PHYTOCHEMICAL, ANALGESIC AND ACUTE TOXICITY STUDIES OF *UNCARIA ATTENUATA* (KORTH.) LEAF EXTRACT

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
Aβ	Amyloid beta
β	Beta
δ	Delta
ε	Molar absorptivity
μ	Mu
κ	Kappa
λ_{max}	Wavelength
°C	Degree Celsius
%	Percent
>	Greater than
<	Less than
1D	One dimension
2D	Two dimension
5-HT1A receptor	Serotonin 1A receptor
A	Absorbance
ANOVA	Analysis of variance
AD	Alzheimer's disease
ALT	Alanine transaminase
b.w.	Body weight
c	Concentration
^{13}C	Carbon
$CDCl_3$	Deuterated chloroform

$CHCl_3$	Chloroform
CH_3COOH	Acetic acid
CD_3OD	Deuterated chloroform
cm	Centimeter
cm^{-1}	1 per centimeter
COSY	Homonuclear correlation spectroscopy
COVID-19	Coronavirus disease 2019
DOP	delta-opioid receptor
DEPT	Distortionless enhancement by polarization transfer
E	*trans* configuration
EBD	Emotional and behavioural disorder
ESI	Electron-sprayed ionization
et al.	Elsewhere or/and other
EtOAc	Ethyl acetate
FTIR	Fourier Transform Infrared
g	Gram
g/kg	gram per kilogram
GC-MS	Gas chromatography-mass spectrometry
h	Hour
^1H	Proton
HMBC	Heteronuclear multiple bond correlation
HR-MS	High resolution mass spectra
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
i.e.	that is

i.p.	Intraperitoneal
IU/L	International unit per liter
J	Coupling constant
KBr	Potassium bromide
kHz	Kilohertz
KOP	kappa-opioid receptor
l	Length
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Lethal dose 50
m	Meter
MeOH	Methanol
mg	Milligram
mg/mL	milligram per milliliter
mg/kg	milligram per kilogram
MHz	Megahertz
min	Minute
mL	Milliliter
mL/kg	milliliter per kilogram
mm	Millimeter
mol dm^{-3}	Molarity
morphine HCl	Morphine hydrochloride
M. speciosa	*Mitragyna speciosa*
MOP	mu-opioid receptor
m/z	mass to charge ratio
n	Number of proton

naloxone HCl	Naloxone hydrochloride
NH_4OH	Ammonium hydroxide
nm	Nanometer
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
OECD	Organisation for Economic Co-operation and Development
p	Probability
p.o	per os
ppm	Parts per million
QToF-MS	Quadrupole time-of-flight mass spectrometry
R_f	Retention factor
RBD–ACE-2	Angiotensin-converting enzyme receptor 2 domain
ROW	Relative organ weight
s	Second
SEM	Standard error of the mean
SD	Sprague Dawley
sp.	species
spp.	species (pluralis)
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
Tween-80	Polyoxyethylene sorbitan monooleate
US	United States
UV-Vis	Ultraviolet visible
U. acida	*Uncaria acida*
U. africana	*Uncaria africana*

U. attenuata	*Uncaria attenuata*
U. bernaysii	*Uncaria bernaysii*
U. borneensis	*Uncaria borneensis*
U. callophylla	*Uncaria callophylla*
U. cordata	*Uncaria cordata*
U. elliptica	*Uncaria elliptica*
U. gambir	*Uncaria gambir*
U. guianensis	*Uncaria guianensis*
U. hirsuta	*Uncaria hirsuta*
U. homomalla	*Uncaria homomalla*
U. kunstleri	*Uncaria kunstleri*
U. laevigata	*Uncaria laevigata*
U. lancifolia	*Uncaria lancifolia*
U. lanosa	*Uncaria lanosa*
U. longiflora	*Uncaria longiflora*
U. macrophylla	*Uncaria macrophylla*
U. nervosa	*Uncaria nervosa*
U. orientalis	*Uncaria orientalis*
U. perrottetii	*Uncaria perrottetii*
U. rhynchophylla	*Uncaria rhynchophylla*
U. scandens	*Uncaria scandens*
U. sessilifructus	*Uncaria sessilifructus*
U. sinensis	*Uncaria sinensis*
U. sterrophylla	*Uncaria sterrophylla*
U. talbotii	*Uncaria talbotii*

U. tomentosa	*Uncaria tomentosa*
U. veluntina	*Uncaria veluntina*
U. villosa	*Uncaria villosa*
µg/mL	microgram per milliliter
µm	micrometer
µL	microliter
v/v	volume by volume
W	Watt

LIST OF APPENDICES

KAJIAN FITOKIMIA, ANALGESIK DAN KETOKSIKAN AKUT EKSTRAK DAUN *UNCARIA ATTENUATA* (KORTH.)

ABSTRAK

Spesies *Uncaria* daripada keluarga Rubiaceae ialah sejenis tumbuh-tumbuhan menjalar yang terdapat di Asia Tenggara, Asia Timur dan Amerika Selatan. Bahagian daun dan dahan (yang berbentuk seperti cangkuk atau cakar) tumbuhan *Uncaria* secara tradisinya digunakan untuk merawat nyeri, gangguan saraf, darah tinggi, strok, reumatik dan lain-lain. Kajian ini bertujuan untuk mengekstrak, memencil dan mencirikan sebatian alkaloid dari daun *Uncaria attenuata* (Korth.). Dalam kajian ini, ujian ketoksikan akut telah dinilai selama 14 hari dengan menggunakan tikus betina Sprague Dawley. Untuk menilai aktiviti analgesik ekstrak *U. attenuata* dan alkaloid terpencil, eksperimen plat panas dengan menggunakan tikus telah dijalankan. Teknik kromatografi seperti kromatografi lajur, kromatografi lapisan nipis dan kromatografi lapisan nipis penyediaan telah digunakan untuk pemencilan dan penulenan alkaloid. Penentuan struktur kimia alkaloid telah dijalankan dengan 1D NMR (^{1}H, ^{13}C, DEPT 90, DEPT 135), 2D NMR (COSY, HMBC, HSQC dan NOESY), UV-Vis, FTIR, GC-MS dan LC-MS. Kajian ketoksikan akut (175, 550 and 2000 mg/kg) telah diberi secara oral berpandukan peraturan OECD 425. Keabnormalan fizikal, jangkitan, perbezaan postur dan pergerakan dan kematian tikus-tikus telah dinilai. Berat organ relatif tikus telah dikira. Seterusnya, untuk kajian analgesik, ekstrak metanol (250, 500 mg/kg), ekstrak alkaloid (50, 100 mg/kg) dan villocarine A (5, 10 dan 20 mg/kg) telah diberi secara oral. Masa (saat) telah direkod sama ada tikus menunjukkan tindak balas menjilat atau melompat. Nalokson hidroklorida (2 mg/kg i.p.) telah digunakan untuk

