UNIVERSITI PUTRA MALAYSIA

ENHANCED ANTINOCICEPTIVE EFFECTS OF MITRAGYNINE IN COMBINATION WITH MORPHINE VIA OPIOID RECEPTORS ACTIVATION

SHAMIMA BINTI ABDUL RAHMAN

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By

SHAMIMA BINTI ABDUL RAHMAN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfillment of the requirements for the degree of Doctor of Philosophy

June 2014

This thesis is specially dedicated to:

My husband:
Abang, Yudi Kurniawan Budi for his patience and stay by my side through all the day

My children:
Muhammad, Ibrahim, Maryam, Adam and Zulaikha for their love and understanding of ummi's doing

My parents:
Mama, Bedah Musooh and Abah, Abdul Rahman Shamsuddin for being with me throughout the up's and down's, through happiness and sorrow...for all the du'a for my success and easinest of my way

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Assoc Prof Datin Sharida Fakurazi, PhD
Faculty : Medicine and Health Sciences

The management of chronic pain is one of the greatest challenges in modern medicine. Opiates such as morphine have been used to treat pain for centuries. However, the long term use of morphine is limited due to its side-effects. To date, a number of natural compounds have been detected to possess analgesic effects. One of these natural compound is mitragynine (MG) which is isolated from *Mitragyna speciosa* Korth. *Mitragyna speciosa* is popularly known as 'ketum' in Malaysia and 'kratom' in Thailand. Over 25 alkaloids are found in *Mitragyna speciosa*, MG being a major one. In this study, we investigated the action of MG as antinociceptive agent and the receptor selectivity effect. The nociceptive effect was estimated in a hot plate test (Ugo Basile model 7280; 50.0 ºC). The latency time was estimated until the mice showed pain responses such as shaking, licking or jumping and the duration of latency was measured for every 15 minutes until 120 minutes. Male ICR mice (n=8/group) were administered intraperitoneally with single dosage of MG (3, 10, 15, 30, and 35 mg/kg), 15 minutes prior to pain induction. The control groups were given appropriate dose of vehicle. For the receptor selectivity test, the treated groups were administered naloxone (non-selective opioid antagonist), naltrindole (δ-opioid antagonist), norbinaltorpimine (κ-opioid antagonist) and AM251 (cannabinoid 1 antagonist) respectively prior to MG injection at the dosage of 35 mg/kg. The groups administered with MG showed an increased in latency time as compared to the control groups in a dose-dependent manner. Meanwhile, 35 mg/kg of MG was found to significantly increase the latency time. The results also showed that naloxone and naltrindole fully blocked the antinociceptive effect of MG, whilst norbinaltropimine partially blocked the effect, but the antinociceptive effect of MG was not antagonized by AM251. These results demonstrated that MG acts through opioid receptor specifically on δ and κ receptor and not through the cannabinoid CB1 receptor. Later on, we investigated the enhancement of analgesic action of this compound when combined with morphine and the effect on the development of tolerance due to morphine acutely and chronically. Male ICR mice (n=7/group) were administered intraperitoneally with a single dose of MG either 15 mg/kg or 25 mg/kg combined with morphine (5 mg/kg) in the acute study, whilst the study was continued for 9 days for the chronic phase. The control groups were given the appropriate dose of a vehicle. The antinociceptive effect was estimated with a hot

plate test (Ugo Basile model 7280; 50.0 ºC). The latency time was assessed until the mice showed a pain response such as shaking, licking or jumping. The expression of cAMP, cAMP response element binding (CREB) protein, ERK and c-fos were analyzed. Liver and kidney function test were also analyzed and compared between groups. In acute study, the administration of MG and morphine showed a significant latency period compared to the vehicle treated groups. The combination of MG and morphine has enhanced morphine-induced analgesia which shows synergism in analgesic action. In the chronic phase, the concurrent administration of MG and morphine showed a significant increase in the latency time when compared to morphine alone groups and the remarkable analgesic effects in the combination regimens were maintained from day 1 until day 9. The result was in contrast when compared to morphine alone groups, where the latency time were reduced from day 5 to day 9. For the protein expressions, there were a significant increment of the cAMP and CREB levels ($p<0.001$) in groups treated with 5 mg/kg morphine but there was no significant changes of cAMP and CREB expression for MG alone groups and groups combined with morphine. There were no significant changes in other proteins (ERK and c-fos) for all groups when compared with the control group. There was also no significant changes in the liver enzymes of the treated groups when compared to the control group except for the AST level. There were no significant changes in the excretion level of urea in all groups when compared to the control groups. Similar results were found for the excretion of creatinine. However, the creatinine excretion was significantly increased when the treatment was combined. This study indicates that MG has antinociceptive properties and act fully via the opioid system. It also indicates that concurrent administration of morphine and MG enhanced the analgesic effects. Following the inclusion of MG, tolerance due to repeated administration of morphine is delayed.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENINGKATAN KESAN ANTINOSISEPTIF GABUNGAN MITRAGYNINE DAN MORFIN MELALUI PENGAKTIFAN RESEPTOR OPIAT

