# LARVICIDAL PROPE... OF ...FFERENT PARTS OF NEEM (*AZADIRACHTA INDICA*) AGAINST *AEDES AEGYPTI* MOSQUITOES' LARVAE

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## ABSTRACT

Dengue is one of the major health problem in many countries. *Aedes aegypti* mosquito is the major vector of dengue fever disease. Search for larvicidal active compound(s) is one of the several attempts to find effective and affordable ways to control this mosquito. The aim of this study is to investigate the toxic effect of different solvent (acetone, chloroform, cold and hot ethanol) extracts from different parts (bark, leaf, root and seed) of Neem (*Azadirachta indica*) against *Aedes aegypti* larvae. For the larvicidal bioassay, four concentrations (50, 100, 500 and 1000 ppm) of plant crude extracts were prepared; 1 mL of DMSO was used to solubilize the extract in water. 10 larvae (second and third instar) were inserted in each solution. 2.0 % Dimethyl sulfoxide (DMSO) and untreated sets of larvae in (tap) water were also run for comparison. Data were evaluated through regression analysis. From the regression line; the $LC_{50}$ and $LC_{90}$ values were read. The larvicidal activities of the crude extracts were varied and the $LC_{50}$ and $LC_{90}$ values ranging from 50-837.5 ppm and 94-950 ppm respectively. Assays showed that leaf acetone extracts were more toxic against larvae and causes 100 % mortality at concentration of 100 ppm, while root, seed and bark extracts achieve 100 % mortality at 1000 ppm. Bioactive groups such as alkaloids and sesquiterpene lactones were screened by Thin Layer Chromatography (TLC); and the results obtained were negative for alkaloids and positive for sesquiterpene lactones. Result obtained in this study shows the potential of the crude extracts of Neem (*Azadirachta indica*) against *Aedes aegypti* larvae and this may warrant further research to determine bioactive compound(s)

# ABSTRAK

Denggi adalah salah satu masalah kesihatan utama di kebanyakan negara. *Aedes aegypti* adalah salah satu vektor utama penyakit demam denggi. Pelbagai kajian dan usaha telah dilakukan untuk mencari sebatian aktif larvasida bagi mencari cara-cara yang berkesan dan sesuaian untuk mengawal nyamuk *Aedes aegypti*. Tujuan kajian ini adalah untuk mengkaji kesan racun terhadap larva *Aedes aegypti* dari bahagian yang berbeza (batang, daun, akar dan biji) dari pokok mambu (*Azadirachta indica*) yang diekstrak dengan pelarut (asetone, klorofom, etanol panas dan sejuk) yang berbeza. Bagi menguji tahap larvasida, empat kepekatan (50, 100, 500 dan 1000 ppm) ekstrak mentah tanaman telah disediakan; 1 mL pelarut Dimethyl sulfoxide (DMSO) telah digunakan untuk melarut ekstrak dalam air. Dalam setiap kepekatan larutan ekstrak, sebanyak 10 larva (instar kedua dan ketiga) telah dimasukkan. Satu set akan disediakan sebagai kawalan dengan menggunakan DMSO dengan kepekatan 2.0 % dan larva yang tidak dirawat dalam (paip) air juga dijalankan untuk tujuan perbandingan. Data dinilai melalui analisis regresi; dan nilai $LC_{50}$ and $LC_{90}$ telah dibaca. Aktiviti larvisida berbeza bagi setiap extrak mentah dan nilai $LC_{50}$ dan $LC_{90}$ adalah diantara 50.0-837.5 ppm dan 94-950 ppm. Kajian menunjukkan bahawa ekstrak mentah asetone daun adalah lebih bertoksid terhadap larva dan menunjukkan 100 % kematian pada kepekatan 100 ppm, sementara itu, ekstrak mentah akar, biji dan batang juga mencatatkan 100 % kematian pada 1000 ppm. Bahan bioaktif seperti alkaloid and sesquiterpene lactone dipisahkan dan/ atau dikenalpasti dengan menggunakan Kromatografi Lapisan Nipis (TLC) dan didapati bahawa ujian tersebut adalah negatif bagi alkaloids dan positif bagi sesquiterpene lactone. Oleh yang demikian, hasil yang diperolehi dalam kajian ini menunjukkan potensi ekstrak mentah pokok mambu (*Azadirachta indica*) terhadap larva nyamuk *Aedes aegypti* dan ini boleh menjamin penyelidikan lanjutan bagi menentukan kompaun bioaktif.

