SELECTED PHARMACOLOGICAL STUDIES OF *Cinnamomum iners* STANDARDIZED LEAVES METHANOLIC EXTRACT

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SELECTED PHARMACOLOGICAL STUDIES OF *Cinnamomum iners* STANDARDIZED LEAVES METHANOLIC EXTRACT

BY

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LIST OF ABBREVIATIONS

CDC- Centre for Disease Control and Prevention

CE- catechin equivalents

CFU- colony forming unit

CSLE- *Cinnomomum iners* standardized leaves methanolic extract

DPPH -2,2-diphenyl -1-picrylhydrazyl

DXP-1-deoxy-d-xylulose 5-phosphate

E-4P -erythrose-4-phosphate

FTIR- fourier transform infrared

GAE- gallic acid equivalents

GC-MS- gas chromatography-mass spectrometry

H_2O_2-hydrogen peroxide

IC_{50}- median inhibitory concentration

INT-p-iodonitrotetrazolium violet

LC_{50}-median lethal concentration

LD_{50}- median lethal dose

MBC- minimum bactericidal concentration

MFC- minimum fungicidal concentration

MHA- Mueller Hinton agar

MHB- Mueller Hinton broth

MIC-minimum inhibitory concentration

MRSA- methicilin resistant *Staphylococcus aureus*

NMR- nuclear magnetic resonance

OECD -The Organization of Economic Co-operation Development

PEP-phosphoenolpyruvate

PPP-pentose phosphate pathway

SDA- sabouraud dextrose agar

SDB- sabouraud dextrose broth

TCA- trichloroacetic acid

TLC-thin layer chromatography

UV-ultraviolet

KAJIAN FARMAKOLOGI TERPILIH EKSTRAK METANOL DAUN *Cinnamomum iners* TERPIAWAI

ABSTRAK

Penyaringan fitokimia, antioksida, kajian ketoksikan, aktiviti analgesik dan antimikrob telah dijalankan ke atas ekstrak daun metanol *Cinnamomum iners* yang telah dipiawaikan (CSLE). Penyaringan fitokimia menunjukkan kehadiran glikosida kardia, flavonoid, fenolik, saponin, tannin dan terpenoid. Kehadiran metabolit sekunder ini di dalam CSLE mungkin penyumbang kepada bioaktiviti CSLE. Secara keseluruhannya, kajian antioksida mendapati CSLE menunjukkan aktiviti antioksida yang sederhana baik. Aktiviti antioksida ini mungkin disebabkan oleh kandungan fenolik yang tinggi di dalam CSLE (211.94 ± 13.04 ekivalen mg asid gallik/g jisim tumbuhan). Penyelidikan juga telah dijalankan bagi menentukan ketoksikan CSLE memandangkan tumbuhan ini telah digunakan sejak lama dahulu sebagai herba tradisi. Penyaringan ketoksikan CSLE dijalankan menggunakan ujian anak udang brin, ketoksikan akut dan histopatologi. Ujian ketoksikan akut dijalankan berpandukan arahan OECD 423 (Organisasi Kerjasama dan Pembangunan Ekonomi). Ujian ketoksikan mendapati CSLE selamat digunakan dengan nilai LD_{50} dan LC_{50} yang tinggi. Penyaringan aktiviti analgesik CSLE dijalankan menggunakan ujian formalin, plat panas dan jentik ekor pada dos 100, 200 dan 500 mg/kg pada mencit. CSLE menunjukkan aktiviti yang signifikan ($P<0.05$) pada tikus bagi fasa terakhir ujian formalin pada dos 200 dan 500 mg/kg. Walaubagaimanapun, CSLE tidak menunjukkan sebarang aktiviti bagi ujian plat panas dan jentik ekor. Kajian ini menunjukkan bahawa CSLE bertindak pada sistem saraf periferi bagi melegakan sakit. Bagi penyaringan aktiviti antimikrob, CSLE, fraksi (heksana, etil asetat, butanol dan akueus) dan sebatian bioaktif yang telah dipencilkan diuji menggunakan

kaedah yang berbeza beza. Kaedah pembauran cakera, nilai perencatan minimum (MIC) dan nilai kepekatan maut minimum (MBC) telah ditentukan bagi CSLE dan fraksi menggunakan bakteria gram positif, gram negatif dan yis. Kajian lanjut bagi mengenalpasti sebatian antimikrob dijalankan menggunakan teknik bioautografi. Kedah bioautografi menunjukkan kehadiran satu sebatian dari fraksi etil asetat (nilai Rf 0.77) yang merencat pertumbuhan MRSA (*Staphylococcus aureus* rintang metisillin). Sebatian yang sama juga didapati merencat pertumbuhan *Escherichia coli*. Sebatian tersebut telah berjaya dipencilkan dan dikenalpasti meggunakan spektroskopi ultralembayung, inframerah, spektroskopi ^{1}H dan ^{13}C resonans magnet nukleus dan spektroskopi jisim. Sebatian bioaktif tersebut dikenalpasti sebagai xanthorrhizol 2-methyl-5-[(2R)-6-methylhept-5-en-2-yl] phenol. Secara keseluruhannya, keputusan kajian ini mendapati daun *Cinnamomum iners* dan sebatian yang dipencilkan (xanthorrhizol) berpotensi sebagai sumber ubatan antimikrob yang baru bagi MRSA dan juga mikroorganisma patogen yang lain.

SELECTED PHARMACOLOGICAL STUDIES OF *Cinnamomum iners* STANDARDIZED LEAVES METHANOLIC EXTRACT

ABSTRACT

Cinnamomum iners standardized leaves methanolic extract (CSLE) was subjected to phytochemical screening, antioxidant, toxicity studies, analgesic and antimicrobial activity. Phytochemical screening of CSLE showed the presence of cardiac glycoside, flavanoid, polyphenol, saponin, tannin and terpenoid. These secondary metabolites might be contributed to the bioactivity of CSLE. The overall antioxidant research provides information that CSLE showed moderate antioxidant activity. This might be due to the high phenolic content (211.94 ± 13.04 mg GAE/g plant material) in CSLE. Our study also investigated the safety parameters (toxicity) of CSLE to provide information regarding long term usage of this plant as traditional herbs. The toxicity screening of CSLE was conducted using brine shrimp assay, acute toxicity screening and histopathologic study. An acute toxicity study was carried out using OECD (The organization of Economic Co-operation Development) guideline 423. CSLE is considered safe for consumption with high LC_{50} and LD_{50} values. The analgesic activity of CSLE was evaluated using formalin, hot plate and tail flick tests at doses of 100, 200 and 500 mg/kg in mice. CSLE showed significant activity ($P<0.05$) in the formalin model (late phase) on the rats at 200 and 500 mg/kg. However, CSLE did not show activity in the hot plate and tail flick test. The results obtained suggest that CSLE act peripherally to relieve pain. As for the antimicrobial activity, CSLE, its fractions (hexane, ethyl acetate, butanol and aqueous) and the isolated compound of CSLE was investigated using different antimicrobial assays. CSLE and fractions were subjected to disc diffusion test,

minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using different gram positive, gram negative and yeast. Subsequent work was focused on the identification of antimicrobial compound by the means of bioautography. Bioautography assay of fractions revealed one compound of ethyl acetate fraction (Rf of 0.77) inhibited MRSA (methicillin resistant *Staphylococcus aureus*). Interestingly the same compound was found to inhibit *Escherichia coli.* The compound was successfully isolated and the isolated compound was identified by using ultraviolet, infrared, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectroscopy. The compound was recognized as xanthorrhizol 2-methyl-5-[(2R)-6-methylhept-5-en-2-yl] phenol. The overall results provide evidence that *Cinnamomum iners* leaves as well as the isolated compound (xanthorrhizol) exhibited potential as new antimicrobial drug for MRSA as well as other pathogenic microorganisms

CHAPTER 1

INTRODUCTION

Cinnamomum iners leaves are widely practised as traditional medicine for various ailments such as fever, headache, breathing problem and cough. In addition, *Cinnamomum iners* leaves are used to treat problem related to digestive system and appetite. Even though this plant has been used for a long time, there is a lack of scientific data regarding this plant. Therefore, scientific data need to be built up to document the potential of this herb. In this context, this work was undertaken to determine the antioxidant capacity, analgesic effect and antimicrobial activity of *Cinnamomum iners* standardized leaves methanolic extract (CSLE). In addition, the safety parameter of CSLE was determined to provide information regarding the long term usage of this plant. The secondary metabolite presence in CSLE was also identified using phytochemical screening procedure.

The antioxidant activity of CSLE was evaluated using scavenging activity and reducing power assay. The analgesic effect of CSLE was investigated using hot plate, tail flick and formalin test. As for the antimicrobial activity, CSLE was subjected to disc diffusion test, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using different gram positive, gram negative and yeast. The findings of the overall research study have provided an insight into the bioactivity of CSLE. This study also has provided scientific evidence to prove the traditional claim of *Cinnamomum iners* leaves against various diseases.

RESEARCH OBJECTIVES

The objectives of the research are listed as follows:

a) To standardize extract of *Cinnamomum iners* leaves by using marker compound.
b) To determine the toxicity of *Cinnamomum iners* standardized leaves methanolic extract by using brine shrimp and acute toxicity studies.
c) To determine the antioxidant activity of *Cinnamomum iners* standardized leaves methanolic extract.
d) To determine the analgesic effect of *Cinnamomum iners* standardized leaves methanolic extract.
e) To determine the antimicrobial properties of *Cinnamomum iners* standardized leaves methanolic extract.
f) To identify the bioactive compound from *Cinnamomum iners* standardized leaves methanolic extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Discovery of Plant- Derived Drugs

2.1.1 Definition, History and Examples of Plant-Derived Drugs

Plants are extremely important matter of our planet which is essential to maintain the balance of nature and plays vital role in human life as they provide us with food, medicine, oxygen, clothing and shelter. Furthermore, medicinal plant is the source of drug which is rich in therapeutic values (Edeoga et al., 2005). The World Health Organization (WHO) estimated that 70 to 80% of the developing countries implemented traditional medicine mainly from herbs as the alternative medicine. Medicinal plants have high economic impact to the world and are widely used by large majority of population to cure disease and illness (Bauman, 2004). The plant extracts and their constituents play major role in traditional medicines and therapies (Willet, 1994). Pang et al. (2000) reported that traditional herbal medicine is considered as an art of healing. The pharmacological activity of herbal medicine is contributed by the specific biologically active compounds and also the synergistic effect of plant extract. Synergistic effect results from the combination of two or more chemical components present in the herbal mixture (Pang et al., 2000).

Ethno pharmacological documentation on the safety of locally used herbal plants can lead to the development of new herbal drugs (Cowan, 1999). There is now much scientific activity on the use of plants by cultural groups (ethnopharmacology) since it is realized that much knowledge is being lost due to the breakdown of many traditional societies due to urbanization (Raman and Houghton, 1998). The term of plant derived drugs can be defined as isolation of substances from plant that possess biological activity. Based on the statistical analysis, approximately 119 pure

compounds isolated from plants are used as medicine throughout the world such as aspirin, quinine and morphine (Farnsworth and Pezzuto, 1983).

Acetylsalicylic acid or commonly known as aspirin is derived from *Salix alba* (white willow). Aspirin which is consumed by 80 million people in US alone is widely practice as medication to relieve pains, inflammation and reduce fever. In addition, aspirin is extremely useful as an anti-platelet agent to prevent heart attacks and strokes. Another good example of plant based drug is quinine. Quinine which originated from *Cinchona ledgeriana* bark is very useful for the treatment of malaria (Farnsworth and Pezzuto, 1983). Serpentine isolated from the root of the Indian plant *Rauwolfia serpentina* was an outstanding contribution for lowering blood pressure and treatment of hypertension (Farnsworth et al., 1967). Vinblastine isolated from the *Catharanthus roseus* is used for neck cancer and leukemia in children. Pophyllotoxin derived from *Phodophyllum emodi* was used for treatment against testicular, small cell lung cancer and lymphomas (Farnsworth and Bingel, 1977). The chemotherapy drug paclitaxel (Taxol), used in breast, ovarian and lung cancer treatment, is derived from *Taxus brevifolia* (Farnsworth and Bingel, 1977). Between 1971 to 1990 new drugs such as ectoposide, E-guggulsterone, teniposide, nabilone, plaunotol and ginkgolides continue to be introduced all over the world.