mengkaji sifat antagonis aktiviti analgesik. Dalam kajian ini, sebatian pertama yang telah dipencilkan ialah alkaloid indol yang telah diketahui iaitu villocarine A, manakala sebatian kedua adalah alkaloid oksindol yang merupakan sebatian baru dan dikenalpasti sebagai isovillocarine D. LD_{50} kajian ketoksikan akut ekstrak metanol daun dan ekstrak alkaloid masing-masing ditentukan pada 2000 mg/kg dan 1190 mg/kg. Tikus yang telah diberi ekstrak alkaloid (2000 mg/kg) menunjukkan tindak balas ketoksikan akut. Ekstrak alkaloid (100 mg/kg) dan villocarine A (20 mg/kg) masing-masing menunjukkan pengurangan sensasi nyeri yang ketara apabila dibandingkan dengan tikus kawalan (tempoh latensi: 100 mg/kg (15-75 minit); 20 mg/kg (30-90 minit)). Aktiviti analgesik ekstrak alkaloid dan villocarine A telah berkurangan dengan ketara dalam tikus pra-rawatan dengan antagonis opioid tak selektif (nalokson hidroklorida) (2 mg/kg i.p.). Hasil penyelidikan ini mencadangkan *U. attenuata* sebagai agen analgesik yang berpotensi dan aktiviti analgesiknya mungkin disebabkan oleh interaksinya dengan reseptor opioid yang terdapat di sistem saraf pusat.

PHYTOCHEMICAL, ANALGESIC AND ACUTE TOXICITY STUDIES OF *UNCARIA ATTENUATA* (KORTH.) LEAF EXTRACT

ABSTRACT

Uncaria species from the family of Rubiaceae is a type of climbing vine found across Southeast Asia, East Asia, and South America. The *Uncaria* leaves and hooks (claw-like) traditionally been used to treat pain, neurological disorders, hypertension, stroke, rheumatism and other ailments. The study aimed to extract, isolate, and characterize the alkaloid constituents from the leaves of *Uncaria attenuata* (Korth.). In the present study, female Sprague Dawley rats were evaluated for acute toxicity for 14 days. In order to evaluate the antinociceptive activity of *U. attenuata* extracts and isolated alkaloid, a rat hot plate model was carried out. Two alkaloids have been successfully isolated and purified using chromatographic techniques: column chromatography, thin-layer chromatography and preparative thin-layer chromatography. Structure elucidation was performed using 1D NMR (^{1}H, ^{13}C, DEPT 90, DEPT 135), 2D NMR (COSY, HMBC, HSQC and NOESY), UV-Vis, FTIR, GC-MS and LC-MS. For the acute toxicity study, the rats were fed orally and employed in series of 175, 550 and 2000 mg/kg following the OECD 425 guideline. The rats were evaluated for physical abnormalities, infection, differences in posture and movement, and mortality. The relative organ weight of the rats' harvested organs was calculated. Subsequently, for the analgesic study, the methanolic extract (250, 500 mg/kg), alkaloid extract (50, 100 mg/kg), and villocarine A (5, 10, 20 mg/kg) were given orally to the rats, respectively. The time (second) was recorded when the rat showed either licking or jumping response. Naloxone hydrochloride (2 mg/kg i.p.) was tested for the

antagonism of analgesic activity. In this research, the first isolated compound was an indole alkaloid, identified and characterized as villocarine A. Interestingly, the second isolated compound is a new oxindole alkaloid identified and characterized for the first time as isovillocarine D. The LD_{50} of the acute toxicity study of the methanolic and alkaloid extracts was 2000 mg/kg and 1190 mg/kg, respectively. The rat given the alkaloid extract (2000 mg/kg) showed an acute toxicity effect. The alkaloid extract (100 mg/kg), and villocarine A (20 mg/kg) showed a considerable antinociceptive effect when compared to the control, respectively (latency period: 100 mg/kg (15-75 min); 20mg/kg (30- 90 min)). The antinociceptive activity of the alkaloid extract and villocarine A was significantly reversed in animals pre-treated with a non-selective opioid antagonist (naloxone hydrochloride). These findings suggested *U. attenuata* as a potential analgesic agent, and the effect is likely mediated by a pathway involving interactions with the central opioid receptors.

CHAPTER 1

INTRODUCTION

1.1 Overview

Pain is a public health problem often related to socioeconomic status, physical and mental stress at work (Goldberg and McGee, 2011). Analgesics remain as the most effective and pertinent option for treating pain. The market-based painkillers used to manage pain are paracetamol, codeine, and morphine. They are prescribed based on the level of illness (Wester et al., 2018). Natural product plays a crucial role as an agent for treating pain disorders. Among them, herbal medicines have gained widespread use owing to their accessibility and availability. Plants are known as rich sources of bioactive secondary metabolites. They possess various medicinal uses with promising effects (Wink, 2015). The bark of the willow tree and the seed of the opium poppy are the two popularly used plants to alleviate pain (McCurdy and Scully, 2005). The World Health Organization has reported that about 70% to 80% of the world's population relies on herbs for their primary health care and Malaysia is not spared either (Tan et al., 2020).

