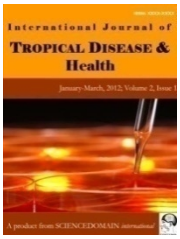

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Biocidal activities of Solvent Extracts of *Azadirachta indica* against Some Endemic Tropical Vector-borne Diseases

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TCNA and EIO designed the study, performed the statistical analysis, wrote the protocol, and the first draft of the manuscript. Authors DVZ, BD and NB managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

The biocidal efficacy of Chloroform (*CEX*), n-Hexane (*HEX*), Acetone (*AEX*) and Ethanol (*EEX*) extracts, of the bark and root of *Azadirachta indica* (Neem), *were* investigated

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against *Anopheles gambiae and Bulinus globosus* in a two-phased rapid and final screening test. Results of the phytochemical screening, indicated phytochemical constituents like; Flavonoids, Tannins, phenols, saponin, phytates, glycosides and triterpenes. All extracts were active (≥500ppm), during the rapid screening phase; hence final screening phase was also carried out. Results of the final screening shows that, the snail (*B. globosus*), were more susceptible to all solvent extracts compared to the mosquito larvae (*An. gambiae*). The ethanol extract of the bark against the snail (*EEXbS*) induced the highest mortality with LC_{50} value of 0.35ppm compared to the least, chloroform extract of the root against the larvae (*CEXrL,* LC_{50}=46.0 ppm). The positive control induced mortality to both snails and larvae at 1ppm, while there was no mortality induced by the negative control. The results confirm solvent extracts of neem bark and root as potential biocidal agent against disease vectors.

Keywords: Vector-borne diseases; Azadirachta indica; Anopheles gambiae; Bulinus globosus; solvent extracts.

1. INTRODUCTION

Over the past decades, malaria and schistosomiasis are becoming hyper-endemic vector-borne diseases in Africa. Malaria is transmitted by female mosquito belonging to the genus *Anopheles* [1,2], while schistosomiasis is transmitted by aquatic snails belonging to the genera *Biomphalaria*, *Bulinus* and *Oncomelia* [3]. There are about 3,500 species of mosquitoes worldwide, which are grouped into about 41 genera [1]. Furthermore, only about 30-40% of the Anopheles mosquitoes transmit malaria [4,1]. Diseases caused by Mosquito are prevalent in more than 100 countries across the world, with a morbidity burden affecting over 700 million people globally Ghosh [5]. A global Statistic by the World Health Organization, shows that more than 3 billion people in endemic area are prone to malaria [6], with about 59, 38 and 3% of clinical cases in Africa, Asia, and America, respectively [7].

In terms of morbidity burden, data show that schistosomiasis is next to malaria in ranking, amongst vector-borne diseases [8,3], schistosomiasis affects about 4-5% of the world population [3]. The morbidity burden of schistosomiasis may vary depending on the intermediate host responsible for the disease [3]. For instance, [3], reported that the genus *Bulinus* host parasites is mostly responsible for urinary schistosomiasis, while the genus *Biomphalaria* is the intermediate host for the parasite responsible for intestinal schistosomiasis. These vectors are endemic in regions such as Africa, Asia, South America and Middle East [3].

Control measures like, chemotherapy and environmental manipulation had been put in place to check vector-borne diseases [3]. Chemotherapy has witnessed several challenges including insect resistance, inadequate resources, vector adaptation [9,1]. Furthermore, chemotherapy only abates the morbidity burden as the risk of re-infection still abounds [10,3,1] and even the use of synthetic pesticides affects non-targeted species which might infringe on the health of the ecosystem [1,11,1].

Multifaceted and eco-friendly vector control measures are recommended for an integrated vector management [3,5] especially the environmental manipulation of vectors prior to maturity [1]. Contemporary, research focuses on eco-friendly vector control measures, especially the use of botanicals with biocidal efficacy [9,1]. Neem products contains over a hundred comprehensive bioactive metabolites [12], that are potentially eco-friendly tools

used for vector control [13,9,14]. Notwithstanding, it is established in literature that the efficacy of a plant-derived pesticide varies, depending on the solvent used during extraction [3], as such it became necessary to investigate the biocidal activities of some Solvent extracts of *Azadirachta indica* against malaria and schistosomiasis vectors.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The fresh bark and root of *A. indica* (Neem plant) were purchased from an alternate therapy center (Amolab Centre for Alternative Therapy), in Yenagoa Metropolis, Bayelsa State, Nigeria in March 2014. The plants were identified at the center by Dr. Adesekan (a Phyto-pathologist), and transported to the Federal Medical Center Yenagoa for bioassay.

