LARVICIDAL ACTIVITY OF *Picralima nitida,* AN ENVIRONMENTAL APPROACH IN MALARIA VECTOR CONTROL

Dibua, Uju Marie-Esther[1], Odo, Greg Ejikeme [2], Nwabor, Ozioma Forstinus[3], Ngwu, Goddy Ikechukwu[4]

[1,2] Department of Microbiology, University of Nigeria, Nsukka, Nigeria.
[3,4] Department of Zoology, University of Nigeria, Nsukka
*Corresponding author E-mail: oby2112@yahoo.co.uk; essuju@gmail.com.
Tel: +2348037792951; +2348033855848

Abstract

Insecticide resistance and high incidence of malaria prompt the search for alternative strategies for malaria vector control. This study evaluated the larvicidal efficacy of *Picralima nitida* on 3rd and early 4th instar larvae of *Anopheles gambiae.* Laboratory Reared Larvae of *Anopheles gambiae* were exposed to varying concentrations of the *Picralima nitida* extracts ranging from 0.5 to 5.0mg/ml, according to WHO Bioassay Methods for susceptibility. Probit analysis using SPSS version 16.0, at (P<0.05) gave LC_{50} and LC_{95} values of 3.141mg/ml and 42.154mg/ml, 0.352mg/ml and 4.730mg/ml and 0.164mg/ml and 2.201mg/ml for aqueous leaf extracts at 24, 48 and 72h. LC_{50} values for methanolic leaf extract were 48.383mg/ml, 15.817mg/ml and 0.333mg/ml at 24h, 48h and 72 h. Methanolic seed extract gave LC_{50} value of 0.87mg/ml, 0.21mg/ml and 0.15mg/ml at 24, 48 and 72 h and an LC_{95} value of 0.739mg/ml, 0.182mg/ml and 0.124mg/ml at same time interval. The 24 h Relative Potency estimate revealed that aqueous leaf extract had 5.479 times the potency of aqueous seed, while the methanolic seed extract had 269.763 times the potency of the methanolic leaf extracts. The potential use of the methanolic seed and aqueous leaf extracts of *P. nitida* as an eco-friendly alternative in malaria vector larviciding is demonstrated.

Keyword: Larviciding, Insecticides, Resistance, Eco-friendly, *Picralima nitida.*

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1. Introduction

Over the years, different strategies have been devised to curb disease transmission by insect vectors but these have suffered various setbacks. Development of resistance to readily available insecticides and simultaneous environmental and health problems posed by synthetic inorganic insecticides necessitated the search for environmentally safe, degradable, affordable and target-specific compounds against these insect-vectors. Mosquitoes are some of such vectors responsible for the spread of some of the world deadliest diseases. It was reported that malaria is endemic in 117 countries with some 3.2 billion people living in risk areas all over the world [1]. The report further stated that there are about 350 to 500 million clinical cases of malaria worldwide each year, with over 1 million deaths. About 59% of all clinical cases occur in Africa, 38% in Asia, and 3% in the Americas.

The Integrated Vector Management has among other strategies, larviciding as a useful approach in vector control. According to [2]. It was suggested that the best time to begin a mosquito management program is before the adult mosquitoes emerge. Control efforts should therefore begin immediately after the mosquito eggs have hatched, the breeding sites have been inspected, and the numbers of larvae present have been quantified to determine whether or not the use of an insecticide is justified. Larviciding is a preferred option in vector control because larvae occur in specific areas and can thus be more easily controlled [3].

In view of the current reliance on insecticide-based strategies for vector control and the inevitability of insecticide resistance arising if selection pressure is maintained, there is an urgent need for a cheap, eco-friendly and active alternatives with improved modes of action. The search for such compounds has been directed to the plant kingdom [4]. Many herbal products have been used as natural insecticides before the discovery of synthetic insecticides [5]. Plants have revolutionized the fields of vector control as they possess different bioactive components and can be used as general toxicants against various developmental stages of the mosquito [6].

Picralima nitida, family Apocynaceae was first described and characterized by T. H. Durand in 1909 [7]. The plant is commonly called the Akuamma plant. The plant, *Picralima nitida*, has provided drugs used in the treatment of many diseases [8]. Extracts of the plant have been used in the treatment of pathogenic diseases [9], protozoan infections [10] and non pathogenic diseases [11]. Diabetes mellitus is a major endocrine disease that is treated with the extracts of

the plant [12]. Larvicidal and antifungal properties of leaf samples *P. nitida* has been investigated and was reported to show a significant larvicidal effect on *Anopheles gambiae* [3]. This work seeks to evaluate the toxic and lethal effect of the leaf and seed samples of this plant as possible options in the search for phytoinsecticides.

