Research Article

In vitro Cytotoxic Effects of Extracts of Fourteen Medicinal Plants of Nigerian Origin on Vero Cells - 🔓

Obi RK[1]*, Chikwendu CI[1] and Shenge JA[2]

[1]*Department of Microbiology, School of Biological Sciences, Federal University of Technology, Owerri, P.M.B 1526, Owerri, Imo State, Nigeria*
[2]*Virology Unit, Department of Biological Sciences, Dominican University, Ibadan, Oyo State, Nigeria*

*Address for Correspondence: Obi RK, Department of Microbiology, School of Biological Sciences, Federal University of Technology, Owerri, P.M.B 1526, Owerri, Imo State, Nigeria, Tel: 234-080-387-575-15; ORCID: 000-000-016-524-694X; E-mail: obi.robert@ymail.com

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ABSTRACT

Background and Objective: Studies on the efficacy and safety of medicinal plants have indicated that there are many phytochemicals that have cytotoxic, genotoxic, and carcinogenic effects. The aim of the present study was therefore to evaluate the non-specific toxicity of extracts of fourteen (14) edible medicinal plants indigenous to Nigeria using African green monkey kidney (Vero) cells.

Materials and Methods: Different parts of some of the plants were collected from the wild in Ijegun and Ikotun communities, while others were bought from Mushin market, Lagos State, South Western Nigeria. They were subjected to Soxhlet extraction using analytical grade methanol and dried in vacuo, under pressure. The resulting extracts were then subjected to cytotoxic analysis using Vero cells. Using probit, the concentration of the extracts that inhibited Cytopathic Effect (CPE) on Vero cells by 50% (IC_{50}), was calculated.

Results: Results showed that the highest percentage of cytotoxic activity of the methanol extracts of the plants was produced by *A. indica* (78.9%) and IC_{50} of 0.011 µg µL^{-1}. *G. kola* with 43.8% cytotoxic activity produced IC_{50} of 0.090 µg µL^{-1}. The result also showed that the extracts with the least percentage toxicities were *M. oleifera* and *V. album*. Acyclovir, an orthodox drug, was found to be milder on the Vero cells with 15.6% and IC_{50} of 4.56 µg µL^{-1}.

Conclusion: The cytopathic activities exhibited by the methanol extracts of the fourteen medicinal plants used in this study shows that they are very toxic to mammalian cells.

Keywords: Cell line; Toxicity; Probit; Medicinal plants; Inhibitory concentration; Herbarium; Vero

INTRODUCTION

Many plants produce toxic secondary metabolites as natural defense mechanisms from adverse conditions. In some plant species, these toxic substances are not distinguished from therapeutically bioactive components [1]. Due to the fact that plants do not move about, they have developed different strategies that will enable them to co-exist with herbivores and potentially pathogenic microorganisms in their environment. In view of this, they synthesize a large number of bioactive compounds known as phytoanticipins that are released in response to specific environmental stimuli [2]. Some of these phytochemicals produced by plants against herbivorous insects also end up being harmful to humans, probably because certain biological similarities between humans and insects [3].

The primary aim of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur. Two important factors which are taken into consideration in evaluating the safety of any herbal drug are the nature and significance of the adverse effect and the exposure level where the effect is observed [4]. Another important objective of toxicity analysis is the detection of toxic plant extracts or compounds in the early and late stages of drug discovery and development from plant sources [5]. This facilitates the identification of toxic metabolites which can be discarded or modified during the process and create an opportunity for extensive evaluation of safer, promising alternatives [6]. For certain compounds, modifications such as dosage reduction, chemical group or structural adjustments may improve their tolerability [7].

Cytotoxicity assays are performed to predict potential toxicity, using cultured cells which may be normal or transformed cells. These tests normally involve incubation of cultured cells and plant extracts, to detect how basal or specialized cell functions may be affected by the substance, before safety studies could be performed using whole organisms. This can also provide insight toward the carcinogenic and genotoxic effect of herb-derived compounds and extracts [8]. The ability of a plant extract to inhibit cellular growth and viability can also be ascertained as an indication of its toxicity [9]. Culturing cells with cytotoxic compound can lead to necrosis involving loss of membrane integrity and rapid death as a result of cell lysis. Furthermore, active cell division may completely stop, leading to a decrease in cell viability, or the cells can activate a genetic program

of controlled cell death or apoptosis [10]. Evidence of cell necrosis is manifested by rapid swelling, loss of membrane integrity, shut down of metabolism and release of cellular contents into the environment [11]. Apoptosis is characterized by well-defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into fragments [12].

In view of the fact that many plants produce toxic and medicinally useful secondary metabolites in their tissues, this study was designed to evaluate the cytotoxicity of partially purified extracts of fourteen medicinal plants indigenous to Nigeria, on uninfected Vero cells.

