CYTOTOXIC AGENTS FROM NIGERIAN PLANTS: A CASE STUDY OF *Spondias mombin* LINN (ANACARDIACEAE*)* LEAVES

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Abstract: In this work, six Nigerian plants traditionally used to manage inflammation and cancer have been screened for cytotoxicity against brine shrimps with the aim of assessing their potential as anticancer agents. The plants included *Spondias mombin* leaves, *Dichrostachys cinerea* root, *Crassocephalum crepidoides* leaves, *Crassocephalumrubens* leaves, *Myriantusarboreus* stem bark and *Maytenus senegalensis* root. Extraction of *Spondias mombin* (SM) leaves, *Dichrostachys cinerea* (DC) root, *Crassocephalum crepidoides* (CC) leaves, *Crassocephalum rubens* (CR) leaves, *Myriantus arboreus* (MA) stem bark and *Maytenus senegalensis* (MS) root with methanol yielded 11.87%, 7.11%, 6.51%, 6.4%,1.87% and 24% of crude extracts, respectively. The crude methanolic extracts showed lethal activities in Brine Shrimps Test(BST) in the order, SM>MS>DC>CC>MA>CR based on their LD_{50} (µg/mL) values of 29.17 ± 3.18, 32.05 ± 3.50, 37.90 ± 6.26, 42.39 ± 5.53, 46.32 ± 5.38 and 56.44 ± 5.01 and their LD_{90} (µg/mL) values of 115.12 ± 7.83, 120.04 ± 11.05, 142.53 ± 21.43, 153.87 ± 9.64, 164.98 ± 10.21 and 173.60 ± 7.75, respectively. Fractionation of SMcrude extract gave SM_1,> SM_2,>SM_4> SM_3 in increasing order of toxicity. Further purification of fraction SM_1 using vacuum liquid chromatography (VLC) gave fractions SM_{1-1}, SM_{1-2}, andSM_{1-3} with LD_{50} values at 24h as 4.63, 6.14 and 5.92 and LD_{90} values of 41.38, 47.72 and 45.68µg/mL, respectively. Column chromatography of fraction SM1-1 gave fractions CC1-CC7 which showed slightly more cytotoxic fractions, particularly fractions CC1, CC3, CC4 and CC6 with LD_{50} (µg/mL) values of 3.72±1.01, 4.26±0.33, 3.20±0.24 and 5.31±0.29, respectively. Thus, the selected plants have been confirmed to possess cytotoxicity. Among the extracts the leaves of *Spondias mombin* have exhibited the greatest activity. Also, fractionation has been shown to enhance the cytotoxicity of the extract in this work and has justified the activity of this plant in ethnomedicine for the management of inflammation and cancer.

Keywords: Nigerian plants, *Spondias mombin* leaves, Brine Shrimps cytotoxicity

Introduction

Plants are used medicinally in different parts of the world as sources of many potent medicines. Traditional medical practitioners use a variety of herbal preparations to treat different types of diseases. Many of the active principles are either primary or secondary metabolites (Kubmarawa *et al*., 2007). Despite the advancement of orthodox medicine, many developing countries of the world, especially in the rural areas, still rely heavily on the use of medicinal plants to meet their basic healthcare needs (Uchendu and Isek, 2008; Umeh *et al.,* 2009). Apart from natural products used as components of modern drugs, natural products are also used directly in the natural products based pharmaceutical industries which are growing rapidly in Europe, North America, Asia and Africa (Satyajit *et al*., 2006).

It is well known that cancer is second to cardiovascular disease as a natural cause of death. Most of the synthetic chemical agents currently being used in cancer therapy today are toxic and therefore potentially cause damage to normal cells (Hussein *et al*., 2013). Many plants have been investigated in order to obtain new, effective and safe antioxidant and anticancer drugs, as well as to study their mode of action of cancer cell inhibition (Hussein *et al*., 2013). In this work six Nigerian plants traditionally used to manage inflammation and cancer have been screened for cytotoxicity against brine shrimps with the aim of assessing their potential as anticancer agents. The plants included *Spondias mombin* leaves, *Dichrostachys cinerea* root, *Crassocephalum crepidoides* leaves, *Crassocephalum rubens* leaves, *Myriantus arboreus* stem bark and *Maytenus senegalensis* root.