2. Materials and Methods

2.1. Collection of Plant Materials

Plant samples (leaves and fruits) of *Picralima nitida* were collected from Umuagwu Akabor in Oguta Local Government Area of Imo State State, Nigeria. The plant was identified by A.O. Ozioko, a professional plant taxonomist at the Herbarium section of the International Center for Ethno-medicine and Drug Development, Nsukka

2.1.2. Sample Preparation and Extraction

The leaves of the test plant were rinsed with water to remove dirt. They were then spread out on a clean surface and allowed to air-dry under shade at room temperature (28 ± 2°C) for about seven days. The seeds were extracted from the fruit; dried leaves and fresh seeds were then pulverized using an electrical blender.

Two kilogram of each pulverized plant samples (leave, seed and pulp of *P. nitida),* were subjected to extraction using soxhlet extractor (Electrothermal heating mantle, Model Ms-9506 with pyrex soxhlet column, condenser and round bottom flask). The samples were packed into the soxhlet column to 2/3 its volume. The column was then inserted into the flask and filled with the solvents. The column was filled until the solvents began to siphon. The soxhlet was then placed on the heating mantle, and heat adjusted to 40°c. The solvents were allowed to reflux repeatedly, until refluxing solvent was clear and free from extracts. 2.5L of 95% methanol and 3.0L distilled water were used respectively. The extracted content was then subjected to rotary evaporator (Bibby Sterlin Ltd, England, RE. 200) until solvents were completely evaporated to get the solidified crude extracts. The crude extracts thus obtained was stored in sterilized amber coloured bottles and maintained at 4°c in a refrigerator.

The percentage yields of the extracts were determined as:

$$\% \text{ Yield} = \frac{\text{Weight of dry extract}}{\text{Weight of sample extracted}} \times \frac{100}{1}$$

A 5000mg each of the methanolic and aqueous extracts were solublized in 5ml of 20% dimethylsulphoxide (DMSO). The extracts were then diluted in 1000ml of distilled water to obtain a stock solution of 5.00mg/ml. From the stock, graded concentrations of 4.00mg/ml, 3.00mg/ml, 2.00mg/ml, 1.00mg/ml and 0.50mg/ml were then obtained.

2.2. Identification and Rearing of Mosquito Larva

Larvae of *A. gambiae* used in this investigation were collected from small water depressions on the soil and also from concrete ponds and transported to the laboratory in plastic containers. In the laboratory, the larvae were transferred to enamel larval trays until adult emergence. After emergence, the mosquitoes were identified by a professional Parasitologist, of the Department of Zoology, University of Nigeria, Nsukka. Cyclic generations of *A. gambiae* were maintained in a 29 cm x 21.5 cm x 56.5 cm cages with potted plants. Mean room temperature of (27± 2^0C) and a relative humidity of 70-80 percent were maintained in the insectary. The adult mosquitoes were fed on ten per cent glucose solution. For continuous maintenance of mosquito colony, the adult female mosquitoes were blood fed with laboratory reared albino rats. Ovitraps were placed inside the cages for egg laying. The eggs laid were then transferred to enamel larval trays maintained in the larval rearing chamber. The larvae were fed with larval food (Quaker oat and yeast in the ratio 3:1). 3rd and 4th instar larvae were then picked for larvicidal bioassay.

2.2.3. Larvicidal Bioassay

Larvicidal bioassay of individual plant extracts was tested against 3rd and 4th instar larvae of *A. gambiae.* The tests were conducted in glass beakers, in accordance with [1] protocol with slight modification. Three replicates and a control were run simultaneously during each trial. For control, 5ml of 20% DMSO in 995ml of distilled water was used. Twenty healthy larvae were released in each glass beaker and mortality was observed at 24, 48 and 72 hrs after treatment with extract concentrations of 5.00, 4.00, 3.00, 2.00, 1.00, and 0.5mg/ml. The treatments were maintained at room temperature. Larvicidal activity of each extract was determined, by counting the number of dead larvae on daily basis (24hrs interval). The moribund and dead larvae in the

three replicates were combined and expressed as percentage mortality for each concentration. Dead larvae were recorded when they failed to move after probing with a needle. Moribund larvae were those unable of rising to the surface within reasonable period of time. The percentage mortality was calculated and analysis of data was carried out by employing probit analysis.

$$\% \text{ Mortality} = \frac{\text{Number of Dead Larvae}}{\text{Number of Larvae Introduced}} \times \frac{100}{1}$$

Correction for Control Mortality

The Corrected percentage mortality was used were a proportion of the insects in the control batches died during the experiment.

