Toxicity of *Ruta angustifolia* (Rutaceae) Leaves Extracts Using Brine Shrimp Lethality Test (BSLT)

By

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A report submitted in fulfilment of the requirement for the degree of Bachelor of Applied Science (Product Development Technology) with Honours

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DECLARATION

I hereby declare that the work embodied in this Report is the result of the original research except for the quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and it is not concurrently, submitted for any other degree at Universiti Malaysia Kelantan or at any other institutions.

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I certify that the Report of this final year project entitled "Toxicity of *Ruta angustifolia* (Rutaceae) Leaves Extracts Using Brine Shrimp Lethality Test (BSLT)" by Nadhirah Afiqah binti Ismail, matric number F13A218 has been examined and all the correction recommended by the examiners have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro Based Industry, Universiti Malaysia Kelantan.

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ABSTRACT

Ruta angustifolia L. (Rutaceae) which commonly known as “Garuda”, “Aruda” or “Inggu” grown as herb and can be found in Malaysia and Indonesia. It has been used for extensively by local people to treat fever, influenza, cough (plant decoction) and headaches (plant decoction). However, there are lacking of scientific data found on this species on its toxicity level and the bioactive components. The objective of this study was to determine the toxicity of *R. angustifolia* leaves extracts using BSLT. The crude ethanol extract was obtained by ultrasonication process in an ultrasonic bath. The ethanol extract later subjected to liquid-liquid partitioning to obtain various partitions; hexane, dichloromethane, ethyl acetate and butanol partitions. The lethality test was performed against brine shrimp nauplii with various concentrations of extracts; 1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/ml. The toxicity activity was determined by calculating the percentage of mortality within 24 hours to obtain LC_{50} values which is the lethal concentration required that can kill half the population of nauplii. Dichloromethane and hexane partitions exhibited the highest toxicity level with the LC_{50} values of 76.00±7.99 µg/ml and 81.21±3.20 µg/ml, respectively, while LC_{50} values for other partitions; ethanol is 125.84±18.21 µg/ml, ethyl acetate is 318.67±30.76 µg/ml and butanol is 3197.74±378.02 µg/ml. Potassium dichromate (the control) showed LC_{50} value of 27.62±3.01 µg/ml. Hence, the findings reveal that this plant is only safe to be consumed at a certain dosage only.

Keywords: *Ruta angustifolia*, Rutaceae, Brine Shrimp Lethality Test, LC_{50}

Ketoksikan Ekstrak Daun *Ruta angustifolia* (Rutaceae) menggunakan Ujian Kematian *Artemia* (UKA)

ABSTRAK

Ruta angustifolia L. (Rutaceae) yang dikenali "Garuda", "Aruda" atau "Inggu" berkembang sebagai herba dan boleh didapati di Malaysia dan Indonesia. Ia telah digunakan untuk secara meluas oleh orang-orang tempatan untuk merawat demam, selesema, batuk (rebusan tumbuhan), sakit kepala (rebusan tumbuhan). Walau bagaimanapun, terdapat kekurangan data saintifik yang ditemui pada spesies ini untuk tahap ketoksikan dan komponen bioaktif. Objektif kajian ini adalah untuk menentukan ketoksikan ekstrak daun *R. angustifolia* menggunakan Ujian Kematian Artemia. Ekstrak etanol mentah telah diperoleh dengan cara proses ultrasonikasi mengunakan rendaman ultrasonik. Ekstrak mentah etanol kemudian dibawa kepada proses pengekstrakan cecair-cecair untuk mendapatkan pelbagai partisi; heksana, diklorometana, etil asetat dan butanol. Ujian kematian dilakukan terhadap nauplius *artemia* dengan pelbagai kepekatan ekstrak; 1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/ml. Aktiviti ketoksikan ditentukan dengan mengira peratusan kematian dalam tempoh 24 jam untuk mendapatkan nilai LC_{50} iaitu kepekatan maut diperlukan yang boleh membunuh separuh daripada komuniti nauplius. Partisi diklorometana dan heksana mempamerkan peringkat tertinggi untuk ketoksikan tertinggi dengan nilai LC_{50} 76.00±7.99 µg/ml dan 81.21±3.20 µg/ml, masing-masing, manakala nilai LC_{50} untuk partisi lain; etanol adalah 125.84±18.21 µg/ml, etil asetat adalah 318.67±30.76 µg/ml dan butanol adalah 3197.74±378.02 µg/ml. Kalium dikromat (kawalan) menunjukkan nilai LC_{50} 27.62±3.01 µg/ml. Oleh itu, penemuan ini menunjukkan bahawa tumbuhan ini hanya selamat untuk dimakan pada dos yang tertentu sahaja.

Kata kunci: *Ruta angustifolia*, Rutaceae, Ujian kematian *Artemia*, LC_{50}

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BSLT	Brine Shrimp Lethality Test
BuOH	Butanol
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EA	Ethyl Acetate
EtOH	Ethanol
LC_{50}	Median lethal concentration
Log C	Log Concentration

LIST OF SYMBOLS

%	Percentage
ºC	Celcius
g	Gram
mg	Milligram
ml	Milliliter
µl	Microliter

CHAPTER 1

INTRODUCTION

In this world, the use of traditional medicine in the world depends on local plants that are easy to be reached and emphasised on a conventional wisdom-store of enlightenment that are affordable and simple (Tesfaye & Demissew, 2009). For the countries that still trying to make its industry and economic system more advanced, the use of medicinal plants as a traditional method helps to incorporate the basic health needs of the people. Furthermore, the developed countries have utilized the use of herbal remedies in the last ten years and more. As mentioned by Kesaran *et al*., in 2007, medicinal plants and herbs still go on as an affluent origin designed to treat an illness. The extensive figures of biological and phytochemical studies by the scientist to the medication productions were possible because of the amazing instalment of plants all over the earth as a cure for the diseases and also as to provide remedies. In addition, it is important to look into the toxicity levels of those plants such as by using the brine shrimp lethality test whether the plant it is safe to consume or not where from that test we can provide credible data on the lethal concentration, LC_{50} of the plants. One of the simplest toxicity tests is brine shrimp lethality test that was contemplated as a competent tool for initial evaluation of toxicity.

1.2 Problem Statement

There are many natural products from the medicinal plants that could fulfil the need of using natural ingredients in medicines as most of the modern medicines using the synthetic ingredients that have many adverse effects. Most of the information available to the consumer regarding to the medicinal plants was not backed by credible scientific data because consumers just rely on the traditional ways of using the plants without any proper dosage. In order to accomplish safe treatment with plant products, many researchers had been done on both pharmacology and toxicity of medicinal plants and they appointed brine shrimp lethality assay as one of the convenient test for monitoring biological activities of various plant species. For this basis, a study was done to investigate the toxicity of *R. angustifolia* L. plant. The leaf parts of *R. angustifolia* L. was chosen to be investigated for the toxicity test. The Brine Lethality Assay were carried out to determine the toxicity of crude ethanol, liquid-liquid partition of hexane, dichloromethane, ethyl acetate and butanol extracts of *R. angustifolia* L. leaves.

1.3 Hypothesis

H_0: The Brine Shrimp Lethality Test of crude ethanol, liquid-liquid partitioned in hexane, dichloromethane (DCM), ethyl acetate (EA) and butanol (BuOH) of *R. angustifolia* L. leaves extracts showed significant cytotoxic activity against brine shrimp nauplii.

H_a: The Brine Shrimp Lethality Test of crude ethanol, liquid-liquid partitioned in hexane, dichloromethane (DCM), ethyl acetate (EA) and butanol (BuOH) of *R. angustifolia* L. leaves extracts do not show significant cytotoxic activity against brine shrimp nauplii.

1.4 Research Question

1. Are the ethanol crude extract and the liquid-liquid partitions extract will show positive results that indicate the test samples are toxic to brine shrimp nauplii?
2. What is the median lethal concentration, LC_{50} of the ethanol crude extract and the partitions by plotting graph of log concentration (log C) versus percentage of mortality (% Mortality)?

1.5 Objectives

The objectives of this study were:

1. To separate the ethanol crude extract into several partitions using a liquid-liquid partition method.

2. To evaluate the toxicity of *R. angustifolia* L. leaves extracts using brine shrimp lethality test.
3. To determine the median lethal concentration, LC_{50} and compare the toxicity among the extracts.

1.6 Scope of Study

The study was carried out to perform a toxicity test of *R. angustifolia* L. leaves. It is focused on the toxicity level of ethanol crude extract and its partitions of leaf of *R. angustifolia* L. The leaves were extracted with ethanol and then were partitioned to hexane, dichloromethane, ethyl acetate and butanol partition by liquid-liquid partition method. Dry extracts were obtained by using rotary evaporator to

remove the solvents. LC_{50} of ethanol crude extract and all the partitions were obtained by using Brine Shrimp Lethality Test (BSLT) for 24 hours.

1.7 Significance of Study

The significance of this study is to reveal the toxicity of *R. angustifolia* L. leaves as to know the LC_{50} which the lethal concentration required from the extracts (crude ethanol and from the fractionation of hexane, butanol, dichloromethane and ethyl acetate) that can kill half of the population of nauplii. The LC_{50} values of toxicity of the herbal extracts were referred by using Meyer's Toxicity Index and also Clarkson's Toxicity Index.

LITERATURE REVIEW

CHAPTER 2

2.1 Rutaceae

According to Cronquist (1993), the Rutaceae family is usually placed in Sapindales because of its characteristics such as woody stem with typically compound, estipulate leaves, have rarely more than two ovules per carpel, a disk that full with nectar, usually no more than twice as many stamens as sepals or petals, and a superior ovary. Species in the Rutaceae family generally have flowers that divide into four or five petals and have strong scents. Engler (1896) identified seven subfamilies in the Rutaceae family that being defined based on the character of the gynoecium (ovule of flower that will change to fruit or seed) which is Rhabdodendroideae, Aurantioidea, Flindersioideae, Spathelioideae, Dictyolomatoideae, Rutoideae, and Toddalioideae. Although in modern classifications Cronquist (1993), Rhabdodendroideae have been excluded from Rutaceae, the other six subfamilies have been retained. Takhtajan (1987) recognized the six subfamilies. Thorne (1992) combined Toddalioideae with Rutoideae, making five subfamilies. Dahlgren (1989) and Cronquist (1993) did not mention subfamilies, but Cronquist did include Flindersiaceae as a synonym of Rutaceae.

2.2 *Ruta angustifolia* L.

According to Marisa in 2013, different names for *R. angustifolia* L. are *R. chalepensis* L. var. *Angustifolia*, *R. graveolens* L. var. *Angustifolia* Hook., *R.*

bracteosa DC., *R. frangiata*. *R. angustifolia* L. plant also has different names according to areas which are aruda (Sumatera), inggu, godong inggu (Jawa), and anruda busu (Sulawesi) (DepkesRI, 1989). In Malaysia, *R. angustifolia* have been recognised as 'garuda' or sadal'. It is also called as 'luru' in Vietnam and Rue in English (Richardson *et al*., 2012)

2.3 Ethnobotanical Studies

R. angustifolia L. belongs to family Rutaceae. It is an herb that lives for two years or more and woody at the base. It can grow up to 0.3-1.5 m tall. The leaves are spirally arranged, 2-3 pinnatisect, obovate to oblong-obovate in outline, measure 4-15cm x 2-9 cm, and obovate-lanceolate to narrowly oblong which are about 8-14 mm x 1.5-3.5 mm. They are conspicucously pale-bluish green, crenate, translucent-punctate-glandular, and strong smelling while the lower leaves are shortly petiolate (Irwanto, 2001).