The Rubiaceae family serves as the primary source of plant alkaloids. Among the genera, the Naucleeae tribe *Mitragyna* and *Uncaria* were traditionally used to relieve pain (Veltri and Grundmann, 2019). Indole and oxindole alkaloids were found as the major chemical constituents that are probably implicated in the pharmacological effects of kratom products (Chear et al., 2021). However, less information is known on *Uncaria* sp., particularly its pharmacological effects on pain relief. The genus *Uncaria* belongs to the family of Rubiaceae (Madder) (Qin et al., 2021). The genus

contains 34 species worldwide and is found predominantly in Southeast Asia, East Asia and South America (Ndagijimana et al., 2013). Among the species, *U. gambir*, *U. rhynchophylla*, *U. tomentosa* and *U. sinensis* are the most common plants. *Uncaria* sp. is a climbing vine plant. It consists primarily of alkaloids, flavonoids, triterpenoids and organic acids. Among the chemical constituents, indole alkaloids possess the most desired pharmacological activity (Heitzman et al., 2005; Carvalho Junior et al., 2017). Traditionally, the leaves and hooks of *Uncaria* are used in treating pain, neurological disorders, hypertension, stroke, rheumatism and other ailments (Zhang et al., 2015). In traditional folk medicine, *Uncaria* has been widely used to treat wounds and ulcers, headaches, fevers and gastrointestinal illness (*U. gambir*); hypertension, convulsions, eclampsia, epilepsy and other cerebral diseases (*U. rhynchophylla*); arthritis, cardiac-related problem and inflammatory disease (*U. tomentosa*) (Amir et al., 2012; Elgawish et al., 2019; Yang et al., 2020). A commercial product known as Japan-Yokukansan was produced by incorporating *U. rhynchophylla* as one of the herbal ingredients (Kanno et al., 2014). Moreover, a botanical product (UP3005) consisting of *U. gambir* as one of the ingredients was shown to produce analgesic and anti-inflammatory effects (Yimam et al., 2015). In Malaysia, the phytochemical and pharmacology studies on *Uncaria* sp. are lacking, particularly on *U. attenuata* (Korth). A few alkaloids have been isolated from the species. Nonetheless, no reports on their pharmacological studies, thus far (Phillipson and Hemingway, 1975; Ponglux et al., 1980; Olivar et al., 2018).

1.2 Problem Statement

A literature survey revealed that alkaloids had been isolated from the leaves of *U. attenuata* (Phillipson and Hemingway, 1975; Ponglux et al., 1980). Nevertheless, the pharmacological activity of the compounds remained unknown. Scientific investigations on *U. attenuata* are scarce to date. Analgesics play a crucial role in the medical field, and natural products could offer a traditional approach treating pain disorders (Rauf et al., 2017). In this effort, plants from *Uncaria* were not spared. There is a possibility that this plant may possess kratom-like analgesic activities. *U. attenuata* is native to Malaysia and is widely found in Negeri Sembilan, Sabah and Sarawak. Initial screening of several plants for analgesics activity, *U. attenuata* was found to exhibit the most promising results. However, there is no scientific evidence to support *U. attenuata* pharmacological activity. Indeed, the phytochemical, analgesic, and toxicity properties are still lacking.

1.3 Hypothesis

The *Uncaria* genus is known for its alkaloid content, and this genus is from the family of Rubiaceae, which is similar to kratom. There is the traditional claim of this genus for the treatment of pain, fevers, hypertension, gastrointestinal illness, inflammatory disease, and arthritis. The extract of this plant and isolation of the major compound may possess opioid-like analgesic activity. Owing to the native use of *Uncaria* in traditional medicine, hence the plant is relatively safe.

1.4 Objectives

The present study pursued the following objectives:

1. To extract, isolate and characterize the alkaloid constituents from the leaves of *U. attenuata* using various chromatographic and spectroscopic techniques.
2. To evaluate the acute toxicity of *U. attenuata* leaves extracts using an *in vivo* (rat) model.
3. To evaluate the antinociceptive activity of *U. attenuata* leaves extracts and isolated alkaloid using a rat hot plate model.

CHAPTER 2

LITERATURE REVIEW

2.1 Plants as a source of medicine

Health remains a top priority in life among other challenges. In this regard, modern medicines play a critical role in treating diseases as a result of the Industrial Revolution and the advancement of pharmaceutical chemistry. Nonetheless, the use of traditional remedies in treating illnesses and maintaining health has been widespread since ancient times (Rates, 2001). Traditional medicine is preferred due to the general belief that it poses lesser adverse effects (Wachtel- Galor and Benzie, 2011). Since ancient times, people have relied on remedies made from natural resources to treat illnesses (Yuan et al., 2016). Almost 40% of medicines have originated from natural sources, either directly or indirectly. According to the literature, approximately 25% of medicines are derived from plants, 13% from microorganisms and 3% from animals (Abdel-Rahman, 2017).

Medicinal plant is one of the branches of traditional medicine which is widely applied by traditional healers to enhance human general fitness and health (Šantić et al., 2017). China and India are the leading countries to practice herbal medicine before Columbia, South Africa, the United States and Malaysia (Chen et al., 2016). According to archaeological discoveries, herbal medicine practice has been traced back 6000 years in Iraq and 800 years in China (Pan et al., 2014). Examples of some traditional medicines that have been used are *Gingko biloba* (asthma, cough, intestinal tract worm infections), *Panax ginseng* (increases energy, antioxidant, anti-depression, inhibits or delays the neurodegenerative process, anti-inflammatory) and *Punica granatum*

(antioxidative, antimicrobial, and anticarcinogenic) (Wang et al., 2018; Wang and Zhang, 2019; Hou et al., 2020).

According to the literature, almost all parts of the plant (leaves, hook, stem bark, flower, tuber, fruits, root) contributed to the preparation of different medicinal formulations (Awan et al., 2021; Lim and Lee, 2020). The most common preparation method for herbal remedies is decoction (22.99%), powder (18.39%), infusion poultice (13.87%), and raw material consumption (11.49%). Various routes are used for administration, such as internal use (66.33%), topical application (23.47%), nasal application (10.2%) and others (Ambu et al., 2020; Awan et al., 2021). The healing outcome from traditional medicine is proven since it has been passed down from generation to generation (Ravipati et al., 2014).

