# CYTOTOXIC ACTIVITY OF DIFFERENT FRACTIONS OF *TINOSPORA CRISPA* STEMS EXTRACTS

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# CYTOTOXIC ACTIVITY OF DIFFERENT FRACTIONS OF *TINOSPORA CRISPA* STEMS EXTRACTS

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Report submitted in partial fulfillment of the requirements for the award of the degree of Bachelor of Applied Science (HONOR) Industrial Chemistry.

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## SUPERVISOR'S DECLARATION

I hereby declare that I have checked this project report and in my opinion this project is satisfactory in terms of scope and quality for the award of the degree of Bachelor of Applied Science (Honor) in Industrial Chemistry.

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| Date | : 29 DECEMBER 2011 |

## STUDENT'S DECLARATION

I hereby declare that the work in this report is my own except for quotations and summaries which have been duly acknowledged. The report has not been accepted for any degree and is not concurrently submitted for award of other degree.

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*Dedicated to my beloved*

*Parents Mr. Mahalingam and Mrs Rajamal, Sibling, Friends*

*and lecturers*

*for their continuous encouragement and support*

*to complete this project successfully.*

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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 cytoxicity 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 $LC_{50}$ value of 41.59 μg/mL and 118.58 μg/mL respectively whereas Fraction 1, 2 and 5 did not exhibited any $LC_{50}$ 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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# ABSTRAK

Patawali" (Melayu) merujuk kepada *Tinospora crispa* (*Menispermaceae*) yang telah dituntut untuk mempunyai banyak kegunaan tradisional. Akar, batang dan daun tumbuhan ini telah digunakan untuk tujuan perubatan seperti demam, kencing manis dan sakit sendi. *Tinospora crispa* mengandungi pelbagai fitokimia. Dalam kajian ini, batang *Tinospora crispa* telah diekstrak menggunakan pelarut dan pH yang berbeza untuk mendapatkan lima pecahan (Pecahan 1, 2, 3, 4 dan 5) dengan polariti yang berbeza. Pecahan ini telah kemudiannya diperiksa untuk ujian toksikasi menggunakan "Brine Shrimp Lethality Assay". Untuk setiap pecahan, 3 kepekatan telah disediakan (10, 100 dan 1000 μg/mL) dan ujikaji ini telah dijalankan secara tiga kali ganda. Keputusan yang diperolehi menunjukkan hanya Pecahan 3 dan 4 adalah toksik dengan nilai $LC_{50}$ 41.59 μg/mL dan 118.58 μg/mL masing-masing, manakala Pecahan 1, 2 dan 5 tidak menunjukkan apa-apa nilai $LC_{50}$ untuk julat kepekatan yang disediakan. Keputusan "Brine Shrimp Lethality Assay" daripada kajian ini boleh digunakan sebagai petunjuk awal kehadiran berkemungkinan sebatian toksik dalam pecahan tertentu. Ujian toksikasi lain dan bioasai khusus yang boleh dilakukan ke atas sebatian bioaktif yang diasingkan kemudian.

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| | |
|---|---|
| ˚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 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| $IC_{50}$ | Inhibitory Concentration |
| $LD_{50}$ | Median Lethal Dose |
| BHT | Butylated hydroxytoluene |
| BSLT | Brine Shrimp Lethality Test |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPPH | 2, 2-Diphenyl-1-Picrylhydrazyl |
| HTS | High-Throughput Screening |
| PAF | Platelet-Activating Factor |
| TLC | Thin Layer Chromatography |

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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*-formylnornucifrine, *N*-acetylnornuciferine, 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 $LC_{50}$ 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 cytotoxicty 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:

**Table 2.1:** Scientific classification of *Tinospora* crispa

| | |
|---|---|
| **Domain** | *Eukaryota* |
| **Kingdom** | *Plantae* |
| **Phylum** | *Tracheophyta* |
| **Class** | *Magnoliopsida* |
| **Order** | *Menispermales* |
| **Family** | *Menispermaceae* |
| **Tribe** | *Tinosporeae* |
| **Genus** | *Tinospora* |
| **Botanical name** | *Tinospora crispa* (L.) |

Source : ZipcodeZoo.Com (2004)

*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, 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*-formylnornuciferine, *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 cris*pa 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 Makabühai 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 b-cell $Ca^{2+}$ concentration is the reason for this property (Noor and Ashcroft, 1998). Insulin secreting clonal ß-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 ß-cell to extracellular $Ca^{2+}$ and

promotes intracellular $Ca^{2+}$ accumulation which in turn causes increased insulin release.

### 2.6.3 Antinociceptive and Anti Inflammatory Activity

The study has showed that the ethanolic *Tinospora crispa* extract of stem exhibited antinoniceptive 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 *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 *Tinospora crispa* had the highest scavenging activity in a dose-dependent manner where the $IC_{50}$ value was 12 μg/mL. Furthermore, methanol crude extract of *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 goniothalamin, 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 pseudobtusifolium* 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,

pesticides and cytotoxicity testing of dental materials (Meyer et al., 1982). It can also be extrapolated for cell-line toxicity and anti tumor activity (Anderson et al., 1991). The brine shrimp assay is very useful for the isolation of biogenic compounds from plant extracts.

The eggs of brine shrimp, *Artemia salina* (Leach), are readily available, especially in pet shop at low cost and remain viable for many years in the dry state. The hatching of the eggs can be observed upon placing the eggs in sea water within 48 h to provide large number of larvae (nauplii) for experimental use.

Brine shrimp nauplii have been used previously in a number of bioassay system, but methods has been developed where by natural product extract, fractions or pure compound tested at initial concentration of 10, 100 and 1000 ppm (mg/ml) in vials containing 5 ml of brine and 10 shrimp in each of three replicates (Mayer et al., 1982 and McLaughlin et al., 1991). Survivors are counted after 24 h. These data are processed in a simple program for probit analysis to estimate $LC_{50}$ values with 95 % confidence intervals for statically significant comparisons of potencies.

Over 300 novel antitumor and pesticidal natural products have been isolated in the laboratory (McLaughlin et al., 1991). Thus, it is possible to detect and then monitor fractionation of cytotoxic active extracts using brine shrimp lethality bioassay rather than more expensive *in vitro* and *in vivo* antitumor assays. The brine shrimp assay has advantages of being rapid (24 h), inexpensive and simple as there is no aseptic techniques are required. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and relatively small amount of sample. Furthermore, it does not require animal serum as is needed for cytotoxicities.

*Tinospora crispa* extract produced no toxic effect on animal cells and does not demonstrate any $IC_{50}$ even up to an extreme concentration of 1 g/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. Besides that, numerous previous studies done on *Tinospora crispa* in

several experimental animals reported no evidence of organ damage. However, there are other factors that are considered as a confounder in this assay and will affect brine shrimp mortality such as lack of oxygen (Hartl and Humpf 2000) and age of the shrimp (Hlywka et al., 1997). The shrimp will barely survive for 72 h alone on their own resource.

## 2.10 BRINE SHRIMP, *ARTEMIA*

The brine shrimp, *Artemia salina* Leach, Anostraca: Artemiidae, has been used for a long time as a test organism in natural products research (Meyer et al., 1982) and also to assess the effect of presence of chemicals in aquatic environments (Persoone and Wells, 1987). The brine shrimp lethality assay is used routinely in laboratories around the world for prescreening of plant extracts with potential medicinal properties such as antimicrobial or antiparasitic, for bioguided fractionation of bioactive constituents of plant extracts and for cytotoxic effects detection.

The major reason why this salt-water anostracan crustacean is used widely for toxicity testing of plant extracts is due to the commercial availability of dormant eggs which is known as cysts. They are harvested in huge amounts in salt lakes and pans. The larvae hatched from the cysts are used worldwide in aquaculture and in aquariology as live food for juvenile fish. Dormant brine shrimp eggs remain viable for many years and are therefore a suitable biological source for rapid, simple and inexpensive bioassays. A drawback of this bioassay, however, is that the saline medium decreases the solubility and bioavailability of some substances, thus limiting the detection of possible bioactive plant constituents.

Several areas of scientific knowledge distribute information related to the genus *Artemia (*brine shrimp). Toxicologists working with this genus are solely relying on the integration of this information. *Artemia* is subdivided into six generally recognized bisexual species and a large number of parthenogenetic populations, is characterized by common features such as adaptability to wide ranges of salinity (5-250 g $L^{-1}$) and temperature (6-35 ˚C), short life cycle, high adaptability

to adverse environmental conditions, high fertility, bisexual/ parthenogenetic reproduction strategy, small body size, and adaptability to varied nutrient resources as it is a non-selective filter feeder. The intrinsic features of this genus turn it into a suitable organism for use in toxicology, guaranteeing reliability, feasibility and cost-effectiveness in routine and/or research toxicity practices.

### 2.10.1 General Features of the Genus *Artemia*

*Artemia* has a wide geographical distribution. The species of this genus possess an uncommon adaptability to extreme conditions, thus being found in environments where other life forms are not sustainable (Triantaphyllidis et al., 1998). The different species of the genus *Artemia* present one common characteristic, that is, their strong adaptability to hypersaline environments, such as permanent salt lakes, coastal lagoons and man-made salt pans where evaporation of seawater results in high sodium chloride concentrations. High salt concentrations can also be found in other habitats colonized by *Artemia*, such as temporal salt lakes, subjected to unpredictable floods. The habitats in which the genus *Artemia* is found are characterized by the absence of predatory animal species. Therefore, in such environments, the evolution of *Artemia* populations is favoured by the abundance of bacteria, protozoa and algae that are the basis of the *Artemia* diet.

Several studies have been performed in order to take advantage of the high adaptation patterns of *Artemia* concerning nutrient resources. For instance, *Artemia* is able to use several nutrient resources, such as wheat bran, soybean meal, rice bran and whey powder (Dobbeleir et al., 1980 and Sorgeloos et al., 1980). Other nutrient sources suitable for *Artemia* production are fishpond effluents (Zmora et al., 2002) or inert commercial diets (Naegel, 1999). In spite of this high adaptability to varied nutrient resources, the concentration of nutrient supplies is highly relevant for *Artemia*, as shown by the works of Evjemo and Olsen (1999), which clearly established a relation between growth and total food intake under controlled abiotic conditions where growth and ingestion rate were positively correlated with nutrient concentrations.

Tolerance to extremely variable oxygen concentrations is another common trait to several species of *Artemia*, which allows specimens to successfully face environmental adversities under extreme conditions. The wide distribution of *Artemia* species and strains can also pose several concerns related to the site where cysts and specimens can be collected. The place of origin of *Artemia* cysts is a most important factor for every of its posterior uses, because different strains with distinct patterns of growth, survival and reproduction occur at distinct sites. On the other hand, in the same natural *Artemia* samples, cysts of several species of the genus *Artemia* may be found. Triantaphyllidis et al. (1998) suggested a simple procedure, which allows the detection of a mixed nature in *Artemia* commercial cysts samples, through their incubation at high temperatures.

