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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.*

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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 *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 1259 µg/ml but was active in the Larvicidal activity test with LC_{50} value 15.14 µg/ml. ACM 2 was proposed to be **N-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl)amine ($C_{25}H_{44}N$)**.

Keywords: Brine Shrimp, Larvicidal, *Combretum molle, Artemia salina, Culexquinquefasciatus Say.*

INTRODUCTION

Combretum molle R. (COMBRETACEAE) is an extremely variable shrub or small tree, usually growing 3 - 10 metres tall, but occasionally to 16 metres. It is evergreen where there is sufficient moisture but can become deciduous in dry seasons. It is common in Tropical Africa and spread widely through drier to moister areas where there are distinct dry seasons. In Nigeria, the plant is known as boodi by the Fulani *and* wuyan damoo (neck of the monitor lizard) by the HAUSA and ànràgba by the *YORUBA*

The leaf is used as painkillers, pulmonary troubles; the stem bark is used as a remedy to nasopharyngeal affections, diarrhea and dysentery. The stem products include building materials while the leaf Products: dyes, stains, inks, tattoos and mordants. The bark-slash exudes a gum, known as mumuye gum, which has been a minor source of the trade from Bornu and Adamawa in N Nigeria. It has been examined for commercial potential and found inferior as a substitute for gum-arabic. Alcoholic extracts of leaves with water extracts of twigs have shown a capacity to reduce sarcoma tumors in animals. Cattle in Senegal do not browse the plant. In the southern part of Senegal, the plant is ascribed with magical properties to promote courage in battle by inhaling smoke emitted from a fire on which bark and branches have been placed.

Medicinal plants have been prescribed and used for centuries, with almost o change in the form or the way they have been used, and with a strong belief in their usefulness in diseases. The study of bioactive compounds from plant sources and extracts in the chemical laboratory is often hampered by the lack of a suitable, simple, and rapid screening procedure. There are many procedures for bioassay that are employed using whole animals, isolated tissues, or biochemical systems. These bioassays can be quite complicated and expensive. A practical procedure for general toxicity screening is, therefore, essential as a preliminary stage in the study of bioactive plants. A model animal that has been used for this purpose is the brine shrimp, *Artemia salina* Leach. Availability of the eggs, the ease of hatching them into larvae, the rapid growth of the nauplii, and the relative ease of maintaining a population under laboratory conditions have made the brine shrimp a simple and effective animal test in biological sciences and toxicology. Combined with a reference standard, the brine shrimp test offers a bioassay that can be rapid, simple, bench-top, and more importantly, inexpensive and reproducible. The brine shrimp (*Artemia salina* Leach) is a simple zoologic organism (an arthropod). The use of the brine shrimp test (BST) as a tool to measure general bioactivity in plant extracts was initiated in 1982 and then modified in 1991 as a simple, rapid, in-house, bench-top, and low-cost prescreen for cytotoxicity and pesticidal activities.

The brine shrimp bioassay has been implemented as a test for the last 20 years and

has led to the discovery of the cytotoxic effects of a wide range of plants and bioactive compounds so diverse in their chemical structure. The safety evaluation of compounds such as drugs, cosmetics, food additives, pesticides and industrial chemicals is growing year by year. Cytotoxicity assays were among the first in vitro bioassay methods used to predict the toxicity of substances to various tissues. They are widely used for the determination of cell proliferation, viability and activation.

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 a 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 the mosquito population at the larval stage.

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 were analyzed at the University of Ibadan and recorded on Varian Mercury 200 MHz Spectrometer in $CDCl_3$. IR and the GCMS were analyzed 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

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 the Biological Sciences Department, BUK. The plant sample was air-dried at 38^{o} Celsius (room temperature) and ground into fine powder.

Extraction of Plant Material

The plant powder (200 g) was percolated with 750 ml of 96 % ethanol for two weeks. Percolate was filtered and evaporated on a rotary evaporator (R110) at 40^{o}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^{o}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^{o}C. The water-soluble extract was only evaporated using a water bath to avoid fungus growth.

Screening of ACM-2 in the Brine Shrimp Lethality Assay.

Artemia salina eggs were added into a hatching chamber filled with ocean seawater. 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 500 µl 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 hours. One drop of dimethylsulphoxide (DMSO) was added separately to each test vial. 4ml ocean water was then added and ten (10) larvae of Artemia salina were introduced into each vial. The volume of seawater in the vials was adjusted to 5ml. After 24 hours of introducing larvae, the number of survivals was counted in triplicate and recorded. LC_{50} values at various dosages were determined using statistical analysis where the percentage mortality of the brine shrimp versus the log of concentrations was 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 a value greater than 1000 µg/ml, suggesting that the extract is nontoxic.

