ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2014; 1 (4): 25-29

Received: 30-07-2014
Accepted: 28-08-2014

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Larvicidal activity of *Annona senegalensis* and *Boswellia dalzielii* leaf fractions against *Aedes aegypti* (Diptera: Culicidae)

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Abstract
The purpose of the present study was to evaluate the larvicidal activity of leaf fractions of *Annona senegalensis* and *Boswellia dalzielii* against fourth instar larvae of *Aedes aegypti*. Fourth instar larvae of *Ae. aegypti* were exposed for 24 hours to various concentrations (312.5-2500 mg/L) of methanolic crude extract and its fractions obtained with n-hexane, chloroform, ethyl-acetate and methanol solvents, following WHO method. The mortalities recorded were subjected to ANOVA test for mean comparison and Probit analysis to determine LC_{50}. Preliminary phytochemical screening test for some components of the plants assessed were also evaluated. The phytochemical screening of the two plants revealed the presence of alkaloids, steroids, phenolic compounds, terpenoids, fats and oils in the crude extracts which, after splitting were most distributed in n-hexane and chloroform fractions. Apart from methanol fraction, all products used showed a significant ($P<0.001$) concentration-dependent toxicity against *Ae. aegypti* larvae. The LC_{50} recorded with crude extract were 759.6 and 830.4 mg/L for *A. senegalensis* and *B. dalzielli* respectively. After fractionation, n-hexane and chloroform fractions of *A. senegalensis* revealed more effective activity than others with CL_{50} values of 379.3 and 595.2 mg/L respectively. As for *B. dalzielli*, n-hexane (LC_{50}=537.1 mg/L) and chloroform (LC_{50}=585.5 mg/L) fractions were also the most effective. These results suggest that the n-hexane and chloroform fractions of these plants as a promising larvicide against *Ae. aegypti* and can constitute the best basic and vital step in the development of a botanical insecticide source.

Keywords: *Aedes aegypti*, *Annona senegalensis*, *Boswellia dalzielii*, Larvicidal activity, Phytochemicals.

1. Introduction
Mosquitoes are the best known class of insect in terms of public health. According to WHO [1], *Aedes aegypti* is confirmed as a redoubtable vector of dengue fever, yellow fever and chikungunya in tropical regions. Worldwide, more than 2.5 billion people are now at risk of dengue and around 30 000 deaths from yellow fever are reported every year [1].
The best way to reduce these arbovirus diseases is to control mosquito vector which is frequently dependent on applications of conventional synthetic insecticides in the breeding and resting places [2]. However, their use in vast and increasing scale has led to the widespread development of insecticide resistance, several environmental and health concerns [3]. Hence, the researches for alternative methods on plants in the development of environmentally safe, biodegradable insecticides for mosquito control are encouraged. Review papers from all over the world have documented the toxic effect of plant extracts on *Ae. aegypti* larvae. Several plant extracts such as *Spathodea campanulata*, *Eclipta alba*, *Cardiospermum halicacabum*, *Pithecellobium dulce*, etc, are recognized to possess larvicidal effect against *Ae. aegypti* [4,5].
Larvicidal effect of plant extracts belonging to the family of Annonaceae including *Annona muricata*, *A. cherimolia*, *A. squamosa*, etc against *Anopheles sp*, *Ae. aegypti* and *Culex quinquefasciatus* was reported [6, 7, 8]. *Annona senegalensis* has shown his insecticidal effect on different development stages of *Caryedon serratus* (Coleoptera: Chrysomelidae) [9].
Earlier, gum from the bark of *B. dalzielii* was thought to protect against termites when rubbed on wood and is also effective against mosquitoes, flies, and clothing pests [10]. *Boswellia carteri*, *B. serrata and Commiphora myrrha* belonging to the family of Burceraceae have also shown larvicidal properties against *Ae. aegypti*, *Culex pipiens and Cx. pipiens pallens* [11, 12]. In northern part of Cameroon, both the leaves of *A. senegalensis* and *B. dalzielii* were used locally to protect maize, millet and sorghum against weevils' attacks [13].

