Research Journal of Medicinal Plant

ISSN 1819-3455

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Medicinal Plants

ISSN 1819-3455
DOI: 10.3923/rjmp.2020.156.166

Research Article
Carissa edulis and *Gmelina arborea* Reduce Cell Viability and Prevent Apoptosis in CaCO-2 and Hep-G2 Cells

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Abstract

Background and Objective: *Gmelina arborea* and *Carissa edulis* are medicinal plants worldwide used and especially in Benin to manage various diseases. The present study aimed to evaluate the cytotoxic activity of extracts and fractions from the *Gmelina arborea* and *Carissa edulis*. **Materials and Methods:** Plants leaves were collected, dried and extracted. The crude extracts were then fractionated using ethyl acetate and butanol. Thin layer chromatography was applied to screen phytochemical components. CaCO-2 and Hep-G2 cells were cultured in DMEM medium for 24 h and exposed to the extracts with concentrations range from 0.05-7.5 mg mL^{-1} for 48 h. Cell viability and cell growth were performed by using the neutral red uptake and MTT colorimetric assays. Furthermore, cytotoxicity mechanism was screened by quantifying Lactate Dehydrogenase (LDH) leakage in the extracellular medium. In addition, the caspase-3/7 activation and DNA fragmentation assays were performed for apoptosis cell death pathway. **Results:** Many secondary metabolites such as; anthracenes, naphthoquinones, alkaloids, saponins and flavonoids were identified in the both plant extracts. An important cell viability reduction and inhibition of the two cells growth were recorded. Necrosis was the major pathway of cell death almost at the highest concentration and on the other hand, neither caspases-3/7 activation nor DNA fragments showing ladder shapes were observed. **Conclusion:** *Carissa edulis* and *Gmelina arborea* contain phytochemical components which reduced significantly cell viability concentration-dependently in the two cell lines in which the cell death pathway may be by necrosis rather than apoptosis.

Key words: *Gmelina arborea*, *Carissa edulis*, cytotoxicity, cell death pathway, apoptosis

Citation: Razack Osseni, Serge Moukha, Habib Ganfon, Théophile Mobio, Pascale Dozolme and Edmond E. Creppy, 2020. *Carissa edulis* and *Gmelina arborea* reduce cell viability and prevent apoptosis in CaCO-2 and Hep-G2 cells. Res. J. Med. Plants, 14: 156-166.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phytotherapia is widely practiced in Africa, especially in Benin. The rural population considers plants as safe since they experienced them many years ago[1]. Plants utilization is favored not only because of their easy availability especially in rural provinces, but also because of the expensive prices of modern medicines. Although, the population well appreciates herbal remedies, most of them lack scientific studies that could stand their efficiency and non-toxic effects. According to the World Health Organization, herbal medicines must scientifically be screened for pharmaco-toxicological studies in order to prove their beneficial and/or adverse effects before any human use. Many modern drugs were discovered after ethnopharmacological investigations[2].

Gmelina arborea Roxb (Verbenaceae) and *Carissa edulis* Vahl (Apocynaceae) are reported in the traditional management of many diseases. *Carissa edulis* Vahl (Apocynaceae) also called arabic num-num is reported to manage many symptoms such as; headache, toothache, cough, chest pains, fever and diseases like rheumatism, epilepsy, sickle cell anemia, gonorrhea, syphilis, helminthiases and rabies[3]. Some pharmacological properties were evaluated on *Carissa edulis*. These include diuretic[3], analgesic[4], anticonvulsant[5], antiviral[6], antimicrobial[7], anti-inflammatory, antioxidant and antitumor effects[8].

In many African countries, *Gmelina arborea* is traditionally used for management of arterial hypertension[9]. Many other usages of this plant in the traditional medicine are known. For example, it is used in Nigeria and in Guinea traditional medicine to treat diarrhea[10]. Several pharmacological properties including; antimicrobial, antidiabetic, antioxidant and vasodilating were described for *Gmelina arborea*[11-14]. Previous phytochemical studies on *Gmelina* genus showed the presence of several compounds such as; phenolic compounds[15,16] and iridoids[17]. It is reported to contain alkaloid, glycoside, lignan and sesquiterpenoid; furthermore, phytochemical screening analysis reveals the presence of carbohydrates, saponins, tannins, anthraquinones and cardiac glycosides[18].

Despite the numerous uses of the two plants in traditional medicine, few data are available on the leaves particularly and on their safety[19-21]. A previous study demonstrated the antihypertensive properties[22,23] and the *in vivo* toxicity of the two medicinal plants on rats[24,25]. The antihypertensive studies showed efficacy of the both crude extracts, the butanol fraction of *Gmelina arborea* and the residual aqueous fraction of *Carissa edulis*. Therefore, these two fractions and the crude extracts were selected for *in vitro* cytotoxic proprieties. The aim of this present study was to screen the cytotoxicity of the extracts and eventually understand mechanisms that may underlie this cytotoxicity on cell lines.

MATERIALS AND METHODS

Plant materials and extractions: This survey was a part of the PhD project and a continuity of the laboratory thematic on the two plants. It took place at the Laboratory of Pharmacognosy and Phytotherapia of the Faculty of Health Sciences, University of Abomey-Calavi, Benin and at Laboratory of Toxicology and Applied Hygiene of Bordeaux, France. It lasted 2 years 6 months from October, 2014-March, 2017.

Gmelina arborea and *Carissa edulis* fresh leaves were collected at Abomey-Calavi in Southern Benin. Specimen were deposited for identification and authentication at the National Herbarium of Benin (N° AA 6337 and N° AA 6482 for *GA* and *CE,* respectively). The leaves were washed with tap water and dried at room temperature 20°C for 2 weeks. The dried leaves were crushed and pulverized into powder. Then, the extraction was made by using the method described by Awede *et al.*[23]. Briefly, 500 g (5x100 g) of the powder with 750 mL of distilled water. The decoction was filtered three times through Whatman paper No.1 and the same operation was repeated until the decoction extractable components were entirely extracted. The filtered extract was evaporated using a Rotavapor "RE-300" at 80°C. The obtained dried extract was stored in refrigerator until use. Then, the dried extract of each plant was entirely fractionated by liquid-liquid partition as follow. About 50 g of the aqueous extract were dissolved with 500 mL of distilled water and then introduced in a 2 L conical flask. A first separation was carried out entirely with ethyl acetate and then with and n-butanol. The organic phases were recovered and evaporated at 30°C on a rotary evaporator under reduced pressure as well as the residual aqueous phase. All extract fractions were evaporated till dryness prior testing.

