HEPATOPROTECTIVE AND PHARMACOLOGY STUDIES OF STANDARDIZED ETHANOLIC EXTRACT OF *CURCUMA XANTHORRHIZA* ROXB.

by

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LIST OF SYMBOLS AND ABBREVIATIONS

ΔA	: absorbance
ABTS	: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ALP	: alkaline phosphatase
ALT	: alanine aminotransferase
AST	: aspartate aminotransferase
$AlCl_3$	: aluminium chloride
ATP	: adenosine triphosphate
BDE	: bond dissociation energy
BSA	: bovine serum albumin
b.w	: body weight
cAMP	: cyclic adenosine monophosphate
CAT	: catalase
CCl_4	: carbon tetrachloride
COX	: cyclooxygenase
CMC	: carboxymethylcellulose
Curcuma xanthorrhiza	: *C. xanthorrhiza*
CXRE	: *C. xanthorrhiza* rhizome ethanolic extract
CXRH	: *C. xanthorrhiza* rhizome hexane fraction
CE	: catechin equivalents
cm	: centimeter
DNA	: deoxyribonucleic acid

DPPH	: 2, 2-Diphenyl-1-picrylhydrazyl
EDTA	: ethylene diaminetetraacetic acid
ELISA	: enzyme-linked immunosorbent assay
EI	: electron impact
eV	: ionization energy
FC	: folin-Ciocalteau
Fe	: ferum
FRAP	: ferric reducing antioxidant power assay
GPx	: glutathione peroxidase
GSH	: glutathione
GR	: glutathione reductase
g	: gram
GCMS	: gas chromatography mass spectrometry
GAE	: gallic acid equivalents
h	: hour
H	: hydrogen
HAT	: hydrogen atom transfer
HPLC	: high performance liquid chromatography
HPTLC	: high-performance TLC
H_2O_2	: hydrogen peroxide
HandE	: hematoxylin-eosin
IP	: ionization potential
IC_{50}	: concentration providing 50% inhibition

K^+	: potassium ion
kg	: kilogram
LC_{50}	: medium lethal concentration
LD_{50}	: median lethal dose
LC	: liquid chromatography
LDL	: low-density lipoprotein
L	: liter
MDA	: malondialdehyde
mL	: mililiter
M	: molarity
mmol	: milimoles
mg	: milligram
min	: minute
nmol	: nanomoles
N	: normality
nm	: nanometer
MS	: mass spectrum
NMR	: nuclear magnetic resonance
NSAIDs	: non-steroidal anti-inflammatory drugs
NaOH	: sodium hydroxide
n	: number of replicates
Na^+	: sodium ion
OPLC	: over-pressured layer chromatography

pH	: negative logarithm of H^+ concentration
p.o	: per oral
ROS	: reactive oxygen species
Rt	: retention time
r^2	: correlation coefficient
ROW	: relative organ weight
rpm	: rotation per minute
SET	: single electron transfer
SOD	: superoxide dismutase
SIM	: single-ion monitoring
S.E.M	: standard error of the mean
Sec	: second
s.c	: subcutanoeus
TPTZ	: 2, 4, 6-Tris (2-pyridyl)-1, 3, 5-triazine
TEAC	: trolox equivalent antioxidant capacity
TLC	: thin layer chromatography
Trig	: triglyceride
TP	: total protein
TBARS	: thiobarbituric acid reactive substances
UV	: ultraviolet
U	: unit
v/v	: volume by volume
wt	: weight

w/v	: weight by volume
w/w	: weight by weight
µL	: microliter
µmol	: micromoles
µg	: microgram

LIST OF PUBLICATIONS

1) Devaraj, S., Esfahani, AS., Ismail. S., Ramanathan, S and Yam, M.F. (2010). Evaluation of the antinociceptive activity and acute oral toxicity of standardized ethanolic extract of the rhizome of *Curcuma xanthorrhiza* Roxb. Molecules, 15(4).

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2) Devaraj, S., Ismail. S., Ramanathan, S (2010). Evaluation of the hepatoprotective activity of standardized ethanolic extract of *Curcuma xanthorrhiza* (Roxb). International Conference on Natural Products (ICNP) 2010.

KAJIAN HEPATOPROTAKTIF DAN FARMAKOLOGI EKSTRAK ETANOL TERPIAWAI *CURCUMA XANTHORRHIZA* ROXB.

ABSTRAK

Sifat hepatoprotektif dan farmakologi ekstrak rizom *C. xanthorrhiza* telah dinilai menggunakan model esei *in vivo* dan *in vitro*. Sebelum bioesei, pengekstrakan cecair terpilih dilakukan ke atas ekstrak etanol daripada rizom *C. xanthorrhiza* menghasilkan fraksi heksana, etil asetat dan air. Xanthorrhizol digunakan sebagai penanda untuk pempiawaian ekstrak etanol dan semua fraksi daripada rizom *C. xanthorrhiza*. Kehadiran terpenoid, flavonoid, glikosida kardiak dan saponin dapat diperhatikan melalui analisis kuantitatif penyaringan fitokimia di dalam ekstrak rizom etanol terpiawai *C. xanthorrhiza* (CXRE). Ketoksikan akut secara oral CXRE menunjukkan LD_{50} melebihi 5,000 mg/kg, menunjukkan CXRE adalah selamat untuk digunakan untuk penyelidikan pre-klinikal pada haiwan. Ujian seterusnya ke atas aktiviti antioksida pada CXRE dan fraksi (heksana, etil asetat dan air) telah dijalankan. Esei FRAP, DPPH dan ABTS menunjukkan aktiviti antioksida yang paling tinggi dalam fraksi heksana berbanding ekstrak etanol *C. xanthorrhiza*, fraksi etil asetat dan fraksi air, selari dengan kandungan fenolik dan flavonoid total. Kandungan fenolik dan flavonoid total fraksi heksana masing-masing ialah 61.00 mg GAE/g dan 92.80 mg CAE/g. Fraksi heksana rizom *C. xanthorrhiza* yang telah dipiawaikan (CXRH) pada dos 125, 250 dan 500 mg/kg telah digunakan untuk menilai aktiviti hepatoprotaktif ke atas tikus yang mengalami kerosakan hepar setelah diinduksi dengan carbon tetraklorida (CCl_4). Enzim-enzim hepar (ALT, AST dan ALP), trigliserida, protein serum total menunjukkan penurunan yang signifikan dengan peningkatan pada paras enzim antioksidatif (SOD, CAT, GPx dan GR) dan kandungan

