EVALUATION OF *Mitragyna speciosa* KORTH. (KETUM) EXTRACTS PREPARED BY ACCELERATED SOLVENT EXTRACTION TECHNIQUE, ON ANTINOCICEPTIVE AND CYTOTOXICITY EFFECTS

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

$AlCl_3.6H_2O$	Aluminium chloride hexahydrate
ANOVA	One-way analysis of variance
ASE	Accelerated solvent extraction
ATCC	American type culture collection
br-dd	Broad doublet-of-doublet
br-s	Broad singlet
br-t	Broad triplet
cm	Centimetre
$CDCl_3$	Deuterated chloroform
CO_2	Carbon dioxide
DAD	Diode array detector
dd	Doublet-of-doublet
DE	Dry extract
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EI	Electron impact
EIMS	Electron-impact mass spectrometry
EMEM	Eagle's minimum essential medium
ESI	Electrospray ionisation
EtOH	Ethanol
eV	Electron in Volt
FBS	Fetal bovine serum
FC	Folin-Ciocalteu

FDA	Food and Drug Administration
g	Gram
GAE	Gallic acid equivalent
Galp	Galanin-like peptide
GC-MS	Gas chromatography-mass spectrometry
Glcp	Glucopyranosyl
HEK-293	Human embryonic kidney cells
HPLC	High performance liquid chromatography
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
ICH	International conference on harmonisation
IITC	Animal monitoring equipment software
J	Coupling constant in Hertz
kg	Kilogram
L	Litre
LC-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
m	Metre
m	Multiplet
M^+	Molecular ion
MAE	Microwave-assisted extraction
MCL-5	Mantle cell lymphoma-5
MeOH	Methanol

MEP	Methylerythritol 4-phosphate
mg	Microgram
MHz	Megahertz
min	Minute
mL	Milli Litre
mm	Millimetre
mm Hg	Millimetre of mercury
MS	Mass spectroscopy
MS/MS	Tandem mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
m/z	Mass-to-charge ratio
Na_2CO_3	Sodium carbonate
$NaNO_2$	Sodium nitrate
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
nm	Nanometre
PBS	Phosphate-buffered saline
pH	Potential of hydrogen
p.o.	Oral administration
QE	Quercetin equivalent
QTOF	Quadrupole Time-of-Flight
Rhap	Rhamnopyranosyl
RP	Reverse phase
rpm	Rotation-per-minute
RSD	Relative standard deviation

s	Singlet
s.c.	Subcutaneous injection
SD	Standard deviation
SEM	Standard error of the mean
SFE	Supercritical fluid extraction
td	Triplet-of-doublet
TFC	Total flavonoid contents
TIC	Total ion chromatogram
TPC	Total phenolic contents
UAE	Ultrasound-assisted extraction
UHPLC	Ultra-high performance liquid chromatography
v	Volume
W	Watt
μ-opioid receptors	Mu opioid receptors
δ-opioid receptors	Delta opioid receptors
κ-opioid receptors	Kappa opioid receptors
β-arrestin	Beta arrestin
^{13}C	Carbon-13
^{1}H	Proton
δ_H	^{1}H-NMR chemical shift in ppm
δ_C	^{13}C-NMR chemical shift in ppm
H α	Alpha hydrogen
H β	Beta hydrogen
$[M + H]^+$	Positive-charged molecular ion
℃	Degree Celsius

%	Percentage
µ	Micro
µM	Micro molar

LIST OF APPENDICES

LIST OF PUBLICATIONS

Goh, Y. S., Karunakaran, T., Murugaiyah, V., Santhanam, R., Abu Bakar, M. H., and Ramanathan, S., 2021. Accelerated Solvent Extractions (ASE) of *Mitragyna speciosa* Korth (Kratom) Leaves: Evaluation of Its Cytotoxicity and Antinociceptive Activity. *Molecules, 26*(12), 3704.

PENILAIAN EKSTRAK *Mitragyna speciosa* KORTH. (KETUM) YANG DISEDIAKAN DENGAN TEKNIK PENGESTRAKAN PELARUT TERPECUT KE ATAS KESAN ANTINOSISEPTIF DAN SITOTOKSIK

ABSTRAK

Daun *Mitragyna speciosa* Korth (Ketum) sangat terkenal dengan kesan psikoaktif dan analgesiknya. Mitraginin merupakan sebatian alkaloid utama dalam daun ketum yang menyumbang besar kepada aktiviti farmakologi ketum tersebut. Kajian ini menekankan penggunaan pelarut dan teknik pengekstrakan yang mesra alam untuk menghasilkan ekstrak ketum yang lebih baik dan selamat. Pada mulanya, ketum diekstrak dengan air tulen menggunakan teknik pengekstrakan konvensional, Soxhlet, dan maserasi, serta teknik pengekstrakan moden iaitu pengekstrakan ultrasonik berbantu (UAE), dan teknik pengekstrakan pelarut terpecut (ASE). Kemudian, dua teknik pengekstrakan mesra alam iaitu ASE, dan UAE telah dipilih. Pelarut seperti air, metanol, etanol dan etil asetat digunakan dalam pengoptimuman ASE dan UAE. Perbandingan antara kedua-dua teknik pengekstrakan menunjukkan hasil kering ASE lebih tinggi (0.53 – 2.91 g) dengan kandungan mitraginin yang konsisten (6.53 – 7.19 %), dan memerlukan masa yang lebih pendek (5 minit), serta penggunaan pelarut yang sedikit (<100 mL). Ekstrak ASE etanol mempunyai jumlah kandungan fenolik (407.83 ± 2.50 GAE mg/g) dan flavonoid (194.00 ± 5.00 QE mg/g) yang tinggi jika dibandingkan dengan ekstrak organik dan air ASE dan UAE yang lain. Berkenaan dengan sitotoksisiti, kecuali ekstrak daun ASE EA, semua ekstrak daun ASE didapati tidak sitotoksik (IC_{50} > 500 µg/mL) dibandingkan dengan ekstrak-ekstrak daun UAE (IC_{50} < 200 µg/mL). Semua ekstrak ASE telah diuji kesan analgesiknya ke atas mencit. Apabila diuji melalui model jentik ekor dan plat panas,