The inflorescence is terminal or axillary in the upper leaf and often combines into a corymb. The bracts are lance-shaped, not or scarcely wider than the subtended branch and the glandular is usually hairy. The flowers are 4 (-50-merous, with deltateovate sepals, measure 2-3 mm x 1-2 mm, sebacute, and hairy glandular. The petals are oblong, 7-10 mm long and fringed with cilia as long as the width of the petal. The capsule is smooth and with acuminate segments (Irwanto, 2001).

Figure 2.1: *Ruta angustifolia* L.

Classification of Garuda plants is:

Division : Spermatophyta

Sub Division : Angiospermae

Class : Dicotyledonae

Nation : Geraniales

Tribe : Rutaceae

Marga : *Ruta*

Type : *Ruta angustifolia* (L) (Pollio, 2008)

Another classification based from (Sequiera *et al*., 2011)

Kingdom : Plantae

Phylum : Spermatophyta

Class : Magniliopsida

Order : Sapindales

Family : Rutaceae

Genus : *Ruta*

Species : *Ruta angustifolia*

2.4 Phytochemical Studies

Based on phytochemical studies of the roots and plant parts of *R. angustifolia* L., the plant contains the active alkaloids, furanocoumarin from the class of coumarin, flavonoids, tannins, volatile oils, sterols and triterpenes (a-pinene, limonene, cineole) as according to (Marisa, 2013). Del Castillo *et al*., (1984) generally stated that the aerial parts of *R. angustifolia* L. contain angustifolin from the class of coumarin. Some alkaloid isolated from the roots, among others arborinine, graveoline, graveolinine, dictamnine, ptelein, skimmianine, isogravacridon-chlorine, maculosidine, 4-methoxy-1-methyl-2\ (1H)-quinolone, cocusaginine, ribalinidine, rutacridone, isotaifine and others.

Tanker *et al*., (1980) stated that odour in *Ruta* plan*ts* are due to methyl-n-nonyl ketone. *Ruta chalepensis* and *Ruta graveolens* are the two main species that frequently used in traditional preparation of medicine (Lauk *et al.*, 2004). Gunaydin (2005) mentioned that the phytochemical screening of *Ruta chalapensis* plants (Rutaceae) obtained alkaloids, terpenoids, coumarin, tannins, saponins, anthraquinone, and terpenoids. A research conducted by Cowan (1999) describes that the leaves and young stems of Rutaceae family contain alkaloids, flavonoids, phenols, aminoacids, furanocoumarins and saponins. In aerial part of *R. graveolens*, there is a content of cardiac glycosides, coumarins, sterols, triterpenes, cyagenic

glycosides, tannins and antraquinones. Then, there are also volatile oil and volatile bases. Based on recent research by Richardson *et al.,* 2016, the chloroform fraction (without chlorophyll) of *R. angustifolia*, contains mostly furanocoumarins such as psoralen, bergapten, and methoxysalen. It is also contains alkaloids which are arborinine, kokusaginine and rutamarin.

2.5 Pharmacological Studies

Ruta angustifolia L. has been traditionally used as an abortifacient, antihelmintic, and ophthalmic (Richardson et al., 2016). The research by Milesi *et al*., (2001) showed that psoralen, furanocoumarin in *Ruta* species and its derivatives (linear furanocoumarins) can be used to treat skin diseases. In this study, *R, chalepensis*, *R. angustifolia*, *R. graveolens* and *R. montana* showed that the potential of those plants for the production furanocoumarins (psoralen, xanthotoxin, bergapten, isopimpinellin). 17 mg g−1 dry weight (DW) of furanocoumarins (FCs) is contained in Ruta species and the concentration of furanocoumarins found in Ruta species is higher than Moraceae, Apiacae and Fabaceae although these families produce the same compounds too.

According to Del Castillo *et al.,* (1984), arborinine alkaloids have anti-inflammatory activity, antihistamines, and spasmolytic effect. Furanocoumarin which are bergapten, and xantotoxine have a spasmolytic effect on smooth muscle and are useful in treating psoriasis phototoxic. They also concluded that, the content of the routine at this plant can be used to reduce capillary permeability, antihypertensives, and prevention of stroke. The ethanol extract of this plant also has the effect of anti-inflammatory, antipyretic, and CNS depressants (Ulubelen *et al*., 1988). Furanocoumarin, bergapten are having xantotoxine spasmolytic effect on smooth

muscle tissue. *Ruta angustifolia* L. has an effect significant inhibition against collagen to induce aggregation platelets from human blood in vitro (Gunaydin, 2005).

A research by Mahyuni *et al.,* in 2014 stated that chalepin and psuedane IX compounds in *R. angustifolia* L. showed strong anti-HCV activities without obvious cytotoxicity. Ribavirin has been widely used for the treatment of HCV but chalepin and pseudane IX have stronger anti-HCV activities. According to research by Norazian *et al*., in 2012, arborinine, graveoline and skimmianine has been identified and isolated and called as antimicrobial active alkaloids. Minimum inhibitory concentration (MIC) ranged between 62.5 µg/ml and 1000 µg/ml showed by the alkaloids when tested on the microbes. For the internal treatment, rue plant has been used as an antispasmodic which is treatment for menstrual problems, as a sedative and abortifacient.

According to research by Kasimala & Tukue in 2014, for the treatment for analgesic, antipyretic, mental disorders and also rheumatism, a decoction of the aerial parts of the plant was used in Saudi Arabia. In India, it is used for the treatment of dropsy, neuralgia, rheumatism, menstrual and other bleeding disorders while in China, it can be used as anti-venom by root decoction. According to Acquaviva *et al*. (2011), the oxidative property of Ruta extracts have can control the colon cancer. The oil and leaves of Rue extracts can cause blister so there are precautions not to apply the oil while under the sun exposure as it can cause dermatitis problem. The leaves of *Ruta* are used for myalgia, cold, whooping cough, abdominal pain, anti-emetic and many more traditionally in Eritrea.

2.6 Extraction techniques

Extraction can be defined as separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites, in liquid or semisolid state or after removing the solvent in dry powder form, and are intended for oral or external use. Some techniques of medicinal plant extraction are maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), counter-current extraction, supercritical fluid extraction and ultrasound extraction (Humprey & Keller, 1997).

In maceration process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, (the damp solid material is pressed, and the combined liquids are clarified by filtration. Then, for infusion technique, fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs (Seader & Henley, 1996).

Digestion is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased while for decoction process, the crude drug is boiled in a specified volume of water for a defined time and then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. This process is typically used in preparation of Ayurvedic extracts. (Handa & Kaul, 1996)

Percolation is the procedure that used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 hours in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 hours. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting. (Handa, 2008)

For hot continuous extraction (Soxhlet), the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper. The extracting solvent is heated, and its vapours condense in condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber containing thimble rises to the top of siphon tube, the liquid contents flowed out of siphon into lower flask. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale. (Handa, 2008)

In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce fine slurry. In this process, the material to be extracted is moved in one direction (generally in the form of fine slurry) within a cylindrical extractor where it comes in contact with extraction solvent. The further the starting material moves, the more concentrated the extract becomes. Complete extraction is thus possible when the quantities of solvent and material and their flow rates are optimized. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end. This extraction process has significant advantages such as a unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, percolation. CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques. The extraction procedure has been rated to be more efficient and effective than continuous hot extraction. (Handa, 2005)

Supercritical fluid extraction (SFE) is an alternative sample preparation that reduced the use of organic solvents and increased sample throughput. The factors to consider include temperature, pressure, sample volume, analyte collection, modifier (cosolvent) addition, flow and pressure control, and restrictors. There are many advantages to the use of Carbon dioxide as the extracting fluid such as favourable physical properties, inexpensive, safe and abundant. But while carbon dioxide is the preferred fluid for SFE, it possesses several polarity limitations. Organic solvents are frequently added to the carbon dioxide extracting fluid to alleviate the polarity limitations. Of late, instead of carbon dioxide, argon is being used because it is inexpensive and more inert. The extraction procedure possesses distinct advantages: such as the extraction of constituents at low temperature, which strictly avoids

damage from heat and some organic solvents. No solvent residues and environmentally friendly extraction procedure. (Abraham, 1997)

In this study ultrasound extraction (sonication) or ultrasonic assisted extraction was used as this increases the permeability of cell walls and produces cavitation that helps to shorten extraction time and then enhanced the efficiency of the extraction. (Schinor *et al.,* 2004)

2.6.1 Ultrasonic Assisted Extraction

According Ou *et a.l,* 1997; Ong *et al.,* 2000 and Shotipruk *et al.,* 2001, they reported that ultrasound extractions have beneficial effects on extraction of medicinal plants such as enhancing efficiency and shortening of extraction time (Schinor *et al.,* 2004). The mass transfer is enhanced as penetration of solvents into the cell of the plant causing disruption of the cell and also to the capillary (Toma *et al.* 2001). The mechanism of ultrasonication process is the bubbles made by the ultrasound on the cell walls of plants can be portrayed by; some plant cells have external glands that are very narrow and can be destroyed by ultrasound. External glands that filled with essential oils will be ruptured thus releasing the content of essential oil into the extraction solvent (Vinotoru, 2001).

2.7 Toxicity Studies

Toxicity studies are the scientific knowledge of the unpleasant effects of chemical and physical substance and the level to which an agent can detriment and organisms. It can be chronic, sub-chronic and acute toxicity. If the toxic substances giving a harmful effects in an organism through a short term or single exposure it is called as acute toxicity while if the toxic substance causing effects for more than one

year but less than the lifetime of the organism it is called as sub-chronic toxicity. Then, chronic toxicity is the ability of the substance or mixture of substances to cause harmful effects over an extended period, usually upon repeated and continuous exposure (Parasuraman, 2011).

The acute toxicity test in which a single dose that used in each animal on one occasion only for the median lethal Dose, LD_{50} and median lethal dose, LC_{50}. The chronic tests in which two species, one rodent and one non rodent are dosed daily for six months while the sub -acute tests in which animals (usually rats and dogs) are dosed daily, starting at around expected therapeutic level and increasing stepwise every two to three days until toxic signs are observed. Acute toxicity tests are generally the first tests conducted to provide data on the relative toxicity likely to arise from a single or brief exposure. Standardized tests are available for oral, dermal, and inhalation exposures (Gupta, 2012).

