Journal of Ethnopharmacology 128 (2010) 501–512

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Ethnobotanical survey and cytotoxicity testing of plants of South-western Nigeria used to treat cancer, with isolation of cytotoxic constituents from *Cajanus cajan* Millsp. leaves

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ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form
28 December 2009
Accepted 3 January 2010
Available online 11 January 2010

Keywords:
Ethnobotanical survey
Cytotoxicity
Cancer
Cajanus cajan
Stilbenes
SRB assay
XTT assay

ABSTRACT

Ethnopharmacological relevance: There is only scant literature on the anticancer components of medicinal plants from Nigeria, yet traditional healers in the area under study claim to have been managing the disease in their patients with some success using the species studied.
Aim of study: To document plants commonly used to treat cancer in South-western Nigeria and to test the scientific basis of the claims using *in vitro* cytotoxicity tests.
Methods: Structured questionnaires were used to explore the ethnobotanical practices amongst the traditional healers. Methanol extracts of the most common species cited were screened for cytotoxicity using the sulforhodamine B (SRB) assay in both exposure and recovery experiments. Three cancer cell lines (human breast adenocarcinoma cell line MCF-7, human large cell lung carcinoma cell line COR-L23 and human amelanotic melanoma C32) and one normal cell line (normal human keratinocytes SVK-14) were used for the screening of the extracts and the fractions obtained. The extract of *Cajanus cajan* showed considerable activity and was further partitioned and the dichloromethane fraction was subjected to preparative chomatography to yield six compounds: hexadecanoic acid methyl ester, α-amyrin, β-sitosterol, pinostrobin, longistylin A and longistylin C. Pinostrobin and longistylins A and C were tested for cytotoxicity on the cancer cell lines. In addition, an adriamycin-sensitive acute T-lymphoblastic leukaemia cell line (CCRF-CEM) and its multidrug-resistant sub-line (CEM/ADR5000) were used in an XTT assay to evaluate the activity of the pure compounds obtained.
Results: A total of 30 healers from S W Nigeria were involved in the study. 45 species were recorded with their local names with parts used in the traditional therapeutic preparations. Cytotoxicity (IC_{50} values less than 50 μg/mL) was observed in 5 species (*Acanthospermum hispidum*, *Cajanus cajan*, *Morinda lucida*, *Nymphaea lotus* and *Pycnanthus angolensis*). *Acanthospermum hispidum* and *Cajanus cajan* were the most active. The dichloromethane fraction of *Cajanus cajan* had IC_{50} value 5–10 μg/mL, with the two constituent stilbenes, longistylins A and C, being primarily responsible, with IC_{50} values of 0.7–14.7 μM against the range of cancer cell lines.
Conclusions: Most of the species tested had some cytotoxic effect on the cancer cell lines, which to some extent supports their traditional inclusion in herbal preparations for treatment of cancer. However, little selectivity for cancer cells was observed, which raises concerns over their safety and efficacy in traditional treatment. The longistylins A and C appear to be responsible for much of the activity of *Cajanus cajan* extract.

1. Introduction

There is currently a global renaissance of ethnobotanical surveys of medicinal plants and the need for screening specific parts of plants (Li and Vederas, 2009; Paterson and Anderson, 2005; Igoli et al., 2005). Although much screening of medicinal plants for potential anticancer activity has occurred in the last fifty years, the study of ethnopharmacological leads from African medicinal plants has not been realized as fully as from other traditional societies such as India and China. However the recent screening of 7500 species from South Africa (Fouche et al., 2008) marks some progress and the present work investigates some species from South-western Nige-

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doi:10.1016/j.jep.2010.01.009

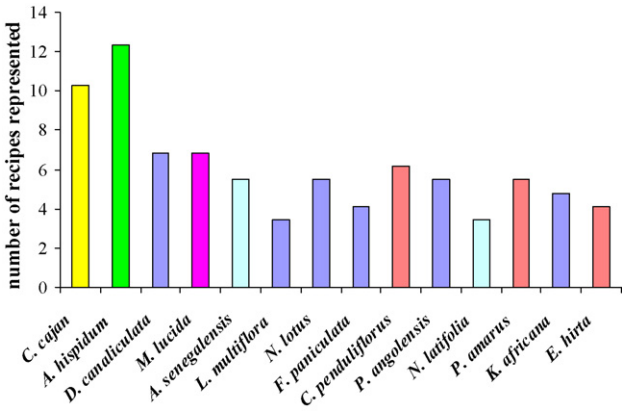

Fig. 1. Species frequency in the recipes commonly used for treatment of various cancer types in South-western Nigeria as evident from the ethnobotanical survey.

ria. The Nigerian government has recently set aside US$1billion for the development of traditional medicine and to encourage its integration at all levels of health care delivery system of the country (Adelaja, 2006).

In South-western Nigeria, traditional medicine is part of the cultural heritage, and is acceptable to the majority of the populace. However the secrecy attached to it has prevented much scientific recording of the knowledge and few literates have been taken into confidence. The present study investigates some plants from this area used to treat cancer by local healers, who divulged the information as part of a survey built on established trust.

2. Experimental

2.1. Ethnobotanical survey

The survey was carried out in four major states [Ogun, Oyo, Lagos and Ekiti] in the South-western region of Nigeria. This area contains savannah, mangrove and rain forest vegetations and has a diversity of ethnic groups including Awori, Egun, Egba, Ekiti, Eyo, Ijebu, Oyo, Yewa, and Yoruba.

Table 1
List of plants that are used in South-western Nigeria for treatment of cancer.