ekstrak daun ketum ASE etanol (200 mg/kg) dapat menahan kesakitan jauh lebih baik daripada ekstrak-ekstrak yang lain. Analisis UHPLC-ESI-QTOF-MS/MS telah mengesahkan adanya sebatian fitokimia yang dilaporkan sebelum ini dalam semua ekstrak ketum ASE yang terutama terdiri daripada alkaloid indol jenis korinanti dan terbitan kuersetin seperti mitraginin (*m/z* 399.2278) dan diastereomernya, spesioginin (*m/z* 399.2302), spesiosilatin (*m/z* 399.2283) dan juga kuersetin (*m/z* 303.0509), dan terbitan kuersetin seperti rutin (*m/z* 611.1621). Ekstrak daun ketum ASE etanol boleh dijadikan sebagai pilihan ekstrak yang lebih baik, selamat, dan jimat berbanding dengan ekstrak metanol dalam penyelidikan ketum. Ekstrak daun ASE etanol bertahan hingga 120 minit manakala ASE metanol hanya bertahan hingga 90 minit dalam ujian jentik ekor. Sejajar dengan prinsip pengekstrakan yang mesra alam, ekstrak daun ketum ASE etanol boleh dipertimbangkan untuk pengembangan selanjut sebagai alternatif kepada ekstrak daun ketum metanol untuk penilaian praklinikal dan klinikal di masa depan.

EVALUATION OF *Mitragyna speciosa* KORTH. (KETUM) EXTRACTS PREPARED BY ACCELERATED SOLVENT EXTRACTION TECHNIQUE, ON ANTINOCICEPTIVE AND CYTOTOXICITY EFFECTS

ABSTRACT

Mitragyna speciosa Korth. (Ketum) leaves are well known for their psychoactive and analgesic properties. Mitragynine, is the principal alkaloid of ketum leaf, is a well-known factor that contributes to ketum's pharmacological activities. The present study underlined the use of green solvent and green extraction technique for the yield of a better and safe ketum extract. At first, ketum was extracted with pure water using conventional extractions, Soxhlet, and maceration, as well as modern extraction techniques, ultrasound-assisted extraction (UAE), and accelerated solvent extraction (ASE) techniques. Subsequently, two green extraction techniques which are ASE, and UAE were selected. Solvents such as water, methanol, ethanol, and ethyl acetate were used in the optimisation of ASE and UAE. Comparing both extractions, ASE demonstrated a better dry yield (0.53 – 2.91 g) with consistent mitragynine content (6.53 – 7.19 %), shorter time (5 minutes), and reduced solvent usage (< 100 mL). ASE ethanol extract had both substantial total phenolic (407.83 ± 2.50 GAE mg/g) and flavonoid (194.00 ± 5.00 QE mg/g) contents when compared to other organic and water extracts of ASE and UAE. In regard to cytotoxicity, except for ASE EA leaf extract, all ASE leaf extracts were found non-cytotoxic (IC_{50} > 500 µg/mL) in comparison with UAE leaf extracts (IC_{50} < 200 µg/mL). All the ASE leaf extracts were tested for their analgesic effects in mice. When tested through the tail-flick and hot plate models, ASE ethanol ketum leaf extract (200 mg/kg) could alleviate the pain much better than the other extracts. UHPLC-ESI-QTOF-MS/MS analysis has affirmed

the presence of the previously reported phytochemicals in all ASE ketum leaf extracts which mainly consist of corynanthe-type indole alkaloids and quercetin derivatives such as mitragynine (*m/z* 399.2278) and its diastereomers, speciogynine (*m/z* 399.2302), speciociliatine (*m/z* 399.2283) as well as quercetin (*m/z* 303.0509), and its derivative rutin (*m/z* 611.1621). ASE ethanol ketum leaf extract offers a better, safe, and cost-effective choice compared to methanol extract for ketum studies. ASE ethanol leaf extract persist up to 120 minutes whereas ASE methanol only persisted to 90 minutes in tail-flick test. In line with the green extraction principle, ASE ethanol ketum leaf extract can be considered for further development as an alternative to methanolic ketum leaf extract for future preclinical and clinical evaluations.

CHAPTER 1

INTRODUCTION

1.1 General introduction

In recent years, there has been increasing demand for complementary and alternative medicines, especially herbal medicinal products, for their health and economic value. Herbal medicinal products remain the popular choice in developing countries for their low cost, ready availability, and cultural and surrounding influences (Khan and Ahmad, 2019). Moreover, herbal medicinal products are cost-effective and have fewer side effects on the human body as compared to synthetic medicine (Nishith and Venkatesh, 2019). WHO reported that 80 % of the health care are relying upon the traditional medicine practice and medicinal plants usage (Nishith and Venkatesh, 2019). Historically, medicinal plants have been utilised and consumed since ancient times in enhancing prosperity and well-being (Abdel-Azim et al., 2011; Khan and Ahmad, 2019). In Malaysia, traditional Malay, Chinese and Indian medicinal plants are widely used among the local people (Zaki et al., 2019). Medicinal plants used for pain relief include *Curcuma longa* (Turmeric), *Papaver somniferum* (Opium poppy), *Petasites hybridus* (Butterbur), *Cannabis sativa* (Marijuana), *Hypericum perforatum* (Saint John's Wort) and *Rosa damascena* (Damask rose) (Sun et al., 2018; Rajapakse et al., 2019). Although some of these plants have been reported for their effective analgesia, adverse effects such as severe nausea, vomiting, and respiratory depression may occur (Soergel et al., 2014).

Ancient use of medicinal plants is widely studied with research and development carried out for their synthesis into botanical drugs. The U.S. Food and Drug Administration (FDA) defines botanical drugs as natural products, contain plant substances, algae, microfungi or combinations of the mentioned items (Ahn, 2017). There are many active constituents present in these botanical mixtures. The characterisation of these constituents and their effects must be clearly studied to foster the field of organic and analytical chemistry, as well as biological and pharmacological evaluation in their drug discovery and development. This is important to assure the safety, quality, and consistency of the product (Wu et al., 2020). The quality and preparations of a product need to be controlled throughout its development. Safety studies such as toxicology and preclinical trials are needed to validate the safety information of the botanical product (Nishith and Venkatesh, 2019; Wu et al., 2020). A few guidelines and assessments were stressed by the WHO and FDA including the testing of quality, stability, safety, and efficacy of botanical products (Kumari and Kotecha, 2016; Wu et al., 2020). The scientific data obtained from the toxicology studies, clinical, standardisation and certification can ensure the effectiveness and safety of developed botanical drugs.

