CYTOTOXIC ACTIVITY OF DIFFERENT FRACTIONS OF TINOSPORA CRISPA STEMS EXTRACTS

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ABSTRACT

"Patawali" (Malay) refers to *Tinospora crispa* (*Menispermaceae*) which has been claimed to have many traditional uses. The root, stem and leaves of this plant have been used for many medicinal purposes such as fever, diabetes and rheumatism. *Tinospora crispa* contains variety of phytochemicals. In this study, the powdered *Tinospora crispa* stems were extracted using different solvents and pH to obtain five fractions (Fraction 1, 2, 3, 4 and 5) with varying polarity. These fractions were then screened for cytotoxicity test using brine shrimp lethality assay. For each fraction, 3 concentrations were prepared (10, 100 and 1000 μg/mL) and the assay was conducted in triplicates. The result obtained showed only Fraction 3 and 4 were toxic with LC₅₀ value of 41.59 μg/mL and 118.58 μg/mL respectively whereas Fraction 1, 2 and 5 did not exhibited any LC₅₀ value for the range of concentrations prepared. Brine shrimp lethality assay results from this study may be used as preliminary indication as anticancer agent of the likely presence of toxic compounds in those particular fractions. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.
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LIST OF SYMBOLS

°C  Degree Celcius
λ  Wavelength
%  Percentage
>  Greater than
±  Uncertainty
cm  Centimeter
h  Hour
g  Gram
kg  Kilogram
min  Minutes
mg  Milligram
mL  Milliliter
nm  Nanometer
ppm  Part Per Million
sec  Seconds
μL  Microliter
C_p  Viscosity
R_f  Retention Factor
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<td>IC\textsubscript{50}</td>
<td>Inhibitory Concentration</td>
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<td>LD\textsubscript{50}</td>
<td>Median Lethal Dose</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
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<td>BSLT</td>
<td>Brine Shrimp Lethality Test</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DPPH</td>
<td>2, 2-Diphenyl-1-Picrylhydrazyl</td>
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<tr>
<td>HTS</td>
<td>High-Throughput Screening</td>
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<td>PAF</td>
<td>Platelet-Activating Factor</td>
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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Medicinal plants and mankind has a strong relationship where they have been used by mankind as a source of medicines for a long time ago. Due to the role as biologically and chemically active resources, the medicinal plants synthesize various chemicals as defense against pests, diseases and predators. Researchers are able to design and synthesize new drugs as they are excellent reservoir of medicines and chemicals leads. In fact, about 25% of the drugs used in modern medicine are from plants (Elliot, 1986). Of more than 120 pure pharmaceutical chemicals isolated from about 100 plant species, currently in use as drugs (Farnsworth and Soejarto, 1991).

In the past five decades, medicinal plant research in Malaysia has been carried out mainly by researchers from various group including government-funded universities and research institutes with little involvement of industries and multinationals. The great public interest and expansion in the use of herbal medicine has led to new emphasis and drive in medicinal plant research. The research approaches taken have recently included activities to develop herbal medicine into quality, efficacious and safe products for human consumption.

The first report of phytochemical survey of plants in Malaysia was carried out by Arthur in 1954, and this was followed by several more similar reports right up to the 90's (Teo et al., 1990). This was, and still is, a proven strategic approach whereby plants with alkaloids are chosen for further detailed investigation. Inadvertently, a trend was set earlier, in Malaysia where by most of the
Phytochemical work concentrated on plants belonging to certain families or genus only because they contain alkaloids. Some examples are the Annonaceae, Apocynaceae, Lauraceae, Menispermaceae, and Rubiaceae. There was only one report (Nakanishi et al., 1965) which made use of bioactivity as a means of selecting plants for further investigation but this was not fully utilized until the present time (Said et al., 1996).

*Tinospora crispa* or 'Akar patawali' as it known to the Malays is a medicinal plant belonging to the family *Menispermaceae*. *Tinospora crispa* can be found distributed from the southwestern part of China to Southeast Asia, including Malaysia. It is widely used in the traditional medicinal practice of peoples living in Malaysia, Indonesia and Thailand to treat ailments like fever, hyperglycemia, wounds, intestinal worms and skin infections. Other than that, *Tinospora crispa* is also used to treat tooth and stomach aches, coughs, asthma and pleurisy (Nik Rahman et al., 1999).

Scientifically, *Tinospora crispa* has been demonstrated to possess antibacterial (Zakaria et al., 2006), antifilarial, antimalarial, antipyretic (Kongkathip et al., 2002) and hyperglycaemic effects. The extracts of *Tinospora crispa* have also been reported to suppress the synthesis and release of nitric oxide, which is known to take part in various physiological processes within the body, including in the pain and inflammation processes.

