THE EFFECT OF MITRAGYNINE ON CYCLIC AMP FORMATION AND mRNA EXPRESSION OF MU-OPIOID RECEPTORS MEDIATED BY CHRONIC MORPHINE TREATMENT IN SK-N-SH NEUROBLASTOMA CELL

By

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TABLE OF CONTENTS

CHAPTER TWO: MATERIAL AND METHOD

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

ABBREVIATION	FULL NAME
%	Percentage
±	Plus/minus
°C	Degree Celsius
µg	Microgram
µl	Microliter
>	Less than
>	Greater than
^{13}C-NMR	Carbon NMR
^{1}H-NMR	Proton NMR
ATCC	American Type Cell Culture
C	Carbon
CAD	Charged Aerosol Detection
CL	Chemiluminescence Detection
cm	centimetre
CO_2	Carbon dioxide
COSY	Correlation spectroscopy
DAD	Diode Array Detector
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g	For example
ECD	Electron Capture Detector
ELCD	Evaporative Light Scattering Detectors

et al	Co workers
FBS	Fetal bovine serum
FD	Flat Detector
FID	Flame Ionization Detector
g	gram
GADPH	Glyceraldehyde 3-phosphate-dehydrogenase
h	Hour
H_2O	Water
HMBC	Heteronuclear multiple-bond correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Multiple-Quantum Correlation
IC_{50}	Inhibition concentration caused 50% cell death
IR	Infrared
IS	Internal standard
L	Litre
M	Molar
m/z	Mass-to-charge
MEM	Minimum essential media
MeOH	Methanol
mg	Miligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	millilitre
mM	Milimolar
MS	Mass Spectrometry

n	Number of replicate
NaOH	Sodium hydroxide
NMR	Nuclear Magnetic Resonance
O	Oxygen
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Negative logarithm of H^+ concentration
ppm	Parts per million
r^2	Determination coefficient
R_f	Retention factor
RID	Refractive Index Detector
Rpm	Revolution per minute
s	Second
SD	Standard deviation
SK-N-SH	Human neuroblastoma cell
TLC	Thin layer chromatography
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume

KESAN MITRAGININ TERHADAP PEMBENTUKAN CYCLIC AMP DAN EKSPRESI mRNA OPIOID MU-RESEPTOR DARIPADA RAWATAN KRONIK MORFIN DALAM SEL NEUROBLASTOMA SK-N-SH

ABSTRAK

Mitraginin adalah sebatian indole alkaloid daripada pokok *Mitragyna speciosa* (*M.speciosa*) Korth. (Famili). Di Thailand, tumbuhan ini dikenali sebagai "Kratom" dan di Malaysia yang dikenali sebagai "Biak-Biak" dan "Ketum". *M. speciosa* telah digunakan sebagai terapi penggantian untuk rawatan kronik opioid dalam menguruskan gejala ketagihan di Malaysia dan Thailand. Sehingga kini lebih daripada 40 sebatian telah diasingkan dari daun pokok ini. Mitraginin adalah sebatian yang paling banyak daripada 25 alkaloid dalam daun *M.speciosa* (66% daripada asas mentah) dan mempunyai kesan terhadap opioid seperti morphine.

Pengasingan sebatian bioaktif mitraginin dengan menggunakan kaedah ekstrak asid-bes dan kaedah turus kromatografi telah dibangunkan. Dalam kajian ini, kami menyiasat mekanisme selular rawatan bersama dan rawatan selepas morfin secara kronik dengan mitraginin dalam pengeluaran adenosina monofosfat siklik (cAMP) ransangan oleh forskolin dan ekspresi reseptor mu-opioid (MOR) semasa pembezaan sel-sel manusia neuroblastoma SK-N-SH, mekanisme yang terlibat dalam perubahan tersebut, dan kesan pembezaan morfin dan mitraginin. cAMP adalah molekul yang dikawal oleh G protein-coupled reseptor pengaktifan dan lain-lain proses selular. Pengukuran cAMP dalam sel digunakan secara meluas sebagai penunjuk fungsi reseptor dalam aplikasi penemuan ubat-ubatan. Manakala perubahan dalam bilangan ekspresi MOR sebagai tindak balas kepada rawatan opioid kronik telah lama menjadi spekulasi secara langsung menyumbang kepada penyahpekaan reseptor dan pembangunan toleransi opioid.

Kesitotoksikan mitraginin terhadap sel SK-N-SH telah ditentukan. Mitraginin memperlihatkan kesan kepada kebergantungan dos dalam 50% perencatan percambahan sel pada kepekatan 77.268 μM. Pembangunan rawatan morfin kronik telah dioptimumkan. Tahap cAMP dalam sel-sel telah meningkat dengan ketara selepas 24 jam pengeraman dengan morfin sulfat berbanding dengan kawalan (sel sahaja). Sementara itu ekspresi reseptor mu-opioid menunjukkan kesan penurunan berbanding dengan kawalan. Menariknya mitraginin juga mempunyai kesan yang sama tetapi tahap kesan peningkatan cAMP dan kesan penurunan ekpresi MOR adalah kurang berbanding morfin. Dalam kajian ini rawatan bersama selama 24 jam diantara morfin sulfat dengan mitraginin menunjukan berkurangan pengeluaran cAMP pada kepekatan mitraginin yang lebih rendah berbanding dengan rawatan dengan morfin sulfat sahaja. Begitu juga pra rawatan dengan morfin sulfat selama 6 jam, kemudian disambung dengan rawatan mitraginin selama 6 jam. Sementara itu, pada kepekatan tinggi, mitraginin akan menghasilkan kesan toleransi dan kebergantungan dengan meningkatkan pengeluaran tahap cAMP pada rangsangan foskolin. Kajian dalam ekspresi MOR, rawatan bersama morfin sulfat dengan mitraginin telah pengurangkan kesan mengurangan ekspresi MOR. Kajian *in vitro* ini menunjukkan mitraginin mempunyai potensi untuk mengelakkan toleransi dan kebergantungan rawatan morfin kronik pada kepekatan yang lebih rendah. Walau bagaimanapun ia akan mengakibatkan toleransi dan pergantungan pada kepekatan tinggi.