Phytochemical analysis: Thin Layer Chromatography (TLC) method was performed to detect phytochemical groups of each fraction[26]. About 5 mg of each extract was dissolve in 1 mL of appropriate solvent (mixture of methanol/water (1:1), dichloromethane or ethyl acetate). The chromatographic plates were then loaded with 10 µL of each extract and the migration was performed using appropriate solvents system according to each chemical group. The phytochemical investigation includes; alkaloids, flavonoids, coumarins, tannins, triterpenes, saponosides, anthracenes, lignans, naphthoquinones and anthocyanins.

Cells culture: Hep-G2 cells were purchased from ATCC (American Type Culture Collection) and $CaCO_2$ cell line was obtained from Dr. Jing Yu (Tuffs School of Medicine, Medford, MA, USA). The two cell lines were cultured in DMEM medium (Sigma-Aldrich, France) supplemented with 10% fetal bovine serum, without antibiotic at 37°C, 5% CO_2.

Reagents includes Phosphate Buffered Saline (PBS), trypsine-0.02%, Ethylene Diamine Tetra Acetic Acid (EDTA) mixture, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) and neutral red were purchased from Sigma-Aldrich (Lyon, France). All other chemicals used were of analytical grade and from either Sigma-Aldrich or Dominique-Dutscher chemicals, France.

MTT test: This assay was used to determine cell sensitivity to the extracts as reflected by the inhibitory concentration 50% (IC_{50}). The IC_{50} is the concentration of plant extract that inhibits cell proliferation by 50%. The IC_{50} values of each extract for a given cell type could then be compared each other. The MTT assay was performed according to the method described by Creppy *et al.*[27].

CaCO-2 and Hep-G2 cells were seeded in 96 well plates [Nunc] at an initial density of 10^5 cells per well and incubated overnight in DMEM medium without antibiotic and without serum supplementation at 37°C and 5% CO in a humidified atmosphere. Adherent cells were treated with extract in an increasing concentration from 0.05-7.5 mg mL^{-1}. Assays were performed in sextuplets. The 0% control wells were empty (without cells), whereas 100% control cells were incubated only with culture medium. After 48 h incubation period under the above-mentioned conditions, cells were treated with 100 µL of MTT [Sigma; 1 mg mL^{-1}] solution. Forty five minutes after, the medium was removed and cells were treated with 200 µL of Dimethyl Sulfoxide (DMSO). After 20 min, cell viability proportional to MTT transformation into formazan was quantitated by using an ELISA reader (LABTECH LT 4000, France) at 540 nm.

Neutral red uptake assay: Cells were seeded in 96-well micro plates (10^6 cells/well), routinely cultured in a humidified incubator for 24 h. Cells were maintained in culture and exposed to the crude aqueous extract over a range of concentrations (0.05-7.5 mg mL^{-1}). After 48 h exposure, Neutral Red Uptake (NRU) test was performed according to the procedure described by Creppy *et al.*[27]. Briefly, at the end of the treatment, the medium was discarded and 150 µL of a freshly prepared neutral red solution (3.3 µg mL^{-1}) was added to every well and re-incubated for an additional 4 h at 37° C. Thereafter, the cells were carefully washed twice with 200 µL of PBS to eliminate extracellular NR. The incorporated dye was eluted from the cells by adding 200 µL elution medium (50% ethanol supplemented with 1% acetic acid, v/v) into each well followed by gentle shaking of micro-plate for 15 min. The plates were then read at 540 nm using a micro-plate reader (LABTECH LT-4000). The number of treated cells was compared to that observed in control cultures and the percentage of viable cells calculated.

Lactate dehydrogenase (LDH) leakage assay: The lactate dehydrogenase (LDH) leakage assay is a colorimetric assay for the quantification of cell death and cell lysis based on the measurement of LDH released from the cytosol of damaged cells into the culture medium supernatant. A kit provided by Promega (CytoTox 96® Non-Radioactive) was used for this assay. The LDH activity can be determined with a 30 min coupled enzymatic assay, which results in the conversion of a tetrazolium salt (iodonitrotetrazolium violet; INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance data are collected using a standard 96-well plate reader.

Hep-G2 and CaCO-2 cells were seeded in 96-multiwell plates in the same conditions as previously described. After 24 h after seeding, the cells were treated with extracts (0.05-10 mg mL^{-1}) and incubated for 48 h. Then, each supernatant (100 µL) was treated with 100 µL of the reaction mixture (as prescribed by the manufacturer) in a 96-wells plate. The reaction was carried out for 45 min in the dark under gentle stirring at 37°C and stopped by the stop solution provided in the kit. The absorbance was read at 492 nm by using a spectrophotometer plate reader (LABTECH LT-4000). Obtained data were expressed in reference to a positive blank prepared by using the lysed solution provided by the manufacturer. The enzyme activity detected in the culture supernatant reflects the membrane integrity and correlates with the proportion of lysed cells. Hence, cell vitality is inversely proportional to the LDH released.

Assessment of apoptosis induction

Caspase-3/7 activation assay: Caspases-3 and 7 are the most frequently activated death protease for apoptosis evaluation which catalyzes specific cleavage of many key cellular proteins. In this assay, caspase-3/7 activation was evaluated by using the caspase-3/7 fluorometric Kit (Promega) following the protocol prescribed by the manufacturer. The assay kit provides all reagents for a fast and sensitive measurement of the activities of caspase-3 and 7. The buffer and substrate are

mixed and added to the sample (cellular lysate). Upon sequential cleavage and removal of the DEVD peptides by caspase-3/7 activity and the rhodamine-110 cleaved-group becomes intensely fluorescent with excitation at 499 nm and emission at 521 nm. Comparison of the fluorescence of rhodamine-110 from the sample with that of the control allows determining the fold of increase in caspase-3/7 activity. Briefly, Hep-G2 cells were cultured in 96-multiwell plates at a density of 10^6 cells/well. Twenty four hours after seeding, the cells were treated with 100 µL of the extracts (0.46, 1.875 and 7.5 mg μL^{-1}) and incubated for 48 h. Cells were treated with 100 µL of a mixture of caspases 3/7 reagents. All assays were performed in triplicate. The plates were gently shaken by using a plate shaker for 30 sec and kept out of light at room temperature for 15 h. Then, the fluorescence was read with a fluorimetric reader with excitation at 499 nm and emission at 520 nm, respectively.