The complete knowledge and control of the origin of cysts is highly important for research. A considerable scientific investment has been made in order to study the taxonomic relationship between *Artemia* species and strains, according to their geographic origin, taking into consideration morphological, reproductive, physiological, morphometrical, biochemical and genetic features. Subdivision of the genus into species, the occurrence of (diploid and polyploid) parthenogenetic and of bisexual strains, and generally the entire problem of genetic flexibility and speciation have very direct implications in the field of aquaculture (Gajardo et al., 2002).

Through multidisciplinary studies both the causes for the nutritional variability in *Artemia* and the methods to improve poor-quality *Artemia* were identified. To optimize the use of the *Artemia* stocks in the market, and to diversify the use of natural resources, a variety of different research initiatives were launched, leading to improved techniques for cyst harvesting, processing and storing, and for nauplii applications, and exploitation of new natural resources (Lavens and Sorgeloos, 1987). In addition, numerous managed ponds and salt works worldwide started providing small quantities of good quality cysts in the volume of 1-20 metric tons each, providing interesting opportunities for commercial development (Dhont and Sorgeloos, 2002).

After harvesting and processing, cysts are available in cans as storable ‘on demand’ live feed. Upon a few hours of incubation in seawater, the free-swimming nauplii can directly be fed as a nutritious live food source to the larvae of a variety of marine as well as freshwater fish and crustaceans, which makes them the most convenient, least labor-intensive live food available for aquaculture (Sorgeloos et al., 2001). Besides, they also can be used for toxicity testing. By bio-encapsulating specific amounts of particulate or emulsified products rich in highly unsaturated fatty acids in the brine shrimp metanauplii, the nutritional quality of *Artemia* can be further tailored to suit the nutritional requirements of fish and shrimp larvae (Lavens and Sorgeloos, 1987).

The distinct patterns evidenced by all species of the genus *Artemia* related to parameters such as 21-day survival, $LT_{50}$, lifespan and reproductive traits, confirmed the high intrinsic variability in physiological responses towards salinity and temperature fluctuations among strains and between species of the same genus (Triantaphyllidis et al., 1998).

### 2.10.2 *Artemia* in Toxicology

Major attempts to use representative organisms in multi nutrition and multispecies assessments have been made in recent years with an aim of seeking more accurate approaches and reliable extrapolations for real scenarios. Standardized laboratory procedures involve culture of animals and the maintenance of constant abiotic conditions to assure the reproducibility of results. Choice of the more adequate animal model should take into account global aspects of biology, life cycle, adaptability to laboratory conditions, ecological relevance, systematic use, and practical conditions of maintenance and sustainability of laboratory cultures.

*Artemia* use in toxicology poses a reasonable number of answerable questions, namely practical considerations of laboratory culture and attainment of cyst, thus making achievable a sustainable development of *Artemia*-based bioassays. Nevertheless, great attention is needed to fully comprehend the complexity of distinct species or strains from geographical distinct sites and the possible

implications of these general characteristics over culture practices and ultimate result interpretation. *Artemia* is by far one of the most striking examples of organisms well adapted to laboratory practice, as long as a strict control over laboratory procedures and methodologies is maintained. On the other hand, its use as test organism is representative of the effort to reduce the scale of test organisms, with resultant reduction in test volumes, amount of waste produced, and space needed to perform testing protocols (Blaise, 1998).

### 2.10.3. Origin of Test Organisms (*Artemia*)

Full characterization of biological samples is a fundamental aspect in toxicity testing. The possibility of simultaneously using several different strains of *Artemia,* due to contamination, deficient geographical localization of origin or incomplete characterization of cyst samples, can lead researchers to erroneous results. A work showed consistent distinct patterns for chlorpyrifos toxicity according to the species, strain and even geographical origin of the cyst lot incubated for the testing purposes. Responses of distinct nature and magnitude can be found among strains as evidenced by Browne (1980), when studying acute toxicity and reproductive traits of five strains of brine shrimp following copper sulphate exposure.

### 2.10.4. Sensitivity of *Artemia*-Based Toxicity Bioassays

In spite of its massive use in toxicological testing, earlier studies refer *Artemia* as a less sensitive species for toxicity studies, when compared to other test organisms under the same experimental conditions such as *Streptocephalus rubricaudatus* and *Streptocephalus texanus* (Crisinel et al., 1994), *Echinometra lucunter* and *Crassostrea rhizophorae* (Nascimento et al., 2000) or algal species, such as *Selenastrum capricornutum* and *Dunaliella tertiolecta* (Gaggi et al., 1994).

Guerra (2001) also reported lower sensitivity of *Artemia*-based assays, when compared to commercially available screening and to the standardized test. Okamura et al. (2000) also reported a diminished sensitivity of *Artemia*, when comparing the responsiveness of several organisms towards the antifouling agent Irgarol 1051.

*Artemia* was considered to be the least sensitive organism among a group, which included varied species of crustaceans, such as *Daphnia magna*, *Daphnia pulex* and *Thamnocephalus platyurus*.

*Artemia* was shown to be more tolerant than *Aedes taeniorhynchus* after exposure to the insecticides aldicarb, dimethoate, imidacloprid and tebufenozide (Song and Brown, 1998). Sensitivity of *Artemia* was also once questioned when a comparative study of the sensitivity of several bioassays to 15 quaternary ammonium compounds was performed. *Artemia franciscana* was reported to be the least sensitive organism tested.

On the other hand, researchers such as Hlywka et al., (1997) noticed similar sensitivities to the mycotoxin fumonisin $B_1$ when simultaneously using the acute screening toxicity assay with *Artemia* and chicken embryo screening test, but preferred the former due to simplicity of procedures and lower volumes of toxicant required to develop the test. *Artemia* was found to be more sensitive than *Daphnia similis* and *Ceriodaphnia dubia* to niclosamide (Oliveira-Filho and Paumgartten, 2000).

### 2.10.5 End-point selection in *Artemia*-based Bioassays

The end-point selection for toxicity testing is sometimes a decisive factor to be discussed, as the information considered vital may or may not be fully obtained when varied end-points are considered. Most frequently, cyst-based toxicity assays involving *Artemia* refer to a well-accepted end point as criterion where the mortality of the recently hatched nauplii when tested with the test samples.

Besides the use of standardized development phases of *Artemia*, several studies investigated the suitability of specimens of different ages, such as the ones described by Barahona and Sanchez-Fortun (1996) where this study evaluated several age classes of *Artemia salina* and the conclusions pointed to a greater relative sensitivity of the 48 h old specimens, for the majority of the tested compounds. Studies were also performed in order to evaluate the suitability of *Artemia* cysts as

test organisms (Vismara, 1998), prior to hatching and without incubation. Viability (hatchability) of cysts after exposure was selected as end-point criterion, and comparisons between the two approaches which were mortality of nauplii and hatchability of cysts were made to assess differences in sensitivity for both tests (Carballo et al., 2002). This study indicated high concordance between results, and considered useful the complementary development of the two assays. Behavioral parameters were also suggested to serve as endpoint for toxicity assays.

*Artemia* can also be used in tests based on biomarkers, such as enzymatic activity. The work of Varo et al. (2002) presented a test based on cholinesterase inhibition with two species of the genus *Artemia* (namely *Artemia salina* and *Artemia parthenogenetica*) for the assessment of effects due to exposure to organophosphorous pesticides such as dichlorvos and chlorpyrifos.

### 2.10.6 Reducing Variability as for Toxicity Testing

Diverse factors can alter the reliability of results in toxicity testing such as environmental conditions, related to non-genetic factors including temperature, pH and chemical composition of the medium, oxygen, photoperiod, nutrients, diseases and population effects as well as genetic characteristics of the organisms used in tests (Soares et al., 1992). These sources of variation may be minimized by a convenient understanding of concepts underlying the use of animals for research purposes where the first source of variation is inherently dependent on maintenance or test conditions, and can be reduced through a standardization of both culture and test protocols.

The second source is strictly dependent on the animal species used, its geographical provenience, and its strain and life cycle characteristics. Natural variability of organisms can be theoretically surpassed through the use of genetically similar individuals, held under strict control and artificial abiotic conditions. This state of cautious controlled environment can end in the maximum similarity as a clone. The use of *Artemia* clones, whenever a parthenogenetic population is selected

to serve as test-organism, might take into account an exhaustive characterization of the clone.

The majority of work performed so far involving *Artemia* as test organism in toxicology is related to the use of cysts (Migliore et al., 1997) and cyst-hatched nauplii of *Artemia,* (Vanhaecke et al., 1980). Cysts are produced both in bisexual and asexual (parthenogenetic) reproduction under stressing environmental conditions.

In the genus *Artemia* there is no cyclic bisexual versus parthenogenetic mechanism as in rotifers and cladocerans. Therefore, cyst production may reflect the occurrence of genetic variation in order to permit higher adaptability to new and adverse environmental conditions; however, genetic variability is not desired in most of toxicity assays since it may be an important source of variability in results. Nevertheless, the use of cysts in toxicology is highly advantageous, whenever short-term bioassays are routinely demanded, for early toxicity screening. Other major advantages are ascertained availability of test organisms, the low cost and simplicity in use.

*Artemia* is commercially available in the form of cysts, small spherical-like structures of high physical and chemical resistance, specialized in subsisting under extremely adverse conditions. *Artemia* cysts are available from varied sources, according to their geographical site of origin. Persoone and Wells (1987) described an intercalibration exercise involving the use of reference cysts of *Artemia* in toxicity assays, aiming at the determination of parameters such as reliability, accuracy and precision of this standardized methodology. This test protocol was then subjected to discussion and validated for routine, screening toxicology tests. Conclusions drawn from years of scientific work, sustained by debate over *Artemia* characteristics as test organism, supported the attainment of a commercially available, off-the-shelf toxicity test.

### 2.10.7 Major Evolutionary Perspectives in Toxicology Testing Using *Artemia*

The early 1980's witnessed large developments in toxicology as a self-directed science in modern scientific world. New ideas were brought to actual research, as a part of an effort to adapt cost-effective procedures, which conform to accuracy, reliability and ecological relevance. These developments intend to extrapolate from laboratory results to holistic visions of ecosystems.

An increasing number of animal species was adapted to laboratory culture conditions. These concepts served as a fundamental for the development of new methods, such as the already mentioned cyst-based screening toxicity assays (Vanhaecke et al., 1980), which represent highly valuable answers to general laboratory needs, due to their general availability and easy manipulation. An international intercalibration exercise, described by Persoone and Wells (1987), stated that 87 % of all the involved participants were able to successfully complete the standardized test with *Artemia*, and evaluated the test results as simple to interpretation, reliable and accurate. Nevertheless, the assumed end-point for the mentioned cyst-based assays such as mortality which is defined as total immobilization after a period of 10 sec, was not always well interpreted, due to the assumption of ''mortality'' for those animals that sunk in the testing medium but still exhibited appendages movements. Other parameters can be considered as reliable end-points, such as growth of *Artemia* individuals, described by Sarabia et al. (2002).

*Artemia nauplii* have been used in marine toxicity testing in coastal areas (Nipper, 2000), and for the establishment of alternative toxicity tests (Parra et al., 2001). Tothill and Turner (1996) also highlight the advantageous use of *Artemia*-based assays for toxicity testing in water treatment and for rapid screening of toxicity of cyanobacterial blooms.