protein total pada hepar dalam tikus yang dirawat dengan berbanding dengan kumpulan yang dirawat dengan CCl_4. CXRH juga didapati memperbaiki aktiviti peroksidasi lipid dan menunjukkan pemulihan pada tisu hepar yang dirosakkan oleh CCl_4. Aktiviti farmakologi seterusnya dikaji untuk aktiviti antinosiseptif dalam tikus dengan menggunakan 3 model yang berbeza iaitu plat panas, jentik ekor dan ujian sakit induksi formalin. CXRE tidak menunjukkan aktiviti antinosiseptif yang signifikan dalam model sakit akut tetapi berupaya menekan fasa awal (mekanisme tindakan pusat) dan inflamasi neurogenik (mekanisme tindakan periferi) yang memerlukan penyelidikan lanjut untuk menjelaskan mekanisma yang terlibat.

HEPATOPROTECTIVE AND PHARMACOLOGY STUDIES OF STANDARDIZED ETHANOLIC EXTRACT OF *CURCUMA XANTHORRHIZA* ROXB.

ABSTRACT

The hepatoprotective and pharmacology properties of *C. xanthorrhiza* rhizome extracts were investigated using *in vivo* and *in vitro* model assays. Prior to the bioassays, *C. xanthorrhiza* rhizome ethanolic extract (CXRE) were subjected to liquid-liquid extraction resulting in hexane, ethyl acetate and water fractions. Xanthorrhizol was employed as the marker to standardize the CXRE and fractions respectively. The presence of terpenoids, flavonoids, cardiac glycosides and saponin were observed in the standardized CXRE through qualitative phytochemical screening analysis. The acute oral toxicity of standardized CXRE showed an LD_{50} of greater than 5,000 mg/kg, indicating CXRE is relatively safe for preclinical investigation in animals. Further, the standardized CXRE and its fractions (hexane, ethyl acetate and water) were tested for antioxidant activity. In FRAP, DPPH and ABTS assay, the highest antioxidant activity was found in hexane fraction compared to the CXRE, ethyl acetate fraction and water fractions in line with their total phenolics and flavonoids content. The total phenolics and flavonoids content of hexane fraction were 61.00 mg GAE/g and 92.80 mg CAE/g respectively. The standardized *C. xanthorrhiza* rhizome hexane fraction (CXRH) at doses 125, 250 and 500 mg/kg was further tested for hepatoprotective activity against CCl_4- induced hepatic damage in rats. The liver enzymes (ALT, AST, and ALP), triglycerides, total serum protein showed significant decrease with a substantial increase in the antioxidative enzyme levels (SOD, CAT, GPx, and GR) and total protein content of the liver in

standardized CXRH treated rats as opposed to CCl_4–treated groups. Standardized CXRH was also found to ameliorate the lipid peroxidation activity and showed a good recovery of the CCl_4 damaged hepatic tissues. The pharmacological activity of standardized CXRE was further studied for its antinociceptive activity in rats using three different models, namely the hot plate test, tail flick test and formalin-induced pain test. CXRE did not show significant antinociceptive in acute pain model but was able to suppress the early phase (central acting mechanism) and neurogenic inflammation (peripheral acting mechanism) which requires further investigation to elucidate the exact mechanism involved.

CHAPTER ONE

INTRODUCTION

1.1 Role of plants as medicine

Medicinal plants have played a key role in world health. According to WHO, 80% of the world's populations rely on plant-derived medicines for their healthcare (WHO, 2002). Medicinal plants are distributed worldwide, but they are most abundant in tropical countries. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants (Majno, 1991; Farnsworth and Morris, 1976). Enhancement of such practices lead to the well-established Asian systems of medicines including Ayurveda and Siddha of India, Unani system of middle and Far East Asia, Ying and Yang principles of Chinese herbal medicines, Jamu of Indonesia and others (Sharma *et al*., 1998; Natesh, 2000). About 400 plant species are used in regular production of Ayurvedic, Unani, Siddha and tribal medicine (Rajasekharan and Ganeshan, 2003). It is also a fact that one quarter of all medical prescriptions are formulations based on substances derived from plants. It is likely that the profound knowledge of herbal remedies in traditional cultures developed through trial and error over many centuries, and was passed on verbally from one generation to another (Bensky and Gamble, 1993).

It is estimated that around 122 pure chemical constituents were isolated from higher plants throughout the world. One of the classic examples is vincristine and vinblastine isolated from *Catharanthus roseus*, Apocynaceae. Vinblastine isolated from the *Catharanthus rosesus* is used for the treatment of Hodgkins, choriocarcinoma, non-hodgkins lymphomas, leukemia in children, testicular and neck cancer (Farnsworth *et al*., 1967). Vincristine is recommended for acute lymphocytic leukemia in childhood advanced

stages of Hodgkins, lymophosarcoma, cervical and breast cancer (Farnsworth and Bingel, 1977). Phophyllotoxin, a constituent isolated from *Phodophyllum emodi* is another good example of drug used against testicular, small cell lung cancer and lymphomas. There are many drugs that are commonly used today, for instances, aspirin, ephedrine, ergometrine, tubocurarine, digoxin, galanthamine and apomorphine which has been derived through indigenous medicine via bioscientific investigations of plants (Duke and Martinez, 1994).

