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CHEMICAL INVESTIGATION AND BIO-EFFECT OF *Erythrophleum Africanum* HARMS (Leguminosaceae) ON SELECTED PESTS

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ABSTRACT

The stem bark of *Erythrophleum africanum* Harms was extracted using ethanol and dichloromethane and the extracts were separately partitioned between water, chloroform, methanol and petroleum ether. The various partitioned fractions were screened for bioactivity against brine shrimp larvae, aphids and nematodes. The water soluble-fraction and the methanol-soluble fraction both of the ethanol extract were found to be moderately active against the brine shrimp larvae with LC_{50} values of $214.4 \mu\text{g}/\text{cm}^3$ and $326.6 \mu\text{g}/\text{cm}^3$ respectively. The methanol-soluble fraction of the dichloromethane extract with an LC_{50} value of $179.8 \mu\text{g}/\text{cm}^3$ was the most active in the brine shrimp test (BST). The petroleum-ether soluble fraction of the ethanol extract was found to be very active against aphids at $4000 \mu\text{g}/\text{cm}^3$ while no extract had any effect on nematodes. Activity-guided fractionation and chromatography of the most active fraction in the BST led to the isolation of a compound whose amount was only enough for ^1H NMR. The ^1H NMR signals suggest a non-aromatic alkaloid.

INTRODUCTION

The destruction of large agricultural products annually by diseases, insects and other pests such as birds and rodents in developing countries, particularly in sub-Saharan Africa cannot be overemphasized. The discovery and consequent use of pesticides have tremendously increased the productivity of farmers in developing countries. Over years, synthetic insecticides such as carbamates, organophosphates and DDT have yielded fruitful results. However the routine application of such synthetic insecticides was found to pose serious environmental hazards (McEwen and Stephenson, 1979). As a result they are highly regulated or totally banned in most affluent countries. To minimize such effects, scientists gear researches towards finding compounds from plants sources that could be used as pesticides because

botanicals are less hazardous and are biodegradable. The use of plants in pest control is an old method around the world. Decoction of the leaves and stem of tomato, *Lycopersicon esculentus* L. Mill (solanaceae) were used in Russia against caterpillars of cabbage butterfly, *Pieris brassicae* (Dieridae) (Martin and Woodcock, 1983). In Sudan Savannah belt of Nigeria, various plants are used to control pests. The extract of the neem tree, *Azadiracta indica* A. Juss (Miliaceae) are used in northern Nigeria to ward off grasshoppers from crops during dry season (Jakai, 1993), while the fresh leaves of African custard-apple, *Annona senegalensis* Pers. (Annonaleae) are used against storage insect pests (Jakai, 1993). In this research work the stem bark of African Blackwood tree, *Erythrophleum africanum* Harms.

('Samberu' Hausa, 'Sachi' Nupe) was extracted using ethanol and dichloromethane and the extracts were separately partitioned between water, chloroform, methanol and petroleum ether. The various partitioned fractions were screened for bioactivity in the brine shrimp test (BST) and further screened against two common agricultural pests in Northern Nigeria; aphids and nematodes. The most active fraction in the BST bioassay was chromatographed in an attempt to isolate the active compound that could be developed as a potent pesticide. BST is chosen in this research work because it has been found to correlate well with many bioassays (Anderson, *et al.*, 1991).

MATERIALS AND METHODS

Plant Material

The stem bark of the plant was collected from Niger state of Nigeria in the last quarter of 2016 and identified by Malam Ali Garko (Hausa name), Malam Nda Musa (Nupe name) and Dr. B. Y. Ali (Scientific name). The material was air-dried at room temperature, ground to powdered form and stored for further use.