To correct for this the Abbott formula was used [13].

$$P = \frac{\% \text{ Po} - \% \text{ Pc}}{100 - \% \text{ Pc}} \times 100$$

Where P is the corrected mortality, Po is the observed mortality and Pc is the control Mortality, all expressed in percentages.

2.3. Phytochemical Screening

Crude methanolic and aqueous extracts of the leave and seed of *P. nitida* were screened for their phytochemical components using the methods described by [14,15].

Test for Alkaloids

About 20ml of 5% sulphuric acid in 50% ethanol was added to about 2g of each of the sample. This was then heated on a boiling water bath for 10 minutes, cooled and filtered. The filtrate was transferred into four test tubes, each containing 2ml of the filtrate and used for the following tests.

a. Few drops of Dragendoff's reagent (a solution of bismuth iodide in potassium iodide) were added to first portion of the filtrate, and homogenized. A brick red precipitate indicated the presence of alkaloids.

b. About two drops of Wagner's reagents (a solution of iodine in potassium iodide) were added to the second portion and swirled for few seconds. A brownish-red precipitate indicated the presence of alkaloids.

c. About two drop's of Meyer's reagent (a solution of mercury iodide in potassium iodide) were added to the third portion and homogenized for few seconds. A creamy, dirty white precipitate indicated the presence of alkaloids.

d. Two drops of picric acid (1%) solution was added to the fourth portion and homogenized for 30 seconds. A reddish precipitate indicated the presence of alkaloids.

Test for Saponins

About 20ml of distilled water was added to 0.5g of each of the sample in a 100cm^3 beaker and boiled gently on a hot water bath for 20 minutes. The mixture was filtered hot and allowed to cool. The filtrate was used for the following test.

a. Frothing Test: A volume of 20ml distilled water was added to 5ml of the filtrate in a test tube, and shaken vigorously. A stable froth (foam) upon standing for about 30 seconds indicates the presence of saponin.

b. Emulsion Test: Two drops of olive oil was added to 5ml of the filtrate in the test tube above. Formation of emulsion indicates the presence of saponin.

Test for Phenols/Tannins

About 100g of each of the sample was extracted in 10ml of distilled water. The solution was heated in a boiling water bath for 3 minutes and filtered. Then 2ml aliquots of the filtrates were placed in test tube and the following tests were performed.

a. Few drops of 10% neutral aqueous $FeCl_3$ was added to the aliquot of the diluted solution. Development of green to blue-black precipitates indicates the presence of tannins.

b. A volume of 1ml of 10% lead acetate solution was added to a portion of the filtrate in a test tube, and homogenized. A coloured precipitate indicates the presence of Phenols.

Test for Glycosides

About 300mg of each of the samples was dissolved in 10ml of distilled water and the resulting solution was filtered. 5ml of equi-volume mixture of Fehling's solution 1 and11 was added to a 2ml aliquot of the aqueous solution obtained above. The mixture was then homogenized and heated in a water bath for not less than 5 minutes. Brick red precipitates indicates the presence of free reducing sugars.

Test for Flavonoids

About 10ml of ethyl acetate was added to about 0.2g of each of the sample and heated on a water bath at 40°C for 3 minutes. The mixture was cooled, filtered and used for the following test.

- Ammonium Test: About 4ml of the filtrate was shaken with 1ml of dilute ammonia solution. Layers were formed and allowed to separate. An intense yellow colour in the ammoniacal layers indicates the presence of flavonoids.
- To the yellow coloured solution, 3 drops of concentrated sulphuric acid was added. Disappearance of the yellow colour indicates the presence of flavonoids.
- 1% Ammonium Chloride Solution Test: To 4ml of the filtrate, 1ml of 1% ammonium chloride solution was added. A yellow colour indicates the presence of flavonoids.

Test for Steroids and Terpenoids

About 5ml of 96% ethanol was added to 1.0g of each of the samples and refluxed for 2 minutes and filtered. The filtrate was concentrated to 2.5ml on a water-bath and 5ml hot water added. The mixture was allowed to stand for 1 hr. Waxy matter observed was filtered off. The filtrate was extracted with 2.5ml chloroform. The layers observed were separated using separating funnel.
A volume of 1ml of concentrated H_2SO_4 was carefully added to 0.5ml chloroform extract and shaken to form lower layers. Reddish Brown interface shows the presence of steroids.