In fact, nature has bestowed some plants with the property to prevent, treat and cure hepatic disturbances with interception of fewer side effects. Hepatoprotectives are a class of therapeutic agents that includes synthetic as well as natural product which offer protection to liver from damage or assist in regeneration of hepatic cells (Craker and Simon, 1990). Medicinal herbs are significant source of hepatoprotective drugs. It has been reported that about 170 phytoconstituents isolated from 110 plants belonging to 55 families do possess hepatoprotective activity (Handa, 1991). In general, liver protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, curcuminoids, lignans, essential oils and terpenoids. Clinical research has also shown that herbals have genuine utility in the treatment of liver diseases. However, only a small portion of hepatoprotective plants as well as formulations used in traditional medicine are pharmacologically evaluated for its efficacy (Handa, 1991).

Scientific research in ethnomedicine is ongoing and is growing rapidly especially in countries like Malaysia which has an abundance of natural resources. In the recent years, Malaysian government has shown interest in Malaysian herbal industry which was in line with the National Agricultural Policy (1998-2010) for herbal cultivation. In addition, the Ministry of Health in 1999 has released a Traditional/Complimentary Medicine (TCM)

Policy with the emphasis of 'rational use'; the philosophy that emphasized on the concurrent use of traditional and modern medicines (Puteh, 1999). Currently, herbal plants such as Tongkat Ali (*Eurycoma longifolia),* Kacip Fatimah (*Labisia pumila),* Misai Kucing (*Orthosiphon stamineus)* and Hempedu Bumi, (*Andrographis paniculata)* hava gained extensive research interest locally. Off these plants, Misai Kucing, *Orthosiphon stamineus* and Hempedu Bumi, *Andrographis paniculata* have been extensively reported to their hepatoprotective activity besides their other medicinal values as well (Arif, 2002). *C. xanthorrhiza,* another local plant which has been widely used for anti-aging has also been traditionally claimed for its hepatoprotective activity (Herman, 1985).

In this context, systematic investigation of the hepatoprotective activity of *C. xanthorrhiza* in *in-vivo* model is required to develop a satisfactory herbal therapy to treat liver diseases as to prove its traditional claims. On the other hand, *C. xanthorrhiza* antinociceptive and antioxidant properties together with its toxicity effects are not well documented. With this in view and as part of this thesis, *C. xanthorrhiza* was selected for pharmacological evaluations.

CHAPTER TWO

LITERATURE REVIEW

2.1 The plant *C. xanthorrhiza*

2.1.1 Overview of *C. xanthorrhiza*

C. xanthorrhiza Roxb. is a plant that belongs to ginger family (Zingiberaceae) of the genus *Curcuma*. *C. xanthorrhiza* is a native Indonesian plant, which is indicated by its synonym, namely *Curcuma javanica* (Arifin and Kardoyono, 1985). It is known as 'Temu Lawak'in Malaysia. Other common names of *C. xanthorrhiza* are Tumulawak, Java turmeric, and gedè.

The cultivation of temulawak has spread over several islands such as Java, Moluccas islands and Kalimantan. Recently, due to its various medicinal qualities, cultivation of the plant is also found in several countries, such as Malaysia, Sri Lanka, India, Philippines and Thailand (Herman, 1985). The official documentation of temulawak in Indonesia was first written by Garcia ab Orta, a Portuguese, in 1563, while its activity was reported in detailed by Bontius, a Dutch doctor from Batavia. The Dutch, realizing its benefits, attempted to introduce *C. xanthorrhiza* as a seasoning and a drug component in Europe.

Simultaneously, the popularity of temulawak began to increase, which was also followed by various researches on its activity as medicine. Prior to World War II, temulawak from Indonesia has been exported to Netherlands and Germany and to date, it is well known in several European countries. In those countries, temulawak is known as liver tea, because of its effects to cure various liver disorders (Herman, 1985).

2.1.2 Botanical description of *C. xanthorrhiza*

C. xanthorrhiza is a low growing plant between 2 to 2.5 meters. It grows as a bush in wet semi-open areas, especially by roads and river with a root (rhizome) that is similar to ginger with aromatic, pungent odor and bitter taste. The large leaves are oblong in shape, thin and usually tufted. Leaves are deep green with purple central feather. Spites peduncled and bracts large ovate forming a cone with upper ones empty and lower ones have several fungacious flowers. Spike is lateral with short scape. Bracts are pale green with red–edged with uppermost red violet and base green striped. Flower of *C. xanthorrhiza* is pinkish with many petals and a green stalk.

Curcumas usually have ordinary annual flowering especially during wet season. Based on the observation, *C. xanthorrhiza* flowering in Malaysia (Penang) occurs between November to December (Arifin and Kadaryono, 1985). It can be harvested after 8-12 months, when the leaves turn yellowish. The tuber grows from stem and has dark-yellowish (brownish) colour, 15 cm of length and 6 cm of diameters. This tuber smells balmy and tastes bitter. *C. xanthorrhiza* grows in low level ground up to 750 m above the sea (Arifin and Kardoyono, 1985).

Figure 2.1: *C. xanthorrhiza* Roxb plant

Figure 2.2: Leaves of *C. xanthorrhiza* Roxb.

Figure 2.3: Rhizome (root) of *C. xanthorrhiza* Roxb.

2.1.3 Traditional uses of *C. xanthorrhiza*

C. xanthorrhiza has been used for centuries in traditional system of medicine to treat several diseases. In folk medicine, *C. xanthorrhiza* is reported to be useful for hepatitis, liver complaints, diabetes, rheumatism, cancer, hypertension and heart disorders. *C. xanthorrhiza* has also shown diuretic, anti-cancer, anti-inflammatory, anti-oxidant, anti-hypertensive, anti-rheumatic, anti-hepatotoxic, anti-dysmenorrheal, anti-spasmodic, anti-leucorrhoea, anti-bacterial and antifungal effects. Traditionally, this plant which is available as an herbal drink prevents blood clots and increases the immune system (Herman, 1985).