Solvents

All solvents were redistilled before use. Column chromatography was performed on silica gel (Merc 70-30 mesh, bet surface area 500 m²/g, pore volume 0.75 cm³). Thin layer chromatography (TLC) was performed on aluminium thin plates coated with silica gel (Merck, TLC grade with gypsum binder and fluorescent indicator). TLC bands were viewed either under ultraviolet (UV) lamps (254nm and 265nm) or by exposure to iodine. Brine shrimp eggs were obtained from Artemia Inc., USA and instant ocean sea salt was purchased from Aquarium Systems, Sareborg, France.

Extraction of Plant Material with Ethanol and Fractionation of the Ethanol Extract

Air-dried and ground material (500g) was extracted by percolation with 95% ethanol (3L) at room temperature for 1 week. The percolate was evaporated to dryness on a rotary evaporator at 35 °C. The residue, F₀₀₁, was partitioned between CHCl₃-H₂O (500 cm³, 1:1). The CHCl₃ soluble fraction, F₀₀₂, and the water soluble fraction, F₀₀₃, were separately evaporated to dryness as described above. The CHCl₃ soluble fraction (F₀₀₂) was further partitioned between 90% methanol and petroleum ether (60°C-80°C) (200cm³, 1:1). The 90% MeOH fraction, F₀₀₄,

and the pet-ether soluble fraction, F₀₀₅, were separately evaporated to dryness and stored in a freezer until needed.

Extraction of Plant Material with Dichloromethane and Fractionation of the Dichloromethane Extract

Air-dried and ground material (700g) was extracted by percolation with CH₂Cl₂ (5L) at room temperature for 48h. The percolate was evaporated to dryness on a rotary evaporator at 35 °C. The residue, F₀₁₁, was washed several times with water, concentrated *in vacuo* and partitioned (7.0g) between hexane and methanol (400 cm³, 1:1). The fractions were separately evaporated to dryness to obtain the hexane soluble fraction, F₀₂₂ (2.0g) and methanol soluble fraction, F₀₃₃ (3.8g) and stored in a freezer until needed.

Column Chromatography of the Methanol Soluble Fraction of the Dichloromethane Extract (F₀₃₃)

The methanol soluble fraction, F₀₃₃, (15.0g) was loaded on a column (silica gel, 60.0g, heaxane slurry id 2cm) and eluted with solvents in the following order, collecting eluents in the portions indicated: hexane (1L), CHCl₃ (1L), CHCl₃:ethylacetate (1:1, 1L), ethylacetate (1L), ethylacetate:ethanol (1:1, 1L) and ethanol (1L). The fractions were stored in a freezer until needed.

Brine Shrimp Lethality Bioassay

A solution of instant ocean sea salt (Aquarium systems, Ohio) was made by dissolving 3.8g of the sea salt in distilled water (100 cm³). Fifty gram (50.0g) of *Artemia salina* Leach eggs (Artemia, Inc. California) was added in a hatching chamber (Meyer *et al.*, 1982). The hatching chamber was kept under fluorescent light for 48h for the eggs to hatch into shrimp larvae. The test sample (20mg) was dissolved in methanol (2.0 cm³) and from this 500, 50 and 5 µL of each solution was transferred in to vials corresponding to 1000, 100 and 10 µg/cm³, respectively. Each dosage was tested in triplicate. The vials (9 per test fraction) and one control containing 500 µL of solvent were allowed to evaporate to dryness in about 48h at room temperature. An instant ocean sea salt solution (4.5 cm³) were added to each vial and 10 larvae of *Artemia salina* (taken 48-72h after the initiation of hatching) were added to each vial. The final volume of solution in each vial was adjusted to 5cm³ with the sea salt solution immediately after adding the shrimp larvae.

24h later, the number of surviving larvae at each dosage was counted and recorded. LC₅₀ values were determined with 95% confidence intervals by analyzing the data on a kingtech IBM-PC loaded with 'Finney program'.

