LARVICIDAL POTENTIALITY OF THE BANDOTAN (Ageratum conyzoides) LEAVES FOR CONTROLLING THE THREE IMPORTANT SPECIES OF MOSQUITOES (Aedes aegypti, Culex quinquefasciatus and Anopheles maculatus)

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FOR THE PARTIAL FULFILLMENT OF MASTER OF SCIENCE IN BIOSCIENCE

Submitted By:
Wafi Ali Mohamed Massuod
S901302009

POSTGRADUATE STUDY PROGRAM
SEBELAS MARET UNIVERSITY
SURAKARTA
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MASTER THESIS

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1. It is going to be declared that the thesis entitled: “Larvicidal Potentiality of The Bandotan (Ageratum conyzoides) For Controlling Three Important Species of Mosquitoes (Culex quinquefasciatus, Aedes aegypti and Anopheles maculatus)” is a research work having free of plagiarism, and there is no scientific papers that have been taken by others to obtain academic degrees and there is no work or opinion ever written or published by another person except in writing used as a reference in this text and a reference source as well as mentioned in the bibliography. If at a later proved the data of this research having similar to another scientific papers i.e. plagiarism, then I am willing to accept sanctions in accordance with the provisions of the legislation.

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Surakarta, October 2014

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Acknowledgement

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Surakarta, October 2014

Keywords: Ageratum conyzoides, Nyamuk, Larvicida, ramah lingkungan
ABSTRACT

Mosquitoes transmit serious human diseases, causing millions of deaths every year. However, mosquito control is facing a threat due to the emergence of resistance to synthetic insecticides. With regard this issue insecticides of plant origin may serve as a promising alternative bio-control techniques. Consequently, the purpose of this study was to assess the larvicidal potentiality of Bandotan (*Ageratum conyzoides*), an annual herb with a long history of traditional medicinal uses in many countries in the world, for controlling three most important species of mosquitoes such as *Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles maculatus*. The crude leaf extract was prepared with different concentration (5%, 10%, 15%, 20%, 50%) of methanol solution using soxhlet apparatus. Afterwards, the crude leaf extract solution was applied on the three species of cultured mosquitoes and observed its effect since the beginning until 24 hours. In the context of quick response and high mortality the 15% methanol solution containing 10 g crude leaf powder showed the best result. Nevertheless, the LC₅₀ value was determined by using PROBIT analysis. In the end, this research findings demonstrated that leaf extract of *A.conyzoides* can be utilized as a highly potential and ecofriendly larvicide for controlling three most important species of mosquitoes.

Keywords: *Ageratum conyzoides*, Mosquito, Larvicide, Ecofriendly.
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CHAPTER I

INTRODUCTION

A. Background of the study

Mosquitoes can transmit more diseases than any other group of arthropods and affect million of people throughout the world. WHO has declared the mosquitoes as “public enemy number one” (WHO report, 1996). To prevent proliferation of mosquito borne diseases and to improve quality of environment and public health, mosquito control is essential. The major tool in mosquito control operation is the application of synthetic insecticides such as organo-chlorine and organophosphate compounds. But this has not been very successful due to human, technical, operational, ecological, and economic factors. In recent years, use of many of the former synthetic insecticides in mosquito control program has been limited. It is due to lack of novel insecticides, high cost of synthetic insecticides, concern for environmental sustainability, harmful effect on human health, and other non-target populations, their non-biodegradable nature, higher rate of biological magnification through ecosystem, and increasing insecticide resistance on a global scale (Brown, 1986; Russell, 2009). Thus, the Environmental Protection Act in 1969 has framed a number of rules and regulations to check the application of chemical control agents in nature (Bhatt, 2009). It has prompted researchers to look for alternative approaches ranging from provision of or promoting the adoption of effective and transparent mosquito management strategies that focus on public education, monitoring and surveillance, source reduction and environment friendly least-toxic larval control. These factors have resulted in an urge to look for environment friendly, cost-effective, biodegradable and target specific insecticides against mosquito species. Considering these, the application of eco-friendly alternatives such as biological control of vectors has become the central focus of the control programmme in lieu of the chemical insecticides.

One of the most effective alternative approaches under the biological control programme is to explore the floral biodiversity and enter the field of using safer insecticides of botanical origin as a simple and sustainable method of mosquito control. Plant materials with insecticidal properties have been used traditionally for generations throughout the world (Belmain et al., 2001). Botanical insecticides compared to synthetic ones may be safer for the environment, are, generally, less expensive, easily processed and used by farmers and small industries (Belmain et
Since these insecticides are often active against a limited number of species, are often biodegradable to nontoxic products, and are potentially suitable for use in integrated pest management, they could lead to the development of new classes of safer insect control agents (Kim et al., 2003). Thus there is very little chance of pests developing resistance to such substances. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management. Botanicals have widespread insecticidal properties and will obviously work as a new weapon in the arsenal of synthetic insecticides and in future may act as suitable alternative product to fight against mosquito borne diseases.

