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Brine Shrimp Lethality Assay and Larvicidal Activity of N-heptadecyl-n-(8-octa-1, 3-diene-6-ynyl)amine ($C_{25}H_{44}N$) Isolated from *Combretum molle R.*

[1]Abdu Zakari, [2]Adoum A. Omar and [3]Dimas Kubmarawa

[1]Department of Chemical Sciences, Federal University Kashere,
PMB 0182, Gombe State, Nigeria
[2]Department of Pure and Industrial Chemistry,
Bayero University Kano, PMB 3011 Kano, Nigeria
[3]Department of Chemistry Modibbo Adama University of Technology Yola PMB 2076 Yola, Nigeria

Abstract: The extracts of leaves of Combretum molle were tested against the larvae of *Artemia salina,* i.e. Brine Shrimp Lethality Test (BST) and *Culexquinquefasciatus Say* (filaria disease vector) i.e. Larvicidal Test, using crude ethanol, n-hexane, chloroform, ethyl acetate and methanol extracts. The methanolic extract proved to be the most effective in inducing complete lethality at minimum doses both in the BST and the Larvicidal activity test. The LC_{50} values obtained are 24.85 µg/ml and 0.4µg/ml respectively. The Bioactivity result was used to guide the column chromatography which afforded the pure compound ACM 2 which was not active in the BST with LC_{50} value of 1259µg/ml but was active in the Larvicidal activity test with LC_{50} value 15.14µg/ml. ACM 2 was proposed to have the structure in figure 3.

Key words: Brine Shrimp · Larvicidal · Combretum molle · Artemia salina · Culexquinquefasciatus Say

INTRODUCTION

Mosquito is any member of the family Culicidae in the insect order Diptera. They are of major importance in both human and veterinary medicine. They can cause severe annoyance and blood loss when they occur in dense population. They act as vectors of three important groups of disease causing organisms: Plasmodium; the protozoa parasite that produces malaria; Filarial worms; parasitic nematodes causing elephantiasis in humans and heartworm disease in canines and Arboviruses which are the causative agents of yellow fever. This study seeks to find a way of reducing mosquito population at larval stage [1].

MATERIALS AND METHOD

Column chromatography was done using silica gel (50 – 200 mesh) and Celite (filter agent). Thin layer chromatography (TLC) was performed on 2 x 20 cm glass plates coated with silica gel (Merck TLC grade with gypsum binder and fluorescent indicator). TLC bands were visualized under ultraviolet (UV) lamp at 254 nm and 356 nm or by exposure to iodine. The mosquito larvae were collected and reared in mosquitories obtained from the Biological Sciences Laboratories, Bayero University Kano (BUK) and identified as Culex by Entomologists, Dr. ZainabTukur of Biological Sciences Department, Bayero University Kano. ^{1}H and ^{13}C NMR wereanalysed at University of Ibadan andrecorded on Varian Mercury 200 MHz Spectrometer in $CDCl_3$. Tetramethylsilane (TMS) was used as internal standard in NMR measurements. IR and the GCMS were analysed at NARICT Zaria and recorded on Nicolet FT-IR (Impact 100D). Identification of the compound was based on the Shimadzu 48602 Library [2].

Collection of Plant Materials: Three plant, *C. molle,* was collected from Kwami in Kwami LGA of Gombe State, Nigeria in June, 2008. The plant was identified by Baba Ali Garko and authenticated by Dr. B.S. Aliyu both of Biological Sciences Department, BUK. The plant sample was air dried at 38° Celsius (room temperature) and ground into fine powder [3].

Corresponding Author: Abdu Zakari, Department of Chemical Sciences, Federal University Kashere, PMB 0182, Gombe State, Nigeria.

Extraction of Plant Material: 200g of the plant powder was percolated with 750ml of 96% ethanol for two weeks. Percolate was filtered and evaporated on a rotary evaporator (R110) at 40°C and was labeled crude extract F001. F001 was macerated using solvents in ascending polarity (n-hexane, chloroform, ethyl acetate, methanol and water. For each solvent 20ml was used to macerate the crude extract 5 times. The n-hexane soluble extract was collected, labeled F02 and allowed to evaporate to dryness at 38°C. The chloroform, ethyl acetate, methanol and the water soluble extracts were in the same way collected labeled as F03, F04, F05 and F06 and allowed to evaporate to dryness at 38°C. The water soluble extract was only evaporated using water bath to avoid fungus growth [4].