Modern science has proven that plants can treat various diseases effectively (Dhama et al., 2018). The effectiveness of medicinal plants is recognized because many of them have entered the official pharmacopoeia and are actively used today (Fitzgerald et al., 2020). Despite the advancement of synthetic drugs, there are still several drugs that only can be derived from plants as the primary raw material, such as artemisinin, paclitaxel, vinblastine, vincristine, podophyllotoxin and camptothecin (Bernath, 1998; Pan et al., 2013). Table 2.1 shows the plant species successfully used in the pharmaceutical industry as commercial drugs. Plants contain bioactive natural substances such as alkaloids, tannins, terpenoids, phenols, and glycosides. They play a vital role in the treatment of human diseases (Akinyemi et al., 2018). This has led to the extensive study of the active constituents of plants, as well as their pharmacological properties, which may pave the way to the discovery of lead molecules (Süntar, 2020).

Table 2.1: Plant species processed by the pharmaceutical industry on a large scale (Source: Bernath, 1998).

Common names	**Scientific name**	**Active agents**	**Pharmacological action**
Red peppers	*Capsicum annuum*	capsaicin	local blood circulation, rheumatism
Hops	*Humulus lupulus*	humulone, lupulone	sedative
Senna	*Cassia senna*	sennoside A sennoside N, rhein	laxative
Milk thistle	*Silybum marianum*	silybin, silymarin	liver protection, antioxidant
Yam	*Dioscorea spp.*	diosgenin	source of steroids
Foxglove	*Digitalis lanata*	lanatoside C, digoxin	heart muscle activity, cardiac arrhythmias
Wormwood	*Artemisia annua*	artemisinin	cerebral malaria
Feverfew	*Chrysanthemum parthenium*	parthenolide	migraine, menstrual disorders
Ginkgo	*Ginkgo biloba*	ginkgolides	cerebral circulation, loss of memory
Thornapple	*Datura stramonium* *Datura metel*	hyoscyamine, atropine, hyoscine	depressant of nerve endings, control of motion sickness
Henbane	*Hyoscyamus niger*	hyoscyamine, hyoscine	sedatives, secretion
Deadly nightshade	*Atropa balladonna*	hyoscyamine, atropine, scopolamine	depressant of nerve endings, control of motion sickness
	Duboisia myoporoides *Duboisia leichhardtii* *Duboisia hopwoodii*	hyoscyamine, hyoscine	sedatives, secretion

Ephedra	*Ephedra sinica* *Ephedra equisetina* *Ephedra gerardiana* *Ephedra intermedia* *Ephedra major*	ephedrine, pseudoephedrine	relief of asthma and fever, anti-inflammatory
Opium poppy	*Papaver somniferum.*	morphine, codeine, narcotine, papaverine	pain relief, hypnotics, allaying coughing, narcotic antagonists
Ergot	*Claviceps purpurea*	ergocristine, ergocornine, ergocryptine, ergometrine	migraine, autonomic nervous system, adrenaline antagonist, action on blood vessels
	Rauwolfia serpentina	reserpine, rescinnamine, ajmaline	hypertension, neuropsychiatric disorders, cardiac arrhythmias
Quinine	*Cinchona succirubra* *Cinchona officinalis* *Cinchona ledgeriana* *Cinchona calisaya*	quinine, quinidine	antimalarial, cardiac arrhythmias, cardiac depressant
Periwinkle	*Catharanthus roseus*	vincristine, vinblastine	Hodkin's disease, nonHodgkin's lymphomas, leukaemia in children
Pacific yew	*Taxus brevifolia*	taxol, baccatin, 10-deacetylbaccatin	ovarian cancers, breast cancers, head and neck cancers
Rue	*Ruta graveolens*	rutin	antihaemorrhagic, emmenagogue, hypotensive
Ipecac	*Cephaëlis ipecacuanha*	emetine, cephaeline, psychotrine	amoebic dysentery, expectorant, antitumor action,

2.1.1 *Uncaria* as natural/alternative medicine and natural dye

Plants from the genus *Uncaria* have been common in tropical countries for a few decades (Keplinger et al., 1998; Almeida et al., 2017). Local populations widely use different plant parts (leaves, hooks, stem) of *Uncaria* to treat epilepsy, seizures, preeclampsia, analgesics, hypertension, inflammation, diabetes, cancer, and certain associated brain diseases and act as sedatives (Zhang et al., 2015). Table 2.2 presents several medicinal properties of different species of *Uncaria* used by indigenous communities. In addition, the curative values of *Uncaria* herbs have been documented in Chinese and Japanese pharmacopoeia (Zhang et al., 2019; Wei et al., 2019).

Table 2.2: Medicinal uses of various *Uncaria* spp. (Source: Ravipati et al., 2014; Zhang et al., 2015).

Uncaria species	Part of plant	Ethnomedicinal use of the plant
U. gambir	Leaf and young twigs	To treat diarrheal disease, sore throat, spongy gums, dysentery, arteriosclerosis, obesity and as astringent
U. guianensis	Inner bark	To treat cancer, arthritis, diabetes, inflammation and intestinal affections
	Root bark Leaf	To treat wounds and abscesses
U. homomalla	Stem and hook	To treat migraine and infantile convulsion
U. hirsuta	Stem and hook	To treat primary hypertension, epilepsy and arthritis
U. macrophylla	Stem and hook	To treat lumbocrural pain, arthritis and osteomyelitis
U. perrottetii	Hook	To treat hematuria, puerperal fever and puerperal sepsis
U. lanosa	Stem and hook	To treat hyperpyrexia, analgesia and spasmolysis

