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Stop malaria by killing its mosquito vector at developmental stage through the use of some eco-friendly botanicals

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Abstract
Malaria remains one of the deadly diseases in Africa in general and Cameroon in particular. Synthetic insecticides have been used to kill its mosquito vector and so to prevent the disease. Though very efficient, those insecticides showed their limits. Plants can be used as alternative mosquitocidal agents as they are made up of several bioactive chemicals. This project aimed at evaluating the larvicidal activity of n-hexane, chloroform, ethyl acetate and methanol fractions of stem bark and root of *Nauclea latifolia* and *Ekebergia senegalensis* and the leaf of *Clerodendrum splendens* against 4th instar larvae of *Anopheles gambiae*, the major malaria vector mosquito. The World Health Organization standard protocol for mosquito larval tests was used. Twenty five 4th instar larvae were treated with 125 to 1000 ppm of methanol crude extract, n-hexane, chloroform and ethyl acetate and methanol fractions as well as 500 ppm of Diclorvos. Dead larvae were registered 24 h post-exposure. The LC_{50} and LC_{90} values were determined by Probit analysis. n-hexane fractions of *N. latifolia* stem bark and root and *E. senegalensis* root were the most active. They globally killed all exposed larvae at all concentrations as compared to the synthetic insecticide (Diclorvos). As for *C. splendens* leaf, n-hexane fraction was still the most active followed by ethyl acetate fraction, with LC_{50} values of 250.7 and 363.2 ppm, respectively. Our findings reveal that n-hexane is the ideal solvent that can be used to extract the most toxic compounds from the three above mentioned plants. However, further studies need to be done in order to identify the bio-efficient chemicals responsible of the toxicity through the bio-guided fractionation.

Keywords: *Anopheles gambiae*, malaria, fractions, *Nauclea latifolia*, *Ekebergia senegalensis* *Clerodendrum splendens*

Introduction
According to the annual report of the World Health Organization 2019, malaria remains one of the killers in the world (228 million cases with 405 thousand deaths). Africa alone has recorded 213 million cases, or 93% with 94% of the total global death toll. Cameroon has recorded 6,228,154 cases with 11,192 deaths [1]. This deadly disease is caused by mosquitoes, especially *Anopheles* species. Synthetic insecticides have been used to kill those vectors at larval, pupal and adult stages. Killing mosquitoes at larval stage may be the easiest way for larvae are susceptible to insecticides. They just breed and develop in artificial or domestic containers such as tin cans, rain barrels, discarded automobiles tyres, cisterns, bottles, earthen pots, flasks, flower vases, jars, overhead tanks, unused water closets, etc. They don't fly as adults [2]. Though those synthetic insecticides are very effective but their effectiveness is accompanied with adverse effects such as resistance, killing of non-target organisms and environmental pollution [3]. That is why researchers have been looking for alternative measures. Plants, due to their biodegradable and apparently safe nature, may be considered as the leading alternative agents to replace synthetic insecticides [4].

Nauclea latifolia Smith (Rubiaceae) is a valuable medicinal plant that is widespread in the humid tropical rainforest or in savannah woodland zone of West and Central Africa. Different parts of the plant possess remarkable therapeutic actions that can support the traditional usage of this plant in the treatment of several ailments [5].

Ekebergia senegalensis A Juss (Meliaceae) is a tree that grows up to a height of 30 feet and commonly found in the Savannah forests. It is distributed from Senegal to Angola but can also be found in Northern parts of Nigeria and Cameroon.

The plant has been reported to be of immense value in folk medicine [6]. In Nigeria, the leaf has shown its very impressive larvicidal activity against *An. gambiae* larvae [7].

Clerodendrum splendens G. Don (Lamiaceae) is a shrub of about 3.7 m high. It has simple and opposite dark green leaves. Its leaves are used in traditional medicine by local people to treat shingles, spleen in children, asthma, rheumatism, ulcers and malaria [8].