Botanical name	Local name(s) (Yoruba)	Voucher number	Family	Part(s) used	Mode of preparation and use (T-topical; O-drunk)
Acanthospermum hispidum D.C.	Dagunro, Dagunro	FHI106989	Asteraceae	Flowering shoots	Poultice (**T**); decoction (**O**)
Acanthus montanus (Nees) T. Anderson	Ahon-Ekun	FHI109031	Acanthaceae	Leaves and roots	Poultice (**T**)
Adenopus breviflorus Benth.	Tagiiri	FHI109040	Cucurbitaceae	Unripe fruit and leaves	Poultice(**T**)
Aframomum melegueta [Roskoe] K. Schum.	Ata-ire	FHI109051	Zingiberaceae	Fruiting shoot	Poultice(**T**)
Allium ascalonicum L.	Alubosa elewe	FHI109046	Liliaceae	Whole plant	Decoction(**O**); infusion(**O**)
Anchomanes difformis (Blume) Engl.	Agooni, Abirisoko	FHI109032	Araceae	Rhizome	Infusion(**O**); poultice(**O**)
Annona senegalensis Pers.	Abo	FHI106990	Annonaceae	Leaves	Infusion(**O**); decoction(**O**)
Cajanus cajan (L.) Millsp.	Otili	FHI106993	Fabaceae	Leaves	Poultice(**T**); concoction(**O**)
Canavalia ensiformis (L.) D.C.	Sese-nla	FHI109033	Papilionaceae	Seeds	Poultice(**O**); decoction(**O**)
Croton penduliflorus Hutch.	Aworo-oso	FHI106988	Euphorbiaceae	Seed and stem bark	Decoction(**O**)
Croton zambesicus Muell. Arg.	Ajeofole	FHI109041	Euphorbiaceae	Stem bark	Decoction(**O**); concoction(**O**)
Curculigo pilosa (Schum. and Thonn.) Engl.	Epa-ikun	FHI109047	Hypoxidaceae	Rhizome	Infusion(**O**)
Cymbopogon citratus (D.C.) Stapf	Ekan	FHI109052	Poaceae	Leaves and rhizome	Concoction(**O**)
Dioclea reflexa Hook. f.	Ebe, Agbarin	FHI109054	Fabaceae	Seeds	Poultice(**T**)
Dioscorea hirtiflora Benth.and Hook.	Isanyinahun	FHI109034	Dioscoreaceae	Leaves	Decoction(**O**)
Diospyros canaliculata De Wild.	Oriloje, Odubu	FHI106994	Ebenaceae	Leaves	Decoction(**T**, **O**)
Entandrophragma macrophyllum A. Chev.	Arunje, Ijebo	FHI109042	Meliaceae	Stem bark	Decoction(**O**)
Euphorbia heterophylla L.	Oro	FHI109053	Euphorbiaceae	Root and leaves	Decoction(**T**)
Euphorbia hirta L.	Emile, Oro-elewe,	FHI109062	Euphorbiaceae	Shoot	Concoction(**O**, **T**)
Euphorbia laterifolia L.	Enu-kopire	FHI109056	Euphorbiaceae	Root and leaves	Infusion(**T**)
Euphorbia poissonii L.	Oro-adete	FHI109035	Euphorbiaceae	Sap	Poultice(**T**)
Flabellaria paniculata Cav.	Lagbolagbo	FHI106996	Malpighiaceae	Leaves	Decoction(**O**)
Funtumia africana (Benth.) Stapf.	Ako-ire	FHI109039	Apocynaceae	Stem bark	Decoction(**O**)
Imperata cylindrica (L.) Räusch.	Ekan	FHI109045	Poaceae	Root	Decoction(**O**)
Jatropha curcas L.	Botuje, Lapalapa	FHI109036	Euphorbiaceae	Root and stem bark	Decoction(**T**, **O**)
Jatropha gossypifolia L.	Botuje-pupa	FHI109050	Euphorbiaceae	Root and stem bark	Decoction(**T**, **O**)
Kigelia africana (Lam.) Benth.	Pandoro	FHI109060	Bignoniaceae	Fruit	Decoction(**T**, **O**)
Lippia multiflora Moldenke	Eforomoba	FHI106995	Verbenaceae	Leaves	Poultice(**T**)
Luffa cylindrica (L.) Roem.	Kankan-ayaba	FHI109037	Curcurbitaceae	Fruit and leaves	Infusion(**T**)
Mezoneuron benthamianum Baill.	Jenifiran, Ekanan-Ekun	FHI109058	Caesalpinaceae	Root	Decoction(**O**)
Microdesmis puberula Hook. f. ex Planch	Esunsun	FHI109038	Euphorbiaceae	Root	Decoction(**O**)
Mitragyna inermis (Willd.) K. Schum.	Okobo	FHI109049	Rubiaceae	Stem bark	Decoction(**O**)
Morinda lucida Benth.	Oruwo	FHI106992	Rubiaceae	Leaves and stem bark	Infusion(**O**); decoction(**O**)
Nauclea latifolia Smith	Ira	FHI109044	Rubiaceae	Root and leaves	Decoction(**O**)
Nymphaea lotus L.	Osibata	FHI106987	Nymphaceae	Leaves	Decoction(**T**, **O**)
Olax subscorpioidea Oliv.	Ifon	FHI109065	Olacaceae	Root	Decoction(**O**)
Phyllanthus amarus Schumach. and Thonn.	Ajelara	FHI109059	Euphorbiaceae	Shoot	Decoction(**T**)
Piptadeniastrum africanum (Hook.f.) Brenan	Agboin	FHI109043	Mimosaceae	Root	Decoction(**O**)
Pycnanthus angolensis (Welw.) Warb.	Akomu	FHI106991	Myristicaceae	Stem bark	Poultice(**O**)
Securidaca longipedunculata Fres.	Ipeta	FHI109048	Polygalaceae	Root	Decoction(**O**, **T**)
Triplochiton scleroxylon K. Schum.	Arere	FHI109063	Sterculiaceae	Stem bark	Decoction(**T**)
Vernonia amygdalina L.	Ewuro	FHI109061	Compositae	Root	Decoction(**O**)
Xylopia aethiopica (Dunal) A. Rich.	Eeru	FHI109057	Annonaceae	Fruits	Decoction(**O**); poultice(**T**)
Zanthoxylum zanthoxyloides Zepern. and Timler	Ata	FHI109064	Rutaceae	Stem bark and root	Decoction(**O**)
Zingiber officinale Roscoe	Ata-ile	FHI	Zingiberaceae	Rhizomes	Concoction(**O**), decoction(**O**)