THE EFFECT OF MITRAGYNINE ON CYCLIC AMP FORMATION AND mRNA EXPRESSION OF MU-OPIOID RECEPTORS MEDIATED BY CHRONIC MORPHINE TREATMENT IN SK-N-SH NEUROBLASTOMA CELL

ABSTRACT

Mitragynine is an indole alkaloid compound of *Mitragyna speciosa* (*M.speciosa)* Korth. (Rubiaceae). In Thailand, this plant is known as "Kratom" and in Malaysia it is known as "Biak-biak" or "Ketum". *M. speciosa* was used as substitution therapy for chronic opioid treatment to manage the withdrawal symptoms in Malaysia and Thailand. To date over 40 compounds have been isolated from the leaves of this plant. Mitragynine is the most abundant of the more than 25 alkaloids of Kratom (66% of the crude base) and is responsible for the substance's opioid effects.

The bioactive compound mitragynine from *M.speciosa* was isolated using acid-base extraction followed by a column chromatography method. In this study, we had investigated the cellular mechanism of chronic morphine treatment after co- and pre-treatment with mitragynine in production of forskolin stimulation cyclic adenosine monophosphate (cAMP) and expression of mu-opioid receptor (MOR) during the differentiation of SK-N-SH human neuroblastoma cells, the possible mechanisms involved in those changes, and the differential effects of morphine and mitragynine. cAMP is a molecule that is controlled by G protein-coupled receptor activation and other cellular processes. Measurement of cAMP levels in cell is widely used as an indicator of receptors function in drug discovery application. Meanwhile changes in expression of MOR number in response to chronic opioid

treatment have long been speculated to directly contribute to receptor desensitization and the development of opioid tolerance

The cytotoxicity of mitragynine on SK-N-SH cell was aslo determined. Mitragynine appeared to be dose-dependent in 50% inhibition of cell proliferation at concentration of 77.268 µM. Development of chronic morphine treatment was optimized. The cAMP level in the cells was significantly increased after 24 hours incubation with morphine sulphate as compared to the control (cell only). Meanwhile expression of mu-opioid receptor showed down-regulation as compared to the control. Interestingly mitragynine also has a similar effect but the up-regulation of cAMP level and down-regulation expression of MOR were less than morphine. In this study co-treatment for 24 hours between morphine sulphate with mitragynine significantly reduced the production of cyclic AMP at lower concentration of mitragynine as compared to treatment with morphine sulphate alone. Similarly pretreatment with morphine sulphate for 6 hours was carried out following treatment with mitragynine for 6 hours. Meanwhile at higher concentration, mitraygnine developed the tolerance and dependence by increasing the cAMP level production in foskolin stimulation. In the expression of mu-opioid receptor study, co-treatment of morphine sulphate with mitragynine had significantly reduced the down-regulation of MOR expression as compared to morphine treatment only. The present study showed mitragynine have the potential to avoid tolerance and dependence of chronic morphine treatment at lower concentration. However it will develop the tolerance and dependence at higher concentrations.

CHAPTER 1

INTRODUCTION

1.1 Medicinal Plant

Plants play an important role in our lives. Besides providing us with food and shelter, they are also sources of medicine. Herbs may be defined as any plant that can be put into culinary or medicinal use and include those we associate with orthodox drugs such as opium poppy, as well as everyday plants like garlic or sage (WHO 2003).

In a previous year, herbal medicines are a common drug to increase and improve the physical performance or to treat human diseases. Despite the move towards technological medicine and the use of sophisticated drugs, traditional plant-based remedies still play an important role in the world's medicine. 80% of the world's population still uses plant-based remedies as their primary form of healthcare (WHO, 2003). The use of plant-based medicines is a popular healthcare approach in Asia, Europe, North America and also being an important form of treatment in many developing countries, where it is part of the diverse local medical system (Heinrich *et al*., 2004). Although there are more than 20,000 herbs being used tody, only few herbal medicines have been proven safe by WHO (Ernst, 2004).

At present, there is an abundance of medicinal plant in the world that has been discovered with the potential to treat various kinds of diseases. The use of medicinal plants from the previous year may provide an indication about diseases and treatment that may conflict with the beliefs of people in the healthcare system (Morgan and Watkins, 1988). Therefore all medicinal plants may have a

recognizable therapeutic effect and also may have a toxic side effect that need to be studied (Keen *et al*., 1994).

1.2 Safety concerns on the use of pharmaceutical from plant

Popular belief has regarded that all medications come from natural resources are safe. Paracelsus, toxicology expert has made a statement concerning safety and his most popular quote is 'all substances are poisons; there is none that is not a poison; the right dose differentiates a poison and a remedy' (Timbrell, 2002). This statement is applies to all product including herbs. Houghton in an editorial note stressed the point that even in 'problem plant' (which refers to plants affecting the central nervous system, e.g opium), the correct dose and usege provide useful pharmaceuticals (Carlini, 2003). Most of the time, people use herbal medicines as dietary supplements to improve their health but the usage without prescription and precaution action. Herbal medicine should in fact be used with caution and advice from an expert to prevent common herbal medicines used in irregular, a high doses and in combination with other medication that may pose toxic side effect.

The toxic side effects can range from an allergic reaction to hepatic failure, cardiovascular problem, neurological and dermatological effect. Sometimes the herb itself is not toxic. Toxicity arise because of adulteration occurs during preparation or processing (e.g. heavy metals), toxic effects may be exhibited such as poisoning by the Chinese herbal medicine *podophyllum* (But *et al*., 1996). Malaysia is a country that has a lot herbal medicines and this herbal medicine or pharmaceuticals from plants are regulated under a government agency, National Pharmaceutical Control Bureau (NPCB) which is also a WHO collaborating Centre for Regulatory Control of Pharmaceuticals.

1.3 Effect of plant on central nervous system

In the last decades, the plant has been used as a source of drugs for treating human diseases (Chin *et al*., 2006). Some of the well-known plants first reported to have such use include poppy capsule latex (*Papaver somniferum*), myrrh (*Commiphora species*) and licorice (*Glycyrrhiza glabra*). The chemical entities derived from opium plant, *P. somniferum* such as papaverine, noscapine (narcotine), codeine and morphine are still used clinically (Newman *et al*., 2000). *P. somniferum* is the species of plant from which opium and poppy seeds are derived and has narcotic properties which mainly affect the function at the central nervous system (CNS). Morphine (and its derivative heroin), thebaine, codeine, papaverine, and noscapine are compounds that derived from this plant. Morphine was used as potent painkillers for severe pain and has made this plant a preferred choice for clinically used drugs. Until now, very few alternative drugs are proven to be as good as morphine as a potent painkiller for chronic pain management.

