

RADICAL SCAVENGING AND CYTOTOXICITY EVALUATION OF EIGHT EXTRACTS OF *CITRUS LIMON* AND *CITRUS AURANTIFOLIA*

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ABSTRACT

Cancer is a global cause of death characterized by uncontrolled proliferation and spread of abnormal cells. Eight extracts composed of the leaf, stem bark, seeds and juice of each of *Citrus limon* and *C. aurantifolia* were subjected to *in vitro* antioxidant assay using DPPH, brine shrimp lethality bioassay (BSL) and cytotoxicity MTT colorimetric assay using human cancer cell lines. Extracts of *C. aurantifolia* stem bark and leaf had IC₅₀ of 28.2±0.11 and 47.2±0.39 µg/mL, respectively and displayed better radical scavenging activity compared to the other extracts, Ascorbic acid, the reference drug, had an IC₅₀ of 9.2±0.14 µg/mL. *Citrus limon* stem bark (LC₅₀=10.0±0.33) and *C. limon* leaf (LC₅₀= 5.0±0.74 µg/mL) extracts were observed to be strongly cytotoxic compared to cyclophosphamide (LC₅₀=98.76±0.15 µg/mL), while the other extracts were either non, weakly, or moderately toxic in BSL assay. *Citrus aurantifolia* leaf extract (CC₅₀=4.02±2.85 µg/mL, CC₅₀=5.45±2.8 µg/mL) retained a comparable cytotoxicity to cyclophosphamide (CC₅₀=2.23±0.14 µg/mL, CC₅₀=2.66±0.8 µg/mL) on Rd and Hep-2c human cancer cell lines, respectively. The other extracts exhibited varying degrees of cytotoxicity. This study demonstrated that the extracts of both *Citrus* species had weak DPPH radical scavenging activity. *Citrus aurantifolia* leaf extract displayed potent toxicity in BSL assay and on the two human cancer cell lines; Rd and Hep-2c used in the study and were selective to cancer cells than the normal cell, Vero.

INTRODUCTION

Cancer is a group of disease characterized by uncontrolled proliferation and spread of abnormal cells and if not controlled, may result in death [1]. In the year 2012 alone, about 14.1 million new cancer cases and 8.2 million cancer deaths were reported, globally [2]. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in under-developed regions of the world and it was projected that 19.3 million new cancer cases will be reported annually by 2025 and under-developed nations will be most devastated [3]. Sadly, most anticancer drugs are not selectively cytotoxic to cancer cells leading to the destruction of normal cells and resulting in toxicity. This lack of selectivity is the bane behind the urgency of discovery and development of new anticancer drugs from plant sources. Hence this makes the search for safer anticancer agents imperative, considering the side effects associated with many of the currently used chemotherapeutic agents [4]. The use of plants for food and nutrition, shelter, clothing and medicines is deeply root in human history [5, 6]. Medicinal plants have recently been recognized as a major source of new compounds for drug discovery [7]. Most of the anticancer drugs such as vincristine, vinblastine, taxol, indicine-N-oxide, etoposide analogs, camptothecine and analogs are derived from natural sources [8-10]. More than half of anticancer compounds in clinical trial are isolated from plant sources [11, 12].

Citrus is one of the most widely known plant species, consisting of about 40 species that are distributed around the world. In Nigeria, lime (*Citrus aurantifolia*), lemon (*Citrus limon*), tangerine (*Citrus tangerina*), orange (*Citrus sinensis*) and grape (*Citrus paradisi*) are among the most commonly consumed citrus fruits [13]. *Citrus limon* and *C. aurantifolia* are known locally in Yoruba in SW