This present work employed the strategy suggested by Heinrich (2000) to evolve a relationship between researcher and the traditional healers. This relationship is built on mutual trust so that a forum is developed to explain cancer to local healers. Pictures of sufferers were used to explain to the traditional healers how cancers, especially solid tumours, are presented. Swellings due to inflammation could usually be distinguished by their smaller size. In addition, the healers frequently treated patients who had had cancer diagnosed in a state hospital but who, for financial, geographical or personal reasons, chose to be treated by a traditional healer. Meetings with all the traditional healers in various districts were then held and those who had at one time or another handled cases, diagnosed first in a hospital, with a degree of success (from patients' testimonies) were enrolled to participate in the survey. Questionnaires were drawn up to explore the practices, claims and bio-data of the respondents (see Appendix A). The survey was conducted in the Yoruba language with most of the healers, but Egun was used in a few cases. One of us (JSA) is fluent speaker in both languages and his training in taxonomy of West African plants helped in the immediate identification of the plants in most cases. Where there was difficulty in identifying the plants, the traditional healers pointed out specimens, which were collected and taken to the herbarium for identification as detailed below. Herb sellers in most cases were not involved since the interview could not be conducted with their clients. In all, a total of 60 questionnaires were distributed and contact was later made with individuals to help them complete the form personally. Those that appeared to have misconstrued other inflammatory conditions for cancer were excluded from the list and finally 30 healers were involved in the study.

2.2. Plant material

Of the 45 species recorded in the survey (Table 1), only the ten with most frequent occurrence (Fig. 1) were collected. Relevant parts of the species used were collected from Ogun State in South-western Nigeria during the rainy season (April–August) 2004. Authentication of the plants was carried out in the herbarium of the Forestry Research Institutes (FRIN), Jericho, Ibadan, Nigeria by Dr. M.O. Soladoye. All plants collected were cleaned immediately of extraneous material and were then air-dried in a moisture-regulated room.

2.3. Preparation of extracts

Since poultices were a common traditional mode of application, the dried powdered plant material (1 kg each) was extracted by percolation with methanol AR. The extracts were concentrated to dryness under reduced pressure at 45 °C. The dried extracts were kept in desiccators. Five species *Acanthospermum hispidum*, *Annona senegalensis*, *Croton penduliflorus*, *Diospyros canaliculata* and *Nymphaea lotus*, are traditionally employed as aqueous extracts, so 150 g of each of were extracted with water using a Soxhlet extractor for 4 days, filtered and the resulting extracts were subsequently freeze-dried.

Yields from both methanol and water extractions are shown in Table 2.

2.4. Fractionation of methanol extract of Cajanus cajan and isolation of compounds

60 g of methanol extract of *Cajanus cajan* dried leaves were adsorbed on silica gel F_{254} and separated into fractions using vacuum liquid chomatography (VLC; 15 cm × 16 cm) employing solvents in increasing order of polarity [hexane (Hex) (1.5 L), dichloromethane (2.0 L), chloroform (2.0 L), ethyl acetate EtOAc (1.5 L), acetone (1.5 L) and methanol (2.0 L)].

Cytotoxicity testing of each fraction showed that the greatest activity lay in the hexane and dichloromethane fractions (Table 5). The TLC profile of these two fractions gave similar profiles so they were combined and subjected to flash chomatography (Biotage flash pack 20 g column) with Hex:EtOAc 80:20, aliquots of 15 mL being collected. Aliquots were monitored by TLC (Silica gel G F_{254} Merck) using 0.5% anisaldehyde in 10% H_2SO_4 followed by heating at 105 °C for 10 min. Aliquots showing the same components were combined and PTLC, using the same solvent system, used to isolate the six pure compounds JK1 from fractions (F) 6–15 (50 mg), JC1 from F16–20 (170 mg), JSLP1 from F21–25 (4.3 mg), JSLP from F21–25 (15.4 mg), JC3b from F21–25 (15.4 mg) and JC4 from F 21–25 (35.4 mg).

2.5. Identification of isolated compounds

Melting points (m.p.) were determined with a Gallenkamp Instrument and are uncorrected. All Low Resolution Electron Impact Mass Spectroscopy (LR-EI-MS) were obtained on a Jeol AX505W. High resolution spectra were from a Bruker Apex III System. ^{1}H NMR and ^{13}C NMR spectra were obtained from a 500 MHz Bruker DRX 500 spectrometer with chemical shift reported in δ (ppm) using TMS as an internal standard and deuterated chloroform ($CDCl_3$) as the solvent. Infra-red (IR) spectroscopy data were recorded on a Perkin–Elmer 1600 Spectrum One FT-IR with a Durasampler II-Diamond attachment. UV spectra were recorded on a Perkin–Elmer Lambda 2 UV/VIS spectrometer. Structures were determined by comparison of the spectral data with those in the literature (Cuong et al., 1996; Hopp and Inman, 2003; Delle Monache et al., 1977).