Cannabinoid, a psychoactive compound from the plant Cannabis sativa also has a good analgesic effect (Watts, 2004) and the potential to treat other neurological illnesses (Fernández-Ruiz *et al*., 2007); however its narcotic effects and undesirable side effects such as addiction and high potential for toxicity are drawbacks of its use and thus made it illegal in most countries. The cannabis plant is widely abused as a recreational drug and is well known as marijuana, ganja and has many other street names (Watts, 2006). Other alternative drugs of the kappa-opioid group such as nalbuphine, pentazocine and butharphanol were clinically available as morphine alternatives but the controversy around the actual analgesic effects of these drugs remain debated (ScienceDaily, 2000)

Other specific plants which are frequently used by the public and have direct and indirect effects on the central nervous system include Ephedra or Ma huang (*Ephedra spp*), which is a good nasal decongestants due to vasodilation effects however can also stimulate CNS side effects from nervousness to imsomnia (Tyler, 1999). Gingko (Gingko biloba L.) a breakthrough herb in the late 1990's, was found to be effective in improving cognitive performance and social functioning of demented patients, with a lower incidence of side effects. St. John's wort (*Hypericum perforatum L.*), one of the five most popular herbs in the U.S.A was found can treating mild to moderate depression states (Tyler, 1999). Kava (*Piper methysticum G. Forst*) which has a narcotic-like effects such as sedation and is effective in treating conditions such as nervous anxiety, stress and restlessness, was reported to have no addictive properties (Tyler, 1999). Valerian (*Valeriana officinalis L.*) which has been in use for a long time, has a reputation as a sleep aid and a mild tranquiliser but has been shown to produce CNS depression (Tyler, 1999). All this plants were reported have an affect on the central nervous system in the human body. Some of these plants have potential as a medicinal plant to treat various human diseases, but most of their misuse to get satisfaction as addiction.

1.4 Drug addiction and withdrawal

Drug addiction affects global health of a large community around the world and Statistical World Drug Report 2012 showed, 5% of the world's adult populations are estimated to use drugs illegally and about 200,000 people about died each years because of drug abuse (UNODC, World Drug Report 2012). Addiction is characterized as a person initiate to force drug intake and followed by repeating of drug intake. This will cause loss of control in drug abuse and development of withdrawal symptoms when drug intake is discontinued. (Koob and Le Moal, 2008).

Drug addiction is a human behavior that consists of two disorders; impulse control disorders and compulsive disorder. In the early stages of addiction, impulsivity is a process that contribute to addiction, meanwhile the combination of impulsivity and compulsivity are involved in later stage of addiction. The impulse control disorder (ICD) is mainly controlled by the positive reinforcement and was characterized by failure to resist a temptation, repetitive engagement followed by increasing tension or arousal in specific behavior and impulse that may harm oneself or others. This mechanism leads to uncontrolled behavior (Grant and Potenza, 2004). Drug abuse will develop compulsivity after series of behavior. On the other hand, negative reinforcement mechanisms and voluntary responses mainly contributed to the compulsive disorder. Compulsive disorder (OCD) is an anxiety disorder which is characterized by uncontrollable, fear, repetitive behaviors and a combination of obsessions and compulsions (Koob *et al*., 2004).

Study of pharmacological and psychological of the drugs should be included in the treatment of addiction behavior. The pharmacological study includes detoxification and attenuation of the drug withdrawal syndrome by traditional replacement therapy (van Dorp *et al*., 2007). Meanwhile psychological treatments of addiction will help by giving an opportunity to talk to the expert specially trained health professional in order to understand the symptoms, dealing with stress, self-medicate and decreasing withdrawal related distress in patients (Baker *et al*., 2004).

Prolonged drug intake, either voluntarily or involuntarily will cause the body system rapidly metabolize and remove the drugs from the body. However, once the body start to adapt to the drug, it will reduce the metabolism of drug release and increase the homeostatic mechanism can cause a phenomenon known as drug

withdrawal (G.A Barr *et al*., 2011). Drug withdrawal syndrome is the discontinuation or reduces the use of drug, type of drug and quantity of drug used. Drug withdrawal syndrome will develop several physiological disorders and mental behavioral disorder. This syndrome also can cause severe symptoms such as flu-like, and include gastrointestinal distress, anxiety, nausea, insomnia, muscle pain, fevers, sweating, impatience depression and anger which began 6 to 8 hours after the last dosage (Cooper and Haney, 2009; Hughes *et al*., 1994).

Detoxification is used in the treatment of patients who experience withdrawal symptoms followed by prolonged drug abuse. Detoxification in the drug withdrawal syndrome is a form of pharmacotherapy process with medical care to minimize and help patients from the drug abuse. This treatment usually used the agonist medication with the control dose in order to reduce the withdrawal symptoms from psychoactive drugs dependence (Margolin *et al*., 1995). After detoxification treatment, the pharmacotherapy treatment will be used to treat the behavioral and treatment long-term rehabilitation. The rehabilitation process is a process more specific to prevent from drug abuse and improve the health of the patients. (McPeake *et al*., 1991; Schuler, 1993). However the combined treatment on pharmacological and physiological does not produce adequate therapeutic efficacies on drug addicts. Therefore, research on natural product for the development of drug that reduce the addiction currently are more toward to the understanding of the cellular, molecular and genetic pathways (Koob and Volkow, 2010)

1.5 Opioid drugs

An opioid is a psychoactive chemical that bind to the opioid receptors such as μ, δ and κ, which are mostly found in the gastrointestinal tract and the central and peripheral nervous system. The receptors in these organ systems mediate both the side effects of opioids which can be both an indication of opioid administration side effect (Pert and Snyder, 1973) and can develop with ongoing administration, leading to a withdrawal syndrome that can produce a feeling of euphoria, motivating some to to recreational use of opioids (Harrison *et al*., 1998). Opioid is a generic term applied to opiates that are limited to natural alkaloids found in the resin of opium poppy (*Papaver somniferum*) and synthetic compound which have morphine-like properties and work at the opioid receptor. (Trescot *et al*., 2008). Most commonly opioids structure is an alkaloid base. Morphine, codeine, thebaine, papaerine and noscapine are natural alkaloid from plant resources. Heroin, methadone, buprenorphine, propoxyphene, pentazocine and oxycodone are synthetic opioid. A statistical report from the United Nation Office on Drugs and Crime (UNODC) in the year 2011, estimated 15 to 25 million aged 12 or older are opiate users in the United States. Heroin is the major contributing drug to the opiate user with 75% of the total number (UNODC, World Drug Report 2011).