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| | |
|---|---|
| % | Percentage |
| ± | Uncertainty |
| °C | Degree celsius |
| °N | North latitude |
| °S | South latitude |
| µL | Microliter |
| µL/L | Microliter per liter |
| cm | Centimeter |
| g | Gram |
| h | Hour |
| mg | Miligram |
| mL | Mililiter |
| mm | Milimeter |

# LIST OF ABBREVATIONS

| | |
|---|---|
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| DDT | Dichlorodiphenyltrichloroethane |
| DMSO | Dimethyl sulfoxide |
| GGT | Gamma glutamyl transpeptidase |
| HPLC | High performance liquid chromatography |
| LC | Lethal concentration |
| $LC_{50}$ | The lethal concentration required to kill 50 % of a sample population |
| $LC_{90}$ | The lethal concentration required to kill 90 % of a sample population |
| NMR | Nuclear magnetic resonance |
| pp | Page |
| TLC | Thin Layer Chromatography |
| UV | Ultraviolet |

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# CHAPTER 1

# INTRODUCTION

## 1.1 INTRODUCTION

The aim of presenting this chapter was to present the background of the study together with some others aspects such as the problem statement, objectives of the research, scope of research, significance of the study. All this aspects would be a foundation in order to further discover the research.

## 1.2 BACKGROUND OF THE STUDY

Mosquitoes are generally a vector that carries disease-causing viruses and parasites from a person to another person. Some of these diseases can be life threatening such as yellow fever and dengue fever. It has been found out that the *Aedes aegypti* is the primary vector of dengue fever (Anees, 2008; Preet and Sneha, 2011). In order to minimize and eradicate the occurrence of these diseases, many steps have been employed to prevent the spread at various levels. For examples, mosquito eradication at the early and later stages, disease prevention via prophylactic drugs and vaccines, prevention of mosquito bites by using repellents and so forth.

There are various methods in controlling the spread of these diseases such as, by the means of biological control and adulticiding. Biological control is carried out by the means of using mosquitoes' natural enemies to control the population of the mosquitoes. Examples of such enemies are the mosquito fish, cyprinids and killifish. Besides that, dead spores of varieties of the natural soil bacterium, *Bacillus thuringiensis* controls the mosquitoes at the larvae level by disrupting the digestion

system of the larvae. These methods may not be viable if to be carried out on a large scale due to the cost and also unpredictable seasonal distribution of adult and larval mosquitoes. For example, the use of soil bacterium is only effective at the larvae stage and has to be dispersed by air into large areas. Soil bacterium dispersion in air may cause complications and therefore they should be dispersed to non-intended areas.

For the control of adult mosquitoes, pesticides are currently being used in modern mosquito-control programs for controlling the adult mosquitoes. One of the most common pesticides used is the dichlorodiphenyltrichloroethane (DDT). Although the usage of DDT has been restricted much, DDT remains in common use in many developing countries for the control of the mosquito population. The usage of DDT may pose serious health effects causing genotoxicity, acute and chronic toxicity and other major complications. If DDT is commonly used in mosquito control, the highly resistant DDT can be absorbed and distributed in the environment and thus causing damage to the biodiversity. Consistent use of DDT in mosquito control will also result in an increase of DDT-resistant mosquitoes thus reducing effectiveness (Chowdhury et al., 2008), and if DDT is continued being used indiscriminately, the mutations may spread faster.