Table 2.1: Examples of plant-derived drugs

Drugs	Properties	Plant
Borneol	Antipyretic, analgesic, antiinflammatory	Several plants such as *Dryobalanops aromatic* and *Blumea balsamifera*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Ectoposide	Anticancer	*Podophyllum peltatum*
E-guggulsterone	Antidiabetic	*Commiphora weightii*
Morphine	Analgesic	*Papaver somniferum*
Nordihydroguaiaretic acid	Antioxidant	*Larrea divaricata*
Pophyllotoxin	Anticancer	*Phodophyllum emodi*
Quinine	Antimalaria	*Cinchona ledgeriana*
Rotundine	Analgesic, sedative, tranquilizer	*Stephania sinica*
Salicin	Analgesic	*Salix alba*
Serpentine	Antiinflammatory	*Rauwolfia serpentina*
Taxol	Analgesic	*Taxus brevifolia*
Tetrahydropalmatine	Analgesic, sedative, tranquilizer	*Corydalis ambigua*
Vinblastine	Anticancer	*Catharanthus roseus*

(Farnsworth and Bingel, 1977)

2.1.2 Importance of Drugs Discovery from Plants

In recent times, the development of synthetic drug is gaining attention from scientist worldwide. Synthetic drugs refer to bioactive substance of plant that is being derived from chemical process (not originated from plants) (Verpoorte, 2000). However, synthesizing some of the plant derived drug such as morphine and cocaine remains a challenge for researchers. Some drugs cannot be synthetically produced such as atropine and reserpine due to high costs (Rahman et al., 2005). Hence, the isolation of plants derived drugs is a key determinant in drug discovery.

2.2 Selection of Plant for Drug Discovery

The estimated higher plant species that exist on this earth is 250 thousands. From this number, only about 15 thousands have been evaluated for their biological activity (Verpoorte, 2000). Realizing the fact that undiscovered plants might have some therapeutic value, many researchers have focused on discovering potential plant sources with medicinal properties. However, there are some broad starting points of selecting plant material of interest. Several reviews had proposed method for selecting plants of choice as the subject of research. Phytochemical screening is a commonly used method by the developing countries which employs random selection of plant materials followed by chemical screening for the identification of bioactive compounds (Farnsworth, 1966). Second approaches are random selection followed by biological assessment of the plants. Biological activity was evaluated by means of experimental animals such as mice and rats (Suffness and Douros, 1982). Plants of interest might also be selected based on the traditional claims for the treatment of diseases (Bannerman et al., 1983). These plants have ethnobotanical background which is related to ancient people natural remedies. Example of plant that was selected based on the ethnobotanical background is turmeric (*Curcuma*

longa) that has been practised in Indian Ayurveda for various ailments. Based on the traditional claim that turmeric exhibit antimicrobial activity, several studies was conducted and lead to the discovery of curcumin (Aggarwal et al., 2005).

2.3 *Cinnamomum iners*

2.3.1 Morphological description

Cinnamomum iners is a genus of evergreen trees and shrubs belonging to the family, Lauraceae. According to the Agroforestry Tree Database, *Cinnamomum iners* is commonly found in India, Burma (Myanmar), Indo-China, Thailand, Peninsular Malaysia, Sumatra, Java, Borneo, Sulawesi and the Southern Philippines. It is a medium-sized evergreen tree found throughout lowland and hill forest of Malaysia. It is locally known as *kayu manis hutan*, *medang kemangi* and *teja*.

Usual appearances of *Cinnamomum iners* is about 60 feet height with dense, bushy and dull green in colour. The leaves are arranged either opposite or nearly opposite with three main longitudinal veins, 7.5-30 cm long, 2-13 cm wide (Choi et al., 2003). Flowers are very small, creamy white or yellowish in long stalked panicles. Fruit is round, blue black in colour when ripening. The bark is greyish brown in colour, rather smooth and even (Corner, 1952).

Taxonomy of *Cinnamomum iners*

Kingdom : Plantae

Division : Magniliophyta

Class : Magnoliopsida

Order : Laurale

Family : Lauraceae

Genus : *Cinnamomum*

Species : *Iners*

Figure 2.1: Leaves of *Cinnamomum iners*

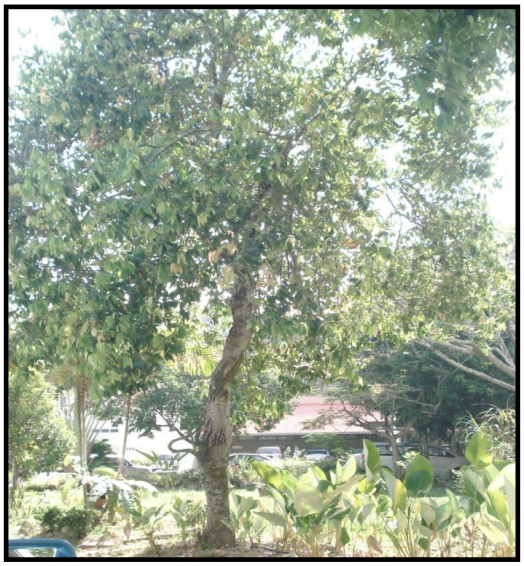


Figure 2.2: Tree of *Cinnamomum iners*

2.3.2 Bioactivity Studies of *Cinnamomum iners*

Although *Cinnamomum iners* is widely cultivated and practised medicinally, the information on its biological properties is still limited. Due to the presence of terpenes, it is widely used as traditional medicine to relieve fever, for digestive system and appetite problem (Pengelly, 2004). In addition, this plant leaves have been used for rheumatism, headache, breathing problem and cough. The major bioactive compounds of this plant leaves are saponin, terpene, cinnamic aldehyde and eugenol (Choi et al., 2003). Based on the traditional claim, several studies had been carried out to prove this claim scientifically. Researchers from India reported that oil obtained from stem bark of *Cinnamomum iners* contain 1, 8-cineole as a major component that possess antinociceptive and anti-inflammatory activity (Baruah et al., 2001). The antioxidant activity of essential oil of *Cinnamomum iners* has also been reported (Phutdhawong et al., 2007). Recently, the analgesic properties of this plant leaves have been published by our research team (Mustaffa et al.,

2010a). The antioxidant activity and toxicity of this plant was recently reported by our research team (Mustaffa et al., 2010b). The ethnobotanical reports offer information on the medicinal properties of *Cinnamomum iners* root extract that include details on their antiplasmodial activity and cytotoxicity (Wan et al., 2007). Furthermore, an amylase inhibitory property of this plant has been revealed (Iida et al., 1997).