A major goal in opioid treating addiction is to reduce the severity of opioid withdrawal symptoms. Methadone and buprenorphine are opioid agonists most commonly used in the pharmacotherapy treatment to alleviate symptoms (Kosten and O'Connor, 2003). Prolonged usage of these treatments may lead to development another phase of addiction and dependence of the drug treatment (Kreek *et al*., 2002). Therefore traditional medicine from natural resources may be comparable to

the existing medicine in treating the opioid withdrawal discomfort and it is found to be safer and has a less adverse effect (Shi *et al*., 2006).

1.6 Molecular mechanisms of opioid addiction and withdrawal

Biological mechanisms of opioid addiction can be divided into mediating tolerance, dependence and addiction. Since the discovery of morphine, researcher have tried to develop opioids with a reduced propensity to cause these negative sequel of prolonged opioid use (Connor *et al*., 2004). Morphine is a modern pain management therapy and the satisfactory treatment of chronic pain. In many cases morphine was used as a drug to prevent frustation by tolerance, dependence and drug addiction (Berger and Whistler, 2010).

An acute opioid effect will activate several potential mechanisms in the specific region of the brains. These mechanisms include the inhibition of calcium conductance, inhibition adenylyl cyclase that cause a reduction in cAMP level, activation of potassium conductance and acute inhibition of transmitters released by opioids (Williams *et al*., 2001). Meanwhile withdrawal from chronic treatment with morphine will superactivate adenylyl cyclase activity that increases the production of cAMP level, deactivate voltage-dependent potassium currents to inhibit potassium release and increases transmitter release through activation of PKA (Williams *et al*., 2001). Another mechanism involved in chronic opioid action is opioid receptor internalization, recycling and downregulation (Koch and Hollt, 2008).

Exposure of drug to the cells may cause persistent changes in the brain such as changes in the protein expression, protein-protein interactions and gene expression. Therefore it will affect the network behavior responses in the subjects.

The repeated exposure to a drug abuser could alter gene expression in the brain such as alteration of gene transcription, alteration of primary RNA transcripts into mature mRNAs, alteration translation of these mRNAs into proteins and processing of protein and alteration of trafficking mature proteins to their intracellular sites of action. (Nestler, 2004).

The most commonly reported actions on drug addiction on a cellular level include regulation of adenylyl cyclase activity, inhibition of transmitter release, activity of calcium conductance, potassium conductance and activation of any of the three opioid receptor subtypes. More recent observations have extended the actions of opioids to include the activation of protein kinase C (PKC), the release of calcium from extracellular stores, the activation of the mitogen-activated protein kinase (MAPK) cascade, and the realization that receptor trafficking plays an important role in receptor's function (Figure 1.1) (Williams *et al*., 2001). Therefore the current trend in the neuroscience research focuses on the basic cellular and molecular pathways in the development of addiction and complex behaviour over drug seeking.

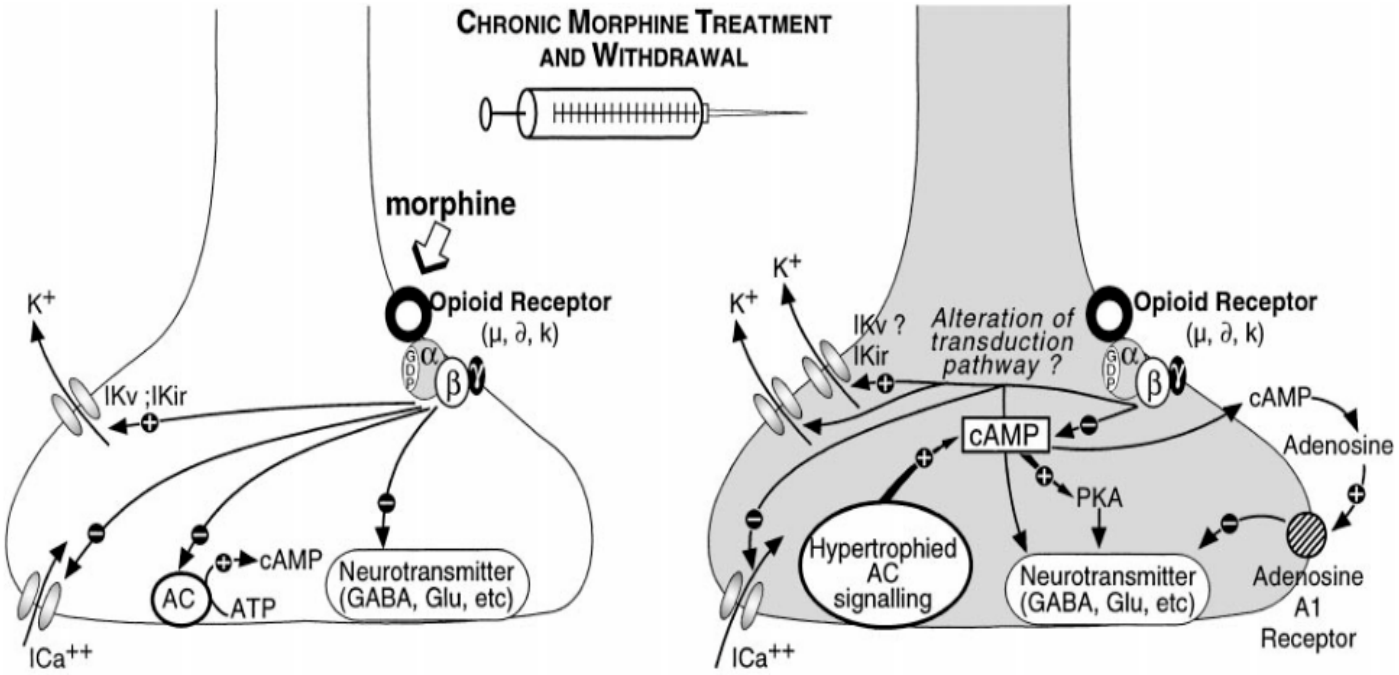

Figure 1.1: The regulation of transmitter release from terminals is changed after chronic opioid treatment. Left: when applied acutely, opioids inhibit transmitter release by several potential mechanisms such as activation of potassium conductance, inhibition of calcium conductance, or an inhibition adenylyl cyclase. Right: after withdrawal from chronic treatment with morphine, the inhibition of transmitter release by opioids is changed in several ways such as opioids no longer activate voltage-dependent potassium currents to inhibit release, an upregulation of adenylyl cyclase that increases transmitter release by activation of PKA, upregulated adenylyl cyclase is sensitive to inhibition by opioids, increased adenylyl cyclase activity increases the production of cAMP that is metabolized to adenosine such that adenosine tone and thus presynaptic inhibition mediated by A_1 adenosine receptors is enhanced at some synapses (Williams et al., 2001).