Chronic toxicity tests determine toxicity from exposure for a substantial portion of a subject's life and it's similar to the sub-chronic tests except that they extend over a longer period of time and involve larger groups of animals. Sub-acute toxicity tests are employed to determine toxicity likely to arise from repeated exposures of several weeks to several months. Standardized tests are available for oral, dermal, and inhalation exposures (Gupta, 2012)

Toxicity testing of new compounds is essential for drug development process. The preclinical toxicity testing on various biological systems reveals the species-, organ- and dose- specific toxic effects of an investigational product. The toxicity of substances can be observed by studying the accidental exposures to a substance, in vitro studies using cells/ cell lines and in vivo exposure on experimental animals (Parasuraman, 2011). In this study, Brine Shrimp Lethality Test was used to screen

the toxicity of the extracts of the plant. It is one of the examples of acute toxicity studies (Meyer et al., 1982; McLaughlin et al., 1998a; Moshi et al., 2010; Ogugu et al., 2012; Gadir, 2012; Solanki and Selvanayagam, 2013; Sharma et al., 2013; Hamidi et al, 2014).

2.7.1 Brine Shrimp Lethality Test

Brine Shrimp (*Artemia salina*) Lethality Test is one of has been used for over 30 years. From the test, the unpleasant effects were occurring within a short time of (oral) administration of a single dose of substances or multiple doses given within 24 hours. Nowadays, because of the fast, cheap and desirable ways rather than to test on bigger animal, many researchers using brine shrimp as a pre-screen for plant extracts. There is a positive correlation prevails between brine shrimp fatality and 9KB (human nasopharyngeal carcinoma) cytotoxity; so it has potential to be used as many pre-screens for potential anti-tumour activity. Furthermore, it also excellently predicts activity of pesticides and also reacts to a broad range of chemical and pharmacological diverse compounds. (McLaughlin, 1991).

Harwig and Scott stated that in 1971, Brine Shrimp Lethality Test is an easy, high throughput acute toxicity test of biochemical compounds. It is based on ability of test compounds or samples to kill the zoological organism-brine shrimp (*Artemia salina*). This assay was first recommended by Michael *et al*., in 1956, and further progressed by several researchers (Chao, 2014). The brine shrimp lethality assay is broadly used for the evaluation of toxicity of heavy metals, pesticides, preliminary toxicity screen for further experiments in mammalian animal models. (Chao, 2014)

As stated by Winnett in 1997, Wilson in 1989 and cited by Etterson in 2013, Brine Shrimp Bioassay or Brine Shrimp Lethality Test are used for many purposes

such as screening potential new biological activity in the plant extracts and also plant chemicals that show on specific biological effects such as effect on growth, survival or reproduction on specific selected research organisms. For example, botanists and biochemists are currently using this assay to screen unknown plants for potentially useful, naturally occurring compounds.

Brine Shrimp lethality assay is a fast and high throughput bioassay for the research of bioactive compounds. Determination of pure compound or their bioactivity can be tested by using this study as well as for natural product extracts and fractions (Asaduzzaman *et al.,* 2015). A simple zoological organism that called as Brine nauplii will be exposed to specific substances that might kill the organism as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. The advantages are being fast for only 24 hours, cheap, and easy because no aseptic techniques are required. It easily uses a big number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less). Moreover, animal serum is not needed for cytotoxicities (McLaughlin et al., 1998).

2.8 Median Lethal concentration, LC_{50}

The study of concentration-response or concentration-effect in toxicology is essential and is used to determine the LC_{50} of drugs and other chemicals (Goodman & Gilman, 2007). The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}). LC_{50} is the concentration of the substances or chemical compound that can kill in half of the test subjects after being exposed to a certain time and it can be determined by linear regression method from plotting % mortality against correspondent log of concentration (Naidu *et al*., 2014).

The toxicity of plant extracts expressed as LC_{50} values was commonly classified either by comparison to Meyer's toxicity index listed in Table 2.1 and Table 2.2 (Meyer *et al*., 1982; Clarkson *et al*., 2004).

Table 2.1: Clarkson's toxicity criterion for the toxicity assessment of plant extracts

LC_{50} (µg/ml)	Toxicity level
Above 1000	Non-toxic
500-1000	Low-toxic
100-500	Medium toxic
0-100	Highly toxic

(Clarkson et al., 2004)

Table 2.2: Meyer's toxicity index

LC_{50} (µg/ml)	Toxicity level
$LC_{50} < 1000$	Toxic
$LC_{50} > 1000$	Non-toxic

(Meyer et al., 1982)

CHAPTER 3

METHODOLOGY

3.1 Materials, Apparatus and Machines

3.1.1 Materials

The materials that had been used in this research are Dimethyl sulfoxide, DMSO (FRiedemann Schmidt Chemical) , hexane (HmbG), ethanol, EtOh (HmBG), dichloromethane, DCM (HmbG), ethyl Acetate, EA (HmbG), butanol, BuOH (DC Laboratory Reagen), Potassium dichromate, K2CR2O7 (Bendosen laboratory), R. angustifolia L. (leaves), distilled water, artificial seawater, brine shrimp (Artemia salina)

3.1.2 Apparatus

The apparatus that had been used in this research are round bottomed flask (1000 ml and 250ml), beaker (1000 ml, 500 ml, 250 ml, 100 ml and 50 ml), media bottle (500 ml), tray, conical flask, Whatman filter paper No. 1, micropipette (1000 µl and 10 µl), petri dish, light bulb, spatula, microcentrifuge tube, micropipette rack, measuring cylinder (500 ml, 250 ml, 50 ml and 10 ml), retort stand, zipper bags, pasteur pipette

3.1.3 Machines

Blender (ELBA Model EBM9761G Blender), Ultrasonic Cleaner Bath (Lab Companion UC-20), Rotary Evaporator (Buchi Rotavapor R-210, Buchi Heating Bath B-491).

3.2 Sample collection and Preparation

R. angustifolia L. were bought fresh from the Ukay Nursery, Ampang in July 2016. The plants were grown in a vase and after one month, the leaves were plucked and then air-dried on the tray at ambient temperature for 4-7 days. The leaves were ground into powder by a blender and kept in closed zipper bag and stored in chiller until further used.

3.3 Sample Extraction

The ground leaves (100 g) were put in media bottle and extracted by ultrasonic-assisted extraction with ethanol with at 25ºC. The extraction process in the ultrasonic cleaner bath was carried out for 40 minutes. Every 40 minutes, a new volume of ethanol was added until the colour of the sample in the ethanol solvent become colourless. After that, the extract solution was filtered by using filter papers to get the filtrate. The volume of filtrate was recorded and the concentrated crude ethanol extract was obtained by using the rotavapour to concentrate the filtrate to dryness under vacuum and reduced pressure at 39 ºC.

3.4 Preparation of Partitions

Liquid-liquid partition was performed by taking 5 g of crude ethanol extract. The solvents used in this preparation of partitions are hexane, dichloromethane, ethyl acetate and butanol. The crude ethanol extract was combined with 300 ml of distilled water and 200 ml of ethanol. Once the mixture is completely mixed, it was poured into the separating funnel. Then, 600 ml of hexane was poured into the separating funnel until two immiscible layers of the mixture were formed. The upper layer was hexane where it is less dense than water. The hexane layer was put into the beaker and the residue was put back into the separating funnel.

The residue was continued with dichloromethane where 300 ml of dichloromethane was added into the separating funnel and the dichloromethane layer was formed at the bottom because it is denser than water. After that, the residue was put back into the separating funnel where 600 ml of ethyl acetate was put into the separating funnel and the layer was formed at the upper layer because ethyl acetate is less dense than water. The layers were put in the respective beakers and the residue was added back into the separating funnel and the partition of butanol was done by adding 400 ml of butanol into the separating funnel until two immiscible layers formed. The upper layer is butanol where it is less dense than water and the biomass left was labelled as residue.

3.5 Preparation of Brine Shrimp Lethality Assay

3.5.1 Preparation of artificial seawater

33.4 g sea salt was weighed and dissolved in 1000 ml of distilled water to get water for the brine.

3.5.2 Hatching of *Artemia salina*

Artemia salina eggs were hatched and used as test organisms. The artificial seawater was poured into the small tank and shrimp eggs were put into the tank. The shrimps were completely hatched after 2 days or 48 hours at room temperature. Through the hatching time, a constant supply of oxygen was used. The hatched shrimps were captivated to the light (phototaxis) and the light bulb was used to supply light thus free the shrimp from its eggs. The nauplii was taken from the tank by using a Pasteur pipette and diluted in fresh, clear sea water to make sure the brine shrimp is visible and 10 brine shrimps were taken by micropipette and put into microcentrifuge tube.

3.5.3 Preparation of stock solution of extracts

The test solution was made for 2 ml which consist of 20 µl or 0.02 ml of extract and 1980 µl or 1.98 ml of artificial sea water as to make the concentration of DMSO in the extract to become only 1% because absolute DMSO is toxic to the brine shrimps. The sample was weighed and then added with 200 µl absolute DMSO in a microcentrifuge tube. The sample was prepared for working concentration in 200 µl. Then, the sample was prepared for different concentrations; 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25 µg/ml and 15.625 µg/ml by serial dilution. The micropipette was set for 980 µl for hunting the brine shrimp nauplii and was put into the microcentrifuge tube that contain 1000 µl of artificial sea water. 20 µl of extract was placed into the microcentrifuge tube in triplicate for each concentration.

3.5.4 Preparation of the positive control group

Widely cytotoxic agent potassium dichromate was used for the positive control study and the result was compared with the result obtained for the brine shrimp lethality test with the samples. As potassium dichromate is highly toxic and it was evaluated at very low concentration (50, 40, 30, 20 and 10 µg/ml).

3.5.5 Preparation of the negative control group

Three microcentrifuge tubes containing 20 µl of 1%DMSO and 1.98 ml of artificial sea water with 10 brine shrimps were used for the negative control test. The negative control was done to make sure that if brine shrimps show a high mortality rate, then the test is not accepted as the brine shrimps died because of other reasons other than cytotoxicity of the compound and the value of dead nauplii will be added according to the amount of dead nauplii that exposed by DMSO for all the triplicates according to concentration.

3.5.6 Counting of brine shrimp

The brine was counted after 24 hours. A magnifying glass was used to inspect the brine shrimp in the micropipette tube. The brine shrimps were inspected against a black background and then were put on the petri dish for further clarification on the number of dead nauplii. The number of survived brine shrimp in each tube was counted. From the data of number of dead nauplii from each concentration, the percentage of mortality (%) was calculated. The mortality was corrected using Abbott's formula (Abbott, 1925)

Percentage of Mortality (%) = (Total nauplii – Alive nauplii) × 100%/ Total nauplii (3.1)

The successful of relationship between concentration and mortality of the plant was expressed as the median lethal concentration which is LC_{50}. The value of LC_{50} were determined by plotting the graph of % of mortality against the log C and by calculation the value of x based on the equation of linear regression and from the graph the concentration of the chemical that produces death in half of the test subjects after a certain exposure time was obtained.