1.2 Problem statements

In recent years, the reported toxicity cases which related to the usage of *M. speciosa* (ketum) have been increasing (Nelsen et al., 2010; Karinen et al., 2014; Henningfield et al., 2019). Eventhough toxicity cases and fatalities were reported on the usage of ketum products, but no direct inferences were made regarding to the toxicity of its extracts and alkaloids (Tay et al., 2016). To date, the available toxicity

reports on the adverse effects of ketum related to the type of phytochemicals and its content are still limited. Some preclinical studies on ketum's toxicity were focused on mitragynine and MeOH extract. For example, mitragynine showed LD_{50} of 477 mg/kg in a single dose on male Swiss albino mice and exhibited hepatic and neurotoxicity on Sprague Dawley rats at 100 mg/kg over 28 days (Sabetghadam et al., 2013). Furthermore, a recent study by Reanmongkol and fellow co-researchers (2017) had shown that the ketum alkaloidal extract exhibited higher toxicity than that of the MeOH extract, with LD_{50} of 173.20 mg/kg and 4900 mg/kg, respectively in the tested rodents. Type of phytochemicals content and solvent extraction techniques play a vital role in determining the bioactivity and toxicity of a botanical extract (Choi and Verpoorte, 2014). Naturally occurring alkaloids and its higher content in botanical extracts are known for its contribution on toxicity effect and numerous reports can be found supporting the claim (Kumar and Kumar Jain, 2016; Heinrich et al., 2017). The toxicity effect of the ketum leaf extracts and alkaloidal content needs to be studied in detailed as higher alkaloidal content especially mitragynine in ketum extracts might have contributed towards its toxicological properties (Kumar and Kumar Jain, 2016). Thus, to produce a non-toxic and antinociceptive active botanical extracts of ketum, a suitable green solvent extraction technique is needed (Sabetghadam et al., 2013).

Numerous preclinical studies strongly support the uses of ketum leaf extracts as herbal analgesics. Generally, methanol and chloroform were used to extract ketum leaf. Nevertheless, methanol and chloroform are toxic solvents and not recommended to be used in herbal extractions by US Environmental Protection Agency (EPA) (Mustafa et al., 2012; Mohamed, 2015). The previous studies reported on the types of organic solvent extractions performed on ketum leaf using ultrasound-assisted

extraction (UAE), Soxhlet and conventional maceration techniques (Harizal et al., 2010; Utar et al., 2011; Orio et al., 2012; Haris et al., 2013 Parthasarathy et al., 2013). Besides, in most cases, the past extraction techniques conducted on ketum leaf are time-consuming, high-energy consumption and some even did not apply the principles of green chemistry such as the usage of green solvents like ethanol. Hence, an efficient green chemistry-based extraction of ketum leaf with low solvent usage and short time consumption should be implemented to provide a yield of a safe and potent ketum extract with substantial antinociceptive effect.

1.3 Objectives

In the advent of green chemistry principles, the use of green solvents and analytical techniques are preferred to yield safer and beneficial plant extracts for the future development of herbal based cocktails or drugs. These green chemistry principles include the use of green solvents such as water, ethanol, and ethyl acetate instead of toxic methanol, chloroform, or acetonitrile solvents. Considering this, the present study has objectively evaluated the effects of different solvent extractions on extraction yield, total phenolic content, total flavonoid content, cytotoxicity, and antinociceptive activity of ketum leaf extracts. These were achieved via the following objectives:

1. To extract ketum leaf using different solvent extraction techniques.
2. To isolate and characterise mitragynine from ketum leaf extracts using spectroscopic techniques such as MS and NMR, in which it will be used as a chemical marker.

3. To quantify the mitragynine content in each ketum leaf extracts using HPLC-DAD analysis.

4. To evaluate TPC, TFC and cytotoxicity of ketum leaf extracts obtained from the optimised UAE and ASE methods.

5. To profile tentatively the prensence of the chemical constituents in ASE ketum leaf extracts using UHPLC-ESI-QTOF-MS/MS analysis.

6. To test ASE ketum leaf extracts for its antinociceptive activity using Swiss albino mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Botany of *Mitragyna speciosa* (ketum)

M. speciosa Korth (ketum) is a medicinal plant under the coffee tree (Rubiaceae) family. It is indigenous to Southeast Asia, more specifically in Malaysia and Thailand. *Mitragyna* is named by the Dutch botanist Korthals, for the shape of the flower's stigmas, which take after a bishop's mitre (Cinosi et al., 2015). The common name of *M. speciosa* is *ketum* or *biak-biak* in Malaysia and *ketum*, *kakuam*, *ithang* or *thom* in Thailand (Gong et al., 2012; Hassan et al., 2013; Suhaimi et al., 2016). Classification of ketum is shown in Table 2.1.

Table 2.1: Classification of ketum

Kingdom	: Plantae
Phylum	: Tracheophytes
Class	: Magnoliopsida
Order	: Gentianales
Family	: Rubiaceae
Genus	: *Mitragyna*
Species	: *M. speciosa*

M. speciosa belongs to the genus *Mitragyna*. Other species of *Mitragyna* include *M. diversifolia, M. hirsuta, M. inermis, M. ledermannii, M. parvifolia, M. rotundifolia, M. rubrostipulata, M. stipulosa* and *M. tubulosa.* For suitable growth conditions must be wet and humid with enough sun exposure (Hassan et al., 2013). The distributed area of *Mitragyna* consist of tropical countries such as Southeast Asia, Africa, Sri Lanka, India, Ceylon, Bangladesh and China (Brown et al., 2017).

Ketum is a tropical evergreen tree with an average height of 50 feet, width up to 15 feet, with a variety of sizes (Chittrakarn et al., 2012; Brown et al., 2017). The tree has straight and branching stems with leaves. The leaves of ketum are dark green, oval or acuminate shape with tapered ends as shown in Figure 2.1. The leaves are usually 180 mm long and 100 mm wide (Hassan et al., 2013). Properties of the leaves depend on their vein colour which can be either green, white or red. Red veined leaves are believed to have stronger psychoactive effects (Chittrakarn et al., 2012; Hassan et al., 2013). The tree has many tiny and globular clusters of deep yellow flowers attached to the leaf axils on long stalks. The flower has a unique mace-like shape, and its stigma resembles a bishop's mitre as shown in Figure 2.2. Each flower can bear up to 120 florets. The fruit capsule contains many small, flat and winged seeds (Hassan et al., 2013; Cinosi et al., 2015).

Figure 2.1: Dry leaves of ketum.

Figure 2.2: Flower of ketum (Wogg, 2000).

2.1.1 **Ethnobotany of ketum**

Ketum is traditionally used in Southeast Asia for its medicinal, cultural, and recreational purposes. Earliest reports of ketum usage dates to 1836 when it was used as an opium substitute in Malaysia (Hassan et al., 2013). Locals would usually consume the leaves by chewing, drinking, or smoking them to relieve fatigue or opioid withdrawal (Ahmad and Aziz, 2012; Brown et al., 2017; Singh et al, 2020). It has also been used in local folklore medicine to treat diseases such as fever, malaria, diarrhoea, cough, and expulsing worms (Watanabe et al., 1997; Vicknasingam et al., 2010; Hassan et al., 2013; Halpenny, 2017).