Oleh

SHAMIMA ABDUL RAHMAN

Jun 2014

Pengerusi : Prof Madya Datin Sharida Fakurazi, PhD
Fakulti : Perubatan dan Sains Kesihatan

Pengurusan sakit kronik adalah salah satu cabaran terbesar dalam perubatan moden. Opiat seperti morfin telah lama digunakan dalam pengurusan sakit kronik. Walau bagaimanapun, penggunaan jangka panjang morfin adalah terhad disebabkan oleh kesan sampingan. Setakat ini, beberapa sebatian semulajadi telah dikenalpasti sebagai memiliki kesan analgesik. Salah satu sebatian semula jadi adalah mitragynine (MG) yang diekstrak daripada *Mitragyna speciosa* Korth. *Mitragyna speciosa* lebih dikenali sebagai ketum di Malaysia dan Kratom di Thailand. Lebih 25 alkaloid terdapat dalam *Mitragyna speciosa* dan MG merupakan komponen utama. Dalam kajian ini, kami mengkaji kesan MG sebagai ejen antinosiseptif dan kesan pemilihan reseptor. Kesan nosiseptif dikenalpasti menggunakan ujian plat panas (Ugo Basile model 7280; 50.0 ºC). Masa laten dianggarkan sehingga tikus menunjukkan tindak balas kesakitan seperti menggigit, menjilat atau melompat dan tempoh laten diukur bagi setiap 15 minit sehingga 120 minit. Tikus jantan ICR (n = 8/group) telah disuntik secara intraperitoneal dengan dos tunggal MG (3, 10, 15, 30, dan 35 mg / kg), 15 minit sebelum induksi kesakitan. Kumpulan kawalan diberi dos kenderaan yang sesuai. Bagi ujian pemilihan reseptor, kumpulan yang dirawat telah diberikan naloxone (antagonis opioid tidak terpilih), naltrindole (antagonis δ - opioid), norbinaltorpimine (κ - opioid antagonis) dan AM251 (cannabinoid 1 antagonis) masing-masing sebelum suntikan MG pada dos 35mg/kg. Kumpulan yang diberi MG menunjukkan peningkatan dalam masa laten berbanding dengan kumpulan kawalan dan kesannya adalah bergantung kepada dos. Sementara itu, 35 mg/kg MG didapati meningkatkan masa latennya secara signifikan. Keputusan juga menunjukkan bahawa naloxone dan naltrindole menyekat sepenuhnya kesan antinosiseptif MG, manakala norbinaltropimine menyekat sebahagian kesannya, tetapi kesan antinosiseptif MG tidak dihalang oleh AM251. Keputusan ini menunjukkan bahawa MG bertindak melalui reseptor opioid khusus pada δ dan κ reseptor dan bukan melalui reseptor cannabinoid CB1. Kemudian, kami menyiasat peningkatan tindakan analgesik sebatian ini apabila digabungkan dengan morfin dan kesan ke atas ketahanan terhadap morfin secara akut dan kronik. Tikus jantan ICR (n = 7/group) telah disuntik secara intraperitoneal dengan dos tunggal MG sama ada 15mg/kg atau 25 mg/kg digabungkan dengan morfin (5mg/kg) dalam kajian akut, manakala kajian diteruskan selama 9 hari untuk fasa kronik. Kumpulan kawalan diberi dos kenderaan yang sesuai. Kesan antinosiseptif dikenalpasti dengan ujian plat panas (Ugo Basile model 7280; 50.0 ºC). Masa laten dikira sehingga tikus