Collection and Rearing of *Culexquinquifasciatus Say* Mosquito Larvae

The eggs of the Culex mosquito were identified by their appearance as always fastened together vertically in batches of about 100-300 forming a raft-like structure that 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 to create the 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 were 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 fresh beakers of sterile water and transferred into "mosquitories" which was the laboratory fume chamber covered with a net to prevent the 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 the 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 a little amount of ascorbic acid to stimulate egg-hatching. Emergent larvae were harvested for the bioassay (Gerberg, 1970).

Larvicidal Test of ACM-2

The test was carried out in four replicates (25 larvae/replicate) with two controls run simultaneously. Results were scored after 24 hours of continuous exposure to ACM-2 test solution and were expressed as percentage mortality (Vibha *et al.*, 2007). The percentage mortality of the Larvicidal test versus the log of concentrations was 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 a value greater than 1000 µg/ml, suggesting that the extract is nontoxic.

Isolation of Pure Compound (ACM-2)

Powdered plant material (800 g) of *C. molle* was percolated with 4 liters of methanol in a 10-liter glass jar for two weeks. The percolate was filtered and the solvent evaporated on a rotary evaporator (R110) at 30OC. The extract (85.0 g) was kept in the freezer until needed. Silica gel (500 g) was washed several times with n-hexane and was packed into a glass column (38" inches length 2" inches diameter) in a 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 21.0 g silica gel and 3.0g celite to form a homogenous mixture. Washed silica gel 240 g 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 (1.5 L), n-hexane: chloroform (1:1, 1.5 L), chloroform (1.5 L), chloroform: ethyl acetate (1:1 1.5 L), ethyl acetate (1.5 L), ethyl acetate: methanol (1:1 1.5 L), and methanol (1.5 L). Eluants were collected in 50.0cm3 portions. Each portion collected was allowed to dry and analyzed on TLC. Similar fractions were pooled (combined) based on their TLC RF patterns. Pooled fraction ACM-51-70 (17.628 g) was re-chromatographed on a smaller column (35 cm length 0.7 cm 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.

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 ^{13}C and ^{1}H NMR of ACM 2 were presented in table 4. Finally, the structure of ACM 2 was proposed to be N-heptadecyl-N-(8-octa-1, 3-diene-6-ynyl)amine(C25H44N)

Table1:Brine Shrimp Lethality Assay Result of ACM-2

Conc. (µg/ml)	**Survivals**			**Death**			**% mortality**	**Log_{10}Conc**	**LC_{50}(µg/ml)**
	V1	V2	V3	V1	V2	V3			
1000	6	4	6	4	6	4	46.67	3	
500	5	6	7	5	4	3	40.00	2.7	
250	7	5	7	3	5	3	36.67	2.4	259
125	7	7	8	3	3	2	26.67	2.1	
62.5	9	9	7	1	1	3	16.67	1.8	
Ctrl (+)	0	0	0	10	9	10	100		
Ctrl (-)	10	10	10	1	0	0	100		

Table 2: Larvicidal Effect of ACM-2 on Culex mosquito larvae.

Conc. (µg/ml)	Initial no. of larvae	Total Deaths				% mortality	Log_{10}Conc	LC_{50}(µg/ml)
		V1	V2	V3	V4			
50	25	20	17	16	18	71	1.70	15.14
25		10	14	12	15	51	1.34	
12.5		12	09	13	15	49	1.10	
6.25		08	10	08	10	36	0.80	
Ctrl (+)		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

