynine showed higher cytotoxicity against WRL 68, HepG2 and Clone 9 hepatocytes after 72 h treatment.

**Conclusion:** The obtained ADME and cytotoxicity data demonstrated that both MSAE and mitragynine have poor bioavailability and have the potential to be significantly cytotoxic.

**Keywords:** ADME-Tox, Cytotoxicity, *Mitragyna speciosa* alkaloid extract, Mitragynine, Ultra-Fast Liquid Chromatography, High Throughput.

1. **INTRODUCTION**

*Mitragyna speciosa* (from the Rubiaceae family) is an indigenous plant which is found mainly in the northern region of the Malay peninsula and in the central and southern parts of Thailand [1]. It is locally known as “Biak-biak” and “Ketum” in Malaysia, and “Kratom” in Thailand [2]. The major indole-alkaloid constituent found in *M. speciosa* is mitragynine, an agonist on \(\mu\)-opioid receptors and antagonist on \(\delta\)- and \(\kappa\)-opioid receptors [3]. Traditionally, leaves from this plant are consumed in small amounts mainly to treat diarrhea, to produce a stimulant and euphoric effect to combat fatigue and to increase tolerance to the hot sun. High doses of *M. speciosa* extract is able to produce analgesic effect in human and animal models [4, 5].

Due to its unique psychoactive properties, it has been proposed to be used as a replacement therapy for opiate addiction [6]. Animal study in zebrafish model showed that mitragynine is able to attenuate the anxiety caused by morphine withdrawal [7]. However, to date, there is no proper clinical evidence to show that mitragynine is safe to be consumed at high doses, so the medical use as an opiate substitute remains uncertain. Nevertheless, mild addiction, opioid abstinence syndrome and mild side effects have been observed among chronic users resulting in anorexia, weight loss, skin darkening and constipation [8]. Reports of misuse of the plant by drug addicts for sedative and euphoric effects have caused major drug abuse concerns in Southeast Asia. Consequently, the kratom plant has been listed as a controlled substance in Malaysia, Thailand and Australia. However, in other parts of the world, kratom is currently not strongly regulated by law. The availability of kratom over the internet has caused a significant drug abuse issue, beyond traditional uses for self-treatment in opioid withdrawal and for chronic pain [6, 9].
Studies of ADME-Tox are critically important in preclinical drug development [10]. The Food and Drug Administration (FDA) of the United States of America has reported that the cost for developing a new drug was as high as 2.6 billion US dollars in 2014 [11, 12]. More than 50 % of the failure of active compounds in the early clinical phases has been due to either a poor pharmacokinetics profile or toxicity. Thus, early assessment of in vitro ADME-Tox properties of a new drug candidate is not only important for pre-formulation assessment, drug candidate selection, but the information is also used in order to increase the success rate in clinical trials. High throughput in vitro ADME-Tox assays have always been the preliminary screening for pharmacokinetic profiling to accelerate the drug discovery process, minimize failure in clinical trials, and reduce time, cost, and animal usage.

Although, the pharmacological effects of kratom in human and experimental animals are well-established, the pharmacokinetics and toxicity data of pure mitragynine and alkaloid extract still remain poorly defined. Some of the basic ADME properties especially MDCK permeability of mitragynine had been described by Manda et al., 2014 [13]. A simple and cost effective ADME-Tox method was developed and applied in this study to determine mitragynine’s suitability, from an ADME-Tox perspective, as a drug candidate for use in opiate withdrawal treatment. The findings will assist in dose determination, route of administration, solvent and toxicity prediction and consideration of the drug for further progression to animal and clinical testing.