3.5.7 Statistical Analysis

All the data were collected in triplicate and expressed as mean ± standard deviation. Statistical Package for the Social Science (SPSS) version 20 was used to analyse the data. The significance differences within groups were determined by One-way ANOVA and post hoc tests which are Duncan Multiple Range Test and Dunnett-test. Duncan test were performed to determine the differences between partitions while Dunnett t-tests treat potassium dichromate as a control, and compare all other samples against it.

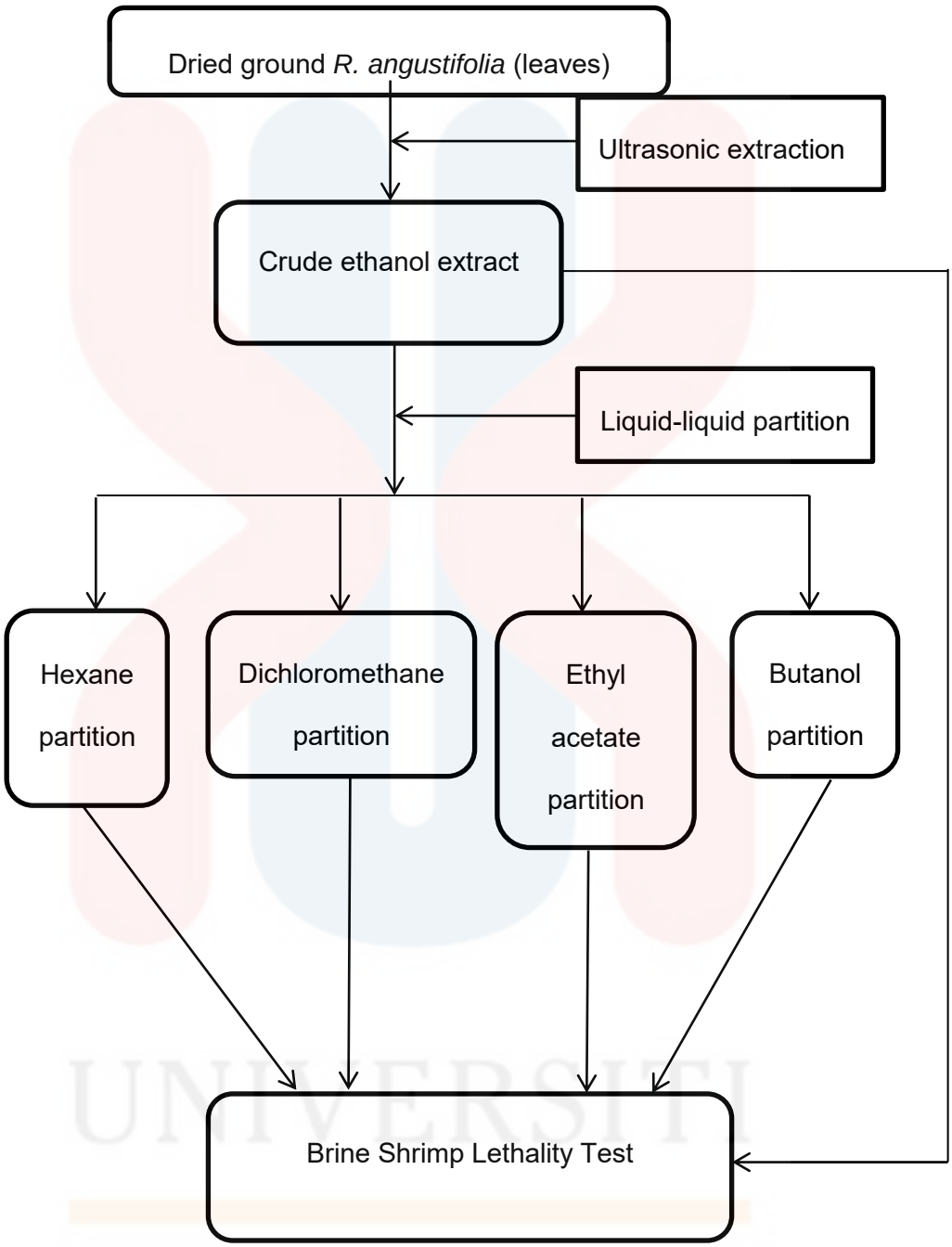

Figure 3.1: Summary of Methodology

CHAPTER 4

RESULT

4.1 Extraction and Liquid-Liquid Partitioning

Extraction of biological compound content in the leaves of *R. angustifolia* L. was performed with ethanol by using ultrasonic-assisted extraction and then continued by liquid-liquid partition. From the extraction using ethanol, dry extract with dark green colour was obtained.

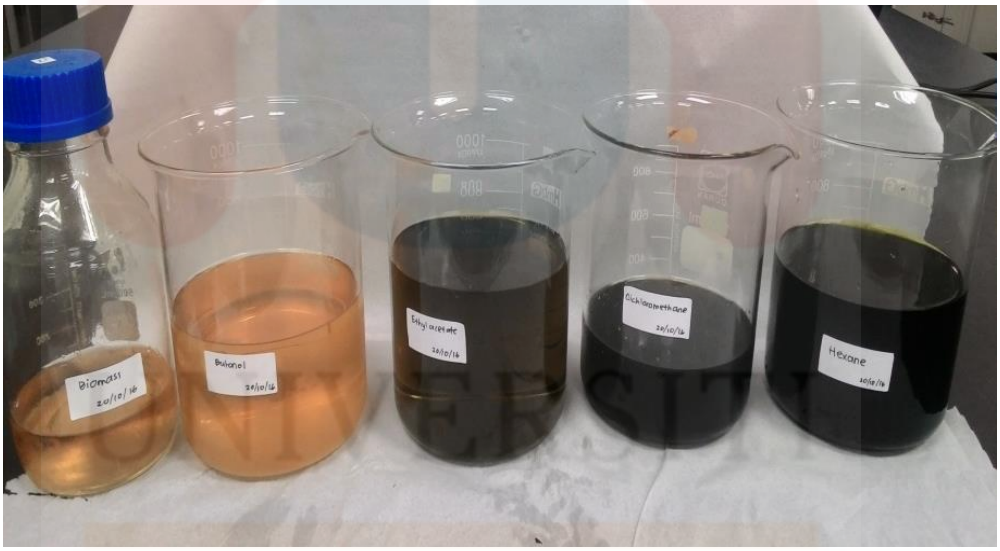

Figure 4.1: Colour range of each partition (From left to right: hexane partition, dichloromethane partition, ethyl acetate partition, butanol partition and residue)

A dark brown colour partition of hexane was formed after it was put in the ethanol-water mixture while dichloromethane partition shown a brown colour and ethyl acetate shown a transparent dark green-yellow while butanol shown a clear gold colour. The colour of residue left was clear yellow.

During liquid-liquid partitioning, when the system reached equilibrium, the two layers were formed and the densities of the solvents will predict which solvent is the top or bottom layer.

4.2 Brine Shrimp Lethality Test

After 24 hours, the number of dead brine shrimps in the microcentrifuge tubes was calculated for each concentration of the samples (crude ethanol, hexane partition, dichloromethane partition, ethyl acetate partition and butanol partition) for the brine shrimp lethality test. The median lethal concentration, LC_{50} for all samples was determined by using Statistical Packages for Social Science (SPSS) version 20.0 (IBM Corp., USA). Toxicity of the samples was expressed by using two toxicity indexes which are Meyers's Toxicity Index (Meyer *et al.,* 1982) and Clarkson's Toxicity Index (Clarkson *et al.,* 2004). Based on Meyer's Toxicity Index, LC_{50}<1000 µg/ml are considered toxic and LC_{50}>1000 µg/ml are considered non-toxic. According to Clarkson's toxicity index where LC_{50} value more than 1000 µg/ml are non-toxic, LC_{50} value between 1000 µg/ml and 500 µg/ml are low toxic, LC_{50} value less than 500 µg/ml and higher than 100 µg/ml are medium toxic and for highly toxic, LC_{50} value is from 0 µg/ml to 100 µg/ml.

The results were shown based on the graph of percentage of mortality against log concentration (µg/ml) of samples after 24 hours. Values for LC_{50} for all samples were obtained from the mean LC_{50} that obtained from the linear regression equation for triplicate data. The significance differences within groups were determined by One-way ANOVA. Duncan test were performed to determine the differences between samples while Dunnett t-tests treat potassium dichromate as a control, and compare all other samples against it.

Table 4.1: LC_{50} values from descriptive ANOVA

Sample	LC_{50} (µg/ml)	Toxicity Level (Meyer's)	Toxicity Level (Clarkson's)
Crude ethanol	125.84 ± 18.21^{a}	Toxic	Medium toxic
Hexane partition	81.21 ± 3.20^{a}	Toxic	Highly toxic
Dichloromethane partition	76.00 ± 7.99^{a}	Toxic	Highly toxic
Ethyl acetate partition	318.67 ± 30.76^{a}	Toxic	Medium toxic
Butanol partition	3197.7433 ± 378.02^{b}	Non-toxic	Non-toxic
Potassium dichromate (control)	27.62 ± 3.01^{a}	Toxic	Highly toxic

4.2.1 Brine Shrimp Lethality Test of Crude Ethanol Extract

The graph shown below dictated the increasing percentage of mortality with increasing concentration of crude ethanol extract of *R. angustifolia* L. leaves. Value for LC_{50} was obtained from the mean LC_{50} that obtained from the linear regression equation for triplicate data. The LC_{50} obtained was 125.84 ± 18.21 µg/ml. The value indicates that crude ethanol extract of *R. angustifolia* L. leaves can kill half of the brine shrimp population when the concentration at 125.84 µg/ml. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there was are significant difference between the ethanol crude extract and butanol partition when $p \leq 0.05$ for the value of LC_{50}.

