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Correlation between brine shrimp test (BST) and some bioassays using Neem (*Azadirachta indica* A. Juss) and Wild custard-apple (*Annona senegalensis* Pers)

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ABSTRACT: The leaves of Neem (*Azadirachta indica* A.Juss) and Wild custard-apple (*Annona senegalensis* Pers) were extracted using ethanol and extracts were screened for bioactivity against brine shrimp larvae. The bioactive extracts in the brine shrimp test (BST) were investigated for correlation with aphid nematode and insect-deterrent bioassays. The BST correlated statistically with insect-deterrent bioassay in both plants (having a product moment correlation of $\gamma xy = 0.9387$) while there is no correlation with aphid assay. No extract was found active against nematodes.
KEY WORDS: Extracts, brine shrimp test, correlation and bioassays.

INTRODUCTION

Agriculture has been providing man with the three basic necessities of life – food, shelter and clothing, for centuries. With the industrial revolution, agriculture plays another great role in providing raw materials for the ever increasing industries (Albert, 1971). As a result, several methods for boosting agriculture in technologically advanced societies and the developing world have been evolved to meet the increasing demand for food and raw material (Martin and Woodcock, 1983). However, these efforts are hampered by the large destruction of agricultural products by diseases and pests such as insects, birds and rodents, particularly in developing countries (McEwen and Stephenson, 1979).

Within the last century, scientists had discovered chemical compounds that could protect crops from diseases and pests – the pesticides. The discovery of the synthetic insecticide Dichlorodiphenyltrichloroethane (DDT), after the Second World War geared researches in to synthesizing many more pesticides, particularly insecticides. Over the years synthetic insecticides such as DDT, carbamates and organophosphorous have been used in agriculture and are yielding fruitful results (Fatope *et al.*, 1993). However, the indiscriminate use of such synthetic pesticides cause serious environmental hazards (McEwen and Stephenson, 1979). As a result, DDT and most synthetic insecticides are banned in affluent countries (Albert, 1971).

Efforts are now geared towards finding compounds from plant sources that could be used as pesticides because botanicals are less hazardous and are biodegradable. Bioassays provide first-hand information on active components in plants and they guide the laboratory isolation of pure active compounds that could be developed to pesticides or drugs. The brine shrimp test (BST) is a simple bench-top bioassay that has been used to screen a number of plants' extracts (Fatope *et al.*, 1993) and it has correlated statistically with cytotoxicity and pesticidal assays (Anderson *et al.*, 1991). Hence, BST is a reliable indicator of biological activities of pesticidal significance.

This work is a continuation of the correlation study of the BST with other assays using leaves extracts of Neem (*Azadirachta indica* A.Juss) and Wild custard-apple (*Annona senegalensis* Pers). The Leaves extract of the Neem tree (*Azadirachta indica* A.Juss) is used in Northern Nigeria to cure malarial fever and to ward off grasshoppers from crops during dry season (Jackai, 1993) while the fresh leaves of Wild custard-apple (*Annona senegalensis* Pers) are used against storage insect pest (Audu, 1994).

MATERIALS AND METHODS

Plant Materials

All plant materials of Neem and Wild custard-apple were collected in Kano in the second quarter of 2006 and identified by local residents (Hausa name) and Dr. L.D. Fagwalawa of the Biological Sciences Department, Kano University of Science and Technology, Wudil (scientific name). A voucher specimen of the

sample was deposited in the herbarium of Bayero University, Kano.

Extraction with ethanol and fractionation of the EtOH extract

Air-dried and ground plant material (200g) 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, F_{001} (or F_{011}), was partitioned (24.0g) between $CHCl_3$-H_2O (500 cm^3, 1:1). The $CHCl_3$ soluble fraction, F_{002} (or F_{022}), and the water soluble fraction, F_{003} (or F_{033}), were separately evaporated to dryness.