In the present project, the stem bark and root of *N. latifolia* and *E. senegalansis* and the leaves of *C. splendens* were extracted and fractionated in four solvents and the extracts/fractions were evaluated against 4th instar larvae of *An. gambiae* to establish the most active fractions.

Materials and Methods

Plant materials

Fresh leaves of *Clerodendrum splendens* were collected from Dang (latitude 7° 22' North and longitude 13°34' East, altitude of 1100 masl), Ngaoundere 3 Sub-division, Vina Division, Adamawa Region of Cameroon and the fresh stem bark and root of *Nauclea latifolia* and *Ekebergia senegalensis* were harvested from Zera (latitude 8° 52' North and longitude 13°56' East, altitude of 392 masl), Lagdo Sub-division, Benoue Division, North Region of Cameroon. These plants were identified for confirmation by Pr Tchobsala, a Pioneer Botanist from the University of Ngaoundere, Cameroon. All plant materials were dried at room temperature of 26 ± 4°C and 80 ± 6% of relative humidity, and ground in powder using a local mortar and pestle until the powder passed through a 0.4 mm mesh sieve. The powders were stored in opaque containers and brought to the Faculty of Pharmaceutical Sciences, Agulu; Nnamdi Azikiwe University, Awka; Anambra State, Nigeria where both extraction and bioassay were carried out and stored in a freezer set at -4°C until needed.

Extraction and fractionation of plant materials

The extraction and fractionation processes were performed in line with the method of Danga *et al*. [9].

Source of *Anopheles gambiae* larvae

The larvae of *An. gambiae* were from the colony reared in the insectarium of the Faculty of Pharmaceutical Sciences, Agulu; Nnamdi Azikiwe University, Awka; Anambra State, Nigeria. The established colony was initially collected from WHO/National Arbovirus and Vector Research Centre, Enugu, Enugu state, Nigeria.

Larvicidal bioassays

The standard procedure recommended by the World Health Organization [10] was followed to determine the toxicity of the plant extracts and fractions against 4th instar larvae of *An. gambiae*. Extracts were emulsified with Tween 80 and stock solutions were made from which four concentrations ranging from 125 to 1000 ppm were chosen. Tap water was used as negative control. Daksh insecticide (Diclorvos 100% EC w/v), a commercial formulation, was bought from the local market at Awka market, Anambra State, Nigeria and used as positive control at 500 ppm. Mortality was recorded 24 h after exposure under room temperature and relative humidity of 28 ± 2 °C and 76 ± 4%. There were four replicates for each product and concentration.

Statistical analysis

Wherever mortality in negative control reached 5%, Abbott's formula [11] was applied to correct it. Mortality data were subjected to ANOVA procedure using SPSS 17.0. Tukey test ($p = 0.05$) was applied for mean separation. Probit analysis [12] was applied to determine lethal concentrations causing 50% (LC_{50}) and 90% (LC_{90}) mortality of larvae 24 h post-exposure as well as their fiducial limits and Chi-square values.

Results and Discussions

In general, both *N. latifolia* stem bark and root yielded similar results of extraction except for ethyl acetate and methanol fractions which were higher with the stem bark and root, respectively. *E. senegalensis* stem bark registered better yields of extraction compared to the root of the same plant, and that is for all solvents (Table 1).

The toxicity of both *N. latifolia* stem bark (Table 2) and root (Table 3) against 4th instar larvae of *An. gambiae* 24h post-exposure showed that the n-hexane fractions were the most active. The same observation was made with the *E. senegalensis* root (Table 5). They globally killed all exposed larvae at all concentrations as compared to the synthetic insecticide (Diclorvos).