Nigeria as *Osanlakuregbe/Osanlaimu* and *Osanwewe*, respectively [14, 15]. *Citrus limon* (L.) Burm and *C. aurantifolia* (Christm) Swingle belong to the family Rutaceae [16]. Traditionally among the Yorubas', a decoction of the *C. limon* fruit, leaves, root and bark is used as an antipyretic, stomachic and as cold remedy. About 100 mL of the decoction is used twice daily. While, a decoction of *C. aurantifolia* leaves, bark, root and fruit is used to treat toothache and abdominal ulcer, also about a 100 mL of the decoction is used twice daily [14]. *Citrus limon* is recognized as an important medicinal plant mainly for its alkaloids content that has been reported to have anticancer and antibacterial activities [17]. Crude extracts of the different parts (leaves, stem, root, juice, peel and flower) of lemon have been reported to show antimicrobial activity against clinical bacterial strains [18]. Flavonoids isolated from *Citrus* have been reported to have numerous biological activities including antibacterial, antifungal, anti-diabetic, anticancer and antiviral activities [19]. Investigation into the pharmacological activities of *C. aurantifolia* extracts have shown that the plant has laxative, anti-inflammatory, anticancer, antiviral and antibacterial properties [5, 20], it has also been reported to have antihypertensive activity [21]. The hydro-alcoholic extracts of *C. aurantifolia* leaves were found to possess significant antibacterial and antifungal activities [22]. The essential oils from *Citrus limon* had been reported to have strong antioxidant activity [23].

The aim of this study is to determine and compare the antioxidant and cytotoxic properties of *C. limon* and *C. aurantifolia* leaf, stem bark, seeds and juice extracts, respectively on human Rhabdomyosarcoma (Rd) and laryngeal carcinoma (Hep-2c) cancer

cell lines.

RESULTS AND DISCUSSION

The voucher number of FHI: 110008 and FHI: 110009 were issued for the *C. limon* and *C. aurantifolia*, respectively at the Forest Herbarium in Forestry Research Institute of Nigeria (FRIN), Ibadan. The yields of the different extracts are displayed in **Table 1**. The extracts of *C. limon* stem bark and *C. aurantifolia* leaf extract had the highest yield.

The DPPH radical scavenging activity of the eight extracts was assessed as shown in **Table 1**. Of eight extracts, *C. aurantifolia* stem bark ($IC_{50} = 28.2 \pm 0.11 \mu\text{g/mL}$) and leaf ($IC_{50} = 47.2 \pm 0.39 \mu\text{g/mL}$) extracts had the highest DPPH radical scavenging activity, while the seed extracts of *C. aurantifolia* ($IC_{50} = 400.0 \pm 1.27 \mu\text{g/mL}$) and *C. limon* ($IC_{50} = 400.0 \pm 0.52 \mu\text{g/mL}$) had comparable activity. However, each extract demonstrated significantly lower DPPH radical scavenging property than *C. aurantifolia* stem bark and leaf extracts. The eight extracts had significantly ($P < 0.05$) lower DPPH scavenging activity when compared to ascorbic acid ($IC_{50} = 9.26 \pm 0.14 \mu\text{g/mL}$) used as the positive control. Ascorbic acid is also a constituent of Citrus juice. *Citrus aurantifolia* and *C. limon* contain 0.425 mg/mL and 0.385 mg/mL of ascorbic acid, respectively in of fresh juice [27]. In the radical scavenging assay, *C. aurantifolia* leaf extract was reported to have up to 87.05% DPPH radical inhibition [28]. The methanol, hexane and ethanol extracts of *C. aurantifolia* leaf had been reported to have IC_{50} of 967, 580 and 736 $\mu\text{g/mL}$ respectively, while ascorbic acid had an IC_{50} of $25.5 \pm 0.75 \mu\text{g/mL}$ in 1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay [29]. This is the first report of DPPH radical scavenging activity of *C. aurantifolia* stem bark and it had the highest scavenging activity.