2.2 Phytochemical Screening

Phytochemical screening of the bark and root extracts was carried out following the standard procedures [15,16,17], with slight modification. Prior to the bioassay, all the prepared plant extracts were subjected to preliminary phytochemical screening for the presence of alkaloids, flavanoids, tannins, saponin, phytates, glucosides, triterpenes and phenolic compounds. The degree of turbidity of the different extracts were ranked using the following scales; abundance (++), present (+) and absent (-).

2.3 Plant Extraction Process

The fresh bark and root of the plant were pulverized with mortar and pestle. Two hundred grams of the pulverized plant were macerated in 500ml chloroform, hexane, acetone and ethanol for 72 hours. The filtrates were concentrated to dryness in a rotary evaporator at 65ºC, and preserved at -4ºC until it was ready for use.

2.3 Vector Collection/Culture of Mosquito

Mosquito Larvae belonging to the genus *Anopheles* (*An. gambiae*), as well as aquatic snails belonging to the genus *Bulinus* (*B. globosus*), were used for the study. The larvae were cultured *in-vivo* in the wild using baits positioned around conspicuous breeding sites (4.94886N 6.34046E; 4.93816N 6.34006E). The baits used were slightly modified as per method described by Ohimain et al. [1], using plastic container half-filled with water, sand and cotton wool. The baits were constantly monitored for the conspicuous emergence of larvae. Prior to their transportation for the i*n-vitro* bioassay, the emerging larvae (wrigglers), were identified as 3rd instar larvae by Dr. Bassey (a Parasitologist at the Niger Delta University). Prior to the bioassay, the larvae were placed on an enamel tray with de-chlorinated water (pH 7.4), and acclimatized to laboratory condition, using methods as described by Dibua et al. [7]. The Snails were collected from the wild and bred in a laboratory-designed aquarium. The aquaria were designed to suit the snail's natural habitat with stick, sand, some stones, and aerators to improve oxygen in the water [3,8]. They were fed with lettuce (salad leaves), and acclimatize to laboratory conditions.

2.5 Experimental Set Up

Triplicate samples of 20 healthy larvae and snails (8±1mm), were distinctly placed in a 500ml solution of the various extracts, in a 24 hours exposure static non-renewal test. It was performed in accordance with the World Health Organization guidelines (WHO, 1965), with slight modification. The average mortality rates of organisms were observed and recorded from the replicates. Copper sulphate (1ppm) was used as the positive control [8,10], while 500ml of distilled water adjusted with 2.5ml of 10% dimethyl sulfoxide (DMSO)at pH 7.3, was used as the negative control [7]. The screening was carried out in two phases; the rapid screening test and the final screening test. These screening methods, with slight modifications are as described by Agboola et al. [10].

2.6 Rapid Screening and Final Screening Test

Triplicate concentrations of 1000ppm and 500ppm were used to screen the larva and snails for total (i.e. 100%) mortality within 24 hours in order to detect the range of activity. The replicates of the extracts showed average mortality (i.e. 100% mortality) on larva or snails at 500ppm during the rapid screening. For the final screening, the extracts which were active at 500ppm and below (≤500ppm), were further screened (at different concentrations in descending order), in order to determine the average minimal lethal concentrations (LC_{100}).

2.7 Statistical Analysis

The median Lethal doses (LC_{50}) of all tested extracts of the bark and root, were estimated from the average minimal lethal concentrations by probit analysis using SPSS Version 20 (IBM SPSS Inc.). Furthermore, the dose-time dependency of the larvae and snails to each of the 4 extracts of the bark and root, were determined using Microsoft 2013 excel package with 5% error [8].

3. RESULTS AND DISCUSSION

The results of the biocidal efficacy of the four solvent (i.e. chloroform, hexane, acetone and ethanol) extracts of the bark and root of neem plant (*A. indica*), against *An. Gambiae* and *B. globosus* are presented in (Tables 1-3) and (Figs. 1 and 2). This was carried out according to the standards methods [6], of assessing the toxicity of the different solvent extracts against the vectors.