MATERIALS AND METHODS

Study design

This is an *in vitro* tissue culture study that involved the collection, methanol extraction, and cytotoxic analysis of fourteen medicinal plants of Nigerian origin using Vero cells. Initial tissue culture study was carried out at the Virology Laboratory in the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos from Jan 2013-Oct 2014. Methanol extraction was done at the Pharmocognosy Department, School of Pharmacy University of Lagos from Oct 2013-June 2016. Evaluation of cellular toxicity was done at the Department of Virology, College of Medicine, and University of Ibadan from Oct 2014-Oct 2016.

Collection and preparation of plant samples

Different plant parts of the selected fourteen (14) medicinal plants were collected from Ijegun, Okota, and Mushin Market, all in Lagos State, South Western Nigeria, between October, 2012 and July, 2014. The plants were authenticated by a Taxonomist at the Herbarium Unit of the Department of Botany, University of Lagos, where voucher specimens were deposited. The voucher numbers were: *Azadirachta indica* (A. Juss.) LUH 5502, *Bambusa vulgaris* (Shrad.) LUH 5493, *Bryophyllum pinnatum* (Lam.) LUH 5500, *Citrus paradisi* (Macfad.) LUH 7659, *Cymbopogon citratus* (D.C) Stapf. LUH 5496, *Garcinia kola* (Heckel.) LUH 7658, *Moringa oleifera* (Lam.) LUH 5498, *Newbouldia laevis* (P. Beauv.) LUH 5696, *Piper guineense* (Schumah.) LUH 5501, *Psidium guajava* (L.) LUH 8146, *Rauvolfia vomitoria* (Afzel.) LUH 5503, *Senna alata* (L.) Roxb. LUH 5496, *Spondias mombin* (L.) LUH 5492, *Viscum album* (L.) LUH 8252. Table 1 shows the medicinal uses of the plants, their local, common and species names [13,14].

Table 1: Plant samples for the cytotoxicity study.

S/No	Species Name	Family Name	Local Name	Common Name	Medicinal uses
1	Azadirachta indica (A. Juss)	Meliaceae	Ochonyeogwo (I) Eke Oyibo (Y)	Neem tree	Malaria, jaundice, laxative, antihelminthic, ringworm, skin disease
2	Bambusa vulgaris (Shrad.)	Poaceae	Achara (I), Oparun (Y)	Bamboo	Gonorrhoes, abortifacient, antihelminthic, emenagogue, skin rashes of HIV/AIDS
3	Bryophyllum pinnatum (Lam.)	Crassulaceae	Odaa opuo (I) Abomoda (Y)	Resurrection plant	Cough, diarrhea, dysentery wound, fever, sedative, epilepsy, antimicrobial,
4	Citrus paradise (Macfad.)	Rutaceae	Mkpuru grepu (I) Osan grepu (Y)	Grape fruit	Antimicrobials, diabetes,astringent, emollient, malaria
5	Cymbopogun citratus (D.C) Stapf.	Poaceae	Achara tee (I), Kooko-oba (Y)	Lemon grass	malaria, cough, sprains, lumbago, stomach tonic, stimulant, diphoretic,
6	Garcinia kola (Heckel)	Guttiferae	Aku ilu (I), Orogbo (Y)	Bitter kola	antimicrobials, dysentery, bronchitis, cough, fever,
7	Moringa oleifera (Lam.)	Moringaceae	Okochi egbu (I) Ewe ile (Y)	Horse radish tree	Inflammatory disease,
8	Newbouldia laevis (P. Beauv.)	Bignoniaceae	Ogirishi (I), Akoka (Y)	Fertility tree	Round worm, dysentery, cough, hernia, earache, infertility, migraine
9	Piper guineense (Schumah.)	Pipareceae	Uziza (I), Iyere (Y)	Climbing black	black Herbal recipe, antipyretic, Pepper stomachache, mental illness
10	Psidium guajava (Linn.)	Myrtaceae	Gova (I), Gilofa (Y)	Guava	Fever, diarrhea, cough, dysentery, malaria, laxative,
11	Rauvolfia vomitoria (Afzel.)	Apocynaceae	Akata (I), Asofeyeje (Y)	Serpent wood	hypertension, insomnia, dysentery, fever, jaundice, scabies, malaria, diarrhea
12	Senna alata (Linn) Roxb	Leguminaceae	Ndichi (I), Asuwon-oyinbo (Y)	Candle bush	Skin disease, itch, measles, antihelminthic, ringworm, venereal disease, eczema
13	Spondias mombin (Linn.)	Anacardaceae	Ijikara (I), Akikan (Y)	Hog plum	Cough, fever, measles, dizziness, sore throat, cold, yaws, diabetes, gonorrhea
14	Viscum album (Linn.)	Lorantheceae	Awuruse (I), Afoma (Y	Mistletoe	All-purpose herb for cancer, diabetes, hypertension, anaemia, stroke, hysteria,

Extraction and concentration of plant materials

Each of the plant parts was dried at 25°C for 7 days. The samples were blended to coarse powder using a laboratory milling machine (Christy and Norris, England). The method of extraction used was hot continuous extraction using the Soxhlet extractor (Thermo Fisher Scientific, USA), using 250 mL of methanol (Emsure, Merck KGaA, Germany) as described by Velmurugan, et al [15]. After extraction, the solvent was concentrated in vacuo at 45°C using a rotary evaporator (Heidolph Laborata 4010 Digita, USA).