In term of chemical constituents successfully isolated from various part of *Tinospora crispa*, the plant contained quartenary alkaloids, including berberine (Bisset and Nawaiwu, 1984), borapetol A and B, borapetoside A and B, tinocrisposide, *N*-formylanondine, *N*-formylhormuciferine, *N*-acetylnormuciferine, *γ*-sitosterol picrotein and tinotubride. In addition, Kongkathip et al., (2002) have also isolated 2 new triterpenes, cycloeucalenol and cycloeucalenone from *Tinospora crispa*.

The *in vivo* lethality in a simple zoological organism, such as the brine shrimp lethality assay, developed for Meyer et al. (1982) might be used as a simple
tool to guide screening and fractionation of physiologically active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. This general bioassay detects a broad range of biological activities and a diversity of chemical structures. One basic premise here is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, a lower, non-toxic, dose might elicit a useful, pharmacological, perturbation on a physiologic system (McLaughlin, 1991). However, it has been demonstrated that brine shrimp lethality assay correlates reasonably well with cytotoxic and other biological properties (McLaughlin, 1991). Cytotoxicity is the ability of a compound to kill a cell. Brine shrimp have been previously utilized in various bioassay systems. There have been many reports on the use of this animal for environmental studies (Sorgeloos et al., 1980), screening for natural toxins (Harwig and Scott, 1971) and as a general screening for bioactive substances in plant extracts.

Based on the traditional uses of Tinospora crispa, the present study was carried out to determine the cytotoxic properties of the different fractions obtained as a result of extraction and fractionation of Tinospora crispa stems.

1.2 PROBLEM STATEMENT

The plant kingdom represents a vast reservoir of biologically active molecule and thus far only small fractions of plant with medicinal activity have been assayed. Nearly 50 % of drugs used in medicine are of plant origin. There is therefore much current research devoted to the phytochemical investigation of plants which has been ethno botanical information related to them. Taking this into account, Tinospora crispa, which is a medicinal plant commonly associated with entho botanical, is therefore interested to be studied on the determination of cytotoxic activities of different fractions obtained from the extraction of Tinospora crispa. The cytotoxicity test will be carried out using brine shrimp lethality assay where if the fraction happened to exhibit toxic behavior, this can be a good indicator as an anticancer agent.
1.3 PROJECT OBJECTIVE

The main objectives of this project are:

i. To extract different fractions of *Tinospora crispa* stems.

ii. To test cytotoxic activity of the fractions using brine shrimp lethality assay.

1.4 PROJECT SCOPE

This research is limited to the investigation of *Tinospora crispa* as the subject. The extract of this plant will be derived from the stem and not from any other part of the plant. The concern of this research is focused on the accomplishment of two major goals. Firstly it is aimed to extract and fractionate different fractions of stems of *Tinospora crispa* based on the polarity which is by using different solvents with different polarity. Secondly, these fractions will be subjected to brine shrimp lethality assay for the determination of cytotoxic activity where LC50 value will be determined in order to rectify the toxicity induced.

1.5 SIGNIFICANCE OF PROJECT

*Tinospora crispa*, locally known as “Patawali” (Noor and Ashcroft, 1989) is a climbing shrub that grows in tropical and subtropical regions. It is known as rejuvenative herbs. *Tinospora crispa* is abundant in Philippines, India, Sri Lanka, China, Thailand, Vietnam, Indonesia and Malaysia. The root, stem and leaves of this plant are used for medicinal purposes, both externally and internally. Via this study it is desired to investigate the cytotoxic properties of different fractions of *Tinospora crispa* stems which obtained as a result of extraction process. If any of this fractions happen to exhibit cytotoxic property thus it can be recommended as an indicator as anticancer agent. This is preferable in a way that the demonstrated cytotoxicity should be mediated through a mechanism that allows healthy cells to survive but not the tumor cells. The primary goal of cytotoxic agent is to prevent the growth of cancer cells. This is actually achieved by targeting mechanism that directly affects DNA replication, transcription or by disturbing functions of the cell that are important in
mitosis. Since cytotoxic compounds affect all cells, there are inevitably side effects but the healthy cells can usually cope with them or repair the resulting damage more easily than tumor cells (Csoka et al., 1994).
CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter consists of the literature review from the available sources related to the research conducted. This includes the literature review about Tinospora crispa, the brine shrimp lethality assay and some information about DMSO.

