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Azadirachta indica LEAF AND SEED EXTRACTS AS POTENTIAL LARVICIDES OF *Anopheles gambiae* FROM NORTHWEST, NIGERIA

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ABSTRACT
Malaria has been a major problem accounting for thousands of death in Nigeria. It is spread by female Anopheles mosquito which injects the Plasmodium parasite into the human system. Chemical control has been the mainstay in control of mosquitoes for decades which have proven to be toxic to humans, animals and the environment in addition to widespread resistance. This study investigated the potential of phytochemicals (Azadirachta indica leaf and seed extracts) in the control of mosquito larvae. The plant leaves were extracted using solvents of varying polarities starting from least polar to most polar (hexane, chloroform, butanol, ethanol, methanol/aqueous) respectively while the seed was extracted using hexane. Varying concentrations (5000ppm, 6000ppm, 7000ppm, 8000ppm, 9000ppm and 10,000ppm) were administered to 20 larvae each, water/acetone were used as control. The result of phytochemicals screening revealed the presence of various compounds of which tritepenes were only detected in butanol extract. Mortality was recorded after 24 hours. The result showed no significant difference (P>0.05) when hexane, chloroform, ethanol, methanol/aqueous leaf extracts and hexane seed extracts were compared with control while there was significant difference (P<0.05) when butanol leaf extract was compared with control. There was significant decrease (P<0.05) in the level of glucose estimated in the susceptible larvae when compared with resistant and control larvae. The result of DNA estimation showed no significant difference (P>0.05) between all comparisons. In conclusion, butanolic extract of Azadirachta indica leaf displayed a promising larvicidal properties which might be due to the presence of triterpenes which may have interference with the normal metabolic processes and could be explored for possible novel bio-insecticide for the control of mosquitoes.
Keywords: Anopheles gambiae, Azadirachta indica

INTRODUCTION

Malaria continues to be a primary cause of morbidity and mortality in Nigeria (Morakinyo *et al.*, 2018; WHO, 2018). In year 2017, an estimated 219 million cases of malaria occurred worldwide (WHO, 2018). About 92% of these cases occurred in the World Health Organization (WHO) African Region where Nigeria accounted for 25% of the global burden. Children under the age of five are the most vulnerable with a child dying every 2 min from malaria infection (WHO, 2018).

Mosquitoes are important vectors of several diseases (An *et al.*, 2020). *Anopheles gambiae* Giles commonly referred to as the African malaria mosquito, is the most common vector of human malaria in the Afro tropical Region (CDC, 2010). *Anopheles gambiae* are recognized malaria vectors due to their inclination to humans as a host, proneness to the *Plasmodium* parasite, and their indoor-feeding pattern (CDC, 2010).

In recent years, the search for newer products and alternatives for mosquito control that are environmentally safe, target-specific and easily degradable is on the rise (Ohia and Ana, 2015). Botanical metabolites are increasingly been realised as potential substitute for chemical insecticides (Vivekanandhan *et al.*, 2018). Quite a number of plant products have been identified to be effective, eco-friendly, biodegradable, user-friendly, inexpensive and pose little or no risk to human and environmental health (Azizullah *et al.*, 2014; Benelli *et al.*, 2015b; Murugan *et al.*, 2015). These plant products have previously been used as insecticides for controlling larvae, adult mosquitoes or as repellents for reducing human-mosquito contact through biting (Prabhu *et al.*, 2011).The plants offer an advantage over synthetic insecticides as they are less toxic, less prone to develop resistance and easily biodegradable (Imam *et al.*, 2018).

Azadirachta indica belong to the family of Meliaceae plants which contain so many varieties of compound that shows insecticidal, antifeedant, growth-regulating, and development-modifying properties (Nakatani *et al.*, 2004). *Meliaazedarach L.* and *Azadirachtin indica* (Sapindales: Meliaceae), commonly known as Chinaberry or Persian lilac tree, are deciduous trees that are native to north western India; and have long been recognized for their insecticidal properties.