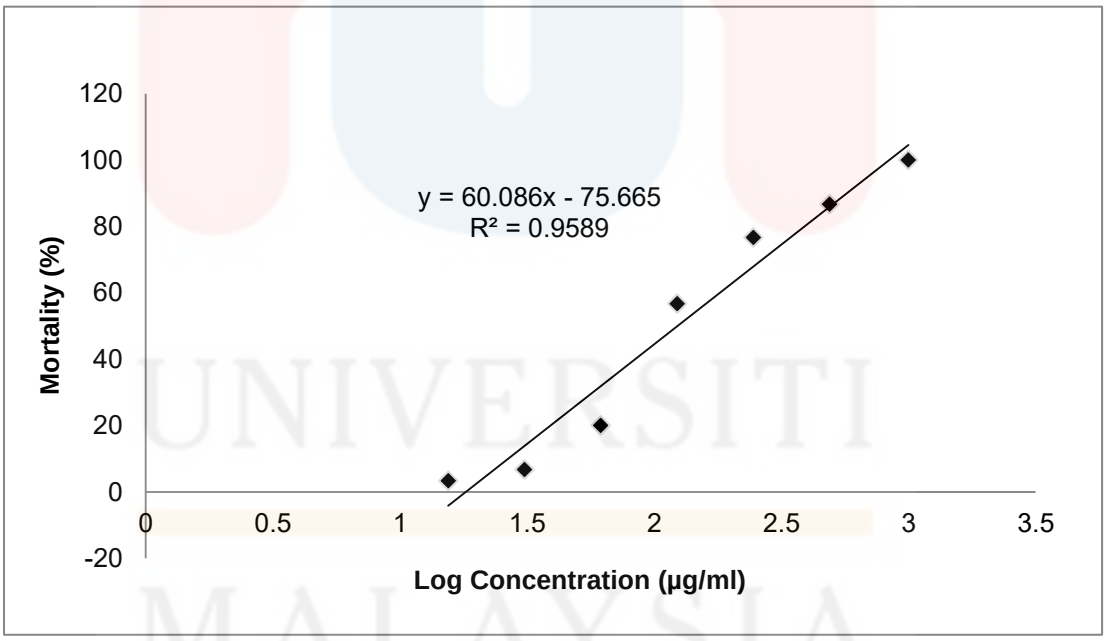

Figure 4.2: Graph of Percentage of Mortality against Log Concentration (µg/ml) of Crude Ethanol Extract after 24 hours

4.2.2 Brine Shrimp Lethality Test of Hexane Partition

The graph shown below dictated the increasing percentage of mortality with increasing concentration of hexane partition of *R. angustifolia* L. leaves. Value for LC_{50} was obtained from the mean LC_{50} that obtained from the regression line equation for triplicate data. The LC_{50} obtained was 81.21 ± 3.19 µg/ml. The value indicates that hexane fraction of *R. angustifolia* L. leaves can kill half of the brine shrimp population when the concentration 81.21 µg/ml. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there was a significant difference between the hexane partition with butanol partition when $p \leq 0.05$ for the value of LC_{50}.

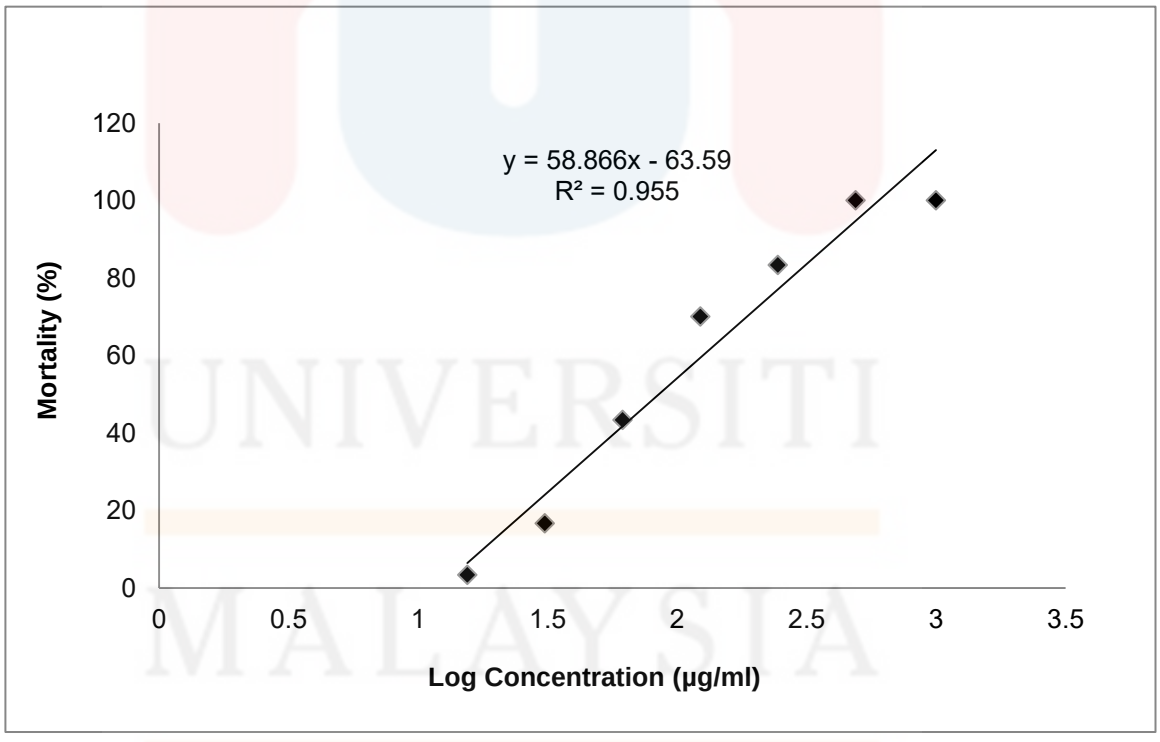

Figure 4.3: Graph of Percentage of Mortality against Log Concentration (µg/ml) of Hexane Partition after 24 hours

4.2.3 Brine Shrimp Lethality Test of Dichloromethane Partition

The graph shown above dictated the increasing percentage of mortality with increasing concentration of crude ethanol extract of *R. angustifolia* L. leaves. Value for LC_{50} was obtained from the mean LC_{50} that obtained from the regression line equation for triplicate data. The LC_{50} obtained was 76.00 ± 7.99 µg/ml. The value indicates that dichloromethane of *R. angustifolia* L. leaves can kill half of the brine shrimp population when the concentration 76.00 µg/ml. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there was a significant difference between the dichloromethane partition with butanol partition when $p \leq 0.05$ for the value of LC_{50}.

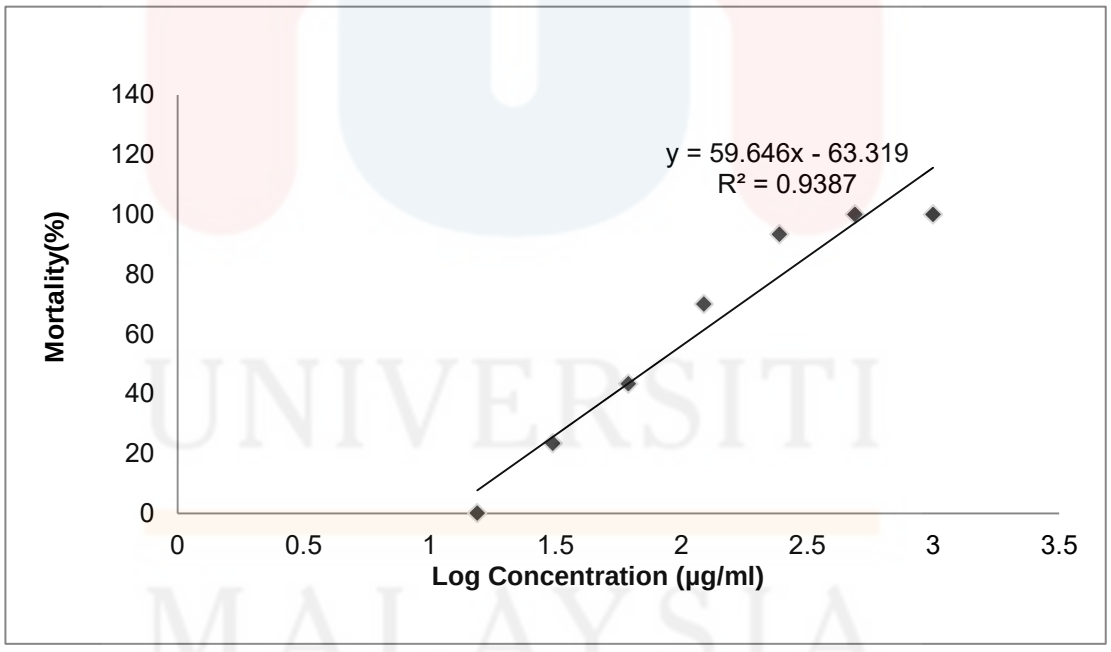

Figure 4.4: Graph of Percentage of Mortality against Log Concentration (µg/ml) of Dichloromethane Partition after 24 hours

4.2.4 Brine Shrimp Lethality Test of Ethyl Acetate Partition

The graph shown below dictated the increasing percentage of mortality with increasing concentration of ethyl acetate partition of *R. angustifolia* L. leaves. Value for LC_{50} was obtained from the mean LC_{50} that obtained from the regression line equation for triplicate data. The LC_{50} obtained was 318.67 ± 30.76 µg/ml. The value indicates that, ethyl acetate fraction of *R. angustifolia* L. leaves can kill half of the brine shrimp population when the concentration 318.67 µg/ml. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there was a significant difference between the ethyl acetate partition with butanol partition when $p \leq 0.05$ for the value of LC_{50}.

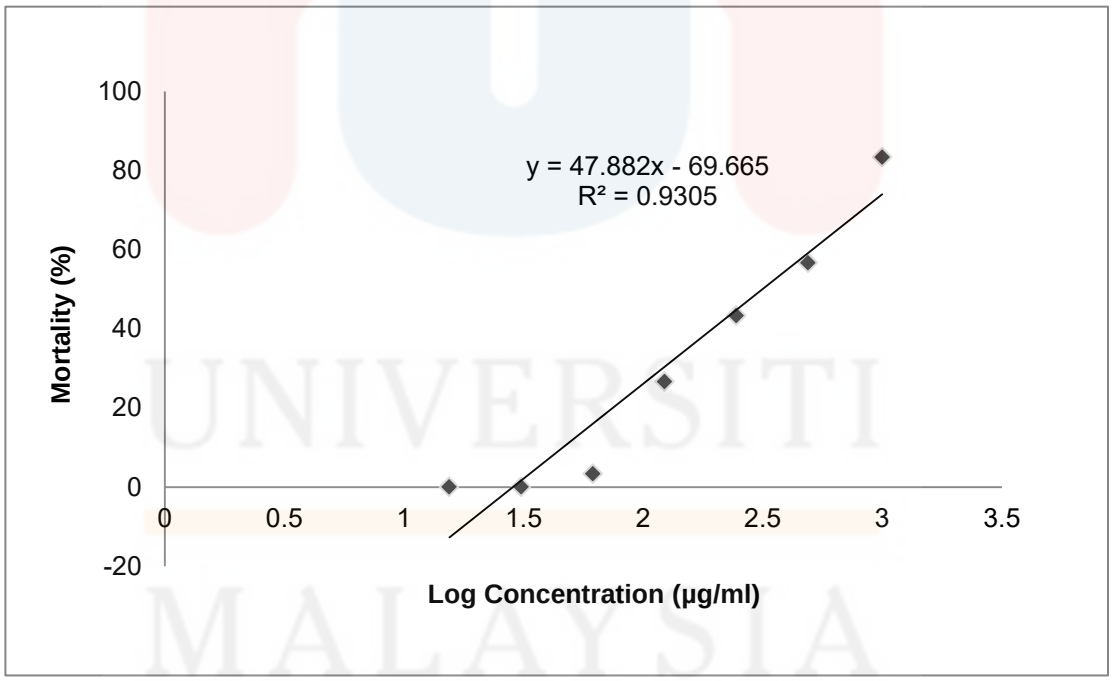

Figure 4.5: Graph of Percentage of Mortality against Log Concentration (µg/ml) of Ethyl Acetate Partition after 24 hours

4.2.5 Brine Shrimp Lethality Test of Butanol Partition

The graph shown below dictated the increasing percentage of mortality with increasing concentration butanol partition of *R. angustifolia* L. leaves. Value for LC_{50} was obtained from the mean LC_{50} that obtained from the regression line equation for triplicate data. The LC_{50} obtained was 3197.74 ± 378.02 µg/ml. The value indicates that butanol fraction of *R. angustifolia* L. leaves can kill half of the brine shrimp population when the concentration 3197.74 µg/ml. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there are significant difference between the butanol partition with other samples when $p \leq 0.05$ for the value of LC_{50}.