Genomic DNA fragmentation assay: Oligo-nucleosome fragmentation of genomic DNA is one of the hallmarks of apoptosis. It can be detected as a ladder pattern on agarose gel electrophoresis. The assay was performed according to method described by Mobio *et al.*[28]. The two cell lines were seeded in 6-multiwell plates at a density of 5.10^6 cells/well. Twenty four hours after, the cells were treated with extracts (0.46, 1.875 and 7.5 µg μL^{-1}) and incubated for additional 24 h. An untreated control was set up in parallel. The extraction procedure was performed as described by the genomic DNA extraction kit manufacturer Promega (Wizard Genomic DNA Extraction kit).

Briefly, cells were washed with PBS, harvested by trypsinization and centrifuged in 1.5 mL tubes (14,000 xg, 1 min). Cells were washed again, vortexed and lysed in the nuclei lysis buffer (50 mM Tris-HCl pH 8.0, 10.0 mM EDTA, 0.5% SDS). Then, 3 µL of RNase were added to the nuclear lysate and mixed by inversion. The mixtures were incubated for 30 min to room temperature. The protein precipitation solution (200 µL) was added to each micro-centrifugation tube. They were vigorously vortexed and centrifuged (14,000 xg) for 1 min. The supernatants containing DNA were transferred into another clean 1.5 mL micro-centrifugation tube containing 600 µL of isopropanol. DNA strands were visible by gently mixing, the tubes were centrifuged (14,000 xg for 1 min and 70%) ethanol (600 µL) was added to the pellet to wash DNA. After centrifugation, (14,000 xg, 1 min) the ethanol was carefully removed and the micro-centrifugation tubes were inverted on absorbent paper and air-dried. DNA pellets were dissolved in the DNA rehydration solution and incubated at 4°C overnight.

DNA samples were quantified using a NanoDrop 2000/2000c Spectrophotometers (Thermo-Scientific, Wilmington, DE, USA) and stored at 4°C. Three microgram of each DNA sample were loaded on 2% agarose gel (containing 10 µg mL^{-1} of ethidium bromide) in TAE (Tris, Acetate, EDTA) buffer and mixed with a blue/orange dye. A 100 base pairs DNA ladder ideal for determining of double-stranded DNA from 100-1500 base pairs was used as a nucleosome sizes marker. Afterward, gels were exposed to UV light (254 nm) for determination of the shape of migration.

Statistical analysis: All experiments were performed three times in duplicate and data was statistically analyzed using linear regression to determine the IC_{50} and the unpaired student's t-test for the other assays by using the GraphPad Prism software Version 6.

RESULTS

The aqueous extraction was carried out from 500 g of the both plant powder. The extraction rates were 16.55 and 19.77%, respectively for *Carissa edulis* and *Gmelina arborea*. Three fractions (Ethyl acetate, Butanol and the residual aqueous fraction) were obtained after the liquid-liquid partition of each plant material.

Phytochemical groups: The phytochemical groups of the two plants are summarized in Table 1 and 2. *Carissa edulis* revealed the presence of lignans, flavonoids, saponins, anthocyanins, anthracenes, alkaloids and tannins in the crude extract. In addition to these chemical groups, naphthoquinones, triterpenes and sterols were found in the ethyl acetate fraction. The butanolic fraction did not reveal the presence of naphthoquinones, triterpenes and alkaloids.

As for extract of *Gmelina arborea*, it revealed the presence of saponins, anthocyanins and flavonoids in the three extracts samples. Bitter principles were not detected in any of the extracts. Moreover, only the ethyl acetate fraction did not show the presence of coumarins and tannins. In the other hand, anthracenes and alkaloids were detected only in the fractions.

Effect of the extracts on cell growth and cell viability: The extracts and fractions of the two plants were submitted to an extensive screening to assess their cytotoxic properties by using MTT and NR assays devoted to evaluation of cell growth and/or cell viability. The two assays resulted in concentration-dependent inhibition of the two cell lines proliferation. The

Table 1: Phytochemical groups of the fractions from *Carissa edulis*

Chemical groups	Crude extract	Ethyl acetate fraction	Butanol fraction	Residual aqueous fraction
Lignans	+	+	+	+
Flavonoids	+	-	+	+
Saponins	+	+	+	+
Naphthoquinones	-	+	-	-
Anthocyanins	+	+	+	+
Triterpenes	-	+	+	-
Anthracene derivatives	+	+	+	+
Alkaloids	+	+	-	+
Tannins	+	+	+	+
Coumarins	+	-	+	+
Bitter principles	-	+	-	-

+: Detected, -: Not detected

Table 2: Phytochemical groups of the fractions from *Gmelina arborea*

Chemical groups	Crude extract	Ethyl acetate fraction	Butanol fraction	Residual aqueous fraction
Lignans	+	+	-	-
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Naphthoquinones	-	+	-	-
Anthocyanins	+	+	+	+
Triterpenes	-	+	-	-
Anthracene derivatives	-	+	+	+
Alkaloids	-	+	+	-
Tannins	+	-	+	+
Coumarins	+	-	+	+
Bitter principles	-	-	-	-

+: Detected, -: Not detected

Table 3: Inhibitory concentrations (IC_{50}) values of the extracts in CaCO-2 and Hep-G2 cells

Extracts	CACO-2 (IC_{50} mg mL^{-1})	Hep-G2 (IC_{50} mg mL^{-1})
Crude extract of *C. edulis*		
MTT	0.39 ±0.015	0.10 ±0.011
NR	0.34 ±0.055	0.07 ±0.030
Aqueous fraction of *C. edulis*		
MTT	1.30 ±0.053	1.14 ±0.024
NR	1.35 ±0.012	1.60 ±0.038
Crude extract of *G. arborea*		
MTT	2.50 ±0.081	2.16 ±0.027
NR	4.50 ±0.16	No inhibition
Butanol fraction of *G. arborea*		
MTT	2.34 ±0.024	2.28 ±0.037
NR	0.05±0.015	1.20 ±0.025

two colorimetric methods revealed comparable results. The concentrations that inhibit 50% of cells viability or cell growth (IC_{50}) were deduced and showed in Table 3.

Briefly, the IC_{50} in MTT test were 0.39 and 0.10 mg mL^{-1}, respectively for CaCO-2 and Hep-G2 cells when exposed to the crude extract of *C. edulis*. As for the NR test, the IC_{50} were respectively of 0.34 and 0.07 mg mL^{-1}. The aqueous fraction of *C. edulis* showed higher IC_{50} values following the two assays and cell lines. In the other hand, the butanol fraction of *G. arborea* highlighted the lowest IC_{50} value 0.05 mg mL^{-1} following NR assay with CaCO-2 cells.

Cytotoxic effect on cell lines: Both crude extracts of *Gmelina arborea* and *Carissa edulis* and their fractions induced LDH leakage in the extracellular medium, mainly at concentration range of 2.5-10 mg mL^{-1} (Fig. 1, 2). CaCO-2 cells showed the largest amount of LDH in the culture medium following exposure to the aqueous fraction of *C. edulis*. The butanol fraction of *G. arborea* exhibited the lowest concentration that induced cytotoxicity.