menunjukkan tindak balas kesakitan seperti menggigit, menjilat atau melompat. Protein cAMP, unsur tindak balas CAMP mengikat (CREB) protein, ERK dan c-fos telah dianalisis. Ujian fungsi hati dan buah pinggang juga ditentukan dan dibandingkan diantara semua kumpulan. Dalam kajian akut, penggunaan MG dan morfin menunjukkan tempoh laten yang ketara berbanding dengan kumpulan kenderaan dirawat. Gabungan MG dan morfin telah meningkatkan kesan analgesik morfin yang menunjukkan sinergi dalam tindakan analgesik. Dalam fasa kronik, suntikan serentak MG dan morfin menunjukkan peningkatan yang ketara pada masa laten berbanding kumpulan morfin sahaja dan kesan analgesik yang luar biasa dalam rejimen gabungan dikekalkan dari hari 1 hingga hari 9. Hasilnya adalah berbeza apabila dibandingkan dengan kumpulan morfin sahaja, di mana masa laten dikurangkan dari hari ke 5 ke hari ke 9. Bagi analisis protein, terdapat kenaikan yang ketara daripada cAMP dan tahap CREB ($p < 0.001$) dalam kumpulan yang dirawat dengan 5 mg/kg morfin tetapi tidak terdapat sebarang perubahan signifikan terhadap cAMP dan CREB protein untuk kumpulan MG sahaja dan kumpulan digabungkan dengan morfin. Tidak ada perubahan ketara dalam protein lain (ERK dan c-fos) untuk semua kumpulan berbanding dengan kumpulan kawalan. Tiada sebarang perubahan penting dalam enzim hati kumpulan yang dirawat berbanding dengan kumpulan kawalan kecuali bacaan AST. Tiada perubahan ketara dalam tahap perkumuhan urea dalam semua kumpulan apabila dibandingkan dengan kumpulan kawalan. Keputusan yang sama diperolehi untuk perkumuhan kreatinin. Walau bagaimanapun, perkumuhan kreatinin telah meningkat dengan ketara apabila rawatan digabungkan. Kajian ini menunjukkan bahawa MG mempunyai ciri-ciri antinosiseptif dan bertindak sepenuhnya melalui sistem opioid. Ia juga menunjukkan bahawa suntikan serentak morfin dan MG meningkatkan kesan analgesik. Kombinasi MG bersama morfin melewatkan toleransi terhadap penggunaan morfin yang berlanjutan.

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I certify that an Examination Committee met on 2012 to conduct the final examination of Shamima Abdul Rahman on her Doctor of Philosophy entitled 'Enhanced antinociceptive effects of mitragynine in combination with morphine via opioid receptors activation' in accordance with Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I hereby confirm that:

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This is to confirm that:

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HPT	Hot plate test
MG	mitragynine
MS	*Mitragyna speciosa*
min	minute
$	Dollar US
h	hour
sec	second
° C	degree Celcius
Rf	retention factor
ED_{50}	effective dose at 50 percent
μ	mu
δ	delta
κ	kappa
MOR	mu opioid receptor
DOR	delta opioid receptor
KOR	kappa opioid receptor
CB_1	cannabinoid type 1 receptor
CB_2	cannabinoid type 2 receptor
norBNI	norbinaltorphimine
NS	normal saline
T80	Tween 80
AM251	1 – (2,4-diclorophenyl) – 5 – (4-iodophenyl) – 4 – methyl – N – 1 – piperidinyl – 1H – pyrazole 3 carboxamide
CNS	central nervous system
%	percentage
MRI	mean relative intensity
LFT	Liver function test
KFT	Kidney function test
c-fos	gene
CREB	cAMP response element binding
PKA	cAMP-dependent protein kinase
MAPK	mitogen-activated protein kinase
ATF-1	activating transcription factor-1
cAMP	cyclic adenosine monophosphate
CREM	cAMP response element modulator
ERK	extracellular signal-regulated kinases
ALT	Alkaline phosphatase
AST	Aspartate transaminase
GGT	Gamma-glutamyltransferase
NSAIDs	non-steroidal anti-inflammatory drugs
WHO	World Health Organization
RVM	rostral ventromedial medulla
PAG	periaqueductal grey
GPCRs	G-protein-coupled receptor
SEM	standard error mean
ELISA	enzyme-linked immunosrbant assay
BSA	bovine serum albumin
ANOVA	One-way analysis variance

i/p	intraperitoneal injection
kg	kilogram
mg	miligram
g	gram
Nal	Naloxone
NTI	Naltrindole
COX-2	cyclooxygenase 2 pathway
mL	milimeter
&	and
b.wt	body weight