These trees grow typically in tropical and subtropical parts of Asia, but nowadays they are also cultivated in other warm regions of the world because of their considerable climatic tolerance. *Azadirachta indica* elicit a variety of effects in insects such as anti feedant, growth retardation, reduced fecundity, moulting disorders, morphogenetic defects, and changes of behaviour (Wandscheer *et al*., 2004). Larvae of Anopheles mosquitoes showed high mortality rate when exposed to undiluted extract of seed oil, leaf and bark of Azadirachtin (neem) (Adobu *et al*.,2018).

The incessant and haphazard use of conventional insecticides for the control of mosquito vectors has led to recent increase in the development of resistance and negative impacts on non-target organisms and the environment. Therefore, there is a need for development of biological effective mosquito control tools (Govindarajan *et al*., 2016). With Nigeria being one of the 10 countries with the highest burden of malaria in the year 2017 (WHO, 2018), the development and adoption of alternative methods of integrated vector management remain the key. However, there is a dearth of information on the bio-insecticidal effects of the seed oil of *Azadirachta indicia* on *Anopheles gambiae* in Nigeria. This study is determined to test the repellent and larvicidal potential of emulsified *Azadirachta indica* leaf and seed extract as a suitable alternative for commercially available insecticides against *Anopheles gambiae* in Nigeria.

MATERIALS AND METHODS

Collection of Plant Materials

Fresh leaves and seeds of *A. indica* were collected on 20th of January, 2017 from the botanical garden of Bayero University, Kano with the assistance of Herbarium keeper. The leaves were authenticated by the Chief Technologist at the Department of Plant Biology, Bayero University Kano, (Herbarium number 242). The leaves and seeds were dried under room temperature and then ground using pestle and mortar to a semi powdered form.

Preparation of the Extracts

Extracts were prepared using solvents of varying polarities (n- hexane, chloroform, butanol, ethanol and methanol/aqueous). Powdered leaves (200g) were extracted in 500ml of the respective solvents using a Soxhlet apparatus by partitioning and freeze dried to yield the crude extracts of A. indica. The extracts were collected in plastic containers and stored at room temperature for further studies. One gram of each residue was dissolved in 100ml of acetone to make a 1% stock solution. Six different concentrations of the extracts (5000, 6000, 7000, 8000, 9000 and 10000ppm) were prepared through serial dilution from the stock solution for subsequent larvicidal assay.

A. indica seed powder (100g) was extracted in 250ml of hexane using a soxhlet apparatus and the extract was allowed to evaporate to yield the oil. The extracts were collected in plastic vials and stored at room temperature for further studies. One gram of the residue was dissolved in 100 ml of acetone to make a 1% stock solution. Six different concentration of the extract (5000, 6000, 7000, 8000, 9000 and 10000ppm) were prepared through serial dilution from the stock solution for subsequent larvicidal assay.

Collection of Larvae

Mosquito larvae of Anopheles gambiae were collected from breeding sites in Auyo Local Government Area, Jigawa State. Late 3rd and early 4th instar larvae were used to screen for the larvicidal activity of the extracts of the leaf and seed. Morphological keys of Gillies and Dermillier (1988) were used for morphological identification and observed at fluctuating temperature of 25-33ºC and relative humidity of 90-95%.

METHODS

Phytochemical Screening

Determination of saponins by Earl (1961) flavanoids, tannins, alkaloids and triterpenes by Sofowara (1993) cardiac glycosides by Keller-Killani test, terpenoids by salkowki's test, phenols by Ferric chlorides test, Steroids by Lieberman Burchard's test and anthraquinones by Felgis, (1975).

Experimental Design

A total of 2,880 larvae were taken and grouped into 6 with each group containing 480 larvae. Each subgroup contained 20 larvae in six replicates, making a total of 100 in each subgroup. 1ml of the extract was mixed in 99ml of dechlorinated water and then 20 larvae were then added to the mixture for each of the solvent and for every concentration. Larvicidal activity (mortality) was observed after 24 hours, LC_{50} and LC_{90} were computed and biochemical parameters (Nucleic acids and Carbohydrates) were assayed.