In Indonesia, Malaysia and Thailand, *C. xanthorrhiza* is considered as a panacea and is recommended in the treatment of abdominal complaints, liver disorders and gout attacks. *C. xanthorrhiza* is also used in traditional products for haemorrhoids, skin rashes, infected wounds and eczema as it contains anti-inflammatory properties. *C. xanthorrhiza* is very often utilized as an ingredient in 'jamus' recipes which is a typical Indonesian kind of elixir or liquid remedy (Perry, 1980).

In Southeast Asia, temulawak is a highly appreciated food ingredient because of its spicy and bitter taste, which is stronger than turmeric (*Curcuma longa*). Sliced rhizomes are cooked with vegetables; on the other hand the dried and cooked pieces are used to enhance flavors in soft drinks (Perry, 1980).

2.1.4 Phytochemistry of *C. xanthorrhiza*

Xanthorrhizol, the major component of the essential oil of *C. xanthorrhiza*, is a bisabolane-type sesquiterpenoid containing a stereogenic centre at the benzylic position. Xanthorrhizol is a potential chiral starting material for the synthesis of bisabolane-type

sesquiterpenoids as shown in Figure 2.4 (Rimpler *et al*., 1970). This compound makes up nearly 46.3% of the total component of essential oil through hydrodistillation technique (Cheah *et al*., 2009). Along with xanthorrhizol, bisabolane sesquiterpenes, a-curcumene, *ar*-turmerone and p-atlantone were isolated from the rhizomes of C. *xanthorrhiza* and they were reported to be the most active compounds (Itokawa *et al*., 1985). Studies have shown that xanthorrhizol can be used as a precursor for the synthesis of several other bisabolane-type sesquiterpenoids, including the first enantioselective syntheses of triols, facile and short syntheses of curcuquinone, curcuhydroquinone, helibisabonol A and the epimer of helibisabonol A, as well as syntheses of the unnatural allylic alcohols (Sirat *et al*., 2007).

Curcumin, a phenol compound is the second essential active chemical entity found in this plant as in Figure 2.5 (Ruslay *et al*., 2007). It comprises almost 1 to 2% in ethyl acetate fraction of *C. xanthorrhiza*. Several diarylheptanoids, curcumin (Kuroyanagi and Natori, 1970; Uehara *et al*., 1987), demethoxycurcumin (Kuroyanagi and Natori, 1970; Jitoe *et al*., 1992), bisdemethoxycurcumin (Kuroyanagi and Natori, 1970; Jitoe *et al*., 1992), curcuminoid (Jitoe *et al*., 1992), dihydrocurcumin, octahydrocurcumin and hexahydrocurcumin (Uehara *et al*., 1987) have been isolated from the rhizomes of *C. xanthorrhiza.* Three non-phenolic diarylheptanoids were also isolated from the hexane extract of the rhizomes of *C. xanthorrhiza* and they were identified as 1, 7 dipheny1-1, 3-heptadien-5-one, l, 7-diphenyl-l-hepten-5-ol and *trans*, l, 7-diphenyl- l, 3-heptadien-5-ol (Shin-Ichi *et al*., 1987).

C. xanthorrhiza contains various active volatile substances such as germacrone, borneol and essential oils which are present in much lower quantities (Simon and Cherry, 2006).

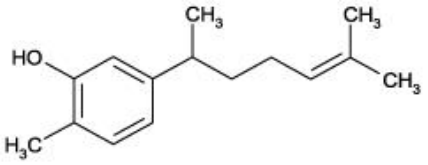

Figure 2.4: Xanthorrhizol chemical structure

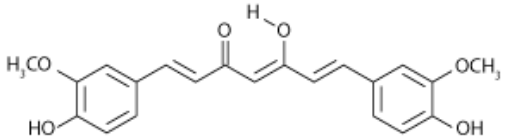

Figure 2.5: Curcumin chemical structure

2.1.5 Pharmacological activites of *C. xanthorrhiza*

C. xanthorrhiza has been documented for its antidiuretic, anticancer, anti-inflammatory, anti-oxidant, anti-hypertensive, anti-hepatotoxic, anti-spasmodic, anti-bacterial and antifungal properties. Besides, it also reduces cholesterol in blood and liver, treats constipation, and increases flow of milk during breast feeding (Herman, 1985).

Pharmacological activities of *C. xanthorrhiza* and its dominant compound, xanthorrhizol have been long recorded and reviewed since 1980's. Ozaki *et al*. (1990) has reported the anti-inflammatory properties of *C. xanthorrhiza* rhizomes where it inhibited the edema induced by carrageenan in rats. *C. xanthorrhiza* methanolic extract was known for its diuretic activities (Mahmood *et al*., 2004). The antioxidant activity of *C. xanthorrhiza* rhizomes was also not spared due to the presence of curcuminoids (Jitoe *et al*., 1992).

There are several claims that *C. xanthorrhiza* has been used for its hepatoprotective purposes in folk medicine. This was supported by lowering of the serum enzyme levels such as alanine aminotransferases (SGPT), aspartate aminotransferases (SGOT) and y-glutamate transferases in cisplastin induced hepatotoxicity rats given the *C. xanthorrhiza* extract (Seong *et al*., 2004). In addition, the hepatoprotective activity of aqueous extract of *C. xanthorrhiza* against β-D-galactosamine induced liver damage and alcohol has been reported by Lin *et al.* (1996) and Yasni *et al.* (1993) respectively.

A number of reports were found on the pharmacological activities of xanthorrhizol and curcumin, which are the major compounds identified in *C. xanthorrhiza*. Xanthorrhizol was investigated for its antibacterial (Hwang *et al*., 2000) and anticandidal activity (Rukayadi *et al*., 2006). Lim *et al.* (2005) has reported on the anti-inflammatory effects of xanthorrhizol against neuronal cells. Potent insecticidal activity also has been reported on xanthorrhizol (Pandji *et al*., 1993). Recently scientists have reported the anti-metastatic potential of xanthorrhizol in experimental mouse lung metastasis models. Studies also showed that the simultaneous treatment of xanthorrhizol and curcumin exhibits synergistic effect towards human breast carcinoma cells (Cheah *et al*., 2009).