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Pain, both acute and chronic, remains a significant health problem despite tremendous progress in understanding its basic mechanism (Gregory et al., 2013). The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (American Pain Society, 2003). Relief from pain has been the paramount objectives of the medical profession throughout history (Dureja, 2010) and nowadays, the management of chronic pain is one of the greatest challenges in medicine. Pain as a whole, is a very active area for pharmaceutical research and development, not only as the cause of frequent mistreatment, but also of unacceptable side effects associated with older and still widely used compounds (Bruehl, 2013).

Plants or plant parts have been used as a source of medicine since prehistoric times (Smith-Hall, 2012). Until now, plants are an important source of chemical compounds that are developed into drugs. Between 1983 and 1994, of the 520 new prescription drugs approved, 39% were derived from plants or animals or natural sources, with 60% to 80% of those comprised of antimicrobials and anticancer drugs (Wecker, 2010). Drugs from plants continue to be a great source of revenue in the United States, with the annual sales of $10 billion in the year 2000. More than 200 organizations worldwide are investigating new uses of plant-derived drugs, especially in the fight against AIDS, cancer, diabetes and cardiovascular diseases (Khan, 2011). Nowadays, drugs are also processed using a synthetic version of the active chemical found in the plant. Besides all these, plants have become the main component of the ever-growing alternative therapy development (Khan, 2011).

The source for opium is the opium poppy plant, *Papaver somniferum*. Morphine was isolated from crude opium in 1806 by Serturner, who named the substance after the Greek god of dreams, Morpheus (Wecker, 2010). Not long after its isolation, morphine was introduced into the medical practice. Subsequent to medicinal properties of opium poppy, many new plants were introduced and studied to increase the discovery of natural plant products as antinociceptive agents (Hajhashemi et al., 2011; Chen et al., 2011).

A number of natural compounds have been detected to have analgesic effect such as *Papaver somniferum, Cannabis sativa, Clematis sanitora* and *Plantanus orientalis*

(Hajhashemi et al., 2011; Chen et al., 2011). One of the most used compound is mitragynine (MG), which has been isolated from *Mitragyna speciosa* (MS) Korth. MS is a plant that is abundantly found in Thailand and Malaysia which is popularly known as 'kratom' in Thailand and 'ketum' in Malaysia. Over 25 alkaloids have been isolated from this plant (Houghton & Said, 1991), where MG was analysed as the major constituent (66.2%) together with its other analogues, paynantheine (8.6%), speciogynine (6.6%), 7-hydroxymitragynine (2.0%) and speciociliatine (0.8%) (Takayama, 2004). MG constitutes an indole structure, with its fourth position is substituted by the methoxy group. The moleculer structure is 9-methoxy-corynantheidine ($C_{23}H_{30}N_2O_4$) with molecular weight of 398.5 (Chee et al., 2008). Studies have indicated that MG plays a role as an antinociceptive agent and acts via opioid receptors (Yamamoto et al., 1999; Takayama et al., 2002; Takayama, 2004; Matsumoto et al., 2006).

Opioids analgesic drugs such as morphine continue to be the mainstream therapy available for the management of acute and chronic pain (Bruehl, 2013). Up till now, morphine is the most important and powerful analgesic. It has long and widely been used to alleviate various types of severe pain, including acute postoperative and chronic cancer pain.

1.2 Problem Statement

No single analgesic is perfect and no single analgesic can treat all types of pain. Each agent has distinct advantages and disadvantages compared to others. A combination is most effective when the individual agents act through different analgesic mechanisms and act synergistically. Combination analgesic can provide more effective pain relief for a broader spectrum of pain, and might also reduce adverse drug reactions (Raffa, 2001). Many combinations analgesic are available and are commonly prescribed for pain. Combination of acetaminophen and codeine, codeine and ibuprofen, and acetaminophen and oxycodone was found to be a safe and effective analgesic (Palangio et al., 2000).