Barahona and Sanchez- Fortun (1996) reported on the exposure of three different life stages of *Artemia salina* to several phenolic compounds where their conclusions indicate that sensitivity to the selected chemicals increased as the test organism developed. Increased age resulted in increased toxicity expression, at least

when comparing $LC_{50}$ values obtained when using specimens with ages comprised between 24 and 48 h. The use of 168 h-old nauplii, did not result in enhanced toxicity expression at least for the majority of the tested compounds. This study allowed comparing the most suitable age class for toxicological testing (48 h for *Artemia*) with other well-established aquatic animal models, such as *Daphnia magna*, which possessed an analogous response between aging and toxicity expression or enhanced sensitivity. These data show that animals of clearly defined age classes must be used for toxicity assays, since the toxicity expression is directly influenced by the development stage of the test organisms.

Parra et al. (2001) assessed the effect of acute treatment of *Artemia salina* larvae and mice with several extracts drawn from autochthonous plants of Cuba. This study intended to develop a low cost methodology applicable to countries where the use of medicines obtained from vegetable species is common and is an affordable manner of fighting disease. The authors calculated $LC_{50}$ values for *Artemia salina* larvae and $LD_{50}$ values for mice and established significant correlations between both parameters, suggesting the use of *Artemia salina* larvae as a suitable, accurate and inexpensive alternative to pre-screening chemical toxicity with mammals.

## 2.11 Dimethyl Sulfoxide, DMSO

Since many environmental chemicals are insoluble in water, it can be difficult to carry out toxicity tests without using organic solvents. DMSO is a common organic solvent and could be used as toxicant vehicle in toxicity test (Layman, 1987).

Thousands of tons of dimethyl sulfoxide (DMSO) have been used in hundreds of industrial plants, laboratories, universities and medical research establishment since 1960. Applications have included in pharmaceutical production, solvent cleaning, and hydrocarbon refining and agricultural formulations.

### 2.10.1 Properties of DMSO

DMSO dissolves a very wide range of organic and inorganic substances and is miscible with most common organic solvents such as alcohol ethers, chlorinated solvents and aromatics. With very high water solubility and active solvency, DMSO offers a high viscosity reduction. Table 2.2 shows the physical properties of DMSO.

**Table 2.2**: Physical properties of DMSO

| Physical Properties | Metric Units |
|---|---|
| Flash point | 87°C |
| Vapor pressure (20°C) | 0.556 mbar |
| Flash point | 87°C |
| Vapor pressure (20°C) | 0.556 mbar |

### 2.11.2 Reactivity of DMSO

DMSO reacts very rapidly and vigorously with a number of materials, particularly with those which also react rapidly with water. The reactions are highly exothermic, with rapid steam or gas evolution. In most cases the reactions can be controlled by rate of addition or by arranging adequate heat removal. The following types of compounds require care to prevent extremely rapid reactions:

i. Strong oxidizing agents such as perchlorates, permanganates, iodine, pentafluoride, silver fluoride and others react very rapidly.
ii. Acid chlorides react with DMSO at about the same rate as with ethyl alcohol.
iii. Carboxylic acid anhydrides react rapidly.
iv. Alkali anhydrides used for making DMSO anion require adequate heat removal.
v. DMSO cannot be used with Ziegler-Natta catalysts or in Friedel-Crafts reactions.

vi. Methyl bromide can react to form HBr and $Br_2$ where uncontrolled reactions will result.

### 2.11.3 Acute Toxicity of DMSO

Evaluation of the degree of hazard due to contact with chemical is usually by its single-dose $LD_{50}$. The $LD_{50}$ is the Lethal Dose in number of grams of DMSO per kilogram of bodyweight which results in 50 % mortality of the test animals under standardized conditions. Dozens of test data reports are available from man laboratories. The reported $LD_{50}$ varies, but the data confirm a low level of toxicity of DMSO.

Using monkey as one the animal model, it would take more than 0.73 kg applied to the skin or 0.27 kg taken by mouth or injected into blood stream, to have a 50 % mortality rate in a group weighing 68 kg each.

Other studies have shown that DMSO has low acute toxicity and it's practically non-toxic ($LD_{50}$ > 5 g/kg) by ingestion or dermal application (Smith et al., 1967). Rat oral $LD_{50}$ is reported from 17.4 to 28.3 g/kg, whereas LD50 for mice has been reported from 16.5 to 24.6 g/kg. Although DMSO can cause skin and eye irritation, it is not a skin sensitizer.

Besides $LD_{50}$, another unit $LC_{50}$ is used to evaluate the hazard from inhalation. $LC_{50}$ is the Lethal Concentration that kills 50 % of the test animals. The acute rat inhalation $LC_{50}$ is greater than 1.6 mg/L (Rubin, 1969).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 INTRODUCTION

This chapter mainly covers the experimental materials, equipments and apparatus used as well as the methods used to carry out the experiment procedures.

## 3.2 MATERIALS

Hexane, chloroform, methanol, Dragendroff reagent and ammonium solution (25 %) were purchased from MERCK, Germany. Sodium chloride, sulfuric acid, dimethyl sulfoxide (DMSO), potassium dichromate, vanillin , sodium hydroxide were purchased from R & M, U.K. Distilled water obtained from laboratory resources. Eggs of brine shrimps purchased from Super Artemia Sdn. Bhd. Malaysia.

### 3.2.1 Plant Material

The stems of *Tinospora crispa* were collected from district of Bentong, Pahang. The plant was identified by Botanist Prof. Dr. Halijah Ibrahim from University Malaya.

## 3.3 APPARATUS

Beaker, empty container bottles of 2.5 L, evaporator flask, separation funnel, conical flask, measuring cylinder, dropper, retort stand, glass volumetric flask, plastic volumetric flask, petri dishes, disposable Pasteur pipette, glass Pasteur

pipette, air pump, rotary evaporator flask, micropipette, glass rod, aluminium foil, filter paper, funnels

## 3.4 INSTRUMENTS

Rotavapor RII (BÜCHI) Switzerland, ultrasonic bath**,** grinder, pH meter, analytical balance and TLC visualizer.

## 3.5 METHODOLOGY

### 3.5.1 Extraction and Fractionation of Stems of *Tinospora crispa*

The separation and fractionation methods were used as described by Harborne (1988) with minor modifications. Initially, 1kg of the dried plant material was grinded to powdered form using grinder. The powdered materials were then soaked in hexane for 1 day as for defatting purposes. The marc was then separated by filtration process using sieve. This step was repeated for 2 cycles. This filtrate was labeled as Fraction 1.

The filtered marc was subjected to an alcohol extraction using methanol-water solvent system with the ratio 3:1 and left for one day. These were then sonicated in ultrasonic bath for 30 min. The mixture was filtered using sieve and followed by tea bag filter. These steps were repeated for 3 cycles.

The filtrate was then evaporated to 1/3 of original volume using rotary evaporator to reduce methanol composition. This was labeled as Fraction 2. With the aid of pH meter, this concentrated extract was acidified to pH 2 using diluted aqueous sulfuric acid solutions (50 %) and then partitioned with chloroform with ratio 2:1 using separation funnel. The separation funnel was shaken vigorously for 30 min. This step was repeated for three cycles. Due to immiscibility factor, two layers were formed in the funnel with one thin layer which was the emulsion.

At this point, the top chloroform layer was the aqueous layer. The bottom layer or the organic layer was collected in a beaker where this was the Fraction 3.

The aqueous layer was basified with ammonia solution (25 %) to obtain pH 10 where pH meter was used to test the pH level. It was then partitioned with chloroform and methanol, solvent system using ratio of 3:1. Two layers were noticed in the funnel. The organic layer which was at the bottom containing basic alkaloids was collected. It was evaporated to dryness till it become thick brownish in color. This was Fraction 4. The remaining aqueous layer was the Fraction 5.

The Figure 3.1 illustrates the overall procedure for extraction of *Tinospora crispa* stems and fractionation into different classes according to polarity.

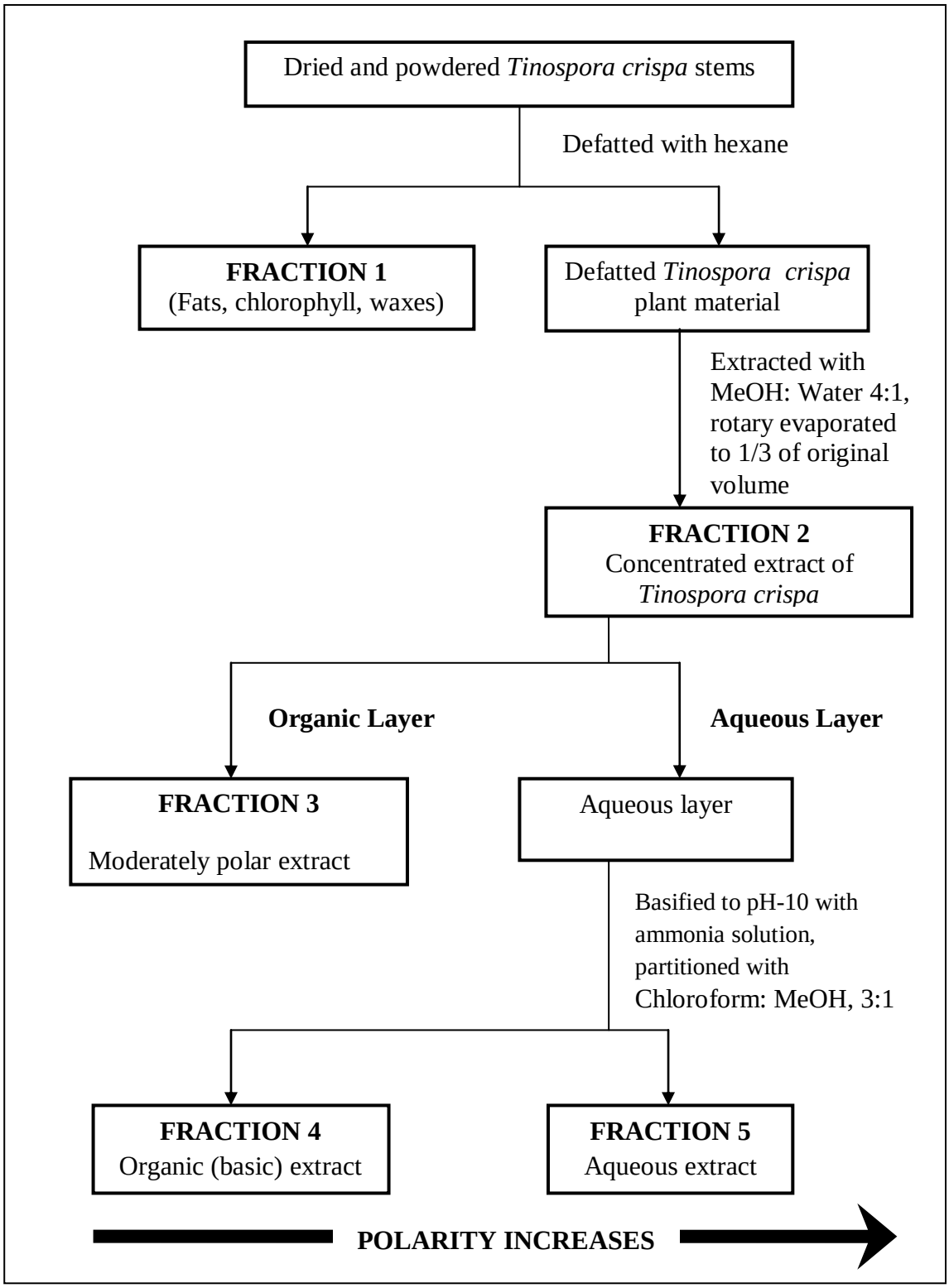


**Figure 3.1**: Flow chart of extraction of stems of *Tinospora crispa*

### 3.5.2 Preparation of Artificial Sea Water

38 g of sea salt (NaCl) was weighed, dissolved in 1000 ml of distilled water and filtered to obtain a clear solution (Persoone and Wells, 1987). This is known as artificial sea water which was used throughout the experiment.