2. MATERIALS AND METHODS

2.1. Reagents

Pure mitragynine was purchased from ChromaDex (Irvin, USA). All other chemicals used in this study such as β-nicotinamide adenine dinucleotide phosphate (NADP+), glucose-6-phosphate dehydrogenase (G6PDH), glucose-6-phosphate, phosphate buffer saline (PBS), reference standard compounds, trifluoroacetic acid (TFA), acetonitrile (ACN), and methanol were obtained from Sigma Aldrich (St. Louis, MO). All other reagents were of analytical grade purchased from different commercial suppliers. Parallel artificial membrane permeability assay (PAMPA) kit was purchased from pION Inc (MA, USA).

2.2. Extraction of M. speciosa

Fresh leaves of M. speciosa were collected from the northern state of Perlis in Malaysia. As described previously, a methanol-chloroform extraction method and an acid-base extraction were used to isolate the MSAE [14]. The presence of alkaloid and mitragynine was further analyzed using Dragendorff’s reagent and bioanalytical analysis.

2.3. UFLC Method Development and Validation

The quantification of selected compounds was carried out using Shimadzu Prominance Ultra-Fast Liquid Chromatography system consisting of a SPD-20A UV-VIS Detector with auto-sampler (UFLC, Shimadzu, Japan). The chromatographic separation was performed on an eclipse plus C18 column, (4.6x250 mm, 5 µm; Agilent, USA) maintained at constant temperature 40°C with a flow rate of 0.2 mL/min. Mobile phase A comprising of 0.1 % TFA in MiliQ water and mobile phase B comprising 100 % ACN were used in a gradient mode 20:80 (v/v). The detector wavelength was set at 254 nm for both the MSAE and mitragynine data quantification. The injection volume for each sample was 10 µL, and 15 min was fixed for every chromatographic run. Shimadzu LC LabSolution software (version 5.55, Shimadzu, Japan) was used for data acquisition and analyzing processing.

Linearity of mitragynine was conducted in the range of 0.4-200 µg/mL. Its linearity and coefficients (R²) were determined using linear regression analysis. Lower limit of quantification (LLOQ), inter-day /intra-day precision, stability, and selectivity were determined. The amount of mitragynine appeared in MSAE was calculated based on the mitragynine calibration curve.

\[
\text{Purity of mitragynine} = \frac{C_{\text{MIT}}}{10 \, \mu\text{g/mL}} \times 100 \, \% \quad (1)
\]

Where \( C_{\text{MIT}} \) is the calculated concentration of mitragynine from the linearity equation.

2.4. Aqueous Solubility Assay

The aqueous solubility assay was performed using high performance liquid chromatography with ultraviolet detection (HPLC-UV). The assay was conducted in 96 deep-well plates with Dulbecco’s phosphate-buffered saline (DPBS) in pH 4 and pH 7.4. MSAE, mitragynine and standard controls (diethylstilbestrol, estriol and furosemide) were dissolved in DPBS in respective pH to get a final concentration of 200 µM with 0.5 % DMSO (v/v). The mixture was allowed to incubate for 16 hours with moderate shaking in room temperature. At the end of the incubation period, all the samples were centrifuged for 20 min at 25°C. The supernatant was subjected to UFLC analysis together with a positive control in 100 % DMSO. Aqueous solubility was then calculated by comparing the dissolved compounds in the supernatant and positive control [15].

2.5. Plasma Protein Binding Assay (PPB)

Plasma protein binding assay was performed using ultrafiltration units (Microcon-10kDa Centrifugal Filter) obtained from Millipore (Bedford, MA). MSAE, mitragynine and reference controls (atenolol, caffeine, and propranolol) were added into the human plasma to obtain a final concentration of 10µM (approximately 4 μg/mL for MSAE). The sample mixture was incubated at 37°C for 60 min. Following the incubation, 400 µL of the mixture was transferred into the micron-10 tube and centrifuged at 2000 g for 45 min to isolate the unbound fraction of the compounds. An aliquot of 200 µL of the remaining plasma (bound fraction) was mixed with 400 µL of cold ACN and then sonicated. The supernatant was collected after centrifugation at 2000 g for 2 min. Lastly, the bound, unbound fractions and controls (known concentration of the test compounds in protein free plasma) were submitted for UFLC analysis. The percentage of bound was calculated using Eq. (2) [16].

\[
\% \text{ of Bound} = \left( \frac{C_T - C_U}{C_T} \right) \times 100 \, \% \quad (2)
\]
Where $C_T$ is the total drug concentration in the protein free plasma and $C_U$ is the concentration of unbound drug in the filtrate.