Accordingly, new alternatives are needed to provide a larvicide which incorporates non-harmful biological products and which can be used safely by humans and animals as well as not harmful to the environment. A screening for larvicidal activity of plant extracts with some known medicinal attributes could lead to the discovery of new agents for pest and vector control (Kamaraj et al., 2008). The extracts of natural sourced products contain low toxicity and since it is available naturally, the process is more cost effective. Besides that, the product (extract) should be effective in killing targeted mosquito larvae, has a longer lasting effect and of lesser toxicity than common and traditional larvicides.

## 1.3 PROBLEM STATEMENT

Dengue fever is a disease caused by a family of viruses that are transmitted by mosquitoes (*Aedes aegypti*). It is an acute illness of sudden onset that usually follows a benign course with symptoms such as headache, fever, exhaustion, severe muscle and joint pain, swollen glands (*lymphadenopathy*) and rash. Recently, a high dengue alert has been issued nationwide due to the total deaths of more than 103 people between January and August 2010. This number increases from 68 deaths that were recorded last year (Cruez, 2010). It has been also identified that dengue fever is one of the major health problems in Malaysia. Therefore, prevention for more deaths should be practiced. The key to mosquito control is larval management. Managing mosquito at larvae stage is easy because mosquitoes in the larval stage are attractive targets for pesticides and at this stage they occur only in specific areas and can be controlled easily by modifying their habitat with insecticides applied to larval breeding sites. Treatments provide control before the biting adults appear and disperse from the breeding sites. Insecticides of botanical origin have been reported as useful for control of mosquitoes. Neem (*Azadirachta indica*) trees from the family of Meliaceae and its derived products have shown a variety of insecticidal properties. Insect growth regulatory activity of Neem weakens the cuticle defence system of the larvae causing easy penetration of pathogenic organisms into insect system. Azadirachtin, a biologically active compound in Neem (*Azadirachta indica*) has been promoted as a new insecticide that is considered more eco-friendly than synthetic insecticides. Through this insecticide the widespread of dengue can be control. The aim of this study is to determine the toxic effects of different parts of Neem (*Azadirachta indica*) extracted by different solvents against *Aedes aegypti* larvae.

## 1.4 OBJECTIVES OF THE STUDY

Neem (*Azadirachta indica*) has rich source of chemically active compounds. Many plants from the same family, Meliaceae, have been reported to have many types of activities such as the antibacterial, anti-inflammatory, hepatoprotective, immunostimulant and larvicidal activity. Thus, this present study will focus on Neem (*Azadirachta indica*) species. The objectives of this present study were;

1.0 To extract the crude from different parts of Neem (*Azadirachta indica*) by using different solvents.

2.0 Screening for bioactive groups such as alkaloids and sesquiterpene lactones by using Thin Layer Chromatography (TLC).

3.0 To study the larvicidal properties of the crude extracts against *Aedes aegypti* larvae and to determine the $LC_{50}$ and $LC_{90}$ value.

## 1.5 SCOPE OF THE STUDY

As a way to accomplish the objective of this study, the scope of this research focuses on the characterization of extracts from different parts of Neem (*Azadirachta indica*) towards 2nd and early 3rd instars larvae *Aedes aegypti* a common vector of dengue, dengue haemorrhagic fever and yellow fever in terms of chemical properties. In this research, Neem (*Azadirachta indica*) samples are collected from Teluk Intan, Perak. The first scope is to separate the Neem (*Azadirachta indica*) according to five different parts which are the barks, flowers, leaves, roots and seeds. The second scope is to extract the components of each part of Neem (*Azadirachta indica*) using acetone, chloroform and ethanol and the extracts of each part are prepared into four different concentrations according to 50, 100, 300 and 500 ppm into glass beakers. The next scope of the study is to test the larvae that were obtained from Institute of Medical Research Kuala Lumpur with the extracts. A number of 10 larvae are released into the glass beakers containing extracts and their mortality are observed within 48 h.