Effect of the two extracts on apoptosis induction in Hep-G2 cells: Following 48 h treatment of Hep-G2 cells with the extracts, caspase-3/7 activities were measured and compared to that of control cells. As shown in Fig. 3a-d, crude extracts and fractions *of Carissa edulis* and *Gmelina arborea* showed a note worthy concentration dependent of caspase-3/7 activity.

Effect on genomic DNA fragmentation: The conventional agarose gel electrophoresis was performed on Hep-G2 cells treated with 1 mg mL^{-1} of plant extract for 48 h. The result showed that inter-nucleosomal DNA cleavage produced no ladder pattern, neither with the crude extracts nor with the fractions treatment (Fig. 4, 5). No DNA fragmentation was detected.

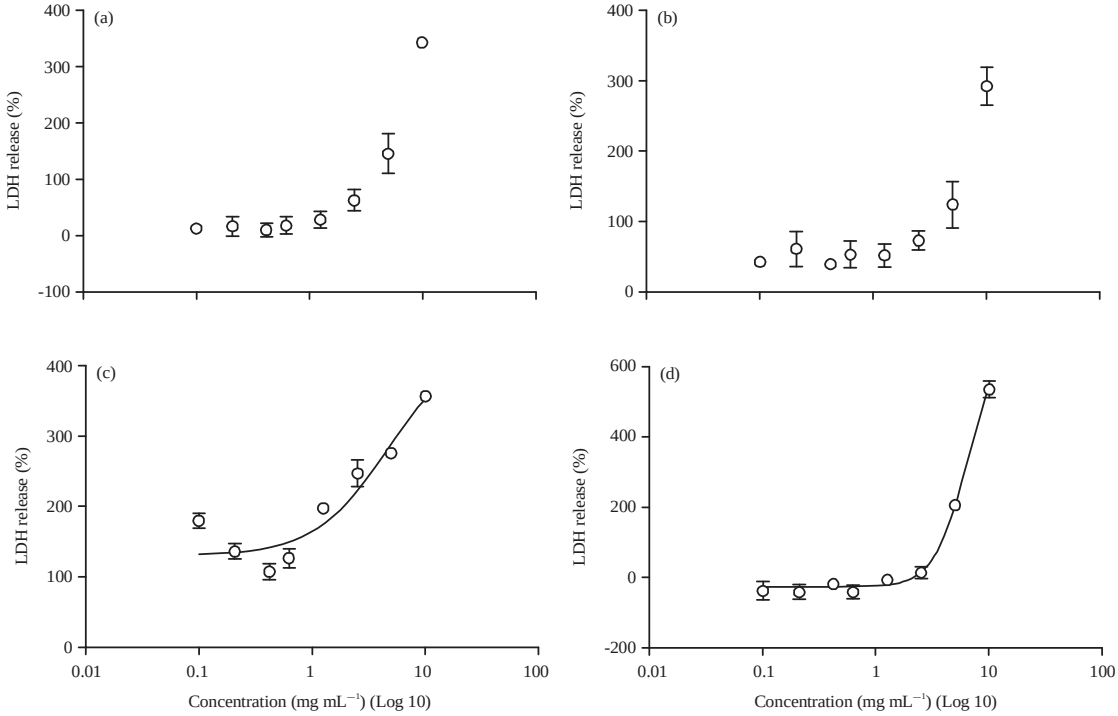

Fig. 1(a-d): Effect of *Carissa edulis* and its aqueous fraction on LDH leakage from CaCO-2 and Hep-G2 cells, (a) *C. edulis* on CaCO-2 cells, (b) *C. edulis* on Hep-G2 cells, (c) Aqueous fraction of *C. edulis* on CaCO-2 and (d) Aqueous fraction of *C. edulis* on Hep-G2

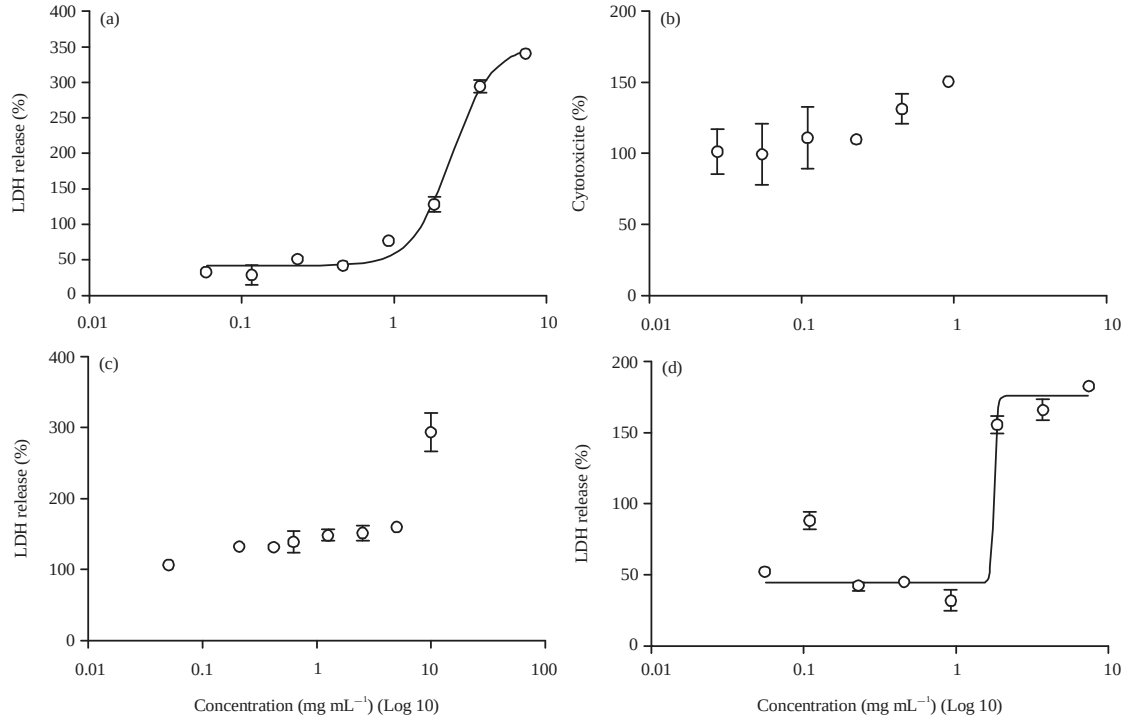

Fig. 2(a-d): Effect of *Gmelina arborea* and its butanol fraction on LDH leakage from CaCO-2 and Hep G2 cells, (a) Crude extract of *G. arborea* in CaCO-2 cells, (b) Crude extract of *G. arborea* in Hep-G2 cells, (c) Butanol fraction of *G. arborea* in and (d) Butanol fraction of *G. arborea* in Hep-G2