Screening of ACM-2 in the Brine Shrimp Lethality Tests: Artemia salina eggs were added into a hatching chamber filled with ocean sea water. The chamber was kept in an open space for 24 hours, after which the eggs hatched into shrimp larvae. ACM-2 (20mg) was dissolved in methanol (2ml) from which 5, 50 and 500µl of each fraction solution was transferred using a micro-syringe into labeled vials corresponding to 10,100 and 100 (µg/ml) respectively in triplicate. The vials (9 per fraction) and one vial containing 500ml of solvent as control were allowed to evaporate at room temperature to dryness in about 24 hour.One drop of dimethylsulphoxide (DMSO) was added separately to each test vials. 4ml ocean water was then added and ten (10) larvae of Artemia salina were introduced into each vial. The volume of sea water in the vials was adjusted to 5ml. After 24 hours of introducing larvae, the number of survivals were counted in triplicate and recorded. LC_{50} values at various dosages were determined using statistical analysis wherethe percentage mortality of the brine shrimp versus the log of concentrations were plotted using the Microsoft Excel spreadsheet application, which also formulated the regression equations. These equations were later used to calculate LC_{50} values for the samples tested with consideration of value greater than 1000 µg/ml, suggesting that the extract is nontoxic [5].

Collection and Rearing of *Culexquinquifasciatus Say* Mosquito Larvae: The eggs of Culex mosquito were identified by their appearance as always fastened together vertically in batches of about 100-300 forming a raft like structure which can float on water. The eggs were placed in a jar of sterile water to which 0.3g/l of ascorbic acid had previously been added in order to create low oxygen tension required to facilitate egg hatching. The larvae were harvested and transferred to several fresh beakers of sterile water to which a few grains of baker's yeast was added daily. Every 2-3 days a Pasteur pipette was used to suck fecal and decomposing dissolved yeast (Mukhtar*et al.,* 2004). As the larvae turned to pupa they were removed and placed in a fresh beakers of sterile water and transferred into "mosquitories" which was the laboratory fume chamber covered with a net to prevent flying adult from escaping or stray mosquitoes from entering. The mosquitories were sterilized by subjection to perpetual ultraviolet radiation 400-300nm for 48 hours in addition to thorough cleaning with disinfectants (Chlorine water) (Arias and Muller, 1975). After 2 days the pupa hatched out into imagoes that were fed with 10% glucose solution. As Culex mosquito is unautogenous, a mouse (for blood meal) was placed in the mosquitories and left to stand overnight. After successful mating some female mosquitoes proceeded to lay eggs in the containers of sterile water. The containers were daily examined and any batch of eggs laid was immediately transferred into beakers of sterile water containing little amount of ascorbic acid to stimulate egg-hatching. Emergent larvae were harvested for the bioassay [6].

Larvicidal Test: The test was carried out in four replicates (25 larvae/replicate) with two controls run simultaneously. Results were scored after 24 hours of continues exposure to the extract test solution and were expressed as percentage mortality (Vibha*et al.,* 2007). The percentage mortality of the Larvicidal test versus the log of concentrations were plotted using the Microsoft Excel spreadsheet application, which also formulated the regression equations. These equations were later used to calculate LC_{50} values for the samples tested with consideration of value greater than 1000 µg/ml, suggesting that the extract is nontoxic [7].

Isolation of Pure Compund (ACM 2): Powdered plant material (800g) of *C. molle* was percolated with 4 liters of methanol in a 10 liter glass jar for two weeks. The percolate was filtered and solvent evaporated on a rotary evaporator (R110) at 30OC. The extract (85.0g) was kept in freezer until needed. Silica gel (500g) was washed several times with n-hexane and was packed into a glass column (38"inches length 2" inches diameter) in slurry of n-hexane. It was then removed from the column after all solvent had drained and allowed to dry. Some quantity of celite was also washed in the same way using a whattman filter paper and allowed to dry. The extract (25.0g) was mixed properly with 21.0g silica gel and 3.0g celite to form a homogenous mixture. Washed silica gel 240g was packed onto a column (86cm length 2cm diameter) and the mixture was carefully loaded on it. An additional portion

of silica gel was packed to form a protective layer on top of the adsorbent. The column was then eluted with n-hexane (1.5L), n-hexane: chloroform (1:1, 1.5L), chloroform (1.5L), chloroform: ethyl acetate (1:1 1.5L), ethyl acetate (1.5L), ethyl acetate: methanol (1:1 1.5L) and methanol (1.5L). Eluants were collected in 50.0cm3 portions. Each portion collected was allowed to dry and analyzed on TLC. Similar fractions were pooled (combined) on the basis of their TLC RF patterns. Pooled fraction ACM-51-70 (17.628g) was re-chromatographed on a smaller column(35cm length 0.7cm diameter) which afforded pure compound ACM-2 which was tested in BST assay and against the mosquito larvae. ACM 2 was finally subjected to spectroscopic analysis (results presented in Table 4) and structural elucidation [8, 9].