Results of the phytochemical screening (Table 1), indicated phytochemical constituents like Flavonoids, Tannins, phenols, saponin, phytates, glycosides and triterpenes. Results of the Bark-extracts of *A. indica* showed stronger presence of most phytochemicals compared to the root-extracts. Notwithstanding, triterpenes was the predominant phytochemicals. Furthermore, it was also worthy of note that the ethanol extract yielded more phytochemicals compared to other solvents (Chloroform, n-hexane, acetone). Results of the rapid screening shows that all extracts tested were active, as a result of the average total mortalities of the replicates (ALC_{100}), within range of 500-1000ppm (i.e. ALC_{100}=100±0.00%), as such all extracts tested proceeded to the final screening phase.

Table 1. Phytochemical screening of solvent extracts of the bark and root of *A. indica*

S/N	Phytochemicals plant organ	Chloroform extracts		Hexane extracts		Acetone extracts		Ethanol extracts	
		Bark	Root	Bark	Root	Bark	Root	Bark	Root
1.	Alkaloids	-	-	-	-	-	-	-	-
2.	Flavonoids	+	+	+	-	+	+	++	+
3.	Tannins	+	-	++	+	-	-	-	-
4.	Phenols	-	-	+	-	+	-	++	-
5.	Saponins	-	-	+	+	-	-	++	+
6.	Phytates	-	-	+	-	+	-	++	-
7.	Glycosides	+	+	++	-	+	+	++	+
8.	Triterpenes	++	-	++	+	++	+	++	++

++: Present in abundance; +: Present; -: Absent

Results of the final screening phase for all solvent extracts of the bark (*SOLEx*^{B}) against the larvae and snails (Table 2) shows that the highest LC_{50} was induced by the ethanol extract (*EEXb*) against the snails (0.35ppm), followed by Hexane Extract of the bark (*HEXb*), whose LC_{50} value was 0.45ppm. The acetone (*AEXb*) and Chloroform (*CEXb*) extracts had LC_{50} values of 0.60ppm and 0.65ppm respectively. On the other hand, the larvae (which were less susceptible compared to the snails), had LC_{50} values of 6.50ppm for the ethanol, 11.02ppm for Hexane, 12.14ppm for Acetone and 13.50ppm for Chloroform extracts. Meanwhile, the snails and larvae survived in the negative control, but the positive control was lethal to both snails and larvae at 1ppm.

Result of the final screening for solvent extracts of the root (*SOLEx*^{R}) is presented in (Table 3). *SOLEx*^{R} were less active compared to *SOLEx*^{B} (Table 2). Notwithstanding, for the *SOLEx*^{R}, the larvae were likewise less susceptible to all extracts compared to the snails, as similarly observed amongst *SOLEx*^{B}. Amongst the *SOLEx*^{R} tested against the snails, Ethanol extract of the root (*EEXr*), was the most active (LC_{50}=9.35ppm), while Chloroform extract of the root (*CEXr*) was least active with LC_{50} value of 15.50ppm. In addition, Hexane extract of the root (*HEXr*), and Acetone extract of the root (*AEXr*) induced mortality with LC_{50} values of 10.31 and 13.00ppm respectively. On the other hand, *SOLEx*^{R} against the larvae induced mortalities with LC_{50} values of 46.00ppm for *CEXr*, 28.03ppm for *HEXr*, 39.82ppm for *AEXr* and 21.50ppm for *EEXr* (Table 3).

Notwithstanding, the biocidal activities of all extracts of neem plant in this studies is comparable to previous studies, wherein the biocidal activities of *A. indica* induced mortality with LC_{50} values of 0.53ppm against *Culex quinquefasciatus* for aqueous extract of the seeds [18], 6ppm for methanolic extract against *An. gambiae* [13], 62.5ppm against *Culex pipiens* after 24 hours [19]. Field trials of neem oil formulation showed 95.5% fecundity suppression of *Cx. quinquefasciatus* under 24hours and subsequently 80% reduction in about 3weeks [20].