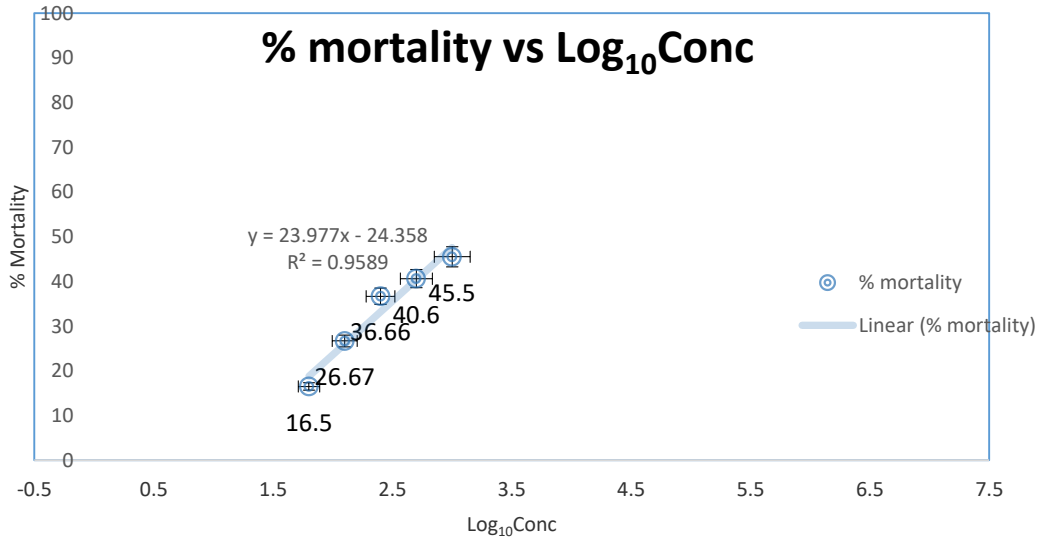

Fig 1: Graph of Percentage mortality versus log_{10}Conc. in the BST of ACM-2

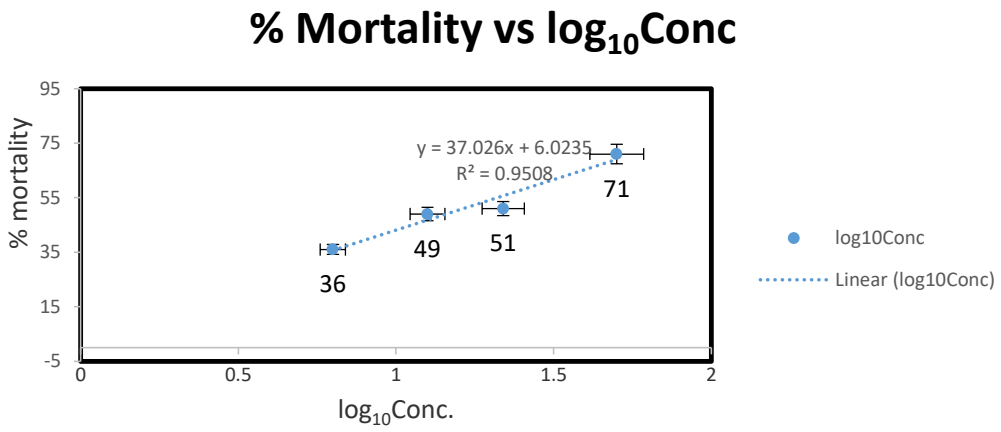

Fig 2: Graph of Percentage mortality versus log_{10}Conc. of ACM-2 in the larvicidal Assay

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

Positions of carbons	**1H** **δH**	**13C** **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	99.380
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}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 (upfield), alkene hydrogens at ð5.5-4.7 ppm, N-CH_2 hydrogens at ð3.8 ppm, saturated long-chain (CH_2)hydrogens at ð value of 0.6-2.9 ppm. The IR spectra of ACM-2 revealed the presence of 2^{o} 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} characteristics of an alkyl group, an alkene (-C=C-) stretching vibrations at 1645.87 cm^{-1}, a weak IR absorption at 2370.18cm^{-1} indicated an alkyne group, a weak absorption at 1173.17cm-1 is characteristic of C-N stretching vibrations of alkylamine. The ð values of 130.517, 130.283, 129.993 and 128.514 ppm in the 13C NMR spectra reveal the presence of a conjugated alkene and the values 99.380, 99.357 reveals an alkyne group. The 13C spectra revealed an overall 25 carbons in the compound. With this 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.

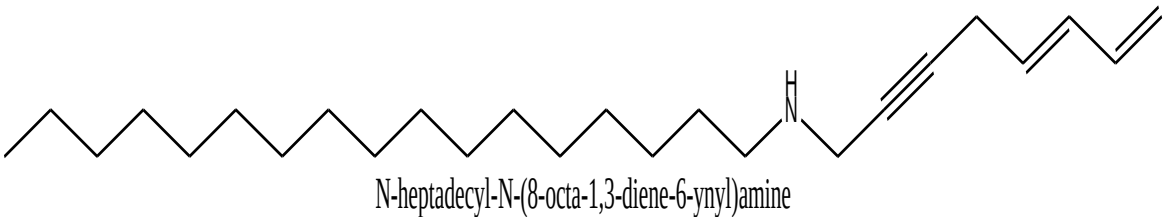

Figure 3: Chemical Structure of ACM 2 chemical formula:($C_{25}H_{44}N$)

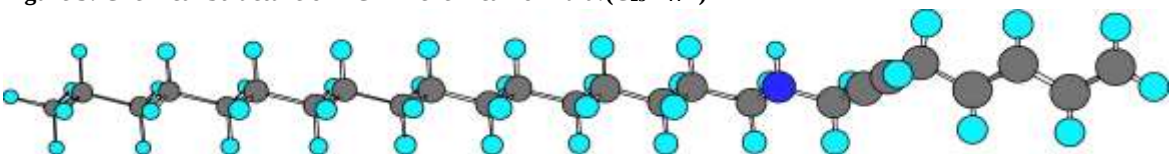

Figure 4: 3D Model of ACM 2

Statistical Analysis

The mean results of mortality percentage of the Brine Shrimp Assay and the Larvicidal Test versus the logarithm to base ten 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 a value greater than 1000 µg/ml, suggesting that the extract is nontoxic. Furthermore the error bars and percentages obtained from the graph supported the submission made in the present study.

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 an insecticide to store grains where conventional insecticides are unaffordable because of the high cost. The reported active (isolated) compound in this study indicates the need for further pharmacological and phytochemical studies 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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