Following preliminary cytotoxicity screening which showed that *S. mombin* is the most cytotoxic among the six selected Nigerian plants more detailed study was carried out on the plant using bioassay-guided fractionation using solvent partitioning and chromatography. *Spondias mombin* Linn, family Anacardiaceae, is commonly known as Hog plum in English language, but in Nigeria it is called 'Ijikara or' Ichikara by the people of south eastern state," Iyeye" in the south western region, and "Tsardarmasar" in the northern region. It is common in the forest and savanna regions of Nigeria and is a medium sized but occasionally large tree (Uchendu and Isek, 2008). *Spondias mombin* is a fructiferous tree that thrives in the rainforest and coastal areas of Africa It is widely found in tropical America, Asia and Africa, and has been recently cultivated in commercial quantities in Mexico (Leon and Shaw, 1990). It also has a wide distribution in Southern America and the West Indies but grows to a limited extent in the Indian subcontinent and Indonesia. It may be propagated by seeds and vegetative using stem cuttings.

All parts of the plant have been reported to be medicinally useful (Onwuka, 1992; Nzegbule and Meregini, 1999; Uchendu and Isek, 2008; Taylor, 2004; Breazile, 1971). Extracts of *S. mombin* L. (Anacardiaceae) are used in the traditional medicines of Africa and Latin America to treat many inflammatory conditions, with repeated claims of efficacy (Nworu *et al*., 2011). Preliminary phytochemical test on aqueous extracts of the plant had revealed the presence of saponins and tannins as the main phyto-constituents of the plant.

These findings demonstrate the possible effectiveness of the plant, especially its leaf extracts, in treating microbial infections. A series of 6-alkenyl-salicylic acids have been isolated from the ethanolic extract of leaves and stems of *Spondias mombin* by a combination of chromatographic methods. These phenolic acids were shown to have a pronounced antibacterial effect against *Bacillus cereus*, *Streptococcus pyogenes*, and *Mycobacterium fortuitum* and a molluscicidal effect against the snail *Biomphalariaglabrata*, an intermediate host in the schistosome life cycle (Corthout *et al*., 1994). Another chemical investigation of *Spondias* leaf

extracts showed the occurrence of quercetin, rutin and ellagic acid, compounds associated with antioxidant and antimicrobial activities (Araujo da Silva *et al*., 2012). Also, the leaves have been reported to contain antiviral ellagitannins and caffeoyl esters and antibacterial and molluscicidal phenolic acids (Corthout *et al*, 1994). The leaves have also been demonstrated to have antihelmintic (Ademola *et al.*, 2005) and abortifacient (Offiah and Anyanwu, 1989) activities.

In our search for cytotoxic plants with potentials for the management of inflammation and cancer we have studied six Nigerian plants used traditionally for that purpose. We now wish to report on the cytotoxicity of the six Nigerian plants against brine shrimps and in particular on the effect of fractionation on the cytotoxicity of the leaves extract of *Spondias mombin* which was the most toxic.

Materials and Methods

Materials

The leaves of *Spondias mombin*, *Crassocephalum crepidoides* and *Crassocephalum rubens* were collected from Odo-Owa, OkeEro Local Government of Kwara State while the roots of *Dischrostachys cinnerea and Maytenus senegalensis* were collected from Minna and Bida, respectively, in Niger State, Nigeria. The stem bark of *Myrianthusarboeus* was collected at Ido-Ekiti in Ekiti State, Nigeria. The species *Spondias mombin* and *Crassocephalum crepidioides* were identified at the Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria with voucher specimen numbers IFE17284 and IFE17253, respectively. *Maytenus senegalensis* and *Dichrostachys cinnerea* were authenticated at the Department of Chemistry, Federal University of Technology, Minna, while *Crassocephalum ruben* and *Myrianthus arboreus* were identified at the Department of Botany. The plants were air-dried and separately milled into powder using a blender. The samples were put in a vial and kept in the fridge prior to analysis. All solvents and chemicals used in this work were of analytical grade and were appropriately purified before use. The materials for the toxicity bioassay included *Artemiasalina* cyst (egg), sea water, an aerator, capillary tubes and 60 watt bulbs. The *Artemiasalina* cyst (egg) and sea water were obtained from the Nigerian Institute of Oceanography and Marine Research, Lagos.