The $CHCl_3$ soluble fraction was further partitioned between aqueous 90% MeOH-petroleum ether (60°C-80°C) (200cm^3, 1:1). The petroleum ether soluble fraction, F_{004} (or F_{044}), and the 90% MeOH soluble fraction, F_{005} (or F_{055}), were also evaporated to dryness. The residues were stored in a freezer until needed.

Column chromatography of the BST bioactive fractionated fractions

Each BST active fractionated fraction (15.0g) was loaded on a column (silica gel, 60.0g, hexane slurry id 2cm) and eluted with solvents in the following order, collecting eluents in the portions indicated: hexane (1L), $CHCl_3$ (1L), $CHCl_3$:ethylacetate (1:1, 1L), ethylacetate (1L), ethylacetate:EtOH (1;1, 1L), ethanol (1L). Each fraction was concentrated and transferred in to a labeled and weighed beaker. 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 in distilled water (100cm^3). 50.0g of *Artemia salina* Leach eggs (Artemia, Inc. California) were 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 (2.0cm^3) or solvent in which it is soluble and, from this 500, 50 and 5µ L of each solution were transferred into vials corresponding to 1000, 100, and 10 µ g/ml, respectively. Each dosage was tested in triplicate. The vials (9 per test fraction) and one control containing 500 µ L of solvent were allowed to evaporate to dryness in about 48h at room temperature. An instant ocean sea salts solution (4.5cm^3) 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 5cm^3 with the sea salt solution immediately after adding the shrimp. 24h later, the number of surviving shrimp at each dosage was counted and recorded. LC_{50} values were determined with 95% confidence intervals by analyzing the data on a kingtech IBM-PC loaded with "Finney program" (Meyer *et al.*, 1982).

Aphids Assay

20.0mg of the test fraction were dissolved or suspended in 5cm^3 of water (concentration: 400µ g/ml). Similarly, 5mg of each chromatographic fraction tested in the brine shrimp assay was dissolved or suspended in 5cm^3 of water (concentration: 1000µ g/ml). Where a test fraction is insoluble in water, 2-3 drops of "Tween 20" were added and shaken. Using syringes, cowpea plants in green house infested with aphids were sprayed with the test solutions after 48h of infestation. Each plant represented a particular test solution. A control plant was sprayed with 5cm^3 of water containing 3 drops of "Tween 20". The plants were observed every 24h over three days for aphids' mortality.

Nematode Assay

A regular funnel (15cm in diameter) attached to 20cm 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 soil sample, collected around the root gall bearing tomato plants was covered with a muslin cloth. The beaker was inverted and placed on the funnel in a manner that allowed contact between water in the funnel and the 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. 20mg of the test fraction were dissolved in 5cm^3 of water, representing a concentration of 4000µ g/ml, and 10 nematodes were counted under a hand lens and introduced in to the test solution. Another 10 nematodes were counted and introduced in to 5cm^3 of water to serve as control. The set up was observed after 24h for

nematodes' mortality. The test was conducted in duplicate.

Insect Deterrent Assay

0.02g of the extract was dissolved in 2cm^3 of the solvent (ethanol or methanol). 250µl of the above solution were transferred in to a vial and allowed to dry overnight. The resultant dry sample was re-dissolved in 5 cm^3 of $CHCl_3$ or acetone. Two sets of 40 healthy beans were counted and each set was placed in a funnel tucked with glass wool. The first set of beans was dressed with the prepared solution. The other set of 40 beans (control) was dressed with the solvent only ($CHCl_3$ or acetone). The dressed beans were placed in a partitioned tray taking care not to mix the two sets of beans. The procedure was repeated with each fraction to be tested and placed in the same partitioned tray in alternate order with the controls. Sufficient live weevils were introduced in to the tray. The tray was then covered with a thin clothing material that would permit the passage of air and prevent escape of the pests. The set up was allowed to stay for 5 weeks (35 days), after which the numbers of infested and uninfested beans were counted. Then the percentage number of infested and uninfested beans was counted for each fraction. Consequently the consumption index, C.I was calculated from the following formula:

$$C.I = \frac{\%\text{number infested}}{\%\text{number infested} + \%\text{number infested in the control}} \times 100$$

