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In vitro Cytotoxicity Studies and Qualitative Investigation of Phytochemicals of Stem Bark Extracts of *Detarium microcarpum (Caesalpinioideae), Echinaceae angustifolia (Compositae) and Isoberlinia doka (Fabaceae)*

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Abstract
Detarium microcarpum (Caesalpinioideae), Echinaceae angustifolia (Compositae) and Isoberlinia doka (Fabaceae) were selected in this study on the bases of their uses in Folk medicine by Lala tribe of Adamawa State - Nigeria to cure Jaundice, malaria and Headache diseases. Extracts prepared by soxhelet extraction with hot methanol from the plant were macerated and screened for the presence of secondary metabolites and activity in the brine shrimp (*Artemia salina* Leach) lethality test (BST). In the phytochemical screening, n-hexane extracts of the stem barks showed the presence of few secondary metabolites with the methanol extracts indicating the presence of most of the secondary metabolites. Extract of the stem barks of *Detarium microcarpum, Echinaceae angustifolia and Isoberlinia doka* exhibited high lethality on brine shrimp larvae with LC_{50} = 158.49µg/ml, 316.23µg/ml and 70.8µg/ml respectively. The leaves extracts exhibited a moderate potent activity at LC_{50} = 436.5 µg/ml. Maximum mortalities occurred at 1000µg/ml concentration while the least mortalities happened to be at 65.5µg/ml concentration. The results of both tests confirm that *Detarium microcarpum, Echinaceae angustifolia and Isoberlinia doka* are toxic and hence not safe especially at uncontrolled high dose.

Keywords: Phytochemicals *Artemia salina*, Brine shrimp test, Folk medicine

Introduction
For centuries medicinal plants have been prescribed and used with almost no change in the form or the way they have been used, and with a strong belief in their usefulness in diseases. The lack of a suitable, simple, and rapid screening procedure often hamper the study of bioactive compounds from plant sources and extracts in the chemical laboratory. 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 in 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 (Khaled, 2005) [5].

Monitoring the lethality is one of the simplest biological responses, since there is only one criterion; either dead or alive. In that case, the statistical analysis is relatively easy. The lethal concentration for 50% mortality after 24 h of exposure (the chronic LC_{50}) is determined as the measure of toxicity of the extract or compound. The choice of time, governed by the solubility of the extract or substance, is largely one of convenience, since the test is to be rapid and kept simple (Abdu, 2011).

The brine shrimp (*Artemia salina* Leach) is a simple zoological organism (an arthropod). The use of 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. This method is now widely used all over the world with a great success (Khaled, 2005) [5].

Detarium microcarpum (Caesalpinioideae), Echinaceae angustifolia (Compositae) and Isoberlinia doka (Fabaceae) were selected in this study on the bases of their uses in traditional medicine by Lala tribe of Adamawa State - Nigeria to cure Jaundice and malaria diseases A large number of Lala people are dependent on traditional medicine as their source of health care; Lala people therefore have a large repository of traditional knowledge which have been adopted from indigenous cultural practice. This knowledge comes gradually through trial and error especially those that served as food and remedies for diseases. One important factor in the survival of well-developed traditional practices in Africa is the oral tradition which results in the knowledge being widely held in the community. The knowledge of traditional medicine is transmitted orally by traditional healers, and elders of the community. This process involves naming the plants to be used and disease to be treated and observing the preparation

and application of the medicaments (Kubmarawa *et al.,* 2013) [6].

Plant collection and identification

The fresh stem bark of *Detarium microcarpum (Caesalpinioideae), Echinaceae angustifolia (Compositae) and Isoberlinia doka (Fabaceae)* were collected from Girei in Gombi Local Government Area of Adamawa State Nigeria in March, 2015. The plants were identified by Mr James of Girei town and authenticated by Botanists in the Department of Biological sciences Modibbo Adama University of Technology Yola.

Preparation of Plant Parts for Extraction

The plant samples were air dried and ground into powder with pestle and mortal and weighed. Around 60g of the powder was packed in a thimble of filter paper prepared manually. The thimble was then inserted into the Soxhlet apparatus, 500ml methanol was transferred down the thimble into the pot. A temperature of 75°C was maintained and extraction continued for 6 hours. Then the methanol extract was collected and the cake recovered from the thimble was kept. The methanolic extract was evaporated on a rotary evaporator (R110) at 40°C. Altogether, 200g of each sample were extracted and labeled F01. The crude extract F01 of the leaves, stem bark and root of isoberlinia doka was macerated using solvents in ascending order of polarity (n-hexane, chloroform, ethylacetate, and methanol). For each solvent 20ml was used to macerate the crude extract 5 times. The n-hexane soluble was collected, labeled as F02 and allowed to evaporate to dryness at 38°C. The chloroform, ethylacetate, and methanol soluble were in the same way collected and labeled as F03, F04, F05 and allowed to evaporate to dryness at 38°C.