Though numerous studies on the *C. xanthorrhiza* pharmacological properties have been reported, however the toxicity, hepatoprotective and analgesic properties are not well documented in literature. In this dissertation, detailed studies on above mentioned pharmacology properties were undertaken.

2.2 Plant extraction method

Extraction is the major step in the herbal drugs preparation. It requires raw material and extraction liquid or solvent. Extraction is the separation of the substances in a mixture, performed by dissolving each component with one or several solvents. It yields at least two components namely the solution extracted in the solvent (solute) and the residue. Extraction is completed when the concentration has reached a balanced level. Methanol or ethanol is commonly preferred solvents for extraction (Wichtl, 2004). There are distinct types of extraction procedures available. Among the extraction methods, the most regularly used is solid–liquid extraction, which consists of the privileged separation of one or more components of a solid mixture by dissolving them in a liquid solvent (Wichtl, 2004).

On the other hand, liquid-liquid extraction is another very common method employed in the organic laboratory. It is often used as the initial step in the work-up of a reaction, before final purification of the product by recrystallization, distillation or sublimation (Houghton and Raman, 1998). Immiscible liquids involved in solvent-solvent extraction resulting in the formation of two different layers. Various component of the mixture possess solubility in each of the two layers and achieves concentration equilibrium at a certain time.

Liquid-liquid extraction will be initiated with low and completed with high polarity solvents in order to extract out specific classes of compounds at each stage (Houghton and Raman, 1998). For example, low polarity solvents such as hexane, light petroleum and toluene will be good choice of solvents to extract waxes, fats and fixed oils. While ethyl acetate, butanol, diethyl ether and acetone are categorized under medium polarity solvents to isolate alkaloids, flavonoids and aglycones (Houghton and Raman, 1998). Ultimately, high polarity solvents like water, aqueous acids and bases are used to separate sugars and amino acids. Some common liquid-liquid extraction solvent pairs are water-ethyl acetate, water-butanol, water-hexane or water-dichloromethane. Each solvent combination contains water since water is highly polar and immiscible with most organic solvents (Houghton and Raman, 1998).

2.3 Standardization of plant sample

Plant drugs are plant-derived medicines containing a chemical compound or more usually mixtures of chemical compounds that act individually or in synergistic manner to maintain health (Gurib-Fakim, 2006). Pure compounds or chemical entities are either isolated from natural products or made by synthesis in the laboratory. Herbal teas,

decoction, alcoholic extracts are also traditional ways of using medicinal plants. These plant materials are often utilized in a non-standardized manner. However, the use of standardized materials in herbal medicine is being emphasized in recent years (Gurib-Fakim, 2006).

Standardization is a method of assuring a consistent level of active ingredients in the extract and only applies to extracts in the field of phytomedicines. It is important as a way of ensuring a consistent supply of high-quality phytopharmaceutical products. It can be defined as the establishment of reproducible pharmaceutical quality by comparing a product with established reference substances. Monographs and pharmacopeias provide the standards for active ingredients to be used in medicinal products. These phytodrugs turn out to be medicines when they are registered and comply with the basic established standards (Gurib-Fakim, 2006)

Standardization can be carried out by obtaining a chemical fingerprint/profile or through bioactivity guided fractionation to obtain marker compounds. It would be ideal to use the active constituent in the plant as the marker compound. Yet, in cases where active constituents are not known, the marker compound can be independent of the therapeutic activity. Furthermore, the plant extracts can also be standardized to class of compounds, for instances, ginsenosides in ginseng, kava lactones in kava, or oxindole alkaloids in cat's claw (Roman, 2001). Such approach would be suitable for situations where the active constituents are not known but expected to belong to a particular class of compounds.

According to European Medicines Agency guidelines, quantification of substances with known therapeutic activity or markers is essential (Roman, 2001). As per the European Pharmacopoeia, marker compounds should be unique for the herbal material or

herbal preparation and includes established chemical structures. Analytical tools such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography (LC), high-performance TLC (HPTLC), over-pressured layer chromatography (OPLC), infrared and UV-VIS spectrometry, nuclear magnetic resonance (NMR), electrophoretic techniques, especially by hyphenated chromatography, are often used for standardization and to control the quality of both the raw material and the finished product. The results extracted from these sophisticated techniques provide a chemical fingerprint as to the nature of chemicals or impurities present in the plant or extract (WHO, 2002; Bilia *et al*., 2002).

Standardization is necessary with the remarkable increase in the global use of medicinal plants related to several concerns that has been raised regarding the efficacy and safety of the herbal medicines. Hence it has become necessary to standardize the quality of the plant material and to increase the level of trust people have in herbal drugs (Brunton, 2006).

2.4 Toxicity and Adverse Effects

Plants contain hundreds of constituents and some of the constituents are very toxic and can be detrimental, for instances; digitalis, the pyrrolizidine alkaloids, ephedrine, phorbol esters which are categorized under the most cytotoxic anti-cancer plant-derived drugs. The adverse effects of most herbal drugs are rather less frequent when the drugs are utilized appropriately compared with synthetic drug (Stewart *et al*., 1998; Schimmer *et al*., 1994).

In short, herbs are medicines which are effective in appropriate quantities and may cause side effects if taken in high amount or prolonged exposure. Evaluation of toxic

properties of a substance is crucial when considering for public health protection. In practice, the evaluation typically includes acute, sub-chronic, chronic, carcinogenic and reproductive effects (Asante-Duah, 2002).

2.4.1 Brine shrimp lethality assay

The brine shrimp assay has been recognized as a safe, practical, and economic method for the determination of the bioactivity of synthetic compounds (De Almeida *et al*., 2002) as well as plant products (Meyer *et al*., 1982; McLaughlin *et al*., 1991; Lhullier *et al*., 2006). It is an *in vivo* lethality test in a simple zoologic organism that can be used as a convenient monitor for screening and fractionation in the discovery of bioactives (Lhullier *et al*., 2006).