Reconstitution of extracts/fractions

Dried/pasty (10 mg) solid plant extracts were introduced into sterile calibrated 50 mL centrifuge tubes. They were then reconstituted in 0.5% Dimethyl Sulfoxide (DMSO) (Sigma) and shaken vigorously to ensure complete dissolution and brought to a final volume of 10 mL with the addition of 9.95 mL of sterile distilled water. They were subsequently filtered; first, with 0.45 µM and then with 0.22 µM membrane syringe filters (Cell Treat USA). A 100 µL of each extract concentration was thereafter used at the concentrations of 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016 and 0.008 µg/µL, to evaluate the cellular toxicity of the extracts on Vero cells using 96-well plates [16].

Evaluation of cellular toxicity

Preparation of cell line: The method used was based on cellular morphologic changes [17]. Vero cells were prepared at a density of 8×10^4 cells mL^{-1} in a 10% MEM in 75 cm^2 tissue culture flasks (Cell Treat, USA). A 100 µL of this cell suspension (containing 8000 cells) was then dispensed into each well of a flat bottomed 96-well tissue culture plate (Cell Treat, USA) and incubated for 24 hours at 37°C. The 10% MEM was aspirated thereafter and discarded.

Preparation and two-fold dilution of plant extract: Eight sterile test tubes labeled with the code name for each extract was arranged on a rack to carry out a 2 fold serial dilution. Unto each tube was introduced 1 mL (1000 µL) of the 1% MEM to serve as diluent. Then 1 mL (1000 µL) of each fully thawed extract was introduced into the first tube containing no medium labeled as Neat (undiluted (100%)) while the remaining 1 mL (1000 µL) was introduced into the second tube containing the 1% MEM diluent and labeled ½ (50%). After proper mixing, 1 mL (1000 µL) of the extract and medium mixture was aspirated and introduced into the third tube labeled ¼ (25%). The dilution continued to the last bottle labeled $^1/_{128}$ (0.8%).

Preparation of positive control: The positive control, Virest 200 mg, a brand of Acyclovir (Hovid Bhd, Malaysia), was prepared by dissolving 200 mg of the tablet in 200 mL of Phosphate Buffered Saline (PBS) and filtered using 0.22µm of 250 mL (Nalgene filter, USA) fixed to electrically controlled suction pump. Then 1 mL of the filtered drug was dispensed into 9 mL of PBS in a sterile test tube. A single two-fold serial dilution of the drug was carried out to evaluate its toxicity on the Vero cells.

Cytotoxicity activity: The 96 well tissue culture plates were also labeled with the different dilution and code name of each extract. Then using different pipette tips, 100 µL of each extract dilution was introduced into each of the 96-wells containing the Vero cells in duplicates. The last row of wells containing cells but no extracts was used as negative control, the row containing solvent only was used as solvent control, while the row containing the Virest drug and cell was used as positive control.

The plates were labeled and incubated at 37°C in a 5% CO_2

incubator and moisture. Cell viability was monitored every day for 14 days to observe any possible immediate changes in morphology (CPE) compared with the control wells containing only medium and no extract, using an inverted microscope (Inverskop 40C, USA) [18,19]. Complete (100%) CPE was scored as 4+ (100%); 3+ (75%); 2+ (50%); 1+ (25%) and 0 (0%) when there was no CPE.

Statistical analyses

Results were presented as Standard Error of Mean (SEM). Data were assessed by one-way Analysis of Variance (ANOVA) followed by Duncan multiple comparison, Turkey's multiple comparison and student's t-test. All statistical analysis were performed at the $p < 0.05$ level of significance. All the statistical analysis were done using GraphPad software version 5.01 (GraphPad Software Incorporated, U.S.A, 2007).

RESULTS

Evaluation of cellular toxicity

Cytotoxicity activity: The toxicity of fourteen crude extracts were evaluated using Vero cells. All the extracts produced varying degrees of toxicity on the cell. The result in figure 1 showed that the highest percentage of cytotoxic activity of the crude extracts of the plants was produced by *A. indica* (78.9%) while *G. kola* produced 43.8% cytotoxic. The result also showed that the extracts with the low percentage toxicities on Vero cells were *M. oleifera* and *V. album* with 34.4% each. Acyclovir, an orthodox drug, was found to be milder on the Vero cells with 15.6%.

Fifty percent (50%) inhibitory concentration (IC$_{50}$): The result of the IC$_{50}$ of the extracts showed that *A. indica* with 0.011 µg µL^{-1} was the most toxic. *Garcinia kola* showed 0.090 µg µL^{-1}. *Moringa oleifera* and *Viscum album* were the least toxic extracts at equal IC$_{50}$ of 0.313 µg µL^{-1}. Acyclovir being a processed drug exhibited very mild toxicity on the Cell at 4.56 µg µL^{-1}.