### 3.5.3 Preparation of the Different Concentrations of Fractions

Initially, each fraction was rotary evaporated to remove the solvent as well as to get the concentrated extract. This followed by evaporation of the concentrated extract to evaporate the remaining solvent where the concentrated extract was placed on a petri dish and left uncovered for one day. After the evaporation process, the extract became semi-solid. These extracts were then dissolved in dimethylsulphoxide (DMSO) and diluted with artificial seawater where 1 % DMSO was used (Choudhary et al., 2001). It was then made in varying concentrations (1000, 100 and 10 μg/mL).

### 3.5.4 Preparation of Positive and Negative Controls

Potassium dichromate was prepared as a 1 mg/mL (1 %) solution in artificial seawater used as a positive control in brine shrimp lethality assay. Artificaial sea water medium containing 1 % of dimethyl sulphoxide was served as negative control for the assay carried out. One more negative control was prepared for the bioassay which was the artificial seawater only. Cytoxocity testing for each control were performed in triplicate ($n$ = 3).

### 3.5.5 Hatching and Preparation of the Nauplii

Simulated seawater was placed in a beaker and the shrimp eggs were added. The shrimps were allowed for two days to hatch and mature as nauplii (larvae). The beaker was well aerated with the aid of an air pump. Constant temperature and sufficient light were provided during the hatching time. After 24 h, the nauplii were separated from eggs by aliquoting them in a small beaker containing seawater.

### 3.5.6 Preliminary Test

Cytotoxic potential of different fractions of *Tinospora crispa* stem extract was assessed using the brine shrimp lethality assay as suggested by Meyer et al. (1982) with a minor modification. The brine shrimp lethality assay was conducted using three different concentrations (10 µg/mL, 100 µg/mL and 1000 µg/mL) of the five different fractions of extract. The freshly hatched free-swimming nauplii were used for the bioassay. The larvae were separated from the eggs and egg shells by aliquoting them three times in small beakers containing seawater. Briefly, the nauplii were attracted to one side of the beaker with a light source and collected with a pipette. 10 brine shrimps were transferred into petri dishes with minimal sea water carried together. 50 mL of concentrations (10 µg/mL, 100 µg/mL and 1000 µg/mL) prepared for each fraction was added into each petri dish. All experimental assays were prepared in triplicates for each concentration.

### 3.5.7 Counting of Nauplii

The petri dishes were observed for the time interval of 1 h, 3 h, 6 h, 12 h and 24 h. The number of surviving nauplii in each petri dish were counted using magnifying glass and recorded. The nauplii were considered dead when there was no movement of the appendages observed within 10 sec.

### 3.5.8 Statistical Analysis

The mean percentage of mortality for each concentration was determined using Statistical Package for the Social Sciences (SPSS) software, version 15.0 where the results were presented as mean percentage mortality $\pm$ standard error. The graphs of mean mortality percentage of the brine shrimp versus log of concentration were plotted using Microsoft Excel spreadsheet application, which also formulated the regression equations. The $LC_{50}$ values for the samples tested were derived from regression equations. $LC_{50}$ is the concentration of the extract or fractions that would kill 50 % of brine shrimps within the 24 h exposure.

### 3.5.9 Pre-Identification of Phenolic Compounds using TLC

The pre-coated silica gel plates were marked with scale, fixing the distance to be travelled by solvent from using sharp pin. The small amount of fractions to be tested was dissolved in chloroform-methanol solvent system using 1:1 ratio. For applying samples on plates, glass capillary was used. The distance between two spots was kept at minimum of 1 cm. Diluted fractions were loaded into glass capillaries and spotted on pre-coated silica gel plates. It was then developed in a TLC tank using chloroform- methanol solvent system with ratio 9:1. The chromatogram was developed by the ascending technique in which plates were immersed in the developing solvent to a depth of 0.5 cm. The chamber used was lined with sheets of filter paper which was dipped into solvent where, this ensures that the chamber is saturated with solvent vapor. Development was allowed to proceed until the solvent front has been traveled the required distance, then the plate was removed from the chamber and solvent front was immediately marked with pointed object. The plate was dried in fumehood. The compounds were separated into several bands with different $R_f$ values. The $R_f$ value was calculated using Eq (3.1).

$$R_f = \frac{\text{Distance traveled by sample (cm)}}{\text{Solvent front (cm)}} \quad (3.1)$$

Vanillin-sulphuric acid spray reagent was used to identify phenols on TLC plates. It was prepared by dissolving 1.0 g of vanillin in 100 ml (98% concentrated sulphuric acid- ethanol, 2:3). The developed plate was covered at one side and sprayed with Vanilin- sulfuric reagent (Harborne, 1988). The presence of phenols was observed with the formation of pink, reddish band under UV light and at the wavelength of 254 and 366 nm under TLC visualizer as for the Fraction 3 which is only a pre-identification.

### 3.5.10 Pre-Identification of Alkaloid Compounds Using TLC

The same method was repeated for identification of alkaloid compounds in Fraction 4 with some changes. As for identification of alkaloids different ratio of the solvent system was used which were chloroform- methanol solvent system with ratio 8:2. Dragendorff's reagent was used to identify the presence of alkaloids (Harborne, 1988). Orange to brick red spots pre-identifies the presence of alkaloids in the Fraction 4.

Figure 3.2 portrays the summary of the overall methodology carried out in this research.

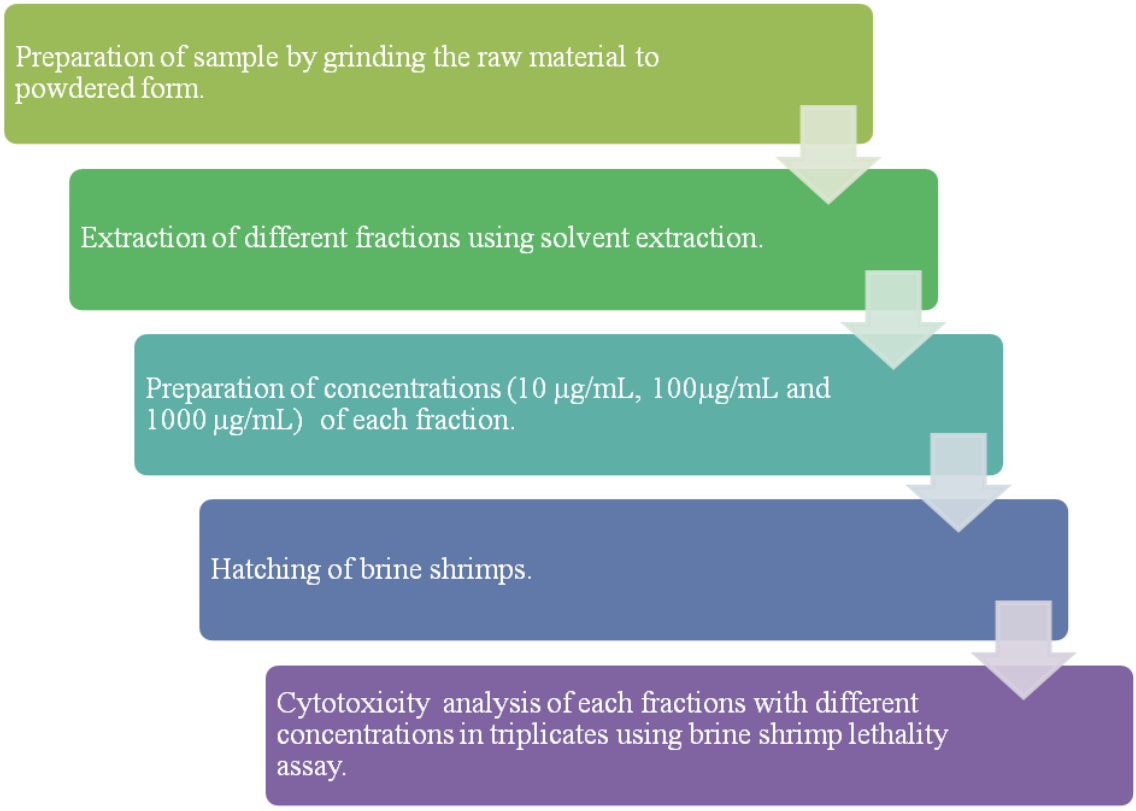


**Figure 3.2:** Flow Chart of Summarized Research Methodology

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 INTRODUCTION

The purpose of this chapter is to provide the results obtained from the research conducted. These results include the yield percentage of the fractions obtained from the extraction and fractionation of the *Tinospora crispa* stems. Besides, the result of the cytoxicity activity of the fractions against brine shrimp has also been presented in this chapter where the result is presented in the form of tables and graphs plotted using Microsoft excel, for a better understanding.

## 4.2 PERCENTAGE YIELD OF THE CRUDE PLANT EXTRACT

Table 4.1 shows the result of percentage yield of fractions obtained from extraction of *Tinospora crispa* stem based on polarity. Fraction 1 which is obtained by soaking the powdered plant material in hexane has percentage value of 0.87. It is a non polar fraction where it contains non-polar compounds such as fats, chlorophylls and essential oils (Harborne, 1988). On the other hand, Fraction 3 recorded higher percentage yield with 39.49 $\pm$ 0.001 g representing 3.95 %. Fraction 3 is the moderately polar fraction which consists of terpenoids, phenolic compounds as well as various glycosidic compounds (Harborne, 1988). The percentage yield of Fraction 4 is 0.51 % with yield obtained 5.08 $\pm$ 0.001 g. It is the basic extract that comprised of alkaloids (Harborne, 1988). In this study, the yield of Fraction 2 and Fraction 5 were not investigated and thus the details are not available.

**Table 4.1:** Percent yield of powdered *Tinospora crispa* stems

| Fraction | Yield obtained (g) | Percent yield (%) |
|---|---|---|
| F1 | 8.74 ± 0.001 | 0.87 |
| F2 | Not investigated | |
| F3 | 39.49 ± 0.001 | 3.95 |
| F4 | 5.08 ± 0.001 | 0.51 |
| F5 | Not investigated | |

The percentage of yield in the table above is obtained using the Eq. 4.1 below.

$$\text{Percentage yield} = \frac{\text{Weight of the sample extract obtained (g)}}{\text{Weight of the powdered sampled used (g)}} \times 100 \quad (4.1)$$

## 4.3 PHYSICAL PROPERTIES OF FRACTIONS OF *TINOSPORA CRISPA* STEMS

As a result of extraction *Tinospora crispa* stems, we obtained 5 different fractions. These five fractions vary in the physical appearance in general and polarity in specific. The first fraction is the filtrate obtained by extracting the powdered plant material with hexane. The physical appearance of this fraction is said to be dark brown in color and sticky. The second fraction is obtained by extracting the defatted plant material with methanol and water with ratio 4:1 and rotary evaporated to 1/3 of original volume. This fraction appeared to be brownish in color. The same physical appearance was observed for both Fractions 3 and Fraction 4. Meanwhile, Fraction 5 formed concentrated extract with the formation of many tiny crystals which are the mineral salts.