U. tomentosa	Bark	To treat diabetes, cancer, chemotherapy side effects, intestinal affections inflammation, abscesses, arthritis, asthma, contraception, disease prevention fevers, gastric ulcers, hemorrhoid inflammation, menstrual irregularity, recovery from childbirth, rheumatism skin impurities, urinary tract inflammation, weakness and wounds
U. cordata	Hook	To treat diabetes and as antioxidant
U. sinensis	Stem and hook	To treat arthritis, headaches, spasmolytic and hypertension
U. longiflora	Leaf	To treat rheumatism, thrush and framboesia
U. sessilifructus	Stem and hook	To treat hypertension, bellyache, hysteresis, rheumatoid arthritis, hemiplegia, sciatica, injuries from falls, ulcer, lumbocrural pain and headaches
U. africana	Bark	To relieve pain
	Leaf	To treat diarrhoea and dysentery
	Root	To treat depression
U. elliptica	Leaf	To treat inflammation
U. rhynchophylla	Stem and hook	To treat convulsive disorders (epilepsy), hyperpyrexia, preeclampsia and hypertension

U. rhynchophylla is known as "Gou teng", a plant indigenous to China. *U. rhynchophylla* is believed to be effective in treating the central nervous system (Lim and Lee, 2020). A study was conducted to inhibit the aggregation of the *β*-amyloid protein (A*β*) aggregation, which is a consistent pathological signature of Alzheimer's disease (AD) (Fujiwara et al., 2006). *U. rhynchophylla* extract was used in thioflavin T binding assays, atomic force microscopy imaging and electrophoresis. The results

showed that *U. rhynchophylla* successfully inhibited the formation of A*β* fibril and dismantles the pre-formed A*β* fibrils. Therefore, *U. rhynchophylla* may be a potential therapeutic medication for AD patients. It may act as a preventative agent for healthy adults or those who have a mild cognitive impairment. A more detailed study was performed to identify the active compounds responsible for the pharmacological action in the brain distortions. Six alkaloids were isolated from *U. rhynchophylla* extract and two of them (rhynchophylline and isorhynchophylline) were found to be the major active ingredients for AD treatment (Xian et al., 2012).

In 2013, a study was conducted by Tanaka and Sakiyama using the Kampo medicine (Japanese traditional medicine) known as Yokukansan (YK) in the treatment of pediatric emotional and behavioural disorder (EBD). The ingredients of Kampo include *U. rhynchophylla*, *U. sinensis* or *U. macrophylla* hooks as the main constituents along with six other herbs. The case study showed YK might be effective for EBD patients. All patients gave positive feedback after the treatment with no adverse effects observed during the treatment. YK is widely used in Japan as medicine for neurosis, insomnia, behavioural and psychological signs of dementia (Kawakami et al., 2011).

A recent study suggested *U. tomentosa* as a complementary therapy and alternative medication for COVID-19 (coronavirus disease 2019) (Yepes-Pérez et al., 2020). All isolated components of *U. tomentosa* could work synergistically through different mechanisms. Possible mechanisms include binding to the angiotensin-converting enzyme receptor 2 domain (RBD–ACE-2) and to the peak protein binding viral interface. In addition, treatment profiles of bioactive components were assessed in terms of drug resemblance. They showed promising results and suggested that *U. tomentosa* may be a potential candidate used to fight the spread of COVID-19.

U. gambir is the only *Uncaria* species widely utilized in the Indonesian textile industry as a natural dye (Failisnur et al., 2018). A variety of colours ranging from light brown to reddish brown and dark brown was obtained after combining *U. gambir* extract with the selected mordant. The major components of *U. gambir* extract that act as a dye are anhydrous catechins, catechins and pyrocatechol. In addition to being taken as an ingredient for betel nut chewing, *U. gambir* also plays an essential role as astringent medicine, anti-inflammatory medicine, analgesic medicine, antibacterial medicine and prolongation of sexual intercourse (Amir et al., 2012; Saad et al., 2020).

2.2 Analgesics

Analgesics (painkillers) are drugs that can alleviate pain (algesia) (Rauf et al., 2017). Analgesics play a crucial role in the medical field. All diseases need to engage with painkillers when it comes to the term pain. The most frequent reason for an individual seeking medical assistance is pain, which is split into two categories: acute and chronic. Acute pain is a warning signal that reacts to acute disease or injury, whereas chronic pain lasts longer than expected and is not always caused by illness or injury (Grichnik and Ferrante, 1991).

Natural product has been used as a natural remedy in treating pain disorders. Examples of plants used in pain therapy are the bark of the willow tree and opium poppy seed (McCurdy and Scully, 2005). Throughout the years, many natural painkillers have been discovered. The chemistry, pharmacology and mechanism of action of these potent chemical substances have been extensively studied (Basri et al., 2017; Luo et al., 2019).

Morphine, codeine, aspirin and thebaine (Figure 2.1) are among the plant-based analgesics with promising health outcomes (Rauf et al., 2017). Morphine and aspirin

are popularly known analgesics used in managing mild to severe pain (Brook et al., 2017; Cadavid, 2017). Historically, kratom, a plant native to Southeast Asia, has been used to manage pain caused by opioid withdrawal. There are about 40 different alkaloids found in kratom, each of which has a unique affinity and activity at opioid receptors (Gutridge et al., 2020). Mitragynine (Figure 2.2) is a major bioactive indole alkaloid in kratom (Ya et al., 2019). Kratom is believed to have a morphine-like analgesic effect (Raffa et al., 2018). This plant, however, has a market barrier because it is outlawed in Malaysia under Section 30 (3) of the Poisons Act 1952 (Han et al., 2019).