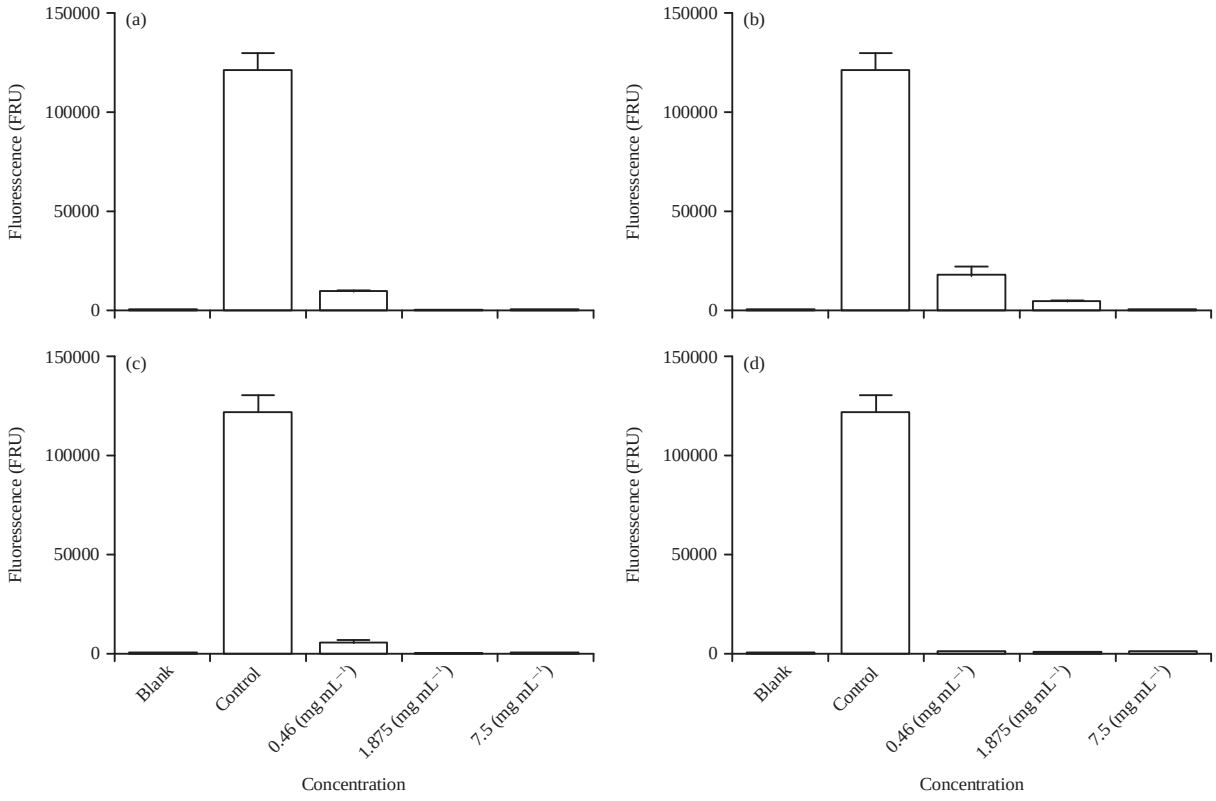

Fig. 3(a-d): Effect of *Carissa edulis* and *Gmelina arborea* and their fractions in Hep-G2 cells, (a) *C. edulis* extract, (b) Crude extract of *G. arborea*, (c) Aqueous of *C. edulis* in and (d) Butanol fraction of *G. arborea*

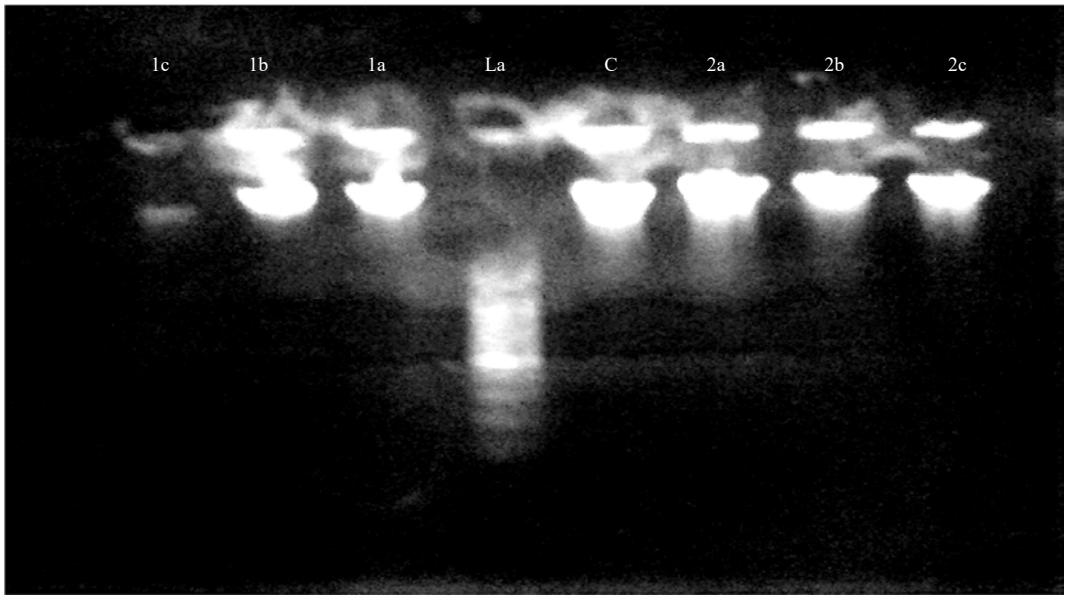

Fig. 4: Agarose gel electrophoresis of DNA

Lanes 1a, b and c are treated respectively with concentrations of 0.46, 1.875 and 7.5 mg mL^{-1} of the aqueous fraction of *Carissa edulis*, Lanes 2a, b and c are from cells treated respectively with concentrations of 0.46, 1.875 and 7.5 mg mL^{-1} of the crude extract of *Carissa edulis*, Lane La is the ladder and C is the control cells that received only medium