GROUPS	SOLVENTS						
	Hexane	Chloroform	Butanol	Ethanol	Methanol /Aqueous	Hexane (Seed)	Control
Group A: 5,000 ppm	A1	A2	A3	A4	A5	A6	A7
Group B: 6,000 ppm	B1	B2	B3	B4	B5	B6	B7
Group C: 7,000 ppm	C1	C2	C3	C4	C5	C6	C7
Group D: 8,000 ppm	D1	D2	D3	D4	D5	D6	D7
Group E: 9,000 ppm	E1	E2	E3	E4	E5	E6	E7
Group F: 10,000 ppm	F1	F2	F3	F4	F5	F6	F7

Larvicidal Bioassay

Using the WHO 2005 guidelines for laboratory and field testing with some modifications by the method of Rahuman *et al.* (2007) for the larvicidal activity, 20 larvae were collected in small containers with 99ml water, to which 1.0 ml of the desired plant fraction concentration was added. The control was set up with water and acetone. Experiments were carried out with a series of concentrations ranging from 5000ppm to 10000ppm.

The numbers of dead larvae were counted after 24 hours of exposure and the percentage of Mortality was recorded from the average of three replicates. LC50 and LC90 values were `calculated.

$$\%Mortality = \frac{\%\ Test\ Mortality\ -\ \%\ Control\ Mortality}{100\ -\ \%\ Control\ Mortality} \times 100$$

Biochemical Parameters

After treatment with plant extracts, the larvae were taken for further evaluation: glucose (Dubios *et al.*, 1986) and nucleic acid estimation (DNA) by Burton *et al.*, (1956).

DNA Isolation:

DNA was isolated using DNA isolation kit (Accu Biomed Co. LTD, Taiwan) following manufacturer's instruction. Proteinase k (20µl) was added to a clean 1.5ml tube and 200µl of the sample was applied to the tube containing proteinase k. 200µl of binding buffer (GC) was then added to the sample and immediately vortexed, the mixture was then incubated at 60^0C for 10mins.

100µl of isopropanol was added and well mixed by pipetting followed by brief spinning. The lysate was then carefully transferred into the upper reservoir of the binding column tube without wetting the rim. Then the tube was closed and centrifuged at 8000rpm for 1min.

The tube was opened and the binding column tube was transferred to a new 2ml tube for filtration and 500µl of washing buffer was added without wetting the rim, the tube was closed and centrifuged at 8000rpm for 1min. The tube was then opened and the solution was poured from the 2ml tube into a disposal bottle, then 500µl of washing buffer 2 was added carefully without wetting the rim, the tube was closed and centrifuged at 8000rpm for 1min.

It was centrifuged once more at 12,000rpm for 1min to completely remove ethanol; it was ensured that there was no droplet clinging to the bottom of the binding column tube. The binding column tube was then transferred to a new 1.5ml tube for elution and 200µl of elution buffer was added onto the binding column tube and waited for 1min at 25^0C until the elution buffer is completely absorbed into the glass fiber of the binding column tube. It was then centrifuged at 8,000rpm for 1min to elute genomic DNA. The eluted genomic DNA was then stored at 4^0C.

DNA Estimation

Working standard (0.5-2.5ml) solution was pipetted into 5 test tubes labelled as s1-s5 with concentration ranging from 50-250µg and 1ml and 2ml of unknown solution was pipetted into two test tubes u1 and u2. The volume in all test tubes was then made up to 3ml with distilled water and 3ml of distilled water alone served as blank. 4ml of diphenylamine reagent was added to all test tubes. The tubes were kept in a boiling water bath at 36^oC for 20mins. They were then allowed to cool and the bluish colour developed and read at 595nm.

A standard curve was obtained with concentration of DNA on x-axis and absorption on y-axis. From the graph, the amount of DNA present in the unknown solution was calculated (50mg Synthetic DNA was dissolved in 50ml Saline Sodium Citrate buffer which was subsequently diluted in 50ml distilled water to obtain the working standard. Concentration 100mg/dl)

Glucose Estimation

Sugar solution (2mg) was pipetted into a colorimetric tube and 0.05ml of 80% phenol added. Then 5ml of concentrated sulphuric acid was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10mins, shaken and placed for 10 to 20 mins in water bath at 30^oC. The absorbance of the characteristic yellow-orange colour is measured at 490nm. Blanks were prepared by substituting distilled water for the sugar solution. The amount of sugar determined by reference to a standard curve (D-glucose was used as standard with concentration range of 0-120mg/dl).