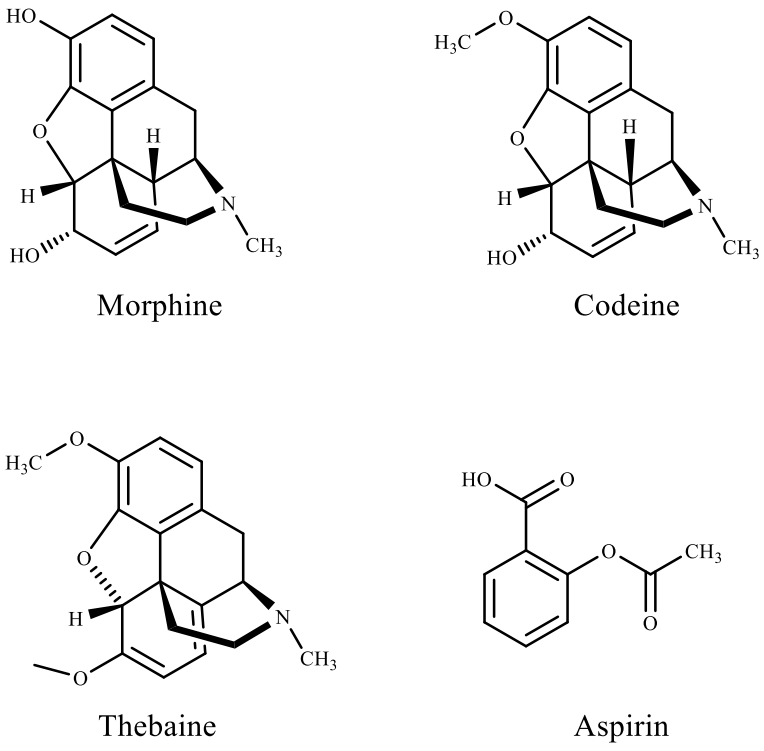


Figure 2.1: Chemical structures of morphine, codeine, aspirin and thebaine.

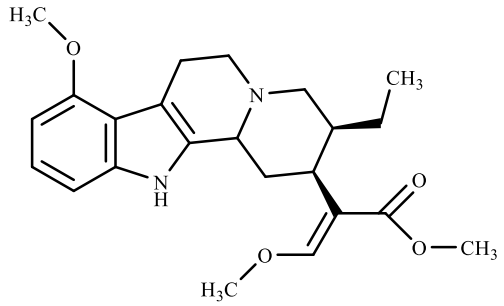


Figure 2.2: Chemical structure of mitragynine.

Heterocyclic compound (with indole group)- C_8H_7N has a bicyclic structure comprising a benzene ring fused with a pyrrole ring group, which is a famous chemical skeleton in the drug molecules (Thanikachalam et al., 2019). Indole derivatives have a wide range of biological effects in pharmaceutical medicine, such as opioid agonist, anti-inflammatory, anticancer and antioxidant (Kumar and Ritika, 2020). This has attracted the interest of researchers in finding new chemical features among the indole derivatives owing to their beneficial properties.

2.2.1 *Uncaria* as analgesics agents

Medicines used to relieve pain or to obtain analgesia are referred to as analgesics or painkillers. As shown in Table 2.1, *Uncaria* plant species are widely used as analgesic agents to treat inflammation, headache, pain, and arthritis. Traditional healers select plants with healing properties and apply them as their medical necessity. A combination of traditional knowledge and scientific advances has allowed the development of a botanical product known as UP3005. The main component of UP3005 comprised of *U. gambir* leaf extract and *Morus alba* root bark extract. This product is aimed to treat osteoarthritis and showed significant results in analgesics and anti-inflammatory activities. The study revealed that carrageenan-induced rats

improved pain resistance while lowering paw edema and ear thickness in mice given extracts at doses ranging from 100 to 400 mg/kg (Yimam et al., 2015).

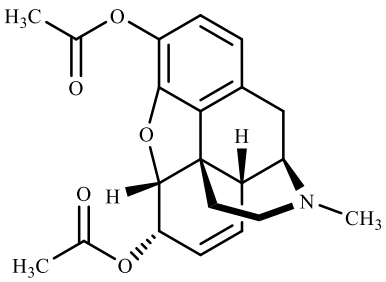
In another study, *U. tomentosa* fraction (95% oxindole alkaloids) was found to have antinociceptive activity (Jűrgensen et al., 2005). The study showed significant results between tested groups in the chemical stimuli of capsaicin, formalin and abdominal writhing tests induced by acetic acid and also caused increasing latencies in the thermal stimuli models (hot plate and tail flick tests). *U. tomentosa* fraction was given to mice via i.p administration at different doses for different indicated activities. The study found that *U. tomentosa* was effective in pain management.

Isorhynchophylline, an alkaloid isolated from *U. rhynchophylla* showed promising antinociceptive effects on tactile allodynia and thermal hyperalgesia (Gao et al., 2020). In mice induced with neuropathic pain, the alkaloid was administered repeatedly at 5, 15, 45 mg/kg (orally) in a dose-dependent manner. According to the findings, isorhynchophylline has the potential to produce sustained antinociception in the presence of chronic neuropathic pain. The study showed significant findings in exploring natural products as novel therapeutic agents, particularly analgesics.

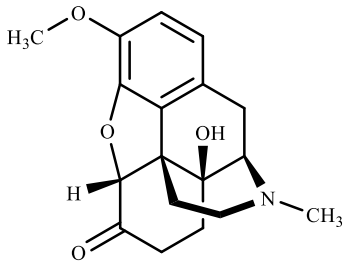
2.2.2 Synthetic analgesics agents

Owing to the discovery of natural active compounds, a variety of semi-synthetic and synthetic analgesic agents are synthesized either by modifying the original analgesic or synthesizing the new drug (Pathan and Williams, 2012). Heroin and oxycodone are semi-synthetic drugs, whereas methadone, fentanyl and propoxyphene are synthetic drugs (Rosenblum et al., 2008) (Figure 2.3). Some of these drugs are well-established to save lives but are often used for illicit purposes. They can be easily purchased on the Internet (“deep web”) and the drug market (Rinaldi et al., 2020). Drug abuse adversely

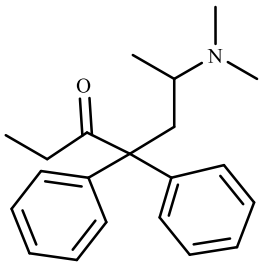
affects society and makes users addicted and intoxicated (Armstrong et al., 2009). The doses of antidote might be higher compared to the traditional analgesics because it has 1) a strong potency compared to morphine; 2) a prolonged effect; 3) a regular combination of other drugs (Pérez-Mañá et al., 2018). Therefore, attention is now being shifted to using natural analgesics as safer substitutes.