Neem oil formulated diet induced an LC_{50} value of 11 ppm against *An. gambiae* after 8 days [9]. Tiwari [21] reported the molluscicidal activities of neem bark formulated feed pellet against *Lymnaea acuminata* in a 24-144 hour exposure investigation with LC_{50} values of 1.35ppm in 24hours, 0.92ppm in 48hours, 0.32ppm in 72hours, 0.20ppm in 96hours, 0.12ppm in 120hours and 0.09ppm in 144hours. Generally, the variation in susceptibility of the snails (to all extracts tested, i.e. *SOLEx*^{B} and *SOLEx*^{R}), compared to the larvae may have been largely attributed to their genetic makeup [3].

Table 2. Results of final screening for solvent extracts of the bark of *A. indica* against the larvae and snail

Concentrations (ppm)	% Mortality rates±SD								Controls	
	Chloroform extract		Hexane extract		Acetone extract		Ethanol extract		+ve	--ve
	Larvae	Snail	Larvae	Snail	Larvae	Snail	Larvae	Snails		
90-100	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	1ppm copper sulphate	Distilled water adjusted with *DMSO*
80-90	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00		
70-80	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00		
60-70	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00		
50-60	****	100±0.00	100±0.00	100±0.00	****	100±0.00	100±0.00	100±0.00		
40-50	****	100±0.00	****	100±0.00	****	100±0.00	100±0.00	100±0.00		
30-40	****	100±0.00	****	100±0.00	****	100±0.00	100±0.00	100±0.00		
20-30	***	100±0.00	****	100±0.00	***	100±0.00	****	100±0.00		
10-20	****	100±0.00	***	100±0.00	****	100±0.00	****	100±0.00		
0-10	****	100±0.00	****	100±0.00	****	100±0.00	****	100±0.00		
ALC_{100}	67.50ppm	3.25ppm	55.10ppm	2.25ppm	60.07ppm	3.00ppm	32.50ppm	1.75ppm	1ppm	0ppm
LC_{50}	13.50ppm	0.65ppm	11.02ppm	0.45ppm	12.14ppm	0.60ppm	6.50ppm	0.35ppm	0.2ppm	0ppm

ALC100= Average Minimal Lethal dose; ** = Average Mortality less than 100%*

Table 3. Results of final screening for solvent extracts of the root of *A. indica* against the larvae and snail

Concentrations (ppm)	% Mortality rates±SD								Controls	
	Chloroform extract		Hexane extract		Acetone extract		Ethanol extract		+ve	--ve
	Larvae	Snail	Larvae	Snail	Larvae	Snail	Larvae	Snails		
225-250	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	1ppm copper sulphate	Distilledwater adjusted with *DMSO*
200-225	****	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00		
175-200	****	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00		
150-175	****	100±0.00	100±0.00	100±0.00	****	100±0.00	100±0.00	100±0.00		
125-150	***	100±0.00	100±0.00	100±0.00	****	100±0.00	100±0.00	100±0.00		
100-125	****	100±0.00	****	100±0.00	****	100±0.00	100±0.00	100±0.00		
75-100	****	100±0.00	****	100±0.00	****	100±0.00	****	100±0.00		
50-75	****	****	****	100±0.00	****	****	****	100±0.00		
25-50	***	****	***	****	****	****	****	100±0.00		
LC_{100}	230ppm	77.50ppm	140.14ppm	51.54ppm	199.1ppm	65.00ppm	***	46.75ppm	1ppm	0ppm
LC_{50}	46.00ppm	15.50ppm	28.03ppm	10.31ppm	39.82ppm	13.00ppm	21.50ppm	9.35ppm	0.2ppm	0ppm

Furthermore, the reason for higher activity of the bark compared to the root might be attributed to the distribution/ quantity of phytochemical in various organs of the plant. The phytochemicals identified in our studies were similar to phytochemicals reported in an earlier studies by other authors [13,22,23,24,9,13], screened and quantified some phytochemicals in neem, which were reported, Alkaloids (3.57%), flavonoids (4.07%) and saponins (1.76%), steroids, tannins, trepenoids and glycocides. The metabolites in neem were attributed to their biocidal efficacy [13,9].

Time-dose dependency assessment are presented in (Figs. 1 and 2). Results for the SOLExB against the snails induce mortality in less than 12 hours of exposure as compared to the larvae whose mortality occurred above 18 hours (Fig. 1). Meanwhile, amongst SOLEx^{R}, the larvae induced mortality above 18 hours as well; the snails were lethal in 18 hours (Fig. 2). These findings are comparable to Arunpandiyan [25], whose investigation showed 30% and 70% mortalities with the leaves of neem plant against *culex* mosquitoes within 6 and 12 hour of exposure respectively.