Statistical Analysis

For the larvicidal bioassay, the percentage mortalities were corrected using Abbott's formula and the average larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90}, 95% confidence limits and one-way analysis of variance (1-Way ANOVA), descriptive statistics were summarized using mean and standard error of mean (SEM) by using the SPSS software (version 20). The statistical analysis of biochemical data was done using GraphPad Instat3 Software. $P < 0.05$ was considered significant.

RESULTS

The results of phytochemical screening of *A. indica* leaves is presented in Table 1 below which indicated the presence of triterpenes, saponins, flavonoids, tannins, steroids, phenols, terpenes, alkaloids, anthraquinones, amino acids and glycoside while quinone was found to be absent. Triterpenes and amino acids were found to be present in butanol and methanolic/aqueous extract respectively. Terpenes, steroid and glycoside were found to be present in all the 4 extracts. Whereas tannins, phenol and anthraquinones were detected in ethanol, butanol and methanolic/aqueous extracts. Alkaloids was present in butanol and methanolic/aqueous extracts while flavonoids were detected in ethanol and methanolic/aqueous extracts.

Table 1: Phytochemical Constituents of *A.indica* Leave Solvents Extract

Phytochemicals	Methanol&H_20	n-Butanol	Ethanol	Chloroform	n-Hexane
Triterpenes	-	+	-	-	-
Tannins	+	+	+	-	-
Phenols	+	+	+	-	-
Flavanoids	+	-	+	-	-
Saponins	+	-	-	-	-
Terpenoids	+	+	+	-	+
Steroids	+	+	+	-	+
Anthraquinones	+	+	+	-	-
Quinones	-	-	-	-	-
Amino acids	+	-	-	-	-
Glycoside	+	+	+	-	+
Alkaloid	+	+	-	-	-

Key: +=Present, -=Absent

Larvicidal Activity

Larvicidal activity of *A.indica* leave and seed solvents extract is presented in Figure 1 below. Significant (P<0.05) larvicidal activity was observed in group treated with butanol extract when compared with the other solvents. The LC_{50} and LC_{90} of the larvicidal activity are presented in Figure 2 and 3 below.

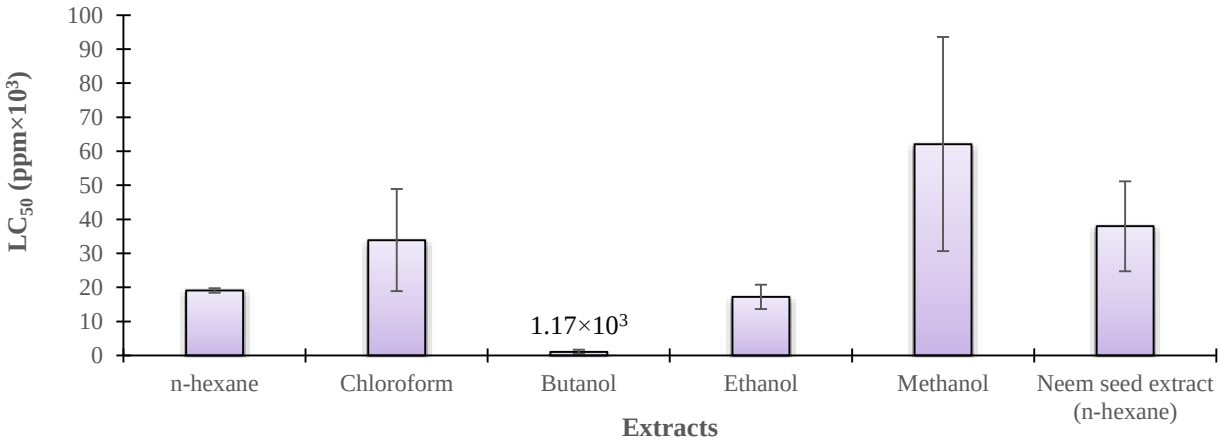

Figure 2: LC_{50} of the different extracts of *A.indica* leaf and seed against larvae of Anopheles mosquito