Brine shrimp *Artemia salina* L. (Artemiidae), is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It plays a significant role in the energy flow of the food chain. Besides, it is being widely utilized in laboratory bioassay to determine the toxicity by estimating the median lethal concentration (LC_{50}) which has been reported for a series of toxins and plant extracts (Meyer *et al*., 1982). Identification of the lethal concentration for 50% mortality after 6 hours of exposure (the acute LC_{50}） makes the test rapid, simple and effective to determine the toxicity level of *C. xanthorrhiza* extract.

2.4.2 Acute oral toxicity

Acute oral toxicity is an initial and a requisite step in the assessment and evaluation of the toxic characteristics of a substance. Acute oral toxicity is the adverse effects occurring within a short time of single or multiple doses of a substance via oral route within 24 h of administration (OECD 401, 2001b). Acute oral toxicity testing in animal models provides information on the biological activity of a substance (plant or chemical)

and gain insight into its mechanism of action. Acute toxicity is distinguished from chronic toxicity, which describes the adverse health effects from repeated exposures, often at lower levels, to a substance over a longer time period (months or years) (OECD 401, 2001b).

It is widely considered unethical to use humans as test subjects for acute (or chronic) toxicity research. However, some information can be attained from investigating accidental human exposures, for example; factory accidents. Otherwise, most acute toxicity data can be obtained from animal testing or *in vitro* testing. The current OECD guidelines recommends the use of small number of animals since authorities today do not usually demand classical LD_{50} tests (OECD 401, 2001b).

The LD_{50} (median lethal dose) value is defined as the statistically derived dose which is expected to cause death in 50% of the treated animals in a given period in an acute toxicity test. This is the basis for toxicologic classification of chemicals. For a classical LD_{50} study, laboratory mice and rats are the species of choice. In this thesis, acute toxicity study was investigated for *C. xanthorrhiza* extract to ensure the safety of this plant product.

2.5 Antinociceptive activity

2.5.1 Pain definition and types of pain

Pain is a complex experience, initiated by unpleasant sensory information from an unpleasant stimulus which is associated with potential tissue damage (Turk and Dworkin, 2004). It is a major symptom in many medical conditions, and can significantly interfere with a person's quality of life and general functioning (Turk and Dworkin, 2004).

In general, pain can be divided into two major categories namely good pain (protective) and bad pain (maladaptive). Good pain is commonly designated as acute pain or known as eudynia. It is protective in nature and reduces in intensity as the tissue returns to normal. Chronic pain states often occur due to structural or functional changes in the nervous system. This type of pain can be defined as maldynia or bad pain. Chronic pain states often occur due to structural or functional changes in the nervous system (Cervero, 2006).

In short, acute pain is an expected symptom of a problem that typically will resolve with time while chronic pain represents an unexpected disease that is a pathologic entity and can be prolonged by triggered stimulus (Cervero, 2006).

2.5.2 Nociceptive Pain Mechanism

Nociceptive pain is related to the processing of noxious stimuli in the nervous system (Lynn, 1996; Schaible *et al*., 2005). It is due to the activation of the nociceptive system by noxious stimuli arising from mechanically, chemically or thermally induced damage to tissue integrity (Woolf, 2004). In general, the nociceptive pain pathway consists of four processes inclusive of *transduction, conduction, transmission,* and *perception* (Woolf, 2004). The nociceptive system originates in peripheral nerve fibers that respond to stimuli exceeding harmful intensity, spans the spinal cord, traverses the brain stem and thalamus and eventually terminates in the cerebral cortex, where the sensation of pain is perceived. Peripheral tissues are innervated by nociceptors, highly specialized primary sensory neurons, which contain specific receptors or ion channels at their peripheral terminals (Woolf, 2004; Scholz and Woolf, 2002).

There are different classes of nociceptors and these react to different types of stimuli depending on the types of receptors or ion channels they contain. The A-delta fiber and the C-fiber nociceptors are the two main classes of nociceptors. C-fibers are the most common which comprises 70 % of the fibers. Their axons are unmyelinated with small cell bodies. C-fibers conduct action potentials slowly when activated, resulting in prolonged burning pain. A-delta fibers consist of thinly myelinated axons with medium- to large-diameter cell bodies. They conduct impulses at a faster rate and produce sharp, pricking pain when activated (Stucky *et al*., 2001). The pain system involves a set of ascending pathways that convey nociceptive information from peripheral nociceptors to higher levels of the central nervous system, as well as descending pathways that modulate that information (Bromm and Desmedth, 1995). This can be referred as 'Gate Control Theory' which was developed by Melzack and Wall (1965).

2.5.3 Peripheral sensitization

Peripheral sensitization involves inflammation which involves a cascade of events. During inflammation, polymodal nociceptors are sensitized. In normal tissue, these fibers contain high mechanical and thermal thresholds in normal tissue. In this case, high intensity stimuli are required to excite those fibers. However, in course of inflammation, this threshold drops where even a mild stimuli is able to activate the fibers. Thus, sensitized "pain fibers" are activated by non-painful stimuli resulting in pain eventually (Handwerker, 1999; Schaible and Schmidt, 1988).

In addition, inflammation is also able to recruit silent nociceptors. These silent nociceptors are known as C-fibers that are inexcitable by noxious mechanical or thermal stimuli in normal tissue. Though, during inflammation, these primary mechanosensitive

fibers are sensitized, and are activated by stimuli (Schaible and Schmidt, 1988). In short, pathophysiological nociceptive input to the spinal cord is generated by the enhanced activity of sensitized polymodal nociceptors and the recruitment of silent nociceptors.

The sensitization during inflammation is induced by the action of inflammatory mediators on the nociceptors. A large number of inflammatory mediators are produced and released in the passage of inflammation and cause several classical signs of inflammation such as swelling, redness, hyperthermia, and pain (Kress and Reeh, 1996). This inflammatory soup includes endothelin, prostaglandin E2, leukotrienes, bradykinin, cytokines, serotonin and adrenaline which are released following tissue injury and causes increased excitability by activating the second messengers cascades (Bevan, 1996). Mast cells, macrophages and neutrophils discharge a number of proinflammatory substances.