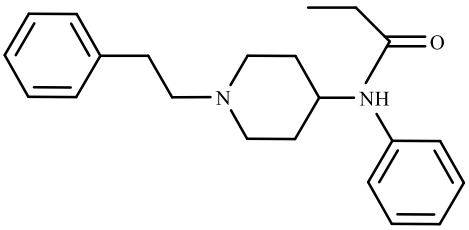
Heroin



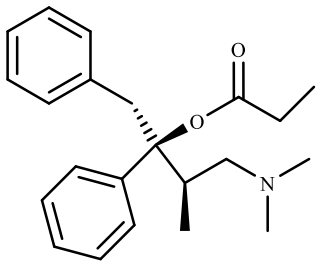
Oxycodone



Methadone



Fentanyl



Propoxyphene

Figure 2.3: Chemical structures of semi-synthetic drugs- heroin and oxycodone; synthetic drugs- methadone, fentanyl and propoxyphene.

2.2.3 Pathophysiology of pain

According to the International Association for the Study of Pain (IASP), pain is defined as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (Malik, 2020). Pain is a signal about tissue damage conveyed by specific receptors and fibre systems that extend from the periphery to the brain (Finnerup et al., 2020). The damage to normal pathways causes immediate consequences, which are loss or reduction of function, including pain (Finnerup et al., 2020).

The perception of pain pathways includes the central and peripheral nervous systems (Khan et al., 2019). The peripheral nervous system consists of nerves and ganglia located outside the brain and spinal cord. Its primary function is to connect the central nervous system to organs and limbs. On the other hand, the central nervous system comprises the spinal cord and the brain. It is responsible for integrating and interpreting the information sent from the peripheral nervous system and coordinating all the activities in bodies before responding to the effector organs (Yam et al., 2018).

The pain pathway involves four major processes: transduction, transmission, modulation, and perception (Christiansen and Cohen, 2018). Transduction is the first step by which tissue-damaging stimuli activate the nerve endings. The process takes place through the conversion of stimulus events to chemical tissue events, followed by chemical tissue and synaptic cleft events to electrical events in the neurons, and lastly, the electrical events in the neurons to chemical events at the synapses. Next, the process is followed by transmission. Transmission refers to the relay functions by transmitting the electrical events along the neuronal pathways, while neurotransmitters in the synaptic cleft transmit information from a post-synaptic terminal of one cell to a pre-synaptic terminal of another. This message is carried from the site of tissue injury

to the brain regions underlying perception. Meanwhile, modulation is the process of altering neural activity along the pain pathway and can suppress or inhibit pain. This process occurs peripherally, within the spinal cord and brain. The final stage is perception where it occurs when the cortexes within the brain receive the nociceptive signal. Perception is the subjective awareness produced by sensory signals; it involves the integration of many sensory messages in which emotional and motor responses are initiated. It produces consciousness to feel pain and displays a complex function of several processes, including attention, expectation, and interpretation (Osterweis et al., 1987; Yam et al., 2018; Lee and Neumeister, 2020).

2.2.4 Opioid receptors

Opioids are analgesic agents widely used in therapeutic practice. Opioids are regulated by opioid receptors, consisting of three common types: the mu-opioid receptor (MOP), the kappa-opioid receptor (KOP) and the delta-opioid receptor (DOP) (McDonald and Lambert, 2005). Agonists, antagonists and partial agonists are all possible effects that opioids can have on these receptors (Martínez and Abalo, 2020).

Agonists bind to the receptor to obtain the best possible response. On the other hand, the antagonists bind to the receptors but do not activate them while simultaneously blocking a binding agonist to that receptor. For partial agonists, it binds to receptors but produces only a partial effect regardless of the quantity of drug administered (Lambert, 2004). Examples of opioids for each type of action are morphine, naloxone and buprenorphine, respectively (Pathan and Williams, 2012).

At present, there is a wide range of analgesics, each with a unique mode of action. They can alleviate pain either through the mechanism of the central nervous system (opioid receptor agonism) or the peripheral nervous system (neuromodulation) (Rauf et al., 2017). MOP can be found in the thalamus, cerebral cortex and periaqueductal gray; KOP is located in the hypothalamus and periaqueductal gray whereas, DOP is located in the basal ganglia (Wang, 2019). Overall, agonists targeting MOP or DOP receptors are pain-relieving and rewarding while agonists targeting KOP are anti-depression and anti-anxiety types (Waldhoer et al., 2004). The most commonly used opioid receptor (morphine, heroin, fentanyl and methadone) in pain management is the MOP system (Trescot et al., 2008; Strain et al., 2022). Studies have been conducted to investigate the rat models of nociception/analgesics employing hot plate, tail-flick, writhing, paw pressure, intradermal formalin, and colonic distension

experiments to get detailed applications of analgesia (Yaksh, 1997; Fan et al., 2014; Scuteri et al., 2020; Deng et al., 2021).

2.3 Acute Toxicity

Toxicity refers to poisonous substances that may cause damage or death (Chaudhary et al., 2019). Recent studies have indicated that although medicinal plants contribute significantly to treat a variety of diseases, many have also shown some adverse effects. (Kharchoufa et al., 2018). There is concern that traditionally used medicinal plants may be toxic. Therefore, determining the toxicological consequences of any herbal extract intended for clinical or pre-clinical use is critical for risk assessment (Porwal et al., 2017).

Early acute toxicological studies can help to determine optimal therapeutic doses. Acute toxicity studies are required to prevent herbal drug overdoses, which would otherwise result in withdrawal from treatments (Bose et al., 2021). Over the years, several species from the *Uncaria* genus have been investigated for their toxicity profile. Previous studies indicated that the LD_{50} for *U. tomentosa* in rats is above 8000 mg/kg, and no lethality was detected in the treatment group (Sheng et al., 2000). In the same study, the acute LD_{50} doses for a commercially available *U. tomentosa* powder and a water/ethanol extract containing 4% alkaloids were determined to be >2000 and >5000 mg/kg, respectively.

Jung et al. (2006) reported the LD_{50} value in acute oral toxicity of the aqueous extract of *U. rhynchophylla* in rats and mice to be more than 2000 mg/kg. Based on the findings, no fatality rates as well as any sign or symptom of toxicity after 14 days of observation. A recent study has shown that an aqueous extract of purified *U. gambir* is relatively non-toxic to the kidneys and liver when given orally to rats at 5-20 mg/kg

for 14 consecutive days (Armenia et al., 2021). The results showed that alanine transaminase (ALT) and liver ratio showed a significant decline (p<0.05). Interestingly, the lower ALT activity is within the standard range of ALT (52-224 IU/L), indicating hepatic protection. Meanwhile, the decreasing liver ratio is due to increasing animal body weight throughout the treatment routine. Therefore, Armenia et al., 2021 concluded that purified *U. gambir* is safe for kidney and liver function.