Table 2
The percentage yield of crude extracts.

Plant name	Plant parts	Abbreviations	% yield MeOH extract	% yield water extract
Acanthospermum hispidum	Shoot	AHLM	2.63	3.45
Cajanus cajan	Leaves	CCLM	12.92	NE
Morinda lucida	Leaves	MLLM	18.34	NE
Morinda lucida	Stem bark	MLSM	20.45	NE
Nymphaea lotus	Whole plant	NLM	3.52	6.67
Annona senegalensis	Leaves	ASW	NE	14.7
Pycnanthus angolensis	Leaves	PALM	20.59	NE
Diospyros canaliculata	Leaves	DIOSP	16.54	20.74
Croton penduliflorus	Seed	CPW	NE	12.84
Lippia multiflora	Root	LMR	16.70	NE
Lippia multiflora	Leaves	LML	18.0	NE
Flabellaria paniculata	Leaves	FPL	17.45	NE

NE = not extracted.

2.6. Bioassay procedure

2.6.1. Human cell lines

Three cancer and one normal human cell lines were used in the initial screening of the extracts. The cancer cell lines were MCF-7 (human breast adenocarcinoma cell line, ECACC no. 86012803), COR-L23 (human large cell lung carcinoma cell line, ECACC no. 92031919), and C32 (human amelanotic melanoma, ECACC no. 87090201), while the normal cell line used was SVK-14 (normal human keratinocytes). The culturing of the cancer cells was as described by Keawpradub et al. (1997) while the SVK-14 cells were cultured as described by Itharat et al. (2004). The leukaemia cell lines were cultured as described (Kimmig et al., 1990; Efferth et al., 2002).

2.7. Cytotoxicity testing

2.7.1. The SRB assay

The SRB assay is widely used for cytotoxicity studies for potential anticancer compounds (Skehan et al., 1990). It was carried out as previously described (Keawpradub et al., 1997). All experiments were done in duplicate with three replicates on each plate.

Viable cells were counted by trypan blue exclusion using a haemocytometer (Freshney, 1994). The cells were plated in 96-well plates for all the experiments and 100 μl of cell suspension used in each well. After initial experiments, the cell densities for optimal growth were found to be were 5000, 1000, 4000 and 5000 cells/well for MCF-7, COR-L23, C32 and SVK-14 respectively which compared favourably with earlier work (Keawpradub et al., 1997; Itharat et al., 2004). 100 μl of medium was added to cell-free wells as background control wells (Monks et al., 1991) and the plate was incubated for 24 h before the introduction of the extracts to ensure that no contamination of the medium had occurred. 20.0 mg of methanol extracts were initially dissolved in 1.0 mL of 100% DMSO and filtered though a 0.2 μm filter to give the sterile stock solution (20.0 mg/mL) used for the experiments. Equal quantities of water extracts dissolved in sterile distilled water were also filtered prior to use in the experiments. Serial dilutions of extracts gave 200, 80, 40 and 10 μg/mL and 100 μl of each were added to each well in the replicates, to give final concentrations of 100, 40, 20 and 5 μg/mL in the wells. The final concentration of the DMSO in the well containing the highest concentration of extract was 0.5%, which had previously been found not to be cytotoxic (Itharat et al., 2004). For each sample two sets of 96-well microtitre plates were used, one for estimating cell growth inhibition after 48 h extract exposure, referred to as the exposure experiment, while the other evaluated cell growth inhibition after a further 48 h period following replacement of the medium plus extract with medium alone; this is referred to as the recovery assay. The plates for both experiments were fixed with 100 μl of ice-cold 40% TCA per well and incubated at 4 °C for 1 h and were subsequently washed with deionised water (five times) to remove TCA, growth medium, low molecular weight metabolites, and serum protein. The fixed plates were then left to dry at room temperature for at least 24 h, after which the SRB assay was performed.

In the selectivity studies, the two stilbenes isolated from the active fraction of *Cajanus cajan*; longistylins A and C were tested on three other cell lines, HepG2 (hepatocellular carcinoma), AR42J-B13 (rat pancreatic tumour cell line) and 16HBE4o (a non-cancer human airway epithelial cell line) with identical culture condition as MCF-7 earlier described.

2.7.2. XTT assay

The SRB assay is not suitable for leukaemia cell lines since they are suspension cells and cannot be fixed easily with 40% TCA (Kim et al., 1996). The XTT assay, which is suitable for cells in suspension, was used for anti-proliferative study with these cells (Konkimalla and Efferth, 2008; Sieber et al., 2009).

The assay is based on the extracellular reduction of XTT by NADH produced in the mitochondria *via* trans-plasma membrane electron transport and an electron mediator (Berridge et al., 2005). All experiments were repeated three times.

Human CCRF-CEM leukaemia cells were maintained in RPMI medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fœtal calf serum (Gibco BRL, Trace) in a 5% CO_2 atmosphere at 37 °C. Cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase. The development of drug-resistant sub-lines was as described previously (Kimmig et al., 1990; Efferth et al., 2002).

All cancer cell lines were seeded into 96-well plates at a concentration of 1.0×10^5 cells/mL and a volume of 100 μL/well. Anti-proliferative studies were performed for pure compounds in 96-well plates. CCRF-CEM and CCRF-CEM/ADR5000 cells were seeded into the wells at 1×10^4 cells/well density (assessed using a haemocytometer). The compounds were added simultaneously at concentrations 1–6 μg/mL in the well. For each set of experiments there were blank controls containing only medium without cells, and another set of wells with the same number of cells as the test wells. The plates were incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2 in air and 100% relative humidity. The

Table 3
The IC_{50} of crude extracts on the cell lines. NB values for vinblastine are in nM.