Phytochemical Screening of Plant Material

All the plant extracts of the leaves, stem bark and root of *Isoberlinia doka* were screened for the presence of alkaloids, saponins, flavonoids, sterols, anthraquinones, phlobanins tannins, etc. using modified procedure described by Ogbeche (1994) and Adoum *et al.,* (2010).

Test for tannins

A small quantity of the extract was mixed with distilled water and heated on a water-bath. The mixture was filtered and a solution of H_2SO_4 followed by few drops of 5% $FeCl_3$ solution are added to the filtrate. A dark green or dark blue precipitate indicates presence of tannins.

Test for glycosides (general test)

Solution of H_2S0_4 (10ml 50%) was added to 1mI of extract in a test tube, this mixture heated in boiling water for 5 minutes. 10mL Fehling's solution A, and B (5 mL each) was added and boiled. Brick red precipitate indicates positive test.

Borntrager's Test for Anthraquinone Glycoside

Small portion of the extract was mixed with dilute hydrochloric acid (HCl), extracted with carbon tetrachloride (CCl_4) and then shaken with dilute ammonia (NH_3). A rose pink to cherry red color indicates the presence of anthraquinone glycoside.

Keller Killiani Test for Cardiac Glycoside

Small portion of the extract was mixed with 3ml of 3.5% solution of ferric chloride ($FeCl_3$) in glacial acetic acid and transferred to the surface of 2ml conc.H_2SO_4. A reddish-brown color at the inter-phase with the upper layer pale-green in color which gradually turn blue was an indication of a deoxy sugar.

Test for Cyanophoric Glycoside

A portion (1ml) of the extract was placed in a test tube, a piece of sodium picrate paper was then suspended above the level of the extract by trapping the edge of the paper between the cork fitting and the wall of the test tube. The set-up was allowed to stand for 30 minutes in a warm water (45ºC). A change in color of the picrate paper from yellow to brick-red indicates the presence of cyanophoric glycoside.

Test for resins

Copper (II) Sulphate solution (2.5ml) was added to 2.5 mL of the extract. The resulting solution was shaken vigorously and allowed to separate. A green colour indicates positive test.

Test for saponins (Frothing test)

The extract solution (2ml) was vigorously shaken in test tube for two minutes followed by heating in a water-bath to boiling point. Frothing indicates positive test.

Test for phlobatannins

Distilled water ($5cm^3$) was added to $5cm^3$ of extract solution and boiled with 1% HC1 for two minutes. A deep green colour indicates positive test.

Test for flavonoids.

Extract solution (2ml) was heated with 10 ml of ethyl acetate on a water bath and allowed to cool for the layers to separate. A layer of red coloration (ammonia) indicates positive test.

Salkowskii Test for Steroidal Nucleus

A small portion of the extract (0.5ml) was dissolved in 2ml $CHCl_3$, followed by 2ml dilute solution of H_2S0_4. A reddish-brown color at the interphase indicates presence of steroidal ring or nucleus.

Liebermann-Burchard Test for Steroidal Nucleus

A small portion of the extract was dissolved in $CHCl_3$ and filtered. The filtrate was mixed with 2ml acetic anhydride and 2 drops of conc. H_2SO_4. A color change from violet to blue or bluish-green indicates the presence of steroidal nucleus or ring.

Test for Phenols

Equal volumes of extract solution and $FeC1_3$ were mixed and allowed to stand for a while. A deep bluish green solution confirms the presence of phenols.

Test for volatile oils

The extract (0.2g) was mixed with 90% ethanol and 3 drops of ferric chloride was added. The appearance of green coloration confirms the presence of volatile oils.

Test for carbohydrate. (Reducing sugars) [Fehling test]
Mixtures of equal volumes (5ml) Fehling solution A and B was added to 2 ml of the extract in a test tube. The resultant mixture was boiled for two minutes. A brick red precipitate of copper oxide indicates a positive test.

Test for alkaloids
Concentrated solution of H_2S0_4 (1ml) was added to 3ml of the extract and then treated with few drops of Wagner reagent. Reddish brown precipitate indicates positive test.

Radulescu Test for Morphine Alkaloid
1ml of the extract was evaporated to dryness and the residue was dissolved in a 0.6ml of 1% H_2SO_4, 2ml of distilled water followed by 2 drops of 10% $NaNO_3$ were added. The solution was made alkaline with dilute ammonia solution. A reddish-brown solution indicates the presence of morphine alkaloid.