Further, voltage-gated sodium channels and the capsaicin receptor are also involved in activation and sensitization of peripheral nociceptors. Besides, cyclic adenosine monophosphate (cAMP), protein kinases and neurotrophic factors (Lewin *et al*., 1994) play a significant role in the sensitizing action of many inflammatory mediators (Chapman and Garvin, 1999).

2.5.4 Testing for antinociceptive properties

The term nociception is used to refer to the studies being conducted on laboratory animals. The crucial in developing laboratory animal models of pain has been attempted to reproduce or mimic pain in humans. Pain can be studied in primates and other mammals, but the rodent models are preferred and most widely used (Bars *et al.*, 2001). The rat or mouse is usually the preferred species in animal models (Fleetwood *et al*., 1999).In conjunction with this, a number of tests have been devised to evaluate basal pain

sensitivity for example, the reaction of a normal or naive animal to graded-strength mechanical, thermal, or chemical stimuli (nociceptive pain) (Woolf and Max, 2001).

Nociceptive pain can be divided into acute nociceptive stimulus (somatic model pain) and those are concerned with medium or long term responses associated with inflammation (visceral or tonic pain). Ultimately, somatic pain is caused by stimuli such as cutting or crushing resulting in acute and sharp pain (Steeds, 2009) .The tests designed for acute nociception includes the tail flick, tail immersion, hot plate, radiant heat and laser irradiation (Siddall and Munglani, 2003).The most common and widely utilized thermal methods in laboratory are hotplate and tail flick tests. Hot plate thermal method is suitable for evaluation of centrally acting and not peripherally acting analgesics. The hot-plate test, initially invented by Eddy and Leimbach (1953), evaluates thermal pain reflexes due to footpad contact with a heated surface. Tail flick tests or known as radiant heat method is used for quantitative measurement of pain threshold against thermal radiation exposed on rat's tails and to discriminate between centrally acting morphine-like analgesics and non-opiate analgesics (D'amour and Smith, 1941). Tail flick depends on both spinal and supraspinal reflexes under different conditions (King *et al*., 1997). Spinal reflex is exhibited when an intense heat source is used while supraspinal system comes when heat intensity decreased (Jensen and Yaksh, 1986). Therefore, different neural system in the central pathway may mediate tail flick responses (Karoly and Jensen, 1987), In short, both hot plate and tail flick assess the central acting acute pain by stimulating the peripheral axons, including thermosensitive and nociceptors fibers (Bars *et al*., 2001).

Visceral pain describes the pain which is persistent and emanated from deep visceral structures. Viscera seem to be most sensitive to distention of hollow muscular-

walled organs (including the gastrointestinal tract, urinary tract, gallbladder), ischemia (the myocardium in human patients), and inflammation (cystitis or pancreatitis) (McMahon *et al*., 1995). The visceral model of pain can be categorized in several ways which includes: (1) persistent central pain (induced by topical capsaicin or subcutaneous formalin); (2) chronic inflammatory pain (induced by Freund's complete adjuvant (FCA), carageenan and turpentine or UV-irradiation); (3) chronic neuropathic pain by damage or disturbance to peripheral nerve (can be induced by Bennett model, Seltzer model, Chung's model) (Fleetwood *et al*., 1999; Siddall and Munglani, 2003). Formalin test has been proposed as a chronic or visceral pain model (Cowan, 1989). The formalin tests identify mainly central and peripheral acting analgesic drugs (Dubuisson and Dennis, 1977). It also allows dissociation between inflammatory and non-inflammatory pain according to their site and mechanism of action (Chau, 1989). Tjolsen *et al.* (1992) described the early phase related to direct activation of peripheral small afferent fibers. The late phase known to be closely related to early phase nociception and is dependent on the activity in primary afferent neurons to produce neurogenic inflammation.

Hence, hot plate and tail flick tests (somatic pain model) along with formalin tests (tonic pain and inflammation model) has been used to screen and evaluate the analgesic activity of *C. xanthorrhiza*.

2.5.5 Plant as analgesics

Analgesics have been estimated to be one of the highest therapeutic categories on which research efforts are concentrated (Elisabetsky and Castilhos, 1990). Analgesic compounds available in the market still outline a wide range of undesired effects (Katzung *et al*., 1998) leaving an open door for novel and better compounds. Natural products are

believed to be a vital source of new chemical substance with potential therapeutic applicability. Several plant species has been traditionally claimed and utilized as analgesics (Mills and Bone, 2000). Reports are available about analgesic effects of medicinal plants in the literatures such as *Mangifera indica*, *Curcuma longa L.* (turmeric), *Zingiber officinale* (ginger), *Salix alba and Sida cordofolia.* (Garrido *et al*., 2001; Ammon *et al*., 1992; Santa Maria *et al*., 1997;Li *et al*., 2002; Suthradhar *et al*., 2006).

Analgesic, also known as a painkiller belongs to the diverse group of drugs used to relieve pain. Analgesic drugs may act in various ways on the peripheral and central nervous systems as described in pain physiology. There are few types of analgesics available including the non-steroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, narcotic drugs such as morphine, synthetic drugs with narcotic properties for example tramadol, and non-acidic anti-pyretic derivatives namely paracetamol (Dworkin *et al*., 2003).

Non-steroidal anti-inflammatory drugs, usually abbreviated to NSAIDs, are drugs containing analgesic and anti-inflammatory properties. Aspirin, one of the most common NSAID drugs used to relieve minor aches and pains, as an antipyretic to reduce fever and anti-inflammatory medication. Aspirin was the first discovered member of the class of drugs as well as non-steroidal anti-inflammatory drugs (NSAIDs), capable of inhibiting the enzyme cyclooxygenase (COX) as their mechanism of action. Today, aspirin is one of the most widely used medications in the world (Reimann and Schneider, 1998).