Extracts	IC_{50} (μg/ml) Mean ± s.d., $n = 3$							
	After exposure (48 h)				After recovery (48 h)			
	MCF-7	COR-L23	C32	SVK-14	MCF-7	COR-L23	C32	SVK-14
AHLM	23.00 ± 5.80	12.25 ± 1.0	22.5 ± 5.3	12.36 ± 3.6	13.50 ± 1.0	8.87 ± 0.9	13.54 ± 0.8	10.31 ± 3.1
AHW	>100	>100	>100	>100	>100	>100	>100	>100
CCLM	26.56 ± 10.0	26.93 ± 0.2	59.83 ± 8.2	28.65 ± 5.2	16.08 ± 3.3	9.81 ± 0.0	33.07 ± 0.7	25.21 ± 8.2
MLLM	43.96 ± 8.8	45.37 ± 9.9	58.66 ± 13.1	36.98 ± 11.9	40.97 ± 4.4	30.02 ± 0.5	43.45 ± 0.0	32.62 ± 3.4
MLSM	57.70 ± 12.8	51.18 ± 0.1	61.60 ± 7.7	42.96 ± ± 5.3	50.89 ± 13.3	37.82 ± 1.6	42.92 ± 17.5	35.01 ± 4.8
NLM	76.00 ± 9.8	61.75 ± 8.4	85.71 ± 1.6	28.78 ± 4.7	51.93 ± 10.4	50.8 ± 6.0	36.26 ± 3.0	29.15 ± 3.2
NLW	31.70 ± 2.9	70.26 ± 1.5	>100	>100	30.5 ± 0.2	50.31 ± 2.4	>100	>100
PALM	52.94 ± 4.9	34.01 ± 2.1	92.88 ± 2.9	41.96 ± 6.8	48.50 ± 1.9	28.31 ± 1.0	67.88 ± 2.0	41.92 ± 6.0
DIOSP	>100	75.55 ± 1.4	>100	61.41 ± 18.1	57.50 ± 2.6	65.98 ± 1.6	>100	40.75 ± 3.9
DIOSPW	>100	>100	>100	>100	>100	>100	>100	>100
CPW	>100	>100	>100	>100	>100	>100	>100	>100
ASLM	58.68 ± 6.7	85.08 ± 16.6	>100	73.72 ± 13.7	59.49 ± 14.9	68.48 ± 4.3	>100	57.60 ± 12.4
ASW	>100	>100	>100	>100	>100	>100	>100	>100
LML	48.24 ± 3.4	40.27 ± 9.3	54.54 ± 3.9	NT	53.43 ± 5.5	65.34 ± 2.3	56.43 ± 4.6	NT
LMR	>100	>100	34.65 ± 6.8	>100	>100	>100	30.43 ± 7.6	>100
Vinblastine	0.98 ± 0.01	0.69 ± 0.02	3.92 ± 0.41	0.95 ± 0.02	0.65 ± 0.05	0.48 ± 0.06	0.56 ± 0.07	0.61 ± 0.05

Table 4
Selectivity of the cytotoxicity of extracts in comparison with SVK-14 cells.

Extract	Selectivity index					
	After exposure (48 h)			After recovery (48 h)		
	MCF-7	COR-L23	C32	MCF-7	COR-L23	C32
AHLM	0.45	1.01	0.55	0.76	1.16	0.76
CCLM	1.08	1.06	0.48	1.57	2.57	0.76
MLLM	0.84	0.82	0.63	0.8	1.09	0.75
MLSM	0.75	0.84	0.7	0.69	0.93	0.82
NLM	0.38	0.47	0.34	0.56	0.57	0.8
NLW	3.27	1.42	–	3.28	1.99	–
PALM	0.79	1.23	0.45	0.86	1.48	0.62
DIOSP	–	0.81	–	0.71	0.62	0.41
ASLM	1.26	0.87	0.73	0.97	0.84	0.58
Vinblastine	0.97	1.38	0.24	0.94	1.27	1.09

The selectivity index is the ratio of the IC_{50} values of extract on SVK-14 cells to those in the cancer cell lines.

experiment was stopped by the addition of 50 μl of XTT solution containing 1% electron coupling reagents (phenazine methosulfate). The plates were further incubated for 12 h to allow the production of formazan. The extent of proliferation was then determined by measuring the absorbance at 490 nm in a 96 well plate reader (BIO-RAD), subtracting the background measurement at 655 nm.

2.8. Statistical analysis

The percentage of cell survival was calculated as {Mean (OD test − OD blank)/Mean (OD control − OD blank)} × 100%. The IC_{50} was calculated using Prism, Graphpad software programme, obtained by plotting the percentage of cell survival against respective concentrations of extracts used in the assay by Cubic Spline and LOWESS curves. One-way ANOVA was used for the comparison of the mean, $p < 0.001$.

3. Results and discussion

The results during the ethnobotanical survey are given in Table 1 and 45 plant species were mentioned by the healers. Preparations were either crude extracts made with water or local spirits, such as gin, or poultices. For decoctions, quantities are not measured accurately and the extracts are made on a large scale by boiling plant material with liquid in large pots over a fire. The resulting extract is kept for about a week, being discarded when a noticeable colour change occurs. Infusions are made by pouring hot water on to the plant material and drinking the resultant extract immediately. Occasionally the patient uses the aqueous extract for bathing the affected area. Poultices are made with mashing the fresh plant material with a small amount of water and palm oil, and then rubbing the resulting mass on to the affected area, or leaving it in contact with the skin for several hours by means of a supporting cloth. In all instances the amount administered to the patient is not very accurately measured, so dosage is very difficult to estimate.