Thalleiquine test for Quinoline Alkaloid
To a portion of the extract was added 0.5ml of conc. HCl and a few crystals of $KClO_3$, it was carefully evaporated and a drop of strong ammonia was added to it. A green coloration indicates a positive test.

Test for Indole Alkaloid
A few drops of conc. H_2SO_4 and few crystals of $K_2Cr_2O_7$ were added to a small portion of the extract. A coloration is an indication of presence of an Indole alkaloid.

Vitalin-Morin Test for Tropane Alkaloid
A small portion of the dry sample of the extract was dissolved in few drops of fuming HNO_3, evaporated and allowed to cool. 2ml of acetone and a few drops of (3%) fresh alcoholic potash solution were added. A violet color indicates positive test.

Test for Terpenoid (Solkowski test)
The extract sample 0.2g was mixed with 2ml chloroform ($CHC1_3$) and carefully followed by conc. H_2S0_4 (3ml) to form a layer. A reddish brown coloration of the interface indicates positive test for the presence of terpenoids.

Preparation of test samples
Samples were prepared by dissolving 2.0mg of the methanolic plant extract in 5 ml of a suitable solvent (Stock solution). Dilution of this stock solution gives the series of concentrations required for testing. Three concentrations (3 replicates) were obtained for each series of tests. The negative and positive control solutions were simply distilled water and methanol respectively without test sample (plant extracts).

Screening of Plants Extract in the Brime Shrimp Lethality Test
Brime shrimp eggs (*Artemia salina* premium grade) was generously provided by Professor O. A. Adoum of the Department of Pure and Industrial Chemistry Bayero University Kano. All the plants fractions were phytochemically screened for carbohydrates, tannins, saponin, anthraquinones, cardiac and cyanogenic glycosides, flavonoids, alkaloids etc using modified procedures outlined by Adoum *et al., 2010*.
Artemia salina eggs was added into a hatching chamber ¾ filled with ocean sea water. The chamber was kept in an open space for 24hours, after which the eggs hatched into shrimp larvae. 4ml ocean water was then added and ten (10) larvae of Artemia salina were introduced into each vial. After 24 hours of introducing larvae, the number of survivals were counted in triplicate and recorded. To each sample vial, a drop of DMSO solvent was added, ten shrimps were transferred using a Pasteur pipette, and artificial seawater was added to make a total volume of 5 ml. The nauplii were counted against a lighted background. Counting for the chronic LC_{50} began 24 hour after initiation of tests. Nauplii were considered dead if they were lying immobile at the bottom of the vials, and the percentage of deaths at each dose and at the control were determined (Adoum *et al.*, 2010).

$$\% \text{ Deaths} = \frac{\text{No of dead shrimps}}{\text{No of survival shrimps in control}} \text{ X } 100$$

Microsoft Excel spreadsheet application was used to formulate the regression equations from the data of mean results of percentage mortality of the brine shrimp versus the log of concentrations. 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.

Results and Discussion

Table 1: BST Assay Results of MeOH extract of Stem bark of *Detarium microcarpum*

Conc. (µg/ml)	Survivals			Deaths			% mortality	LC_{50}(µg/ml)
	V	V	V	V	V	V		
1000	0	0	3	10	10	7	90.00	
500	2	3	2	8	7	8	76.67	
250	4	3	3	6	7	7	66.67	
125	6	7	7	4	3	3	33.33	158.49
62.5	9	10	8	1	0	2	10.00	
Ctrl(+)	0	0	0	10	10	10	100	
Ctrl(-)	10	10	10	0	0	0	0.00	

Table 2: BST Assay Results of MeOH extract of Stem bark of *Echinaceae angustifolia*

Conc. (µg/ml)	Survivals			Deaths			% mortality	Log_{10}Conc	LC_{50}(µg/ml)
	V	V	V	V	V	V			
1000	1	3	3	9	7	7	76.9	3	
500	5	5	3	5	5	7	56.67	2.7	
250	8	8	7	2	2	3	23.33	2.4	
125	8	7	9	2	3	1	19.33	2.1	316.23
62.5	9	10	8	1	0	2	10.90	1.8	
Ctrl(+)	0	0	0	10	10	10	100.00		
Ctrl(-)	10	10	10	0	0	0	0.00		

Table 3: BST Assay Results of MeOH extract of *Isoberlinia doka* Stem Bark

Conc. (µg/ml)	Survivals			Deaths			% mortality	Log_{10}Conc	LC_{50}(µg/ml)
	V	V	V	V	V	V			
1000	3	0	1	7	10	9	86.67	3	
500	4	6	0	6	4	10	66.67	2.7	
250	4	3	4	6	7	6	63.33	2.4	70.8
125	4	5	4	6	5	6	56.67	2.1	
62.5	6	4	5	4	6	5	50.00	1.8	
Ctrl(+)	0	0	0	10	10	10	100		
Ctrl(-)	10	10	10	0	0	0	0.00		