With regard this issue extract of *Ageratum conyzoides*, used as a botanical insecticides, plays an important role to control the dangerous diseases carrier mosquitoes. As there is high variability in the secondary metabolities of *A. conyzoides* which include flavonoids, alkaloids, cumarins, essential oils, and tannins. Many of these are biologically active. Essential oil yield varies from 0.02% to 0.16% (Jaccoud, 1961). Vyas and Mulchandani (1984) identified conyzorigum, a cromene, Borthakur and Baruah (1986) identified precocene I and precocene II, in a plant collected in India. These compounds have been shown to affect insect development, as antijuvenile hormones, resulting in sterile adults (Borthakur and Baruah 1987).

Moreover, changes in the larvicidal efficacy of the plant extracts occurred due to geographical origin of the plant (in *Citrus* sp (Mgbemena, 2010), *Jatropha* sp (Sakthivadivel and Daniel, 2008), *Ocimum sanctum* (Rahuman and Venkatesan, 2008), *Momordica charantia* (Rahuman and Venkatesan, 2008), *Piper* sp (Das et al., 2007) and *Azadirachta indica* (Mgbemena, 2010); response in the different mosquito species in *Curcuma domestica* (Choochate et al., 2005), *Withania somnifera* (Sakthivadivel and Daniel, 2008), *Jatropha curcas* (Rahuman et al., 2007), *Piper retrofractum* (Chansang et al., 2005), *Cestrum diurnum* (Ghosh and Chandra, 2006) *Citrullus vulgaris* (Mullai et al., 2008), and *Tridax procumbens* (Kamaraj et al., 2011); due to variation in the species of plant examined in *Euphorbia* sp (Singh et al., 2007), *Phyllanthus* sp (Rahuman et al., 2007), *Curcuma* sp (Maniafu et al., 2009), *Solanum* sp Rawani et al., 2010), *Ocimum* sp (Maurya et al., 2009), *Eucalyptus* sp (Rahuman et al., 2008), *Plumbago* sp (Rahuman et al., 2007), *Vitex* sp (Kannathasan et al., 2011), *Piper* sp (Das et al., 2007), *Annona* sp (Kamaraj et al., 2010), and *Cleome* sp (Aly and Badran, 1996) , and between plant parts used to study the larvicidal efficacy in *Euphorbia tirucalli* (Singh et al., 2007), *Solanum*
xanthocarpum (Mohan et al., 2006), Azadirechta indica (Mgbemena, 2010), Solanum villosum (Chowdhury et al., 2008), Annona squamosa (Kamaraj et al., 2010), Withania somnifera (Sakthivadivel and Daniel, 2008), Melia azedarach (Senthil et al., 2006), Moringa oleifera (Kamaraj et al., 2010), and Ocimum sanctum (Anees, 2008). Variation of the larvicidal potential of the same plant changed with the solvents used as evidenced in case of Solanum xanthocarpum (Mohan et al., 2006), Euphorbia tirucalli (Singh et al., 2007), Momordica charantia (Rahuman et al., 2008), Eucalyptus globules (Maurya et al., 2007), Citrullus colocynthis (Sakthivadivel and Daniel, 2008), Azadirechta indica (Mgbemena, 2010), Annona squamosa (Kamaraj et al., 2011) and Solanum nigrum (Rawani et al., 2010)

B. Problem Statement:

Though Ageratum conyzoides has huge potential capability as an insecticidal resource yet only very few study has been conducted about the efficacy of A. conyzoides as a larvicide on mosquitoes.

C. Objectives:

To find out the potent larvicidal role of Ageratum conyzoides against life intimidation mosquitoes particularly Aedes aegypti, Culex quinquefasciatus and Anopheles maculatus species.

D. Research Contribution

Herb Bandotan (Ageratum conyzoides) is a widely distributed in tropical and sub-tropical environmental condition. It is easily found home ground area and no special care is needed for cultivation. Consequently, if high larvicidal potentiality is obtained from bandotan plant then it will be utilized as one of the cheapest source for controlling life threatening mosquitoes. This is key contribution of this research.
CHAPTER II

LITERATURE REVIEW AND HYPOTHESIS

1. Bandotan (*Ageratum conyzoides*)

*Ageratum conyzoides* has bioactive activity that may have agricultural use, as shown by several research investigations in different countries. Pereira in 1929, cited by Jaccoud (1961), reported use of the leaves as an insect (moth) repellent. The insecticide activity may be the most important biological activity of this species. The terpenic compounds, mainly precocenes, with their antijuvenile hormonal activity are probably responsible for the insecticide effects.

Assays conducted in Colombia by Gonzalez et al. (1991) showed activity of this species against *Musca domestica* larvae, using whole plant hexane extract (Gonzales et al., 1991). Vyas and Mulchandani (1986) reported the action of cromenes (precocenes I and II), isolated from *Ageratum* plants, which accelerate larval metamorphosis, resulted in juvenile forms or weak and small adults.