2.2 TINOSPORA CRISPA

Tinospora crispa, known by various vernacular names such as ‘akar patawali’ or ‘akar seruntum’ is an indigenous plant which grows wild in Malaysia (Noor and Ashcroft, 1989). The taxanomy of Tinospora crispa is listed in Table 2.1 as follows:

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<tr>
<td>Phylum</td>
<td>Tracheophyta</td>
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<td>Class</td>
<td>Magnoliopsida</td>
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<td>Order</td>
<td>Menispermales</td>
</tr>
<tr>
<td>Family</td>
<td>Menispermaceae</td>
</tr>
<tr>
<td>Tribe</td>
<td>Tinosperae</td>
</tr>
<tr>
<td>Genus</td>
<td>Tinospora</td>
</tr>
<tr>
<td>Botanical name</td>
<td>Tinospora crispa (L.)</td>
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Tinospora crispa is a woody and glabrous belongs to the family of Menispermaceae (Dweck, 2006). Tinospora crispa is widely used in Thailand, Malaysia and Indonesia as a bitter tonic, antipyretic and oral hypoglycemic agent.
The aqueous extract of stems of *Tinospora crispa* can be used to treat stomach troubles, indigestion, diarrhea, coughs and asthma. (Nik Rahman et al., 1999). Besides, the crude extract of the stem can also be used in the treatment of fever, cholera, diabetes and rheumatism.

Traditional folklore attributes the use of its stem to various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites. Among the Malays, an infusion of the stems is consumed as a vermifuge and a decoction of the whole plant is used as a general tonic. It is also used as an anti-parasitic agent in both humans and domestic animals (Noor et al., 1989 and Pathak et al., 1995). Despite its long usage as testified in traditional folklore, the biological properties of *Tinospora crispa* and the scientific evidence of its effects in free-radical mediated diseases such as carcinogenesis is scant.

### 2.3 BIOLOGICAL PROPERTIES OF *TINOSPORA CRISPA*

The proximate analysis showed that *Tinospora crispa* had high contents of protein, carbohydrate and moisture. Prior studies also confirmed that chemical substances in plants including protein, carbohydrate, vitamin and fiber also contribute to the antioxidant capacity (Betancur-Ancona et al., 2004). The amount of ash in *Tinospora crispa* extract is considered low compared to other herbs that were examined by Maisuthisakul et al. (2007). Low ash content indicates that *Tinospora crispa* contains low pro-oxidant substances. A report by Hlywka, Beck and Bulleman (1997) indicated that there is correlation between number of dead shrimps and concentration of extract. *Tinospora crispa* extract produced no toxic effect on animal cells and does not demonstrate any IC$_{50}$ even up to an extreme concentration of 1g/ml. This data is in accordance with the findings by Hartl and Humpf (2000), where there are associations between toxicological level of the herb extracts and the mortality of brine shrimp.
2.4 PHYTOCONSTITUENT

A number of chemical constituents have already been isolated from this plant, such as phenolic acid amides (Fukuda et al., 1985), a phenolic glucoside (Fukuda et al., 1985), and some furanoid diterpenes and furanoid diterpene glucosides of the clerodane type (Fukuda et al., 1985; Fukuda et al., 1986; Fukuda et al., 1993). *Tinospora crispa* also contains quartenary alkaloid compounds and chemical constituents such as borapetol A, borapetol B, borapetoside A, borapetoside B, tinocrisposide, *N*-formylanondine, *N*-formynornuciferine, *N*-acetyl nornuciferine, γ-sitosterol, picrotein, tinotubride (Pathak et al., 1995). All of these chemical substances especially alkaloids, contain anti-cancer properties which can interfere with microtubule function. Alkaloids are widely used in combination with chemotherapy regimens for treating many solid tumours (Rowinsky and Donehower, 1997).

2.5 TRADITIONAL USES

*Tinospora crispa* has been used by Murut community in Sabah, for the treatment of various illnesses which include diabetes, hypertension and lumbar pain (Fasihuddin, 2000).

It also has been widely used as Thai traditional medicine, mainly for maintaining good health. The extraction of the stems, leaves and roots are used for the treatment of fever, cholera, diabetes, rheumatism and snake-bites. It also said to reduce thirst, internal inflammation as well as increases appetite (Umi Kalsom et al., 1999). Besides, *Tinospora crispa* has been consumed in a different ways for different treatment which includes, the decoction of the stem is used for washing sore eyes and syphilitic sores while the crushed leaves are applied on wounds.