In view of the importance of these plants for their insecticidal properties, it will be preferable to assess the potential of these plants extracts and further, their fractions following the solvents polarity on mosquito larvae. The aim of the present study is to assess larvicidal activity of these two Cameroonian plants against *Ae. aegypti*.

2. Materials and Methods

2.1 Plant materials collection

The fresh leaves of *Annona senegalensis* were collected from Dang (latitude 7°24.949'N, longitude 13°32.870'E and altitude 1093 masl), Ngaoundere in the Adamaoua region of Cameroon in November of 2011 while *Boswellia dalzielii* leaves collected from Midjivin (10°10.800'N, longitude 14°20,070'E and altitude 456 masl), Maroua, Far North region of the same country in December of 2011. The two plant specimens were identified by a plant taxonomist, Prof. Mapongmetsem Pierre Marie, Department of Biological Sciences, University of Ngaoundere, Cameroon and then confirmed at the National Herbarium in Yaoundé, Cameroon, where voucher samples were deposited. The leaves were dried at room temperature, then pulverised with an electric grinder and then stored at -18 ^{0}C in a deep freezer until use.

2.2 Extraction and fractionation

The method of Gueye *et al.* [9] was used for the process. For each plant species, 500 grams of powder are macerated in 2500 mL of methanol for 72 h at room temperature. The filtrate was concentrated in the Rotary Evaporator apparatus to obtain a crude extract. Part of this crude extract was separated successively by the method of differential solubility in four solvents of different polarity: n-hexane, chloroform, ethyl-acetate and methanol. The crude extract was mixed with silica gel (70-260 mesh size) and macerated in n-hexane, then filtered with Whatman nº1 filter paper after phase separation. N-hexane fraction and marc (1) were recovered. Marc (1) was dried in the open air and then soaked in chloroform; phase chloroform fraction filtrated and marc (2) were also recovered. Marc (2) after dried in open air was soaked in ethyl-acetate; phase ethyl-acetate fraction filtered and marc (3) were also recovered. Marc (3) was finally taken up in methanol to recover the polar compounds in the methanol fraction after filtration. Each fraction has then concentrated using Rotary evaporator and the solid fractions obtained were stored at -4 ^{0}C until bioassays.

2.3 Phytochemical screening

The extracts and fractions of both plants were used for the qualitative phytochemical screening for the identification of the various classes of active chemical constituents including alkaloids, flavonoids, saponins, tannins, phenolic compounds, steroids, terpenoids, oil and fats using standard prescribed methods [14].

2.4 Aedes aegypti

Larvae from a local strain mosquito colony with no insecticide history were used in the bioassays. The colony was established in June 2012 with specimens originally collected from Department of Zoology laboratory, University of Nigeria, Nsukka, Enugu State, Nigeria. The larvae were reared in mosquito insectary of the Faculty of Pharmaceutical Science, Nnamdi Azikiwe University, Agulu, Anambra State, Nigeria, maintained at fluctuating temperature and relative humidity of insectary under 12:12 light. Early fourth instar larvae were used for the experiments.

2.5 Larvicidal bioassay

The extract or fractions of each plant were dissolved in Tween 80 solvent and were tested to determine the larvicidal activity by making serial dilutions from 2500 to 312.5 mg/L in bioassays against four instar larvae *Ae aegypti* mosquito species according to the WHO [15] standard procedure. The bioassays were performed at a room temperature by exposing 25 fourth instar larvae in each concentration (2500, 1250, 625 and 312.5 mg/L) of n-hexane, chloroform, ethyl-acetate, methanol fraction and crude extract in the final volume of 100 mL in 250 mL beaker. Each experiment was conducted with (alongside) four replicates and a concurrent control group. A control group consisted of 0.5 mL of Tween 80 and 99.5 mL of tap water. The mortality of larvae in each concentration and control was recorded after 24 h exposure.

2.6 Statistical analysis

The corrected mortality was determined using Abbott's formula [16] whenever required. The percentage of mortality data were subjected to the ANOVA procedure using SPSS 16.0. Duncan test (P=0.05) was applied for mean separation. Probit analysis (Finney [17]; SPSS 16.0) was applied to determine lethal dosages causing 50% (LC_{50}) mortality of *Ae. aegypti* larvae 24 h after treatment application.