Psychoactive effects of ketum are dose-dependent as it has cocaine-like stimulant effects at small doses but has morphine-like sedative and narcotic effects at large doses that can be abused as an opium substitute (Ilmie et al., 2015). Ketum is often used because it is milder compared to opioid drug and has fewer side effects. However, usage can induce symptoms such as skin discolouration, constipation, weight loss, irritability, rhinorrhoea, insomnia, lachrymation, and lethargy (Halpenny,

2017). Currently in Malaysia, the use of this plant has been restricted under the Poison Act because of its narcotic effects and abuse potential (Gong et al., 2012; Hassan et al., 2013; Suhaimi et al., 2016).

2.2 Phytochemical review of ketum

2.2.1 Phytochemistry of ketum

Previous phytochemical studies in ketum have reported the presence of secondary metabolites such as alkaloids, phenolics, triterpenoids, saponins, and glycosides (Raffa, 2015).

Seventy-nine alkaloids have been found in ketum (Brown et al., 2017) with total alkaloid content ranging from 0.5 to 1.5 % depending on its region of growth and plant maturity (Hassan et al., 2013). Corynanthe-type indole alkaloid, mitragynine **(1),** is the major alkaloid found in ketum leaf making up 66 % of its total constituents in the Thailand species and 12 % for the Malaysian species (Hassan et al., 2013; Brown et al., 2017). Figure 2.3 and 2.4 shows the chemical structure of indole alkaloids and oxindole alkaloids reported in the ketum plant (Raffa, 2015).

Furthermore, the presence of phenolic groups has been reported, especially flavonol groups in the ketum leaf, shown in Figure 2.5 (Raffa, 2015). Flavonoids such as apigenin **(43)** and apigenin 7-glycosides **(44** & **45)** have been elucidated from ketum leaf. Flavonol derivatives including quercetin **(46)**, quercetin glycosides such as quercitrin **(47)**, rutin (**48**), isoquercitrin **(49)**, hyperoside **(50)**, quercetin-3-galactoside-7-rhamnoside **(51)**, kaempferol **(52)**, kaempferol 3-glucoside derivative **(53)**, and

epicatechin **(54)** were reported in ketum leaf (Raffa, 2015). Table 2.2 displays the types of alkaloids and phenolics discovered in ketum leaf.

Table 2.2: Alkaloids and phenolics reported in ketum plant.

Phytochemicals	Type	Plants part	Compounds	Previous report
Alkaloid	Indole	Leaf	Mitragynine **(1)**	Raffa, 2015; Brown et al., 2017
		Leaf	7-hydroxymitragynine **(2)**	Raffa, 2015; Brown et al., 2017
		Leaf	Speciogynine **(3)**	Raffa, 2015; Brown et al., 2017
		Leaf	Speciociliatine **(4)**	Raffa, 2015; Brown et al., 2017
		Leaf	Mitraciliatine **(5)**	Raffa, 2015; Brown et al., 2017
		Leaf	Corynantheidine **(6)**	Raffa, 2015
		Leaf	3-isocorynantheidine **(7)**	Raffa, 2015; Brown et al., 2017
		Leaf	Paynantheine **(8)**	Raffa, 2015; Brown et al., 2017
		Leaf	3-isopaynantheine **(9)**	Raffa, 2015; Brown et al., 2017
		Leaf	3-dehydromitragynine **(10)**	Raffa, 2015; Brown et al., 2017
		Leaf	3,4,5,6-tetrahydromitragynine **(11)**	Raffa, 2015; Brown et al., 2017
		Leaf	Mitrasulgynine **(12)**	Raffa, 2015; Brown et al., 2017
		Leaf	Mitragynaline **(13)**	Raffa, 2015; Brown et al., 2017

Table 2.2 (cont.): Alkaloids and phenolics reported in ketum plant.

Phytochemicals	Type	Plants part	Compounds	Previous report
Alkaloid	Indole	Leaf	Corynantheidaline **(14)**	Raffa, 2015; Brown et al., 2017
		Leaf	Corynantheidalinic acid **(15)**	Raffa, 2015; Brown et al., 2017
		Leaf	Mitragynalinic acid **(16)**	Raffa, 2015; Brown et al., 2017
		Leaf	Mitralactonal **(17)**	Raffa, 2015; Brown et al., 2017
		Leaf	Mitralactonine **(18)**	Raffa, 2015
		Leaf	9-methoxymitralactonine **(19)**	Raffa, 2015; Brown et al., 2017
		Fruit	7-hydroxyspeciociliatine **(20)**	Raffa, 2015
	Oxindole	Leaf	Mitraphylline (**21**)	Raffa, 2015; Brown et al., 2017
		Leaf	Isomitraphylline (**22**)	Raffa, 2015; Brown et al., 2017
		Leaf	Speciophylline (**23**)	Raffa, 2015
		Leaves	Speciofoline (**24**)	Raffa, 2015
		Leaf	Isospeciofoline (**25**)	Raffa, 2015; Brown et al., 2017
		Leaf	Mitrafoline (**26**)	Raffa, 2015
		Leaf	Isomitrafoline (**27**)	Raffa, 2015
		Leaf	Rotundifoleine (**28**)	Raffa, 2015
		Leaf	Isorotundifoleine (**29**)	Raffa, 2015; Brown et al., 2017

Table 2.2 (cont.): Alkaloids and phenolics reported in ketum plant.

Phytochemicals	Type	Plants part	Compounds	Previous report
Alkaloid	Oxindole	Leaf	Ciliaphylline (**30**)	Raffa, 2015; Brown et al., 2017
		Leaf	Mitragynine oxindole A (**31**)	Raffa, 2015; Brown et al., 2017
		Leaf	Mitragynine oxindole B (**32**)	Raffa, 2015; Brown et al., 2017
		Leaf	Rhynchociline (**33**)	Raffa, 2015; Brown et al., 2017
		Leaf	Specionoxeine (**34**)	Raffa, 2015
		Leaf	Isospecionoxeine (**35**)	Raffa, 2015
		Leaf	Corynoxine (**36**)	Raffa, 2015; Brown et al., 2017
		Leaf	Corynoxine B (**37**)	Raffa, 2015; Brown et al., 2017
		Leaf	Rhynchophylline (**38**)	Raffa, 2015; Brown et al., 2017
		Leaf	Isorhynchophylline (**39**)	Raffa, 2015
		Leaf	Corynoxeine (**40**)	Raffa, 2015
		Leaf	Isocorynoxeine (**41**)	Raffa, 2015
		Leaf	Ajmalicine (**42**)	Raffa, 2015; Brown et al., 2017
Phenolic	Flavonoid	Leaf	Apigenin (**43**)	Raffa, 2015
		Leaf	Apigenin 7-glycoside (**44**) & (**45**)	Raffa, 2015
		Leaf	Quercetin (**46**)	Raffa, 2015
		Leaf	Quercitrin (**47**)	Raffa, 2015

Table 2.2 (cont.): Alkaloids and phenolics reported in ketum plant.