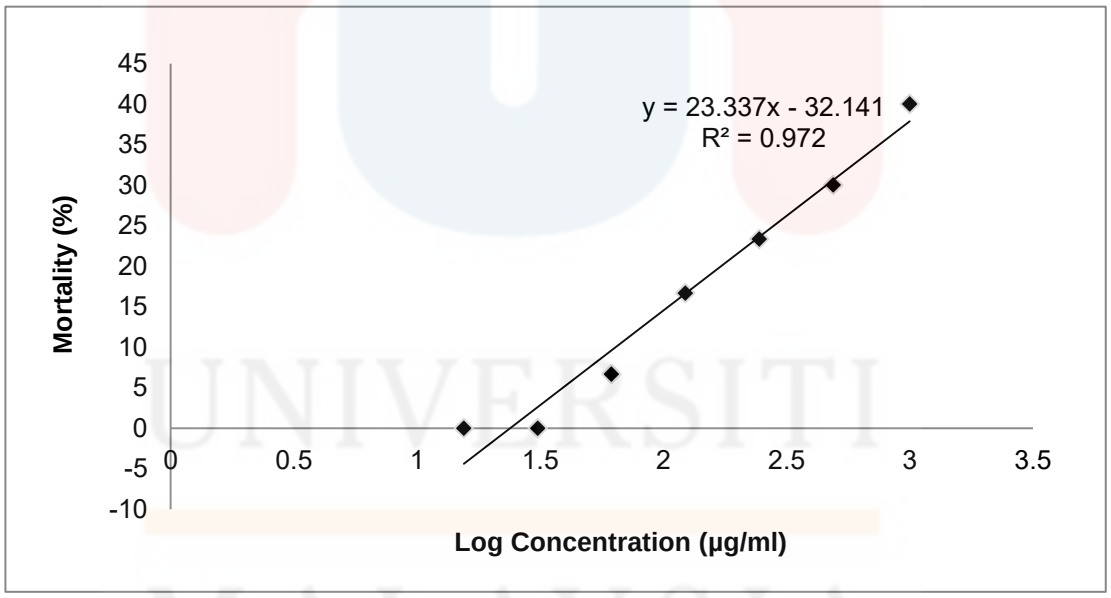

Figure 4.6: Graph of Percentage of Mortality against Log Concentration (µg/ml) of Butanol Partition after 24 hours

4.2.6 Brine Shrimp Lethality Test of Potassium Dichromate

The graph shown below dictated the increasing percentage of mortality with increasing concentration of potassium dichromate. Value for LC_{50} was obtained from the mean LC_{50} that obtained from the regression line equation for triplicate data. The LC_{50} obtained was 27.62 ± 3.01 µg/ml. The value indicates that potassium dichromate can kill half of the brine shrimp population when the concentration 27.62 µg/ml. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there was a significant difference between the potassium dichromate with butanol partition when $p \leq 0.05$ for the value of LC_{50}.

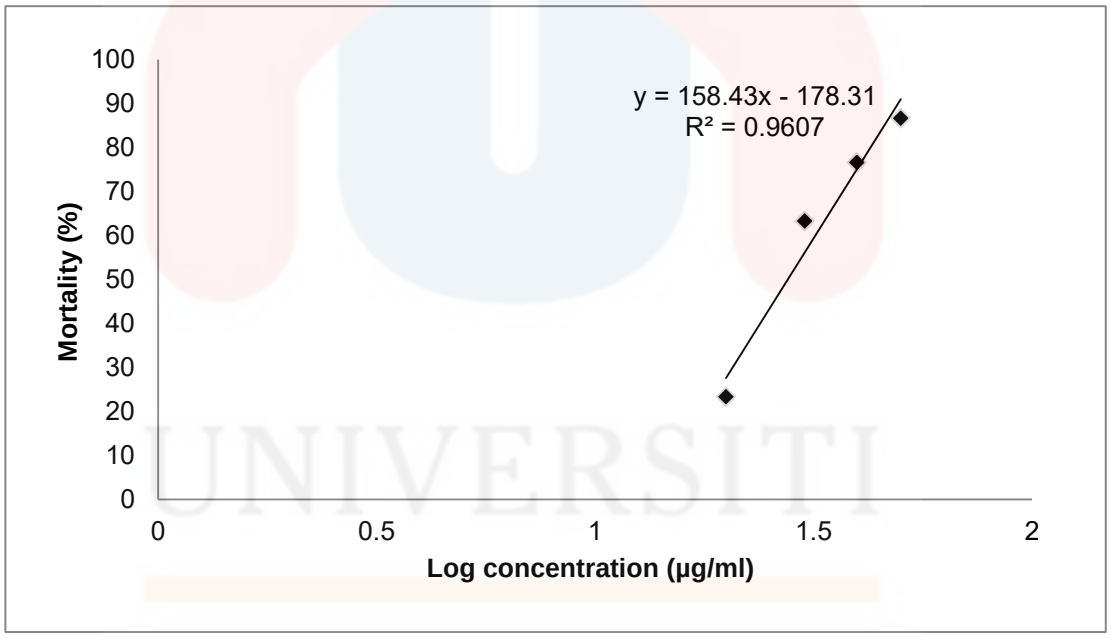

Figure 4.7: Graph of Percentage of Mortality against Log Concentration (µg/ml) of Potassium Dichromate after 24 hours

4.2.7 One-Way ANOVA

A one-way anova was conducted to compare the LC_{50} of each partition. There was a significant difference of the type of samples for LC_{50} values at the $p \leq 0.05$ level for the six conditions [$F(5, 12) = 197.521$, $p = 0.000$]. Since $p = 0.000$ then H_a was rejected.

4.2.8 Post Hoc Analysis

Post-hoc analysis using Multiple Range Duncan Test showed that there were no significant differences between LC_{50} of crude ethanol, hexane partition, dichloromethane partition, ethyl acetate partition and potassium dichromate. However there are significant differences found between butanol and all other extracts.

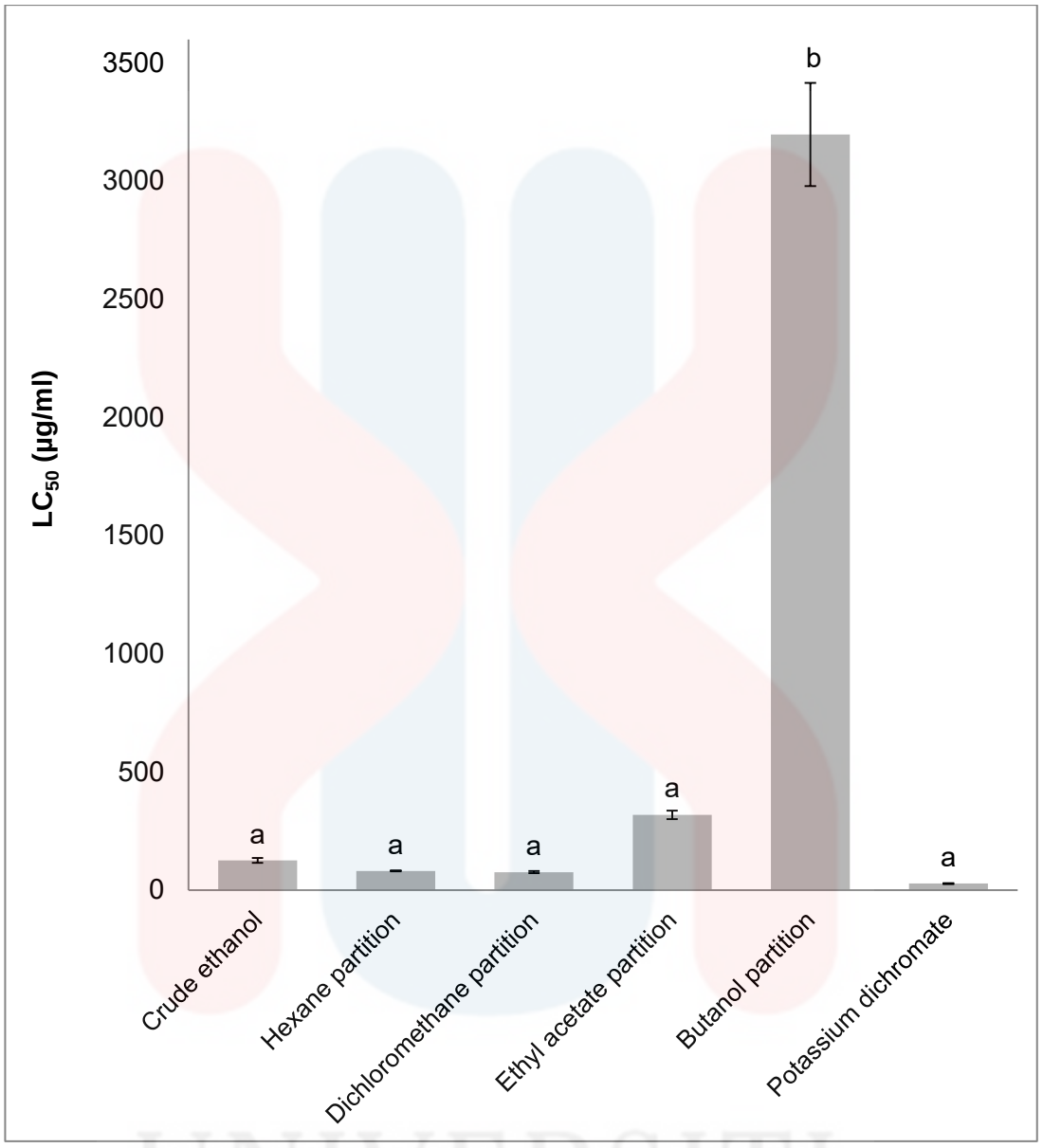

Figure 4.8: Graph of homogenous subsets group of LC_{50} value

CHAPTER 5

DISCUSSION

5.1 Ultrasonic-assisted extraction

A low frequency ultrasound was used to extract the compound as when high frequency ultrasound was employed as the extraction yield did not increase significantly however the degradation of the herb constituents was diminished. The alkaloid compounds in the extract can be saved as lower frequencies can help to prevent the degradation of toxic alkaloids during the process (Vinatoru, 2001). While using the ultrasonic cleaner bath, the hands must be avoided from entering the water in the bath to avoid electric shock. Ultrasonic-assisted extraction reduced the extraction time and also increase higher yield of extracts (Falleh *et al.,* 2012). From this extraction process, the extract did not have to be stirred as sonication speed up dissolution by breaking intermolecular interactions and disrupt cell membranes and release cellular contents.

