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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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 LC_{50} 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: Bandotan, Soxhlet, Probit, Larvicide.

INTRODUCTION

Mosquitoes, declared as “public enemy number one” (WHO, 1996) and most undeniable arthropod vector of diseases (Kumar *et al.*, 2014), can threat millions of people life throughout the world (Vatandoost and Vaziri, 2001). They transmit parasites and pathogens which continue to have disadvantageous impact on human beings (Maheswaran *et al.,* 2008). Mosquito borne diseases are prevalent in more than 100 countries across the world, infecting over 700,000,000 people every year globally. They act as a vector for most of the life threatening diseases like malaria, yellow fever, dengue fever, chikungunya ferver, filariasis, encephalitis, Wet Nile virus infection, *etc*., in almost all tropical and subtropical countries and many other parts of the world (Ghosh *et al.*, 2012). 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). Similarly, one another disease-causing mosquito agents is *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. Apart from *Aedes aegypti Culex quinquefasciatus,* another significant public health problem species in the genus *Anopheles* are known as malarial mosquitoes because they are involved in the development and transmission of the protozoan parasites that cause human malaria. In addition to malaria, certain species of *Anopheles* are known to transmit dog heartworm, but none are thought to be important vectors of West Nile Virus.

To impede the rapid increase of mosquito borne diseases and to improve quality of environment and public health, mosquito control is essential. The most reliable tool in mosquito control operation is the application of synthetic insecticides such as organo-chlorine and organophosphate compounds. However, this has not been very successful due to human, technical, operational, ecological, and economic factors (Ghosh *et*

al., 2012). 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). Therefore, identification of novel effective mosquitocidal compounds is essential to combat increasing resistance rates, concern for the environment and food safety, the unacceptability of many organophosphates and organochlorines and the high cost of synthetic pyrethroids (Shaalan *et al.*, 2005). 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-friedly alternatives such as biological control of vectors has become the central focus of the control programmme in lieu of the chemical insecticides.

In contrast, plant materials with insecticidal properties have been used traditionally for generations throughout the world (Belmain *et al.*, 2001). Currently an increasing number of researchers are reconsidering botanicals containing active phytochemicals in their efforts to address some of these problems (Shaalan et al., 2005). Consequently, one of the most effective alternative approaches under the biological control program 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. As, the search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable remains a top research issue for scientists associated with alternative vector control (Redwane *et al.*, 2002). Additionally, 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 vital role to control the dangerous diseases carrier mosquitoes*. As* there is high inconstancy in the secondary metabolities of *A. conzyoide* which include flavonoids, alkaloids, cumarins, essential oils, and tannins. Many of these are biologically active. Essential oil yield ranges from 0.02% to 0.16% (Jaccoud, 1961). Vyas and Mulchandani (1984) identified conyzorigum, a cromene, as well as 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).

Table (1): The scientific classification of Bandotan

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Asterids
Order:	Asterales
Family:	Asteraceae
Tribe:	Eupatorieae
Genus:	*Ageratum*
Species:	A. conyzoides

Figure (1): A View of Bandotan *(Ageratum conyzoides)* plant

(Source: http://kamijarawulung.blogspot.com/2012/12/bandotan-serta-manfaatnya.html)

Figure (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)***

MATERIALS AND EQUIPMENT

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

METHODOLOGY

The research was carried out by following steps

Collection of plant materials*:*

The leaves of *Ageratum conyzoides* were collected from different places of Sebelas Maret University campus and confirmation of species was ensured by executing appropriate morphological study.

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 bottle and stored at room temperature.

Culture of Mosquitoes

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

Dose- response bioassay

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

Statistical analysis

The Lc_{50} was calculated by using Probit analysis to find out the economical dose of leaf extract.