Another 0.5ml of the chloroform extract was evapourated to dryness on a water bath at 40°C. This was heated further with 3ml concentrated H_2SO_4 for 10 minutes on a water bath at 40°C. Grey colouration indicated the presence of terpenoids.

2.4. Statistical Analysis

The Median Lethal Concentration values (LC_{50} and LC_{95}), Median Lethal Time (LT50) and Relative Potency were estimated using probit analysis as described by [16]. SPSS version 16.0

3. Results

3.1. Percentage Yield of Extracts

The percentage yield of the samples is elucidated in Table 1. The leaf extract showed a greater yield for both aqueous and methanol. However there was no significant difference in yield whereas the methanolic seed sample showed a relatively higher yield compared to the aqueous seed extract.

Table 1: % Yield of Samples

Samples	Methanolic	Aqueous
Leaf	21.65	21.37
Seed	8.33	3.85

3.2. Phytochemistry

The phytochemicals constitutes of crude methanolic and aqueous extracts of *P. nitida* leaf and seed are here presented. The presence of alkaloids, cardiac glycosides, saponins, tannins, flavonoids, terpenes and steroids was observed.

3.3. Lethality/Mortality Assay

The mortality assay, describing the lethality pattern of the aqueous leaf extract of *P. nitida* on the test *Anopheline* Larva is presented in Figure 1. At extract concentration of 5.00mg/ml and 24hs exposure time, the test organism exhibited a mortality profile of 50%. However 100% mortality could only be achieved by extract concentration of 4.00mg/ml at 72 h of exposure

Table 2: Phytochemical Results of *P. nitida* samples

Samples	Alkaloids	Cardiac Glycosides	Flavonoid	Saponin	Tannins	Terpenes	Steroids
Methanolic Leaf Extract	+	+	-	-	+	+	-
Methanolic Seed Extract	+	+	+	+	+	+	+
Aqueous Leaf Extract	+	+	-	+	+	+	-
Aqueous Seed Extract	+	+	+	+	+	+	+

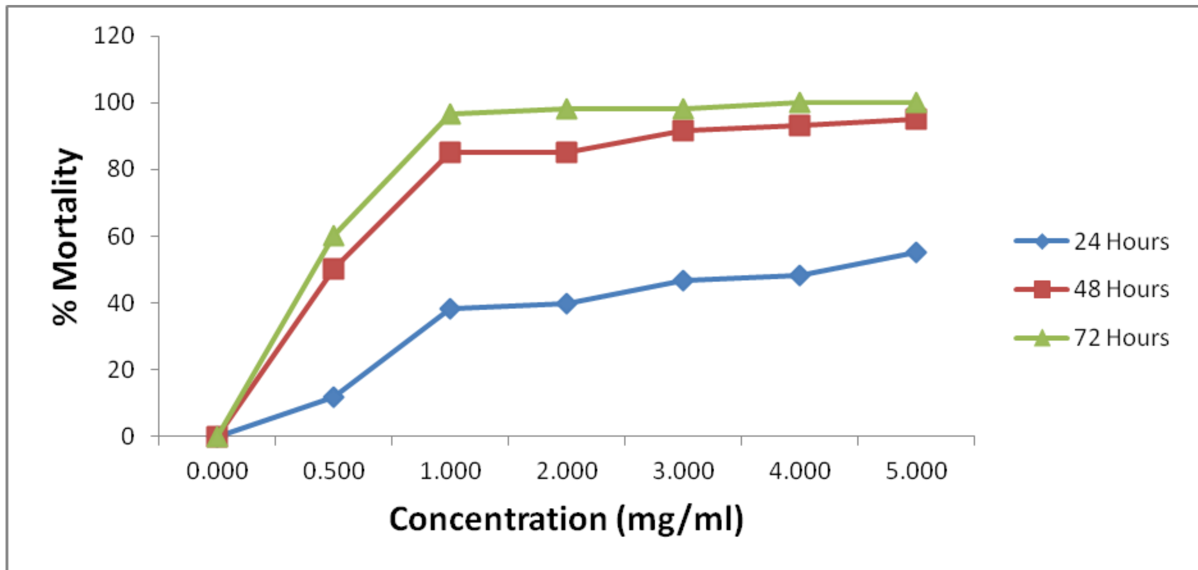

Figure 1: Mortality of *A. gambiae* to aqueous leaf extract of *P. nitida.*

Result of the lethality assay of the methanolic leaf extract of *P. nitida* on *Anopheline* larva indicated the inability of the methanolic leaf extract to achieve an appreciable level of larvicidal activity (50% lethality of test organism) at lower concentrations ranging from 0.5mg/ml to 5.00mg/ml at 24 and 48h time intervals. However at 72, 95% mortality was exhibited at a concentration of 3.00mg/ml (Figure 2).