Level toxicity: the level of toxicity represents the extent at which the extracts caused cytopathic effect on Vero cell line. The result in table 2 represents the level of toxicity and the maximum concentration at which the extracts showed no evident CPE on the cell line. Methanol extract of *A. indica* showed its Maximum Non-Toxic Concentration (MNTC) at 0.008 µg µL^{-1}. In *G. kola,* methanol extract, the MNTC was 0.063 µg µL^{-1}. The methanol extracts of *M. oleifera* and *V. album* also showed an MNTC of 0.063 µg µL^{-1}. Acyclovir was mild on the cell throughout but had its MNTC at 0.063 µg µL^{-1}.

DISCUSSION

Medicinal plants are typically presumed to be non-toxic and regarded as safe due to their natural origin and long use in traditional medicine to treat various forms of diseases [20,21]. However, scientific studies on efficacy and safety of some medicinal plants indicated that there are many phytochemicals that have cytotoxic, genotoxic, and carcinogenic effects when used for longer periods [22,23] and even at shorter times. Cytotoxic agents unselectively kill and damage both normal and cancerous cells by interfering with either, the cellular process or mechanical process [24]. Cytotoxicity testing is therefore important for the sole purpose of determining the potential toxicity of the compounds being studied [25].

This study demonstrated cytotoxicity indices as a measure of cell mortality as observed under the inverted microscope. The reduction in viable cell numbers was visibly evident with all the extracts after incubation. Similarly, the morphological effects were very prominent with all the extract-treated cells manifesting extensive rounding, shrinking, and vacuolation. The extracts and fractions were able to inhibit the proliferation of Vero cells at IC$_{50}$ values as recommended by the National Cancer Institute (NCI) for crude extracts. The recommended guideline set the IC$_{50}$ limit of activity for crude plant extracts at < 20 µg/mL after an exposure time of 72 hours [26]. The lower the IC$_{50}$ values of a given crude extract, the higher will be its toxicity potential [27]. This observation is in agreement with the findings of this present study which showed that the IC$_{50}$ of 4.56 µg/µL recorded for Acyclovir, a processed and purified drug, was far higher than that recorded for all the crude extracts and their fractions. In addition, a higher IC$_{50}$ and low MNTC indicated that the extracts could be considered safe to be applied directly to mammalian cells without activation of cell death, or indirectly, as pharmaceutical raw materials for drug production [26].

Previously, studies have confirmed the high toxicity of methanol extracts to cancer cell lines (HeLa), and attributed this to its toxic target to their cell membrane [28,29]. As reported by Sentkowska, et al. [30], the high toxicity of methanol extracts could be due to the polar nature of methanol and its capability to extract various polar and sometimes non-polar bioactive compounds with varying toxicities. Methanol molecule consists of a single atom of a tetraedric carbon, linked to 3 hydrogens, and a hydroxyl (OH) group. The -OH group is polar in nature, and the three hydrogens, the water-insoluble hydrocarbon chain. This gives methanol dual properties, and the capability to dissolve both polar and non-polar molecules [31].

The cytotoxicity activities of the extracts ranged from high, mid, and low. The potentiality (78.9%) of the crude leaf extract of *A. indica* as a highly active cytotoxic agent against Vero cells was revealed in its very low IC$_{50}$ of 0.011 µg µL^{-1}. The cytotoxic properties of *A. indica* could be attributed to its possession of methyl stearate, which Bai and Krishnakumar [32] reported to possess antiproliferative and cytotoxic properties. The toxicity profile of *A. indica* has also been reported by several other workers [33-35]. Although the toxicity of this plant was reported to be harmful to mammalian cells, it has been
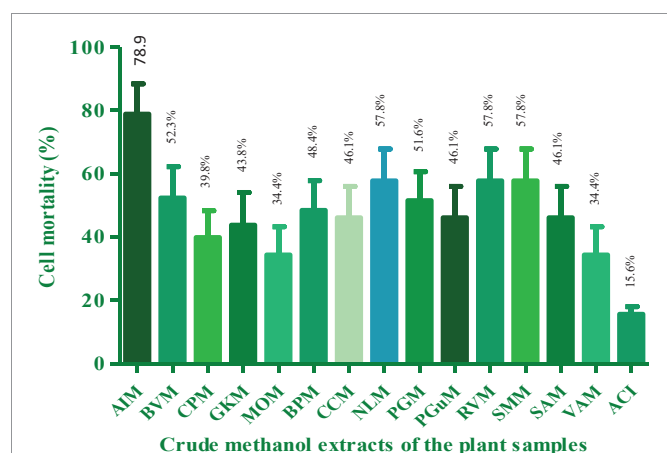