## 1.6 SIGNIFICANCE OF THE STUDY

In this study, extracts of Neem (*Azadirachta indica*) will be used to identify the larvicidal properties of larvae *Aedes aegypti* larvae. For many years, Neem (*Azadirachta indica*) has been identified as one of the natural source that has a variety of larvicidal properties. The extraction method of Neem (*Azadirachta indica*) extracts is considered as a cost effective method in laboratory and all the feeds and raw materials proves that this is one of the best method in terms of applicability. Insecticide industry can benefit from this concept since this can be considered as one

of the effective and cheapest natural source that can be used to overcome the dengue problem.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 INTRODUCTION

The purpose or the aim of presenting this chapter was to present a review of past research attempts related to internal to natural product, Neem (*Azadirachta indica*), crude extracts of Neem (*Azadirachta indica*), biological activities of Neem (*Azadirachta indica*) and mosquito. The review was done so that this present study attempt can be appropriately adapted to include to the present literature in order to fulfill the scope and direction of the present research attempt.

## 2.2 IMPORTANCE OF NATURAL PRODUCT

Nowadays, it has been reported that vector borne diseases that are caused by mosquitoes are one of the major health problems that lead millions of human towards death (Dua et al., 2009). Extensive and indiscriminate use of chemical insecticides for the control of vector borne diseases has created lots of problems. These problems can be related to health effects, environmental effects and high operational cost among the community (Dua et al., 2009 and Shanmugasundram et al., 2008). In view of this, an eco-friendly approach to control mosquito larvae is defensible (Shanmugasundram et al., 2008). Therefore, there has been a major concern for the promotion of plants or botanicals as they have the capability in producing environmental friendly pesticides as well as an insecticides, microbial sprays, and insect growth regulators (Alouani et al., 2009). Keeping an unpolluted and hazardless environment in mind, some of the numerous plant products have been reported either as insecticides for killing larvae or adult mosquitoes or as repellents

for mosquito biting (Dua et al., 2009 and Shanmugasundram et al., 2008). As a result, these are one of the best alternatives for mosquito control because they are easily managed on their larvae stage (Dua et al., 2009). Furthermore, natural products from the botanicals are preferred as effective control agent to reduce the mosquito population irrespective of their side effects. Recent studies do also stimulated the investigation of insecticidal properties of plant-derived extracts and are concluded that they are environmentally safe, degradable, and target specific (Alouani et al., 2009).

## 2.3 THE BENEFICIAL PLANT SPECIES, NEEM (*Azadirachta Indica*)

Flora contains many biologically active compounds which have potential for development as medicinal or curative agents (El-Mahmood et al., 2010). For example, Neem (*Azadirachta indica*) trees, native of India. They are widespread in huge numbers in tropical and subtropical regions of the world, including semi-arid and wet-tropical regions. Neem (*Azadirachta indica*) seeds contain approximately 99 biologically active compounds of which azadirachtin, nimbin, nimbidin and nimbolides are major molecules (Dua et al., 2009).

The components of nimbin and azadirachtin are the most active insecticidal ingredient and they are present in huge amount in the seeds, leaves and other parts of the Neem (*Azadirachta indica*) tree (Mondali et al., 2009). Allelochemicals such as azadirachtin, nimbin, nimbidin, nimbolides, nimolic acid, salannin, melianttriol and azadirachtol present in Neem (*Azadirachta indica*) do also affect the biochemical and physiological processes of insect system. All these allelochemicals nullifies the insect detoxification mechanism and as a result the pest or insects are stopped from further development (Dua et al., 2009).