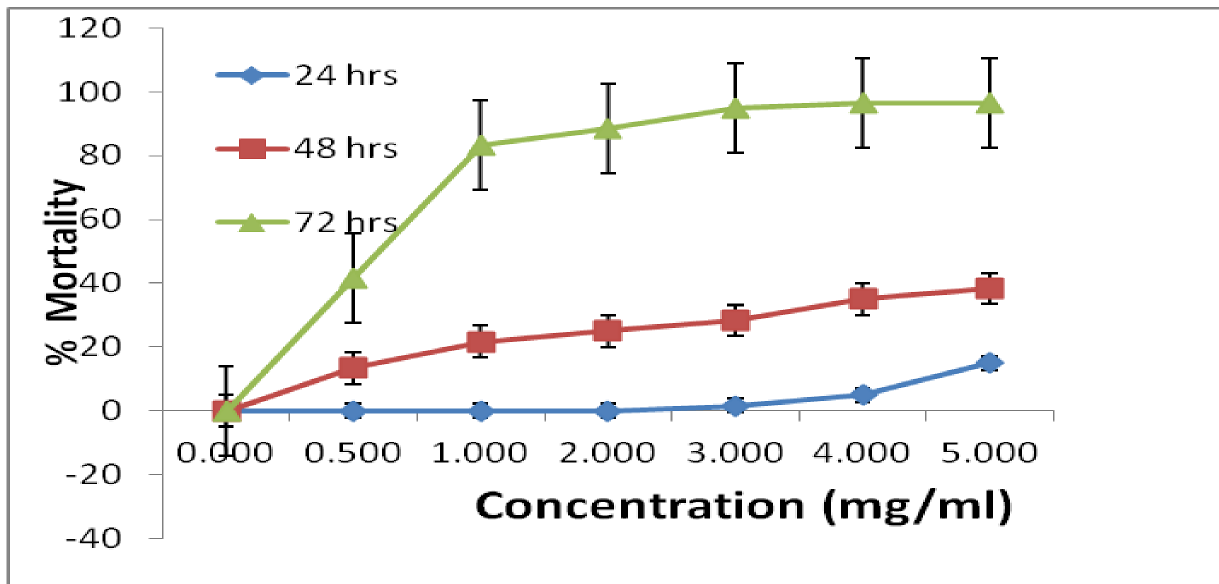

Figure 2: **Dose-Dependent Mortality Profile of *A. gambiae* to methanolic leaf extract of *P. nitida*.**

Analysis of the aqueous seed extract of *P. nitida* on *Anopheline* indicated a minimal level of larvicidal activity at varying concentrations of extract and at different time intervals (Figure 3).