5.2 Liquid-liquid Partition

Separatory funnel was the most common laboratory device for liquid-liquid partition. According Belter *et al.*, in (1988) and Gue (2000), for a very high recovery yield, liquid-liquid partition can be used to extract a solute out of an aqueous phase or organic phase repeatedly with a solvent such as water. A good solvent system must be consisting of two immiscible solvent and each combination must include water because water is immiscible with most organic solvents and highly polar. In this research, distilled water was added to crude ethanol extract to make sure that there

will be a layer when solvent is added to the mixture as water is not miscible with most organic solvents. Hexane, dichloromethane, ethyl acetate and butanol are organic solvents that are not miscible with water. The liquid-liquid partition procedure was started with a non-polar solvent first to break the hydrophilic barriers and then with a polar solvents. This is because, polar solvent will wash out all non-polar and polar compounds in the extract and then, there will nothing left to be extracted by a non-polar solvent.

The position of two immiscible layers formed in the separatory funnel was depended on the density of the solvents. Solvents that have a density that smaller than water will separate as the top layer, and solvents that have a greater density than water will separate into the lower layer. While conducting the liquid-liquid partition, the funnel was vigorously shaken for a few seconds, so the pressure would be released from the funnel. A half minute of vigorous shaking was crucial to allow solutes to come to equilibrium between the two solvents.

5.3 Brine Shrimp Lethality Test

Brine shrimp nauplii have been used as a test for a variety of toxic substances and also the method has also been applied to plant extracts in order to facilitate the isolation of biologically active components. The brine shrimp lethality bioassay is commonly used for the determination of toxicity of heavy metals, medicines and especially natural plant extracts and the technique is based on the ability of the extracts to kill the organism (brine shrimp nauplii) such as *Artemia salina* at certain hours and this technique is easy, use a little cost and only less amount of apparatus and materials needed (Ghisalberti, 1993).

At first, all the extracts were weighed and diluted in 200 µl absolute DMSO. The working concentration in 200 µl was done for all samples for the stock solution.

Then, the stock solutions of the samples were prepared for different concentration by serial dilution. 1000 µl of seawater was place in all tubes and 980 µl of seawater with 10 brine shrimp was pipetted into the test tube. The 100% DMSO is not suitable to use to test on the brine shrimp because the concentration is too high. The brine shrimp might die because of the DMSO not because of the solvent so only 1% of DMSO was used in this test. Only 20 µl of extract was put into the tube containing 1980 µl seawater with brine shrimp to get the 1% concentration for DMSO. Recent research by Ahmed *et al.*, 2013, dimethyl sulfoxide is widely used as solvent for dry extracts, because there was no significant sensitivity of the solvent to brine shrimp up to 11% concentrations. The positive control was done to make sure that the results from the all the samples are relevant but only low concentration is used as organic solvent might be toxic for this zoology invertebrate and interfere in the experimental outcomes.

After 24 hours, for the negative control by using DMSO, there was no death of the brine shrimps recorded. It can be concluded that DMSO did not kill the brine shrimps at 1% concentration. Then, by using the potassium dichromate for the positive control, the result showed that there was death of the brine shrimps. Even though the concentration used in this study is low but because of potassium dichromate which is a widely cytotoxic agent, therefore it contributed to the death of brine shrimps.

5.4 Determination of LC_{50}

From the results, it shows that the increase in concentration will also increase the number of dead of *Artemia salina* in all the extracts. The LC_{50} for the crude ethanol extract is 125.84 ± 18.21 µg/ml, hexane partition is 81.21 ± 3.20 µg/ml, dichloromethane partition is 76.00 ± 7.99 µg/ml, ethyl acetate is 318.67 ± 30.76

µg/ml, butanol partition is 3197.7433 ± 378.02 µg/ml and for the positive control potassium dichromate is 27.62 ± 3.01 µg/ml. According to Meyer's Toxicity Index (Meyer *et al*., 1982) where LC_{50}<1000 µg/ml is considered toxic and LC_{50}>1000 µg/ml is considered non-toxic. According to Clarkson's toxicity index (Clarkson *et al*., 2004) where LC_{50}>1000 µg/ml is non-toxic, 500 µg/ml<LC_{50}<1000 µg/ml are low toxic, 100 µg/ml<LC_{50}<500 µg/ml are medium toxic and 0 µg/ml<LC_{50}<100 µg/ml are highly toxic. In this brine shrimp lethality test of *Ruta angustifolia* L. leaves, partition of butanol is considered non-toxic while the others are toxic as according to Meyer's Toxicity Test. For further classification by Clarkson's, hexane partition and dichloromethane partition are highly toxic, ethanol crude extract and ethyl acetate partition is medium toxic while butanol partition is non-toxic.

In Hippocratic medicine, *Ruta* spp. leaves, roots and seeds, were administered for internal use by Hippocratic physicians after having been soaked in wine or mixed with honey (Pollio *et al.,* 2008). Decoction of *R. angustifolia* L. is commonly used to cure cramps, flatulence and fever. In Indonesia, *R. angustifolia* L. has been known as traditional medicine for liver disease and jaundice (Wahyuni *et al.,* 2014).

Butanol partition is not toxic to the brine shrimp. Based on the previous studies, the plant was used by soaking it in wine. Wine is an alcoholic beverages and alcohol group is polar. Butanol is a polar solvent so it can be determined that polar compounds that being extracted are not toxic to the brine shrimp.

5.5 Statistical Analysis of ANOVA and Post-Hoc Test

Based on analysis of variance, ANOVA of LC_{50}, there are significant differences between all groups when $p \leq 0.05$. The post-hoc test was determined to identify sample means that are significantly different from each other. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there was a significant difference between the crude ethanol extract, hexane partition, dichloromethane partition, ethyl acetate partition and potassium dichromate with butanol partition when $p \leq 0.05$ for the value of LC_{50}. According to Dunnett Test, crude ethanol extract, hexane partition, dichloromethane partition and ethyl acetate partition have significant differences on potassium dichromate while butanol partition has no significant differences on potassium dichromate.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Brine Shrimp Lethality Test is only a pre-screening test for bioactive compounds which need to be subjected to many more complicated assays such as sulforhodamide (SRB) cytotoxicity assay against HCT-116, A549, Ca Ski and MRC5 cell lines. It also can be tested using other toxicity assays such as by using *in-vivo* acute toxicity studies by using rats or rabbits to observe the mortality rate in certain dose. Then, phytochemical screening can be done to detect major functional groups such as by using Wagner's and Dragendorff's tests for alkaloids and lead acetate for flavonoids and also using different method of extraction and also different type of solvents.

From this research it can be concluded that the Brine Shrimp Lethality Test of dichloromethane partition and hexane partition of *R. angustifolia* L. are highly toxic to brine shrimps. The highly toxic extracts can be further analysed by using Gas Chromatography Mass Spectrometry to identify the compounds that contribute to the toxicity in the extracts. The ethyl acetate partition and crude ethanol extract shared the same toxicity levels, which are medium toxic to the brine shrimps. The positive control, potassium dichromate is highly toxic to brine shrimps. The concentration of a compound is the most important determinant of the outcome: if it reaches a sufficiently high concentration in the susceptible biological system, it could lead to toxic effects.

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APPENDIX A

BRINE SHRIMP COUNT

Table A.1: Number of dead brine shrimp exposed with ethanol crude extract

Conc. On (µg/ml)	Log conc.	Brine Shrimp Count(EtOH)				Mortality (%)
		1	2	3	Mean	
15.625	1.19	0	1	0	0.33	3.33
31.25	1.49	1	1	0	0.67	6.67
62.5	1.79	2	2	2	2.00	20.00
125	2.09	6	6	5	5.67	56.67
250	2.39	7	8	8	7.67	76.67
500	2.69	9	8	9	8.67	86.67
1000	3.00	10	10	10	10.00	100.00

Table A.2: Number of dead brine shrimp exposed with hexane extract

Conc. On (µg/ml)	Log conc.	Brine Shrimp Count(Hexane)				Mortality (%)
		1	2	3	Mean	
15.625	1.19	1	0	0	0.33	3.33
31.25	1.49	2	1	2	2.33	23.33
62.5	1.79	4	5	4	4.33	43.33
125	2.09	7	7	7	7.00	70.00
250	2.39	9	8	8	9.33	93.33
500	2.69	10	10	10	10	100

1000	3.00	10	10	10	10	100

Table A.3: Number of dead brine shrimp exposed with dichloromethane partition

Conc. On (µg/ml)	Log conc.	Brine Shrimp Count (DCM)				Mortality (%)
		1	2	3	Mean	
15.625	1.19	0	0	0	0	0
31.25	1.49	2	3	2	2.33	23.33
62.5	1.79	5	4	4	4.33	43.33
125	2.09	7	7	7	7.00	70
250	2.39	9	9	8	93.33	93.33
500	2.69	10	10	10	10.00	100
1000	3.00	10	10	10	10.00	100

Table A.4: Number of dead brine shrimp exposed with ethyl acetate partition

Conc. On (µg/ml)	Log conc.	Brine Shrimp Count(EA)				Mortality (%)
		1	2	3	Mean	
15.625	1.19	0	0	0	0	0
31.25	1.49	0	0	0	0	0
62.5	1.79	1	0	0	0.33	3.33
125	2.09	3	3	2	2.67	26.67
250	2.39	5	4	4	4.33	43.33
500	2.69	6	6	5	5.67	56.67
1000	3.00	8	8	9	8.33	83.33

Table A.5: Number of dead brine shrimp exposed with butanol partition

Conc. On (µg/ml)	Log conc.	Brine Shrimp Count(BuOH)				Mortality (%)
		1	2	3	Mean	
15.625	1.19	0	0	0	0	0
31.25	1.49	0	0	0	0	0
62.5	1.79	1	0	1	0.67	6.67
125	2.09	2	2	1	1.67	16.67
250	2.39	2	3	2	2.33	23.33
500	2.69	3	3	3	3.00	30
1000	3.00	4	4	4	4.00	40

Table A.6: Number of dead brine shrimp exposed with potassium dichromate

Conc. On (µg/ml)	Log conc.	Brine Shrimp Count($K_2CR_2O_7$)				Mortality (%)
		1	2	3	Mean	
10	1	0	0	0	0	0
20	1.30	2	2	3	2.33	23.33
30	1.48	6	7	6	6.33	63.33
40	1.60	8	8	7	7.67	76.67
50	1.70	9	9	8	8.67	86.67

ANOVA

Table A.7: ANOVA of LC_{50}

LC50

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	23744958.646	5	4748991.729	197.521	.000
Within Groups	288516.268	12	24043.022		
Total	24033474.914	17			