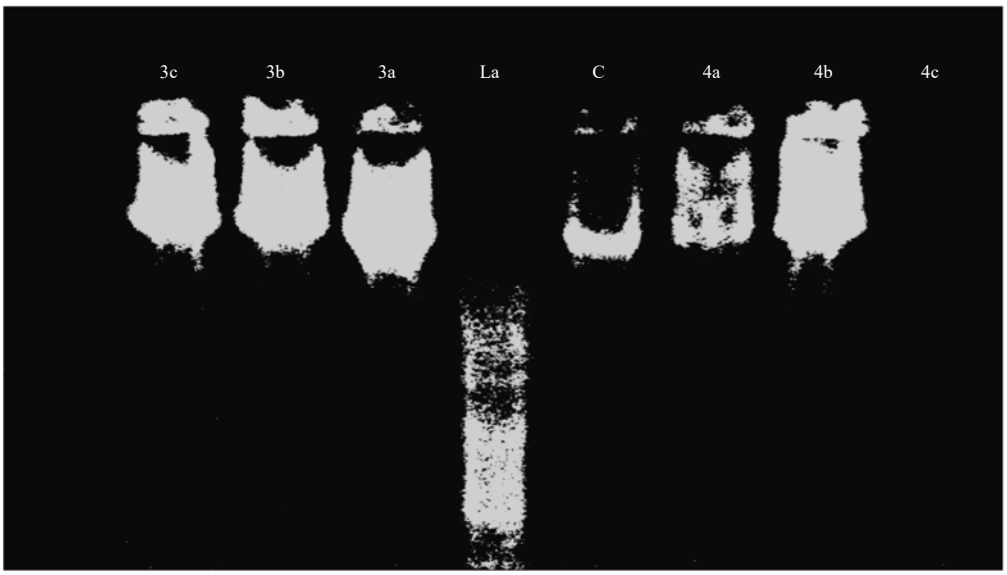

Fig. 5: Agarose gel electrophoresis of DNA

Lanes 3a, b and c are at concentrations 0.46, 1.875 and 7.5 mg mL^{-1} of the butanol fraction of *Gmelina arborea*, respectively, Lanes 4a, b and c are at concentrations at 0.46, 1.875 and 7.5 mg mL^{-1} of the crude aqueous extract of *Gmelina arborea*, respectively. Lane La is the ladder and C is the control cells that received only medium

DISCUSSION

In this study, the extraction method used was the aqueous decoction according to the tradi-practitioners advices. In fact, decoction remains the most widely method of drug preparation in the traditional medicine management. Many data are available on the medicinal benefits of the detected phytochemical components in *Gmelina arborea* and *Carissa edulis* (Table 1, 2). Thus, flavonoids, saponins, naphthoquinones, anthraquinones and alkaloids found in the two plants may act either individually or synergistically to inhibit cell proliferation observed. Some studies have demonstrated the side effects of polyphenolics which are considered as prooxidative when accumulated at high concentrations in cells[29,30]. Saponins present therapeutic effects as well as toxic properties[31]. As regards naphthoquinones, they are pharmacologically active and generally cytotoxic[32] which made their usage in the treatment of cancer. Anthraquinones are good cytotoxic and anti-cancerous drugs, but they are unfortunately pro-carcinogen to cells[33,34]. *Gmelina arborea* and *Carissa edulis* are two medicinal plants used in the management of high blood pressure in Benin by herbal traditional healers. This preliminary work aimed to investigate the cytotoxic proprieties of the two plants. Caco-2 and Hep-G2 cell lines are used as screening tools, since they allowed the assessment of intestinal permeability and hepatic metabolism. Therefore, they may help predicting any pharmacological effect of present extracts. Two different tests were performed to assess the effect of these substances on cells viability. The MTT test is a colorimetric assay measuring the metabolic activity of living cells by assessing the activity of mitochondrial dehydrogenases which is used for cell growth or cell proliferation assessment. Otherwise neutral red test is a cytotoxicity assay on which the principle is based incorporation of a vital dye (neutral red) by viable cells[35]. The results showed a cell viability reduction depending on concentration and an anti-proliferative activity by all extracts and fractions. The extract concentrations that reduced cell viability less than 50% were considered as harmless[36]. There was no significant difference between the two cell-lines sensitivity by using the same colorimetric method for similar concentrations of tested substances. Generally, IC_{50} value equal or above 100 µg mL^{-1} is considered as cytotoxic[37]. In this study, cell viability was mostly inhibited by the butanol fraction of *G. arborea* using NR assay (50 µg mL^{-1}) followed by the crude extract of *C. edulis* in all conditions. A careful analysis of the results revealed, on the one hand, that NR assay was more accurate than the MTT test, thus, suggested that cell viability was mostly altered by a mechanism targeting lysosomes and membranes. On other hand, Hep-G2 cells seemed to be more resistant to all the extracts and fractions than CaCO-2 cells. A study conducted by Ghareeb *et al.*[38] showed the cytotoxic activity of isolated flavonoids

compounds of *G. arborea* using Hep-G2 assay with IC_{50} ranged from 3.38-15.70 μg mL^{-1}. The cytotoxicity of the methanol extracts of *G. arborea* in several cell lines was demonstrated[39] at 1.25 mg mL^{-1}, whereas with the ethanol extract the cytotoxicity[40] was at 12 mg mL^{-1}. Despite the numerous studies evaluating several potential effects of *C. edulis*, mainly *in vivo* pharmacological studies[5,41], very few focused its cytotoxicity on cultured cell lines. The present study is therefore, one of the first to examine the *in vitro* cytotoxicity of extracts of *C. edulis*.

Apoptosis and necrosis are the two major forms of cell death. The lactate dehydrogenase test is a biomarker frequently used in cytotoxicity evaluation since it exhibits the cellular barrier permeability as a consequence of membrane damage[42]. In the present study, extracts of both two plants showed concentration-dependent LDH release in the culture medium. The butanol fraction of *G. arborea* was the most cytotoxic. Regarding these results, since the extracts induced a huge release of LDH in the culture medium, it might be a sign of cell membranes alteration which is an indicator of necrotic pathway which occurred during incubations with the extracts[43]. An imbalance or inactivation of important apoptotic pathway could be harmful and lead to the carcinogenesis process[44]. Apoptosis is triggered through effector caspases (Caspase-3-7)[45]. The results revealed no activation of these caspases in Hep-G2 cells after 48 h incubation with extracts and fractions (Fig. 3a-d). The inhibition of caspase activity was concentration-dependent. Moreover, the high was the extract concentrations; the less was the activity of caspases. The butanol fraction of *G. arborea* (Fig. 3d) induced the greatest caspase inhibitory effect. This observation confirmed somehow that cells might be killed by necrosis pathway through LDH release. To complete this hypothesis, DNA fragmentation assay was carried out on agarose gel electrophoresis. DNA cleavage results in inter-nucleosome fragments (100 bp and multiples) which is a sign of apoptotic cell death. Agarose gel electrophoresis of DNA showed no ladder formation for any of the extracts. There was no difference between the control untreated cells and Hep-G2 cells treated with extracts, whereas the nucleosome sizing mixture showed clear inter-nucleosomal fragments. These results confirmed that caspases activation plays a minor role, if any, in the extracts cytotoxicity.