2.4 Plant Secondary Metabolites

2.4.1 Definition of Plant Secondary Metabolites

Most of the available medicines are derived of plants, it shows that they contain organic compounds with pharmacological activities that can act as pharmaceutical drugs. Plant organic compounds are synthesized from complex metabolism processes. The biosynthetic pathways are universal in plants and are responsible for the occurrence of both primary metabolites and secondary metabolites. Primary plant metabolites are directly involved in the growth, reproduction and development of the plant itself. Secondary metabolites are not directly involved in those processes but are classified as organic compounds that are biologically active (Chakraborty and Brantner, 1999). Examples of plant secondary metabolites are alkaloids, terpenoids, tannins and steroids. In fact there are numerous studies which have revealed the presence of such compounds with antimicrobial, antioxidant and analgesic activities (Arima and Danno, 2002). Terpenoids consist of important groups of active compounds with over 20 000 known structures. Examples are gossypol, parthenolide and artemisinin. Gossypol was derived from *Gossypium herbaceum* and used as an antifertility agent in human (Huang, 1993). Parthenolide from feverfew (*Tanacetum parthenium*) has been used for the treatment of migraine,

while artemisinin from *Artemisia annua* is widely used for malaria (Beekman et al., 1998).

Table 2.2: Examples of secondary metabolites from plants and its pharmacological activities

Plants	Secondary metabolites	Pharmacological activities
Andrographis paniculata	Diterpenoid, flavonoids, polyphenol	Anticancer, anti-inflammatory, hepatoprotective, anti-hyperglycaemic
Curcuma xanthorrhizol	Sesquiterpenoid	Antibacterial, antiinflammatory
Cyanara scolymus	Polyphenol	Hepatoprotective
Eurycoma longifolia	Triterpene, saponin	Antimalaria, antibacterial, antioxidant, treatment of impotence
Ficus deltoidea	Polyphenol, flavonoid, tannin	Antidiabetic, antinociceptive, antioxidant
Garcinia mangostana	Polyphenol, tannin	Antioxidant, antitumoral, anti-inflammatory, antiallergy, antibacterial, antimalarial
Morinda citrofolia	Alkaloid, flavonoid	Anticancer, antitumour, antinociceptive, antihypertension
Orthosiphon stamineus	Flavonoid, polyphenol	Antidiabetic
Punica grantum	Polyphenol	Antioxidant, antinociceptive

(Arima and Danno, 2002)

2.4.2 Plant Secondary Metabolism Pathways

Recent studies continue to describe some of the secondary metabolic pathways leading to active phytochemicals. The pentose phosphate pathway (PPP) is important in the sense that it produces reducing equivalents in the form of NADPH which is used in the synthesizing substances such as nucleic acids and protein. PPP also produces erythrose-4-phosphate (E4P), used in the synthesis of aromatic amino acids. The aromatic amino acids are converted into phenolic compound via shikimic acid pathway (Ruhland, 1958).

The shikimic acid pathway, regarded as the important biosynthetic pathway is the reaction between phosphoenol pyruvate (PEP) and erythrose-4-phosphate (E-4P) to yield phenylalanine-related alkaloids, polyphenol such as flavonoids and condensed tannins (Street and Cockburn, 1972) (Figure 1.3). The shikimic acid pathway also converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids (Herrmann and Weaver, 1999). The 1-deoxy-d-xylulose 5-phosphate **(**DXP**)** pathway is responsible for the formation of essential oil monoterpenes (such as menthol in peppermint), sesquiterpenes, diterpenes, carotenoids and polyphenol. The mevalonate pathway operating in the cytosol gives rise to triterpenes, sterols and most sesquiterpenes (Ruhland, 1958).