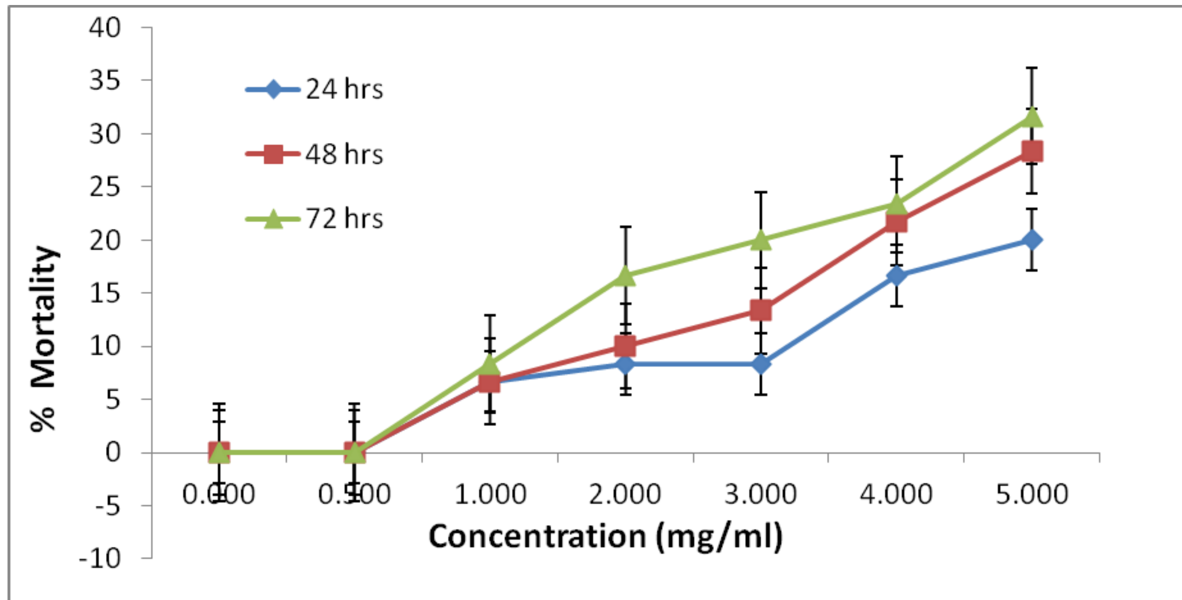

Figure 3: **Mortality Profile of *A. gambiae* to aqueous seed extract of *P. nitida.***

Comparatively, the methanolic seed extract of *P. nitida* however showed an excellent larvicidal activity against the test organism: 100% lethality was observed at concentrations of 3.00mg/ml, 1.00mg/ml and 0.5mg/ml at 24 h, 48 h and 72 h respectively as elucidated in Figure 4

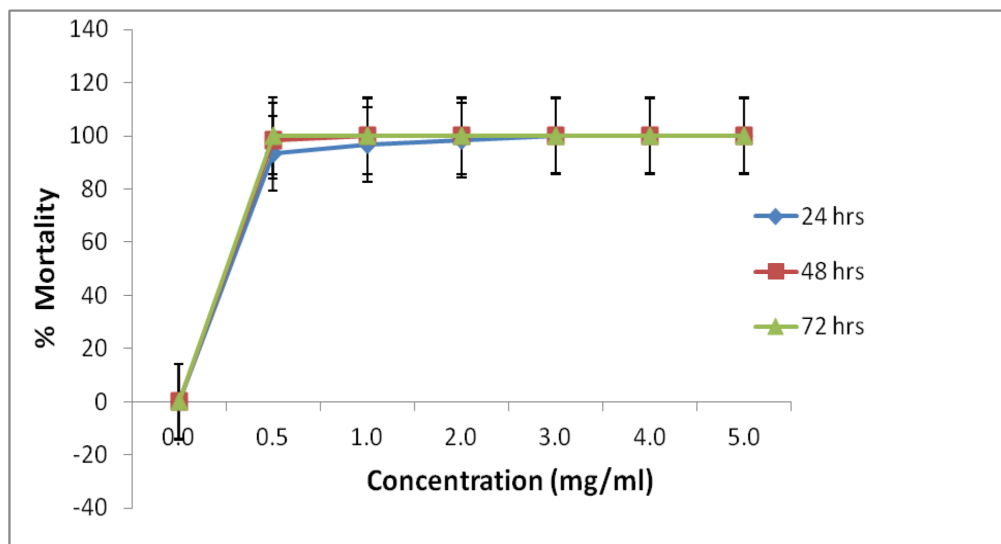

Figure 4: Dose Dependent Mortality Profile of *A. gambiae* to methanolic seed extract of *P. nitida.*

3.4. Relative Median Potency

The Relative Median Potency of the various test extracts at different time intervals is presented in Tables 3 to 8. The methanolic seed extract was observed to exhibit a higher level of potency than the aqueous seed extract at the varying exposure time intervals. Nevertheless, a more significant level of potency was observed with the aqueous leaf extract than its methanolic counterpart.