2.6. Parallel Artificial Membrane Permeability Assay (PAMPA)

The assay was carried out according to the protocol provided by the kit (pION Inc, MA, USA) [17, 18]. In brief, the 96-well filter plate (acceptor) was manually coated with a clear phospholipid layer using pION bilayer lipid mix. A 96-well receiver plate was used as donor. MSAE, mitragynine and standard reference controls (metoprolol, furosemide and carbamazepine) were diluted in pION solution to obtain final concentration of 25 µM (10 µg/mL of MSAE). The DMSO concentration was maintained at 0.5%. A volume of 200 µL of test compounds and clear pION solution was added into the acceptor and donor wells. Then, the acceptor plate was coupled with donor plate and incubated in a humidified chamber for 16 h at room temperature. The permeability of the compounds was assessed in triplicate at pH 4.0 and pH 7.0. At the end of the incubation, a volume of 150 µL of test compounds from each donor and acceptor was transferred to clear glass vial and analyzed using UFLC system. The permeability ($Pe; \text{cm/s}$) was calculated using equation (3) and (4) [19].

\[
\text{Permeability (cm/s), } Pe = -\ln \frac{1-C_A}{C_{\text{equilibrium}}} \frac{1}{S} \left( V_D + 1/N_A \right)^3 \tag{3}
\]

Where $V_D$ is the donor volume (0.2 cm$^3$); $V_A$ is the acceptor volume (0.2 cm$^3$); S is the membrane area (0.3 cm$^2$); t is the incubation time (in seconds)

\[
C_{\text{equilibrium}} = \frac{(C_D^*V_D - C_A^*V_A)}{(V_D^*-V_A^*)} \tag{4}
\]

$C_{\text{equilibrium}}$ is defined as the balance in the concentration between the donor and acceptor wells if the membrane is 100% permeable to the compound, where $C_A$ is the final acceptor concentration; $C_D$, is the final donor concentration.

2.7. Metabolic Stability Assay

The in vitro metabolic stability assay was carried out at a single time point. MSAE, mitragynine and standard reference compounds (propranolol and verapamil) were prepared in 50 mM of potassium phosphate buffer pH 7.4 and pre-incubated with 0.5 mg/mL of rat liver microsomes (RLM) at 37°C for 10 min. Final concentration of the test compounds was maintained at 10 µM with 0.2% DMSO and 2% ACN. After the incubation, the reaction was initiated by adding 20 µL of pre-warmed NADPH regenerating system (NRS) which consist of 1 mM NADP, 5 mM glucose 6-phosphate, 2 mM MgCl$_2$ and 0.06 U/well of glucose-6-phosphate dehydrogenase. After 30 min incubation at 37°C, the reaction was terminated by adding 100 µL of cold ACN. Precipitation of microsome protein was removed by centrifugation at 3000 rpm for 10 min in room temperature. Supernatant was then removed and sent for UFLC analysis [20, 21]. Based on the peak area of parent compounds at time zero ($T_0$), the amount of drug remaining after 30 min ($T_{30}$) was determined.

2.8. Cytotoxicity Assay Using Cell Proliferation XTT Assay

All the normal and cancer liver cell lines, WRL 68 (ATCC® CL 48™), Clone 9 (ATCC® CRL1439™) and HepG2 (ATCC® HB 8065™), were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). WRL 68 and HepG2 cells were maintained in Minimum Essential Media (MEM), while Clone 9 cells were maintained in Ham’s F-12K media as recommended by ATCC. Cells were seeded in respective optimal cell density in a 96-well cell culture plate. Subsequently, the cells were treated with 3x serial diluted MSAE (0.05 -300 µg/mL) and mitragynine (0.01-120 µg/mL) for 24, 48 and 72 h. Doxorubicin was used as positive control. At the day of termination, the number of viable cells was measured using XTT assay according to the manufacturer (Sigma Aldrich, MO) protocol [22]. The cell cytotoxicity was defined as the percent inhibition of cell proliferation.