Phytochemicals	Type	Plants part	Compounds	Previous report
Phenolic	Flavonoid	Leaf	Rutin (**48**)	Raffa, 2015
		Leaf	Isoquercitrin (**49**)	Raffa, 2015
		Leaf	Hyperoside (**50**)	Raffa, 2015
		Leaf	Quercetin-3-galactoside-7-rhamnoside (**51**)	Raffa, 2015
		Leaf	Kaempferol (**52**)	Raffa, 2015
		Leaf	Kaempferol 3-glucoside (**53**)	Raffa, 2015
		Leaf	Epicatechin (**54**)	Raffa, 2015

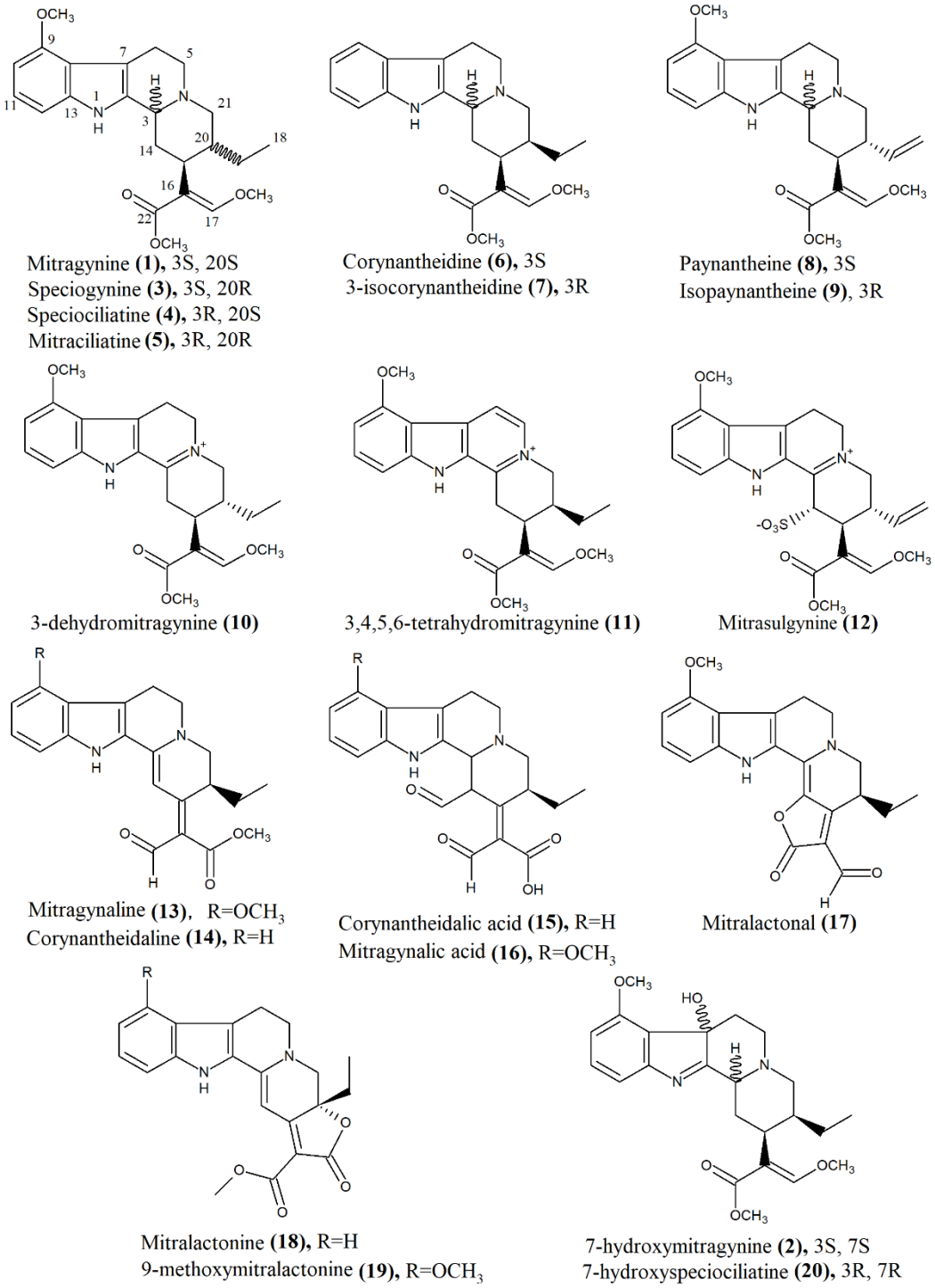
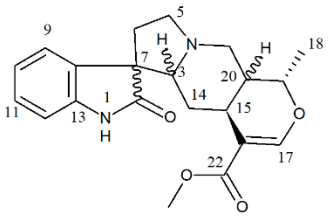
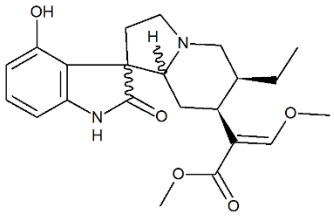


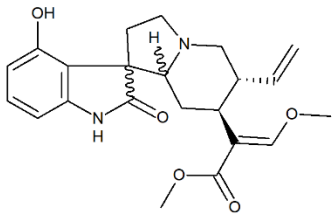
Figure 2.3: Chemical structures of indole alkaloids in ketum.