RESULTS

Though literature of reviews suggest that taking data after 24 hours of different doses being exposed on larva of mosquito, nevertheless in this experiment the data was started recording the data immediately after disclosure as this study used extremely higher dose of extract to get quick response. The remarkable research finding was obtained after 30 minutes, 2 hours and 24 hours of exposure, which are mentioned in below.

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 moquitoes likely *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

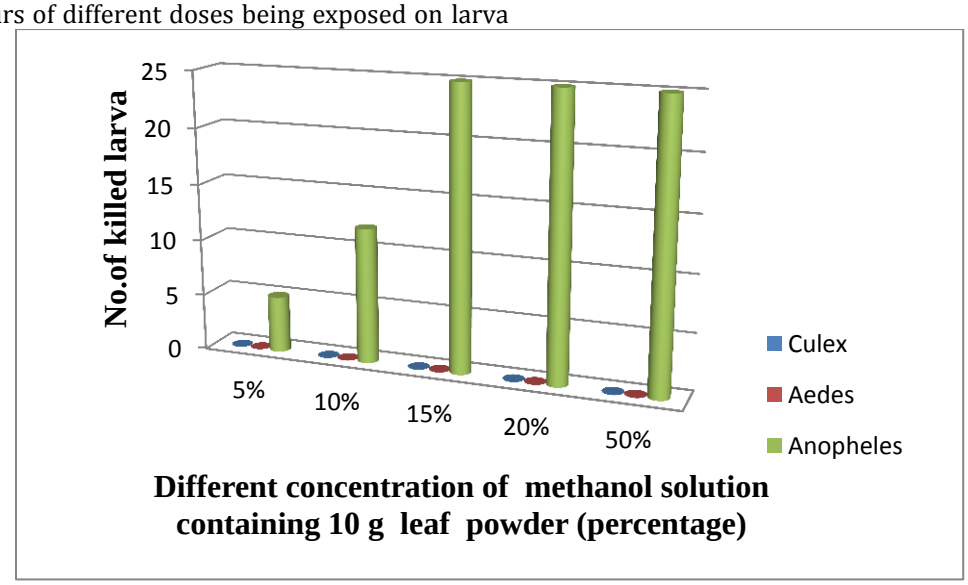

Figure (3): The effect of Bandotan leaf extract on larva of three species of mosquito after 30 minutes of first installment at different concentration

From figure 3 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 at 15%, 20%, and 50% concentration, respectively though the number of killed mosquito only 5 and 12 , by 5% and 10% , respectively.

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.

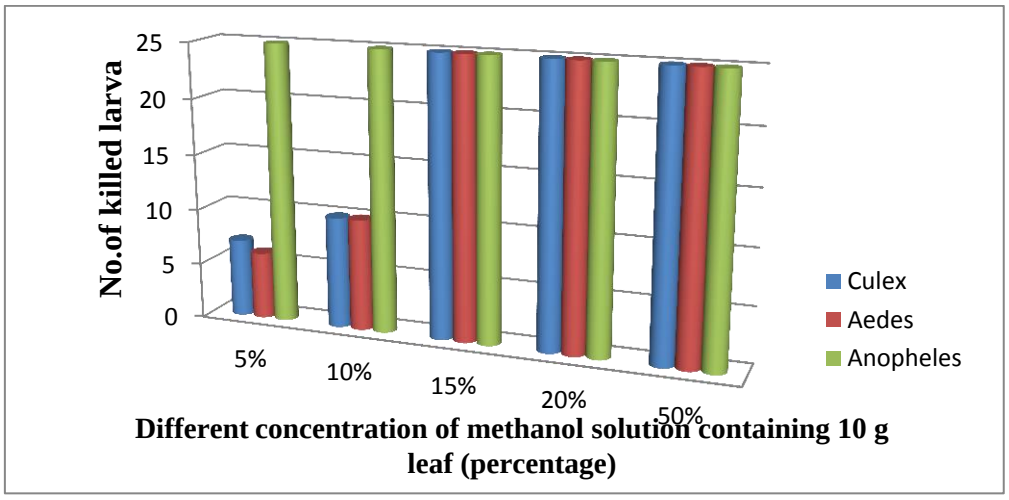

Figure (4): The effect of Bandotan leaf extract on Larva of three species of mosquito after 2 hours of first installment