RESULTS

The Brine Shrimp test, larvicidal test resultsand physical properties of ACM 2 were presented in Tables 1, 2 and 3 while Figures 1 and 2 are the graphs respectively. The ^{13}C and ^{1}H NMR of ACM 2 were presented in Table 4. Finally the structure of ACM 2 was proposed to be Fig. 3.

Table 1: Brine Shrimp Lethality Assay Result of ACM 2

	Survivals.			Death%				
Conc. (µg/ml)	V.	V.	V.	V.	V.	V	Mortality	$Log_{10}ConcLC_{50}$(µg/ml)
1000.	6	6	4	4	4	6	46.67	3
500.	6	5	7	4	5	3	40.00	2.7
250.	7	7	5	3	3	5	36.67	2.4
125.	7	8	7	3	2	3	26.67	2.1
62.5.	9	7	9	1	3	1	16.67	1.8
Ctrl(+)	0	0	0	10	10	10	100	
Ctrl(-)	10	10	10	0	0	0	0.00	

Table 2: Larvicidal effect of ACM 2 on Culex mosquito larvae

Compound G	Conc.Of extract µg/ml	Initial no of larvae	Total deaths in each test vial			% mortality in each vial	Log_{10}Conc.	LC_{50} (µg/ml)
	50	25	20	17	16	18	71	1.70
	25	25	10	14	12	15	51	1,34
	12.5	25	12	9	13	15	49	1.10
	6.25	25	8	10	8	10	36	0.80
	0.00 ctrl+	25	00	00	00	00	00	

Table 3: Physical Properties of ACM 2

Compound	Weight (g)	Rf Value	Color
ACM-2	0.0338	0.85	Light green

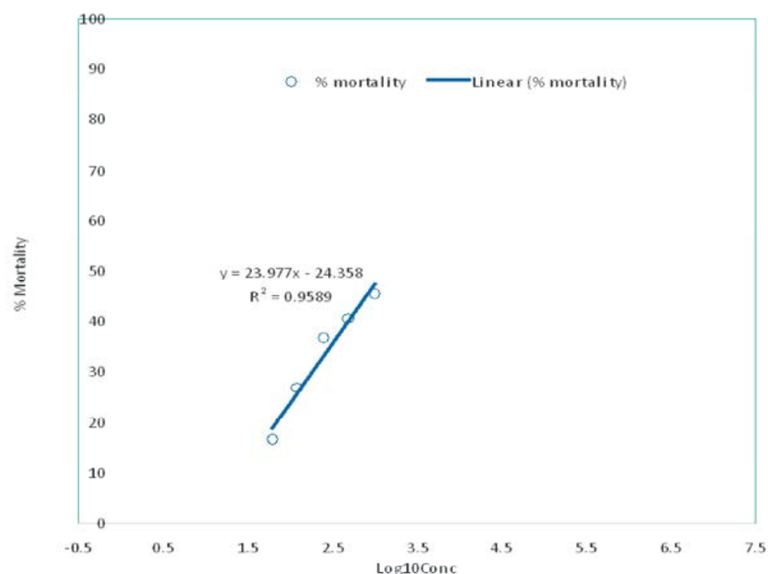

Fig. 1: Graph of Percentage mortality versus log_{10}Conc. in the BST of ACM 2

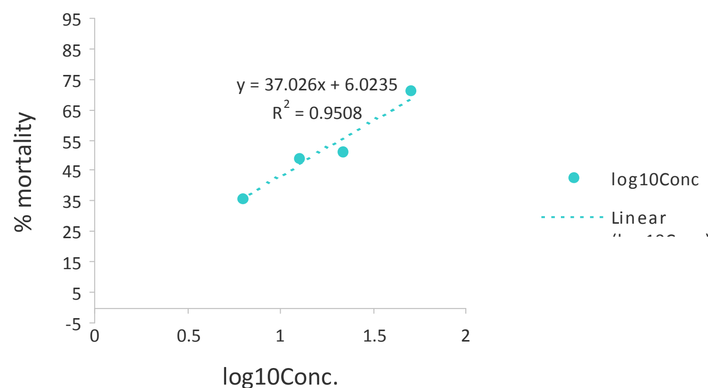

Fig. 2: Graph of Percentage mortality versus log10Conc. of ACM 2 in the larvicidal Assay