Jamu is a main product of traditional medicine in Java. It is prepared using more than 1200 species which include *Tinospora crispa* where it is known as Brotowali. This medicine is commonly used for the treatment of diabetes. In Vietnam, *Tinospora crispa* is used vastly to treat malaria where it is described as "vine with genie's intelligence" (*day than thong*).
Among Filipinos and Malays, *Tinospora crispa* is considered as universal medicine due to its capability to treat many diseases. In Filipina, this plant known as Makabuhai which is prescribed in the form of aqueous extract to treat stomach trouble, indigestion and diarrhea. For the treatment of fever, it is prescribed in the powder form. For the treatment of rheumatism and flatulence of children, it is prepared with coconut oil.

For the treatment of rheumatism, a decoction of fresh root is mixed with pepper and goat milk. The leaves become sticky when agitated in water, which will then be added with sugar and consumed as a cure for gonorrhea.

### 2.6 THERAPEUTIC APPLICATIONS

#### 2.6.1 Anti Malarial Activity

A report showed the extract of *Tinospora crispa* was effective against malaria. (Hashimah et al., 1991). It also showed considerable antimalarial activity against *Plasmodium Falciparum*. The blocking of protein synthesis in *Plasmodium Falciparum* is said to be probably due to the presence of quarternary alkaloids including berberine in the extract of stem and root of *Tinospora crispa* (Elford, 1986).

#### 2.6.2 Anti Diabetic /Hyperglycemic activity/ Insulinotropic Activity

The efficacy of *Tinospora crispa* (*Menispermaceae*) extract for the treatment of diabetes has previously been verified in animal models. The results obtained by Noor et al. (1989) explained the interference with intestinal glucose uptake or uptakes of sugar into the peripheral cell are not responsible for the antihyperglycaemic effects of *Tinospora crispa*. The stimulation of insulin release via modulation of β-cell Ca\(^{2+}\) concentration is the reason for this property (Noor and Ashcroft, 1998). Insulin secreting clonal β-cell line and HIT-T15 cells were used for the mechanism of insulinotropic action of *Tinospora crispa*. The mechanism works in a way that the aqueous extract sensitizes the β-cell to extracellular Ca\(^{2+}\) and...
promotes intracellular Ca\textsuperscript{2+} accumulation which in turn causes increased insulin release.

2.6.3 Antinociceptive and Anti Inflammatory Activity

The study has showed that the ethanolic \textit{Tinospora crispa} extract of stem exhibited antinociceptive and anti-inflammatory activities in various animal models. The extract is said to have strong analgesics characteristic due to their ability to inhibit chemically and thermally-induced nociception (Hunskaar and Hole). It is suggested that the antinociceptive and anti-inflammatory activities of the extract was attributed to the presence and synergistic action of alkaloids and triterpenes contained in the plant material (Sulaiman et al., 2008).

2.5.4 Antioxidant Activity

Zulkhairi et al. (2008) concluded that the \textit{Tinospora crispa} extract has the ability to scavenge DPPH free radicals in a concentration dependant manner. Cavin et al. (1998) also support this, where the DPPH assay conducted showed that the methanol extract of \textit{Tinospora crispa} had the highest scavenging activity in a dose-dependent manner where the IC\textsubscript{50} value was 12 µg/mL. Furthermore, methanol crude extract of \textit{Tinospora crispa} had higher total phenolic and flavonoid content and free radical scavenging activity compared to water extract and chloroform extract.

2.7 BIOASSAY METHODS

The development and utilization of a series of specialized bioassays in many laboratories in Malaysia started in the early nineties. This was largely due to the increase in active participation of biological scientists especially pharmacologists, biochemists and microbiologists and the availability of substantial aids from the government through research grants known as Intensification of Research in Priority Areas (IRPA) program which announced in 1985. Medicinal plant research was given attention when development and production of biopharmaceuticals from plants was identified as one of the priority areas.
The new bioassay methods include the use of *in vitro* systems such as cultured cells for anticancer, antiviral and antiparasitic assays, *ex vivo* systems involving isolated tissues and organs, *in vivo* systems involving whole animal experiments and the mode of action assays based on specific enzymes or receptors. Some examples of reports published in the eighties on biological activities of medicinal plants are the *in vitro* antihypertensive activity and cardiovascular effects of alkaloids isolated from several plants notably *Uncaria callophylla* (Goh et al., 1986 and Chang et al., 1989). In the following years the number of papers published on the biological activities of plant extracts and isolates from the local plants increased greatly. Some examples are the anti-tumor promoting activities of *Zingiberaceae rhizomes* (Vimala et al., 1999), the antimalarial activity of the extracts of *Piper sarmentosum, Andrographis paniculata* and *Tinospora crispa* (Nik Rahman et al., 1999), the antimicrobial, antioxidant, antitumour-promoting, cytotoxic and antifungal activities of *Garcinia atroviridis* (Mackeen 2000, 2002). The mode of action assays were becoming more popular because they were fast, easy to perform, quantitative and could selectively detect biologically active molecules at very low levels.