RESULTS AND DISCUSSION

Result of the preliminary BST screening of the solvent partitioned extracts of the plants' leaves shows that the water soluble (F_{003}) and the pet. ether soluble (F_{004}) of *Azadirachta indica* are moderately active against the brine shrimp larvae. They have BST LC_{50} values of 212 µg/cm^3 and 72.8 µg/cm^3 respectively (Table 1); while the $CHCl_3$ soluble (F_{022}), water soluble (F_{O33}) and pet. ether soluble (F_{044}) of *Annona senegalensis* are also active having BST LC_{50} values of 85.6 µg/cm^3, 280 µg/cm^3 and 106 µg/cm^3 respectively (Table 2). All other solvent partitioned fractions have BST LC_{50} values greater than 1000 µg/cm^3 and are therefore not active. The five active solvent partitioned fractions were screened for bioactivity against aphids, nematodes and the cowpea storage insect pest, *Callosobruchus maculatus*. The result of the screening shows that no extract was active against nematodes, while only the pet. ether soluble fraction (F_{004}) of *Azadirachta indica* was active against aphids (Table 3).

The result of the insect-deterrent bioassay on the five BST active fractions shows an interesting correlation between the two bioassays. All the five fractions have low C.I values ranging from 13.5 – 20.8 which suggests that they are also active in the insect-deterrent bioassay (Table 4). All the controls have > 95% infestation which makes the result valid. For a completely non-toxic fraction (i.e. 100% infestation in both the test fraction and the control), it will have a maximum C.I value of 50. As the C.I value approaches zero the fraction is more toxic. This result therefore shows a perfect correlation between the BST and the insect-deterrent bioassay in the solvent partitioned fractions.

To investigate the result further, some portions of the most bioactive solvent partitioned fractions in both plants, which are the pet. ether soluble, F_{005} (BST LC_{50} 72.8 µg/cm^3) and the $CHCl_3$ soluble, F_{022} (BST LC_{50} 85.6 µg/cm^3) were chromatographed on silica gel. Fractions were pooled and screened using the BST. A total of seventy chromatographic fractions in each plant were screened. Seven fractions from *Azadirachta indica* were found active while four fractions from *Annona senegalensis* were active (Table 5). All the eleven BST active fractions were investigated for correlation with aphid, nematode and insect-deterrent bioassays. None of the fractions was active in the aphid and nematode bioassays while all the eleven chromatographic fractions were active in the insect-deterrent bioassay (Table 5). The product moment correlation coefficient between the BST LC_{50} values and the insect-deterrent bioassay C.I. values (Table 5) gives $\gamma xy = 0.9387$. This supports the earlier observation that the BST correlates well with the insect-deterrent bioassay. The fractions from aphid and nematode assays have LC_{50} values of greater than 1000 and cannot therefore be correlated.

CONCLUSION AND COMMENTS

The results of the screenings of solvents partitioned fractions and column chromatographic fractions of both *Azadirachta indica* and *Annona senegalensis* shows a correlation exists statistically (γxy = 0.9387) between the brine shrimp lethality test (BST) and insect-deterrent bioassay. The other two bioassays investigated, that is aphid and nematode bioassays, show no correlation with the BST.

The plants *Azadirachta indica* A. Juss (Meliaceae) and *Annona senegalensis* Pers. (Anonaleae) show moderate activity to brine shrimp larvae in the BST bioassay and the cowpea storage insect pest *Callosobruchus maculates* Geyer (Lepidoptera: Pyrillidae) in the insect-deterrent bioassay. It is hoped that further work will lead to isolation of pure active components in both plants.

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Table 1: Brine shrimp test (BST) result of the fractionated ethanol extract of *Azadirachta indica*.