Chloroform and methanol fractions of *N. latifolia* stem bark were more efficient than the same solvents in the same plant's root with the LC_{50} values of 368.8 and 521 ppm (Table 2), respectively against 906.7 and 792.4 ppm (Table 3), respectively. In the same vein, the methanol crude extract and the methanol fraction of *E. senegalensis* root were more active against the treated larvae than the same plant's stem bark and the same solvents, registering LC_{50} values of 327.3 and 341.6 ppm (Table 5), respectively against 1192.1 and 870.4 ppm (Table 4), respectively.

As for the *C. splendens* leaf extracts against 4th instar larvae of *An. gambiae* 24h post-treatment, n-hexane fraction was the most active followed by the ethyl acetate fraction and methanol crude extract, with LC_{50} values of 250.7, 363.2 and 499.2 ppm, respectively (Table 6).

Plants are made up of hundreds of secondary metabolites (Alkaloids, Flavonoids, Steroids, Tannins, Saponins, Essential Oil, Phenolics, etc.) [4; 9; 13]. These metabolites can independently or jointly be effective against mosquito species [14]. Getting plants metabolites which are as efficient as the synthetic insecticides is what researchers have been and are still looking for. Some most toxic metabolites can be mostly concentrated on one part of the plant or the other (leaf, bark, root, flower, seed or fruit) [4]. That is why in the present project, fractionations were carried out on both stem bark and root of the same plants in order to know the appropriate solvent that extracts the most effective metabolites from an adequate part. Most n-hexane fractions recorded bold efficacy against 4th instar larvae of Malaria vector, *An. gambiae*. They globally killed all exposed larvae and within 24 h of exposure. It has been proved that n-hexane extracts mostly essential oil from within the plants. This hypothesis has been confirmed by several authors in different plant species [8; 13].

Table 1: Yield of the plants' extracts

Extracts	Yields (%)				
	N. latifolia **(stem bark)**	***N. latifolia*** **(root)**	***E. senegalensis*** **(stem bark)**	***E. senegalensis*** **(root)**	***C. splendens*** **(leaf)**
Meth. cr. ext.	7.1	8.2	12.2	9.5	5.4
Hexane frac.	1.7	1.3	5.3	3	3.9
Chlo. frac.	3.7	4.2	29.8	11.4	8.5
Eth. ac. frac.	15.6	11.9	27.1	13.4	9.4
Meth. frac.	12	26	23.1	13.1	27.2

Table 2: Toxicity of *Nauclea latifolia* stem bark against 4th instar larvae of *Anopheles gambiae*

Extracts	Concentrations (ppm)	Mortality (mean ± SD)	LC_{50} (95% FL)	LC_{90} (95% FL)	χ^2
Methanol crude extract	0	0 ± 0^a	908.2 (772.5-1134.2)	1402.9 (1166.4-1910.4)	1.9ns
	125	0 ± 0^a			
	250	3 ± 3.8^a			
	500	22 ± 5.1^b			
	1000	56 ± 6.5^c			
	F value	137.9***			
n-hexane fraction	0	0 ± 0^a	#	#	#
	125	98 ± 2.3^b			
	250	100 ± 0^c			
	500	100 ± 0^c			
	1000	100 ± 0^c			
	F value	7428***			
Chloroform fraction	0	0 ± 0^a	368.8 (279.6-471)	747.7 (608.2-1045.5)	1.5ns
	125	27 ± 5^b			
	250	29 ± 6.8^b			
	500	63 ± 5^c			
	1000	100 ± 0^d			
	F value	305.4***			
Ethyl acetate fraction	0	0 ± 0^a	681.3 (-)	1463.9 (-)	12.4**
	125	0 ± 0^a			
	250	2 ± 2.3^a			
	500	28 ± 6.5^b			
	1000	59 ± 5^c			
	F value	183***			
Methanol fraction	0	0 ± 0^a	521 (441.6-632.7)	818.6 (691.4-1061)	2.1ns
	125	4 ± 5.6^a			
	250	18 ± 4^b			
	500	37 ± 6.8^c			
	1000	100 ± 0^d			
	F value	351.3***			

df: 4, 15 for all cases; #: impossible due to total mortality at relatively all concentrations; -: no fiducial limits; Number of replicates: 4; *** $P<0.001$.