The mode of action assays, employed in high-throughput screening (HTS) techniques, allow a large number of compounds to be screened for a wide range of bioactivities including pharmacological, biochemical, microbiological, toxicological and immunological activities, in a relatively short period of time. Most research groups were showed an interest to carry out bioactivity studies on crude extracts or isolated pure compounds of the plant material. This approach has continued over the years and is still widely practiced until today by many research groups. Examples of effort taken based on this approach are the inhibitory effects of xanthones, previously isolated from some *Guttiferae* species, on platelet-activating factor (PAF) binding to receptor *in vitro* (Ibrahim et al., 2004), the mechanisms of apoptosis induced by gonoiothalamin, isolated from *Goniothalamus andersonii*, in the leukemic T-cell line Jurkat and promyelocytic HL-60 leukemia cells (Inayat-Hussain et al., 2003), the effects of iridiods previously isolated from *Saprosma scortechinii* and *Rothmannia macrophylla* on lipoxygenase and hyaluronidase activities and their activation by beta-glucosidase in the presence of amino acids.
2.7.1 BIOASSAY-GUIDED ISOLATION TECHNIQUES

Recently, bioassay-guided isolation techniques became matter of interest and were gradually adopted by many researchers to isolate bioactive compounds. In fact, many multinational drug companies involved in the systematic drug development programs from natural resources based on the bioassay-guided isolation techniques. Fractionations of active extracts, followed by isolation of active compounds are linked with bioassays and most of the time the compounds isolated is responsible for the biological activity of the plant.

Bioassay-guided isolation of active compounds involves a strong collaboration between the chemist who is involved in the isolation and the biological scientist who is performing the assay. In some cases both the isolation and bioassay activities are carried out by the same scientist. The most active compound will be evaluated against the entire spectrum of molecular targets available in the laboratories to determine whether the compound is specific for the desired target.

If the compound is found to interact with the entire family of related targets, its potential side effects or toxicity will be determined. However, in the case of Malaysian plants, very few publications resulted from the performance of this approach. Some examples of bioassay-guided isolation work carried out on Malaysian plants are isolation of reticulatacin, a new bioactive acetogenin from *Annona reticulate* (Saad et al., 1991), antimitotic and cytotoxic flavonols from *Zieridium pseudobtusfolium* and *Acronychia porteri* (Lichius et al., 1994), griffipavixanthone, a novel cytotoxic bixanthone from *Garcinia griffithi* and *Garcinia pavifolia* (Xu et al., 1998), anti-inflammatory agents from *Sandoricum koetjape* (Mat Ali et al., 2004) and a potent PAF antagonist, a new alkenyl resorcinol from *Ardisia elliptica* (Jalil et al., 1995).

2.8 TOXICOLOGICAL STUDIES

The most active compound isolated from a plant by the bioassay-guided isolation will be subjected to pharmacological evaluation and rigorous safety
assessment procedures, involving testing against a large variety of different in vitro and in vivo tests which are designed to reveal different types of toxicity.

There are several toxicity testing available which includes acute toxicity, chronic toxicity, fetal toxicity, and effect on fertility, mutagenic and carcinogenic responses. The compound will be tested on cell cultures and isolated tissues to examine any effects on cell reproduction and to identify its carcinogenic potential. Several species of animals are administered with various levels of doses of the compound to check for toxicity over a period of months. Further study on the compound will be discontinued, if there is significant toxicity in the test animals, even at very high doses of compound.

The activity of structurally related compounds will be similarly evaluated to determine whether the observed toxicity is due to any of the functional groups present in the class of compounds. In order to discover the most promising compound, more analogues of the compound need to be synthesized. The compound which passes the toxicity testing is elevated or proposed to a drug candidate and is suitable to move on to clinical trials. Example of toxicological studies on Malaysian plants are the teratogenic activity of goniothalamin and goniothalamin oxide from Goniothalamus opacus in mice (Sam et al., 1987), the toxic activities of the essential oils of Cinnamomum species (Ibrahim et al., 2004) and the tumour promoting activity of plants used in Malaysian traditional medicine (Ilham et al., 1995).

2.9 BRINE SHRIMP LETHALITY ASSAY

Bioactive compounds are almost always exhibit toxicity in high doses. Pharmacology is simply toxicology at lower dose, and toxicology is simply pharmacology at higher dose. Thus, in vivo lethality in a simple zoological organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products (Sam, 1993).

The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of toxicity, detection of fungal toxins, heavy metals,