Ekundayo et al. (1987) also demonstrated the juvenilizing hormonal action of precocene I and II in insects, the most common effect being precocious metamorphosis, producing sterile or dying adults. Raja et al (1987), using *A. conyzoides* methanolic extract from fresh leaves (250 and 500 ppm) in the fourth instar of *Chilo partellus* (Lepidoptera, Pyralidae), a sorghum pest, observed the presence of a dark stain in the insects' cuticle and immature pupae formation, both symptoms of deficiency of juvenile hormone.

*A. conyzoides* also induces morphogenetic abnormalities in the formation of mosquitoes larvae (*Culex quinquefasciatus*, *Aedes aegypt*, and *Anopheles stephensi*). This has been verified using intermediary stages between larvae–pupae, discolored and longer pupae, as well as incompletely developed adults (Sujatha et al., 1988). Extracts of the flowers of this species showed activity against mosquitoes (*Anopheles stephensi*), in the last instar, showing DL 50 with 138 ppm (Kamal and Mehra, 1991).
Cetonic extracts of the species produced significant effects against the mosquito, *Culex quinquefasciatus*, in India, when applied to fourth instar larvae and adult females. In larvae, the extracts produced altered individuals, intermediate between larvae and pupae, unmelaninized and with inhibition of development, as well as adults with deformed wings muscles. In female adults, there was loss of fecundity, lower eggs production, and production of defective eggs (Saxena and Saxena, 1992). Similar results were observed in larvae of *Anopheles stephensi* and *Culex quinquefasciatus* in others essays, confirming the antijuvenile potential of *A. conyzoides* (Saxena and Saxena, 1992; Saxena et al., 1994).

How the toxicity of *A. conyzoides* is created within insect body reported by some experiments such as Assays conducted in India showed high nymphal mortality 91% of the oil to the Nymphs of *Schistocerca gregaria* (Okunade and Adewole, 2002). Calle et al., 1990 showed that the hexane extract of the whole plant showed activity against *Musca domestica* larvae (Okunade and Adewole, 2002). Methanolic extract from fresh leaves 250 and 500 ppm also produced deficiency of juvenile hormone in the fourth instar of Chilo partellus, a sorghum pest (Okunade and Adewole, 2002). Antijuvenile hormonal activity of Precocenes I and II have been demonstrated on a variety of insects which include *Sitophilus oryzae*, *Thlaspida japonica*, *Leptocarsia chinesis* (Okunade and Adewole, 2002) and *Dysdercus flavidus* (Okunade and Adewole, 2002). The results from these assays include precocious metamorphosis of the larvae, production of sterile, moribund and dwarfish adults. The two chromenes have been reported to act synergistically and they survived metabolism for at least 12 days (Okunade and Adewole, 2002). Preliminary study on the mode of action of precocene II on *Musa domestica L.* and *Lucilia caesar L.* have been carried out (Okunade and Adewole, 2002). While the precocenes have been seen as fourth-generation insecticides, the drawback is that they have been shown to cause hepatotoxicity in rats (Okunade and Adewole, 2002). This is an important factor bearing in mind the human health hazard in field applications of precocenes as large-scale insecticidal agents. The mechanism of action has been carried out by a number of researchers. Some workers demonstrated that the toxicity was due to a highly reactive precocene-3,4-epoxide, a metabolite produced in insect species from cytochrome P-450 (28,29). Others, like Darvas and colleagues (Okunade and Adewole, 2002), Casas et al. (Okunade and Adewole, 2002) reported that the 3, 4...
double bond played no significant role in the toxicity but that the oxidative dealkylation process at C7 position, as a tocopherol-like antioxidants, might be responsible for the cytotoxicity.

![Figure 1.1: A View of Bandotan (Ageratum conyzoides) plant at UNS Campus](image)

2. Mosquitoes

Mosquito borne diseases are prevalent in more than 100 countries across the world, infecting over 700,000,000 people every year globally and 40,000,000 of the Indian population. They act as a vector for most of the life threatening diseases like malaria, yellow fever, dengue fever, chikungunya fever, filariasis, encephalitis, Wet Nile virus infection, etc., in almost all tropical and subtropical countries and many other parts of the world. Among them Dengue fever is one of the most rapidly rising mosquito transmitted infections in the world (Lam, 1993) and has been identified as a re-emerging diseases in southeast Asia (WHO, 1999). Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) are viral diseases transmitted by Aedes mosquitoes, usually Aedes aegypti virus causing dengue infects nearly 120 million of people living 110 countries of the world (Halstead, 2000). Besides, Aedes aegypti, one another mosquito Culex quinquefasciatus, that responsible for the Disease Transmitted of Wet Nile virus and St Louis encephalitis. They also transmit the organisms causing bird malaria, fowl pox, and heartworm of dogs.
Figure 1.2: The Image of three experimented species of mosquito; Left to Right- Culex, Aedes; and Anopheles (Courtesy to James Gathany, Center for Disease Control and Prevention)

3. Phytochemicals

Phytochemicals are botanicals which are naturally occurring insecticides obtained from floral resources. Applications of phytochemicals in mosquito control were in use since the 1920s (Shahi et al., 2010), but the discovery of synthetic insecticides such as DDT in 1939 side tracked the application of phytochemicals in mosquito control programme. After facing several problems due to injudicious and over application of synthetic insecticides in nature, re-focus on phytochemicals that are easily biodegradable and have no ill-effects on non-target organisms was appreciated. Since then, the search for new bioactive compounds from the plant kingdom and an effort to determine its structure and commercial production has been initiated. At present phytochemicals make upto 1 per cent of world's pesticide market (Isman, 1997).