Opiates such as morphine have been used to treat pain for centuries. However, the long term use of morphine is limited due to its side-effects, which include nausea, vomiting, being in a state of euphoria and mental detachment (Macadante *et al*, 2006). Among other side-effects of morphine, development of tolerance and dependence are the most difficult to overcome.

Active compounds such as MG have been shown to have analgesic properties (Matsumoto et al., 1996, 1998; Takayama et al., 2002; Takayama et al., 2004; Horie et al., 2005; Matsumoto et al., 2006). Many studies have been conducted and revealed that MG can give antinociceptive activity without developing toxicity effects (Macko et al.,

1972; Janchawee et al., 2007; Reanmongkol et al., 2007). Besides, MG is reported to be comparable to codeine as an analgesic (Macko et al., 1972; Jansen & Prast, 1988). Eventhough MS have been regarded as an unsafe plant under the Dangerous Drug Act 1953 if it is used repeatedly until the development of addiction by Malaysian government, perhaps the combination of potent morphine and MG will reduce the side effect of morphine (Raffa, 2001).

1.3 Significance of Study

According to the American Pain Society, prevalence of chronic pain in the United States is estimated to be 35.5% or equivalent to 105 million people (Datamonitor, 2009). This costs more than US$100 billion per year in direct health care expenditure and the loss of work productivity time. Current pain management relies heavily on agents that have analgesic properties. Non-narcotic analgesics (acetominophen and aspirin), narcotic analgesics (opioids), non-steroidal anti-inflammatory drugs (NSAIDs), and thermal agents continue to be the mainstays of pain management. More recently, other medicines have been added, such as antidepressants, anticonvulsants and selective cyclooxygenase 2 (COX-2) inhibitors (Katzung, 2010).

The global pain market in 2009 was valued at over US$50 billion in seven major countries namely United States (US), Japan, France, Germany, Italy, Spain and United Kingdom. In US alone, the expenditure is US$27 billion out of the US$50 billion. Figure 1.1 shows the US market share of major drug classes of pain agents. Strong opioid such as morphine become the biggest contributor in the shares (US$7.83). From this chart, it can be deduced that pain killer was the most costly among major drug classes in pain market.

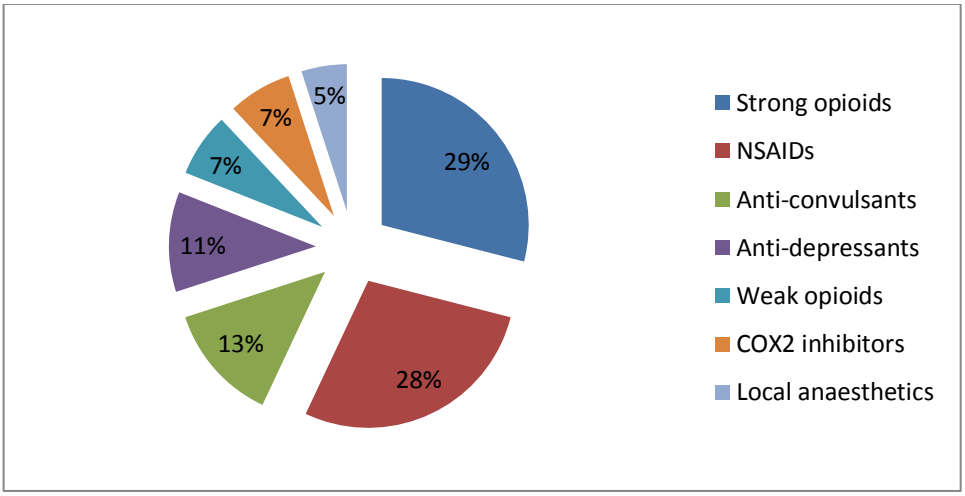

Figure 1.1: Market shares of major drug classes in pain market of US (Sources: Datamonitor, IMS Health, Decision Resources, 2009)

In Malaysia, the Malaysia Statistic of Medicine (2007) reported that strong opioids such as morphine have been used tremendously even though it is costly and induced many side effects. The total opioid consumption in Malaysia in 2007 was 0.4184 defined daily dose (DDD)/1000 population/day. Strong opioids have been widely consumed compared to weak opioid such as tramadol. In reducing the use of morphine, the medical cost in Malaysia will be much reduced as well. Thus, in finding an alternative source to pain treatment from natural products, the side effects and high cost of morphine may be reduced as well.