Aphid Assay

Twenty milligram (20.0mg) each of the water soluble (F₀₀₃), methanol soluble (F₀₀₄) and petroleum ether soluble (F₀₀₅) residues were separately dissolved or suspended in 5 cm³ of water (concentration: 4000 µg/cm³). Similarly, 5mg of each chromatographic fraction tested in the brine shrimp bioassay was dissolved or suspended in 5 cm³ of water (concentration: 1000 µg/cm³). Where a test fraction is insoluble in water, 2-3 drops of 'Tween 20' were added and shaken. Using syringes, cowpea plants in a green house infested with aphids were sprayed with the test solutions after 48h of infestation. Each plant represented a particular test fraction. A control was sprayed with 5cm³ of water containing 3 drops of 'Tween 20'. The plants were observed every 24h over a period of 3 days (74h) for aphids' mortality.

Nematode Bioassay

A regular funnel (15cm in diameter) attached to 20 cm teflon rubber tubing was clamped on a stand. The free end of the tube was closed with a clip. The funnel was filled with water. A plastic beaker containing

some soil sample, collected around the root gall bearing tomato plants was covered with a muslin cloth. The set-up was allowed to stand for 24h. The water in the funnel, now containing some nematodes, was drained in to a beaker. 20 mg of the test fraction was dissolved in 5cm³ of water representing a concentration of 4000 µg/cm³, and 10 nematodes were counted by the aid of a hand lens and introduced in to the test solution. Another 10 nematodes were counted and transferred in to 5 cm³ of water to serve as control. The set up was observed after 24h for nematodes' mortality. The test was conducted in duplicate.

¹H NMR Spectrum

The ¹H NMR spectrum was obtained using CDCl₃ as solvent on a JOEL asx-400 MHz machine with an automated sample changer. Chemical shifts for hydrogen are given on the δ scale relative to TMS (tetramethylsilane, $\delta = 0$ ppm). Coupling constants were measured in Hz.

The ¹H NMR spectrum of the purified most active chromatographic fraction isolated from the stem bark of the plant under study exhibits the following signals: ¹H NMR (400 MHz; CDCl₃) δ , 0.95 [6H, d, *J* 6.4, (CH₃)₂CH-], 1.03 (3H, s, CH₃-C), 1.70 (3H, d, *J* 6.0, CH₃CH-), 1.80-1.90 (2H, m), 2.30-2.50 (2H, m), 4.70 (1H, s), 4.80 (1H, s).

RESULTS AND DISCUSSION

Table 1: Brine shrimp test (BST) result of the fractionated ethanol and dichloromethane extracts

Fractions	Weight of crude partitioned (g) interval)	Weight of fraction recovered (g)	BST LC ₅₀ (Low- high 95% confidence µg/cm ³)
Water soluble ^a	20.4	20.4	214.4 (126.9-382.5)
F ₀₀₃ Methanol soluble ^a	24.0	1.2	326.6 (173.9-736.6)
F ₀₀₄ Pet. ether soluble ^a	24.0	1.0	>1000
F ₀₀₅ Hexane soluble ^b	7.0	2.0	>1000
F ₀₂₂ Methanol soluble ^b	7.0	3.8	179.9 (100.9-341.6)

a-Fraction from ethanol extract

b-fraction from dichloromethane extract

Table 2: Effect of solvent partitioned fractions on aphids

Fraction	Code	No. of aphids before spray	No. of aphids after 24hr of spray	No. of aphids after 48hr of spray	No. of aphids after 72hr of spray
Water soluble ^a	F ₀₀₃	80	80	135	136
Methanol Soluble ^{a*}	F ₀₀₄	-	-	-	-
Pet. Ether Soluble ^a	F ₀₀₅	102	10	08	08
Hexane Soluble ^b	F ₀₂₂	85	87	87	87
Methanol soluble ^b	F ₀₃₃	98	98	97	99
Control		85	85	87	87

a-Fraction from ethanol extract

b-fraction from dichloromethane extract

* Not soluble in either water or water/emulsifier mixture

Table 3: Effect of chromatographic fractions on aphids

Fraction	Code	No. of aphids before spray	No. of aphids after 24hr of spray	No. of aphids after 48hr of spray	No. of aphids after 72hr of spray
Chloroform fraction	EA-2	89	83	83	83
CHCl ₃ /acetone (1:1)	EA-30	82	82	84	84
CHCl ₃ /acetone (2:3)	EA-33	85	40	47	49
CHCl ₃ /acetone (1:4)	EA-43	93	93	153	152
Acetone / MeOH (3:2)	EA-48	92	52	55	64
Control		85	87	90	90