Necrosis is usually considered to be an uncontrolled and accidental cell death, which, mostly due to an inflammatory process on which future investigation will be focused. In contrast, apoptosis is the major cell death pathway for removing unwanted cells in a best manner. Considering the consequences of necrosis and the lack of apoptosis in this study, one could speculate that higher concentration usages of the extracts could be harmful to cells.

CONCLUSION

This present study confirmed the phytochemical components of *Gmelina arborea* and *Carissa edulis*. The IC_{50} were determined and they revealed inhibition of cell viability and cell proliferation. The butanol fraction of *G. arborea* was the most cytotoxic. None of all tested extracts induced apoptosis.

SIGNIFICANCE STATEMENT

This study is the first survey to investigate on the *in vitro* cytotoxicity of the two and mostly to understand mechanism of cell death. This study can be beneficial for researchers not only to standardize their extracts and conveniently adapt doses they may use, but also the butanol fraction could be explored as a candidate in cancer treatment arsenal.

ACKNOWLEDGMENT

We would like to acknowledge the great support from the laboratory of toxicology and applied hygiene of Bordeaux, the University of Abomey-Calavi and the support of the "Agence Universitaire de la Francophonie" the program of which supported the training in Bordeaux [grant number (Contrat N°78 046 014) - (HTA-PHYS-PHAR)].

REFERENCES

1. Petrovska, B.B., 2012. Historical review of medicinal plants' usage. Pharmacogn. Rev., 6: 1-5.
2. Heinrich, M., S. Edwards, D.E. Moerman and M. Leonti, 2009. Ethnopharmacological field studies: A critical assessment of their conceptual basis and methods. J. Ethnopharmacol., 124: 1-17.
3. Omino, E.A. and J.O. Kokwaro, 1993. Ethnobotany of Apocynaceae species in Kenya. J. Ethnopharmcol., 40: 167-180.
4. Nedi, T., N. Mekonnen and K. Urga, 2004. Diuretic effect of the crude extracts of *Carissa edulis* in rats. J. Ethnopharmacol., 95: 57-61.
5. Ibrahim, H., E.M. Abdurahman, M. Shok, N. Ilyas, K.Y. Musaand and I. Ukandu, 2007. Comparative analgesic activity of the root bark,stembark fruits and seeds of *Carissa edulis* VAHL. Apocynaceae. Afr. J. Biotechnol., 6: 1233-1235.

6. Ya'u, J., A.H. Yaro, M.S. Abubakar, J.A. Anuka and I.M. Hussaini, 2008. Anticonvulsant activity of *Carissa edulis* (Vahl) (Apocynaceae) root bark extract. J. Ethnopharmacol., 120: 255-258.
7. Ibrahim, H., E.M. Abdurahman, M. Shok, N. Ilyas and R. Bolaji, 2005. Preliminary phytochemical and antimicrobial studies of the leaves of *Carissa edulis*. Chem. Class J., 2: 15-18.
8. Woode, E., C. Ansah, G.K. Ainooson, W.M. Abotsi, A.Y. Mensah and M. Duweijua, 2007. Anti-inflammatory and antioxidant properties of the root extract of *Carissa edulis* (Forsk.) Vahl (Apocynaceae). J. Sci. Technol., 27: 6-15.
9. N'Guessan, K., M.S. Tiebre, E. Ake-Assi and G.N. Zirihi, 2009. Ethnobotanical study of plants used to treat arterial hypertension in traditional medicine, by Abbey and Krobou populations of Agboville (Cote-d'Ivoire). Eur. J. Sci. Res., 35: 85-98.
10. Gutierrez, S.P., M.A.Z. Sanchez, C.P. Gonzalez and L.A. Garcia, 2007. Antidiarrhoeal activity of different plants used in traditional medicine. Afr. J. Biotechnol., 6: 2988-2994.
11. Jabbar, S., M.T. Khan, M.S. Choudhuri and B.K. Sil, 2004. Bioactivity studies of the individual ingredients of the Dashamularishta. Pak. J. Pharmaceut. Sci., 17: 9-17.
12. El-Mahmood, A.M., J.H. Doughari and H.S. Kiman, 2010. *In vitro* antimicrobial activity of crude leaf and stem bark extracts of *Gmelina arborea* (Roxb) against some pathogenic species of Enterobacteriaceae. Afr. J. Pharm. Pharmacol., 4: 355-361.
13. Pattanayak, P., P.K. Parhi, S.K. Mishra and P.K. Khandei, 2011. Screening of anti-diabetic activity of bark extracts of *Gmelina arborea* in streptozotacin induced diabetic rats. Int. J. Pharmaceut. Sci. Rev. Res., 8: 130-132.
14. Wansi, S.L., P. Nyadjeu, T.B. Nguelefack, S.F. Fodouop, A.A. Donatien and A. Kamanyi, 2012. *In vivo* antioxidant and vasodilating activities of *Gmelina arborea* (verberaceae) leaves hexane extract. J. Complement. Integr. Med., Vol. 9. No. 1. 10.1515/1553-3840.1623.
15. Shankar, S.R., R. Girish, N. Karthik, R. Rajendran and V.S. Mahendran, 2009. Allelopathic effects of phenolics and terpenoids extracted from *Gmelina arboreaon* germination of Black gram (*Vigna mungo*) and Green gram (*Vigna radiata*). Allelopathy J., 23: 323-331.
16. Dighe, V., S. Adhyapak, D. Mestry and N. Shambhu, 2011. High performance liquid chromatography method for quantization of apigenin from dried root powder of *Gmelina arborea* Linn. Int. J. Pharma Biosci., 2: 742-749.
17. Helfrich, E. and H. Rimpler, 2000. Iridoid glycosides from *Gmelina philippensis*. Phytochemistry, 54: 191-199.
18. Kaur, N., S. Kaur, P.M.S. Bedi and R. Kaur, 2012. Preliminary pharmacognostic study of *Gmelina arborea* bark. Int. J. Nat. Prod. Sci., 1: 184-184.
19. Ngulde, S.I., U.K. Sandabe, M.B. Tijjani, A.A. Barkindo and I.M. Hussaini, 2013. Phytochemical constituents, antimicrobial screening and acute toxicity studies of the ethanol extract of *Carissa edulis* Vahl. root bark in rats and mice. Am. J. Res. Commun., 1: 99-110.
20. Sahu, R., G. Divakar and K. Divakar, 2010. *In vivo* rodent micronucleus assay of *Gmelina arborea* Roxb (Gambhari) extract. J. Adv. Pharmaceut. Technol. Res., 1: 22-29.
21. Kulkarni, Y.A. and A. Veeranjaneyulu, 2012. Toxicological evaluation of the methanol extract of *Gmelina arborea* Roxb. bark in mice and rats. Toxicol. Int., 19: 125-131.
22. Lawson, R., B. Awede, R. Osseni, F. Gbaguidi, J. Gbenou and A. Laleye, 2016. Effects of *Gmelina arborea*, Roxb (Verbenaceae) aqueous extract on arterial pressure of Wistar rats. J. Physiol. Pathophysiol., 7: 1-6.
23. Awede, B., R. Osseni, L. Lagnika, M. Adjagba, C. Kpadonou, R.B. Darboux and A. Laleye, 2015. Antihypertensive effects of *Gmelina arborea* Roxb (Verbenaceae) leaves crude aqueous extract fractions in Wistar rats. J. Physiol. Pharmacol. Adv., 5: 803-810.
24. Osseni, R., B. Awede, M. Adjagba, C. Kpadonou, M. Fall, A. Laleye and R. Darboux, 2015. Acute and subchronic toxicity of *Gmelina arborea* Roxb, (Verbenaceae) in Wistar rat. Int. J. Toxicol. Pharmacol. Res., 7: 116-122.
25. Osseni, R., S. Akoha, M. Adjagba, S. Azonbakin and L. Lagnika *et al*., 2016. *In vivo* toxicological assessment of the aqueous extracts of the leaves of *Carissa edulis* (Apocynaceae) in Wistar rats. Eur. J. Med. Plants, 15: 1-10.
26. Wagner, H. and S. Bladt, 2001. Plant Drug Analysis: A Thin Layer Chromatography Atlas. 2nd Edn., Springer, Berlin, Germany, ISBN-13: 978-3540586760, Pages: 384.
27. Creppy, E.E., P. Chiarappa, I. Baudrimont, P. Borracci, S. Moukha and M.R. Carratu, 2004. Synergistic effects of fumonisin B_1 and ochratoxin A: Are *in vitro* cytotoxicity data predictive of *in vivo* acute toxicity? Toxicology, 201: 115-123.
28. Mobio, T.A., I. Baudrimont, A. Sanni, T.W. Shier and D. Saboureau *et al*., 2000. Prevention by vitamin E of DNA fragmentation and apoptosis induced by fumonisin B1 in C6 glioma cells. Arch. Toxicol., 74: 112-119.
29. Lambert, J.D., S. Sang and C.S. Yang, 2007. Possible controversy over dietary polyphenols: Benefits vs risks. Chem. Res. Toxicol., 20: 583-585.
30. Halliwell, B., 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? Arch. Biochem. Biophys., 476: 107-112.
31. Price, K.R., I.T. Johnson, G.R. Fenwick and M.R. Malinow, 1987. The chemistry and biological significance of saponins in foods and feedingstuffs. Crit. Rev. Food Sci. Nutr., 26: 27-135.