Table 3: Relative Median Potency Estimates of Methanolic Extracts at 72h

Extracts	Relative Median Potency at 95% C.L			LOG Transform Relative Median Potency at 95% C.L		
	Potency	Lower Bound	Upper Bound	Log Potency	Lower Bound	Upper Bound
ML MS	25.302	2.977	364.351	1.403	0.474	2.562
MS ML	0.040	0.003	0.336	-1.403	-2.562	-0.474

Key: ML - .Methanolic Leaf; MS -Methanolic Seed

Table 4: Relative Median Potency Estimates of Methanolic Extracts at 48h

Extracts	Relative Median Potency at 95% C.L			LOG Transform Relative Median Potency at 95% C.L		
	Potency	Lower Bound	Upper Bound	Log Potency	Lower Bound	Upper Bound
ML MS	3.553E3	71.978	4.007E9	3.551	1-857	9.603
MS ML	0.000	2.496E-10	0.014	-3.551	-9.603	-1.857

Table 5: Relative Median Potency Estimates of Methanolic Extracts at 24 h

Extracts	Relative Median Potency at 95% C.L			LOG Transform Relative Median Potency at 95% C.L		
	Potency	Lower Bound	Upper Bound	Log Potency	Lower Bound	Upper Bound
ML MS	269.763	16.390	2.247E7	2.431	1.215	7.352
MS ML	0.004	4.450E-8	0.061	-2.431	-7.352	-1.215

Table 6: Relative Median Potency Estimates of Aqueous Extracts at 72h

Extracts	Relative Median Potency at 95% C.L			LOG Transform Relative Median Potency at 95% C.L		
	Potency	Lower Bound	Upper Bound	Log Potency	Lower Bound	Upper Bound
AL AS	0.044	0.003	0.165	-1.352	-2.471	-0.782
AS AL	22.515	6.052	295.533	1.352	0.782	2.471

AL, = Aqueous Leaf, AS, = Aqueous Seed

Table 7: Relative Median Potency Estimates for 48 h (Aqueous Extracts)

Extracts	Relative Median Potency at 95% C.L			LOG Transform Relative Median Potency at 95% C.L		
	Potency	Lower Bound	Upper Bound	Log Potency	Lower Bound	Upper Bound
AL AS	0.053	0.012	0.135	-1.276	-1.925	-0.871
AS AL	18.901	7.429	84.123	1.276	0.871	1.925

Table 8: Relative Median Potency Estimates of Aqueous Extracts at 24 h

Extracts	Relative Median Potency at 95% C.L			LOG Transform Relative Median Potency at 95% C.L		
	Potency	Lower Bound	Upper Bound	Log Potency	Lower Bound	Upper Bound
AL AS	0.183	0.049	0.393	-0.739	-1.312	-0.406
AS AL	5.479	2.546	20.501	0.739	0.406	1.312

3.5. Lethal Concentration (LC_{50} and LC_{95}) assay

Available results of the LC_{50} and LC_{95} assay of the various extracts at different time intervals indicated that the methanolic seed extract has the lowest median lethal concentration at 24, 48 and 72 h respectively. On the other hand, the aqueous seed extract was observed to exhibit a higher activity potential (LC_{50} value of 17.668), compared to the methanolic leaf extract (LC_{50} value of 48.383). t However, the methanolic leaf extract exhibited a significantly higher activity than the aqueous seed extract with LC_{50} values of 5.817 and 0.333 at 48 and 72 h as against 13.461 and 10.557 for aqueous leaf extract (Table 9).

Table 9: Time Dependent Median Lethal Concentrations of the Various Extracts

SAMPLES	24 hrs Lethal Conc. (mg/ml)		48 hrs Lethal Conc. (mg/ml)		72 hrs Lethal Conc. (mg/ml)	
	LC_{50}	LC_{95}	LC_{50}	LC_{95}	LC_{50}	LC_{95}
Methanolic Seed	0.087	0.739	0.021	0.182	0.015	0.124
Aqueous Seed	17.668	216.064	13.461	164.614	10.557	129.102
Methanolic Leaf	48.383	657.786	5.817	79.089	0.333	4.523
Aqueous Leaf	3.141	42.154	0.352	4.730	0.164	2.201

The summary of the overall larvicidal efficacy of the test extracts elucidated below demonstrated the outstanding activity of the methanolic seed extract on test organism (Figure 5). The methanolic seed extract was observed to achieve a significant larvicidal activity on test larvae following exposure to a concentration of 0.5mg/ml. However, no appreciable effect was observed from the aqueous seed and methanolic leaf at same concentration.