Table 4: ¹H NMR summary for compound EA-2

Proposed Signal	¹ H NMR Band	δ_H multiplicity J Hz (CDCl ₃)
	a ₁	δ_H 4.80 (1H, s)
	a ₂	δ_H 4.70 (1H, s)
-CH ₂ -CH ₂ -N<	b ₁	δ_H 2.30-2.50 (2H, m)
-CH ₂ -CH ₂ -N<	b ₂	δ_H 1.80-1.90 (2H, m)
$\text{H}_3\text{C}-\text{CH}-$	c	δ_H 1.70 (3H, d, J = 6.0Hz)
$\text{H}_3\text{C}-\text{C}-$	d	δ_H 1.03 (3H, s)
$\text{H}_3\text{C}-\text{CH}-$ CH ₃	e	δ_H 0.95 (6H, d, J = 6.4Hz)

Erythrophleum africanum (Welw) Harms. (Leguminosaceae) ('Samberu' Hausa, 'Sachi' Nupe) is a tree that is widespread in the Savannah region of tropical Africa. It is highly regarded as a poisonous plant and its aqueous extract is used to kill *Thryonomys swinderianus* (African spiny rat), a field pest of rice and maize (Nwude and Chineme, 1981). Several cases of deaths were reported in East Africa when the bark extract was used for medicinal purposes (Vencourt and Trump, 1958) and decoctions of its leaves and stem were used as an ordeal poison among a native of East Africa during criminal trials. The toxic effect of the leaves on sheep has also been reported (Nwude and Chineme, 1981). Three diterpene alkaloids; Erythrophlamide, Erythrophlamine and Erythrosthachamide have been isolated from the stem bark (Janson and Crunland, 1976). We have examined the stem bark of *Erythrophleum africanum* for bioactive compounds that could be potent to agronomic pests. The result of the preliminary screening of solvent partitioned extracts of *Erythrophleum africanum* against brine shrimp larvae in the brine shrimp lethality test (BST) showed that the water soluble (F_{003}) and the methanol soluble (F_{004}) fractions are weakly active. They have BST LC_{50} values of $214 \mu\text{g}/\text{cm}^3$ and $326 \mu\text{g}/\text{cm}^3$ respectively (Table 1). The methanol soluble fraction (F_{033}) of the dichloromethane extract is also weakly active having a BST LC_{50} value of $179.8 \mu\text{g}/\text{cm}^3$ (Table 1). None of the crude extracts or any of the partitioned fractions was found to be active on nematodes at a dosage of $4000 \mu\text{g}/\text{cm}^3$. Only the petroleum ether fraction (F_{005}) was found active against aphids at a concentration of $4000 \mu\text{g}/\text{cm}^3$ in which case the aphids population drastically reduced from 102 to only 10 in just 24h after spray and stayed at 08 over a period of 72hr after spray of the test fraction (Table 2). The same result was confirmed after repeating the experiment with the same fraction and there was no build-up of the aphid colony when the plant was visited after seven days. A portion of the methanol soluble fraction of the CH_2Cl_2 extract (F_{033}), which is the most active to brine shrimp (Table 1) was chromatographed on silica gel column. Fractions were pooled and screened against brine shrimp larvae and aphids. Two fractions EA-2 (eluted with CHCl_3) and EA-30 (eluted with CHCl_3 : acetone, 1:1) are toxic to brine shrimp having BST LC_{50} values of $3.4 \mu\text{g}/\text{cm}^3$ and $11.4 \mu\text{g}/\text{cm}^3$ respectively.