1.7 Cyclic adenosine monophosphate (cAMP) as potential drug targets

Adenylate cyclase is a two-component enzyme system. It ultimately catalyses the cyclase reaction, but only when it is associated with the hormone-bound receptor and a regulatory protein called stimulatory G-protein (guanylate nucleotide binding protein), which activates adenylate cyclase. The G-protein is the intermediate between the receptor and the synthesis of cyclic AMP (CliffsNotes.com.). cAMP was discovered in the 1960s and the cloning of the first adenylyl cyclase (AC) began in the 1990s. cAMP and its generating enzymes have been shown to play crucial roles in both physiological and pathophysiological settings and have evolved as targets for drug development for numerous diseases (Williams, 2004). The formation of cyclic AMP can be activated by a very large number of cell stimuli, mainly neurotransmitters and hormones. All these stimuli are detected by G-protein-coupled receptors (GPCRs) that use heterotrimeric G proteins, which are the transducers that are responsible for either activating or inhibiting the enzyme AC (Pierre et al., 2009).

The best studied to determine to tolerance and withdrawal effect is inhibition of adenylyl cyclase as an assay (Christie, 2008). Opiates inhibit the functional activity of the cAMP pathway by inhibiting adenylyl cyclase, the enzyme that catalyzes the synthesis of cAMP. The inhibition of the cAMP pathway will recoverto normal with continued exposure of an opioid drug, which can be viewed as a sign of tolerance. This recovery can also be viewed as a sign of dependence. After removal of the opioid drug (e.g., by administration of an opioid receptor antagonist, such as naloxone), the activity of the cAMP level increases above control levels, which can be viewed as a sign of withdrawal. (Figure 1.2) (Nestler, 2004).

A chronic opioid treatment that induced adaptive sensitization or an overshoot adenylate cyclase activity and increased cAMP level has been observed in NG108-15 cells (Musacchio and Greenspan, 1986), SK-N-SH cells (Wang *et al*., 1994), SHSY-5Y cells (Yu *et al*., 2003) and mu-opioid receptor-transfected CHO (Avidor-Reiss *et al*., 1995), and HEK293 cells (Blake *et al*., 1997).

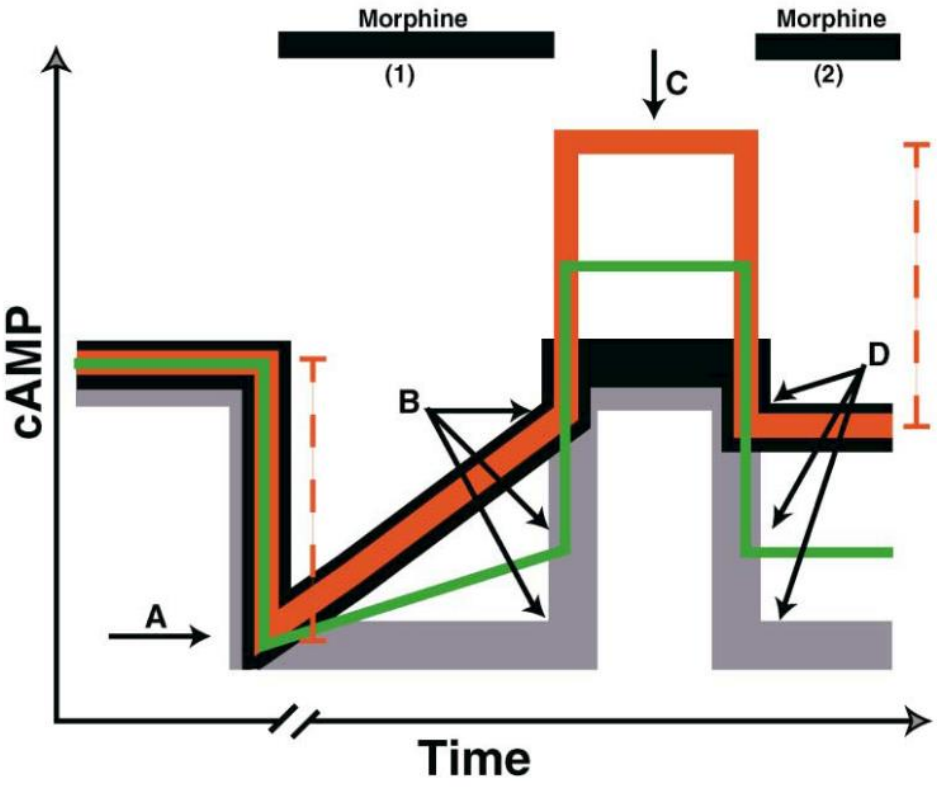

Figure 1.2: Model of cAMP responsiveness in cells treated chronically with morphine. Gray bar represents an idealized situation, in which cells respond robustly to morphine (A) and retain equivalent responsiveness as long as the drug is present (B, gray bar). When the drug is withdrawn, cAMP returns to the same baseline as in naive cells (C, gray bar). In the idealized situation, reapplication of drug (2) leads to equivalent inhibition as naive cells (D, gray bar) (Finn and Whistler, 2001)

1.8 Opioid receptor downregulation

1.8.1 Opioid receptors

Opioid drug produces a mechanism effect on neurons by acting on receptors located on the cell membranes. Opioid receptors are receptors belonging to the seven transmembrane spanning (7TM) G protein-coupled receptors (GPCRs). Physiological importance of these receptors include mediating the actions of the majority of known neurotransmitters and hormones (Waldhoer *et al*., 2004). Three major types of opioid receptor's μ, δ and κ, had been defined pharmacologically several years ago. Activation of opioid receptors is from opioid peptides that were produced endogenous by when the opioid drug was administered. Morphine is an agonist compound that has a higher affinity for mu receptor than other opioid receptor and exhibited the most effective analgesic activities. (Hughes and Kosterlitz, 1983). μ, δ and κ-receptors have analgesia effect when an opioid bind to them. However, activation of κ-receptors do not produce as much physical dependence as activation of μ-receptors (Waldhoer *et al*., 2004).