The results for cytotoxicity testing of the extracts are shown in Table 3 and their selectivities between cancer and non-cancer cell lines are shown in Table 4.

The *Cajanus cajan* methanol extract showed the greatest cytotoxicity of all the extracts tested (Table 3). Greatest sensitivity was observed for MCF-7, COR-L23 and SVK-14 cell lines but the growth of C32 was only inhibited moderately. The fact that the non-cancerous cell line SVK-14 was susceptible to the extract shows that it has no selectivity between cancer and non-cancer cells. Although some biological activities of *Cajanus cajan* extracts have been reported (Duker-Eshun et al., 2004; Hopp and Inman, 2003), toxicity towards cancer cell lines is still ill-defined.

Cajanus cajan is grown for food and medicinal purposes in Nigeria. The interviews with the healers disclosed that the boiled leaves, together with fruits of *Xylopia aethiopica* and the roots of *Securidaca longipedunculata*, are boiled and given to women that have pains and lumps in their breast. The decoction is both drunk and used to wash the breast very early in the morning before talking to anyone. It was the second most-frequently cited plant in the survey conducted (Fig. 1).

The methanol extract of *Acanthospermum hispidum* showed significant cytotoxicity but the water extract was not very cytotoxic as the percentage survival of the cells at 100 μg/mL in all cases was >50%. The observed activity of the methanol extract may be due to the terpenoid and polyphenolic components of the plant since earlier studies describe the *in vitro* and *in vivo* cytotoxicity of melampolides and *cis*,*cis*-germacranolides obtained from the related species *Acanthospermum glabratum* (Nair et al., 1985; Jakupovic et al., 1986; Cartagena et al., 2000).

Nymphaea lotus is traditionally claimed to be very effective in the treatment of breast cancer. The results (Table 3) show that methanol and water extracts have significant cytotoxicity to the MCF-7 breast cancer cell line used, with the methanol extract having some toxicity towards the normal keratinocytes. There is no previous study on the cytotoxicity of *Nymphaea lotus*, although some phenolic constituents of *Nymphaea alba* have been reported to be anti-proliferative and anti-angiogenic in other studies (Hertog et al., 1992; Khan and Sultana, 2005).

Morinda lucida leaf and stem bark extracts showed significant but non-selective cytotoxicity on MCF-7 and COR-L23 after 48 h recovery (Table 3). *Morinda lucida* had been reported to have anti-neoplastic activity in a rat model (Durodola, 1975) but no active compounds were isolated. Anthaquinones may be responsible since damnacanthal in *Morinda citrifolia* was shown to be responsible for the cytotoxic activity of an extract (Hiramatsu et al., 1993).

The activity of methanol extracts of the dried leaves of *Pycnanthus angolensis* has not previously been reported. The methanol extracts of *Diospyros canaliculata* and *Annona senegalensis* showed weak activity and selectivity, especially on MCF-7 and COR-L23 cell lines, at 100 μg/mL. Fatope et al. (1996) found *ent*-kaur-16-

Table 5
The IC_{50} of the fractions of *Cajanus cajan* on the cell lines.

Fractions	IC_{50} (μg/ml) Mean ± s.d., $n = 3$							
	After exposure (48 h)				After recovery (48 h)			
	MCF-7	COR-L23	C32	SVK-14	MCF-7	COR-L23	C32	SVK-14
Hexane	21.3 ± 3.3	12.2 ± 0.8	49.1 ± 22.1	21.9 ± 10.3	16.2 ± 0.7	10.3 ± 0.6	37.4 ± 3.0	20.0 ± 10.5
DCM	15.4 ± 3.8	11.9 ± 1.7	21.7 ± 6.8	10.7 ± 0.1	10.3 ± 0.1	9.31 ± 0.8	11.9 ± 0.1	8.7 ± 1.9
$CHCl_3$	47.3 ± 14.8	29.6 ± 7.6	80.4 ± 5.1	34.0 ± 6.0	29.7 ± 0.2	23.2 ± 4.6	77.4 ± 0.1	22.6 ± 9.1
EtOAc	31.82 ± 13.7	32.5 ± 18.6	81.9 ± 4.7	25.4 ± 4.7	19.4 ± 0.2	23.8 ± 6.7	78.9 ± 15.3	40.3 ± 19.2
Acetone	82.7 ± 2.5	76.2 ± 7.0	>100	66.8 ± 17.3	60.6 ± 8.8	44.3 ± 21.5	94.0 ± 6.1	62.5 ± 12.2
Methanol	>100	>100	>100	>100	>100	>100	>100	>100

Table 6
The cytotoxic effect (IC_{50} (μg/ml) Mean ± s.d., $n = 3$) of longistylins A and C and pinostrobin on the cancer cell lines.