Methods

Extraction of plant materials and fractionation of crude extracts

The powdered plant materialswere cold extracted each with methanol (1L x 6 days). The extracts were concentrated *in-vacuo* to drynessand the crude extracts were coded SM_0, DC_0, CC_0, CR_0, MS_0 and MA_0, respectively. Each of the crude extracts was dissolved in 100 mL of distilled water and successively partitioned with n-hexane, chloroform, ethyl acetate and water. The organic phase residues were coded as SM_1, SM_2, SM_3 and SM_4; DC_1, DC_2, DC_3 and DC_4; MS_1, MS_2, MS_3 and MS_4; CC_1, CC_2, CC_3 and CC_4; MA_1, MA_2, MA_3 and MA_4, respectively (Djilana and Legseir, 2005).

Preliminary phytochemical screening of the methanolic extract of Spondiasmombin

The crude extract of *S. mombin* (SM_0), as the most cytotoxic was selected for further work and was then subjected to preliminary phytochemical screening using the procedures of Sofowora (1993) and Evans (1996).

Chromatographic purification of fraction SM_1

The hexane-soluble fraction (33.0 g) of the crude extract of *S. mombin* (SM_1) was subjected to chromatographic purification using vacuum liquid chromatography (VLC) on silica gel (60-120 mesh) and eluting successively with hexane, hexane-chloroform, chloroform-ethylacetate and methanol. The cytotoxic fraction $SM_{1\text{-}1}$ (3.79 g) was obtained with hexane and hexane/chloroform. This was further purified by column chromatography on silica gel 60G using hexane, hexane-chloroform, chloroform/methanol and methanol as eluents to give seven fractions, CC_1-CC_7.

Artemiasalina cyst bioassay

Each of the crude extracts and fractions was subjected to cytotoxicity screening using the procedure of Mclaughlin *et al*. (1998).

Preparation of shrimp nauplii

Artemiasalina cysts (eggs) (2.0 g) were placed in 800 mL sea water (salt solution: 38.0 g of NaCl per litre of distilled water) in 1L conical flask under 60 watt bulbs with an aerator and aeration stone connected with a rubber capillary. The aeration and the illumination were done for 48h for the hatching of the *A. salina* cysts (eggs) into shrimp nauplii. The shrimps were left under aeration and illumination for another 48 hours before the cytotoxicity bioassay.

Preparation of samples for Brine Shrimp cytotoxicity bioassay

The sample (20 mg) was dissolved in 2 mL of solvent to give 1000 µg/mL. Serial dilutions of the extract were prepared by taking 0.2 ml from 1000 µg/mL into 1.8 mL solvent to give 100 µg/mL. Furthermore, 0.2 mL was taken from 100 µg/mL into 1.8 mL solvent to give 10µg/mL (recommended final dilutions of 10, 100, 1000 µg/mL). Each of the samples was prepared by weighing 2.0 mg into 1.8 mL of the solvent to give 100µg/mL and dilutions of 10 and 1.0 ppm were prepared from it.

2.2.4.3 Cytotoxicity bioassay using shrimp nauplii

Sea water (3 mL) was dispensed into a calibrated 5ml vial and then 10 shrimp nauplii were counted into it. A transparent glass capillary was used for the counting into the vials. To each of the vials the 0.5 mL of the prepared extract or fraction or isolate was added and then made up to 5 ml with sea water. All the bioassay experiments were performed in triplicate. After 24 h the number of shrimp nauplii that survived (lethality estimate) and number of shrimp nauplii with sluggish motion (sedative estimate) were determined. The analyses of the lethality data and the LD_{50} and LD_{90} at 95% confidence limit using the Finney Computer program were determined.

Results and Discussion

The results of the plant yield are presented in Table 1. From Table 1 *Spondiasmombin* leaves gave the highest yield of crude methanolic extract. The crude extracts were investigated for cytotoxicity against brine shrimps (*Artemiasalina*) in a search for plants with potential anti-cancer activity. The results of preliminary cytotoxicity screening are shown in Table 2.

Table 1: **Yields of crude methanolic extracts of six Nigerian plant materials**

Extract	Plantweight (g)	Extract (g)	Yield (%)
SM_0	610.00	72.31	11.85
CC_0	1000.0	65.1	6.51
CR_0	1000.0	5.84	5.84
MA_0	1000.0	18.72	1.87
DC_0	1000.0	71.1	7.11
MS_0	1000.0	270.0	24.55

Spondias mombin = SM_0, *Dichrostachys cinerea* = DC_0, *Crassocephalum crepidoides* = CC_0, *Crassocephalum rubens* = CR_0, *Myriantus arboeus* = MA_0, Maytenus *senegalensis* = MS_0

Table 2:Results of preliminary cytotoxicity screening of crude methanolic extracts of six Nigerian plants against *Artemiasalina*