From figure 4, it is clear that the highest performance of bandotan was showed at 15%, 20% and 50% dose level in comparison to dose level by 5% and 10%. The whole number of experimental mosquitoes was died by 15%, 20% and 50% concentration, whereas the number of killed mosquito at 5% and 10% concentration was 6,10,5,10 in terms of *Culex quinquefasciatus* and *Aedes aegypti though the total number of testes Anopheles maculatus mosquitoes was died at same concentration after 2 hours of exposure .*

A small difference was found regarding to toxicity effect since 2 hours to next couple of hours , consequently the final data was taken after 24 hours that is most supported period for data collection.

In figure below shows the effect of different doses of bandotan leaves extract on larval stage of three key mosquitoes after 24 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 indicate three different species of moquitoes likely *Culex* points out by blue, *Aedes* and *Anopheles* show by red and green, respectively.

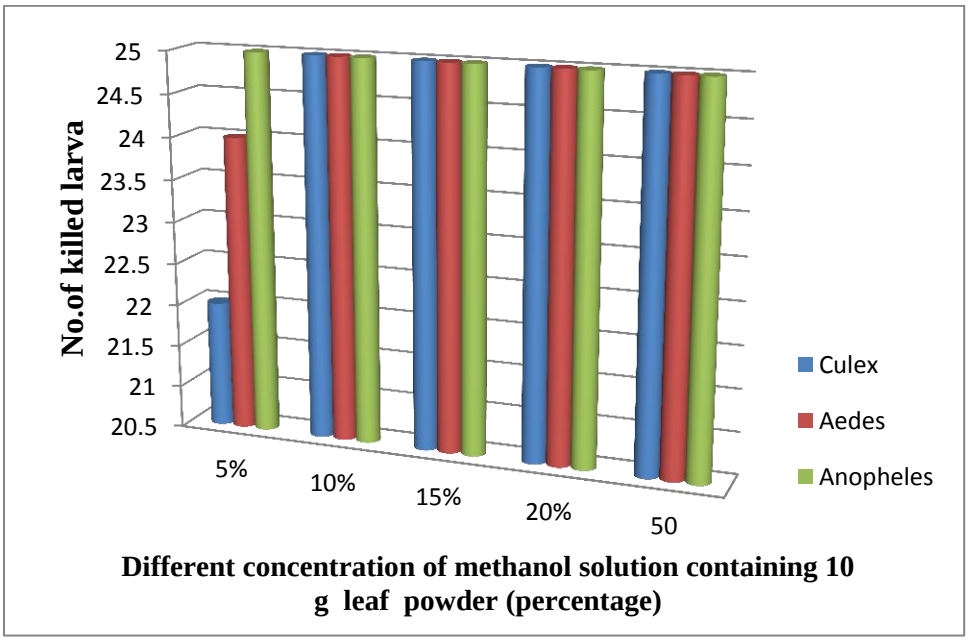

Figure 5: The effect of Bandotan leaf extract on Larva of three species of mosquito after 24 hours of first installment.

From figure 5 it is obvious that the dose level of 5% and 10% showed its highest larvicidal activity after 24 hours of first installment. During this time, the number of killed larva was 22 and 24 respectively, whereas other findings were same as like as results obtained after 2 hours.