Figure 1: Cytotoxicity activities of methanol extracts of the medicinal plants on Vero cells
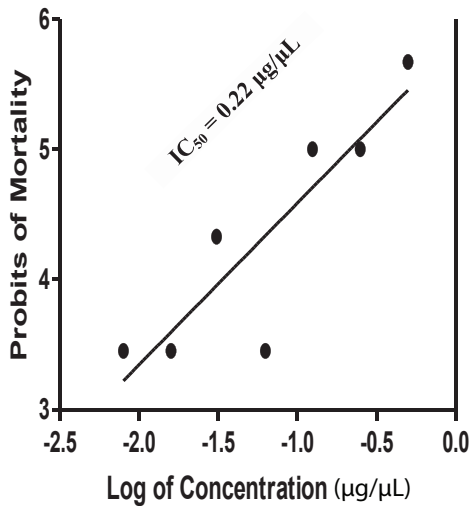
Key: AIM: A. indica methanol; BVM: B. vulgaris methanol; CPM: C. paridisi methanol; GKM: G. kola methanol; MOM: M. oleifera methanol; BPM: B. pinnatum methanol; CCM: C. citrates methanol; NLM: N. laevis methanol; PGM: P. guineense methanol; PGuM: P. guajava methanol; RVM: R. vomitoria methanol; SMM: S. mombin methanol; SAM: S. alata methanol; VAM: V. album methanol; ACI: Acyclovir.
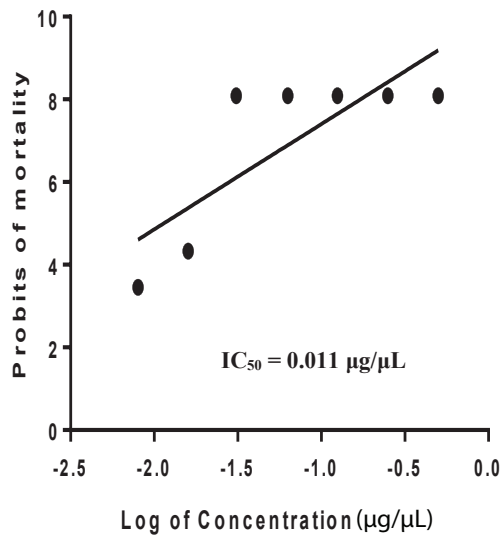
A. indica
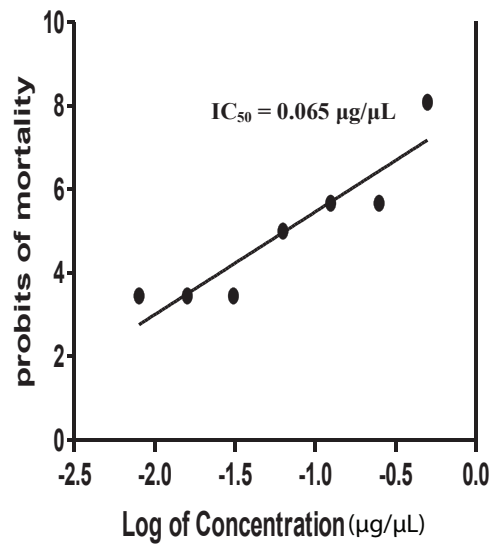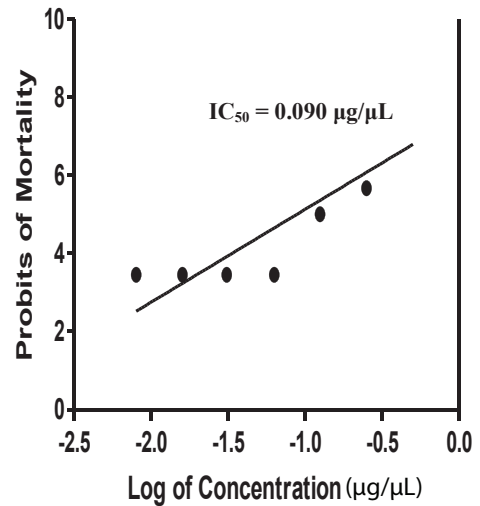