Table A.8: Descriptive ANOVA

Descriptives

LC50

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Crude ethanol extract	3	125.8400	18.20929	10.51314	80.6056	171.0744	104.84	137.25
Hexane partition	3	81.2100	3.19150	1.84262	73.2819	89.1381	77.72	83.98
Dichloromethane partition	3	76.0000	7.99308	4.61481	56.1441	95.8559	66.91	81.93
Ethyl acetate partition	3	318.6667	30.75636	17.75719	242.2636	395.0697	287.27	348.74
Butanol partition	3	3197.7433	378.01785	218.24871	2258.6949	4136.7917	2806.43	3560.89
Potassium Dichromate	3	27.6233	3.00562	1.73529	20.1570	35.0897	25.21	30.99
Total	18	637.8472	1189.00539	280.25126	46.5688	1229.1257	25.21	3560.89

POST HOC TESTS

Table A.8: Duncan Test

LC50

Duncan

Sample	N	Subset for alpha = 0.05	
		1	2
Potassium Dichromate	3	27.4200	
Dichloromethane fraction	3	76.0000	
Hexane fraction	3	81.2100	
Ethanolic extract	3	125.8400	
Ethyl acetate fraction	3	318.6667	
Butanol fraction	3		3197.7433
Sig.		.057	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.9: Dunnett (2-sided)

Multiple Comparisons

Dependent Variable: LC50

Dunnett t (2-sided)

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude ethanol extract	Potassium Dichromate	98.21667	126.60443	.896	-269.0955	465.5289
Hexane partition	Potassium Dichromate	53.58667	126.60443	.990	-313.7255	420.8989
Dichloromethane partition	Potassium Dichromate	48.37667	126.60443	.994	-318.9355	415.6889
Ethyl acetate partition	Potassium Dichromate	291.04333	126.60443	.140	-76.2689	658.3555
Butanol partition	Potassium Dichromate	3170.12000*	126.60443	.000	2802.8078	3537.4322

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

APPENDIX B

Table B.1: Preparation of working concentration in 200 µl

Sample	After measure	Amount for 20mg/200µl	Amount of DMSO needed
EtOH crude extract	25mg/200µl	(25mg/200µl)(V)=(20mg/200µl)(200µl) V=160 µl	40 µl
Hexane partition	28mg/200µl	(28mg/200µl)(V)=(20mg/200µl)(200µl) V=142.9 µl	57.1 µl
DCM partition	26mg/200µl	(26mg/200µl)(V)=(20mg/200µl)(200µl) V=153.9 µl	46.1 µl
EA partition	25mg/200µl	(25mg/200µl)(V)=(20mg/200µl)(200µl) V=160 µl	40 µl
BuOH partition	27mg/200µl	(27mg/200µl)(V)=(20mg/200µl)(200µl) V=148.2 µl	51.8 µl

Table B.2: Sample calculation in 1% DMSO

(100%) (V) = (1%) (2 ml)

V = 0.02ml/ 20 µl

V = 20 µl stock+ 1980 µl artificial sea water

V = 2 ml

APPENDIX C

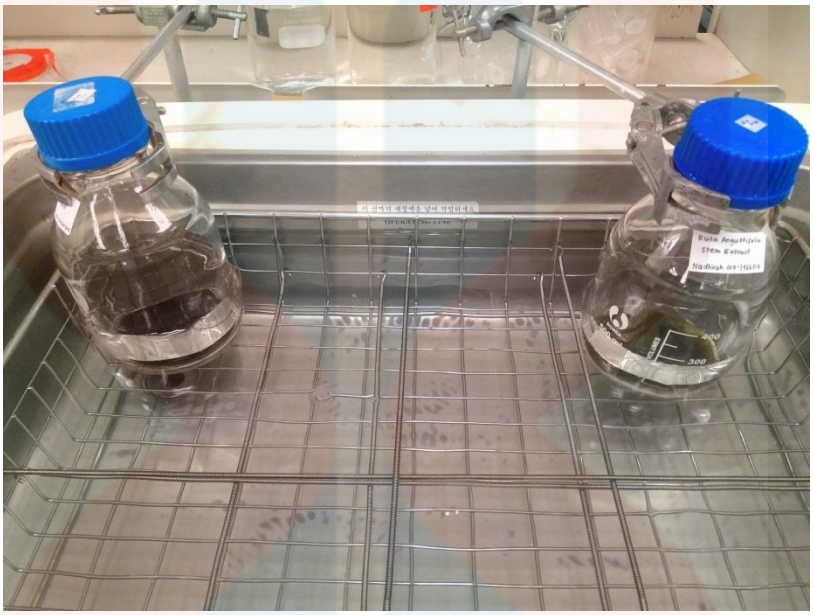

Figure C.1: Ultrasonic-Assissted Extraction

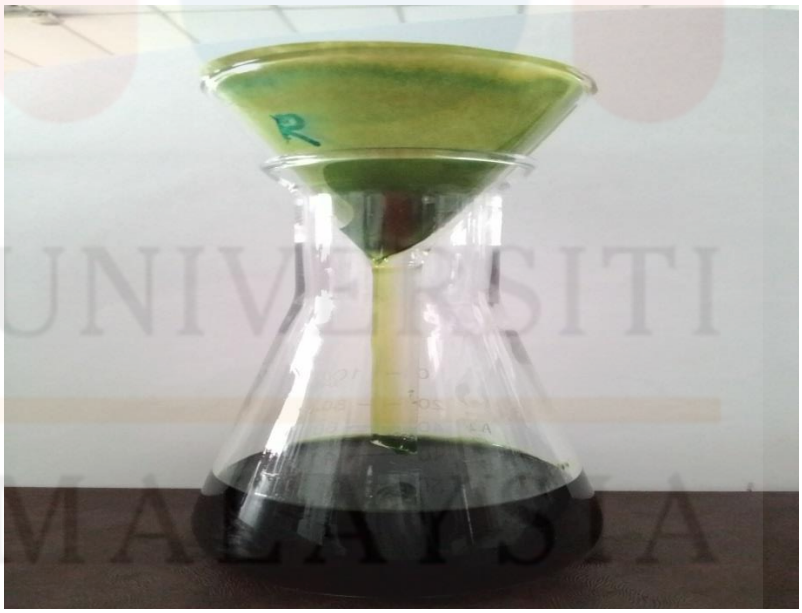

Figure C.2: Filration process of *R. angustifolia* L.

Figure C.3: The drying of solvent in the extract by concentrated to dryness under vacuum and reduced pressure using a rotary evaporator.

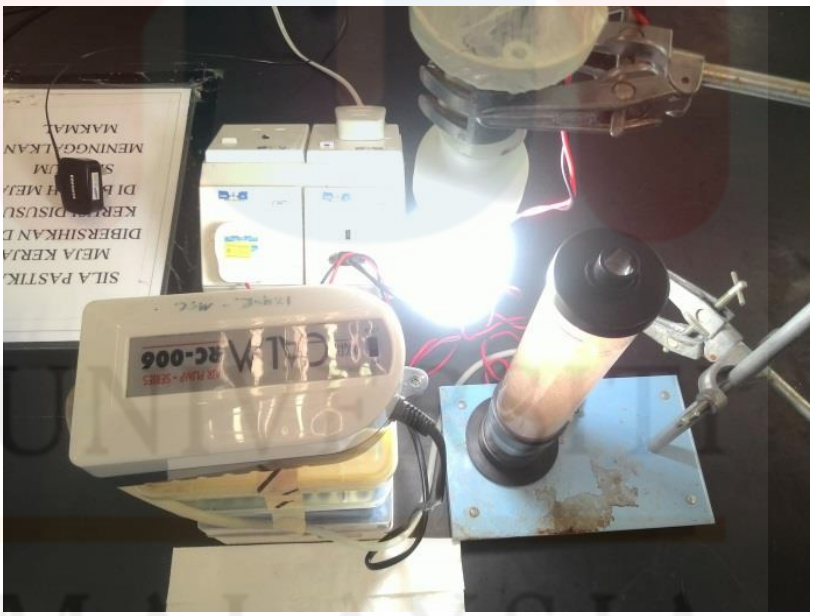

Figure C.4: Brine shrimp hatching process

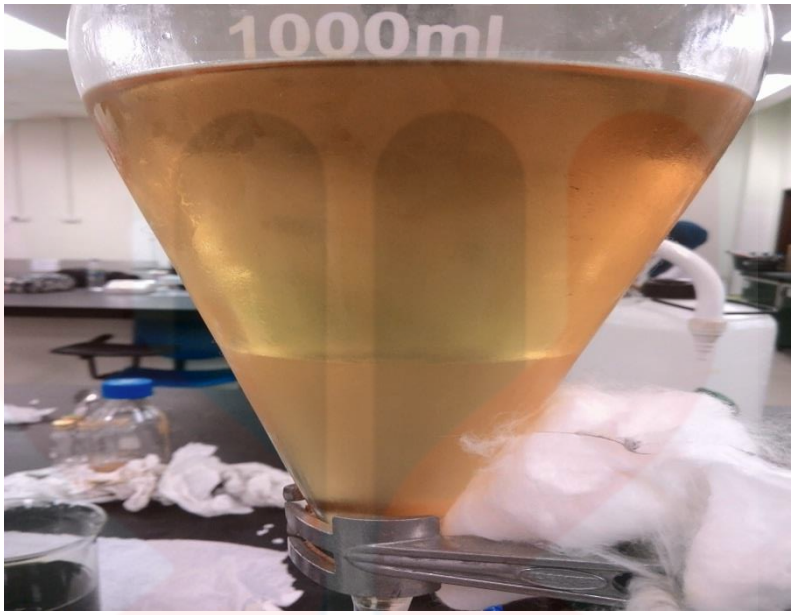

Figure C.5: Butanol partition in separatory funnel

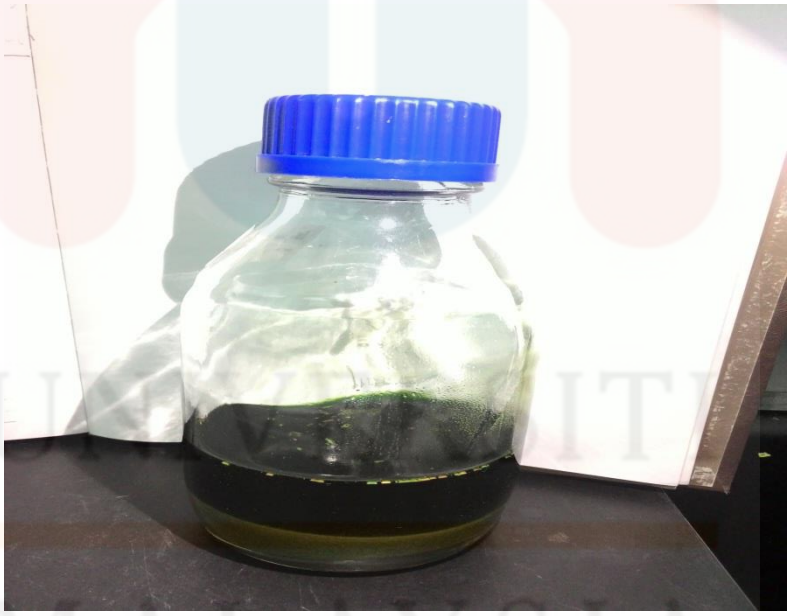

Figure C.6: *R. angustifolia* L. leaves in ethanol