1.8.2 μ-opioid receptor (MOR) internalization, recycling and down-regulation

At the cellular level, loss of responsiveness to an agonist is also a recurrent theme in the field of G-protein coupled receptors (GPCR), including the μ-opioid receptor (MOR). Three major processes of loss of responsiveness to be involved have been characterized. These ligand-induced processes include loss of G-protein coupling to the receptor, receptor internalization, where the receptor is sequestered from the cell surface, and receptor down-regulation, which is defined as the reduction in the total number of receptors (Horner and Zadina, 2004). Desensitization was defined as the loss of responsiveness of receptor's function under continuing or increasing exposure to an agonist. The key to the initial event for acute receptor

desensitization is receptor's phosphorylation in the cells. This suggestion has been supported and demonstrated in an increased MOR phosphorylation as proven in transfected Chinese hamster ovary (CHO) cells (Zhang *et al*., 1996) and HEK293 cells (Schulz *et al*., 2004) as well as in MOR expressing neuronal cells in prolonged opioid exposure (Deng *et al*., 2001).

Mechanisms for the desensitization of opioid receptor not only comprised of phosphorylation and uncoupling of the receptor from G proteins, but they are followed by receptor internalization and downregulation of receptors. Receptor internalization is a first process in downregulation of a receptor after chronic exposure to an agonist. In this process, receptors were internalized into endosomes and moved to the lysosomes for a degradation process. (Ferguson *et al*., 1996). The process of internalization of GPCR by an agonist occurs before the downregulation process of a receptor. After internalization, opioid receptors are either recycled from endosomes to the plasma membrane or trafficked to lysosomes/proteasomes for degradation process. Downregulation of opioid receptors after chronic opioid treatment is a long-term-adaptive process that may result from degradation of internalized receptors and/or from a decrease of receptor synthesis (Koch and Hollt, 2008). A previous report showed more than 80% of internalized receptors were recycled after 30 min of DAMGO treatment, but receptors could be substantially down-regulated (i.e. >60%) after only 2 h of DAMGO treatments (Polakiewicz *et al*., 1998).

Receptor desensitization and changes in the MOR number after chronic opioid treatment have long been speculated to directly contribute to the development of opioid tolerance. First of all, receptor down regulation after chronic opioid

exposure has been clearly demonstrated only *in vitro* (Yabaluri and Medzihradsky, 1997), whereas *in vivo* results are highly variable (Zadina *et al*., 1995). Following chronic treatment with various agonists, opioid receptors in the brain have been reported to decrease (Tao et al., 1987; Zadina et al., 1995) or to remain unchanged (Hollt *et al*., 1975) indicating that down regulation of opioid receptors depends on opioid species, and brain regions tested. Moreover, there are ligand-specific differences in the ability of μ-opioids to cause receptor down regulation that do not correlate with differences in tolerance (Patel *et al*., 2002).

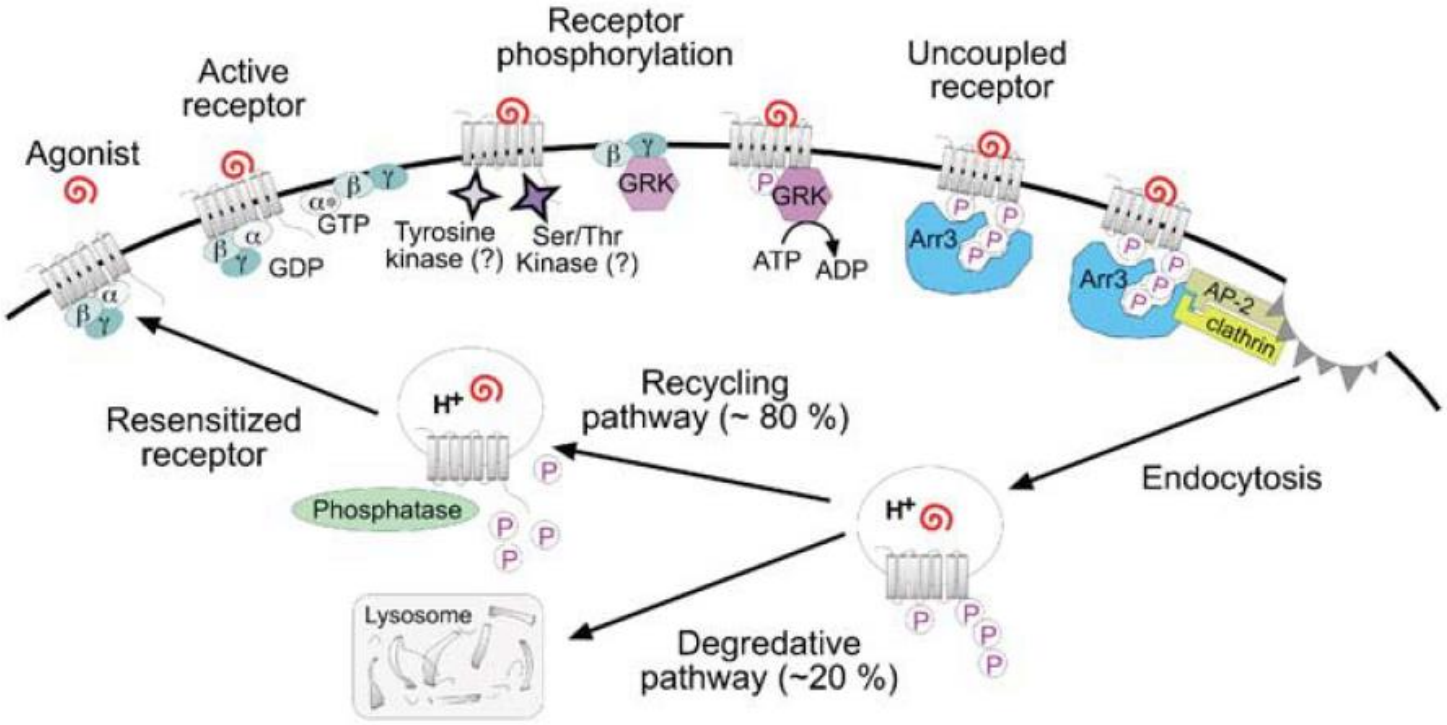

Figure 1.3: The generally accepted pathway for acute m-opioid receptor regulation. The phenomena of opioid receptor activation, uncoupling and internalization are well described. Adapted from figures in von Conner et al., 2004.