Brine shrimp lethality (BSL) assay had been reported to have significant correlation with other antitumor assays. The assay is often employed in preliminary screening of extracts/compounds in drug design and synthesis of cytotoxic compounds [30]. Padmaja *et al.*, [31] classified the lethality of extracts on brine shrimps, where it was reported that extracts with $LC_{50} \leq 1000 \mu\text{g/mL}$ are non-toxic, $LC_{50} = 500$ to $1000 \mu\text{g/mL}$ are weakly toxic, $LC_{50} = 100$ to $500 \mu\text{g/mL}$ are moderately toxic and $LC_{50} \leq 100 \mu\text{g/mL}$ are strongly toxic [31]. The BSL of the eight extracts is shown in Table 2. The extracts of *C. aurantifolia* ($LC_{50} = 40 \pm 2.11 \mu\text{g/mL}$) and *C. limon* ($LC_{50} = 10 \pm 0.33 \mu\text{g/mL}$) stem bark in addition to the leaf extracts of *C. aurantifolia* ($LC_{50} = 55 \pm 3.18 \mu\text{g/mL}$) and *C. limon* ($LC_{50} = 35 \pm 0.74 \mu\text{g/mL}$) were observed to be strongly cytotoxic. However, the other extracts were observed to be either weakly, moderately or non-toxic in the BSL assay compared to cyclophosphamide, the positive control ($LC_{50} = 98.76 \pm 0.15 \mu\text{g/mL}$). The results of this study is unlike a previous work where the juice extracts of both *C. aurantifolia* ($LC_{50} = 26.09 \mu\text{g/mL}$) and *C. limon* ($LC_{50} = 14.47 \mu\text{g/mL}$) were reported to be strongly cytotoxic [32]. In another report, the extract of the aerial part of *C. aurantifolia* (secondary branches with tender and young leaves, flower buds, flowers and small unripe fruits) was found to be moderately toxic with LC_{50} of $376.85 \pm 4.90 \mu\text{g/mL}$ [33]. This study provides the first report of the BSL of *C. limon* and *C. aurantifolia* stem bark and seed extracts.

The extracts were also observed to exhibit varying degrees of cytotoxicity on the various human cancer cell lines as shown in **Table 3**. Interestingly, the extracts of *C. aurantifolia* leaf ($CC_{50} = 4.02 \pm 2.85 \mu\text{g/mL}$) and the juice ($CC_{50} = 4.02 \pm 2.85 \mu\text{g/mL}$) and *C. limon* juice extract ($CC_{50} = 5.80 \pm 2.48 \mu\text{g/mL}$) had comparable cytotoxicity to cyclophosphamide ($CC_{50} = 2.23 \pm 0.14 \mu\text{g/mL}$) on Rd cell line. While on Hep-2c cell line, the extract of *C. aurantifolia* leaf ($CC_{50} = 5.45 \pm 2.8 \mu\text{g/mL}$) had a higher cytotoxicity than leaf extract of *C. limon* ($CC_{50} = 8.49 \pm 4.8 \mu\text{g/mL}$). However, the seed extracts of both *C. aurantifolia* ($CC_{50} = 8.05 \pm 3.12 \mu\text{g/mL}$) and *C. limon* ($CC_{50} = 10.17 \pm 0.2 \mu\text{g/mL}$) and stem bark extracts of *C. aurantifolia* ($CC_{50} = 10.12 \pm 2.6 \mu\text{g/mL}$) and *C. limon* ($CC_{50} = 9.63 \pm 3.5 \mu\text{g/mL}$) displayed similar cytotoxicity on Hep-2c cell line. The juice extract of *C. aurantifolia* ($CC_{50} = 66.09 \pm 8.3 \mu\text{g/mL}$) elicited weaker cytotoxicity than the juice extract of *C. limon* ($CC_{50} = 47.33 \pm 1.7 \mu\text{g/mL}$) on the Hep-2c. Only the leaf extracts of both *C. limon* ($CC_{50} = 8.49 \pm 4.8 \mu\text{g/mL}$) and *C. aurantifolia* ($CC_{50} = 5.45 \pm 2.8 \mu\text{g/mL}$) were observed to have comparable cytotoxicity to cyclophosphamide ($CC_{50} = 2.66 \pm 0.8 \mu\text{g/mL}$) on the Hep-2c. It is worthy of note that, only *C. aurantifolia* leaf extract retained a comparative cytotoxicity to cyclophosphamide on both cancer cell lines used in this study. Isolated compounds from *C. aurantifolia*, such as Hydroxycinnamates, flavonoid glycosides, and polymethoxylated flavones have been shown to inhibit the proliferation of some cancer cell lines and in many cases the compounds had an IC_{50} below 10 μM [34]. The extract of *C. aurantifolia* juice was reported to exhibit maximum cytotoxicity, with an IC_{50} of 81.20 $\mu\text{g/mL}$ and after 72 h apoptosis is induced in Panc-28 human cells line used in the study [35]. The juice extract of *C. limon* had also shown significant inhibition of the spontaneously proliferating RPMI-8866 cell line [36]. In another study, *C. limon* essential oils was observed to have an IC_{50} between 5.75-7.92 $\mu\text{g/mL}$ against LIM-1863 colorectal cancer cell line [37]. The cytotoxicity of *C. aurantifolia* leaf extract has not been reported in literature before now and this study appears to be the first report that compares cytotoxicity of *C. aurantifolia* and *C. limon* extracts on Hep-2c and Rd cancer cell lines. Medicinal plants used traditionally for management of oxidative stress had been reported to be cytotoxic on cancer cells [38]. The extracts with the highest radical scavenging activity (*C. aurantifolia* stem bark and leaf) were observed to display a significant cytotoxicity on the two cancer cell lines.