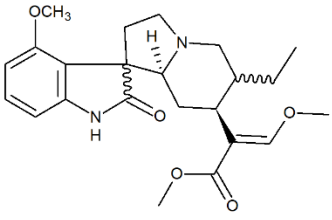
Mitraphylline (**21**), 3S, 7R, 20R
Isomitraphylline (**22**), 3S, 7S, 20R
Speciophylline (**23**), 3R, 7S, 20S



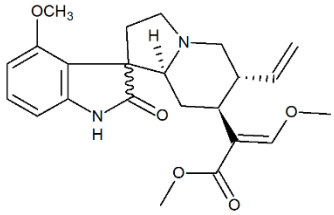
Speciofoline (**24**), 3R, 7R
Isospeciofoline (**25**), 3R, 7S
Mitrafoline (**26**), 3S, 7S
Isomitrafoline (**27**), 3S, 7R



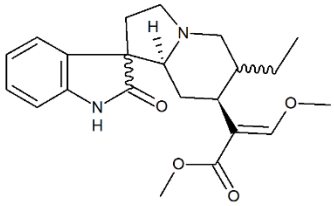
Rotundifoleine (**28**), 3S, 7R
Isorotundifoleine (**29**), 3S, 7S



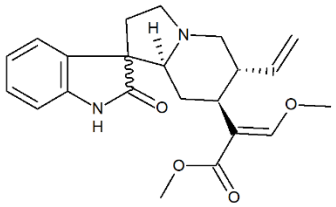
Ciliaphylline (**30**), 7R, 20R
Mitragynine oxindole A (**31**), 7S, 20S
Mitragynine oxindole B (**32**), 7R, 20S
Rynchociline (**33**), 7S, 20R



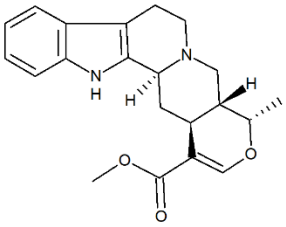
Specionoxeine (**34**), 7R
Isospecionoxeine (**35**), 7S



Corynoxine (**36**), 7S, 20S
Corynoxine B (**37**), 7R, 20S
Rynchophylline (**38**), 7R, 20R
Isorynchophylline (**39**), 7S, 20R

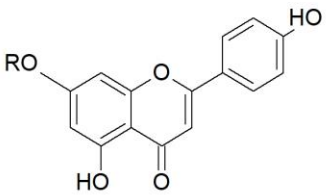


Corynoxeine (**40**), 7R
Isocorynoxeine (**41**), 7S

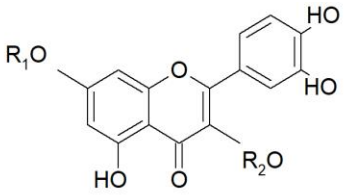


Ajmalicine (**42**)

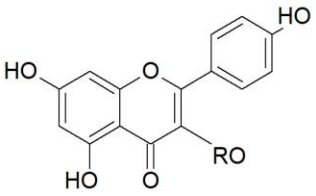
Figure 2.4: Chemical structures of oxindole alkaloids in ketum.



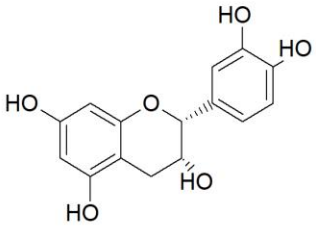
Apigenin **(43)**, R=H
Apigenin 7-glycoside **(44)**, R=ß-D-Glcp
Apigenin 7-glycoside **(45)**, R=ß-D-Rhap



Quercetin **(46)**, R_1=R_2=H
Quercitrin **(47)**, R_1=H , R_2=ß-D-Rhap
Rutin **(48)**, R_1=H, R_2=α-L-Rhap (1→6)ß-D-Glcp
Isoquercitrin **(49)**, R_1= H, R_2=ß-D-Glcp
Hyperoside **(50)**, R_1=H, R_2=ß-D-Galp
Quercetin-3-galactoside-7-rhamnoside **(51)**,
R_1=ß-D-Galp, R_2=ß-D-Rhap



Kaempferol **(52)**, R=H
Kaempferol 3-glycoside **(53)**, R=ß-D-Glcp



Epicatechin **(54)**

Figure 2.5: Chemical structures of flavonoids in ketum.

2.2.2 Chemistry of mitragynine

Mitragynine is a corynanthe-type indole alkaloid found in the ketum leaf. Mitragynine is also known as 9-methoxy-corynantheidine, with a chemical formula of $C_{23}H_{30}N_2O_4$. Mitragynine is insoluble in water but soluble in organic solvents such as acetone, acetic acid, alcohols, chloroform, and diethyl ether. Mitragynine evaporates at 235 °C at 5 mm Hg and melts at 104 °C to form a white, amorphous crystal (Hassan et al., 2013).

Mitragynine was first isolated and identified by Hooper in 1907. The structure of the 9-methoxylated corynanthe skeleton was first determined by Zacharias in 1965 using X-ray crystallography and further confirmed by Liu in 2010 using a computational study (Raffa, 2015). Its chemical structure is similar to yohimbine and

Uncaria alkaloids (Matsumoto et al., 2004). It has a methoxy group at position C-19 and an open E ring with a substitution at position C-9, or oxindoles with a closed E ring with no substitution at position C-9 (Takayama et al., 2002; Hassan et al., 2013).

Mitragynine is produced through the biosynthesis of tryptamine and secologanin using tryptophan and loganin as precursors to form strictosidine. Strictosidine then undergoes a few intramolecular reactions to become mitragynine (Raffa, 2015). The biosynthesis pathway of mitragynine is shown in Figure 2.6.