1.9 Historical overview of *Mitragyna speciosa*

1.9.1 *Mitragyna Speciosa*

Mitragyna speciosa (*M. speciosa*) Korth belongs to the family Rubiaceae and genus mitragyna and can usually be found in tropical and subtropical Asia such as Malaysia, Thailand, Laos and Cambodia (Houghton and Said, 1986). In Malaysia, this plant is locally known as ‘ketum’ or ‘biak-biak’ and is indigenous in the swampy area especially in the northern and west coast part of Peninsular Malaysia, in the states of Perlis, Kedah, Kelantan and Terengganu and also in the west coast states like Selangor and Perak. In Thailand, this plant is well known as ‘ithang’, ‘thom’, ’kakuam’ or ‘kratom’. However, people globally recognise this plant as ‘Kratom’. The genus was given its name by Pieter Willem Korthals, official botanist with the Dutch East India Service from 1831 to 1836, because the flower stigmas in the first species he examined resembled the shape of a bishop's miter.

M. Speciosa (Figure 1.4) reaches heights of 30-60 feet with a branch spread of over 10-20 feet. Flowers are yellow and grow in ball-shaped clusters and the stem is erect and branching. Leaves are dark glossy green in colour, smooth, ovate-acuminate in shape, and opposite in growth pattern. Leaves can grow over 7 inches long and 4 inches wide. *M .speciosa* is evergreen rather than deciduous, and leaves are constantly being shed and being replaced, but there is some quasi-seasonal leaf shedding due to environmental conditions. During the dry season of the year, leaf fall is more abundant, and new growth is more plentiful during the rainy season. When grown outside their natural tropical habitat, leaf fall occurs with colder temperatures, around 4° Celsius. There are two main varieties of this plant which can be differentiated by its leaves.

The scientific classification of *M. Speciosa* (Korthals, 1839) is shown as follows:

Kingdom:	Plantae
Division:	Magnoliophyta
Order:	Gentianales
Family:	Rubiaceae
Genus:	Mitragyna
Species:	*M. speciosa*

Figure 1.4: Classification of *M. speciosa* leaves and morphology of plant.

The leaves have special characteristics which are easily distinguishable in which the petiole (vein) could either be red or white-greenish and it was believed that they produced different strength of effects (Murple, 2006). The leaves with red-greenish type of vein were suggested to have stronger effects (Suwanlert, 1975).

1.9.2 Chemical constituents of the *M. speciosa*

More than 45 compounds have been isolated (Adkins *et al*., 2011) and over 25 alkaloids have been isolated from *M. speciosa*, most of which are yohimbe-type indoles and oxindoles (Figure 1.5) (Houghton and Said, 1986). Alkaloids are a group of compouds containing the basic nitrogen atom in the naturally chemical compound. This group also includes some related compounds with neutral and even weak acidic properties. A rough estimate is that more than one-quarter of all known alkaloids are indoles. All approaches in clarifying pathways of alkaloid metabolism have been applied to the indole alkaloids and perhaps because of their rich complexity, these compounds seem to have been endowed with a greater variety of hypothetical metabolic pathways than any other groups (Hilaire, 1981).

There are six indoles alkaloids found in *M. speciosa*; mitragynine, 7-hydroxymitragynine, paynanthine, speciogynine, speciociliatine and corynantheidine (Figure 1.6). Mitragynine and 7-hydroxymitragynine are unique to this species and most studied. The two oxindoles are mitraphylline and speciofoline. Other alkaloids present include other indoles and oxindoles such as ajmalicine, akuammigine, corynanthedine, mitraversine, rhynchophylline, speciociliatine (also unique to *M. speciosa*) and stipulatine (Hassan et al., 2013).

Table 1.1: Alkaloid profile of Mitragyna speciosa Korth. The percentage is the estimated content in the alkaloid extracts (Hassan et al., 2013)

Alkaloid	Percentage	Effect
Mitragynine	66%	Analgesic, antitussive, antidiarrheal, adrenergic, antimalarial
Paynantheine	9%	Smooth muscle relaxer
Speciogynine	7%	Smooth muscle relaxer
7-Hydroxymitragynine	2%	Analgesic, antitussive, antidiarrheal
Speciociliatine	1%	Weak opiod agonist
Mitraphylline	<1%	Vasodilator, antihypertensive, muscle relaxer,diuretic, antiamnesic
Isomitraphyline	<1%	Immunostimulant, anti-leukemic
Speciophylline	<1%	Anti-leukemic
Rhynchophylline	<1%	Vasodilator, antihypertensive, calcium channel blocker, antiaggregant, anti-inflammatory, antipyretic, anti-arrhythmic, antithelmintic
Isorhynchophylline	<1%	Immunostimulant
Ajmalicine	<1%	Cerebrocirculant, antiaggregant, anti-adrenergic, sedative, anticonvulsant, smooth muscle relaxer
Corynantheidine	<1%	Opioid agonist
Corynoxine A	<1%	Calcium channel blocker, anti-locomotive
Corynoxine B	<1%	Anti-locomotive
Mitrafoline	<1%	
Isomitrafoline	<1%	
Oxindale A	<1%	
Oxindole B	<1%	
Speciofoline	<1%	Analgesic, antitussive
Isospeciofoline	<1%	
Ciliaphylline	<1%	Analgesic, antitussive
Mitraciliatine	<1%	
Mitragynaline	<1%	
Mitragynalinic acid	<1%	
Corynantheidalinic acid	<1%	