Compound	Test	MCF-7	COR-L23	C32	HepG2	16HBE4o	AR42J-B13	CCRF-CEM	CEM/ADR5000
Longistylin A	Exposure	5.2 ± 0.0	5.0 ± 0.05	14.7 ± 0. 4	0.7 ± 0.0	4.5 ± 0.05	1.8 ± 0.2	9.8 ± 0.9	10.3 ± 1.1
	Recovery	5.9 ± 0.1	5.1 ± 0.0	3.3 ± 0.05	1.1 ± 0.0	2.5 ± 0.0	0.7 ± 0.1	NA	NA
Longistylin C	Exposure	4.4 ± 0.3	2.8 ± 0.3	17.3 ± 0.4	1.6 ± 0.4	4.2 ± 0.3	7.1 ± 0.3	10.0 ± 0.8	10.5 ± 0.9
	Recovery	5.4 ± 0.0	3.5 ± 0.05	4.1 ± 0.4	1.7 ± 0.0	2.4 ± 0.05	4.5 ± 0.2	NA	NA
Pinostrobin	Exposure	>30	>30	>30	>30	>30	>30	10.2 ± 1.1	>30
	Recovery	>30	>30	>30	>30	>30	>30	NA	NA
Vinblastine	Exposure	0.9 ± 01	0.7 ± 0.0	4.0 ± 0.2	0.3 ± 0.02	0.7 ± 0.02	0.7 ± 0.03	NT	NT
	Recovery	0.6 ± 0.02	0.5 ± 0.02	0.6 ± 0.01	0.2 ± 0.02	0.6 ± 0.03	0.5 ± 0.04	NA	NA

Values for vinblastine are in nM; NA = not applicable since leukemia cell line; NT = not tested.

en-19-oic acid from the chloroform extract of *Annona senegalensis* to have selective and significant cytotoxicity against MCF-7 breast cancer cells (ED_{50} 1.0 μg/mL). There has been a previous report of the cytotoxicity of pondaplin, a cyclic prenylated phenylpropanoid isolated from *Annona glabra*, showing moderate and selective *in vitro* cytotoxicities amongst six human solid tumour cell lines (Liu et al., 1999). Achiwa et al. (1997) reported the inhibitory effect of *Diospyros kaki* extract on the growth human lymphoid leukaemia cells while Chen et al. (2007) reported the isolation of kakispyrone and kakisaponin A and another 11 compounds from the leaves of *Diospyros kaki* L. with cytotoxic effects against some cancer cell lines (A549, HepG2 and HT29). The related species *Diospyros montana* has yielded diospyrin, a bisnaphthoquinonoid plant product, showing inhibitory activity against murine tumour *in vivo* and human cancer cell lines *in vitro* (Hazra et al., 2005). *Lippia multiflora* extract has previously shown toxicity in the brine shimp test (Ajaiyeoba et al., 2006). The present results show moderate non-selective cytotoxicity (Table 3). *Flabellaria paniculata* has not been previously investigated for cytotoxicity but only weak activity was observed in this study (Table 3).

The traditional healers in South-western Nigeria use the species investigated for the treatment of breast cancer and claim that they are highly effective in the treatment of this cancer type. The results of the present study provide some justification for the traditional use of less polar extracts of *Acanthospermum hispidum* leaves, *Cajanus cajan* leaves, *Morinda lucida* leaves, *Nymphaea lotus* leaves and *Lippia multiflora* leaves for breast cancer, although it should be noted that the absence of selectivity precludes these extracts from serious investigation as a source of compounds of clinical interest (Table 4).

3.1. Constituents of Cajanus cajan extract cytotoxic fractions

The isolated compounds were identified as hexadecanoic acid methyl ester, α-amyrin, β-sitosterol, pinostrobin, longistylins A and C by direct comparison of their spectroscopic data (^{1}H NMR, ^{13}C NMR, NOESY, COSY, EIMS) with those in the literature (Cuong et al., 1996; Hopp and Inman, 2003; Delle Monache et al., 1977). Three of the compounds: pinostrobin and longistylins A and C (Fig. 2), found in the most active fractions, were tested on a variety of cancer and non-cancer cell lines.

Duker-Eshun et al. (2004) reported the presence of the two hydroxyl stilbenes longistylins A and C in *Cajanus cajan* from the same geographical zone and showed that they had anti-plasmodial activity. In the present work, these two compounds appear to make a major contribution to the observed cytotoxicity of the methanol extract and its dichloromethane fractions (Table 6). On 48 h exposure of C32 cells to the stilbenes there seems to be initial resistance which was later subdued, as revealed in the significant reduction in the IC_{50} after another 48 h recovery period. HepG2 cells are the most susceptible of the solid tumour cell lines used, with an IC_{50} of approximately 0.5 μg/mL both during exposure and recovery times.

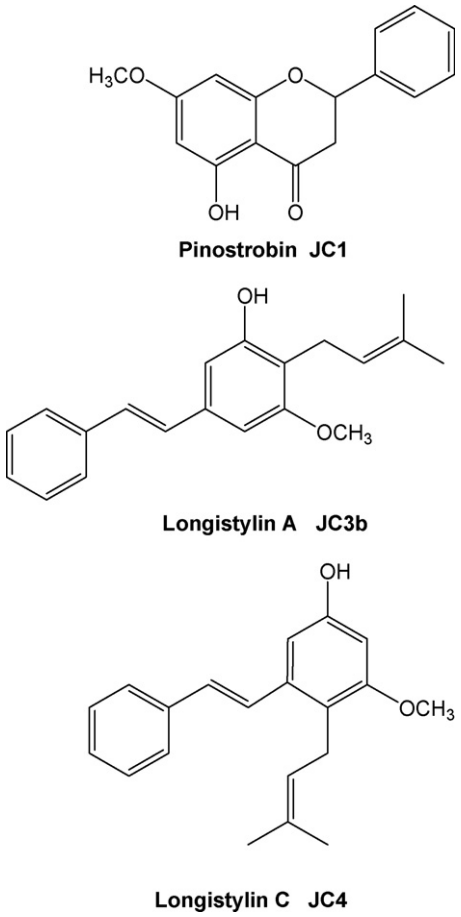

Fig. 2. Chemical structure of some cytotoxic constituents isolated from *Cajanus cajan*.