Table 4: ^{1}H NMR (199.97 MHz $CDCl_3$) and ^{13}C NMR (50.28 MHz $CDCl_3$) for compound ACM-2

1H		13C
Positions of carbons	δH	δC
Octa-1,3-diene-6-ynyl Carbons		
1	5.5d	130.517
2	5.4t	130.283
3	4.9t	129.993
4	4.7q	128.514
5	3.8d	99.38
6	-	99.357
7	-	77.886
8	3.7s	77.249
NH	7.2s	-
Heptadecyl Carbons		
1	2.9t	76.612
2	2.5m	51.674
3	2.5m	40.088
4	2.5m	34.353
5	2.5m	32.16
6	2.0m	29.922
7	2.0m	29.687
8	1.9m	29.588
9	1.7m	29.489
10	1.5m	29.383
11	1.3m	27.433
12	1.2m	25.848
13	1.2m	25.188
14	1.0m	25.028
15	0.9m	22.919
16	0.9m	19.975
17	0.7t	14.354

*see appendix for the IR, ^{13}C,^{1}H NMR spectra and GCMS

DISCUSSION

The ^{1}H NMR of compound ACM-2 indicated the presence of NH hydrogens at ð7.2 ppm (up field), alkene hydrogens at ð5.5-4.7 ppm, N-CH_2hydrogens at ð3.8 ppm , saturated long chain (CH_2)hydrogens at ð value of 0.6-2.9ppm. The IR spectra of ACM-2 revealed the presence of 2° amine (R_2N-H) functional group. N-H stretching frequency at 3745.80cm^{-1}, R-CH_2-NH_2 stretching at 2857.61cm^{-1}, C-H stretching vibration (strong absorption) at 2959.54 cm^{-1} characteristic of an alkyl group, an alkene (-C=C-) stretching vibrations

at 1645.87cm^{-1}, a weak IR absorbsion at 2370.18cm^{-1} indicated an alkyne group, a weak absorbtion at 1173.17cm-1 is characteristic of C-Nstretching vibrations of alkyl amine . The ð values of 130.517, 130.283, 129.993 and 128.514 ppm in the 13C NMR spectra reveals the presence of a conjugated alkene and the values 99.380, 99.357reveales an alkyne group. The 13C spectra revealed an overall 25 carbons in the compound. With these information and that obtained from the GCMS spectra therefore, ACM-2 was finally proposed to beN-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl) amine [10, 11].

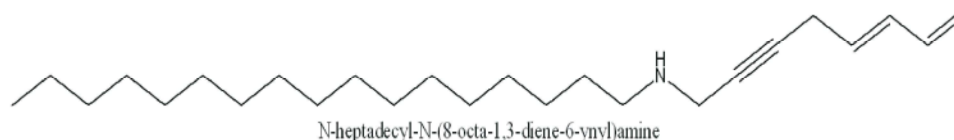

Fig. 3: Chemical Structure of ACM 2 chemical formula: ($C_{25}H_{44}N$)

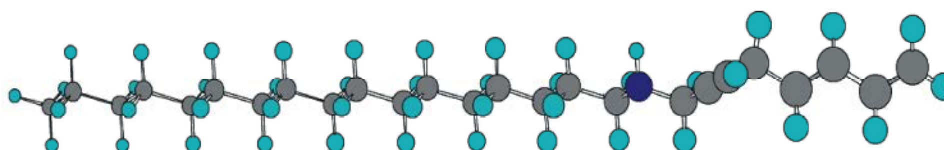

Fig. 4: 3D Modelof ACM 2 chemical formula:

Statistical Analysis: The mean results of mortality percentage of the brine shrimp versus the log of concentrations were plotted using the Microsoft Excel spreadsheet application, which also formulated the regression equations. These equations were later used to calculate LC_{50} values for the samples tested with consideration of value greater than 1000 µg/ml, suggesting that the extract is nontoxic.

CONCLUSION

Our results indicated that N-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl) amine is notactive in the Brine Shrimp Assay but active in the larvicidal test. A World Health Organization survey indicated that about 70-80% of the world's population relies on non-conventional medicine, mainly from herbal sources; hence the result of this study justifies the use of *C. molle* in rural communities as insecticide to store grains where conventional insecticides are unaffordable because of the high cost. The reported active (isolated) compound in this study indicate the need for further pharmacological and phytochemical studies in order to define what kind of cytotoxic and larvicidal activity they have (if any) and to isolate more active constituents, which are responsible for the activity. Studies of this type are needed before a phytotherapeutic agent can be generally recommended for pharmaceutical use.

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