Botanicals are basically secondary metabolites that serve as a means of defence mechanism of the plants to withstand the continuous selection pressure from herbivore predators and other environmental factors. Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Shaalan et al., 2005). Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varities and parts used, but also due to extraction methodology adopted and the polarity of the solvents used during extraction. A wide selection of plants from herbs, shrubs and large trees was used for extraction of mosquito toxins. Phytochemicals were extracted either from the whole body of little herbs or from various
parts like fruits, leaves, stems, barks, roots, etc., of larger plants or trees. In all cases where the most toxic substances were concentrated upon, found and extracted for mosquito control. Plants produce numerous chemicals, many of which have medicinal and pesticidal properties. More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programmes. Members of the plant families- Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae and Rutaceae have various types of larval, adulticidal or repellent activities against different species of mosquitoes (Shaalan et al., 2005).

4. Application of phytochemicals as mosquito larvicide: An essential component of IMM

Human beings have used plant parts, products and secondary metabolites of plant origin in pest control since early historical times. Vector control has been practiced since the early 20th century. During the pre-DDT era, reduction of vector mosquitoes mainly depended on environmental management of breeding habitats, i.e., source reduction. During that period, some botanical insecticides used in different countries were Chrysanthemum, Pyrethrum, Derris, Quassia, Nicotine, Hellebore, Anabasine, Azadirachtin, d-limonene camphor, Turpentine, etc (Shaalan et al., 2005).

From the early 1950s, DDT and other synthetic organochloride and organophosphate insecticides were extensively used to interrupt transmission of vector borne diseases by reducing densities, human-vector contact and, in particular, the longevity of vector mosquitoes. In the mid-1970s, the resurgence of vector borne diseases, along with development of insecticide resistance in vector population, poor human acceptance of indoor house spraying and environmental concerns against the use of insecticides led to a rethinking in vector control strategies (WHO, 2005). As a result, emphasis was given on the application of alternative methods in mosquito control as part of the Integrated Mosquito Management (IMM) (Rose, 2001). Integrated Mosquito Management (IMM) is a decision-making process for the management of mosquito populations, involving a combination of methods and strategies for long-term maintenance of low levels of vectors. The purpose of IMM is to protect public health from diseases transmitted by mosquitoes, maintain healthy environment through proper use and disposal of pesticides and improve the overall quality of life through practical and effective pest control strategies. The main approaches of IMM include: 

1. Source reduction and habitat
2. Commit to user
management by proper sanitation, water management in temporary and permanent water bodies, and channel irrigation. Vegetation management is also necessary to eliminate protection and food for mosquito larvae; (ii) Larviciding by application of dipteran specific bacteria, insect growth regulators, surface films and oils, expanded polystyrene beads, phytochemicals, organophosphates and organochlorides, (iii) Adulticiding by application of synthetic pyrethroids, organophosphates and synthetic or plant derived repellents, insecticide impregnated bed nets, genetic manipulations of vector species, etc., (iv) Use of mosquito density assessment in adult and larval condition and disease surveillance; and (v) Application of biological control methods by using entomophagous bacteria, fungi, microsporidians, predators and parasites.

Of the above avenues of IMM, larviciding approach is the more proactive, pro-environment, target specific and safer approach than controlling adult mosquitoes. Application of larvicide from botanical origin was extensively studied as an essential part of IMM, and various mosquito control agents such as ocimenone, rotenone, capillin, quassin, thymol, eugenol, neolignans, arborine and goniothalamin were developed (Shaalan et al., 2005).

5. Nature of active ingredients responsible for larval toxicity

The plant world comprises a rich untapped pool of phytochemicals that may be widely used in place of synthetic insecticides in mosquito control programme. Kishore et al, 2011 reviewed the efficacy of phytochemicals against mosquito larvae according to their chemical nature and described the mosquito larvicidal potentiality of several plant derived secondary materials, such as, alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpanes and lignans. They also documented the isolation of several bioactive toxic principles from various plants and reported their toxicity against different mosquito species (Table I).
Table I: the isolation of several bioactive toxic principles from various plants and their toxicity against different mosquito species