Cancer chemotherapy is coupled with life threatening adverse drug effects and the major challenge of cancer chemotherapeutic drug development, is the toxicity of the drugs to non-cancerous cells resulting from lack of selectivity to cancerous cells. In this study, a normal African green monkey kidney epithelial cell line (Vero) was included to evaluate the selectivity of the extracts to the cancer cell lines used in this study.

The eight extracts elicited varying degrees of cytotoxicity on the Vero cell line as shown in **Table 3**. The selective indices (SI) of the extracts were estimated as shown in **Table 4**. Extract with SI greater than 1 is considered selective for cancer cells. Extracts of *C. aurantifolia* seed ($SI = 1.91 \pm 0.92$), stem bark ($SI = 7.54 \pm 1.00$) and juice ($SI = 37.94 \pm 19.88$) have better selectivity for Rd cancer cell line than the extracts of *C. limon* seed ($SI = 0.83 \pm 0.06$), stem bark ($SI = 1.97 \pm 0.09$) and juice ($SI = 14.09 \pm 3.78$), respectively. While, on

the Hep-2c cell line, extracts of *C. aurantifolia* seed (SI = 2.56±0.58) and stem bark (SI = 8.67±1.00) had better selectivity than the extracts of *C. limon* seed (SI = 0.79±0.03) and stem bark (SI = 1.06±0.05), respectively. On the contrary, *C. limon* leaf extract (SI = 2.17±0.02 on Rd, SI = 2.46±0.19 on Hep-2c) had better selectivity on the two cancer cell lines than *C. aurantifolia* (SI = 1.39±0.23 on Rd, SI = 1.39±0.19 on Hep-2c). *Citrus limon* juice (SI = 14.09±3.78) also displayed better selectivity than *C. aurantifolia* juice extract (SI = 1.51±0.02). Nevertheless, juice extracts of both *Citrus* species

are selectively not toxic to normal cell line and no wonder the fruits are safe for food. However, only *C. limon* seed extract appeared to be more toxic on the normal cells than the cancer cell lines, on Rd (SI = 0.83±0.06) and Hep-2c (SI = 0.79±0.03). *Citrus aurantifolia* juice (SI = 37.94±19.88) and stem bark (SI = 8.67±1.00) were observed to have the highest selectivity on Rd and Hep-2c cancer cell lines, respectively. This study provides the first report of the selective indices of *C. aurantifolia* and *C. limon* extracts on the two cancer lines used.