## 4.4 BRINE SHRIMP LETHALITY ASSAY OF FRACTION 1

The Table 4.2 is the mean mortality obtained for each concentration at the time interval of 1, 3, 6, 12 and 24 h. It also indicate that the results of the brine shrimp test with Fraction 1, did not produce $LC_{50}$ value but the percentage mortality of 16.67 ± 3.33 %, 26.67 ± 3.33 %, and 30.00 ± 0.00 % for various concentrations

prepared (10,100,1000 µg/mL respectively) obtained, has been tabulated in Table 4.2. Refer appendix A1 for the raw data obtained.

**Table 4.2**: Brine shrimp lethality bioassay of Fraction 1

| **Extract concentration ( µg/mL)** | **Log concentration** | **Post exposure (h)** | | | | | **% of mortality** | **$LC_{50}$** |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **3** | **6** | **12** | **24** | | |
| 10 | 1 | 1 | 3 | 5 | 5 | 5 | 16.67 ± 3.33 | Not Available |
| 100 | 2 | 1 | 4 | 6 | 7 | 8 | 26.67 ± 3.33 | |
| 1000 | 3 | 2 | 7 | 8 | 9 | 9 | 30.00 ± 0.00 | |

The Figure 4.1 is the log concentration versus percentage mortality graph plotted for the Fraction 1. Plotting of mortality percentage versus log concentration for Fraction1 demonstrates an approximate linear correlation. Furthermore, there is a direct proportional relation between the concentration of the extracts and the degree of lethality which is confirmed by the study carried out by Hartl and Humpf (2000). The finding of this study also revealed the effect of the time of exposure to the brine shrimp. Prolonged exposure to the extract resulted in increasing percentage of mortality, although the mortality rate is much lower.

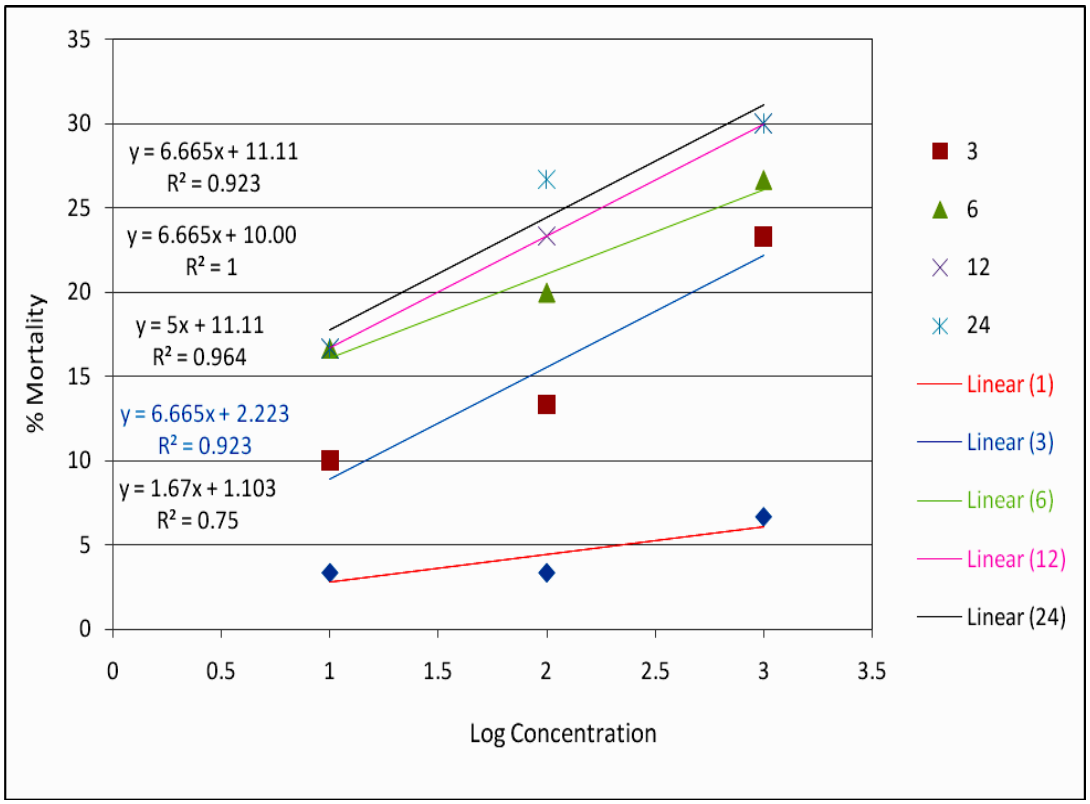


**Figure 4.1**: Graph of Log Concentration versus Percentage Mortality for Fraction 1.

From the Figure 4.1, it can be seen Fraction 1 is not toxic in the range of concentrations used where the $LC_{50}$ value could not be obtained for this fraction as the mortality rate is too low.

This could be due to the non polar characteristic of the fraction where it contains non-polar compounds such as fats, chlorophylls and essential oils. Thus, non polar compounds do not cause any significant effect towards brine shrimp tested.

## 4.5 BRINE SHRIMP LETHALITY ASSAY OF FRACTION 2

Table 4.3 demonstrates the mean mortality percentage of the triplicate assay for each concentration of Fraction 2. It also explains that the Fraction 2 had percentage mortality rates of 3.33 $\pm$ 3.33 %, 23.33 $\pm$ 3.33 %, and 43.33 $\pm$ 3.33 % respectively for the concentrations of 1000, 100, 10 µg/mL and there was no $LC_{50}$ value could be determined due to low mortality. These results indicate that the

Fraction 2 is also not toxic at the respective concentrations. Refer appendix A2 for the raw data obtained.

**Table 4.3**: Brine shrimp lethality bioassay of Fraction 2

| Extract concentration (µg/mL) | Log concentration | Post exposure (h) | | | | | % of mortality | $LC_{50}$ |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 6 | 12 | 24 | | |
| 10 | 1 | 0 | 1 | 1 | 1 | 1 | 3.33 ± 3.33 | Not Available |
| 100 | 2 | 1 | 1 | 7 | 7 | 7 | 23.33 ± 6.67 | |
| 1000 | 3 | 4 | 5 | 9 | 13 | 13 | 43.33 ± 3.33 | |

The Figure 4.2 is the log concentration versus percentage mortality graph plotted for the Fraction 2. From this graph, it can be clearly seen that the $LC_{50}$ value could not be obtained for this Fraction 2 due to low mortality of the brine shrimps when tested with 3 different concentration of Fraction 2.

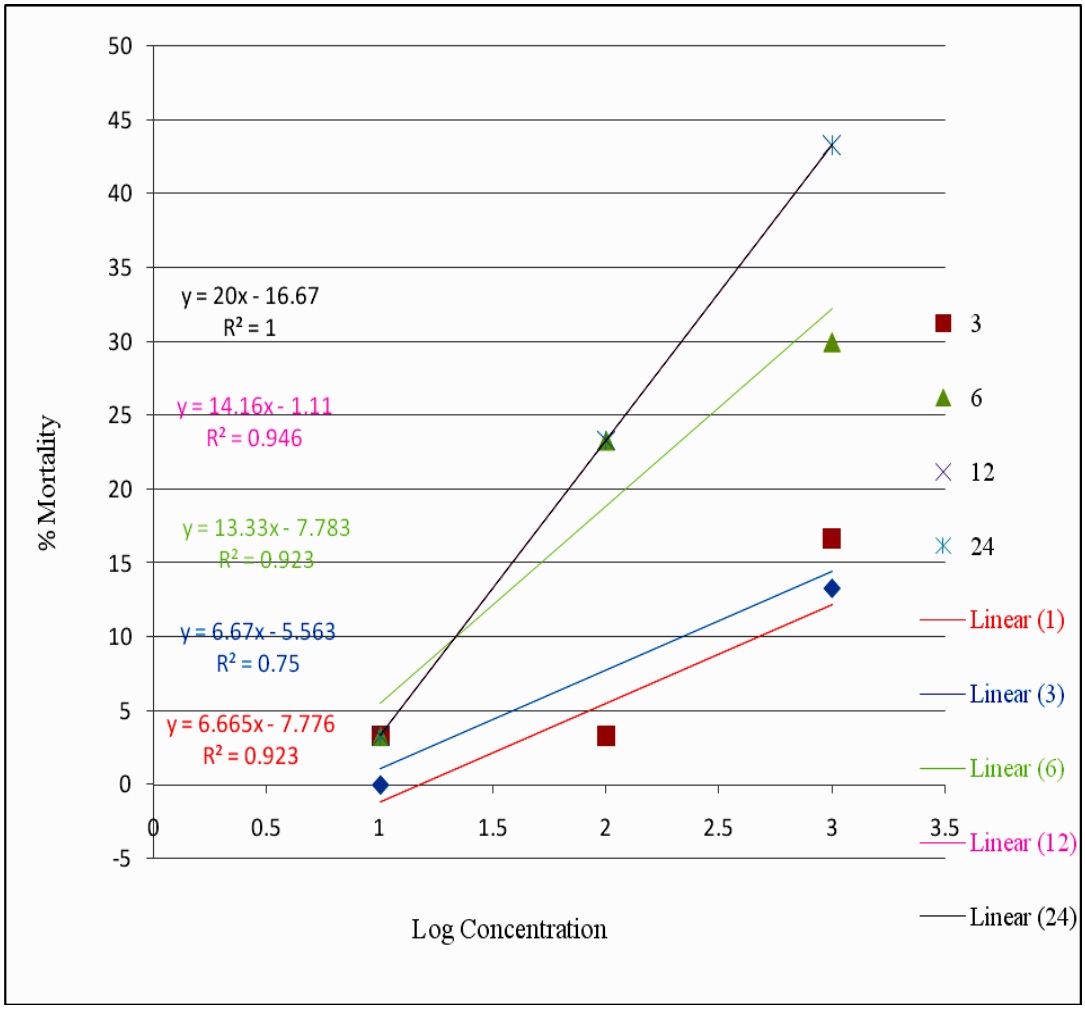


**Figure 4.2**: Graph of Log Concentration versus Percentage Mortality for Fraction 2.

It can be seen that, maximum mortalities took place at a concentration of 1000 µg/ml whereas least mortalities were at 10 µg/ml concentration but the higher mortality percentage obtained in Fraction 2 is only 43.33 $\pm$ 3.33 %. But the cause of the mortality of the brine shrimp could be due to the fact that during fractionation some compounds such as phenols or alkaloids might have remained in the Fraction 2 which could have caused the death of the nauplii.