2.9. Data Analysis

The statistical analysis was performed using GraphPad Prism (version 5.04; CA, USA) and all the data were expressed as mean ± standard error of the mean (SEM). All the experiments were performed in triplicates for two independent experiments.

3. RESULTS

3.1. Quantitative UFLC Method Development and Validation

The MSAE and pure mitragynine were subjected for UFLC method development and analysis. Representative chromatograms obtained for the MSAE and mitragynine are depicted in Fig. (1). A single peak of mitragynine was eluted at 5.8 min in the chromatographic run. The calibration curve of the pure mitragynine was plotted from 0.4 to 200 µg/mL and linearity equation was determined as $Y = 54226X + 139261$ with a regression coefficient ($R^2$) of 0.999 (Fig. 2). The lowest limit of quantification (LLOQ) of mitragynine was set at 0.4 µg/mL which showed signal to noise (S/N) ratio of more than 10 and the % RSD below than 15%. Intra-day and inter-day analysis showed accuracy greater than 95% and imprecision (% RSD) less than 15% at 1, 50 and 100 µg/mL mitragynine. Both MSAE and mitragynine in respective buffer demonstrated high stability (>90 %) after 2 h and 24 h incubation at room temperature. No matrix effect was observed. The calculated concentration of mitragynine in the MSAE was approximately 4.09 µg/mL (purity 40.9 %) based on the calibration equation.

3.2. In vitro ADME Properties

The aqueous solubility, plasma protein binding, permeability and metabolic stability of MSAE and mitragynine are summarized in Table 1. In all the ADME studies, at least two to three reference compounds, low to high level permeability standards, were applied in order to validate the accuracy of the assays.
3.2.4. Metabolic Stability

Rat liver microsomes (RLM) were used to assess metabolic stability. After 30 min of incubation with RLM and NADPH regeneration system (NRS), MSAE and mitragynine were metabolically stable, with 89.79 ± 1.47 % and 84.49 ± 1.85 % remaining unchanged after 30 min of incubation. Hence, drug elimination half-life (T1/2) determination using serial of time points is unnecessary due to extremely low metabolism rate, as more than 50 % parental compound was restrained after 30 min of single time point screening.

3.2.5. Cell Cytotoxicity

In the present study, the cytotoxic effect of MSAE and mitragynine were evaluated using three liver cell lines namely human hepatocarcinoma cells (HepG2), rat hepatocytes (Clone 9) and normal human hepatocytes (WRL 68). As depicted in Table 2, potential cytotoxicity was observed in all three cell lines after 24, 48 and 72 h of treatment. Time-dependent cell proliferation inhibition study showed cytotoxicity level increased from 24 to 72 h. The cytotoxic severity of mitragynine was listed in the order WRL 68 > HepG2 > Clone 9. Mitragynine showed higher cytotoxic effect compared with MSAE where WRL 68 normal liver cells are more sensitive to mitragynine. The cytotoxicity level of compound was categorized into 4 major categories [23] (Table 3). The finding was correlated to the reported severe hepatotoxicity [24]. Further in vivo toxicity analysis is needed to evaluate mitragynine toxicity.

4. DISCUSSION

Opioid analgesics, especially morphine, remain the most effective analgesic for chronic pain management. Unfortunately, long-term treatment of morphine is associated with severe opioid addiction, tolerance, as well as misuse and abuse cases. As an alternative treatment, mitragynine and M. speciosa products have successfully attracted the attention of chronic users since it is always claimed to be “natural” with less toxicity and addictive potential as compared to morphine. In fact, there is no clinical evidence to prove its pharmacological safety. Although mitragynine has been potentially developed as a candidate for opioid substitution and opioid withdrawal syndrome treatment, the high chance of abuse due to the sedative and euphoric effects cannot be neglected. Hence, a balance between the medical benefits and the potential to cause addictiveness as well as the overdose risk need to be investigated intensively [25].