Table 3: Toxicity of *Nauclea latifolia* root against 4th instar larvae of *Anopheles gambiae*

Extracts	Concentrations (ppm)	Mortality (mean ± SD)	LC_{50} (95% FL)	LC_{90} (95% FL)	χ^2
Methanol crude extract	0	0 ± 0^a	770.3 (639.8-972.4)	1323.6 (1086-1822.5)	3.2ns
	125	0 ± 0^a			
	250	15 ± 3.8^b			
	500	35 ± 5^c			
	1000	66 ± 8.3^d			
	F value	142.4***			
n-hexane fraction	0	0 ± 0^a	#	#	#
	125	95 ± 6^b			
	250	100 ± 0^c			
	500	100 ± 0^c			
	1000	100 ± 0^c			
	F value	1086.1***			
Chloroform fraction	0	0 ± 0^a	906.7 (751.3-1193.8)	1500.7 (1208.3-2185.1)	2.4ns
	125	0 ± 0^a			
	250	9 ± 3.8^b			
	500	27 ± 5^c			
	1000	54 ± 7.6^d			
	F value	106.7***			

Ethyl acetate fraction	0	0±0[a]	395.9 (327.1-488.9)	671.5 (557.9-915.2)	2.4ns
	125	5±5[a]			
	250	35±5[b]			
	500	64±5.6[c]			
	1000	100±0[d]			
	F value	106.7***			
Methanol fraction	0	0±0[a]	792.4 (-)	1305.4 (-)	4.3ns
	125	0±0[a]			
	250	7±6[b]			
	500	37±6.8[c]			
	1000	64±3.2[d]			
	F value	170.4***			

df: 4, 15 for all cases; #: impossible due to total mortality at relatively all concentrations; -: no fiducial limits; Number of replicates: 4; *** $P<0.001$.

Table 4: Toxicity of *Ekebergia senegalensis* stem bark against 4th instar larvae of *Anopheles gambiae*

Extracts	**Concentrations (ppm)**	**Mortality (mean ± SD)**	**LC_{50} (95% FL)**	**LC_{90} (95% FL)**	χ^2
Methanol crude extract	0	0±0[a]	1192.1 (973.9-1810.4)	1775.4 (1379.6-3082.4)	2.1ns
	125	0±0[a]			
	250	0±0[a]			
	500	12±3.2[b]			
	1000	31±6[c]			
	F value	78.7***			
n-hexane fraction	0	0±0[a]	529.5 (433-647.8)	947.8 (795.6-1225.6)	1.1ns
	125	12±3.2[b]			
	250	14±7.6[b]			
	500	53±8.2[c]			
	1000	91±3.8[d]			
	F value	185.8***			
Chloroform fraction	0	0±0[a]	375.8 (304.1-468.4)	668.7 (551.6-922.1)	0.4ns
	125	12±3.2[b]			
	250	33±6.8[c]			
	500	68±6.5[d]			
	1000	100±0[e]			
	F value	339.1***			
Ethyl acetate fraction	0	0±0[a]	1104.4 (913.8-1557.4)	1677 (1328.3-2665.6)	3.3ns
	125	0±0[a]			
	250	0±0[a]			
	500	17±3.8[b]			
	1000	37±6.8[c]			
	F value	87.6***			
Methanol fraction	0	0±0[a]	870.4 (745.6-1062.6)	1331.8 (1122-1757.2)	1.5ns
	125	0±0[a]			
	250	3±2.8[a]			
	500	22±4[b]			
	1000	61±6.8[c]			
	F value	176.8***			

df: 4, 15 for all cases; Number of replicates: 4; *** $P<0.001$.