## 4.6 BRINE SHRIMP LETHALITY ASSAY OF FRACTION 3

Table 4.4 is the mean mortality rate obtained for the triplicate data for the 3 concentrations used. Maximum mortality rate of 100 % was observed with 1000 µg/mL while the minimum or least mortality rate was recorded at 10 µg/mL where the percentage mortality rate were 100 ± 0.00 and 30.00 ± 0.00 respectively. Refer appendix A3 for the raw data obtained.

**Table 4.4**: Brine shrimp lethality bioassay Fraction 3

| **Extract concentration (µg/mL)** | **Log concentration** | **Post exposure (h)** | | | | | **% of mortality** | **$LC_{50}$** |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **3** | **6** | **12** | **24** | | |
| 10 | 1 | 1 | 1 | 2 | 6 | 9 | 30.00 ± 0.00 | 41.59 µg/mL |
| 100 | 2 | 3 | 3 | 4 | 9 | 18 | 60.00 ± 5.77 | |
| 1000 | 3 | 2 | 5 | 6 | 8 | 14 | 100.00 ±0.00 | |

The Figure 4.3 is the Log concentration versus Percentage mortality graph plotted for the Fraction 3 to determine the $LC_{50}$ value. From the figure, linear regression formula at 24 h was used to calculate for the $LC_{50}$ value and it was found to be 41.59 µg/mL.

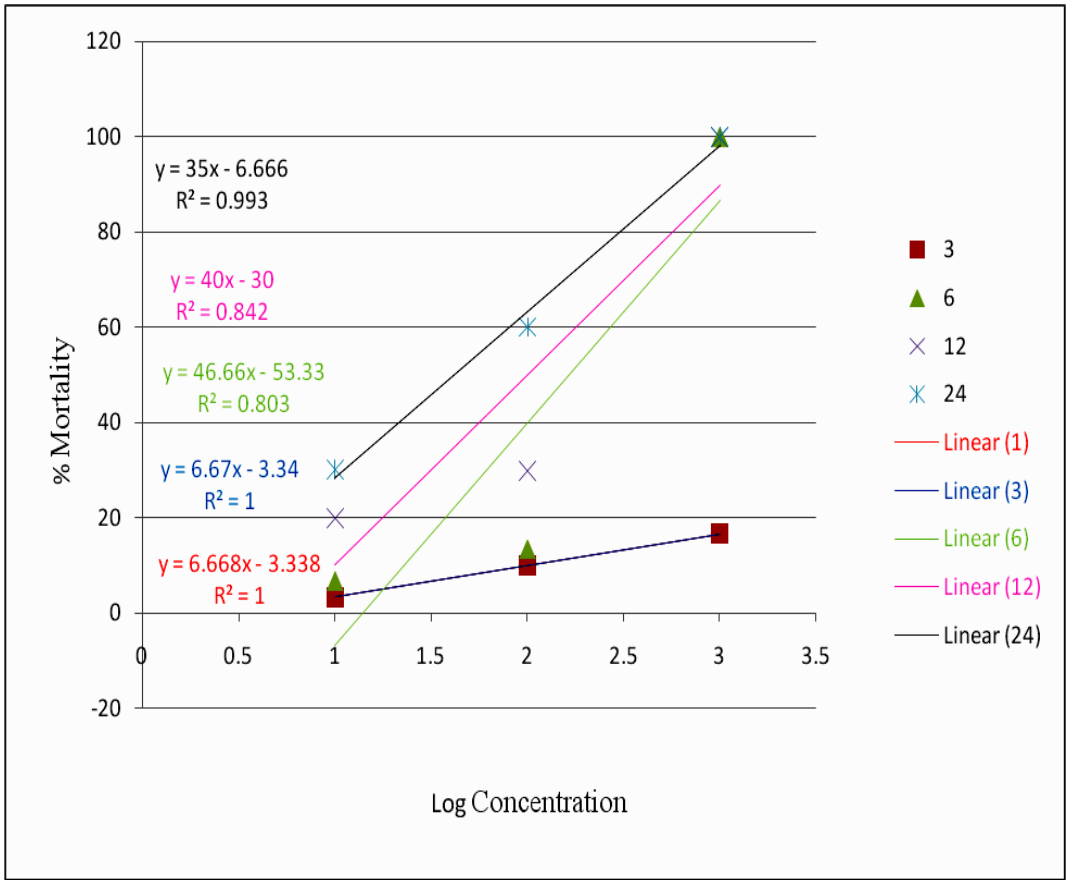


**Figure 4.3**: Graph of Log Concentration versus Percentage Mortality for Fraction 3.

The extract, fraction or isolated compounds were considered bioactive when the $LC_{50}$ value was 1000 µg/mL or less (McLaughlin, 1991). In this study, Fraction 3 happened to exhibit $LC_{50}$ value of 41.59 µg/mL. This value indicates that this fraction is mildly toxic. The cytotoxic effect of the *Tinospora crispa* in this study is slightly different from other study conducted where they investigated this activity by using *Tinospora crispa* aqueous crude extract of the stem which was prepared by soaking 100 g of the powdered stem in 900 ml distilled water (Zulkhairi et al., 2008). The results revealed that *Tinospora crispa* extract is not toxic to biological systems as the $IC_{50}$ of the extract was found to be higher than 1000 µg/ml.

### 4.6.1 Pre-Identification of Fraction 3 Using TLC

Different type of extraction methods contributes to separation of different active compounds of *Tinospora crispa* due to polarity of the solvent used. Regarding *in vitro* study on anti-malarial activity, chloroform extract of *Tinospora crispa* provided best result compared to methanol extract (Nik Rahman et al., 1999). Thus, in this study, Fraction 3 is the moderately polar fraction (Harborne, 1988). According to pytocontituent of *Tinospora crispa*, a number of moderately polar chemical constituents have already been isolated from this plant, such as phenolic acid amides, phenolic glucoside and some furanoid diterpenes and furanoid diterpene glucosides of the clerodane type (Fukuda et al., 1993).

In order to verify the presence of phenolics in Fraction 3, TLC pre-identification was done on the Fraction 3 extract which identified the presence of phenolics when sprayed with vanillin-sulphuric acid reagent as depicted in Figure 4.4. It is proper to mention that although the reduced forms of phenolic compounds act as antioxidants, the oxidized ones which are the phenoxyl radicals may exert cytotoxic, pro-oxidant activity when the lifetime of the radicals is prolonged by effectors of spin-stabilization (Sakihama et al., 2002). Thus, the cytotoxic activity observed could be due to the presence of phenoxyl radicals.

Figure 4.4 is result of pre-identification of phenolic compounds from Fraction 3 at254 and 365 nm using TLC, visualized under TLC visualizer.

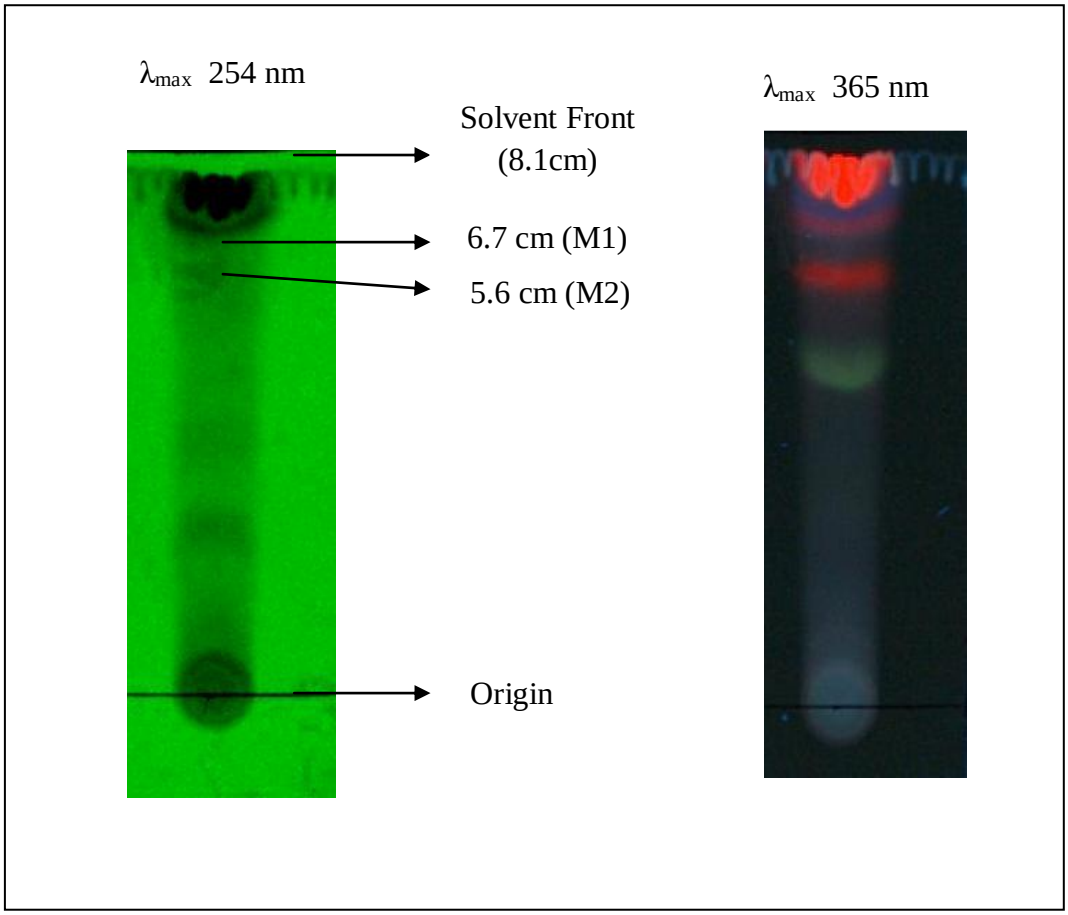


**Figure 4.4:** Pre-identification of phenolic compounds from Fraction 3 at 254 and 365 nm using TLC**.**

From the Figure 4.4, two bands were spotted namely M1 and M2 which turned to pink-reddish spots when sprayed with vanillin-sulphuric. The $R_f$ value obtained for M1 and M2 were 0.8 and 0.7 respectively. Since the $R_f$ values for this bands are more than 0.2, thus this compounds are separable.

Though the presence of triterpenoids in Fraction 3 is not verified but its presence can be assumed since triterpenoids are moderately polar compounds. They have a range of unique and potentially usable biological effects and reference to the use of plants with high triterpenoid content can be found in the first written herbarium. Triterpenoids are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoal infections and for their cytostatic effects. The disadvantage of using

triterpenoids is the toxicity associated with their hemolytic and cytostatic properties. Hand in hand with ongoing extraction and isolation of natural products therefore, is the development of synthetic derivatives with lower toxic and higher therapeutic potential (Dzubak et al., 2006). Thus, it can be said triterpenoids could be one of the reason for the Fraction 3 to be toxic.

## 4.7 BRINE SHRIMP LETHALITY ASSAY OF FRACTION 4

Table 4.5 illustrates the mean mortality of the 3 concentration used at the respective hours for the Fraction 4. According to the table, Fraction 4 had percentage mortality rates of 36.67 $\pm$ 3.33 %, 43.33 $\pm$ 8.82 %, and 66.67 $\pm$ 3.33 % respectively for the concentrations of 1000, 100, 10 µg/mL prepared. Refer appendix A4 for the raw data obtained.