Fractions	Weight of crude extract partitioned (g)	Weight of fraction recovered (g)	BST LC_{50} (95% confidence interval) µg/ml	Remark
Crude extract, F_{001}	200	70.0	> 1000	Inactive
$CHCl_3$ soluble, F_{002}	24.0	8.2	> 1000	Inactive
Water soluble, F_{003}	24.0	14.0	212 (126.9-382.5)	Active
Pet. Ether soluble, F_{004}	7.0	3.0	72.8 (122.9-321.6)	Active
Methanol soluble, F_{005}	7.0	4.8	> 1000	Inactive

Table 2: Brine shrimp test (BST) result of the fractionated ethanol extract of *Annona senegalensis*.

Fractions	Weight of crude extract partitioned (g)	Weight of fraction recovered (g)	BST LC_{50} (95% confidence interval) µg/ml	Remark
Crude extract, F_{011}	200	68.0	> 1000	Inactive
$CHCl_3$ soluble, F_{022}	21.0	7.5	85.6 (129.5-342.61)	Active
Water soluble, F_{033}	21.0	10.2	280 (121.8-355.6)	Active
Pet. Ether soluble, F_{044}	8.0	3.2	106 (129.5-356.5)	Active
Methanol soluble, F_{055}	8.0	3.9	> 1000	Inactive

Table 3: Effect of BST – active solvent partitioned fractions in aphid assay.

Fractions	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	Remark
Water soluble [a]	F_{003}	88	88	97	107	Inactive
Pet. Ether soluble [a]	F_{004}	100	52	20	04	Active
$CHCl_3$ soluble [b]	F_{022}	80	80	82	87	Inactive
Water soluble [b]	F_{033}	85	89	89	91	Inactive
Pet.ether soluble [b]	F_{044}	92	92	90	98	Inactive
Control		95	95	95	97	

a – *Azadirachta indica* extract. b – *Annona senegalensis* extract.

Table 4: Effect of BST – active solvent partitioned fractions in insect-deterrent bioassay.

Fraction	% No. of infected	% No. of infected in the control	C.I value
Water soluble [a], F_{003}	18	98	15.5
Pet.ether soluble [a], F_{004}	15	96	13.5
$CHCl_3$ soluble [b], F_{022}	19	98	16.2
Water soluble [b], F_{033}	25.5	97	20.8
Pet.ether soluble [b], F_{044}	22	97	18.5

a –*Azadirachta indica* extract. b – *Annona senegalensis* extract.

Table 5: Correlation of BST - active chromatographic fractions with aphid, nematode and insect-deterrent bioassays.

Solvent fraction	Code	BST LC_{50} (μg/ml)	Aphid assay LC_{50} (μg/ml)	Nematode assay LC_{50} (μg/ml)	Insect-deterrent assay
hexane	AI-5	60	>1000 (Inactive)	>1000 (Inactive)	24.6 (Active)
$CHCl_3$	AI-20	66	>1000 (Inactive)	>1000 (Inactive)	25.5 (Active)
$CHCl_3$	AI-35	87	>1000 (Inactive)	>1000 (Inactive)	26.7 (Active)
$CHCl_3$:ethylacetate	AI-39	11.5	>1000 (Inactive)	>1000 (Inactive)	9.7 (Active)
Ethylacetate:EtOH	AI-46	26.7	>1000 (Inactive)	>1000 (Inactive)	18.5 (Active)
Ethylacetate:EtOH	AI-55	55.6	>1000 (Inactive)	>1000 (Inactive)	27.0 (Active)
EtOH	AI-68	27.5	>1000 (Inactive)	>1000 (Inactive)	17.9 (Active)
Hexane	AS-11	71	>1000 (Inactive)	>1000 (Inactive)	29.8 (Active)
$CHCl_3$	AS-25	12.6	>1000 (Inactive)	>1000 (Inactive)	13.8 (Active)
$CHCl_3$	AS-32	85	>1000 >1000 (Inactive)	>1000 >1000 (Inactive)	30.4 (Active)
Ethylacetate:EtOH	AS-57	61.7	>1000 (Inactive)	>1000 (Inactive)	24.5 (Active)

AI – Chromatographic fractions of *Azadiracta indica*
AS – Chromatographic fractions of *Annona senegalensis*

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