3. Results

The preliminary phytochemical screening carried out on the methanolic crude extracts revealed the presence of a wide range of phytoconstituents including alkaloids, flavonoids, saponins, tannins, phenolic compounds, terpenoids, oils and fats in methanolic crude extract of *A. senegalensis* while, the same phytochemical components are found in the methanolic crude extract of *B. dalzielii* excepted saponins (table 1). After splitting the methanolic crude extract of both plants used, the majority of these phytochemicals were most distributed in n-hexane and chloroform fractions. In the n-hexane fraction of *A. senegalensis*, alkaloids, flavonoids, tannins, phenolic compounds, terpenoids, oils and fats are present. Alkaloids, flavonoids and tannins were found in the chloroform fraction while flavonoids, saponins and tannins were also found in ethyl-acetate fraction of this plant. The presence of alkaloids, flavonoids and phenolic compounds were revealed in the n-hexane and chloroform fractions of *B. dalzielii*. Fats and oils were also present in n-hexane fraction of the plant.

The results of larvicidal activity of the leaves of *A. senegalensis* and *B. dalzielii* methanolic crude extracts and their fractions against fourth instar larvae of *Ae. aegypti* are presented in table 2. The methanolic crude extract of both plants assessed have shown a significant variation of mortality percent ranging from 14.00 to 100% ($F=455.53$, $P<0.001$) with the LC_{50} value of 759.6 mg/L for *A. senegalensis* and from 09.00 to 99.00% ($F=348.67$, $P<0.001$) with the LC_{50} value of 830.4 mg/L for *B. dalzielii*. By splitting the methanolic crude extract of *A. senegalensis*, three fractions including n-hexane, chloroform and ethyl-acetate fractions presented higher larvicidal activity on *Ae. aegypti* larvae. Among these fractions, n-hexane fraction was the most effective followed by chloroform and ethyl-acetate fractions with LC_{50} values of 379.3, 595.2 and 1240.3 mg/L respectively. Among the four

fractions obtained by fractionation of the methanolic crude extract of *B. dalzielii*, n-hexane and chloroform fractions exhibited a stronger larvicidal effect with LC_{50} values of 537.1 and 785.5 mg/L respectively. The lowest larvicidal property was recorded with ethyl-acetate fraction of the plant when, only 08.00% mortality was observed at 2500 mg/L.

Table 1: Qualitative phytochemical screening of some components of extracts/fractions of *A. senegalensis* and *B. dalzielii*.

Photochemical Components	*Annona senegalensis*					*Boswellia dalzielii*				
	MCE	NHF	CHF	EAF	MTF	MCE	NHF	CHF	EAF	MT
Alkaloids	+	+	+	–	–	+	+	+	–	–
Flavonoids	+	+	+	+	–	+	+	+	–	–
Saponins	+	–	–	+	+	–	–	–	–	–
Tannins	+	+	+	+	+	+	–	–	+	+
Phenolic compounds	+	+	–	–	–	+	+	+	–	–
Steroids	–	–	–	–	–	–	–	–	–	–
Fats and oils	+	+	–	–	+	+	+	–	+	–
Terpenoids	+	+	–	+	+	+	–	–	–	+

MCE= methanolic crude extract, NHF= n-hexane fraction, CHF= chloroform fraction, EAF= ethyl-acetate fraction, MTF= methanol fraction, += present, - = absent.

Table 2: Mortality (%) of *Ae. aegypti* larvae (mean ± SE) after 24 h exposure to methanolic crude extract, n-hexane, chloroform, ethyl-acetate and methanol fractions of *A. senegalensis and B. dalzielii* in the laboratory condition (T=25±2 ^{0}C; 76±5% RH).