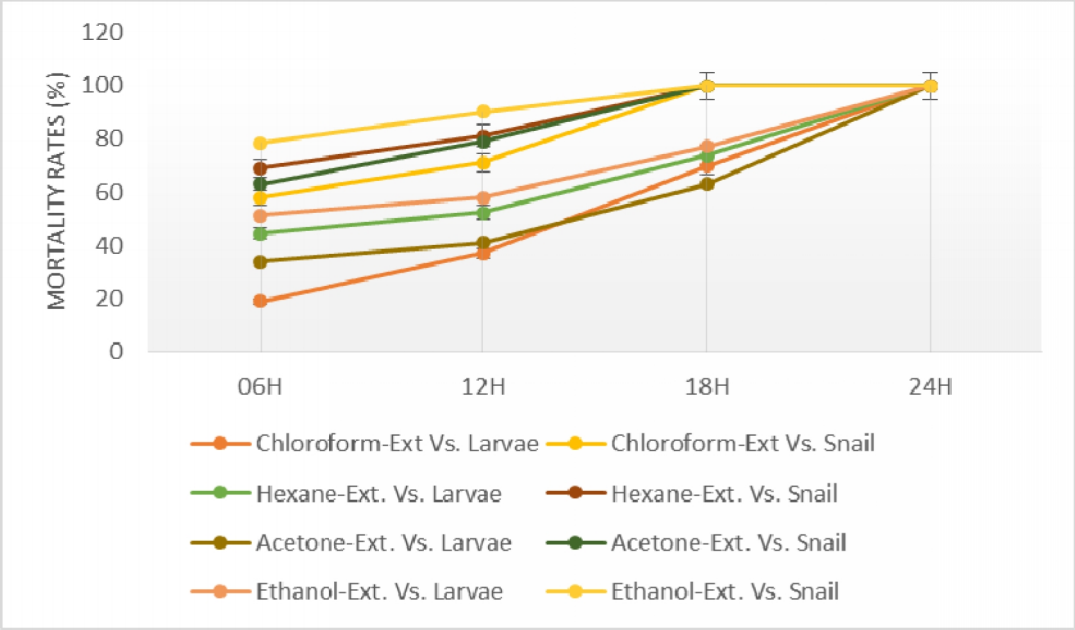

Fig. 1. Result of final screening for SOLEx^{B} based on exposure

Result of the time-dose mortality assessment of the SOLEx^{B} (Fig. 1), shows that none of the extracts induced total mortality within 06-Hours, however the highest mortality was induce by Ethanol extract of the bark with mortality rate of 78.50% (i.e. Ethanol ext. vs. snail). As the exposure increased (12-Hours), the ethanol extract further against the snail induced 90.33% mortality. Furthermore, as the exposure approached the 18-Hour mark; Ethanol ext. vs. snail, Acetone ext. vs. snail, Hexane ext. vs. snail and chloroform ext. vs. snail had all induced total mortality. At the end of (24-Hours), all extracts had induced mortality.