Table 5: Toxicity of *Ekebergia senegalensis* root against 4th instar larvae of *Anopheles gambiae*

Extracts	**Concentrations (ppm)**	**Mortality (mean ± SD)**	**LC_{50} (95% FL)**	**LC_{90} (95% FL)**	χ^2
Methanol crude extract	0	0±0[a]	327.3 (208.9-437.2)	798 (636.3-1165.4)	2.8ns
	125	37±8.8[b]			
	250	39±7.5[b]			
	500	58±8.3[c]			
	1000	100±0[d]			
	F value	129.1***			
n-hexane fraction	0	0±0[a]	#	#	#
	125	91±5[b]			
	250	96±5.6[bc]			
	500	100±0[c]			
	1000	100±0[c]			
	F value	657.8***			
Chloroform fraction	0	0±0[a]	736.8 (565.1-1067.8)	1546.5 (1170.4-2624)	0.4ns
	125	17±7.5[b]			

	250	25±8.8^{bc}			
	500	30±7.6^{c}			
	1000	68±6.5^{d}			
	F value	53***			
Ethyl Acetate fraction	0	0±0^{a}	1331.1 (1069.9-2626)	1875 (1413.9-4488)	0.4ns
	125	0±0^{a}			
	250	0±0^{a}			
	500	4±3.2^{a}			
	1000	21±5^{b}			
	F value	46.1***			
Methanol fraction	0	0±0^{a}	341.6 (-)	727.1 (-)	4.5ns
	125	17±5^{b}			
	250	53±6.8^{c}			
	500	60±6.5^{c}			
	1000	100±0^{d}			
	F value	266.7***			

df: 4, 15 for all cases; #: impossible due to total mortality at relatively all concentrations; -: no fiducial limits; Number of replicates: 4; *** P<0.001.

Table 6: Toxicity of *Clerodendrum splendens* leaf against 4th instar larvae of *Anopheles gambiae*

Extracts	**Concentrations (ppm)**	**Mortality (mean ± SD)**	**LC_{50} (95% FL)**	**LC_{90} (95% FL)**	χ^2
Methanol crude extract	0	0±0^{a}	499.2 (423.6-608.3)	779.4 (656.7-1022.8)	2.3ns
	125	2±2^{a}			
	250	20±6.5^{b}			
	500	43±6.8^{c}			
	1000	100±0^{d}			
	F value	359.5***			
n-hexane fraction	0	0±0^{a}	250.7 (206-306.3)	408.1 (342.1-552.5)	2.1ns
	125	21±5^{b}			
	250	40±3.2^{c}			
	500	100±0^{d}			
	1000	100±0^{d}			
	F value	1169***			
Chloroform fraction	0	0±0^{a}	1072.8 (-)	1948.2 (-)	5.2ns
	125	2±2^{a}			
	250	10±5.1^{b}			
	500	35±5^{c}			
	1000	39±3.8^{c}			
	F value	95.1***			
Ethyl acetate fraction	0	0±0^{a}	363.2 (303.9-438.4)	590.5 (498.8-779.4)	1.8ns
	125	4±3.2^{a}			
	250	35±6.8^{b}			
	500	75±5^{c}			
	1000	100±0^{d}			
	F value	466.1***			
Methanol fraction	0	0±0^{a}	624.1 (502.3-788.3)	1184.8 (968.8-1630)	2.9ns
	125	5±5^{a}			
	250	23±5^{b}			
	500	49±5^{c}			
	1000	76±6.5^{d}			
	F value	170.6***			

df: 4, 15 for all cases; -: no fiducial limits; Number of replicates: 4; *** P<0.001.

Conclusion

In conclusion, n-hexane fractions of *Nauclea latifolia* and *Ekebergia senegalensis* stem bark and root and *Clerodendrum splendens* leaves were boldly effective and so may be suitable candidates for the development of new potential eco-friendly mosquito larvicides. However, further studies are required to determine the active compounds of the n-hexane fractions and their mode of action toward the overall toxicity.

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