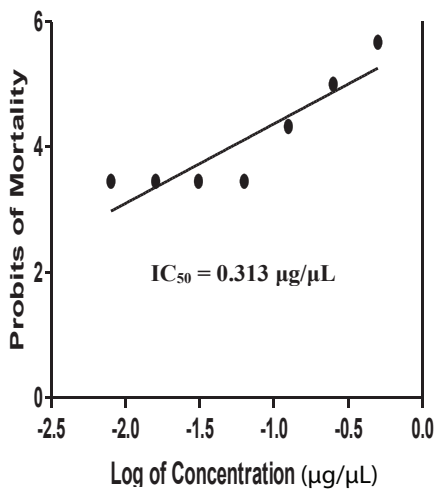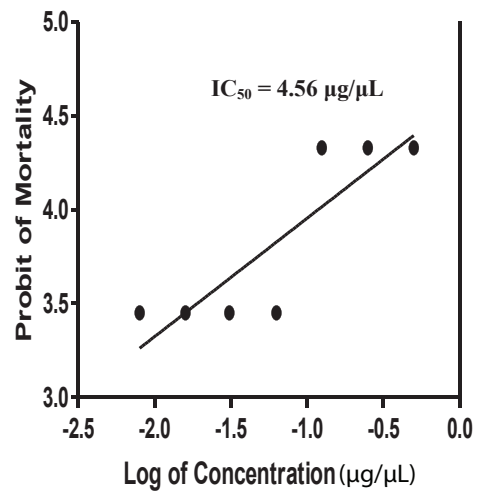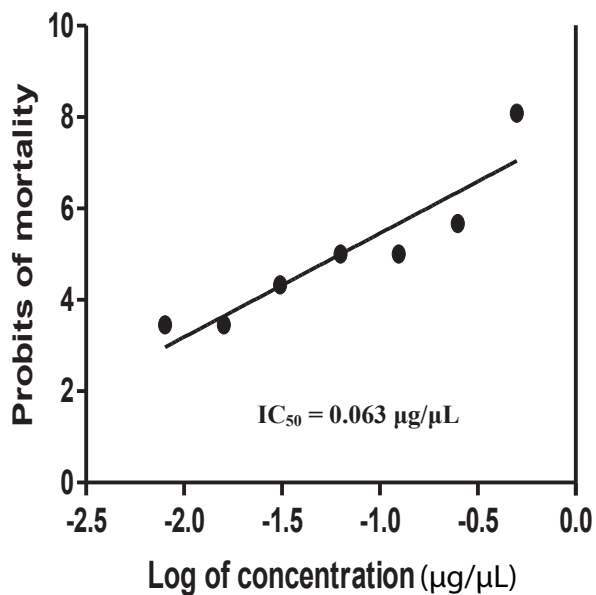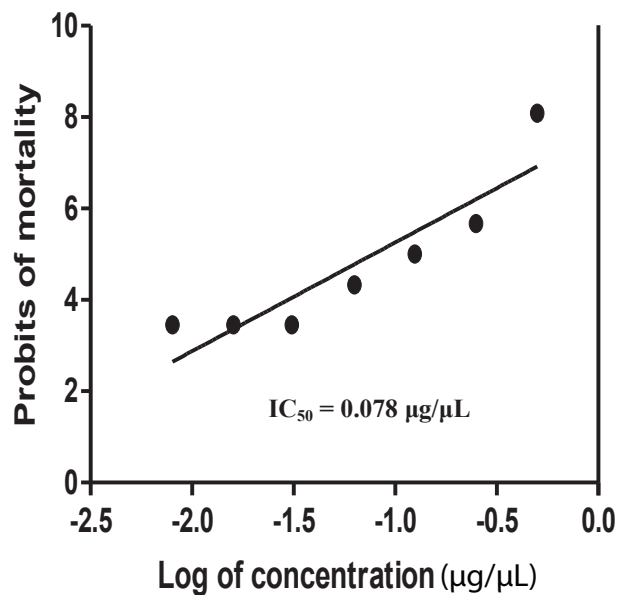*B. vulgaris*