Different Neem (*Azadirachta indica*) parts and products have found widespread use as mosquito repellants (Atawodi and Atawodi, 2009). Seed oil appeared to be the most lethal among the various parts tested because seed oil extract might be attributed to deficiency of dissolved oxygen in the water and they possess significant insect repellant motion, antifeedancy, high inhibition besides insect

growth regulation against insects (Aliero, 2003; Atawodi and Atawodi, 2009 and Dua et al., 2009). Nivoletti et al. (2010), has found out that the Neem (*Azadirachta indica*) cake is a promising low-cost, easily obtain natural resource that can be developed as bioinsecticide as it can show good insecticidal effect when tested against *Aedes albopictus* (Skuse) eggs and larvae. In traditional method, smoke of leaves of *Azadirachta indica* was found to repel mosquito by up to 70 %. Some of the derived products of Neem (*Azadirachta indica*) oil are combined with coconut oil and applied to the exposed body parts of humans and it has been found out to provide protection for around 12 h from the bites of all *Anopheline* mosquito species. Other than that, Neem (*Azadirachta indica*) oil in wood scraping balls prevents the breeding of *Anopheles stephensi* and *Aedes aegypti* in overhead tanks (Atawodi and Atawodi, 2009). The agronomy of this plant is shown in Table 2.1.

**Table 2.1:** Plant Classification of Neem (*Azadirachta indica*)

| | |
|---|---|
| Kingdom | Plantae (Plants) |
| Subkingdom | Tracheobionta (Vascular plants) |
| Superdivision | Spermatophyta (Seed plants) |
| Division | Magnoliophyta (Flowering plants) |
| Class | Magnoliopsida (Dicotyledons) |
| Subclass | Rosidae |
| Order | Sapindales |
| Family | Meliaceae (Mahogany family) |
| Genus | *Azadirachta* A. Juss. (Azadirachta) |
| Species | *Azadirachta Indica* A. juss. (Neem) |

**Source:** USDA, United States Department of Agriculture (2011)

## 2.4 THE CRUDE EXTRACTS OF NEEM (*Azadirachta Indica*)

The crude extract of Neem (*Azadirachta indica)* tree has been reported to be eco-friendly and non-toxic to vertebrates. It has been proven that crude or partially-purified plant or botanicals extracts are less expensive and highly effective for the control of mosquitoes that contributes too many serious vector borne diseases rather than the purified compounds or extracts of the plant (Alouani et al., 2009). In fact, a

variety of crude extracts obtained from seed, bark and leaf of the Neem (*Azadirachta indica*) tree, have been identified as environmentally acceptable bioinsecticides used in crop protection and control of mosquito's larvae (Khalafalla et al., 2007). Therefore, plant derived crude extract are priceless sources of potential insecticides. The *Meliaceae* plant family of *Azadirachta indica* is used as growth regulator against many insect pests (Alouani et al., 2009). Crude aqueous or alcoholic extracts of Neem (*Azadirachta indica*) seed kernels and leaves cause disorders in metamorphosis of insects (Zebitz, 1984). The effect of these crude plant extract on the biology, reproduction, and adult emergence of the mosquitoes are very efficient. For example, 88 % of the adult mortality was observed by the use of *P. citrosa* leaf extracts at 2 % concentration (Alouani et al., 2009).

Besides that, it has been found out that the petroluem ether, ether, chloroform and alcohol crude extracts of Neem (*Azadirachta indica*) leaves to be toxic to fourth instar larvae of *Culex pipiens fatigans* Wied and *Anopheles stephensi* List (Zebitz, 1984). Plant allelochemicals may be useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase their resistance to insect attack based on the fact that compounds of plant origin are safer in usage, without any side effects to the environment (Alouani et al., 2009). The crude extract that contains cardenolide, azadirachtins as well as salannin, nimbin and 6-desacetylnimbin is the larvicidal component of Neem (*Azadirachta indica*) extracts that shows good efficiency (Atawodi and Atawodi, 2009). The choice of crude extract was to ensure flexibility by the public and low-cost involvement strategy (Khalafalla et al., 2007).

## 2.5 THE BIOLOGICAL ACTIVITY OF NEEM (*Azadirachta indica*)

It has been identified that Neem (*Azadirachta indica*) contributes towards several types of biological activities. Each of these biological activities is influenced by the biological active compounds that are present in Neem (*Azadirachta indica*). Table 2.1 represents some bioactive compounds of Neem (*Azadirachta indica*) and its biological activity.