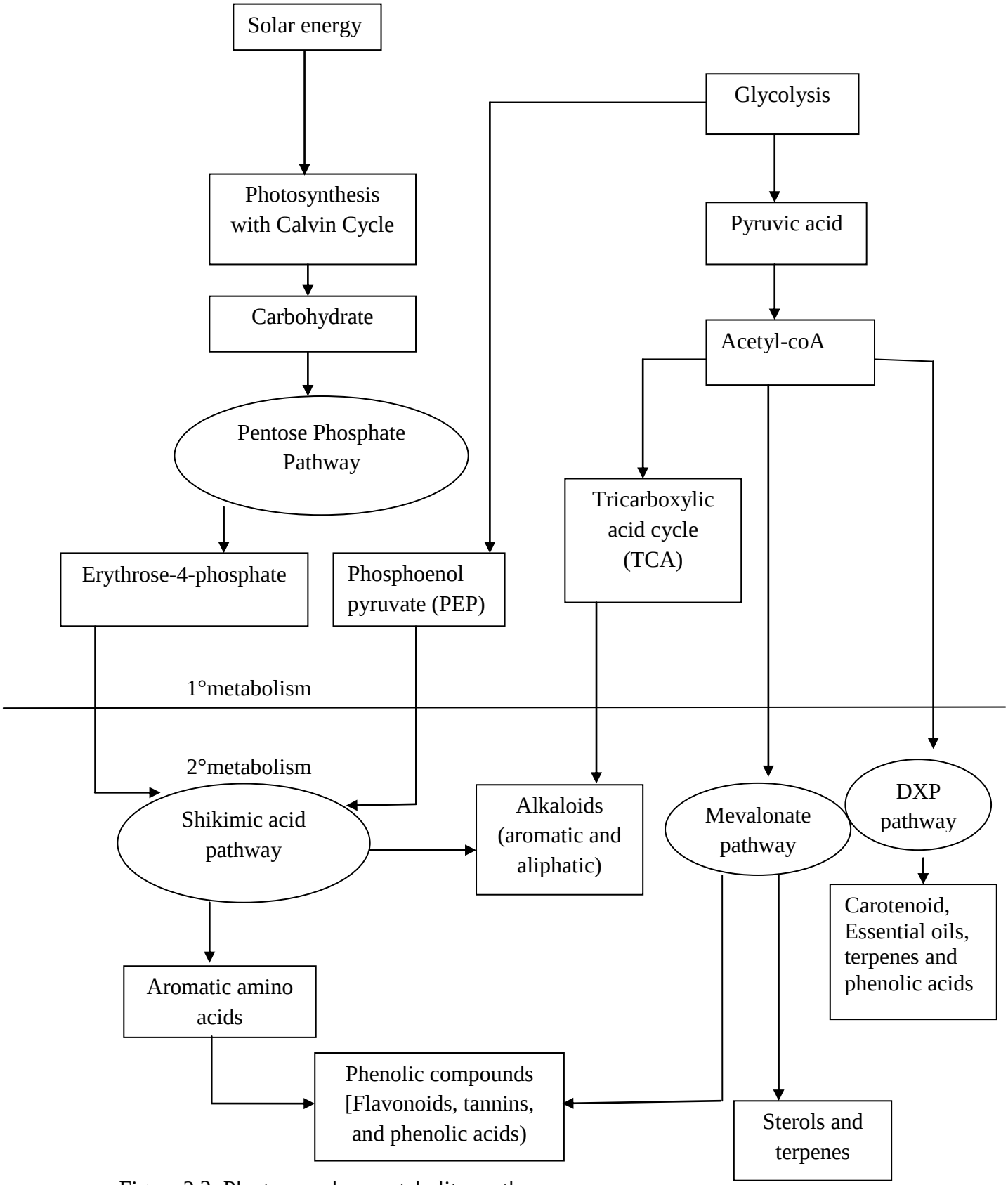


Figure 2.3: Plant secondary metabolites pathway

(Street and Cockburn, 1972)

2.5 Standardization and Isolation of Plant Compounds

Approximately 80% of people living in developing countries still rely on herbal plant for their health care (Verma and Singh, 2008). However, the quality control of herbal medicine remains to be a difficult task because of the variability of active constituents involved (Lijuan et al., 2007). Hence, WHO has approved fingerprint technique to obtain standardized herbal extract for quality assurance purposes which results in the consumer confidence (WHO, 1991). Standardized extract which identified one or two classes of bioactive compounds is the basis for herbal product development (Heinrich, 2007). Quality control of herbal medicines by the means of chromatography has become the common technique because of its consistency. It served as a technique for identification and authentication of herbal drugs which will then assured the quality control of these drugs (Gong et al., 2003). Various standardization procedures using thin layer chromatography, gas chromatography and high performance liquid chromatography are widely applied for this purposes.

Standardization or chemical fingerprint represents a chromatographic pattern which can demonstrate both “sameness” and “differences” among various batches of sample based on the quantity of the marker compounds (Pang et al., 2000). Standardization as well as isolation of plant compounds is a key determinant in development of herbal medicines.

Recently investigators have focused on isolation and characterization of plant-derived compound without regards to bioactivities. For the identification of novel compound from plant, bioassays must be carried out. The sequence of identification of bioactive compound begins with screening of extracts for biological

activity, followed by fractionations directed bioassays and identification of active substances (Mc Laughlin, 1998).

2.6 Antioxidant Activity

Cancer is the leading cause of death in United States. Similarly, in Malaysia, around 70 thousand of cancer cases were diagnosed among Malaysians between 2000 to 2005 (Wu et al., 2005). Around 32% of death caused by cancer could be avoided by the intake of antioxidant rich food source (Willet, 1994; Chen et al., 2006; Haliwell and Gutteridge, 1999). Several studies had proven that cancer and other diseases such as coronary heart diseases, artherosclerosis and aging is related to reactive oxygen species (ROS). Examples of ROS are superoxide radical ($O^{\cdot\bullet-}_2$), hydroxyl radicals ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$). These free radicals are being derived from the normal respiration processes (Madhavi et al., 1996). Exogenous factors such as pollution may also contribute to the formation of the free radicals (Subashree et al., 2009). Free radicals consist of unpaired electron which causes them to be chemically reactive. These chemically reactive radicals will interact with other molecules such as DNA, lipid and protein and will lead to oxidative damage to those molecules (Cadenas and Davies, 2000). Minimizing oxidative damage is the essential step in the prevention of ROS associated diseases. Human body had developed defence system to completely remove ROS by the means of enzymes such as gluthatione S-transferase, glutathione peroxidise, superoxide dismutase and catalase (Diplock, 1994). However, the imbalance between free radicals and antioxidants will lead to oxidative stress, resulting in protein damage and lipid peroxidation.

Lipid peroxidation also may occur in food and cause food spoilage which contributes to decrease in food quality. Lipid peroxidation of food is an alarming

issue since it affects both the consumer and producer due to the rancid odours and flavours which it creates. As a solution, synthetic antioxidant such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butyhydro-quinone (TBHQ) are widely employed in food industry to combat lipid peroxidation. However, scientific results have proven that BHA and BHT can cause liver damage and carcinogenesis (Ito et al., 1986 and Whysner et al, 1994). So as an alternative a large body of research had focused on medicinal plants as alternatives for antioxidant source.

Natural antioxidants present in the plants are closely linked to their ability to combat reactive oxygen species. Since ancient time people has been using various plants which possess antioxidant properties as medicines. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals. Antioxidants may also enhance immune defence system, thus reducing the risk of cancer and infection (Kumaran and Karunakaran, 2007 and Wu et al., 2005). Moreover, it has been revealed that the antioxidant potential of plant products against various diseases is mainly due to the presence of phenolic compounds such as phenolic diterpenes, phenolic acids and flavonoids (Chua et al., 2008 and Atmani et al., 2009). Therefore, many researchers have focused on screening, characterization and isolation of potent polyphenolic compounds. As these compounds are predominantly present in plant and serve as indicators for antioxidant capacity, it is therefore worthwhile to quantify the phenolic content in the plant material (Mokbel and Hashinaga, 2005). Hence, antioxidant assays are widely used for assessing medicinal properties of plant material (Ozsoy et al., 2008). Some general assay for evaluating the antioxidant capacity of plant material or pure compound include DPPH (2,2-diphenyl -1-picrylhydrazyl), reducing power, H_2O_2 (hydrogen peroxide), ESR (Electron Spin Resonance), ORAC (Oxygen Radical

Absorbance Capacity) and ABTS [2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate)] (Scherer and Godoy, 2009).

2.7 Toxicity

Plants commonly used in traditional medicine are assumed to be safe as they are formed naturally. This safety is also based on their long history of usage in the treatment of diseases over centuries. However, scientific evidence has shown that many traditional herbs are toxic and can lead to cancer (Schimmer et al., 1988). For example, mushroom and stone fruit have been documented by Fenton (2002) for its toxicity and presence of potential toxins that cause death. Toxicology studies contributed greatly in the area of therapeutic research yet the information regarding toxicity of traditionally used plant is very limited. Furthermore, the prescription and use of traditional medicine is currently not regulated, with the result that there is always the danger of misadministration, especially of toxic plants. The prolonged use of certain popular herbal remedies may contribute to the potential genotoxic effects, hence toxicity study is required (Maiga et al., 2005 and Schimmer at al., 1988).