Aspirin is widely utilized as an anti-inflammatory drug due to its ability to suppress the production of prostaglandins and thromboxanes. Prostagladins and thromboxanes are

mediators involved in peripheral sensitization resulting in inflammation ultimately. COX is required for prostaglandin and thromboxane synthesis. In this case, aspirin plays a role in the irreversible inactivation of the COX enzyme by acting as an acetylating agent where an acetyl group is covalently attached to a serine residue in the active site of the COX enzyme. Basically, aspirin inhibits COX-1 and modify the enzymatic activity of COX-2 which suppresses the inflammation injury (Bamigbade *et al*., 1997).

Opiates are narcotic type of opioid alkaloids found in opium poppy, *Papaver somniferum*. Morphine is the most prevalent alkaloid in opium, comprising of 10% to 16% of the total mass and has been reported to possess narcotic analg esic properties. It exhibits analgesic effects by binding to opioid receptors in the central nervous system and the gastrointestinal tract. There are three main classes of opioid receptors, μ, κ, δ (mu, kappa, and delta) respectively. Morphine binds strongly to the μ-opioid receptors resulting in analgesic and sedation effects. κ-opioid is associated to spinal analgesia and δ-opioid plays a role in analgesia (Klawe and Maschke, 2009). Aspirin and morphine have been used as standard reference drugs to evaluate antinociceptive properties of *C. xanthorrhiza* extract in this work.

2.6 Antioxidant activity

2.6.1 Plant antioxidants as natural defense

Fundamentally, an antioxidant can be referred as a molecule capable of inhibiting the oxidation of other molecules. Oxidation is defined as a chemical reaction that transfers electrons from a substance to an oxidizing agent resulting in free radicals (Sies, 1997). Consecutively, these radicals are able to start chain reactions in a cell, which can lead to

damage or death. Here, antioxidant plays an important role in terminating these chain reactions by eradicating free radical intermediates, and inhibit other oxidation reactions. This eradication is done by antioxidants by oxidizing themselves, therefore antioxidants are often reducing agents namely thiols, ascorbic acid or polyphenols (Sies, 1997).

Since oxidation reactions can be damaging, plants and animals maintain a complex antioxidant systems with multiple types of naturally occurring antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes, for example catalase, superoxide dismutase and various peroxidases (Valko *et al*., 2007).

As oxidative stress appears to be a vital part of many human diseases, the use of antioxidants in pharmacology is extensively studied. Antioxidant compounds of foods and plants play an imperative role as a health protecting factor. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables (Cao *et al*., 1993). Plant sourced food antioxidants such as vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been known to have the potential to lessen disease risk. Most of the antioxidant compounds found in typical diet are derived from plant sources (Kuhnau, 1976). They belong to various classes of compounds with a wide variety of physical and chemical properties. For example, compounds such as gallates, possess strong antioxidant activity, while others, like the mono-phenols are weak antioxidants (Cao *et al*., 1993).

Besides, antioxidant compounds categorized in phenolic acids, polyphenols and flavonoids groups are able to scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and eventually constrain the oxidative mechanisms that lead to degenerative diseases (Kuhnau, 1976). There are a number of clinical studies and ongoing research

being conducted on the antioxidant capacity of herbs, food and natural products. Some studies have reported that the antioxidants in fruits, vegetables, tea and red wine are the main factors for their efficiency in reducing the incidence of chronic diseases including heart disease and some cancers (Miller *et al*., 2000). The antioxidant activity of *C. xanthorrhiza* has been reported by Jitoe *et al*. (1992).

2.6.2 Reaction mechanisms in antioxidant assays

Principally, there are two major mechanisms involved in the deactivation of radicals by antioxidants namely hydrogen atom transfer (HAT) and single electron transfer (SET). In this both cases, same end results can be achieved; however kinetics and potential for side reactions differ. Bond dissociation energy (BDE) and ionization potential (IP) are two foremost factors that determine the mechanism and the efficacy of antioxidants (Wright *et al*., 2001).

HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. This antioxidant reactivity or capacity measurements are based on competition kinetics. HAT reactions are solvent and pH independent. They are usually quite speedy, normally can be accomplished in sec to min. Yet, the presence of reducing agents, including metals, is a difficulty in HAT assays and can lead to erroneously high apparent reactivity (Wright *et al*., 2001).

Whereas, *SET-based methods* detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Wright *et al*., 2001). SET reactions are usually slow and can necessitate longer time to reach completion (Sartor *et al*., 1999). SET methods are very sensitive to ascorbic acid and uric acid, which are significant in maintaining plasma redox tone, and reducing polyphenols are

also spotted. Prominently, trace components and contaminants (particularly metals) interfere with SET methods and can account for high variability and poor reproducibility and reliability of results. SET and HAT mechanisms almost always take place together in all samples, with the balance determined by antioxidant structure and pH respectively (Ou *et al*., 2002).

Various *in-vitro* antioxidant methods with distinct reaction mechanism have been employed to monitor and compare the antioxidant activity of foods, plants, natural products and biological fluids. These methods require special equipment and technical skills for the analysis. Such methods that are performed to explore the antioxidant activity of *C. xanthorrhiza* are total phenolics and flavonoids content based on oxidation or reduction reaction, FRAP assay which is based on SET mechanism, DPPH scavenging activity and ABTS assay that complies both HAT and SET mechanism.

2.6.3 Total phenolic and flavonoids assays

Phenolics are ubiquitous secondary metabolites in plants. They comprise a large group of biologically active ingredients, above 8000 compounds which varies from simple phenol molecules to polymeric structures with molecular mass above 30,000 Da (Harbone, 1982). Basically, the modern classification forms two basic groups of phenolics namely simple phenols and polyphenols. The group of simple phenols includes 'phenolic acids' or phenols with carboxyl group underlying the specificity of their function. While, polyphenols contains at least two phenol rings structurally.

Based on their carbon skeleton, polyphenols were classified in non-flavonoid compounds (eg: stilbenes, hydroxycinnamic acids and benzoic acids) and flavonoid compounds. More than 4,000 flavonoids have been identified in variety of higher and