Table (2): Data for PROBIT analysis

Concentration (' 000 ppm)	Tested Mosquitoes	No. of Killed Mosquitoes after 24 hrs (Average value of two replication)
0	25	0
105	25	20
110	25	23
115	25	25
120	25	25
150	25	25

Table (3) : Parameter Estimates

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	C	2.857	4.075	.701	.483	-5.129	10.843
	Intercept	-4.288	8.395	-.511	.609	-12.683	4.107

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

Table (4) Confidence Limits

	Probability	95% Confidence Limits for C			95% Confidence Limits for log(C)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	4.860	.	.	.687	.	.
	.020	6.055	.	.	.782	.	.
	.030	6.960	.	.	.843	.	.
	.040	7.730	.	.	.888	.	.
	.050	8.418	.	.	.925	.	.
	.060	9.052	.	.	.957	.	.
	.070	9.647	.	.	.984	.	.
	.080	10.213	.	.	1.009	.	.
	.090	10.756	.	.	1.032	.	.
	.100	11.281	.	.	1.052	.	.
	.150	13.745	.	.	1.138	.	.
	.200	16.082	.	.	1.206	.	.

.250	18.401	.	.	1.265	.	.
.300	20.767	.	.	1.317	.	.
.350	23.230	.	.	1.366	.	.
.400	25.837	.	.	1.412	.	.
.450	28.638	.	.	1.457	.	.
.500	31.690	.	.	1.501	.	.
.550	35.067	.	.	1.545	.	.
.600	38.868	.	.	1.590	.	.
.650	43.230	.	.	1.636	.	.
.700	48.358	.	.	1.684	.	.
.750	54.576	.	.	1.737	.	.
.800	62.445	.	.	1.795	.	.
.850	73.060	.	.	1.864	.	.
.900	89.017	.	.	1.949	.	.
.910	93.368	.	.	1.970	.	.
.920	98.335	.	.	1.993	.	.
.930	104.102	.	.	2.017	.	.
.940	110.944	.	.	2.045	.	.
.950	119.298	.	.	2.077	.	.
.960	129.920	.	.	2.114	.	.
.970	144.283	.	.	2.159	.	.
.980	165.863	.	.	2.220	.	.
.990	206.615	.	.	2.315	.	.

a. A heterogeneity factor is used.

b. Logarithm base = 10.

Discussion:

Effect of Solvent

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). 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 *Azadirechta 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 squamosal* (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)

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 arbotristis* (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)

In this study the effect of using methanol 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.

Potentiality of *Ageratum conyzoides* as an Insecticide

Ageratum conyzoides has bioactive activity that may have agricultural use, as revealed by several research investigations in different countries. 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 carried out in Colombia by Gonzalez et al. (1991) showed activity of this species against *Musca domestica* larvae, using whole plant hexane extract**.** Vyas and Mulchandani (1986) referred the action of cromenes (precocenes I and II), isolated from *Ageratum* plants, which enhance 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 immature metamorphosis, producing sterile or dying adults **.** Raja et al (1987), utilizing *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 procures morphogenetic abnormalities in the formation of mosquitoes larvae (*Culex quinquefasciatus*, *Aedes aegypt,* and *Anopheles stephensi*). This has been verified using petroleum ether extracts (5 and 10 mg/L) of the whole plants. The larvae showed 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 generated within insect body mentioned by some experiments such as Assays conducted in India found high nymphal mortality 91% of the oil to the Nymphs of *Schistocerca gregaria* **(**Okunade and Adewole, 2002). Calle et al. showed that the hexane extract of the whole plant showed activity against *Musca domestica* larvae **(**Okunade and Adewole, 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 referred 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 conducted **(**Okunade and Adewole, 2002). While the precocenes have been seen as fourth-generation insecticides, the disadvantage 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 mentioned that the toxicity was due to a highly reactive precocene-3,4-epoxide, a metabolite generated 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.

Conclusion

In conclusion, this research findings showed that leaf extract of *A. conyzoides* can be developed as environment friendly larvicide. Additionally, the results uncover the possibility for further investigations of the efficacy of larvicidal properties of natural product extracts.

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2002) **.** Methanolic extract from fresh leaves 250 and 500 ppm also generated 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

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