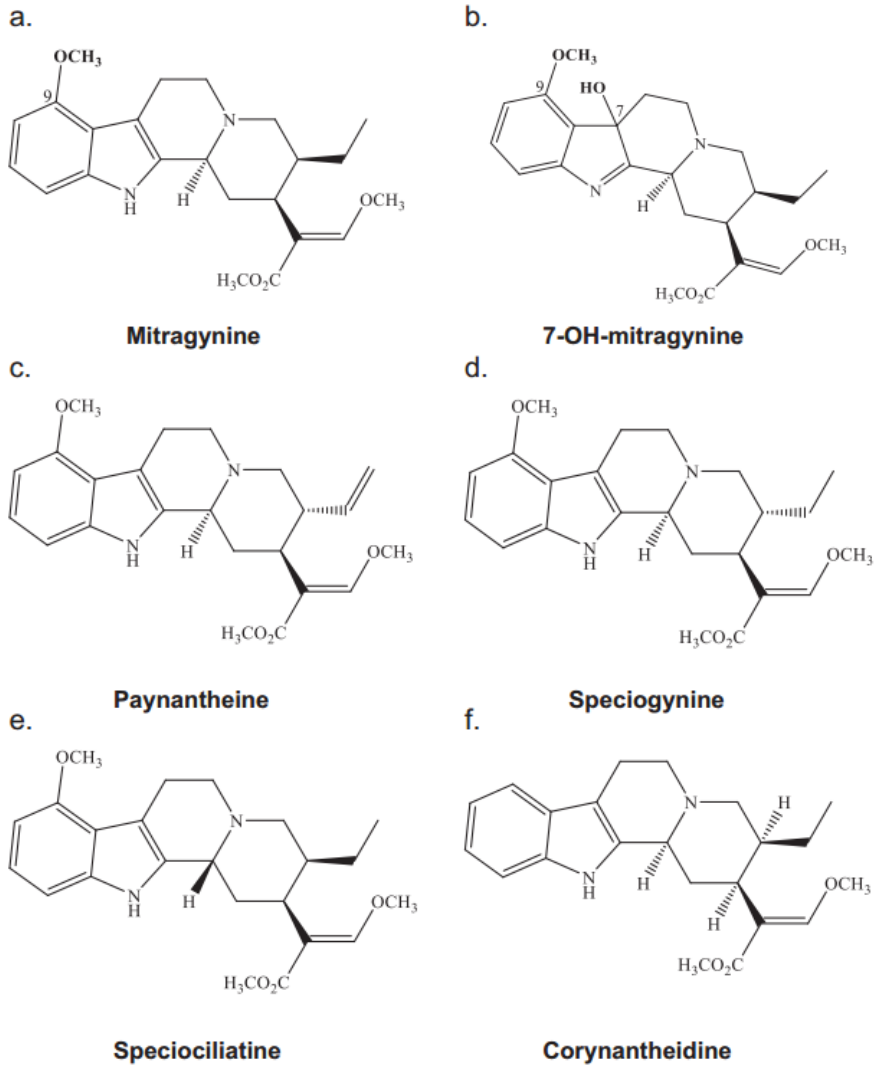

Figure 1.5: Chemical structure of mitragynine and its major analogues (Hassan et al., 2013)

Mitragynine is the major compound with 66% of the total alkaloid mixture derived from these leaves. Most studies suggest mitragynine as the main chemical responsible for the effects of the activity of this herb. Mitragynine was isolated in 1907 by Hooper followed by Field (1921) and the structure of mitragynine was determined by Zacharias (1964). In the 1960s, the Chelsea group in the U.K. reported the isolation of several indole alkaloids from the leaves of *M. speciosa* from Thailand. Mitragynine compound was synthesized by Takayama *et al*., 1995 and alternative synthesis was reported later by Ma *et al.* (2009). A computational study recently identified the lowest energy conformation of mitragynine which was in excellent agreement with X-ray crystal structure geometry (Hassan et al., 2013). Mitragynine is a white amorphous powder, it is soluble in alcohol, chloroform and acetic acid. The chemical structure of mitragynine is related to both yohimbine and voacangine. Chemically, mitragynine is 9-methoxy-corynantheidine.

1.9.3 Pharmacology of *M. speciosa* and mitragynine

M. speciosa Korth plant, especially its leaves has been consumed since time immemorial, where village people such as farmers and labourers chew the fresh leaves, smoke the dry leaves or drink as a tea suspension or even eat it in the form of resin, for the stimulant effects to overcome the burden of hard work under the scorching sun. It was way back in 1897 when the leaves and the bark of this plant were reported by Ridley as a cure for opium habit and was further quoted by Hooper in 1907. In the same year, Holmes also referred to its leaves as an opium substitute (Shellard, 1974). This plant has unique dual opioid properties which exert a stimulant effect at low doses and sedative and analgesic effects at higher doses in humans (Suwanlert, 1975). These effects have also been observed in animal models as reported by Macko *et al* (1972).

It was also reported that *M. speciosa* has effect of depression such as cannabis and opium and psychostimulant effects such as coca. (Jansen and Prast, 1988). Mitragynine has weaker effects than morphine, less harmful than cocaine and has a milder withdrawal syndrome compared to opioids. In Malaysia and Thailand, local people used *M.speciosa* leaveas as a substitution therapy for chronic opioid treatment to manage withdrawal symptoms especially on treatment substitution of morphine and heroin (Matsumoto et al., 2004b; Vicknasingam et al., 2010). Besides that, it has been used as a traditional medicine for the treatment of common illnesses such as hypertension, coughing, muscle pain, diarrhoea and also used as a tonic to regain the energy (Jansen and Prast, 1988).

Mitragynine showed unique effects on the phytochemical and pharmacological study (Chee *et al*., 2008). *In vivo* and *in vitro* pharmacological investigations revealed that *M. speciosa* has antinociceptive, antidepressant, antioxidant, antibacterial and antitussive activities (Chittrakarn *et al*., 2008; Idayu *et al*., 2011; Matsumoto *et al*., 2005; Parthasarathy *et al*., 2009; Sabetghadam *et al*., 2010). Crude extract of *M. speciosa* and mitragynine were also reported to have analgesic properties both *in-vivo* and *in-vitro* (Matsumoto et al., 2004a; Reanmongkol, 2007). The crude methanol (MeOH) extract was used in an *in-vitro* assay on electric stimulation of guinea-pig ileum in which the opioid antagonist, naloxone, successfully inhibited the contraction. This implies that the crude extract is an opioid agonist (Takayama, 2004).

Crude extract of *M.speciosa* and mitragynine were also reported to successfully act via supraspinal μ, δ and κ opioid receptors (Thongpradichote *et al*.,