<table>
<thead>
<tr>
<th>Active Ingredient</th>
<th>Mosquitoes</th>
<th>Plants</th>
<th>LC/LD Values</th>
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<tr>
<td>Octacosane</td>
<td>Cx. quinquefaciatus</td>
<td>Moschosma polystachyum</td>
<td>LC$_{50}$ value of 7.2±1.7 mg/l</td>
</tr>
<tr>
<td>(E) - 6-hydroxy-1,6-dimethyl 1,3-heptane-2-one</td>
<td>Aedes aegyptii</td>
<td>Ocimum sanctum</td>
<td>LD$_{100}$ value of 6.25 µg/ml</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>Aedes aegyptii</td>
<td>Eucalyptus camaldulensis</td>
<td>LC$_{50}$ value of 147 µg/ml</td>
</tr>
<tr>
<td>Geranial</td>
<td>Aedes aegyptii</td>
<td>Magnolia salicifolia</td>
<td>LD$_{100}$ value of 100 ppm</td>
</tr>
<tr>
<td>Germaerene D</td>
<td>Anopheles gambiae; Cx. quinquefaciatus; Aedes aegyptii</td>
<td>Chalaroxylon swietenia</td>
<td>LD$_{50}$ value of 1.8, 2.1 and 2.8 x 10$^{-3}$</td>
</tr>
<tr>
<td>Hugorosenone</td>
<td>Aedes aegyptii</td>
<td>Hugonia castaneifolia</td>
<td>LC$_{50}$ value of 0.3028 mg/ml</td>
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</table>

6. Mode of action of phytochemicals in target insect body

Generally the active toxic ingredients of plant extracts are secondary metabolites that are evolved to protect them from herbivores. The insects feed on these secondary metabolites potentially encountering toxic substances with relatively non-specific effects on a wide range of molecular targets. These targets range from proteins (enzymes, receptors, signaling molecules, ion-channels and structural proteins), nucleic acids, biomembranes, and other cellular components (Rattan, 2010). This in turn, affects insect physiology in many different ways and at various receptor sites, the principal of which is abnormality in the nervous system (such as, in neurotransmitter synthesis, storage, release, binding, and re-uptake, receptor activation and function, enzymes involved in signal transduction pathway). Rattan, 2010 reviewed the mechanism of action of plant secondary metabolites on insect body and documented several physiological disruptions, such as inhibition of acetylcholinesterase (by essential oils), GABA
gated chloride channel (by thymol), sodium and potassium ion exchange disruption (by pyrethrin) and inhibition of cellular respiration (by rotenone). Such disruption also includes the blockage of calcium channels (by ryanodine), of nerve cell membrane action (by sabadilla), of octopamine receptors (thymol), hormonal balance disruption, mitotic poisoning (by azadirachtin), disruption of the molecular events of morphogenesis and alteration in the behaviour and memory of cholinergic system (by essential oil), etc. Of these, the most important activity is the inhibition of acetylcholinesterase activity (AChE) as it is a key enzyme responsible for terminating the nerve impulse transmission through synaptic pathway; AChE has been observed to be organophosphorus and carbamate resistant, and it is well-known that the alteration in AChE is one of the main resistance mechanisms in insect pests.

7. Scope for future research: isolation of toxic larvicidal active ingredients

Several studies have documented the efficacy of plant extracts as the reservoir pool of bioactive toxic agents against mosquito larvae. But only a few have been commercially produced and extensively used in vector control programmes. The main reasons behind the failure in laboratory to land movements of bioactive toxic phytochemicals are poor characterization and inefficiency in determining the structure of active toxic ingredients responsible for larvicidal activity. For the production of a green biopesticide, the following steps can be recommended during any research design with phytochemicals: (i) Screening of floral biodiversity in search of crude plant extracts having mosquito larvicidal potentiality; (ii) Preparation of plant solvent extracts starting from non-polar to polar chemicals and determination of the most effective solvent extract; (iii) Evaporation of the liquid solvent to obtain solid residue and determination of the lethal concentration (LC$_{50}$/LC$_{100}$ values); (iv) Phytochemical analysis of the solid residue and application of column chromatography and thin layer chromatography to purify and isolate toxic phytochemical with larvicidal potentiality; (v) Determination of the structure of active principle by infrared (IR) spectroscopic, nuclear magnetic resonance (NMR) and gas chromatography and mass spectroscopy (GCMS) analysis; (vi) Study of the effect of active ingredient on non-target organisms; and (vii) Field evaluation of the active principle before its recommendation in vector control programme and commercial production.
B. Hypothesis:

The hypothesis of this research was considered likely would leaves extract of bandotan (Ageratum conyzoides) have larvicidal potentiality against mosquitoes or not.
CHAPTER III
RESEARCH METHODOLOGY

A. Materials and Equipment

The materials used for this were leaves of bandotan, pure methanol, filter paper, cultured three species of mosquitoes, plastic bottle, rope, Ice. The used equipment for this research was Erlenmeyer flask, beaker, soxhlet apparatus, weight balance, spatula, petridish, electronic blender.

B. Methodology

The research was carried out by following steps

1. Collection of plant materials:

The leaves of Ageratum conyzoides were collected from different places of Sebelas Maret University campus.