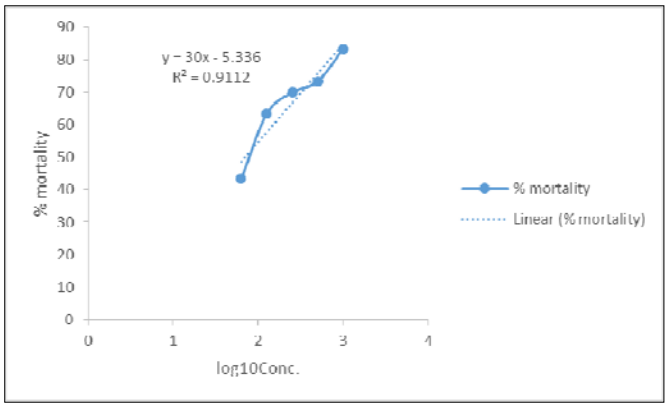

Fig 1: graph of % mortality versus log_{10}Conc. of Stem bark of *Isoberlinia doka*

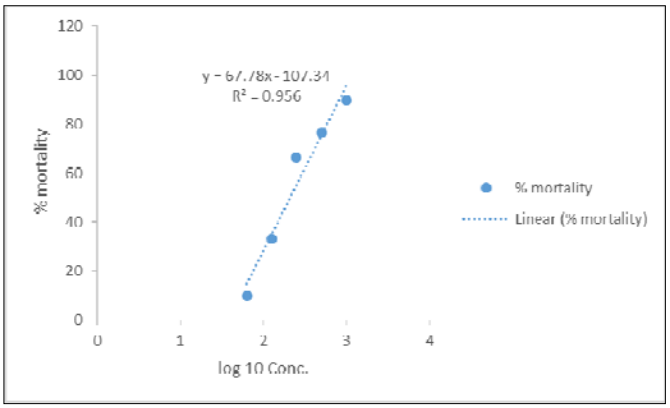

Fig 2: graph of % mortality versus log_{10} Conc. For Stem bark extract of *Detarium microcarpum*

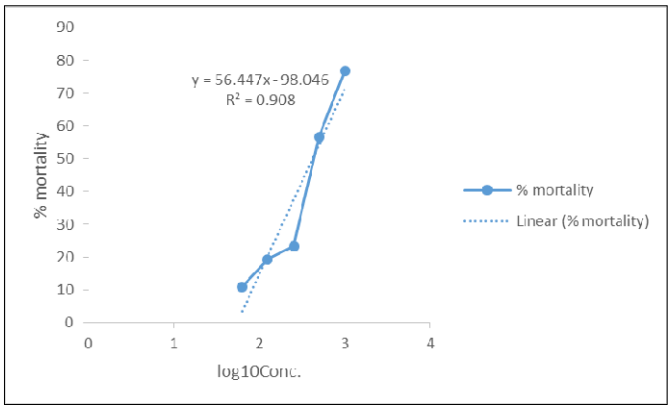

Fig 3: graph of % mortality versus log10 Conc. For Stem bark extract of *Echinaceae angustifolia*

In the phytochemical screening, n-hexane extracts of the leaves, stem bark and root showed the presence of few secondary metabolites with the methanol extracts indicating the presence of most of the secondary metabolites.

Brine shrimp LC_{50} values for the plant extracts evaluated in this study are reported in tables 1-3. All the extracts were found cytotoxic (LC_{50} < 1000 µg/ml) in the brine shrimp bioassay. Based on the percentage of the mortality, the concentration that led 50% lethality (LC_{50}) to the nauplii was determined by using the graph of mean percentage mortality versus the log of concentration.

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.

Conclusions

Our results suggest that methanol extracts of the stem bark of *Detarium microcarpum, Echinaceae angustifolia* and *Isoberlinia doka* showed apparent *in vitro* toxicity in the Brine Shrimp Assay. The results of the current study does not concur with the use of stem bark of these plants by traditional healers in Adamawa State of Nigeria especially at uncontrolled high dose. A World Health Organization survey indicated that about 70-80% of the world's population rely on non-conventional medicine, mainly from herbal sources, in their primary healthcare, hence *Detarium microcarpum, Echinaceae angustifolia* and *Isoberlinia doka* may be used as a medicinal agent only in low dosages, especially in rural communities where conventional drugs are unaffordable because of the high cost. The reported active (cytotoxic) extracts in this study are worth of further pharmacological and phytochemical studies in order to define what kind of antitumor and antimalarial activity they have (if any) and to isolate the natural 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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