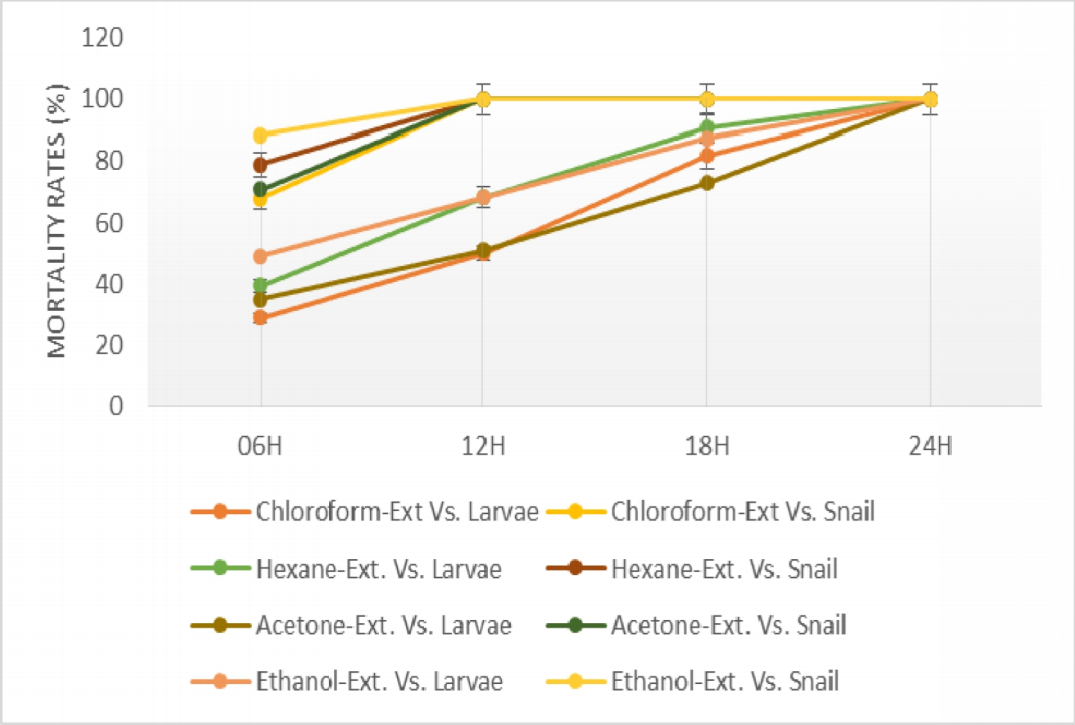

Fig. 2. Result of final screening for SOLEx^{R} based on exposure

Compared to the SOLEx^{B}, results of the SOLEx^{R} was less toxic to the snails and larvae. In 06-Hour experiment, ethanol extract of the bark induced the highest mortality against the snail with a value of 88.50%. Furthermore, at the 12-Hour, total mortality were observed in the Ethanol ext. vs. snail, Acetone ext. vs. snail, Hexane ext. vs. snail and Chloroform ext. vs. snail. As the exposure increased to the 18-Hour, mortality rates remain constant, but at 24-Hour all extracts against the snail and larvae induced total mortality.

Results of this study showed that SOLEx^{B} and SOLEx^{R} produced secondary metabolites (Table 1), which were capable of inducing larvicidal and molluscicidal properties as reported by other authors [22,23,24,9,13]. Whereas we used the solvent extracts of the bark and root in this study; the pressed cake, bark, leaf and oil have been reported for their potent molluscicidal activities against *Lymnaea acuminata* and *Indoplanorbis exustus* [26,27].

In other studies, the chemical constituents of Chloroform, Hexane, ethyl acetate and methanol extracts of neem leaves were reported for their anti-oxidant activities with the chloroform extract yielding more chemicals compounds (order of activities: chloroform >ethyl acetate> hexane> methanol extracts); compared to our study. It has been established in literature that the activities of neem plant are solely reliant on their bioactive chemicals [28,24,30,22,9].

Furthermore, Sofowora [22] reported that neem phytochemicals possessed larvacidal and insecticidal properties. In addition, the crude extract of neem plant as well as the oil were reported to inhibit insect metamorphosis [24,13], and pupation [24,30,9]. In another study, some chemicals also found in neem (Nimbinin, nimbandiolnimbidin, nimbinnimbidolas and

Azadirachtin), have been shown to possess insecticidal, nematicidal, and molluscicidal properties [29]. Others include quercetin which has anti-protozoal, antioxidant properties, as well as Salanin with repellant and anti-feedant.

Neem plant, in an earlier study showed anti-fecundity properties against mosquitoes [30,9,24], as well as anti-feedant properties to snails [21]. It is capable of disrupting the prothoracic gland from the synthesis and release of ecdysteroids, which is a molting hormones [31]. Coincidentally, the vectors responsible for malaria and schistosomiasis begins their metamorphosis in water which is favorable medium for larviciding and molluscicide [1]. Besides, neem plant is less toxic to most aquatic non-targeted species; hence their application as biocidal agents.

4. CONCLUSION

The morbidity and mortality burden of vector-borne diseases in developing nation has become a global challenge. The ecosystem has suffered inimical damages induced by the application of synthetic pesticides, as such contemporary research now focuses on cost-effective and eco-friendly plant-derived pesticides. Consequent upon our findings, the bark and root of neem possess moderate toxicity against the malaria and schistosomiasis vectors. We therefore recommend further investigation of the field application especially with regards to their toxicities against non-targeted species.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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