Table 1:Percentage Yield and DPPH Antioxidant Activity of *Citrus limon* and *Citrus aurantifolia* Extracts

Plant extracts	Voucher Number	Percentage Yield (%)	IC ₅₀ (µg/mL)
<i>C. aurantifolia</i> (SD)	FHI: 110008	6.25	400.0 ± 1.27**
<i>C. limon</i> (SD)		5.41	400.0 ± 0.52**
<i>C. aurantifolia</i> (SB)		4.80	28.2 ± 0.11**
<i>C. limon</i> (SB)		10.27	138.9 ± 1.23**
<i>C. aurantifolia</i> (L)	FHI: 110009	9.50	47.2 ± 0.39**
<i>C. limon</i> (L)		5.66	250.35 ± 1.84**
<i>C. aurantifolia</i> (J)		0.02	400 ± 0.71**
<i>C. limon</i> (J)		0.03	308.46 ± 1.50**
Ascorbic acid			9.26±0.14

KEY

SD- Seed, SB- Stem bark, J- Juice part and L- Leaf

*standard drug / positive control

* = No significant difference (P> 0.05)

** = Significant difference (P< 0.05)

FHI: 110008= Voucher Number of *Citrus limon*

FHI: 110009= Voucher Number of *Citrus aurantifolia*

Table 2:Brine Shrimp Lethality of *Citrus limon* and *Citrus aurantifolia* Extracts

Plant extracts	LC ₅₀ (µg/mL)
<i>C. aurantifolia</i> (Seed)	348 ± 1.30**
<i>C. limon</i> (Seed)	1000 ± 0.00**
<i>C. aurantifolia</i> (Stem bark)	40 ± 2.11**
<i>C. limon</i> (Stem bark)	10 ± 0.33**
<i>C. aurantifolia</i> (Leaf)	55 ± 3.18**
<i>C. limon</i> (Leaf)	35 ± 0.74**
<i>C. aurantifolia</i> (Juice)	500 ± 6.80**
<i>C. limon</i> (Juice)	420 ± 2.18**
Cyclophosphamide*	98.76±0.15

KEY

*standard drug / positive control

* = No significant difference (P> 0.05)

** = Significant difference (P< 0.05)

Table 3: *In vitro* Cytotoxicity Activity of *Citrus limon* and *Citrus aurantifolia* Extracts on Rd, Hep-2c and Vero Cell Lines

Methanol Plant Extracts	CC ₅₀ (ig/mL)		
	Rd	Hep-2c	Vero
<i>C. aurantifolia</i> (Seed)	10.15±2.14**	8.05±3.12**	19±0.7**
<i>C. limon</i> (Seed)	8.69±2.52**	10.17±0.2**	8±0.7*
<i>C. aurantifolia</i> (Stem bark)	11.64±3.2**	10.12±2.6**	84±4.7**
<i>C. limon</i> (Stem bark)	5.02±1.1**	9.63±3.5**	10±2.9*
<i>C. aurantifolia</i> (Leaf)	4.31±3.73*	5.45±2.8*	7±2.3*
<i>C. limon</i> (Leaf)	11.03±2.1**	8.49±4.8*	24±5.0**
<i>C. aurantifolia</i> (Juice)	4.02±2.85*	66.09±8.3**	92±1.4**
<i>C. limon</i> (Juice)	5.80±2.48*	47.33±1.7**	71±0.1**
Cyclophosphamide*	2.23±0.14	2.66±0.8	8±0.6

KEY

*standard drug / positive control

* = No significant difference (P> 0.05)

** = Significant difference (P< 0.05)

Table 4: Selective Indices (SI) of *Citrus limon* and *Citrus aurantifolia* Extracts on Rd and Hep-2c Cancer Cell Lines

Extracts	Rd	Hep-2c
<i>C. aurantifolia</i> (Seed)	1.91±0.92	2.56±0.58
<i>C. limon</i> (Seed)	0.83±0.06	0.79±0.03
<i>C. aurantifolia</i> (Stem bark)	7.54±1.00	8.67±1.00
<i>C. limon</i> (Stem bark)	1.97±0.09	1.06±0.05
<i>C. aurantifolia</i> (Leaf)	1.39±0.23	1.39±0.19
<i>C. limon</i> (Leaf)	2.17±0.02	2.46±0.19
<i>C. aurantifolia</i> (Juice)	37.94±19.88	1.23±0.1
<i>C. limon</i> (Juice)	14.09±3.78	1.51±0.02
Cyclophosphamide*	3.15±0.34	3.25±0.52

KEY

SI value > 1 indicates selectivity.