Sample	N	LD_{50} µg/mL	LD_{90} µg/mL
CC_0	6	42.39 ± 5.53^{abc}	153.87 ± 9.64^{ab}
CR_0	6	56.44 ± 5.01^{ab}	173.60 ± 7.93^{ab}
SM_0	6	29.17 ± 3.18^{ab}	115.12 ± 7.88^{b}
MA_0	6	46.32 ± 5.38^{ab}	164.98 ± 10.21^{a}
MS_0	6	32.05 ± 3.50^{bc}	120.04 ± 11.05^{bc}
DC_0	6	37.90 ± 6.26^{bc}	142.53 ± 21.43^{b}

Mean on the same column with different superscripts are significantly different from each other ($P \leq 0.05$), while those with the same superscript are not significantly different ($P > 0.05$); N= Number of replicates; *Spondiasmombin*= SM_0, *Dichrostachyscinerea*= DC_0, *Crassocephalumcrepidoides*=CC_0,*Crassocephalumrubens*= CR_0,*Myriantusarboeus*= MA_0, *Maytenussenegalensis*= MS_0

The activities of the extracts are in the order of SM_0> MS_0> DC_0> CC_0>MA_0>CR_0. Thus, SM_0 is the most active at the crude level among the six Nigerian plants (Déciga-Campos *et al*., 2007, Amara *et al*., 2008,Shoeb*et al*., 2014) with cytotoxicity values of LD_{50} $29.17^c \pm 3.18$ and LD_{90} $115.12^c \pm 7.83$. It was then selected for further work including phytochemical screening, fractionation and cytotoxicity screening of the sub-fractions. The results of phytochemical screening are presented in Table 3, but contrary to results obtained by some researchers saponins and volatile oils were not observed(Uzama*et al*., 2016). The crude extract of *S. mombin*leaves was fractionated into hexane, chloroform, ethyl acetate and water to give fractions SM_1-SM_4 which were subjectedto further cytotoxicity screening against Brine shrimps. The results are reported in Table 4.

Table 3: Qualitative phytochemical screening of the crude methanolic extract of *Spondiasmombin* leaves

Phytochemicals	Remarks
Tannins	+++
Flavonoids	+++
Saponins	-
Alkaloids	++
Steroids	++
Cardiac Glycosides	+
Anthraquinones	+++
Phlobotannins	+
Volatile Oils	-
Chlorogenic Acids	-
Coumarins	++
Terpenoids	+++

+++ = very high; ++=high; += low; -=nil

Table 4: The results of cytotoxicity screening of fractions of crude extract of *S. mombin* (SMo)

Fractions	No of rep	LD_{50} µg/mL	LD_{90} µg/mL	Wt of fraction (g)
SM_0	6	29.17 ± 3.18^{ab}	115.12 ± 7.88^{b}	**72.31**
SM_1	3	3.00 ±1.151	13.3913± 3.27	39.68
SM_2	3	38.65±15.90	$1.03x10^5$±32.25	0.76
SM_3	3	111.81±33.99	710.41±45.65	9.6
SM_4	3	57.69±23.51	$3.95x10^4$±124.25	18.74

n-hexane=SM_1, chloroform=SM_2, ethylacetate=SM_3, aqueous=SM_4

The n-hexane-soluble fraction (non-polar components) constitutes about 50% of the crude extract of the leaves of *S. mombin* which is the highest yield. Thus, the order of yield is hexane>aqueous>ethyl acetate>chloroform. The phytochemical screening revealed the presence of tannins, flavonoids, alkaloids, steroids, cardiac glycosides, anthraquinones, phlobotannins, coumarins and terpennoids which supports the findings of Omotayo and Borokini (2012) and Arauja da Silva *et al.* (2012).

The crude methanol extract and the fractions from solvent partitioning, the VLC and column chromatography were subjected to cytotoxicity screening using Brine Shrimp Test. The results are recorded in Tables 5 and 6.