**Table 2.2:** Some bioactive compounds of Neem (*Azadirachta indica*)

| Neem Compound | Source | Biological Activity |
|---|---|---|
| Nimbidin | Seed oil | Anti-inflammatory, Antiarthritic, Antipyretic, Hypoglycaemic, Antigastric ulcer, Spermicidal, Antifungal, Antibacterial, Diuretic |
| Sodium nimbidate | | Anti-inflammatory |
| Nimbin | Seed oil | Spermicidal |
| Nimbolide | Seed oil | Antibacterial, Antimalarial |
| Gedunin | Seed oil | Antifungal, Antimalarial |
| Azadirachtin | Seed | Antimalarial |
| Mahmoodin | Seed oil | Antibacterial |
| Gallic acid, (-) epicatechin and catechin | Bark | Anti-inflammatory immunomodulatory |
| Margolone, margolonone and isomargolonone | Bark | Antibacterial |
| Cyclic trisulphide and cyclic tetrasulphide | Leaf | Antifungal |
| Polysaccharides | | Anti-inflammatory |
| Polysaccharides Gla, Glb | Bark | Antitumor |
| Polysaccharides Glla, Gllla | Bark | Anti-inflammatory |
| NB-II peptidoglycan | Bark | Immunomodulatory |

Source: Girish and Shankara Bhat (2008)

### 2.5.1 Antibacterial Activity

Major and common antibacterial activity in human includes the eye and ear infections. These infections occur due to the migration of the affected areas due to pathogenic strains of bacteria. Neem (*Azadirachta indica*) is one of the medicinal plants that have been identified in curing these infections. The roots, stems, barks, seeds, flowers and fruits of Neem (*Azadirachta indica*) have chemically bioactive substances such as peptides, alkaloids, tannins, phenols, sterols, flavonoids and glycosides that contributes in fighting the bacteria's. The oil from the leaves, seeds and bark contains extensive spectrum against antibacterial action due to the eye and ear infections. Among all the parts of Neem (*Azadirachta indica*), the seeds are listed as one of the most popular source of medicaments in antibacterial activity. In the northern parts of Nigeria, to overcome the eye and ear infections, the seeds are

crashed and pressed several times to obtain an effective dosage so that the out coming juices will passed into the unhygienic area of the ear or the eye. The crude extracts of the Neem (*Azadirachta indica*) seeds are able to fight against several pathogenic bacteria related with ear and eye infections (El-Mahmood et al., 2010).

### 2.5.2 Antifeedant Activity

One of the important biological active compounds of the Neem (*Azadirachta indica*) tree is the tetranortriterpenoids. Tetranortriterpenoids are the compound that contains the azadirachtin-A which behaves as the antifeedant towards the insects. Among all the parts of Neem (*Azadirachta indica*), seed kernels of the tree contains the high percentage of the antifeedant compound. Test against the desert locusts have been conducted by using the Neem (*Azadirachta indica*) to prove the antifeedant action and the test proves to be positive. Besides that, several other Neem triterpenoids, especially the C-seco limonoids, and pointed that salannin was as equally efficient as azadirachtin as an insect antifeedant against *Epilachna varivestis* (Govindachari et al., 2000). Therefore, there are several biologically active compounds in Neem (*Azadirachta indica*) that can act as an antifeedant.

### 2.5.3 Antifertility Effect

Neem (*Azadirachta indica*) tree has also been studied as a solution to prevent pregnancy. This prevention can be done through intravaginal application of Neem (*Azadirachta indica*) oil. Spermicidal test have been conducted against rhesus monkey and human spermatozoa through in vitro and it has been established to be true and the seed extract of Neem (*Azadirachta indica*) avert pregnancy in babbons and bonnet monkeys. Besides the oil and the seed extract, the leaf extracts of Neem (*Azadirachta indica*) act to be antifertily in mice and the test is carried out against mice oral administration. Prevention of Neem (*Azadirachta indica*) is due to the activation of cell-mediated immune reaction where the oil and the exracts act as non-hormonal (Biswas et al., 2002).