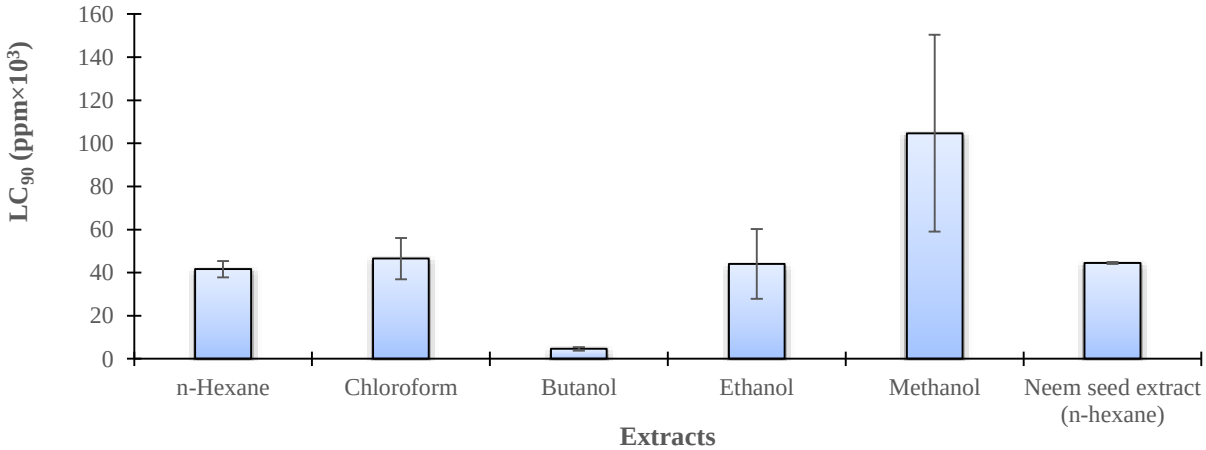

Figure 3: LC_{90} of the different extracts of *A.indica* leaf and seed against larvae of Anopheles mosquito

Biochemical Parameters

Glucose and DNA Estimation

The results of glucose estimation between susceptible and resistant larvae is shown in Figure 4 below. There was significant decrease (P<0.05) in the glucose level of the susceptible larvae when compared with the resistant and control larvae. The result of DNA estimation revealed no significant difference (P>0.05) between the susceptible, resistant and control larvae as shown in Figure 5.

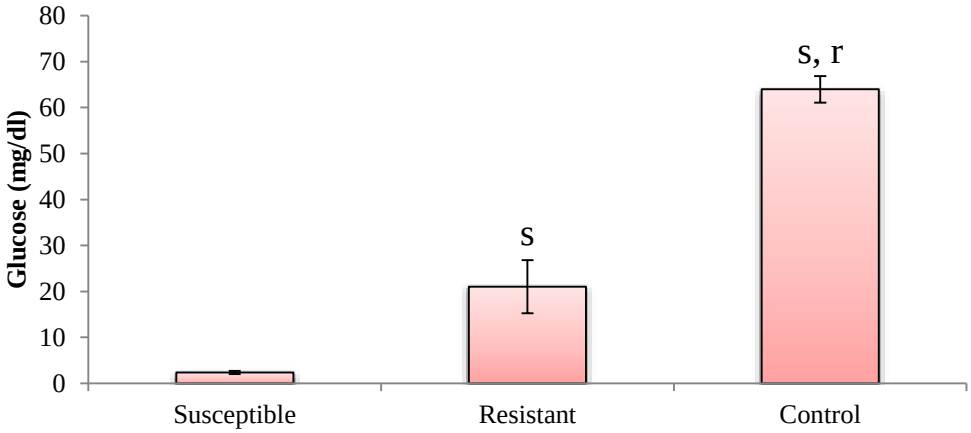

Figure 4: Effect of butanol extract of *A. indica* plant on the glucose content of Anopheles mosquito