C. paradisi

G. kola

M. oleifera

Acyclovir

B. pinnatum

C. citrates
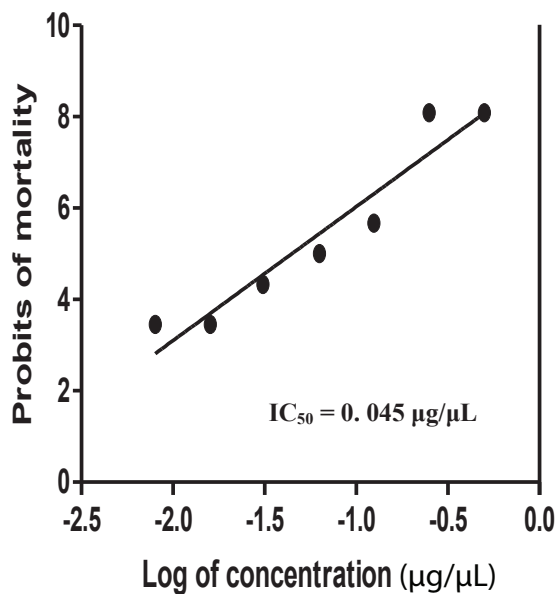
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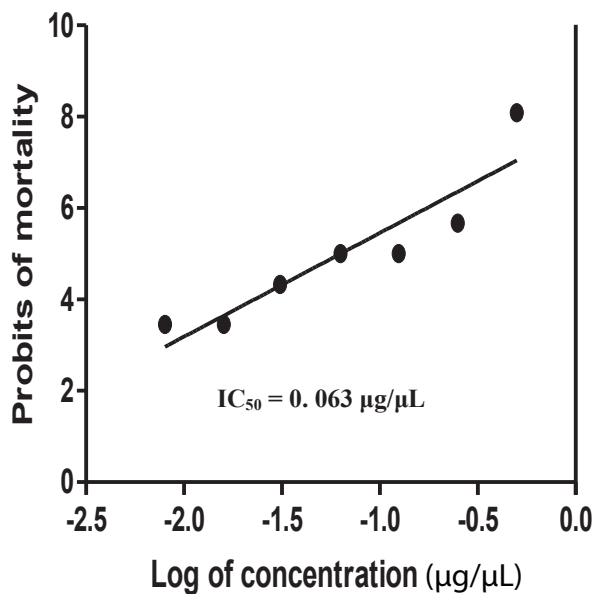
P. guineense
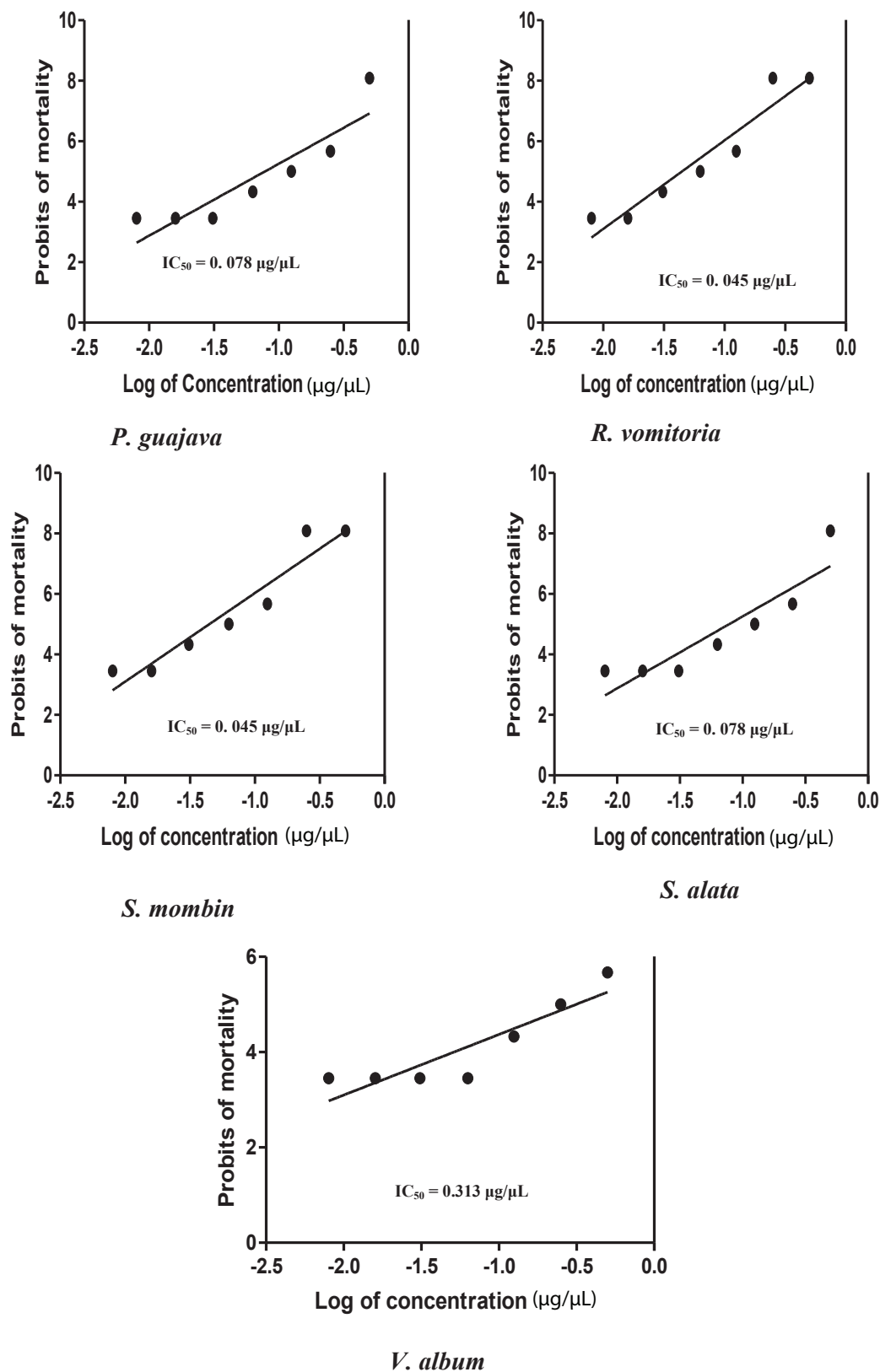
Figure 2: Probit analyses of the toxicity of methanol extracts of the plant samples on Vero cells. Legend: A. indica- Azadirachta indica; B. vulgaris- Bambusa vulgaris; B. pinnatum- Bryophyllum pinnatum; C. paradise- Citrus paradise; C. citrates- Cymbopogon citrates; G. kola- Garcinia kola; M. oleifera- Moringa oleifea; N. laevis- Newbouldia laevis; P. guineense - Piper guineense; P. guajava - Psidium guajava; R. vomitoria- Rauvolfia vomitoria; S. alata- Senna alata; S. momobin- Spondias mombin; V. album- Viscum album.

Table 2: Level of toxicity (μg μL^{-1}) of the crude extract of the plant samples on Vero cell 4+ Complete (100%) cytopathic effect (CPE); 3+ 75% CPE; 2+ 50% CPE; 1+ 25% CPE; 0 No CPE.