**Table 4.5**: Brine shrimp lethality bioassay of Fraction 4

| **Extract Concentration (µg/mL)** | **Log concentration** | **Post exposure (h)** | | | | | **% of mortality** | **$LC_{50}$** |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **3** | **6** | **12** | **24** | | |
| 10 | 1 | 1 | 3 | 3 | 6 | 11 | 36.67 $\pm$ 3.33 | 118.58 |
| 100 | 2 | 2 | 3 | 5 | 7 | 13 | 43.33 $\pm$ 8.82 | µg/mL |
| 1000 | 3 | 3 | 7 | 9 | 15 | 20 | 66.67 $\pm$ 3.33 | |

The Figure 4.6 is the log concentration versus percentage mortality graph plotted for the Fraction 4. The $LC_{50}$ value obtained was 118.58 µg/mL using the linear regression equation y= 15x +18.89.

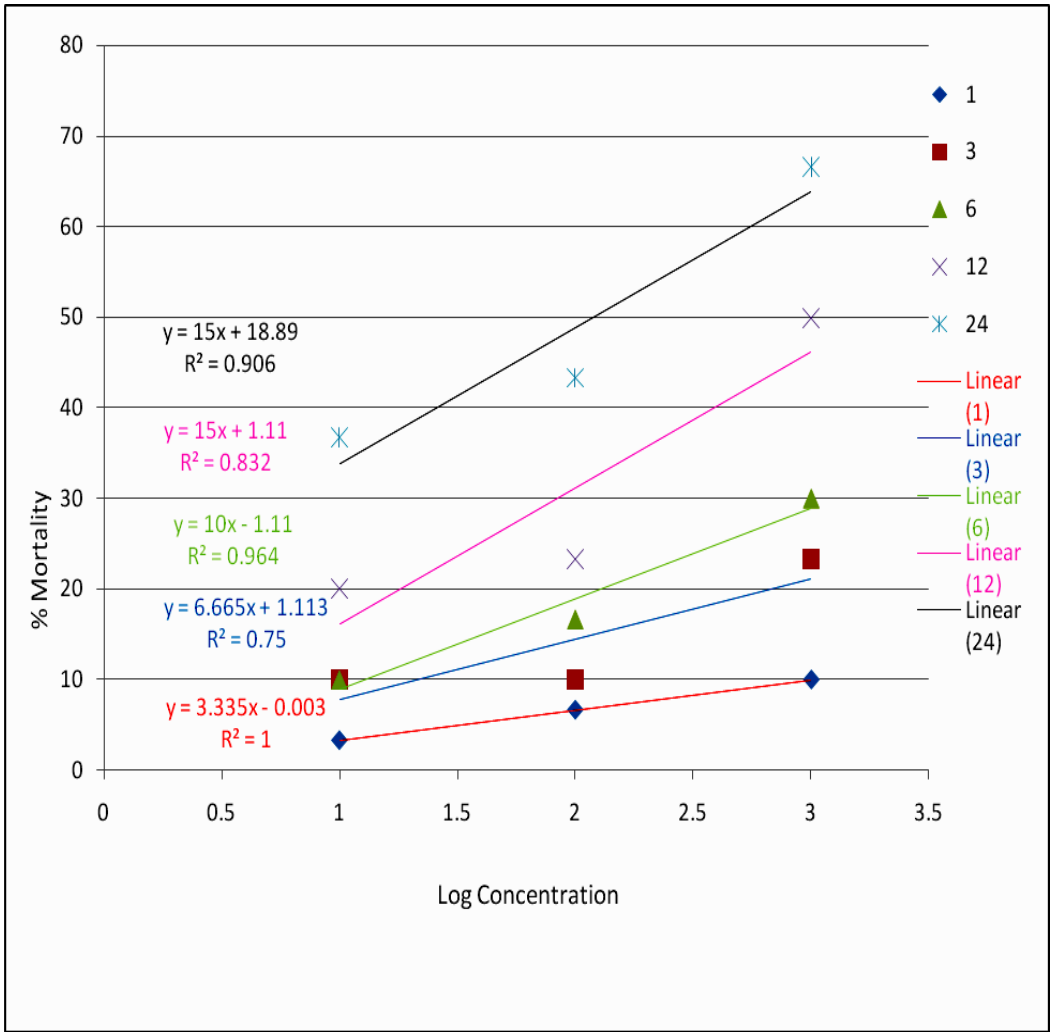


**Figure 4.5**: Graph of Log Concentration versus Percentage Mortality for Fraction 4.

From the figure above, the $LC_{50}$ value of Fraction 4 indicate that this fraction is toxic towards the brine shrimps tested. Fraction 4 is a basic extract obtained from extraction that contains alkaloids (Harborne, 1988) and this could probably have exerted the toxicity towards the brine shrimps tested.

### 4.7.1 Pre-Identification of Fraction 4 Using TLC

In order to verify the presence of alkaloid in Fraction 4, TLC pre-identification of alkaloid was done and presented as a Figure 4.6. The orange- brown spots indicates or pre-identifies the presence of alkaloids in the Fraction 4 when sprayed with Dragendorff's reagent (Harborne, 1988). From the Figure 4.6, there

were 2 bands spotted namely M3 and M4 with the distance from origin 5.4 and 4.6 cm respectively. The $R_f$ value calculated for these 2 compounds were 0.7 and 0.6 cm respectively. Thus, it can be said these alkaloid compounds are separable since their $R_f$ values are greater than 0.2 cm. Thus, it can be assumed that the cytotoxic activity observed in Fraction 4 is probably due to the presence of alkaloids.

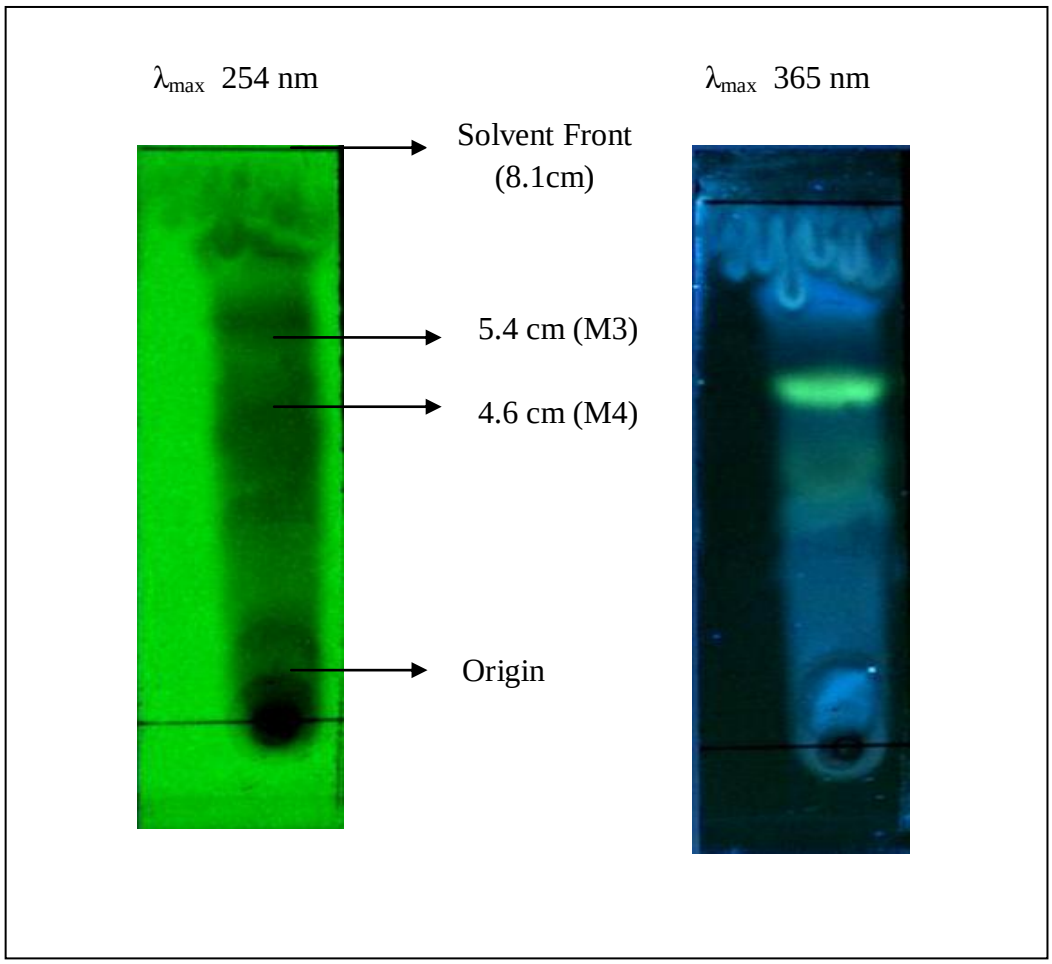


**Figure 4.6:** Pre-identification of alkaloid compounds from Fraction 4 at 254 and 365 nm using TLC**.**

## 4.8 BRINE SHRIMP LETHALITY ASSAY OF FRACTION 5

Table 4.6 presents the mean mortality of the brine shrimps at respective concentration and respective hours of Fraction 5. From the table, the Fraction 5 had percentage mortality rates of 23.33 $\pm$ 3.33 %, 26.67 $\pm$ 3.33 %, and 30.00 $\pm$ 0.00 %

respectively for the concentrations of 1000, 100, 10 µg/mL .The mortality rates found to be lower. However, the mortality percentage was found to be increasing with increasing concentrations. Refer appendix A5 for the raw data obtained.

**Table 4.6**: Brine shrimp lethality bioassay of Fraction 5

| **Extract Concentration (µg/ mL)** | **Log concentration** | **Post exposure (h)** | | | | | **% of mortality** | **$LC_{50}$** |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **3** | **6** | **12** | **24** | | |
| 10 | 1 | 0 | 3 | 4 | 5 | 7 | 23.33 ± 3.33 | Not Available |
| 100 | 2 | 3 | 4 | 5 | 6 | 8 | 26.67 ± 3.33 | |
| 1000 | 3 | 1 | 5 | 6 | 7 | 9 | 30.00 ± 0.00 | |

The Figure 4.7 is the log concentration versus percentage mortality graph plotted for the Fraction 5. From the graph, the $LC_{50}$ value was not able to be determined as low mortality rates observed when tested the Fraction 5 with brine shrimps as the highest mortality percentage obtained is only 30.00 ± 0.00 % which did not reach 50 % death of the nauplii tested.