2. Preparation of plant extracts

The collected leaves were dried for 7-10 days in the shade at the environmental temperatures (27-37°C day time). The dry leaves were powdered mechanically using commercial electrical stainless steel blender. The different concentration of methanol solution such as 5% (95 ml distilled water and 5 ml methanol), 10% (90 ml distilled water and 10 ml methanol), 15% (85 ml distilled water and 15 ml methanol), 20% (80 ml distilled water and 20 ml methanol), 25% (75 ml distilled water and 25 ml methanol) was prepared. 10 gm of crude leaf powder was added each of the prepared methanol solution and obtained solution was set up into soxhlet apparatus for leaf extraction. Afterwards, the leaf extract was collected in a plastic bottole and stored at room temperature.

3. Culture of Mosquito

The three species of mosquitoes were cultured at the Reservoir Laboratory of B2P2VRP Salatiga, Indonesia. They were cultured by following the WHO protocol developed by B2P2VRP. The food was provided for mosquito namely Brewer’s yeast, dog biscuits and algae.
The appropriate environmental condition was checked for the proper growth and development of mosquito.

4. Larvicidal bioassay

The 25 cultured larvae of each of the mosquito species were transferred into 100 ml of prepared leaf extract solution. The effect of leaf extract on mosquitoes was observed next 24 hours.

5. Dose-response bioassay:

The number of dead larvae was counted from first 30 minutes to 24 hours of exposure. One time replication was done to ensure the validity of result derived from first installment.

6. Statistical analysis:

The \( \text{Lc}_{50} \) was calculated by using probit analysis to find out the economical concentration of leaf extract.
Figure 3.1: A Schematic Diagram of Research Methodology
CHAPTER IV
RESULT AND DISCUSSION

A. Results

In figure below illustrates the influences of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 30 minutes of first installment. The X- axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y- axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like Culex points out by blue, Aedes and Anopheles show by red and green, respectively.

![Graph showing the effect of Bandotan leaf extract on larval stage of mosquitoes](image)

**Figure 4.1:** The effect of Bandotan leaf extract on Larva of three species of mosquito after 30 minutes of first installment at different concentration

From Figure 4.1 it is obvious that during the first 30 minute there was no impact of bandotan leaves extract on Culex *quinquefasciatus* and *Aedes aegypti*. However, within this time significant effect was found on *Anopheles maculatus*. As all the trial mosquitoes were killed within first 30 minute by 15%, 20%. And 50% concentration, respectively, though by 5% and 10% the number of killed mosquito only 5 and 12, respectively.

In brief, it can be said that the larvicidal potentiality of Bandotan is more quicker on *Anopheles maculatus* than two other species.
In figure below depicts the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 45 minutes of first installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

**Figure 4.2:** The effect of Bandotan leaf extract on Larva of three species of mosquito after 45 minutes of first installment

From Figure 4.2 it is clear that the highest performance of bandotan was showed at almost 15%, 20% and 50%, respectively in comparison doses level by 5% and 10%. The whole number of experimental mosquitoes were died by 15%, 20% and 50% concentration, whereas, the number of killed mosquito at 5% and 10% concentration was around 5 in terms of *Culex quinquefasciatus* and *Aedes aegypti*. However, this number was considerable higher approximately to 16 in case of *Anopheles maculatus*.

To sum up, the effective concentration is 15 % to show the highest larvicidal performance of bandotan leaves on all the three highly important species of mosquito.

In figure below shows the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 1 hour of first installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-
axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of moquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

![Graph showing the effect of Bandotan leaf extract on Larva of three species of mosquito after 1 hour of first installment.](image)

**Figure 4.3: The effect of Bandotan leaf extract on Larva of three species of mosquito after 1 hour of first installment.**

From Figure 4.3 it can be noted that compare to the effect after 45 minutes, the most distinctive feature in this graph is the increase number of killed larva at maximum level at both 5% and 10% concentration in case *Anopheles maculatus*, while the amount of killed larva for *Culex quinquefasciatus* and *Aedes aegypti* was almost same as like as just 15 minutes before. The entire number of tested mosquitoes were died at all concentration in case of *Anopheles maculatus*, whereas the same result was found for *Culex quinquefasciatus* and *Aedes aegypti* by 15%, 20%, and 25% concentration level.

In short, it can be mentioned that the fastest larvicidal activity of bandotan leaves was found on *Anopheles maculatus* compare to two other species of mosquito.

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In figure below explains the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 2 hours of first installment. The X- axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

**Figure 4.4: The effect of Bandotan leaf extract on Larva of three species of mosquito after 2 hours of first installment**

From Figure 4.4 it can be seen that there was a slightly rise of the number of killed larva for *Culex quinquefasciatus* and *Aedes aegypti* in comparison to the result having been found after 1 hour. The number of killed mosquito was around 7 and 10 for both species by 5% and 10%, while other results were same as like as 1 hour result.