Concentration (mg/L)	% mortality (Mean±SE)-Extract/Fractions					
A. senegalensis	**MCE**	**NHF**	**CHF**	**EAF**	**MTF**	**F value**
0	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	-
312.5	14.00±2.58^{Bb}	46.00±2.58^{Db}	31.00±3.41^{Cb}	07.00±1.91^{ABb}	00.00±0.00^{Aa}	895.74***
625.0	41.00±1.91^{Cc}	62.00±2.58^{Ec}	50.00±4.16^{Dc}	13.00±1.91^{Bb}	00.00±0.00^{Aa}	216.75***
1250	64.00±3.26^{Cd}	93.00±1.91^{Ed}	76.00±2.82^{Dd}	53.00±2.51^{Bc}	00.00±0.00^{Aa}	107.03***
2500	100.00±0.00^{Ce}	100.00±0.00^{Ce}	97.00±1.91^{Ce}	82.00±2.58^{Bd}	00.00±0.00^{Aa}	61.09***
F value	455.53***	501.46***	204.04***	448.74***	-	
LC_{50} (95% FL)	759.6 (662.8-866.3)	379.3 (315.1-438.4)	595.2 (508.0-684.8)	1240.3 (1110.1-1397.6)	-	
B. dalzielli						
0	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	00.00±0.00 ^{Aa}	-
312.5	09.00±1.91^{Bb}	27.00±3.00^{Cb}	10.00±1.15^{Bb}	00.00±0.00^{Aa}	00.00±0.00^{Aa}	3755.0***
625.0	38.00±4.16^{Bc}	49.00±3.41^{Cc}	34.00±2.58^{Bc}	00.00±0.00^{Aa}	00.00±0.00^{Aa}	338.78***
1250	61.00±2.51^{Bd}	87.00±1.91^{Dd}	70.00±3.82^{Cd}	00.00±0.00^{Aa}	00.00±0.00^{Aa}	72.64***
2500	99.00±1.00^{Ce}	100.00±0.00^{Ce}	100.00±0.00^{Ce}	08.00±1.63^{Bb}	00.00±0.00^{Aa}	43.46***
F value	348.67***	388.00***	456.53***	2706.00***	-	-
LC_{50} (95% FL)	830.4 (732.1-941.6)	537.1 (477.5-597.6)	785.5 (712.5-865.68)	78.35E4 -	-	

MCE= methanolic crude extract, NHF= n-hexane fraction, CHF= chloroform fraction, EAF= ethyl-Acetate fraction, MET= methanol fraction. Means ± S.E. in the same column for the same category of extract or fraction, followed by the same small letter and in the same row for the same category of concentration, followed by the same capital letter do not differ significantly at P = 0.05 (Duncan's test). Each datum represents the mean of four replicates of 25 larvae each. FL= Fiducial Limit, LC= Lethal concentration, *** P<0.001.
-: the values have not been determined because of the low or no mortality

4. Discussion

Insecticides of botanical origin have been reported as useful for control of mosquitoes. In the present study, a significant Larvicidal concentration-dependent activity was demonstrated on fourth instar larvae of *Ae. aegypti* tested with the extracts or fractions of *A. senegalensis* and *B. dalzielii*. The efficacy of both plants assessed might be due to various compounds, including phenolic, terpenoides, flavonoids, tannins, steroids, fats, oils and alkaloids acting jointly or independently on mosquito larvae. Bilal and Hassan [18] reported that different plant secondary metabolites including alkaloids, phenolic, terpenoids, rare amino acids, plant amines and glycosides are confirmed to have biological activity and that can be helpful in protecting the plants from a diseases and insect pests. Orozco *et al.* [19] also reported that among the metabolites with biological activities against insects, flavonoids, terpenoids, alkaloids, steroids and phenols stand out.

Results of the present investigation indicated that, the effectiveness of fractions decreased from the non-polar to the polar solvents. The efficacy of n-hexane and chloroform fractions were supported by Anupam *et al.* [20] when reported that n-hexane, the most non polar mainly extracts essential oil and Chloroform was moderately polar extracts steroids, alkaloids, etc. Larvicidal and growth inhibition activity of the alkaloid of *Annona squamosa* against *Anopheles stephensi* was reported by Saxena *et al.* [6]. From India, Kamaraj *et al.* [21] recorded 78, 73, 75 and 100% with n-hexane, chloroform, ethyl-acetate and methanol bark extracts of *Annona squamosa*