32. Bolton, J.L., M.A. Trush, T.M. Penning, G. Dryhurst and T.J. Monks, 2000. The role of quinones in toxicology. Chem. Res. Toxicol., 13: 135-160.
33. Fry, D.W., 1991. Biochemical pharmacology of anthracenediones and anthrapyrazoles. Pharmacol. Therapeut., 52: 109-125.
34. Weiss, R.B., 1992. The anthracyclines: Will we ever find a better doxorubicin? Semin. Oncol., 19: 670-686.
35. Borenfreund, E. and J.A. Puerner, 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol. Lett., 24: 119-124.
36. Machana, S., N. Weerapreeyakul, S. Barusrux, A. Nonpunya, B. Sripanidkulchai and T. Thitimetharoch, 2011. Cytotoxic and apoptotic effects of six herbal plants against the human hepatocarcinoma (HepG2) cell line. Chin. Med., Vol. 6. 10.1186/1749-8546-6-39.
37. Cantrell, C.L., M.A. Berhow, B.S. Phillips, S.M. Duval, D. Weisleder and S.F. Vaughn, 2003. Bioactive crude plant seed extracts from the NCAUR oilseed repository. Phytomedicine, 10: 325-333.
38. Ghareeb, M.A., H.A. Shoeb, H.M. Madkour, L.A. Refahy, M.A. Mohamed and A.M. Saad, 2014. Antioxidant and cytotoxic activities of flavonoidal compounds from *Gmelina arborea* Roxb. Global J. Pharmacol., 8: 87-97.
39. Vijay, T., M.D. Rajan, K. Sarumathy, S. Palani and K. Sakthivel, 2011. Cardioprotective, antioxidant activities and phytochemical analysis by GC-MS of *Gmelina arborea* (GA) in doxorubicin-induced myocardial necrosis in Albino rats. J. Applied Pharmaceut. Sci., 1: 198-204.
40. Punitha, D., A. Thandavamoorthy, K. Arumugasamy, U. Danya and M. Ramanathan, 2012. Potent *in vitro* cytotoxic effect of *Gmelina arborea* Roxb. (Verbenaceae) on three human cancer cell lines. Int. J. Pharma Sci. Res., 3: 357-363.
41. Maina, G.S., J.K. Kelvin, M.B. Maina, N.J. Muriithi and M.J. Kiambi *et al*. 2015. Antinociceptive properties of dichloromethane: Methanolic leaf and root bark extracts of *Carissa edulis* in rats. J. Phytopharmacol., 4: 106-112.
42. Decker, T. and M.L. Lohmann-Matthes, 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and Tumor Necrosis Factor (TNF) activity. J. Immunol. Methods, 115: 61-69.
43. Renehan, A.G., S.P. Bach and C.S. Potten, 2001. The relevance of apoptosis for cellular homeostasis and tumorogenesis in the intestine. Can. J. Gastroenterol. Hepatol., 15: 166-176.
44. Creppy, E.E., A. Diallo, S. Moukha, K. Eklu-Gadegbeku and D. Cros, 2014. Study of epigenetic properties of poly(hexamethylene biguanide) hydrochloride (PHMB). Int. J. Environ. Res. Public Health, 11: 8069-8092.
45. Budihardjo, I., H. Oliver, M. Lutter, X. Luo and X. Wang, 1999. Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol., 15: 269-290.