Several reviews reported that for every 10 000 newly synthesize bioactive compound in US, 20 will be evaluated for the *in vivo* toxicity and out of this figure only 10 will reach for the clinical studies (Vagelos, 1991). This suggests that some bioactive compounds are toxic and demand toxicity studies before further development. Thus, brine shrimp lethality bioassay that does not require too much specialization and is cheap can be used as alternative for preliminary toxicity screening. Brine shrimp assay enables to determine the value of medium lethal concentration (LC_{50}) for the active compounds presence in the extract in 24 hours (Massele and Nshimo, 1995). Haecke and Persoone (1982) have proposed this

bioassay as a standard test for toxicity assessment of natural toxins in environment and plant extracts. A simple zoologic organism that has been used for this purpose is brine shrimp or scientifically known as *Artemia salina* (Mc Laughlin et al., 1998). The first approach on the use of brine shrimp for the toxicity evaluation was reported in 1956. Since then researchers have widely used this animal for laboratory purposes (Michael et al., 1956). Furthermore, the simplicity of this method had established this bioassay as a general preliminary toxicity screening for plant extracts (Sam, 1993).

Another routinely performed method for monitoring toxicity of bioactive plant product is by the determination of LD_{50} (the dose which is lethal to 50% of the experimental animals). LD_{50} serves as the basis for further development of certain drugs. LD_{50} value has been assessed by using subchronic, chronic and acute oral toxicity studies. Subchronic and chronic toxicity studies involved repeated administration of tested substances and observation period of 28 days and three months respectively. In contrast, acute oral toxicity is the short term toxicity studies in 14 days after single administration of tested substances (Akhila et al., 2007). Acute oral toxicity studies are used as references for selecting starting doses for other animal work studies. Acute poisoning due to traditional medicines are not uncommon, yet estimates of mortality is 43% in South Africa (Stewart and Steenkamp, 2000). In Malaysia many cases of poisoning are not reported, hence the mortality rate could not be estimated (Thomsan, 2002 and Popat et al., 2001). Most of investigators carry out the *in vivo* toxicity assay in accordance to OECD (Organisation for Economic Co-operation and Development) guideline (Singh et al., 2009; Mustaffa et al., 2010a; Ridtitid et al., 2008 and Reanmongkol et al., 2007). The OECD guideline for testing up chemical was established by using internationally agreed testing methods used by government, industry and laboratories to assess the

safety of chemical products. It is required for all the animal based research to be carried out according to this guideline. Generally, acute toxicity assay is followed by histopathological analysis of various organs (Chen et al., 2007). All animals surviving to the end of the study and all animals dying during the period of observation will be subjected for autopsy and histopathological analysis. Histopathology analysis will be utilized as a useful tool for detection of organ damage or any adverse effects after the use of chemical substances.

Table 2.3: Example of medicinal plants and their toxic effects

Botanical name	Traditional uses	Toxic signs reported
Acacia senegal	Sexual diseases, haemorrhoids	Diarrhoea, abdominal pains, vomiting
Anogeissus leiocarpus	Malaria, wound, constipation	Salivation, nausea, vomiting
Cassia alata	Malaria, constipation	Diarrhoea
Cassia sieberiana	Abdominal pains, malaria, jaundice, headache	Diarrhoea, vomitting
Entada africana	Malaria, jaundice	Vomiting
Gardenia ternifolia	Malaria, jaundice	Diarrhoea, vomiting
Optilia celtidifolia	Malaria, abdominal pain, constipation	Diarrhoea, trembling, vomiting
Securidaca longepedonculata	Haemorrhoids, dermatitis, snake bite, abdominal pains, headache	Diarrhoea, vomiting
Trichilia roka	Haemorrhoids,fever, malaria, wounds	Diarrhoea, vomitting

(Maiga et al., 2005)

2.8 Analgesic

2.8.1 Pharmaceutical Definition of Pain

Pain is defined as an unpleasant sensory and emotional experience caused by tissue damage. Pain is initiated by stimulation of nociceptors in the peripheral nervous system or central nervous system (Woolf and Mannion, 1999). Around one third of the world population suffer from pain. In the United States $100 billion was spent per year for the treatment of pains (Loeser, 2000). Medicinal plants are important sources of pharmacologically active compounds for the treatment of pains. Almost 80% of people living in developing countries depend on traditional medical practise as drug for therapeutic effects (Calixto, 2005). By definition, a drug is any natural or artificial substance that affects the process or function in the living organism (WHO, 1969).

Pain can be stimulated by thermal, chemical or mechanical nociceptors. There are two basic forms of pain, acute and chronic. Chronic pain is due to mental and physical abnormalities and related to conditions such as back injury, migraine headaches, diabetic, neurophathy and cancer (Stucky et al., 2000). Physical effects include tense muscles, lack of energy and changes in appetite. Emotional effects include depression, anger and anxiety. Chronic pain persists for weeks, months or years (Shipton and Tait, 2005). In contrast, acute pain such as sprain and tissue injury lasts for only a few hours to a few days. Improper treatment of acute pain may lead to chronic pain (Hasford et al., 2004). According to the Malaysian Statistics on Medicine (2006), total opioid consumption for relief of chronic pain is 0.7268 defined daily dose (DDD)/ 1000 population/day (Chye et al., 2008). This figure is very much lower than the opioid consumption in the Nordic countries which ranged

from 14.0 DDD/ 1000 population/day in Finland and 21.8 DDD/ 1000 population/day in Sweeden (Nordic medico, 2004).