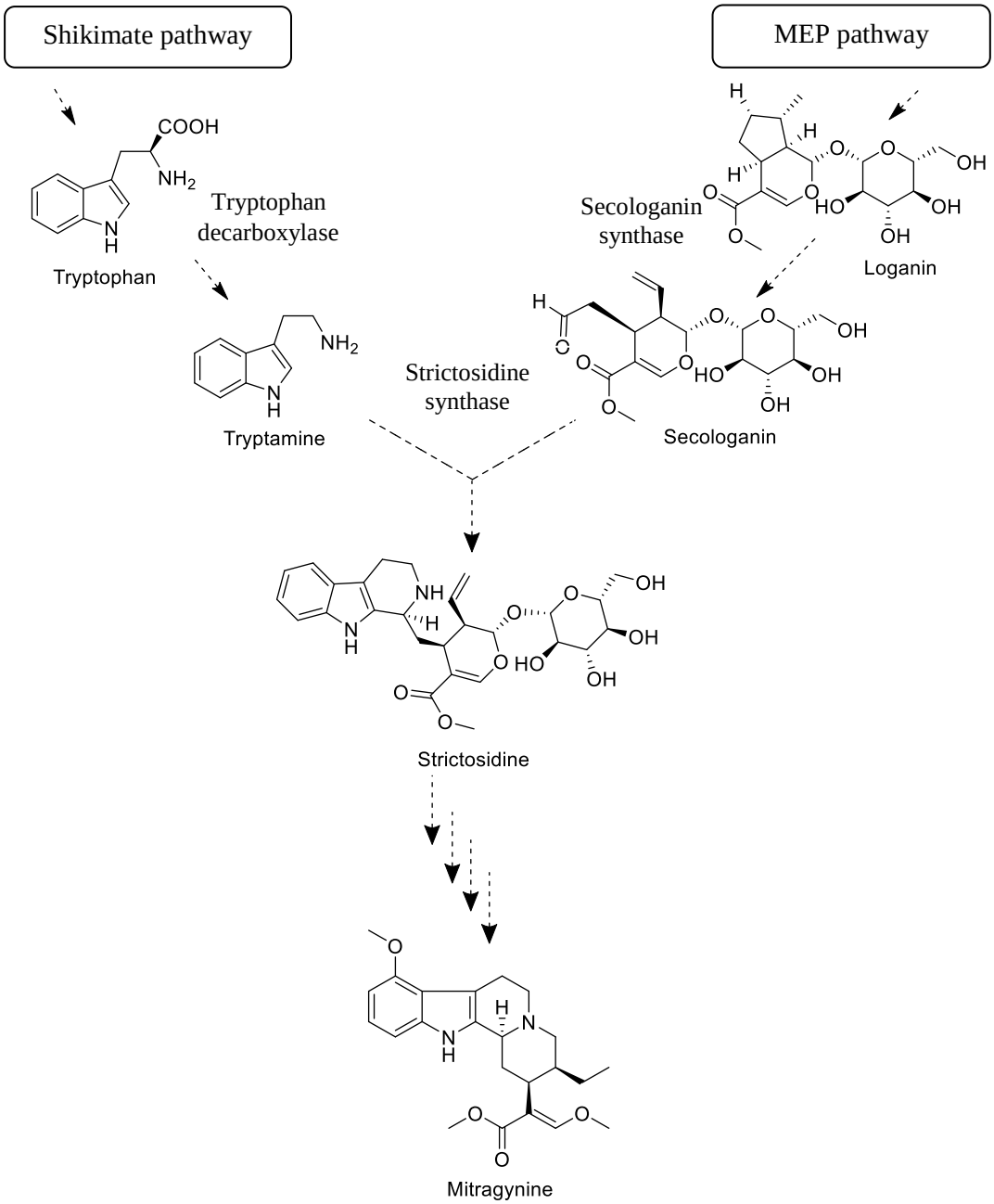
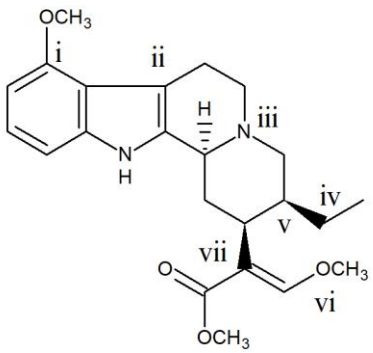


Figure 2.6: Biosynthesis pathway of mitragynine

Structural-activity relationship (SAR) of mitragynine was first studied by Adkins et al. (2011). SAR is important in determining opioid binding affinity with the opioid binding receptors, which include benzene residue, phenolic hydroxyl, and tertiary hydrogen. The presence of substituents on C-7 and C-9, alternations on the β-methoxyacrylate group and modifications on the nitrogen lone pair are factors for SAR (Takayama, 2004; Taufik et al., 2010). Even though 7-hydroxymitragynine has high similarity in structure with mitragynine, 7-hydroxymitragynine has shown 46-fold and 13-fold higher potency affinity towards opioid receptors as compared to mitragynine and morphine, respectively (Matsumoto et al., 2004; Adkins et al., 2011; Raffa, 2015). This property is due to the addition of a hydroxyl group at position C-7 on 7-hydroxymitragynine. Additionally, 7-hydroxymitragynine has higher polarity and lipophilicity than mitragynine. However, this makes 7-hydroxymitragynine more difficult to cross the blood-brain barrier as compared to mitragynine (Takayama, 2004; Matsumoto et al., 2006; Raffa, 2015), though more research must be carried out to study the potency of 7-hydroxymitragynine in this regard (Hassan et al., 2013).



i. Alkyl ester chain enhances opiod activity
   Presence of CH_3 enhances activity
   Presence of OH group reduces its activity
ii. Presence of OH group enhances activity
iii. N-oxidation enhances activity
iv. Double bond reduces activity
v. R-configuration increases activity
vi. NH_3 replacement increases activity
vii. Ester reduction decreases activity

Figure 2.7: SAR of mitragynine by Adkins et al. (2011).

2.3 Ultra-High-Performance Liquid Chromatography coupled with Electrospray Ionisation Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-ESI-QTOF-MS/MS) analysis technique.

The botanical drug is a substance or mixture that consist of active compounds with desirable medicinal properties. The botanical drug is subjected for consumption in their complex mixture form for its biological and pharmacological effects. Identification of the constituents present in the drug using advanced spectroscopic methods need to be carried out for safety purposes and pharmaceuticals properties (Xu et al., 2013).

UHPLC-ESI-QTOF-MS/MS applies an ionisation technique that uses a beam of high-energy electron to bombard a vapourised sample into ions which later being evaluated by a mass spectrometer. The spectrum obtained from the fragmented ions is useful for the structural characterisation of a compound (Dass, 2007). UHPLC-ESI-QTOF-MS/MS technique can be used for qualitative and quantitative analysis. It can also be used to identify unknown compounds, to determine the isotopic composition of elements in a molecule, and the structure of a compound by observing its fragmentation, and to quantify the amount of a compound in a sample (Cappiello and Palma, 2018). In terms of high sensitivity and selectivity, UHPLC-QTOF/MS provides fast resolving power and high-resolution performance (Xiao et al., 2012). The schematic diagram of UHPLC-ESI-QTOF-MS/MS was shown in Figure 2.8.