*standard drug / positive control

* = No significant difference (P> 0.05)

** = Significant difference (P< 0.05)

CONCLUSION

Only the extracts of *C. aurantifolia* stem bark and leaf had better DPPH radical scavenging activity than the other six (6) extracts. All the extracts displayed weak radical scavenging properties which were not comparable to ascorbic acid. The stem bark and leaf extracts of *C. aurantifolia* and *C. limon* were observed to be

strongly toxic on brine shrimps than cyclophosphamide. While the seed and juice extracts were not significantly toxic on brine shrimps. *Citrus aurantifolia* leaf extract had a comparative cytotoxicity to cyclophosphamide on both human cancer cell lines used in this study. All the extracts were selectively toxic to the

two cancer cell lines, except *C. aurantifolia* seed extract. *Citrus aurantifolia* juice and leaf along with *C. limon* juice and stem back extracts had the highest cytotoxicity on Rd cancer cells. On the other hand, only *C. aurantifolia* leaf extract had cytotoxicity on Hep-2c cancer cells comparable to cyclophosphamide. This study underscores the use of both juices in the traditional management of cancer and this gives a scientific basis for their use. Other plant parts whose extracts have shown cytotoxicity need to be exploited for cancer treatments.

MATERIALS AND METHODS

Plant Collection

The leaf, stem bark and mature fruits of both *C. limon* (L.) Burm and *C. aurantifolia* (Christm) Swingle were collected in January, 2015 from Fiditi forest in Afijio Local Government Area of Oyo State, South-West, Nigeria. The plants were identified and authenticated at the Forest Herbarium in Forestry Research Institute of Nigeria (FRIN), Ibadan.

Plant Preparation

The leaf, stem bark and mature fruits of *C. aurantifolia* and *C. limon* were washed with distilled water separately. The rinsed leaf and stem bark were air-dried under the shade and extracted into distilled methanol. While the fruits were cut into halves to collect juice and seeds. The seeds were rinsed with distilled water, air-dried under the shade, pulverized, and extracted into distilled methanol. The extracts collected were concentrated *in vacuo*, weighed and refrigerated at -4°C until needed for analysis. Whereas, the juice collected were filtered through two layer of Whitman no. 1 filter paper packed in Buchner Funnel under pressure. The filtrate was freeze-dried, weighed and refrigerated at -4°C until needed for further use.

Biological assays

In vitro DPPH radical scavenging assay

The method described by Reena and co-worker (2015) [24] was used. The ability of the extracts to scavenge 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radicals was evaluated and the concentration of the extract that inhibits 50% of the DPPH free radicals was estimated. Extract (2 mg) was dissolved in distilled methanol to achieve the concentration of 1000 µg/mL and serial dilutions of 6.25, 12.5, 25, 50, 100 and 200 µg/mL were prepared in a 96 well-plate by diluting the extract solutions (100 µL each) with a 150 µL of DPPH (0.04 µg/mL) in methanol solution. The same procedure was repeated for the other eight extracts. The negative control wells contained only 250 µL of DPPH (0.04 µg/mL) in methanol solution. The positive control wells contained various concentration (6.25, 12.5, 25, 50, 100 and 200 µg/mL) of 100 mL of ascorbic acid. The micro-plate was incubated for 30 minutes in the dark at RT (25°C) after which the absorbance of micro-plate was read at 517 nm using the UV spectrophotometer (Paul Bucher Analytik und Biotechnologie, Switzerland). The experiment was done in three replicates. The absorbance obtained was expressed as percentage inhibition using by the formula below:

$$\text{Inhibition (\%)} = \frac{A-B}{A} \times 100$$

Where: A = the mean optical density of control wells.