However, they have a percentage recovery of only about 0.01%. All other chromatographic fractions tested have BST LC_{50} values $> 1,000 \mu\text{g}/\text{cm}^3$ and are therefore inactive to the brine shrimp larvae. Fraction EA-2 that shows very high toxicity to brine shrimp larvae was found to have very little effect on aphids (Table 3). This supports our earlier work using different plants which suggests that the brine shrimp test (BST) does not correlate with aphid assay (Kutama, 2008), although it has been reported to correlate well with many cytotoxicity tests and pesticidal assays (Anderson, *et al.*, 1991). Fractions EA-33 eluted with CHCl_3 / acetone 2:3 and EA-48 eluted with acetone / methanol 3:2 are fairly active against aphids at $1,000 \mu\text{g}/\text{cm}^3$, although a rebuild of the aphid colony after 72hr spray was observed with fraction EA-48 (Table 3). Other chromatographic fractions tested had no effect on the aphids. An attempt was made to purify the most bioactive chromatographic fraction in the brine shrimp test using TLC. However, the amount of the purified fraction was only enough for ^1H NMR. The proton NMR spectrum shows signal at δ_{H} 0.95 (6H, d, $J = 6.4\text{Hz}$), 1.03 (3H, s) and 1.70 (3H, d, $J = 6.0\text{Hz}$) which are probably due to the presence of $(\text{CH}_3)_2\text{CH}$ -, $\text{CH}_3\text{-C}$ and $\text{CH}_3\text{-CH}$ moieties respectively. There are two signals around δ_{H} 4.70 (1H, s) and 4.80 (1H, s). These signals are not coupled suggesting perhaps a mixture of diastereomeric compounds. There are two broad similar multiplet peaks around δ_{H} 2.30-2.50 (labelled as b_1 in the appendix) due to 2H and δ_{H} 1.80-1.90 (labelled as b_2 in the appendix) also due to 2H probably due to $-\text{CH}_2\text{-CH}_2\text{-N}<$ resonances, again supporting the probability of diastereomeric mixture. The spectrum did not reveal signals attributable to aromatic hydrogens. The three diterpene alkaloids previously isolated from the literature account are also not aromatic but have alkanooates (esters) and methylamines that are absent in the spectrum of this isolated compound (Nwude and Chineme, 1981). The unpurified EA-2 fraction also showed positive test to alkaloid (Meyer's test).

CONCLUSION

From the brine shrimp test the water soluble (F_{003}), the methanol soluble (F_{004}) fractions of the ethanol extract and the methanol soluble (F_{033}) of the dichloromethane extract showed activity.

The chromatographic fraction from the dichloromethane extract labeled as EA-2 showed good activity to brine shrimp (BST LC₅₀ 3.4 µg/cm³) but no activity to aphids. The petroleum ether fraction (F₀₀₅) of the ethanol extract was found to be very lethal to aphids. The structure of the compound isolated from fraction EA-2 could not be confirmed due to absence of complete spectroscopic data (¹³C NMR, IR, Mass and 2D NMR). However, from the ¹H NMR signals it could be a non-aromatic (aliphatic)

alkaloid. This work suggests that a correlation does not exist between the brine shrimp test and the aphid assay. Fractions that showed toxicity to brine shrimp larvae showed little or no toxicity to aphids colony used in this work. The plant *Erythrophleum africanum* (Welw) Harms. is very toxic to animals and humans but showed low level of activity to brine shrimp larvae and aphids. Perhaps the poisonous substances associated with this plant require a metabolic activation.

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Appendix

Appendix 1: ¹H NMR of the isolated compound EA-2

Appendix 2: Expanded ¹H NMR of the isolated compound EA-2 showing multiplicity of signals