The crude methanolic extract of *S. mombin* leaves showed lethal activity against Brine shrimps at LD_{50} (µg/mL) of $29.17^c \pm 3.18$ and LD_{90} (µg/mL) of $115.12^c \pm 7.83$, respectively (Table. 4). Table 4 showed that fractionation apart from re-distributing the phytochemicals in the fractions also enhanced the toxicity drastically in the order SM_1> SM_2> SM_4> SM_3. The toxicity of the n-hexane fraction is about ten times that of the crude methanolic extract. This is in agreement with the findings of some workers on the effect of fractionation ((Huang *et al.*, 2001). Their activities at LD_{50} at 24 hour were 3.004 ± 1.151, 38.649 ± 15.901, 57.69 ± 23.51 and 111.81 ± 33.29 µg/mL while their LD_{90} values were in the order SM_1>SM_3> SM_4> SM_2 ; 13.39 ±3.27, 710.41 ± 251.0, $3.9500.00x10^4$, $1.03x10^5$ ± $0.066x10^5$, µg/ml, respectively (Table 4). The work has also demonstrated that further purification of the extract after the initial solvent fraction using chromatographic fractionation though did not dramatically increase activity it continued to slightly improve in one or more fractions (Tables 5 and 6).

Table 5: Results of cytotoxicity screening of crude methanolic extract and VLC fractions against *Artemiasalina*

Samples	LD_{50} µg/mL	LD_{90} µg/mL
SM_o	29.17±3.18	115.12±7.83
SM_1	3.00 ±0.066	13.39±1.89
SM_{1-1}	4.63±0.76	41.38±3.71
SM_{1-2}	6.14± 0.45	47.07 ±1.31
SM_{1-3}	5.92± 0.29	45.68 ±2.15
SM_{1-4}	$1.24x10^4$ ±00	$1,12x10^5$±$1.12x10^5$

Table6: Results of toxicity screening of column chromatographic fractions of SM_{1-1}

Column fractions	LD_{50} µg/mL	LD_{90} µg/mL	Wt. (g)
CC1	3.72 ± 1.01^{b}	$1001 \pm 240,35^{a}$	1.1845
CC2a	9.72 ± 6.22^{ab}	$1.09x10^6 \pm 91.06^{b}$	0.1652
CC2b	12.96 ± 4.85^{ab}	587.61 ± 53.24^{ab}	0.3389
CC3	4.26 ± 0.33^{b}	33.32 ± 0.78^{b}	0.3143
CC4	3.20 ± 0.24^{b}	24.55 ± 1.97^{b}	0.1400
CC5	16.11 ± 4.65^{a}	53.99 ± 7.02^{b}	0.5968
CC6	5.31 ± 0.29^{ab}	38.54 ± 0.39^{b}	0.2570
CC7	9.58 ± 4.51^{ab}	40.81 ± 7.81^{b}	0.5523

According to the National Cancer Institute (NCI, 2011/2012), the acceptable cytotoxic activity for crude extracts tested against HepG2 cell line is an IC_{50} value of 20 µg/mL (Shoeb *et al*., 2014). Thus, based on NCI standards the crude extract (MS_0) was very toxic and therefore active (Déciga-Campos *et al*., 2007, Amara *et al*., 2008, Shoeb *et al*., 2014). Cytotoxicity screening using BST has been used to evaluate the potentials of plants in the management of various diseases, including cancer and the toxicity of plants is due to the presence of phytochemicals (Rice-Evans, 2004).The solvent partition scheme according to some workers, has been used to search for new bioactive compounds even from plants that previously exhibited poor activity (Hostettmann *et al*., 2010, Mitscher *et al*., 1987). Thus, in this work the SM_1 fraction harbors a very high percentage of the active constituents

against brine shrimps and with LD_{50} 3.00±0.66 µg/ml was the most toxic. The other fractions SM_2, SM_3 and SM_4 however did not fail the NCI benchmark for brine shrimp toxicity test because only LC_{50} values above 1000 µg/mL are considered to be non-toxic (Déciga-Campos *et al*., 2007; Amara *et al*., 2008; Shoeb *et al*., 2014). Thus, the non-polar, medium polar and polar fractions of the leaves of *S. mombin* are appreciably toxic and have the potentials of being used as anti-inflammatory and anti-cancer agents.

Conclusion

The results obtained from this work revealed that the six plants screened were active at crude level in Brine Shrimps Test. Two of the plants (*C. crepidoides* and *C. rubens*) which can be classified as vegetables showed reduction in activity when fractionated. Therefore, these plants could be used at crude level to manage inflammation and cancer by incorporating them in the daily diet of *patients* with tumor symptoms. The plant *S. mombin* however showed progressively improved cytotoxicity with successive fractionations.

This work therefore supports the basis for the ethnomedicinal uses of these plants and in particular *S. mombin*, for the management of inflammation and cancer. In future work the column chromatographic fractions will be examined to isolate and characterize the cytotoxic components.

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