In brief, it can be referred that 5% and 10% bandotan leaf extract concentration are less effective act as a larvicide of *Culex quinquefasciatus* and *Aedes aegypti* compare to other concentrations that are used this experiment.

As a small difference had been found last 1 hour, so the final data was taken after 24 hour that is most suitable period for data collection.
In figure below explains the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 2 hours of first installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of moquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

![Figure 4.5](image)

**Figure 4.5**: The effect of Bandotan leaf extract on Larva of three species of mosquito after 24 hours of first installment.

From Figure 4.5 it is obvious that the highest larvicidal activity of the doses level of 5% and 10% for was demonstrated after 24 hour of first installment. During this time, the number of killed larva was 22 and 24 respectively, whereas other other findings were sama as like as 1 hour result.

In a nut shell, it can be suggested that on the basis of quick larvicidal response on three species of mosquito, the most quickly dose level is 15% which showed the ability to kill all the tested mosquitoes after 45 minutes of first installment, though the maximum efficacy by 5% and 10% dose level was obtained after 24 hours of first installment.
In figure below illustrates the influences of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 30 minutes of second installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of moquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

![Diagram showing the effect of Bandotan leaf extract on larval stage of three species of mosquitoes](image)

**Figure 4.6:** The effect of Bandotan leaf extract on larva of three species of mosquito after 30 minutes of second installment

From Figure 4.6 it is obvious that during the first 30 minute there was no impact of bandotan leaves extract on *Culex quinquefasciatus* and *Aedes aegypti*. However, within this time significant effect was found on *Anopheles maculatus*. As all the trialed mosquitoes were killed within first 30 minute by 15%, 20%. And 50% concentration respectively, though by 5% and 10% the number of killed mosquito only 5 and 12, respectively.

In brief, it can be said that the larvicidal potentiality of Bandotan is more quicker on *Anopheles maculatus* than two other species.
In Figure below depicts the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 45 minutes of second installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

![Figure 4.7: The effect of Bandotan leaf extract on Larva of three species of mosquito after 45 minutes of second installment](image)

From Figure 4.7 it is clear that the highest performance of bandotan was showed at almost 15%, 20% and 50%, respectively in comparison doses level by 5% and 10%. The whole number of experimental mosquitoes were died by 15%, 20% and 50% concentration, whereas, the number of killed mosquito at 5% and 10% concentration was around 5 in terms of *Culex quinquefasciatus* and *Aedes aegypti*. However, this number was considerable higher approximately to 16 in case of *Anopheles maculatus*.

To sum up, the effective concentration was 15% that showed the highest larvicidal performance of bandotan leaves on all the three highly important species of mosquito.
In figure below shows the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 1 hour of second installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like Culex points out by blue, Aedes and Anopheles show by red and green, respectively.

**Figure 4.8:** The effect of Bandotan leaf extract on Larva of three species of mosquito after 1 hour of second installment

From Figure 4.8 it can be noted that the most distinctive feature in this graph is the increase number of killed larva at maximum level at both 5% and 10% concentration in case Anopheles maculatus compare to the effect after 45 minutes, while the amount of killed larva for Culex _quinquefasciatus_ and _Aedes aegypti_ was almost same as like as just 15 minutes before. The entire number of tested mosquitoes were died at all concentration in case of Anopheles maculatus, whereas the same result was found for _Culex quinquefasciatus_ and _Aedes aegypti_ by 15%, 20%, and 25% concentration level.

In short, it can be mentioned that the fastest larvicidal activity of bandotan leaves was found on _Anopheles maculatus_ compare to two other species of mosquito.
In figure below explains the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 2 hours of first installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

![Graph](image.png)

**Figure 4.9**: The effect of Bandotan leaf extract on Larva of three species of mosquito after 2 hours of second installment

From Figure 4.9 it can be seen that there was a slightly rise of the number of killed larva for *Culex quinquefasciatus* and *Aedes aegypti* in comparison to the result having been found after 1 hour. The number of killed mosquito was around 7 and 10 for both species by 5% and 10%, while other results were same as like as 1 hour result.

In brief, it can be referred that 5% and 10% bandotan leaf extract concentration are less effective act as a larvicide of *Culex quinquefasciatus* and *Aedes aegypti* compare to other concentrations that are used this experiment.

As a small difference had been found last 1 hour, so the final data was taken after 24 hour that is most suitable period for data collection.
In figure below explains the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 2 hours of first installment. The X-axis represents the different concentration of methanol solution containing 10 g leaf powder (percentage) and Y-axis indicates the no. of killed larvae. Furthermore, three different colours are used to understand three different species of mosquitoes like *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

![Graph showing the effect of Bandotan leaf extract on larva of three species of mosquito after 2 hours of second installment.](image)

**Figure 4.10**: The effect of Bandotan leaf extract on larva of three species of mosquito after 2 hours of second installment

From figure 4.10 it is obvious that the highest larvicidal activity of the doses level of 5% and 10% for was demonstrated after 24 hour of first installment. During this time, the number of killed larva was 22 and 24 respectively, whereas other other findings were sama as like as 1 hour result.
B. Discussion:

The usage of phytochemicals against mosquito larvae can vary remarkably depending on plant species, plant parts used, age of plant parts (young, mature or senescent), solvent used explained the existence of variations in the level of effectiveness of phytochemical compounds on target mosquito species vis-à-vis plant parts from which these were extracted, responses in species and their developmental stages against the specified extract, solvent of extraction, geographical origin of the plant, photosensitivity of some of the compounds in the extract, effect on growth and reproduction (Ghosh et al., 2012). It has been demonstrated that the extraction of active biochemical from plants confides upon the polarity of the solvents utilized. Polar solvent will extract polar molecules and non-polar solvents extract non-polar molecules. This was gained by using mainly eleven solvent systems ranging from hexane/ petroleum ether, the most non-polar (polarity index of 0.1 that mainly extracts essential oil) to that of water, the most polar (polarity index of 10.2) that extracts biochemical with higher molecular weights such as proteins, glycans, etc. Chloroform or ethyl acetate are moderately polar (polarity index of 4.1) that mainly extracts steroids, alkaloids, etc. It has been obtained that in most of the studies solvent with minimum polarity have been used such as hexane or petroleum ether or that with maximum polarity such as aqueous/steam distillation. However, those biochemical that were extracted using moderately polar solvents were also seen to give good results as reported by a few bioassay. Thus, different solvent types can significantly affect the potency of extracted plant compounds and there is difference in the chemo-profile of the plant species. The lowest LC$_{50}$ value was reported in Solenostemma argel against Cx. pipiens (Al-Doghairi et al., 2004). Several other plants such as Nyctanthes arboristis (Khatune et al., 2001), Atlantia monophylla (Chowdhury et al., 2009), Centella asiatica (Matasyoh et al., 2008), Cryptotaenia paniculata (Rawani et al., 2009) were also reported with promising LC$_{50}$ values. These extracts may be fractioned in order to locate the particular bioactive toxic agent responsible for larval toxicity. It also reported that most of the studies were carried out on Culex mosquitoes and Aedes was the least frequently chosen mosquitoes for all the experiments. In several studies, instead of a particular solvent, combination of solvents or serial extraction by different solvents according to their polarity has also been tried and good larvicidal potentiality found as a result (Chowdhury et al., 2007)
Exploring bioactive medicinal plants in vector and insect pest management program is one of the eco-friendly approaches because they are easily biodegradable in nature. Naturally plants are rich store houses for potential bioactive compounds which are gaining appreciation in recent times among the scientific communities (Nagappan, 2012). As for instance, according to Berenbaum, 1985 crude extracts of the plants may have mixtures of active compounds which act synergistically and their overall bioactivity was also greater than individual compounds (Chen et al., 1995). From above results it can be suggested that there is a heavily importance of using solvent for extraction as well as quick response of larvicidal potentiality. In this study the quickest larvicidal response on three species of mosquito was found at 15% methanol solution containing 10g crude powder, 115 ppm, which showed the ability to kill all the tested mosquitoes after 45 minutes of observation time whereas the maximum efficacy of 5% and 10% methanol solution occupying same amount of crude powder was determined after 24 hours of first installment. However, this dose (15%) is a highly concentrated. So it cannot be used for economical point of view and might be threat for non-target animal such as cats, dogs. In the end the LC$_{50}$ was calculated by PROBIT analysis.

In this study the effect of methanol using as a solvent was found. As the 15% methanol solution showed the quicker response and better performance compare to 5% and 10% concentrated methanol solution. Additionally, the most susceptible species among the three species was *Anopheles maculatus*. It might be because of the habitat. As *Anopheles maculatus* does not live in dirty or closed areas compare to two other species rather they prefer to live at mountaneous area. Consequently, solvent based bandotan solution striked on them heavily and quickly, while two other species such as *Culex quiquefasciatus* and *Aedes aegypti* showed a bit resistance initially against bandotan leaves extract. As they found a similar environmental condition like dirty water based extraction in bandotan leaves extract.
CHAPTER V

CONCLUSION AND SUGGESTION

Conclusion

In conclusion, this research findings showed that leaf extract of *A. conyzoides* can be developed as environment friendly larvicide because it is a bio-degradable and not dangerous for non-target organisms. Additionally, the results, LC$_{50}$ $31.69 \times 10^3$ ppm, uncover the possibility for further investigations of the efficacy of larvicidal properties of natural product extracts.

Suggestion

In this research only the larvicidal efficacy has been tested. However, which chemical compound such as alkaloids, flavonoids, chromenes, benzofurans is responsible for larvicidal activity has not been examined. So the biochemical test for active ingredients using HPLE (High Pressurized Liquid Chromatography) and NMR (Nuclear Magnetic Resonance) can be executed in future for further advancement of this research findings.
Reference


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Data for PROBIT analysis determining the LC50 value

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Parameter Estimates

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a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)
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b. Logarithm base = 10.