Over the past 15 to 20 years, high throughput in vitro ADME studies were routinely applied in the early phase of pre-clinical drug discovery programs [26]. Data of in vitro ADME and in vivo pharmacokinetics are not only relevant for drug candidate selection and structural modification but also in prediction of pharmacokinetic/pharmacodynamic (PK/PD) properties in human [26]. Due to various disadvantages of in vivo studies including issues of ethical use of animals, cost and being time-consuming, numerous in vitro and in silico models have been established for human pharmacokinetic predictions [26]. In this study, we reported the fundamental physiochemical and drug properties of mitragynine, in which the information can be applied in
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A good drug candidate should have good physicochemical properties such as satisfactory solubility, absorption, clearance, and reasonable metabolic stability [23].

Aqueous solubility of a drug is crucial during the early drug development process where it is a key physicochemical property that significantly influences the absorption, permeability and distribution of a compound. Poor compound dissolution in an aqueous solution always results in many undesirable problems which include false positive results, poor oral bioavailability, decrease of drug efficacy, undesirable drug-drug interactions and sometimes induced toxicity [27]. In practice, drug candidates with low aqueous solubility are usually excluded at the beginning of candidate selection. In this study, apparent solubility of mitragynine was tested in DPBS buffer system with minimal presence of DMSO (0.5%). MSAE and mitragynine showed moderate aqueous solubility in pH 7.4. There is no aqueous solubility issue at pH 4.0. Since mitragynine is a weakly basic compound owing to the presence of an amine group, which tends to ionize in acidic media, it is predicted to have higher aqueous solubility in acidic gastric fluid than in basic intestinal fluid in humans [28, 29]. On the other hand, the moderately high lipophilicity of mitragynine in neutral media is likely to aid its ability to effectively cross the blood brain barrier (BBB).

### Table 1. Summary of aqueous solubility, plasma protein binding, PAMPA and metabolic stability of mitragynine and *M. speciosa* alkaloid extract (MSAE).

<table>
<thead>
<tr>
<th>ADME assays</th>
<th>MSAE</th>
<th>Mitragynine</th>
<th>Internal References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous solubility</td>
<td></td>
<td></td>
<td>DES:19.31±0.51 (Low)</td>
</tr>
<tr>
<td>pH 7.4 (µM)</td>
<td>54.93±1.06</td>
<td>82.71±1.92</td>
<td>Estriol: 86.46±2.96 (Medium)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Furosemide:197.52±3.06 (High)</td>
</tr>
<tr>
<td>pH 4.0 (µM)</td>
<td>118.61±2.44</td>
<td>129.98±0.10</td>
<td>DES:24.16±4.24 (Low)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estriol: 89.81±8.17 (Medium)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Furosemide:187.84±0.93 (High)</td>
</tr>
<tr>
<td>PPB (%)</td>
<td>92.19±2.12</td>
<td>85.35±1.22</td>
<td>Caffeine: 13.14±2.115</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Atenolol: 54.54±4.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Propranolol: 92.47±2.65</td>
</tr>
<tr>
<td>PAMPA</td>
<td></td>
<td></td>
<td>DES:19.31±0.51 (Low)</td>
</tr>
<tr>
<td>pH 7.4 (cm/s x 10^{-6})</td>
<td>11.67±0.008</td>
<td>11.14±0.06</td>
<td>Carbamazepine:0.13±0.002 (Low)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Furosemide:0.75±0.003 (Medium)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metoprolol: 4.49±0.007 (High)</td>
</tr>
<tr>
<td>pH 4.0 (cm/s x 10^{-6})</td>
<td>0.19±0.0007</td>
<td>0.23±0.0005</td>
<td>Carbamazepine:0.21±0.005 (Low)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Furosemide:0.72±0.008 (Medium)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metoprolol: 4.55±0.002 (High)</td>
</tr>
<tr>
<td>Metabolic stability</td>
<td>89.79±1.47</td>
<td>84.49±1.85</td>
<td>Propranolol: 21.41±0.60</td>
</tr>
<tr>
<td>(% remained unchanged)</td>
<td></td>
<td></td>
<td>Verapamil: 69.04±1.62</td>
</tr>
</tbody>
</table>