respectively at 1000 mg/L on larvae of *Anopheles subpictus*. The larvicidal potential of different solvent crude (hexane, chloroform, ethyl acetate, acetone and methanol) leaf extracts of *Blepharis maderaspatensis*, *Elaeagnus indica*, *Maesa indica*, *Phyllanthus wightianus* and *Memecylon edule*) was tested against the fourth-instar larvae of *Ae. aegypti* and the maximum larval mortality was detected in acetone extract of *E. indica* and *M. indica* with LC_{50} value of 90.89 and 173.21mg/L respectively [22]. A significant larvicide activity of *Cestrum nocturnum* leaf extract/fraction was reported against *Ae. aegypti* with the LC_{50} value 6 mg/L for the active fraction (Hexane-Ethyl-acetate 1:1) compared to LC_{50} value of 14 mg/L of methanol extract [23]. Larvicidal potential of the crude benzene, chloroform, ethyl acetate and methanol solvent extracts of the medicinal plant *Impatiens balsamina* were assessed against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* and the highest activity was observed in leaf methanol extract with LC_{50} values of 98.04, 119.68 and 125.06 mg/L respectively [24]. Murthy and Rani [25] reported that the acetone extracts of *Delonix regia* and *Limonia acidissima* showed toxicity up to 100 % on *Ae. aegypti* larvae at 2000 mg/L. The leaf ethanolic extract of *Calotropis gigantea* showed a concentration dependent larvicidal activity against *Ae. aegypti larvae* with a LC_{50} value of 351.43 mg/L [26]. The larvicidal action of acetone leaf extracts of 11 plants were evaluated against *Ae. aegypti* larvae and out of the plants tested, leaf extracts of *Millingtonia hortensis* was found to possess the most effective larvicidal activity (LC_{50} of 123 mg/L) followed by *Annona squamosa* (LC_{50} of 190.5 mg/L), *Bauhinia variegata* (LC_{50} of 204.2 mg/L), *Plumeria alba* (LC_{50} of 218.8 mg/L), *Psidium guajava* (LC_{50} of 223.9mg/L), *Syzygium cumini* (LC_{50} of 223.9 mg/L) and *Alstonia scholaris* (LC_{50} of 239.9 mg/L) [27]. The larvicidal activity of *Lantana camara* and *Catharanthus roseus* acetone leaf extracts showed that they are toxic to the fourth instar larvae of *Ae. aegypti* with LC_{50} values of 203.49 and 230.76 mg/L respectively [28]. The larvicidal activity of crude hexane, benzene, chloroform, ethyl acetate, and methanol solvent leaf and seed extracts of *Pithecellobium dulce* (Roxb.) Benth. (Fabaceae) were evaluated against the larvae of two important vector mosquitoes where, the highest larvicidal activity was observed in leaf methanol extract against *An. stephensi* followed by against *Ae. aegypti* with the LC_{50} and LC_{90} values of 145.43, 155.78 mg/L and 251.23, 279.73 mg/L respectively [5]. Ramanathapuram *et al.* [29] reported that the aqueous leaf extract of *Spathodea campanulata* exhibited larvicidal, activity with LC_{50} value of 16.12 mg/L against fourth instar larvae of *Ae. aegypti*.

It can be concluded from the present study that n-hexane and chloroform fractions, which were obtained from the methanolic crude extract of *A. senegalensis* and *B. dalzielii*, showed enormous resources to find out the new plant products with larvicidal activities against *Ae. aegypti* mosquito species. Further studies on synergistic combinations and isolation of bioactive fraction/constituent may provide futuristic lead products for the laboratory test and moreover, for field application of mosquito control.

5. Acknowledgements

The authors are grateful to the staff of Pharmaceutical Sciences, Nnamdi Azikiwe University, Nigeria, where the laboratory experiments were conducted for providing the required facilities. The authors are also thankful to the laboratory of National Arbovirus Research Center (NAVRC), Enugu, Nigeria for the mosquito species supplied and are also grateful to the help of the botanist, Prof. Mapongmetsem Pierre Marie of the Department of Biological Sciences, University of Ngaoundere, Cameroon, for plants identification.

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