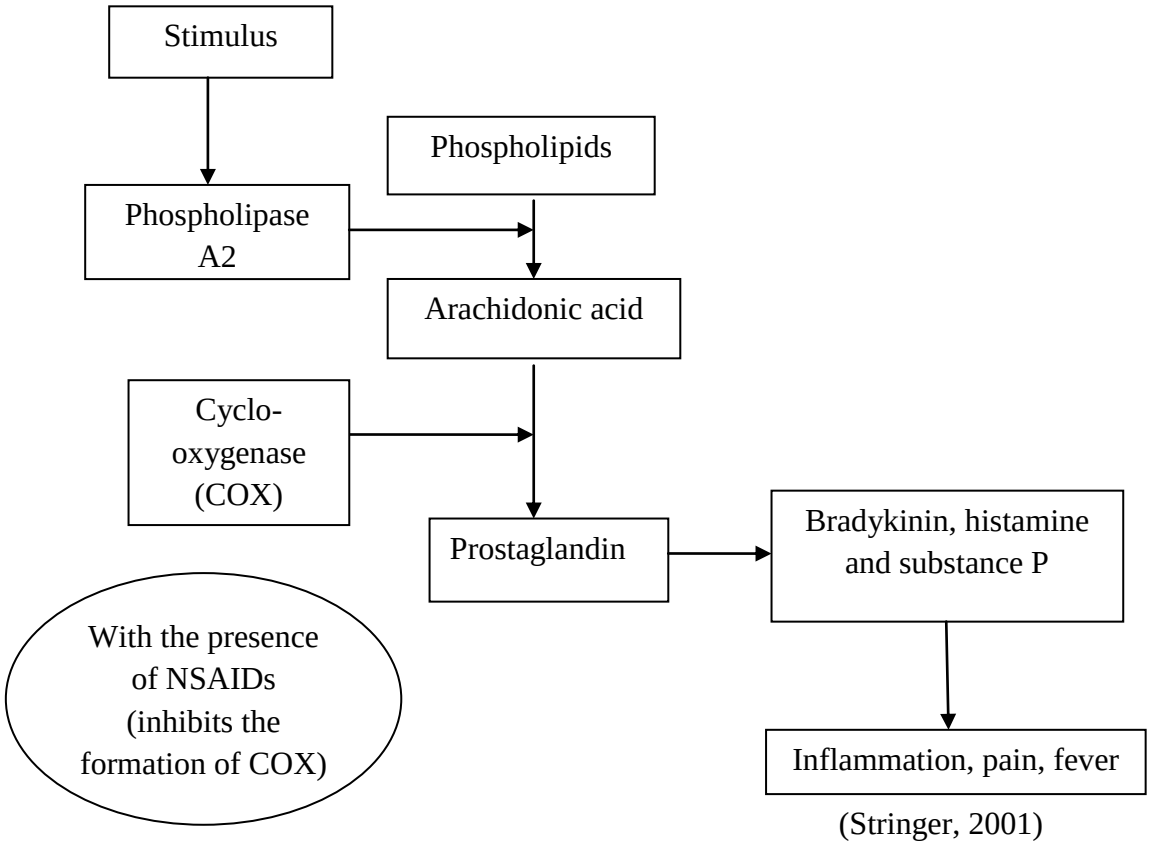
There is no test or instrument to measure the intensity of pain specifically. However, instrument such as electromyography (EMG), magnetic resonance imaging (MRI) and X-rays can detect the cause of pain in muscle and tissues. A neurological examination is used to determine whether the nervous system is impaired (Oomen, 2008).

Drugs which result in relieving pains are categorized as analgesic drugs. Kaufman et al. (2000) reported that in the United States, the three most commonly used drugs were analgesic type of drugs which are acetaminophen, ibuprofen and aspirin. Analgesic drugs are classified as narcotic and non narcotic drugs. The non narcotic drugs are non steroidal anti-inflammatory drugs (NSAIDs) and paracetamol. Opioids are categorized as narcotic drugs.

2.8.2 Non steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs have analgesic, antipyretic and anti-inflammatory actions. Examples of these drugs are aspirin, ibuprofen and naproxen (Stuart and Warden, 2010). NSAIDs are effective for the treatment of mild to severe pain including cancer and post surgical pain. The mechanism of analgesic action of NSAIDs is mediated peripherally via inhibition of cyclooxygenase (COX) enzyme (Burian and Geislinger, 2005). COX enzyme catalyzes the conversion of arachidonic acid to prostaglandin (Figure 2.4). Prostaglandin modulates components of inflammation (bradykinin, histamine and substance P) as well as involvement in body temperature and pain transmission (Stringer, 2001). With the presence of NSAIDs the formation of COX

will be inhibited (Figure 2.4). Two isoforms of the COX enzyme have been identified which are COX-1 and COX-2. COX-2 is expressed constitutively in brain and kidney and is induced at sites of inflammation. COX-1 is used for platelet formation and for the protection of the stomach. Inhibition of COX-1 will cause peptic ulcer as well as alteration of platelet function. Specific COX-2 inhibitors such as rofecoxib and celecoxib (NSAIDs) show anti-inflammatory activity without affecting COX-1 (Moore, 2009). NSAIDs are being metabolized by the liver and were proven to be more effective than paracetamol (Zhang and Po, 1998). Overdose of NSAIDs might cause gastrointestinal complication (Henry and Gettigan, 2003).



(Stringer, 2001)

Figure 2.4: Pathway of NSAIDs action

2.8.3 Opioids

By definition, an opioid is a chemical that works by binding to opioid receptors and used to treat moderate to severe pain. Opioids have actions at the central and peripheral nervous system. Examples of natural opioids are morphine and codeine (Moore, 2009). Synthetic opioids are pethidine and fentanyl (Hodgson et al., 1999). Narcotic agents are potent analgesics which are effective for the relief of severe pain such as cancers. Within the central nervous system, opioids have effects in many areas including the cerebrum, medulla and spinal cord. The action of opioid in the peripheral nervous system is in the tissues such as joints, smooth muscle and gastro intestinal tract (Moore, 2009).

The presynaptic inhibition by opioid is considered to be the major effect in the nervous system by preventing primary afferent fiber from releasing neurotransmitter. The mechanism of action for opioid is by binding to specific pain receptors in the central nervous system (CNS). Hence, it reduces the perception of pain (Stringer, 2001). Three major types of opioid receptor include μ, δ and k (mu, delta and kappa). Once tissue injury happen, neuron releases signalling chemicals called neurotransmitter in the synapse. Synapse is the space between two neurons. Receptors are able to detect the neurotransmitter, hence perform an action of pain transmission (Ray and Ksir, 2002). One analogy that has been used to describe the interaction between the neurotransmitter and its receptor is that of lock and key, the neurotransmitter fits into a receptor as a key fits into a lock (Willis and Westlund, 1997). Some examples of neurotransmitter that is inhibited by psychoactive drugs are acetylcholine, norepinephrine, dopamine, serotonine, γ-aminobutyric acid (GABA) and the endorphins. Morphine, by an action on μ receptors, inhibits release

of several different neurotransmitters including noradrenaline, acetylcholine and the neuropeptide (substance P). The opioid antagonist, naloxone, inhibits all opioid receptors, but has highest affinity for μ receptors (Chahl, 1996).