Data was expressed as mean ± SEM. PPB, plasma protein binding; PAMPA, Parallel artificial membrane permeability assay; DES, diethylstilbestrol.

### Table 2. Cytotoxic IC_{50} values of MSAE and mitragynine on three different liver cell lines.

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC_{50} of cell proliferation (µg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSAE</td>
<td>Mitragynine</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>HepG2</td>
<td>41.7±1.8</td>
<td>31.2±2.2</td>
</tr>
<tr>
<td>WRL 68</td>
<td>55.4±8.7</td>
<td>49.8±9.9</td>
</tr>
<tr>
<td>Clone 9</td>
<td>125.1±7.7</td>
<td>94.5±8.9</td>
</tr>
</tbody>
</table>

Each value was expressed as average of triplicates ± SEM.
Table 3. Classification of cytotoxicity for natural product [23].

<table>
<thead>
<tr>
<th>Category</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Toxic</td>
<td>IC₅₀ &lt;10 μg/mL</td>
</tr>
<tr>
<td>Potentially Toxic</td>
<td>10 &lt; IC₅₀ &lt;100 μg/mL</td>
</tr>
<tr>
<td>Potentially Harmful</td>
<td>100 &lt; IC₅₀ &lt;1000 μg/mL</td>
</tr>
<tr>
<td>Potentially Non-toxic</td>
<td>IC₅₀ &gt;1000 μg/mL</td>
</tr>
</tbody>
</table>

for gastrointestinal model with artificial phospholipid membrane which mimics intestinal transcellular membrane was used to determine the passive diffusion of mitragynine. Instead of active transporter, more than 80% of the oral drug was absorbed into the systemic circulation by passive diffusion through the intestinal epithelium [20]. Hence, understanding the passive diffusion of a drug will be extremely useful to predict the intestinal absorption and bioavailability. Mitragynine showed extremely low permeability in acidic phase (pH 4.0) and the results indicated that mitragynine will be poorly absorbed in the stomach due to its ionized state. This finding correlates well with the reported in vivo low bioavailability (3.03 %) [1]. In the hotplate test study, 35 mg/kg of mitragynine was administrated intraperitoneally to produce significant antinociceptive effects in mice [30]. However, 6 times of the dose was required to produce the similar effects by given orally [31]. Hence, the low bioavailability of mitragynine could be the reason for the individual variations in the reported pharmacological effects on the central nervous system (CNS) [32]. Higher permeability at pH 7.4 is consistent with a neutral form of the compound being more predominant and because the compound is lipophilic (LogP =3.4). Similar findings were reported in cell-based Caco-2 and MDR-MDCK permeability assays, in which mitragynine was mainly transported by passive diffusion in both intestinal membrane and BBB with efflux ratios of 1.0 [13]. However, further in vivo BBB permeability studies are needed to confirm the predicted high BBB penetration, though one might surmise from its in vivo effects that BBB permeability is indeed high due to the small mitragynine molecular size and high lipophilicity.

