

## Brine shrimp lethality assay and larvicidal activity of N-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl) amine (C<sub>25</sub>H<sub>44</sub>N) isolated from *Combretum molle* R.

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### Abstract

The extracts of leaves of *Combretum molle* were tested against the larvae of *Artemia salina*, i.e. Brine Shrimp Lethality Test (BST) and *Culex quinquefasciatus* 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<sub>50</sub> 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<sub>50</sub> value of 1259 µg/ml but was active in the Larvicidal activity test with LC<sub>50</sub> value 15.14µg/ml. ACM 2 was proposed to have the structure in figure 3.

**Keywords:** Brine Shrimp, Larvicidal, *Combretum molle*, *Artemia salina*, *Culex quinquefasciatus* Say

### 1. 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.

### 2. 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. Zainab Tukur of Biological Sciences Department, Bayero University Kano. <sup>1</sup>H and <sup>13</sup>C NMR were analysed at University of Ibadan and recorded on Varian Mercury 200 MHz Spectrometer in CDCl<sub>3</sub>. 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.

### 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.

### 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.

### Screening of ACM-2 in the Brine Shrimp Lethality Test

*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<sub>50</sub> values at various dosages were determined using statistical analysis where the 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<sub>50</sub> values for the samples tested with consideration of value greater than 1000 µg/ml, suggesting that the extract is nontoxic.

### Collection and Rearing of *Culex quinquefasciatus* 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 "mosquitaries" which was the laboratory fume chamber covered with a net to prevent flying adult from escaping or stray mosquitoes from entering. The mosquitaries 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) [4]. 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 mosquitaries 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 (Gerberg, 1970).

### 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) [10]. 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<sub>50</sub> values for the samples tested with consideration of value greater than 1000 µg/ml, suggesting that the extract is nontoxic.

### Isolation of Pure Compound (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 whatman 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 50.0cm<sup>3</sup> 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.

### 3. Results

The Brine Shrimp test, larvicidal test results and physical properties of ACM 2 were presented in tables 1, 2 and 3 while figures 1 and 2 are the graphs respectively. The <sup>13</sup>C and <sup>1</sup>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

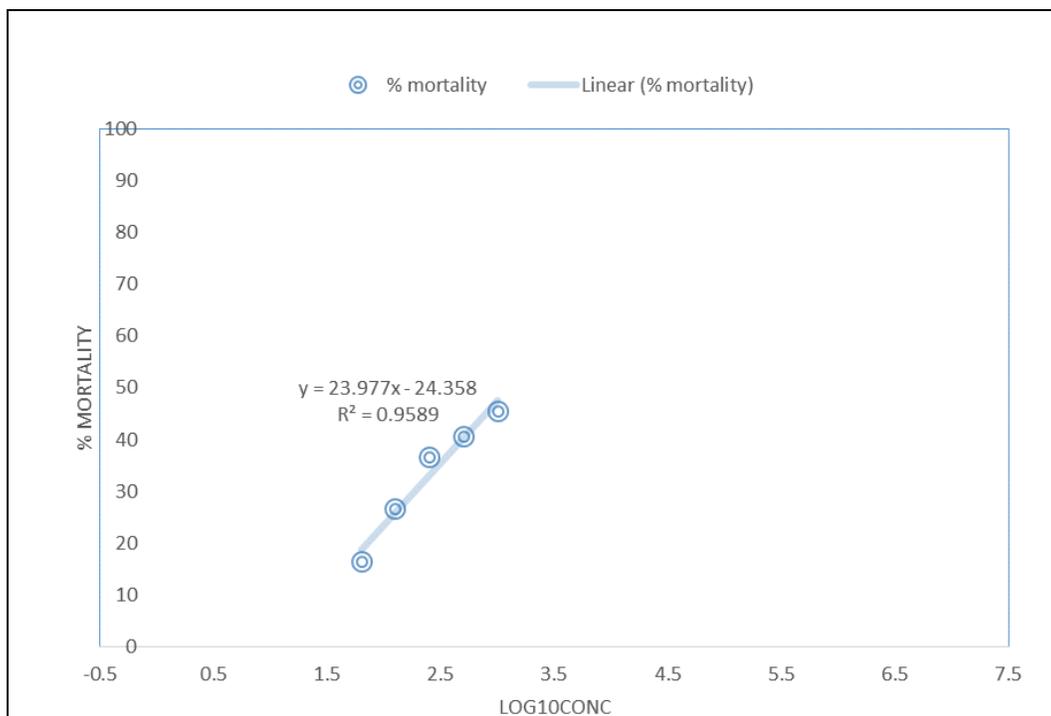
Conc. (µg/ml)	Survivals.			Death			% mortality	% mortality	% mortality
	V.	V.	V.	V.	V.	V.			
1000.	6	6	4	4	4	6	46.67	3	
500.	6	5	7	4	5	3	40.00	2.7	
250.	7	7	5	3	3	5	36.67	2.4	
125.	7	8	7	3	2	3	26.67	2.1	
62.5.	9	7	9	1	3	1	16.67	1.8	
Ctrl(+)	0	0	0	10	10	10	100		
Ctrl(-)	10	10	10	0	0	0	0.00		