According to the literature, *U. attenuata's* phytochemical was explored in 1975 and 1980 with the isolation of numerous alkaloids (Phillipson and Hemingway, 1975; Ponglux et al., 1980). However, its toxicological effects have not been studied thus far.

2.4 Rubiaceae family

The Rubiaceae family is one of the largest classes of Magnoliopsida (flowering plant), which ranks fourth among angiosperms for species diversity (Davis et al., 2009). It is found in the tropical and subtropical regions with over 620 genera and 13000 species (Bremer and Eriksson, 2009). Rubioideae, Ixoroideae and Cinchonoideae are the three subfamilies of Rubiaceae (Bremer and Eriksson, 2009). This family's most prominent distinguishable feature is the mix of simple opposing leaves, stipules and an inferior ovary (Verdcourt, 1958; Davis et al., 2009). The species is predominantly woody plants, shrubs and rarely herbs (Mongrand et al., 2005). Iridoids, anthraquinones, triterpenes, indole alkaloids and other alkaloid subclasses are the most prevalent compounds isolated from Rubiaceae species. These compounds were mostly isolated from *Uncaria*, *Hedyotis*, *Psychotria*, *Morinda* and *Ophiorrhiza* (Martins and Nunez, 2015). Plants in the Rubiaceae family have a long history in medical use, exhibiting antioxidant, anti-inflammatory, antihypertensive, antimicrobial, antidiabetic and antimalarial activities (Kala, 2015).

2.4.1 Genus of *Uncaria*

The genus *Uncaria* comprises 34 species. Among them, three are found in Africa and Madagascar; two are in tropical America, and 29 are distributed in Asia and Australia (Liang et al., 2020). The species of *Uncaria* are easy to recognize as they climb the vines with pair of hooks. The presence of circular flower heads and capsule fruits of *Uncaria* species release small and winged seeds (Turner, 2018). Figure 2.4 shows the general characteristics of *Uncaria* species. Many compounds have been identified in this genus, including alkaloids, flavonoids, terpenoids, organic acids and glycosides, of which many compounds are from the *U. tomentosa* (Heitzman et al., 2005; Zhang et al., 2017). Over the past two decades, the development of *Uncaria*'s ethnobotany, phytochemistry, pharmacological, biological activity and biosynthesis has been extensively studied (Laus, 2004; Qin et al., 2021). The extracts and isolated compounds from *Uncaria* were evaluated for their bioactivity following the traditional uses from the native (Ravipati et al., 2014).

Figure 2.4: Part of *Uncaria* sp. a. General habit. A plagiotropic branch showing origin from the orthotropic axis and bearing flowering heads on first and second-order lateral branches stimulating a compound thyrse; b. Flowering head; c. Flower; d. Interflora bracteoles and young corolla; e,f. Details of Interflora bracteoles. (Source: Ridsdale, 1978).

2.5 Alkaloids

2.5.1 Alkaloids from Rubiaceae family

In the early nineteenth century, W. Meisner coined the term alkaloid to describe basic compounds, also known as alkali-like (Jayakumar and Murugan, 2016). Some alkaloids-rich orders that have been reported include Gentiales (Rubiaceae, Loganiaceae, Apocynaceae), Magnoliales (Magnoliaceae, Lauraceae), Papaverales (Fumariaceae, Papaveraceae), Ranunculales (Ranunculaceae, Berberidaceae, Menispermaceae) and Tubiflorae (Solanaceae, Convolvulaceae, Boraginaceae) (Hussein and El-Anssary, 2019).

Alkaloid is known as a nitrogen-containing compound according to the C-N fundamental skeleton (Ungogo et al., 2020). They are classified into several groups, including pyrrole, pyrroline, pyrrolidine, pyrrolizidine, indole, pyridine, pyrimidine, piperidine, quinoline, isoquinoline, quinolizidine, imidazole and tropane (Figure 2.5). Depending on the structural properties, each of them is further divided into various subgroups (Bribi, 2018).

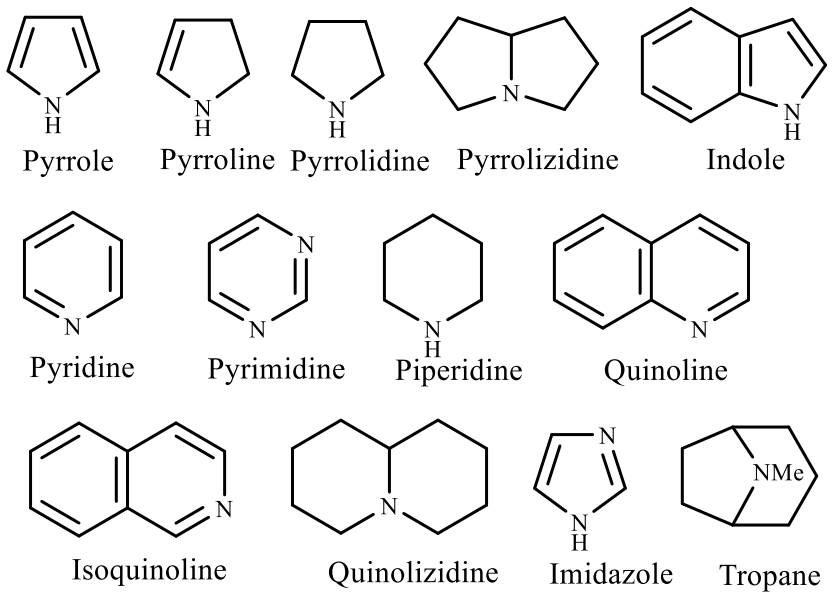


Figure 2.5: Basic heterocyclic structure of skeleton constituting the group of alkaloids.