Heaptic clearance is the major drug clearance mechanism in the human body. The usual practice in the pharmaceutical industry is that, for compounds with low metabolic stability (<30%), it is not advisable for it to be carried further into in vivo studies [33]. Drugs which are highly metabolized will have short half-life (T₁/₂) and low bioavailability. A metabolic stability assay can be conducted using rat, mice, human liver microsomes, S9 fraction and hepatocytes. Compounds with low metabolism are not considered to be disadvantageous in early drug development since the dosing frequency is less, thereby minimizing the potential toxicity. However, it is important to understand that variations in pharmacokinetics do exist between animal species and this will affect the prediction of the rate of metabolism in humans [33]. Hence, studies using human liver microsomes will be more directly related to the clinical development, whereas, rodent metabolism are useful in early drug discovery, particularly to design protocols for animal models. In RLM, both MSAE and mitragynine were very stable, with > 80% of the parent compound unchanged after 30 min of incubation. Together with high plasma protein binding, slow elimination rate (long T₁/₂ > 20 h) was observed in a human pharmacokinetic study.

In the systemic circulation, the drugs will appear either in free form or bound to plasma proteins such as albumin, α-glycoprotein, lipoproteins and globulins. Plasma protein binding will limit the drug distribution to tissues and organs though the kinetics of protein binding (on- and off- rates). Drug efficacy, clearance, and hepatic metabolism are strongly associated with the amount of the unbound drug in the plasma. The direct determination of unbound drug at the site of action is difficult [34]. With regard to this, several attempts have been made to predict drug distribution in the body. In vitro plasma protein binding using ultrafiltration method is one of the most common assays used to measure the unbound drug concentration in the plasma with the assumption that equilibrium of free drug occurs between plasma and tissues [35]. Again, mitragynine was found to be moderately bound to plasma protein, with approximately 90% being bound. The results predict poor distribution and low systemic exposure. Further investigation on PPB dissociation constants is needed to understand the impact of PPB on the drug’s distribution properties.

As recommended by FDA, cell-based models that can predict toxicity in rodents should be incorporated into early stage of lead optimization in order to rule out the high risk molecules, minimizing development time, cost and animal usage [36]. The use of animals in toxicological studies has always been limited by ethical concerns, thus cellular toxicity is often used as a rough approximation of in vivo toxicity. Cancer cell lines have many advantages over primary cell lines for primary cytotoxicity screening in vitro [37, 38]. Multi-tiered in vitro screening is needed to provide better predictions of organ specific and species-specific toxicity in animal studies [39]. In general, moderate cytotoxicity was observed for mitragynine in all three cell lines. Hence, its potential to cause toxicity in humans cannot be excluded. In addition, the low elimination rate of mitragynine as reported previously could further enhance the risk of toxicity by drug accumulation of repeated doses [40].

In vivo animal toxicity studies demonstrated that a standardized methanol extract of M. speciosa caused severe hepatotoxicity and mild nephrotoxicity at doses higher than 1000 mg/kg [24]. A lethal dose of MSAE of approximately 200 mg/kg has been reported in rats and mice, respectively [41, 42]. In a 40 years cohort study in humans, the toxicity of mitragynine is claimed to be relatively low among the chronic users [43]. However, more recent incidents of seizures and deaths have been observed which were presumably related to mitragynine overdose and have been associated with drug-drug interactions with O-desmethyltramadol, alcohol, and propylhexedrine [44-46].

CONCLUSION

This study evaluated the ADME and the cytotoxic properties of MSAE and mitragynine using in vitro high throughput approaches. MSAE and mitragynine have high aqueous solubility at pH 4.0 but relatively low solubility at
pH 7.4. Poor passive diffusion across the phospholipid bilayer membrane was observed at pH 4.0 and the reverse at pH 7.4. Furthermore, mitragynine showed high plasma protein binding and high metabolic stability in rat liver microsomes. In a cytotoxicity study, mitragynine demonstrated moderate to high time- and dose-dependent cytotoxicity against both normal and cancerous liver cells. The cytotoxic level of mitragynine is higher compared to its cytotoxicity against both normal and cancerous liver cells. Although mitragynine is potentiality useful as an opioid substitute, the balance between drug efficacy and potential for abuse should be taken into consideration.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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