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

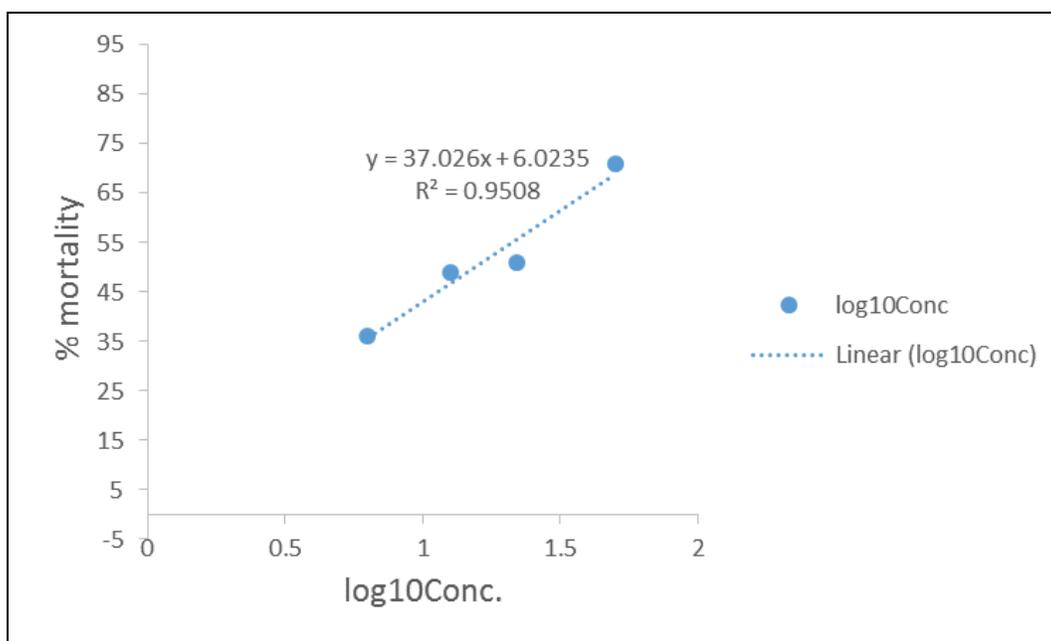
Compound G	Conc. Of extract µg/ml	Initial no of larvae	Total deaths in each test vial				% mortality in each vial	Log <sub>10</sub> Conc.	LC <sub>50</sub> (µg/ml)
	50	25	20	17	16	18	71	1.70	
	25	25	10	14	12	15	51	1.34	
	12.5	25	12	9	13	15	49	1.10	
	6.25	25	8	10	8	10	36	0.80	
	0.00 ctrl+	25	00	00	00	00	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<sub>10</sub> Conc. in the BST of ACM 2