Extract	Extract Concentration (μg μL^{-1})							
	1	0.5	0.25	0.125	0.063	0.031	0.016	0.008
AIM	4$^+$	4$^+$	4$^+$	4$^+$	4$^+$	4$^+$	1$^+$	0
BVM	4$^+$	4$^+$	3$^+$	3$^+$	2$^+$	0	0	0
CPM	4$^+$	3$^+$	2$^+$	2$^+$	1$^+$	0	0	0
GKM	4$^+$	4$^+$	3$^+$	2$^+$	0	0	0	0
MOM	4$^+$	3$^+$	2$^+$	1$^+$	0	0	0	0
BPM	4$^+$	4$^+$	3$^+$	2$^+$	2$^+$	1$^+$	0	0
CCM	4$^+$	4$^+$	3$^+$	2$^+$	1$^+$	0	0	0
NLM	4$^+$	4$^+$	4$^+$	3$^+$	2$^+$	1$^+$	0	0
PGM	4$^+$	4$^+$	3$^+$	2$^+$	2$^+$	1$^+$	0	0
PGuM	4$^+$	4$^+$	3$^+$	2$^+$	1$^+$	0	0	0
RVM	4$^+$	4$^+$	4$^+$	3$^+$	2$^+$	1$^+$	0	0
SMM	4$^+$	4$^+$	4$^+$	3$^+$	2$^+$	1$^+$	0	0
SAM	4$^+$	4$^+$	3$^+$	2$^+$	1$^+$	0	0	0
VAM	4$^+$	3$^+$	2$^+$	1$^+$	0	0	0	0
ACI	1$^+$	1$^+$	1$^+$	1$^+$	0	0	0	0
CELL Con	0	0	0	0	0	0	0	0

Key: AIM = *A. indica* Methanol; BVM = *B. vulgaris* Methanol; CPM = *C. paradisi* Methanol; GKM = *G. kola* Methanol; MOM = *M. oleifera* Methanol; ACI = Acyclovir; BPM = *B. pinnatum* Methanol; CCM = *Cymbopogun citrates* Methanol; NLM = *N. laevis* Methanol; PGM = *Piper guineense* Methanol; PGuM = *P. guajava* Methanol; RVM = *R. vomitoria* Methanol; SMM = *S. mombin* Methanol; SAM = *S. alata* Methanol; VAM = *V. album* Methanol; CELL CON = Cell control.

shown to be beneficial as a pesticide [36] and a larvicide [37,38]. *A. indica* has also been reported to be toxic to spermatocytes [39].

The percentage cytotoxicity of seed extract of *G. kola* (43.8%) and IC$_{50}$ of 0.090 μg μL^{-1} produced marked apoptotic effect on Vero cells. One of the constituents of *G. kola* which could have been responsible for this cytopathic effect on Vero cell is methyl stearate, reported to possess antiproliferative effect on mammalian cells. The toxicity profile of *G. kola* on mammalian cells has further been manifested in its ability to antagonize ovulation, and prevent progesterone production and fetal implantation in pregnant mammals [40]. In addition, the ability of *G. kola* to interfere with sperm production in both men and animals due to its toxic profile to mammalian cells has been extensively documented [41]. Other workers have reported caution in the use of *G. kola* [42] due to its high toxic activity to mammalian cells.

Although leaf extract *M. oleifera* was not as toxic as *A.* indica, it still produced remarkable cytopathic effect on Vero cells, manifested in its percentage toxicity of 34.4% and IC$_{50}$ of 0.313 μg μL^{-1}. Jafarain, et al. [43] has previously reported the cytotoxicity of leaf extract of *M. oleifera* on HeLa cells and attributed this to the presence of phenols in the plant tissues. It is possible also that the presence of this phytochemical, which has been previously identified to be a constituent in Nigerian *M. oleifera* plants, contributed in no small way to the cytopathic activities of *M. oleifera* observed in this study. In a similar manner, Kasolo, et al. [44] reported that *M. oleifera* leaf extract possess phytochemicals which could cause toxicity to mammalian kidney, liver and heart if half lethal doses are administered orally to Swiss Albino mice for thirty days.

Several other reports have been made about the cytotoxic activities of the crude extracts of the plants used in this study. According to Reppas [45], two adult cattle died of myocardial degeneration within 48 h after they were fed with a large amount of *B. pinnatum* (L.), probably due to the presence presence of cardiac and bufadienolide in the plant tissues. Similarly the fruits of most *V. album* (L.) species were described as toxic berries which could lead to vomiting, hypotension, cerebral dysfunctions and death by a heart attack if ingested in large quantity [46]. However very little information is available on the cytotoxicity of *N. laevis* to Mammalian cells which this study has also shown to be very toxic.

CONCLUSION

This toxicity study has shown that, although all the plants are edible, they should be consumed with caution since it was observed that their methanol extracts could damage mammalian cells. However this high cytopathic activity of the crude extracts against a transformed cell line could be exploited in drug production against cancerous cells.

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