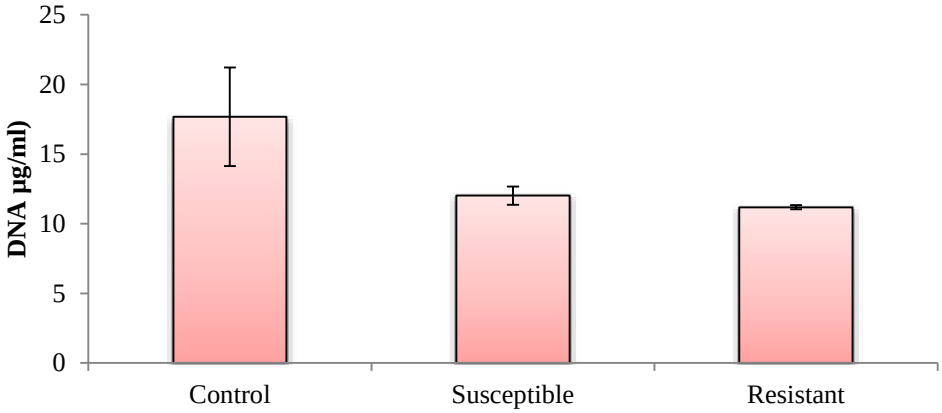

Figure 5: Effect of butanol extract of *A. indica* plant on the DNA content of Anopheles mosquito

DISCUSSION

In the present study, phytochemical screening of all solvent extracts showed the presence of varying bioactive compounds which may have a wide range of actions. Similar findings were reported by Zainab *et al*. (2016) which shows the presence of Triterpenes, tannins, phenols, terpenoids, steroids, anthraquinones, glycosides and alkaloids were present in butanol extract while flavonoids, saponins, quinones and amino acids were absent in *A. Indica* leaves. In addition to having insecticidal properties, neem products have antiviral, antibacterial, antifungal properties, that are effective against pest of field crops (El atta *et al*., 2011; Degu and Sodangi, 2013; Shannag *et al*., 2014; Kamaraj *et al*., 2009) and stored grains (Maina and Lale, 2004). Although all parts of the *A. Indica* tree possess insecticidal activity, the seed kernel is reported to be the most effective and has pesticidal active ingredient called triterpene (Debashi and Tamal, 2012). The larvicidal activity observed in this study might be associated with the presence of triterpenes in butanol extract. This finding is in line with that of Ramanibai *et al*. (2014) and Fredros *et al*. (2007) whose study showed high activity when butanol extract of *A. indica* was administered to larvae of *Anopheles gambiae* and attributed this effect to the inhibition of the development of the larvae. It has been established that the main active component responsibe for insecticidal activity of *A. Indica* plant is azadirachtin which is a precursor of secondary metabolite triterpenes (Estefania *et al*., 2016).

Biochemical analysis showed decrease in glucose content of the susceptible larvae, and this can be due to the presence of triterpenes in butanol extract. This result is in accordance with the work of Gnanamani and Dhanasekaran (2017) that reported depletion in glucose content when *A. indica* was administered to *Pericallia ricini*. Preeti *et al*. (2010) reported depletion in glucose content when *A. indica* extracts were administered to *Culex* sp. This depletion in glucose content may be due to utilization of the reserved glucose sources of larval tissues as a result of insecticidal stress. However, nucleic acid analysis showed no significant difference in DNA content when compared with the control and when susceptible larvae were compared with resistant larvae. This is in contrast with findings of Vinayagam *et al*., who reported depletion in DNA content when some plants extract were administered to larvae of *Anopheles stephensi*, the varying results was probably due to the difference in level of toxicity among the larvicidal ingredients of the plant.

CONCLUSION

In conclusion, the larvicidal activity observed in this study may be attributed to the presence of triterpenes in the butanol extract which exhibited potent larvicidal activity against *Anopheles* mosquito by interfering with metabolic processes of the larvae.

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