**Supplementary information**

**Metabolite profiling and identification of enzymes responsible for the metabolism of mitragynine, the major alkaloid of *Mitragyna speciosa* (kratom)**

**Liquid chromatography-mass spectrometry (LC-MS) Analysis**

**Metabolite profiling:** LC-MS analysis for metabolite profiling was carried out using an Water Acquity I class ultraperformance liquid chromatography (UPLC) systems (Waters, Milford, USA) equipped with binary pump manager, sample manager, column oven and degasser with Acquity photodiode detector (PDA) and QDa (single quadrupole mass detector) detectors to collect the UV data of the samples. Chromatographic separations were performed on Acquity UPLC CSH C18 (100×2.1 mm; 1.7 µ, Waters, Milford, USA) column maintained at 45OC temperature. The mobile phase consisted of solvent A (5 mM ammonium acetate with 1% acetic acid in water) and solvent B (acetonitrile) delivered at a flow rate of 0.3 ml/min with a linear gradient as follows: 15% B till 2 min, 15% to 40% B till 10 min, 40% to 90% B from 10 to 12 min, maintained at 90% B till 13 min, decreased to 15% B from 13 to 14 min and maintained at 15% B till 15 min. The detection wavelength used was 280 nm. The data was acquired using Waters MassLynx software.

The high resolution mass spectrometry data was collected using an Agilent 1290 Infinity series UPLC systems (Agilent Technologies, Santa Clara, USA) equipped with a binary pump, a thermostatted column compartment, an autosampler, and a degasser connected to Agilent 6540 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, USA) equipped with quadrupole-time-of-flight (Q-TOF) mass spectrometer and a JetStream electrospray ion (ESI) source. The data was acquired with MassHunter B.05 software (Agilent Technologies). The chromatographic conditions used were same as mentioned above. The optimized operating parameters in the positive ion mode were as follows: nebulizing gas (N2) flow rate, 13.0 L/min; nebulizing gas temperature, 280 °C; Jet Stream gas flow, 12 L/min; sheath gas temperature, 300 °C; nebulizer, 40 psi; capillary, 2000 V; skimmer, 45 V; Oct RFV, 750 V; and fragmentor voltage, 120 V. The data was acquired in full scan mode from 100 to 1000 Da and MS/MS mode. The collision energy for Auto MS/MS analysis was 30 V.

**Reaction phenotyping:** The reaction phenotyping sample analysis was performed using a Waters Acquity UPLC system coupled with Waters Xevo TQ-S micro mass spectrometer (Waters, Milford, USA) using MassLynx V4.1 software (Waters, Milford, USA). Chromatographic separations were performed on a UPLC column Acquity, CSH, C18, 1.7μm, 2.1×50 mm; maintained at 40°C temperature. The mobile phase consisted of solvent A (5 mM ammonium acetate with 1% acetic acid in water) and solvent B (acetonitrile) delivered at a flow rate of 0.3 ml/min with a linear gradient as follows: 15% B till 1 min, 15% to 40% B over 5 min, 40% to 90% B from 5 to 6 min, maintained at 90% B till 6.5 min, decreased 15% B from 6.5 to 7 min and maintained at 15% B till 7.5 min. The detection wavelength used was 280 nm. The data was acquired using MassLynx software. The MS was operated in the positive electrospray mode at a capillary temperature 450°C, source temperature 150°C, capillary voltage 0.5 kV, desolvation gas flow 900 L/hr and cone gas flow was 50 L/hr; MS/MS analysis was performed using nitrogen as the collision gas and the collision energy was set from 30 V. The analysis was conducted in multiple reaction monitoring mode (MRM), the details of the same are shown in table S1.

Table S1. MRM details for LC-MS/MS analysis of mitragynine and its metabolites

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Analyte | Q1 (m/z) | Q3 (m/z) | Cone Voltage (V) | Collision energy (V) | Dwell time (s) |
| Mitragynine | 399.19 | 174.09 | 66 | 30 | 0.020 |
| Met2 | 415.19 | 190.09 | 40 | 30 | 0.020 |
| Met4 | 415.19 | 190.09 | 40 | 30 | 0.020 |
| Met5 | 385.21 | 160.07 | 40 | 30 | 0.020 |
| Met6 | 415.19 | 190.09 | 40 | 30 | 0.020 |
| Met7 | 415.19 | 190.09 | 40 | 30 | 0.020 |
| Met8 | 385.21 | 174.09 | 40 | 30 | 0.020 |
| Met11 | 415.19 | 190.09 | 40 | 30 | 0.020 |

Figure S1. Comparison of overlain UV chromatogram (top panel) and MS/MS spectra of Met2 and synthetic standard of 7-hydroxymitragynine

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Figure S2. Collision induced dissociation MS/MS fragmentation spectra of monooxidative metabolites (Met2,4,6,7 and 11) of mitragynine

Figure S3. Collision induced dissociation MS/MS fragmentation spectra of *O*-demethylated metabolite (Met5) of mitragynine



Figure S4. Collision induced dissociation MS/MS fragmentation spectra of *O*-demethylated/hydrolyzed metabolite (Met8) of mitragynine

Figure S5. Collision induced dissociation MS/MS fragmentation spectra of hydration+monooxidative metabolite (Met3) of mitragynine



Figure S6. Collision induced dissociation MS/MS fragmentation spectra of *O*-demethylated+monooxidative metabolite (Met1) of mitragynine



Figure S7. Collision induced dissociation MS/MS fragmentation spectra of monooxidative+dehydrogenated metabolites (Met9 and Met10)of mitragynine

Figure S8. Collision induced dissociation MS/MS fragmentation spectra of dehydrogenated metabolite (Met12)of mitragynine



Figure S9. Collision induced dissociation MS/MS fragmentation spectra of glutathione conjugated metabolite (Met13) of mitragynine