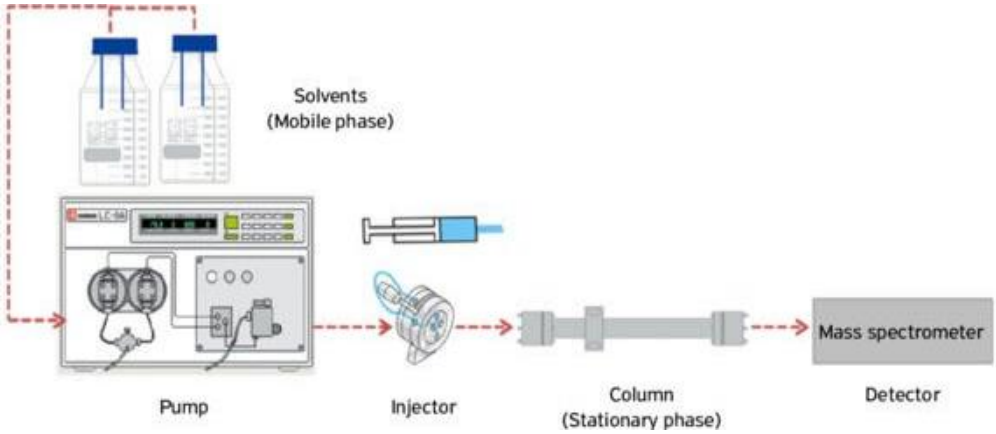


Figure 2.8: Schematic diagram of UHPLC-ESI-QTOF-MS/MS (Torre et al., 2015). The instrument consists of a solvent system, a pump, an injector, a column, and ESI and mass spectrometer.

2.3.1 Ketum extract characterisation using LC-MS/MS analysis

Phytochemicals in ketum leaf extract have been characterised tentatively using different types of LC-MS methods including UHPLC-QTOF-MS/MS, LC-ESI-TOF-MS, and UPLC-QTOF-MS. The bioactive constituents identified include indole alkaloids and oxindole alkaloids were reported by previous literature (Avula et al., 2015; Raffa, 2015; Veeramohan et al., 2018; Sharma et al., 2019). Table 2.3 shows the types of LC-MS analysis used in metabolite profiling of phytochemicals that present in the ketum leaf extracts and fractions.

Table 2.3: LC-MS analysis used in phytochemical profiling of ketum extracts and fractions.

No.	Phytochemical compound	Elemental composition	$[M+H]^+$	Types of LC/MS	Ref.
1	Mitragynine	$C_{23}H_{30}N_2O_4$	399.2276	UHPLC-QTOF-MS/MS	Avula et al., 2015
			399.2680	LC-ESI-TOF-MS	Veeramohan et al., 2018
			399.2500	UPLC−QTOF	Sharma et al., 2019
2	Speciogynine	$C_{23}H_{30}N_2O_4$	399.2278	UHPLC-QTOF-MS/MS	Avula et al., 2015
			399.2680	LC-ESI-TOF-MS	Veeramohan et al., 2018
			399.2500	UPLC−QTOF	Sharma et al., 2019
3	Speciociliatine	$C_{23}H_{30}N_2O_4$	399.2278	UHPLC-QTOF-MS/MS	Avula et al., 2015
			399.2680	LC-ESI-TOF-MS	Veeramohan et al., 2018
			399.2500	UPLC−QTOF	Sharma et al., 2019
4	Paynantheine	$C_{23}H_{28}N_2O_4$	397.2124	UHPLC-QTOF-MS/MS	Avula et al., 2015
			397.2110	LC-ESI-TOF-MS	Veeramohan et al., 2018
			397.1600	UPLC−QTOF	Sharma et al., 2019
5	3-isopaynantheine	C23H28N2O4	397.2123	UHPLC-QTOF-MS/MS	Avula et al., 2015
			397.2110	LC-ESI-TOF-MS	Veeramohan et al., 2018
6	Corynantheidine	$C_{22}H_{28}N_2O_3$	369.1600	UHPLC-QTOF-MS/MS	Avula et al., 2015
			369.1600	LC-ESI-TOF-MS	Veeramohan et al., 2018
			369.1600	UPLC−QTOF	Sharma et al., 2019
7	Isocorynantheidine	$C_{22}H_{28}N_2O_3$	369.1600	UPLC−QTOF	Sharma et al., 2019
8	7-hydroxymitragynine	$C_{23}H_{30}N_2O_5$	415.2217	UHPLC-QTOF-MS/MS	Avula et al., 2015
			415.2210	LC-ESI-TOF-MS	Veeramohan et al., 2018
			415.1900	UPLC−QTOF	Sharma et al., 2019

Table 2.3 (cont.): LC-MS analysis used in phytochemical profiling of ketum extracts and fractions.

No.	Phytochemical compound	Elemental composition	$[M+H]^+$	Types of LC/MS	Ref.
9	7β-hydroxy-7H-mitraciliatine	$C_{23}H_{30}N_2O_5$	415.2224	UHPLC-QTOF-MS/MS	Avula et al., 2015
			415.2210	LC-ESI-TOF-MS	Veeramohan et al., 2018
			415.2210	UPLC−QTOF	Sharma et al., 2019
10	Corynoxine	$C_{22}H_{28}N_2O_4$	385.2116	UHPLC-QTOF-MS/MS	Avula et al., 2015
			385.2100	LC-ESI-TOF-MS	Veeramohan et al., 2018
			385.1900	UPLC−QTOF	Sharma et al., 2019
11	Corynoxine B	$C_{22}H_{28}N_2O_4$	385.2119	UHPLC-QTOF-MS/MS	Avula et al., 2015
			385.2100	LC-ESI-TOF-MS	Veeramohan et al., 2018
			385.1900	UPLC−QTOF	Sharma et al., 2019
12	Isospeciofoline	$C_{22}H_{28}N_2O_5$	401.2066	UHPLC-QTOF-MS/MS	Avula et al., 2015
			401.2050	LC-ESI-TOF-MS	Veeramohan et al., 2018
13	Isospeciofoleine	$C_{22}H_{28}N_2O_5$	399.1913	UHPLC-QTOF-MS/MS	Avula et al., 2015
14	Isorotundifoline	$C_{22}H_{28}N_2O_5$	401.2066	UHPLC-QTOF-MS/MS	Avula et al., 2015
			401.2050	LC-ESI-TOF-MS	Veeramohan et al., 2018
15	Mitragynaline	$C_{21}H_{20}N_2O_5$	380.3330	UHPLC-QTOF-MS/MS	Avula et al., 2015
16	Corynantheidaline	$C_{20}H_{18}N_2O_4$	350.0600	UHPLC-QTOF-MS/MS	Avula et al., 2015
17	Caulerpin	$C_{24}H_{18}N_2O_4$	399.1260	LC-ESI-TOF-MS	Veeramohan et al., 2018
18	Yohimbine	$C_{21}H_{26}N_2O_3$	355.1990	LC-ESI-TOF-MS	Veeramohan et al., 2018
19	Mitraphylline	$C_{21}H_{24}N_2O_4$	369.2200	UPLC−QTOF	Sharma et al., 2019