Previous findings have suggested that the combinations of opioid analgesics and other analgesics can be used to control pain (Lauretti *et al*, 2003; Miranda et al., 2006; Smith et al., 2007). The use of several combinations of potent opioids were suggested to reduce the toxic effects of opioid treatment, to improve analgesia and to reduce opioid tolerance (Lauretti *et al*, 2003; Morita *et al*, 2003; Mercandante *et al*., 2004). Combination of opioids with other classes of analgesics can also help to reduce sensitization processes and optimize pain therapy, as opioids such as morphine will keep their central role in postoperative, traumatic or tumor pain therapy (Wolfang, 2007). Thus, the combinations of medications that offer analgesic synergism should allow a reduction in required dosage which gives the maximum analgesic effects and a decrease in the incidence of adverse effects.

The leaf of MS has been used in Thailand and Malaysia for its opium-like effect (Burkill, 1935). It is also commonly abused due to its stimulant ability to combat fatigue (Grewal, 1932; Suwanlert, 1975). Besides, the Thailand people use the leaf to alleviate pain, coughing and diarrhea (Suwanlert, 1975). Mitragynine is the major indole alkaloid in MS (Takayama, 2004). A study by Matsumoto et al. in 2006 has found that this compound has shown some opioid activities.

Since it has been proven to have antinociceptive effects (Yamamoto et al., 1999; Takayama et al., 2002; Takayama, 2004; Matsumoto et al., 2006) MG could be a potential pain relief alternative to morphine. Thus, a combination of this compound and morphine is predicted to minimize tolerance by reducing the dosage requirement of morphine. Furthermore, the combination might have synergistic effect probably by acting at the same site of action.

Apart from that, the existence of an endogenous cannabinoid system, comprising of cannabinoid CB_1 and CB_2 receptor subtypes together with their signaling pathways and endogenous ligands, is now well recognized (Martin et al., 2004). Cannabinoids have been shown to exert a broad pharmacological action, including the central and peripheral effects through receptor-mediated mechanisms (Howlett et al., 2002). Pharmacological and molecular biological studies have identified at least two types of cannabinoid receptors, cannabinoid type 1 (CB_1) receptor and cannabinoid type 2 (CB_2)

receptor both coupled to the G-protein (Takayuki et al., 2006). The cannabinoid CB_1 receptor is predominantly found in the central nervous system. To date, cannabinoid CB_1 receptor has been shown to play a role in managing pain. However, the study on the effects of MG on opioid receptors especially CB_1 which are involved in pain management has still not been explored.

1.4 Conceptual Framework of the Study

This study consists of several phases of experiment related to one another. Figure 1.2 shows the conceptual framework of this study. Briefly, the first phase was the isolation of pure compounds which is MG from MS Korth **(CHAPTER 3)**. The second phase was to determine the antinociceptive action of MG together with the receptor selectivity **(CHAPTER 4)**. In this phase, opioid receptors as well as the CB_1 receptor have been selected for the determination of action. In Chapter 4, morphine was used as a positive control drug.

The third phase was to determine the effects of MG counteracting the tolerance effect of analgesic morphine. For chapter 5, the analgesic used was morphine. In **CHAPTER 5**, acute and chronic study has been conducted. In acute study, the combination regimen (MG + morphine) was given once whilst in chronic study, the combination regimen (MG + morphine) was carried out for 9 days. This was to confirm and to evaluate the development of tolerance to morphine, the analgesic of choice throughout the study.

The changes of protein expression in relation to tolerance was analysed and carried out for the groups that received 9 days of combination treatment **(CHAPTER 6).** Finally, in the final chapter **(CHAPTER 7)**, the liver and kidneys were analysed for determination of toxicological changes to the metabolic and excretory organs respectively.