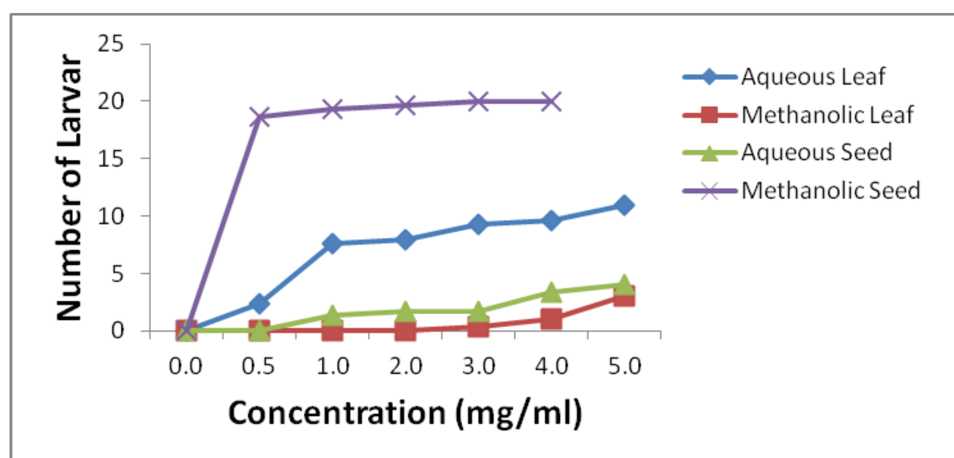

Figure 5: 24hrs larvicidal Activity of Extracts.

4. Discussion

Larviciding as a subset of the Integrated Mosquito Management (IMM), Integrated Vector Management (IVM) and Integrated Pest Management (IPM) programs is gaining grounds with researchers who reported that various phytochemicals are potent adulticidal and larvicidal agents. The use of conventional pesticides in the water sources, however, introduces many risks to people and/or the environment and due to the continuous increase in resistance of mosquitoes to familiar synthetic insecticides, better alternative means are therefore sought [17]. Plant extracts are potential sources of novel insecticidal compounds. Application of larvicide from botanical origin was extensively studied as an essential part of IMM, and various mosquito control agents such as ocimenone, rotenone, capllin, quassin, thymol, eugenol, neolignans, arborine and goniothalamin were developed [18]. Plants with good insecticidal values were previously reported [19], while the plant species that only exhibited mosquitocidal activity were indicated [20]. In line with the ongoing search for efficient, eco-friendly compounds which might serve as suitable alternatives to the present day synthetic insecticides, this work evaluated the larvicidal potentials of crude methanolic and aqueous extracts of the Leaf and Seed of the plant *Picralima nitida* on the malaria vector *Anopheles gambiae.* The plant extracts were found to be a good potential against the larvae of the test malaria vector. This report is in consonance

with previous studies which demonstrated the efficacy of the crude ethanolic and aqueous extracts of the plant leaves [3]; however, there is hitherto no study on the seed extracts. We have reported the presence of important phytochemical and/or bioactive compounds: alkaloids, cardiac glycosides, saponins, tannins, flavonoids, terpenes and steroids in the test plant extracts, as was also observed in previous studies [21, 8]. These compound were similarly reported as vital elements of the bio-activity potential of the plant [22, 23, 24], which thereby confirms the efficacy potential reported in the present study. Nevertheless, all test extracts exhibited varying levels of toxicity on the larvae, with 100% mortality exhibited by the methanolic seed extract at a concentration of 3.0mg/ml following 24 h exposure; thus demonstrating its potential for use as a possible alternative in the mosquito larvae control.

The aqueous extract was less potent than the methanol extract. The observed low activity of the aqueous seed could be attributed to the inability of the aqueous solution to extract more of the bioactive compounds (given its high oil content) which were readily available using the methanol which provided a more complete extraction, including less polar compounds, many of which possess larvicidal properties. It has also been reported that the geographical origin and/or location might affect the larvicidal and general bioactivity of a plant species [25,26,27]. This view might explain the reported larvicidal activity of the ethanolic and aqueous extracts of the leaf of *P. nitida* against *Anopheles gambiae* larvae [3].

The extracts showed a relatively low LC_{50} values at 24 hours, suggesting their highly efficacious activity against the test organism. However, their LC_{50} values decreased with increase in the time of exposure, showing a better activity at 72 h trial. Relative potency estimate demonstrated by the extracts was observed to be both concentration and time dependent: at 72 h, the potency of Methanolic seed was 25.302 times that of the methanolic leaf extract; aqueous leaf extract showed a potency of 25.515, 18.901 and 5.479 times that of the aqueous seed at 24, 48 and 72 h respectively. Our findings on the degree of relative potency exhibited by the extracts lend credence to the traditional and/or folkloric uses of the extract for the control of mosquito, and further buttress their use as an eco-friendly alternative to synthetic insecticides in the elimination of the deadly malaria vector.

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