**Fig 2:** Graph of Percentage mortality versus log<sub>10</sub> Conc. of ACM 2 in the larvicidal Assay

**Table 4:**  $^1\text{H}$  NMR (199.97 MHz  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  NMR (50.28 MHz  $\text{CDCl}_3$ ) for compound ACM-2

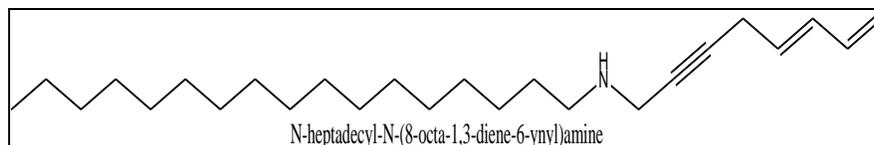
<b><math>^1\text{H}</math></b>	<b><math>^{13}\text{C}</math></b>	
<b>Positions of carbons</b>	<b><math>\delta\text{H}</math></b>	<b><math>\delta\text{C}</math></b>
Octa-1,3-diene-6-ynyl Carbons		
1	5.5d	130.517
2	5.4t	130.283
3	4.9t	129.993
4	4.7q	128.514
5	3.8d	99.380
6	-	99.357
7	-	77.886
8	3.7s	77.249
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	32.160
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,  $^{13}\text{C}$ ,  $^1\text{H}$  NMR spectra and GCMS

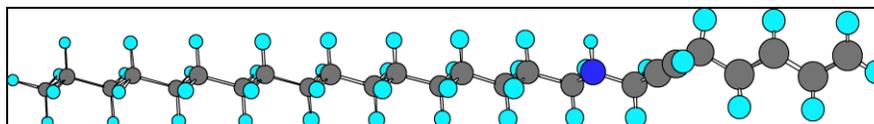
#### 4. Discussion

The  $^1\text{H}$  NMR of compound ACM-2 indicated the presence of NH hydrogens at  $\delta 7.2$  ppm (up field), alkene hydrogens at  $\delta 5.5$ - $4.7$  ppm, N- $\text{CH}_2$  hydrogens at  $\delta 3.8$  ppm, saturated long chain ( $\text{CH}_2$ ) hydrogens at  $\delta$  value of  $0.6$  -  $2.9$  ppm. The IR spectra of ACM-2 revealed the presence of  $2^\circ$  amine ( $\text{R}_2\text{N-H}$ ) functional group. N-H stretching frequency at  $3745.80\text{cm}^{-1}$ , R- $\text{CH}_2$ - $\text{NH}_2$  stretching at  $2857.61\text{cm}^{-1}$ , C-H stretching vibration (strong absorption) at  $2959.54\text{cm}^{-1}$  characteristic of an alkyl group, an alkene ( $-\text{C}=\text{C}-$ ) stretching vibrations at  $1645.87\text{cm}^{-1}$ ,

a weak IR absorption at  $2370.18\text{cm}^{-1}$  indicated an alkyne group, a weak absorption at  $1173.17\text{cm}^{-1}$  is characteristic of C-N stretching vibrations of alkyl amine. The  $\delta$  values of 130.517, 130.283, 129.993 and 128.514 ppm in the  $^{13}\text{C}$  NMR spectra reveals the presence of a conjugated alkene and the values 99.380, 99.357 reveals an alkyne group. The  $^{13}\text{C}$  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 be N-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl) amine.



**Fig 3:** Chemical Structure of ACM 2 chemical formula: ( $\text{C}_{25}\text{H}_{44}\text{N}$ )



**Fig 4:** 3D Model of ACM 2 chemical formula:

#### 5. 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  $\text{LC}_{50}$  values for the samples tested with

consideration of value greater than  $1000\text{ }\mu\text{g/ml}$ , suggesting that the extract is nontoxic.

#### 6. Conclusions

Our results indicated that N-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl) amine is not active 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 rely 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.

## 7. References

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