B = the mean optical density of the wells with plant extracts.

Brine shrimp lethality assay

Artemia salina eggs were used for the Brine shrimp lethality (BSL). The eggs were hatched in natural seawater (obtained from the Bar Beach, Ikoyi, Lagos) for 48 h. Modified McLaughlin (1991) method was used to conducted the BSL assay [25]. Extract (10 mg), was diluted to 1000 µg/mL by adding 50 µL of DMSO prior to the addition of sea water. Serial dilutions of the extracts were prepared in a 96-well microplate in triplicates. Negative control wells contained only 0.5% DMSO in sea water. Cyclophosphamide was used as the positive control. Suspension of nauplii (250 µL) in the extract was added to each well. The plates were incubated at RT (25°C) for 24 h. The dead nauplii in each well were counted thereafter.

Cytotoxicity assay

Human cancer and normal cell lines culture

Cell lines used for the study were obtained from WHO Polio laboratory, Department of Virology, University of Ibadan, Nigeria. This consisted of two human cancer cell lines; Rhabdomyosarcoma (Rd) and laryngeal carcinoma (Hep-2c) and a normal cell line (Vero) from African green monkey kidney. All the cells were grown in minimum essential medium (MEM); supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin and maintained in MEM. The MEM was also supplemented with 0.01 mg/mL insulin and 1 mM sodium pyruvate. Cells were incubated in a 5% CO₂ humidified incubator at 37°C and split bi-weekly.

The MTT colorimetric assay

The viability of cell lines treated with the extracts was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay using a method described by Raja and co-workers (2011) [26]. Each of the eight extracts was dissolved in DMSO to give a concentration of 10 mg/mL overnight and 0.1 mL of the stock was added to 0.9 mL of maintenance medium to obtain a dilution of 1000 µg/mL. This was designated as “neat” and a serial ten-fold dilution of the extract was prepared from the “neat” using maintenance medium as the diluent. A volume of 50 µL of each extract dilution was dispensed into 96-well microtitre plates containing a 24 h cultured monolayer of Rd, Hep-2c or Vero cells. The plates were incubated at 37°C in a CO₂ environment and the cells observed under microscope after 72 h. Thereafter, supernatants were skilfully removed from all wells, leaving the cells attached to the wall surface. Then, 25 µL of MTT solution (2 mg/mL) in phosphate buffer saline (PBS) was added to each well and the plates were incubated for additional 2 h at 37°C. Lastly, 125 µL of DMSO was added into each well to solubilize crystalline formazan formed by viable cells. The plates were shaker for 15 min

after which absorbance was read at a wavelength of 492 nm by a UV spectrophotometer (Paul Bucher Analytik und Biotechnologie, Switzerland). Experiment was done in triplicates.

The percentage cytotoxicity was calculated as:

$$\% \text{cytotoxicity} = \frac{(A-B)}{A} \times 100$$

Where: A = the mean optical density of untreated cells.

B = the mean optical density of cells treated with plant extracts/ cyclophosphamide.

The selective index (SI) of the four extracts and cyclophosphamide were estimated using Vero cells. Data were expressed as the means $\pm$ SEM. A selective Index (SI) greater than 1 is indicative of selectivity.

$$\text{Selective index (SI)} = \frac{\text{CC}_{50} \text{ on Vero cells}}{\text{CC}_{50} \text{ on cancer cells}}$$

Data analysis

Date was analyzed by Graphpad prism computer software. A non-linear regression curve was used to calculate the concentration with 50% DPPH radical scavenging activity (IC_{50}), 50% brine shrimp lethality (LC_{50}) and 50% cytotoxicity (CC_{50}). The results are displayed as mean $\pm$ SEM of three replicates. Statistical significance was evaluated by one-way analysis of variance (ANOVA) using Dunnett's multiple comparisons test with Graphpad prism version 6.0. Values of P $\leq$ 0.05 were considered significant.

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