Pinostrobin (JC1) showed selective dose-dependent antiproliferative activity on the acute T-lymphoblastic leukaemia cell line (CCRF-CEM) which was cross resistant to the doxorubicin resistant sub-line CEM/ADR5000 (Table 6). It has previously been shown to exhibit anti-leukemic activity (Smolarz et al., 2006) and to affect estrogen metabolism (Le Bail et al., 2000).

No previous work has been reported on the cytotoxicity of the lignans although the stilbene resveratrol is active in this respect (Jang et al., 1997).

4. Conclusion

The cytotoxicity of some of the extracts lends some support to their use in the traditional medicine of South-western Nigeria to treat cancer. However, the methanol extracts tened to be more active than the aqueous extracts and these would not approximate to the aqueous preparations generally employed, so whether

tradition preparations would have clinically-significant activity must be open to doubt. The lack of selectivity in cytotoxic effect between cancer cell lines and non-cancerous cell lines minimises the prospect that these plants contain compounds which could serve as leads for novel anticancer drugs.

Acknowledgements

The authors are grateful to the Commonwealth Scholarship Commission UK, for funding of the research work and the traditional medical practitioners for volunteering vital information about their rich heritage which is their means of livelihood. Amongst them are: Alhaji T.O. Oduntan (late), Chief A. Adebambo, Chief J.S. Suru, Chief K. Hunbabe, Chief E.K. Oke, Mr. T. Jaiyeola and Mrs. M.M. Okuneye (late). Dr. M.O. Soladoye and Mr. Seun Osiyemi of the Forestry Herbarium Ibadan also helped in the preparation of voucher specimens. We also thank Mrs. Jane Hawkes of the Bruker AMX 400, and 500 University of London Intercollegiate Research Service for NMR and Mr. R. Tye, ULIRS Mass Spectrometry Service, King's College, London, for NMR spectra and mass spectra, respectively.

Appendix A. Questionnaire on the ethnobotanical survey of plants commonly used for cancer phytotherapy in South-western Nigeria (used as basis for the study in King's College London)

A.1. Introduction

We please request that you kindly assist us in providing the following useful information on the treatment methods and remedies used traditionally in our environment for the cure/management of cancer and the associated diseases. The information provided shall be treated as confidential and will only be used for research purposes and not for private practice.

The ultimate aim is for drug development from potential anticancer plants indigenous to Africa, South-Western Nigeria in particular.

GENERAL DATA

NAME……Family name…….

Age: …….. Sex: ( ) Male ( ) Female Date of Birth: ……..

Position in your village;

( ) 1, Priest

( ) 2, Family Head

( ) 3, Opinion Leader

( ) 4, Chieftancy Title Holder

( ) 5, Others (please specify)

HOME ADDRESS

BUSINESS/WORKPLACE ADDRESS

VILLAGE NAME……CLOSEST TOWN……

LOCAL GOVERNMENT AREA……

STATE……

HIGHEST EDUCATIONAL LEVEL (Please tick one)

( ) 1 Primary School ( ) 2, Secondary School

( ) 3 Technical/Teacher's college ( ) 4 Diploma

( ) 5 National Certificate of Education ( ) 6 First Degree

( ) 7 Higher Degree ( ) 8 No Formal Education

OCCUPATION (Please tick one)

Main Occupation Other Occupation (please specify)

( ) Farming

( ) Traditional healer

( ) Artisan

( ) Private sector employee

( ) Government official

Average Monthly Income

( ) 1, Less than 5000 naira ( ) 2, 5000--------10,000 naira

( ) 3, 10,000------20,000 naira ( ) 4, 20,000------40,000 naira

( ) 5, 40,000 naira and above.

KNOWLEDGE ABOUT TRADITIONAL MEDICINE

Which of the following do you have knowledge about? (Tick as many as apply)

( )1 Delivery

( ) 2 Massaging

( ) 3 Massaging with decoction

( ) 4 Teaching how to use medicinal plants for treatment

( ) 5 Treating patients using medicinal plants

( ) 6 Treating patients using incantation

( ) 7 Treating patients using incision

( ) 8 Preparing drugs using medicinal plants

( ) 9 Treating patients by divination

( )10 Others (Please specify)

Do you use above knowledge in your occupation? (Please tick)

( ) Yes ( ) No

How did you obtain the above knowledge ? (Please tick)

( ) 1 Formal training ( ) 2 Apprenticeship

( ) 3 From your Elders ( ) 4 Family herbal practice records

( ) 5 Others (Please specify)

Are you a registered Tradimedical practitioner?

( ) Yes ( ) No

With which of the following council are you registered?

Community council of traditional healers

Traditional healers association of Nigeria

Herb sellers Association

Others (Please specify)

In which of the following do you specialise ?

( ) Child care ( ) General practice ( ) Antenatal care ( ) Orthopaedic

( ) Others (Please specify)

KNOWLEDGE ABOUT MEDICINAL PLANTS FOR CANCER

Do you have any knowledge about the use of medicinal plants for the following ailments?

a) Tumours? ( ) Yes ( ) No

b) Loss of weight ( ) Yes ( ) No

c) Weakness? ( ) Yes ( ) No

What medicinal plants or traditional recipes do you use for the following? (you may describe the recipes)

Loss of weight

Medicinal plants or traditional recipes:

……

……

……

Usage:

……

……

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……

Dose:……

……

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Tumours

Medicinal plants or traditional recipes:

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Usage:

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Dose:……

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Weakness:

Medicinal plants or traditional recipes:

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Usage:

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Dose:……

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Please provide any other additional useful information. You may use extra sheets for more recipes information.

Thank you.

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