If the opioid drug is discontinued, withdrawal symptoms such as sweating, nausea and addiction are experienced by chronic users. Opioid addiction test carried out by Ghodse et al. (1999) reported that out of 127 opioid users, 103 (81.1%) developed addiction behaviour. Opioid also may cause several adverse effects such as pupillary constriction and constipation. Patients receiving chronic opioid therapy require continuous laxative therapy for the treatment of constipation (Ballantyne, 2007).

2.8.4 Paracetamol

Acetaminophen or paracetamol is an antipyretic drug used to treat acute pain. It possesses anti-inflammatory action via the inhibition of cyclo-oxygenase enzyme in the central nervous system (Hinz et al., 2008). Paracetamol does not work in peripheral nervous system due to the presence of peroxides during inflammation that inhibits the action of paracetamol. Overdose usage of paracetamol can lead to hepatotoxicity which can cause death (Larson, 2007).

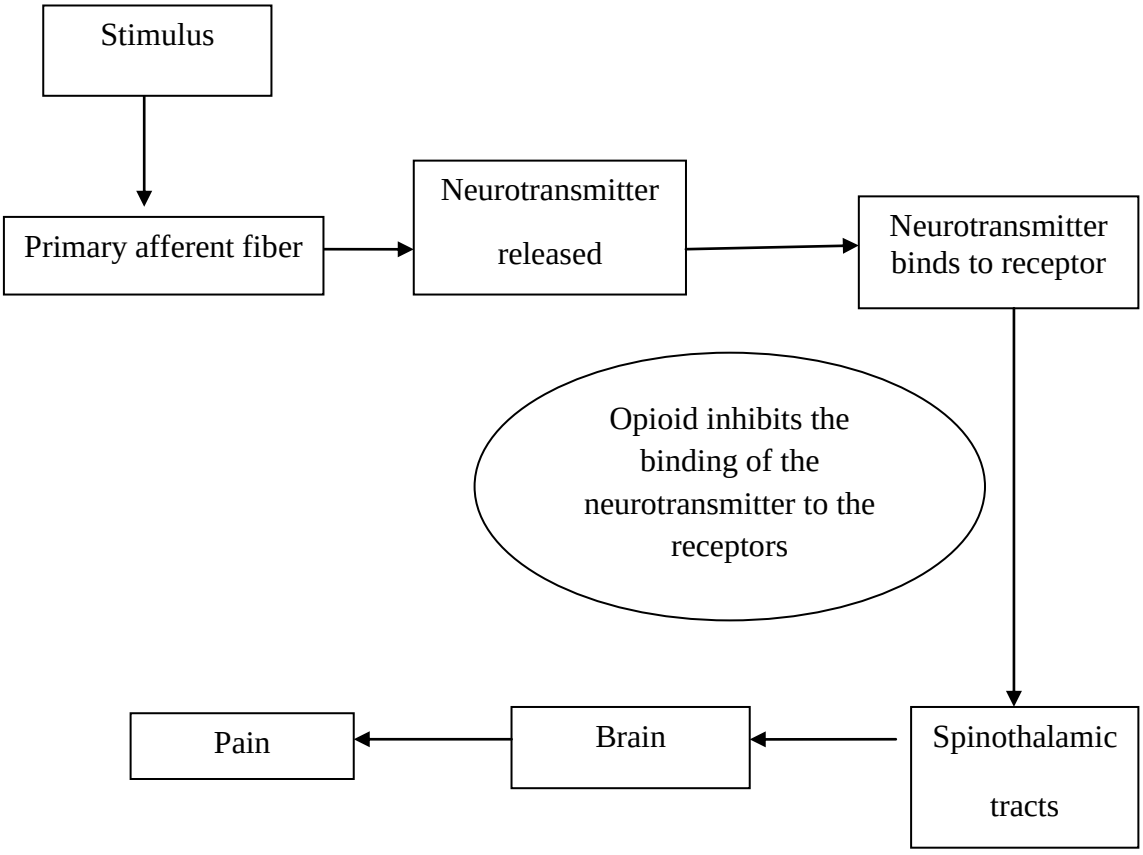


Figure 2.5: Mechanism of opioid. Stimulus will cause primary afferent fiber to release neurotransmitter. Without the presence of the opioid, neurotransmitter will bind to the specific receptor and sent chemical message via the spinothalamic tracts at the spinal cord to the brain. In the brain, chemical messenger was interpreted as pain. With the presence of opioid, it binds at the specific opioid receptors and inhibits binding of neurotansmitter. Hence, pain transmission is blocked.

2.9 Antimicrobial

2.9.1 Antimicrobial Susceptibility Testing

Antibiotics are substances produced by bacteria and fungi that inhibit the growth of other bacteria (Davey, 2000). The discovery of antibiotics that was once regarded as novel in the 20th century is helpful in treating infectious diseases.

However, microbial resistance to antibiotic had developed due to its indiscriminate use for the treatment of infectious diseases. So, scientist worldwide focused on discovery of new antimicrobial drugs from medicinal plants (Kumar et al., 2006). The screening of plants with potential antimicrobial activities against pathogenic microorganisms serves as the first step in discovery of antimicrobial drug. A variety of methods are found for the screening purposes such as bioautography, diffusion and dilution methods (Valgas et al., 2007). The qualitative antimicrobial methods which indicate the presence or absence of antimicrobial activity or compounds are disc diffusion and bioautography.

The disc diffusion method makes use of the filter Whatman, which is composed of cellulose [ß-(1-4) linked glucose monomer]. The bioautography method it employs the use of chromatograms and is a useful tool for identification of antibacterial compounds. However, incomplete removal of acidic or alkali solvents from TLC plate might interfere with the results by inhibiting the growth of bacteria (Hamburger and Cordell, 1987). Inhibition zones (white spots) on a purple-red background of TLC plates indicate conversion of p-iodonitrotetrazolium violet (INT) to coloured formazan did not occur due to the presence of antibacterial compound (Eloff and Masako, 2005).

The quantitative antimicrobial assay includes dilution method which determines the minimal inhibitory concentration (MIC) of test sample (Vanden and Vlientick, 1991). The MIC values vary with the test organism, inoculums size, incubation time and aeration. The use of dye such as p-iodonitrotetrazolium violet (INT), triphenyl tetrazolium chloride (TTC) and methyl thiazolyl tetrazolium (MTT) serve as an indicator for microbial growth (Eloff, 1998). The dye was used based on the fact that as the bacteria grow, it produces an electron which is transferred to the