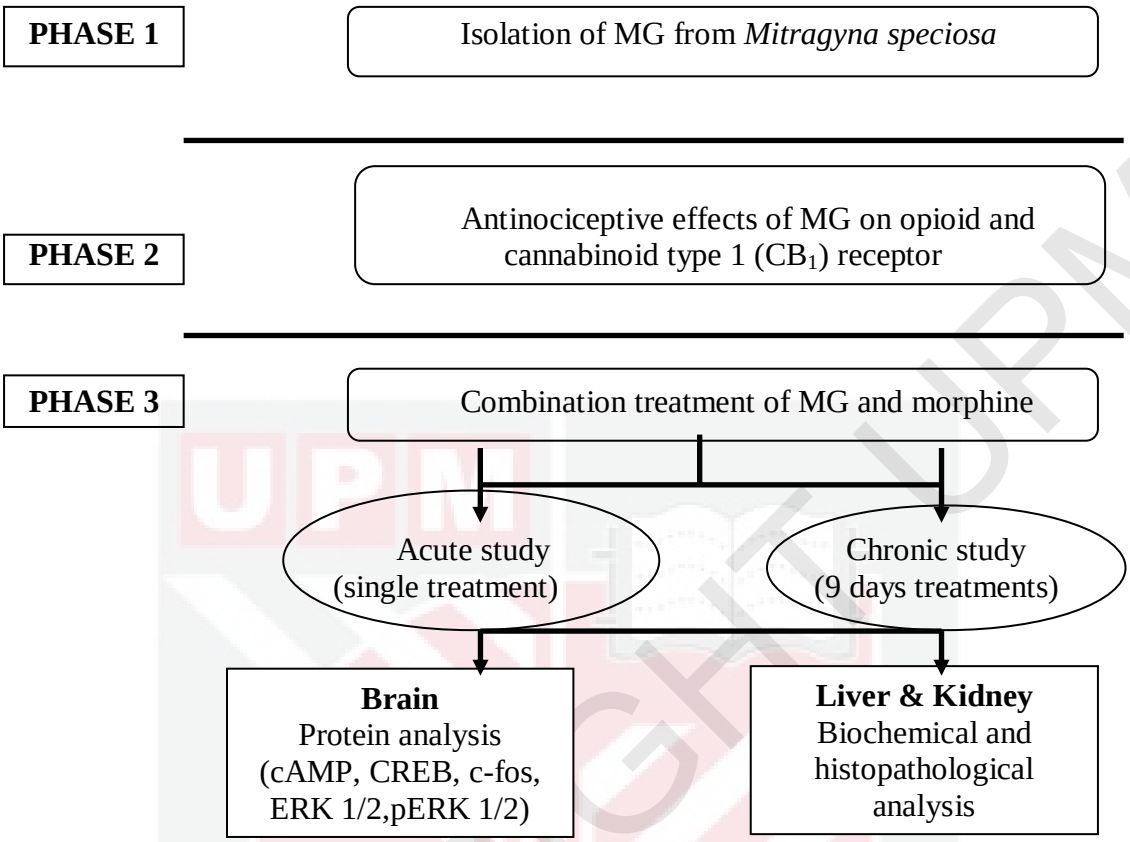

Figure 1.2: Conceptual framework of the study

1.5 Study Hypotheses

1. Mitragynine (MG) isolated from *Mitragyna speciosa* (MS) will have antinociceptive effect on opioids and cannabinoid receptor.

2. The combination treatment of morphine and MG will give synergistic antinociceptive effect.

3. There are no pathological changes in liver and kidney following the combination treatment.

1.6 Objectives of the Study

1.6.1 General objectives

1. To investigate the antinociceptive activities of MG isolated from local MS on opioid and cannabinoid receptors.

2. To determine the antinociceptive activity of MG and morphine as a potential combination to reduce tolerance.

3. To investigate any pathological changes in liver and kidney following combination treatment of MG and morphine.

1.6.2 Specific Objectives

1. To isolate MG from MS leaves obtained from North Peninsular Malaysia.

2. To investigate the effective dose at 50 percent (ED_{50}) of MG with the hot plate test.

3. To determine the antinociceptive effects of MG on opioids (μ,κ,δ) and cannabinoid (CB_1) receptor.

4. To evaluate the antinociceptive effects of MG in combination with morphine in acute and chronic study by using hot plate test.

5. To assess changes on cAMP, CREB, c-fos and ERK 1/2 protein expression following combination treatments.

6. To investigate the effect of the combination therapy on liver function test (LFT) and kidney function test (KFT).

7. To conduct histopathology analysis of liver and kidney following combination treatments.

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