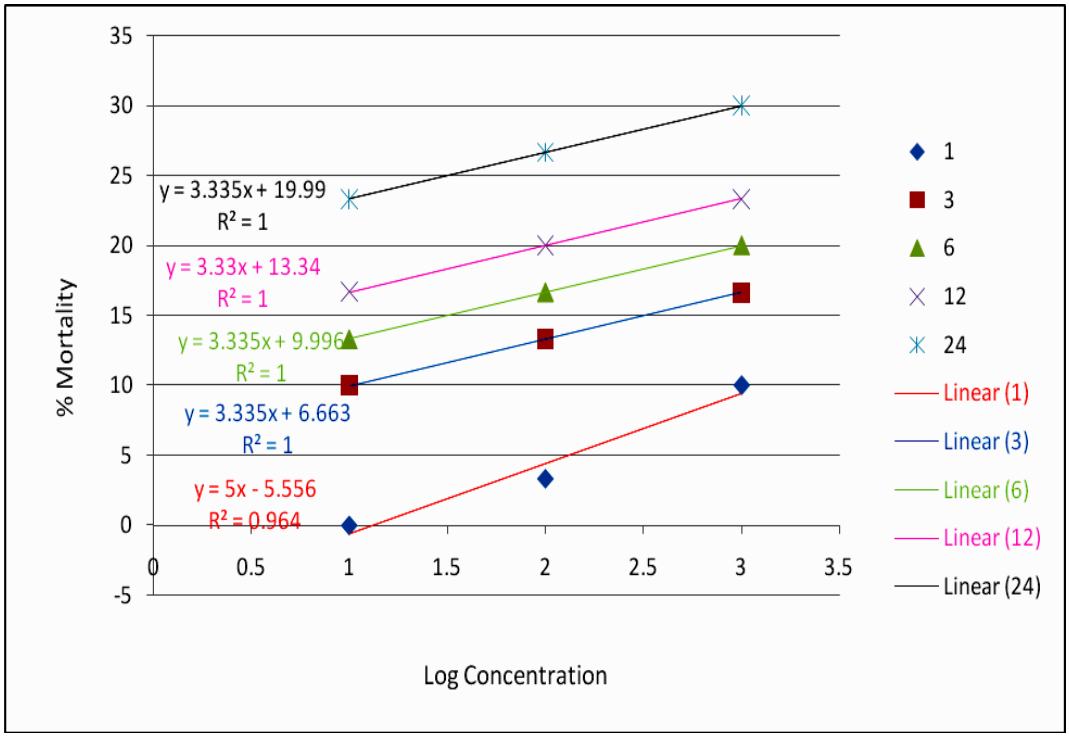


**Figure 4.7**: Graph of Log Concentration versus Percentage Mortality for Fraction 5.

## 4.9 BRINE SHRIMP LETHALITY ASSAY OF CONTROLS

Table 4.8 is shows the percentage mortality rates of the three different controls used. Choudhary et al. (2001) suggested that the compound should be prepared by dissolving in DMSO in the suggested maximum volume range of 2 % to prevent possible false effects originated from DMSO's toxicity to the experimental results. Thus, 1% DMSO was used as a negative control for the toxicity assay. Sea water also served as negative control while potassium dichromate was used as positive control. From the table, the both negative controls did not cause any mortality to the brine shrimps tested thus the percentage of mortality is not obtained. On the other hand, the positive control showed mortality percentage of 100 %. Refer appendix A6 for the raw data obtained.

**Table 4.7**: Brine shrimp lethality bioassay of controls (DMSO, Sea water and Potassium Dichromate)

| Controls | Post exposure (Hours) | | | | | % of mortality | $LC_{50}$ |
|---|---|---|---|---|---|---|---|
| | 1 | 3 | 6 | 12 | 24 | | |
| DMSO | 0 | 0 | 0 | 0 | 0 | 0 | Not investigated |
| Sea water | 0 | 0 | 0 | 0 | 0 | 0 | Not investigated |
| Potassium dichromate | 7 | 15 | 30 | 30 | 30 | 100.00 | Not investigated |

Potassium dichromate served as the positive control for this brine shrimp lethality assay and it has shown that it exhibits toxic expressions against the brine shrimps. In this research, the $LC_{50}$ value of potassium dichromate was not investigated as the purpose of positive control in this research was merely to confirm the positive result which was, to cause the death of nauplii tested. Thus, this shows that the experiment set up carried out in this research was capable of producing proper results.

# CHAPTER 5

# CONCLUSION AND RECOMMENDATIONS

## 5.1 INTRODUCTION

This chapter concludes the overall achievement of the research conducted.

## 5.2 CONCLUSION

As a conclusion, via this study, 5 different fractions of *Tinospora crispa* stems were obtained through extraction and fractionation. These fractions obtained as a result of fractionation into different classes according to the polarity using solvent system. When these 5 fractions tested against brine shrimps only 2 fractions exhibited biological activity which were Fraction 3 and Fraction 4 with $LC_{50}$ value of 41.59 and 118.58 μg/mL for respectively. Fraction 3 and 4 are therefore said to be toxic towards the brine shrimps tested. The degree of lethality was found to be directly proportional to the concentration of the extract and also to the prolonged exposure to the extract. According to the percentage of mortality, the fraction can be arranged as F3>F4>F2>F5, F1. Thus, from this findings of this research, Fraction 3 and 4 can be recommended as an indicator as an anticancer agent.

## 5.3 RECOMMENDATIONS

For future research studies, there are a few recommendations to improve the quality of the research and the testing standards. Firstly, the results of brine shrimp lethality assay obtained in this research may be used to guide the researchers; on the fraction of the *Tinospora crispa* plant extract to be prioritized for isolation of the

bioactive compounds since toxicity against brine shrimp larvae is merely a preliminary indication of the likely presence of toxic compounds in an extract. Thus, it is recommended that column chromatography to be employed to fractionate and isolate the active compounds and then subject it for further identification using High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR).Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later on.

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# APPENDICES

## APPENDIX A

**Appendix A1**: Number of Dead Nauplii Counted For Fraction 1

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 10 µg/mL | 2 | 1 | 1 | 2 | 2 | 2 |
| | 3 | 0 | 1 | 2 | 2 | 2 |
| | Total | 1 | 3 | 5 | 5 | 5 |

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 1 | 1 | 0 | 0 | 1 | 1 | 3 |
| 100 µg/mL | 2 | 0 | 2 | 3 | 4 | 3 |
| | 3 | 1 | 2 | 2 | 2 | 2 |
| | Total | 1 | 4 | 6 | 7 | 8 |

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 1 | 1 | 1 | 2 | 3 | 3 | 3 |
| 1000 µg/mL | 2 | 1 | 2 | 2 | 3 | 3 |
| | 3 | 0 | 3 | 3 | 3 | 3 |
| | Total | 2 | 7 | 8 | 9 | 9 |

**Appendix A2**: Number of Dead Nauplii Counted For Fraction 2

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| 10 μg/mL | 2 | 0 | 1 | 1 | 1 | 1 |
| | 3 | 0 | 0 | 0 | 0 | 0 |
| | Total | 0 | 1 | 1 | 1 | 1 |

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 2 | 1 | 0 | 0 | 3 | 3 | 3 |
| 100 μg/mL | 2 | 1 | 1 | 3 | 3 | 3 |
| | 3 | 0 | 0 | 1 | 1 | 1 |
| | Total | 1 | 1 | 7 | 7 | 7 |

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 2 | 1 | 1 | 1 | 2 | 4 | 4 |
| 1000 μg/mL | 2 | 2 | 2 | 4 | 5 | 5 |
| | 3 | 1 | 2 | 3 | 4 | 4 |
| | Total | 4 | 5 | 9 | 13 | 13 |

**Appendix A3**: Number of Dead Nauplii Counted For Fraction 3

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 3 | 1 | 0 | 0 | 1 | 1 | 3 |
| 10 µg/mL | 2 | 0 | 0 | 0 | 2 | 3 |
| | 3 | 1 | 1 | 1 | 3 | 3 |
| | Total | 1 | 1 | 2 | 6 | 9 |

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 3 | 1 | 1 | 1 | 1 | 3 | 6 |
| 100 µg/mL | 2 | 0 | 0 | 0 | 3 | 5 |
| | 3 | 2 | 2 | 3 | 3 | 7 |
| | Total | 3 | 3 | 4 | 9 | 18 |

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 3 | 1 | 2 | 2 | 10 | 10 | 10 |
| 1000 µg/mL | 2 | 1 | 1 | 10 | 10 | 10 |
| | 3 | 2 | 2 | 10 | 10 | 10 |
| | Total | 5 | 5 | 30 | 30 | 30 |

**Appendix A4**: Number of Dead Nauplii Counted For Fraction 4

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 4 | 1 | 0 | 2 | 2 | 3 | 4 |
| 10 µg/mL | 2 | 0 | 2 | 2 | 4 | 4 |
| | 3 | 1 | 1 | 1 | 1 | 3 |
| | Total | 1 | 5 | 5 | 8 | 11 |

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 4 | 1 | 0 | 0 | 1 | 2 | 4 |
| 100 µg/mL | 2 | 1 | 2 | 2 | 2 | 3 |
| | 3 | 0 | 1 | 3 | 3 | 6 |
| | **Total** | **1** | **3** | **6** | **7** | **13** |

| Sample | Number of death occurred due post exposure (h) | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 4 | 1 | 1 | 3 | 3 | 5 | 7 |
| 1000 µg/mL | 2 | 2 | 2 | 3 | 4 | 6 |
| | 3 | 0 | 2 | 3 | 6 | 7 |
| | Total | 3 | 7 | 9 | 15 | 20 |

**Appendix A5:** Number of Dead Nauplii Counted For Fraction 5

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 5 | 1 | 0 | 1 | 1 | 2 | 2 |
| 10 µg/mL | 2 | 0 | 1 | 2 | 2 | 3 |
| | 3 | 0 | 1 | 1 | 1 | 2 |
| | Total | 0 | 3 | 4 | 5 | 7 |

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 5 | 1 | 0 | 1 | 1 | 2 | 3 |
| 100 µg/mL | 2 | 2 | 2 | 3 | 3 | 3 |
| | 3 | 1 | 1 | 2 | 2 | 2 |
| | Total | 3 | 4 | 6 | 7 | 8 |

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Fraction 5 | 1 | 1 | 2 | 2 | 2 | 3 |
| 1000 µg/mL | 2 | 0 | 2 | 2 | 2 | 3 |
| | 3 | 0 | 1 | 1 | 1 | 3 |
| | Total | 1 | 5 | 5 | 5 | 9 |

**Appendix A6**: Number of Dead Nauplii Counted For Controls Used

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Negative | 1 | 0 | 0 | 0 | 0 | 0 |
| Control | 2 | 0 | 0 | 0 | 0 | 0 |
| DMSO | 3 | 0 | 0 | 0 | 0 | 0 |
| | Total | 0 | 0 | 0 | 0 | 0 |

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Negative | 1 | 0 | 0 | 0 | 0 | 0 |
| Control | 2 | 0 | 0 | 0 | 0 | 0 |
| Sea water | 3 | 0 | 0 | 0 | 0 | 0 |
| | Total | 0 | 0 | 0 | 0 | 0 |

| **Sample** | **Number of death occurred due post exposure (h)** | | | | | |
|---|---|---|---|---|---|---|
| | **Replicate** | **1** | **3** | **6** | **12** | **24** |
| Positive | 1 | 2 | 10 | 10 | 10 | 10 |
| Control | 2 | 3 | 10 | 10 | 10 | 10 |
| Potassium | 3 | 2 | 10 | 10 | 10 | 10 |
| Dichromate | Total | 7 | 30 | 30 | 30 | 30 |