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Potential Antiradical Activity and Cytotoxicity Assessment of *Ziziphus mauritiana* and *Syzygium polyanthum*

Shanmugapriya Perumal, Roziahanim Mahmud, Suthagar Pillai Piaru,
Lee Wei Cai and Surash Ramanathan
School of Pharmaceutical Sciences, Centre for Drug Research, Universiti Sains Malaysia,
11800 Minden, Penang, Malaysia

Abstract: The present study was aimed at evaluating the antioxidant and cytotoxic activities of methanolic extracts from two ethnomedicinal plants growing wild in Malaysia and often used as both herbal medicine and food: *Ziziphus mauritiana* and *Syzygium polyanthum*. The plant extracts were tested for total phenolic and flavonoid contents as well as for *in vitro* antioxidant activities by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay, reducing power and β-carotene/linoleic acid bleaching assay. The stem bark extract of *Z. mauritiana* showed a potent free radical scavenging activity as evidenced by low EC_{50} at 20.09±0.19 μg mL^{-1}. Interestingly, *Z. mauritiana* leaf extract demonstrated higher reducing power activity (EC_{50}: 20.62±0.34 μg mL^{-1}) than ascorbic acid (EC_{50}: 20.69±0.49 μg mL^{-1}). The oxidation of linoleic acid was effectively inhibited by *S. polyanthum* leaf extract with 91.43±2.52%. In addition, both plant extracts exhibited no cytotoxic effect against Vero cell line. The results revealed that the methanolic extracts of *Z. mauritiana* and *S. polyanthum* possess promising antiradical properties and therefore can be used as a safe, reliable and economical natural source in pharmaceutical application and food industry.

Key words: *Ziziphus mauritiana*, *Syzygium polyanthum*, Vero cell, antioxidant activity, β-carotene

INTRODUCTION

The past decade has witnessed the renewed interest in evaluating the antioxidant activity of various indigenous medicinal plants. Medicinal plants are important therapeutic aid for various ailments because they contain numerous secondary metabolites with therapeutic values (Chanda *et al*., 2011). Antioxidant compounds provide protection to the cells and tissues against the impairment of Reactive Oxygen Species (ROS). Oxidative stress caused by free radicals leads to a number of degenerative disorders including cancer, stroke, cardiovascular, Alzheimer's disease and Parkinson's disease (Giasson *et al*., 2002; Thetsrimuang *et al*., 2011). Therefore, plant derived antioxidants which can stabilize or neutralize free radicals, may be of central importance in the prevention of such diseases (Wang *et al*., 1996). *Ziziphus mauritiana* is a tropical fruit tree species belonging to the family Rhamnaceae and most commonly found in the tropical and sub-tropical regions of the world. It is commonly known by many vernacular names such as 'jujube', 'Chinese apple', 'Indian plum' and 'ber'. The extract from this plant are commonly used to treat various conditions such as diarrhea, ulcer, amoebic dysentery and gastrointestinal disorders. The young leaves of the plant are sometimes used in folk remedies for treating fever (Adamu *et al*., 2006). *Syzygium polyanthum* is a member of Myrtaceae family distributed in the temperate, subtropical and tropical regions of the world. Most species are evergreen trees and generally known as lilly pillies, brush cherries, Indian bay-leaf, or satinash. The leaves of this plant are usually added in local dishes for its pleasant aroma. Besides, the leaf and bark extract are also consumed traditionally to treat diarrhea and infections (Hermana *et al*., 2008).

As far as our literature survey could ascertain, less studies have been carried out with the *Z. mauritiana* and *S. polyanthum*. The antioxidant activities of these plants have not been studied extensively although, Asian people have been consuming it as food and medicine for a long time. The present research was aimed at investigating the traditional claims of the antioxidant potentials of *Z. mauritiana* and *S. polyanthum*. To further validate the safe consumption of these ethnomedicinal plant extracts, a toxicity evaluation in cell culture was carried out against Vero cells.

MATERIALS AND METHODS

Plant collection and authentication: The fresh plant of *Z. mauritiana* and *S. polyanthum* were obtained from plants and herbs garden in Relau, Penang city, Malaysia.

Corresponding Author: Roziahanim Mahmud, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

The plants were authenticated by Mr. Shunmugam, the botanist of the School of Biological Sciences, Universiti Sains Malaysia, where voucher specimens (11254 and 11255) were deposited in the Herbarium Unit of the school.

Extraction of plant material: Leaves and stem bark of *Z. mauritiana* and *S. polyanthum* were air-dried and ground into fine powder and macerated individually by ratio of 10 g of ground plant material in 100 mL of methanol. The powdered leaves and stem bark were extracted solely with methanol which was in accordance with folkloric preparation. Extraction was done for 6 days under occasional shaking and the process was repeated three times. The combined extracts obtained were filtered and concentrated to dryness with a rotary evaporator (Rotavapor® R-200, Buchi, Switzerland) under reduced pressure. The extracts obtained were eventually freeze-dried (FreeZone®, MO) to remove any residual water. The yield of each extract was calculated. The extractive procedures were performed in dim lighting and all the dried extracts stored at 4°C until use.

Determination of antioxidant contents

Sample preparation: All the extracts were re-dissolved in methanol at a concentration 1 mg mL^{-1} for determination of antioxidant contents.

Total phenolic content: The concentration of phenolic compounds in the extract was estimated by a colorimetric assay described by Slinkard and Singleton (1977) with some modifications. Fifty microliters of methanolic extract samples were mixed with 1 mL of distilled H_2O in test tubes. After mixing well, 0.5 mL of Folin and Ciocalteu's phenol reagent was added the mixture. After 3 min, 2.5 mL 20% (w/v) Na_2CO_3 solution was added and the volume was filled up to 10 mL with distilled H_2O. The reaction was kept in the dark for 90 min incubation at ambient temperature after which the absorbance was measured at 735 nm against blank. The same procedure was repeated for standard gallic acid solutions and total phenolic content was calculated using a calibration curve of gallic acid (0.05-1.0 mg mL^{-1}, y = 0.0012x-0.0141, R^2 = 0.9985, y is the absorbance, x is the solution concentration). The results were expressed as mg of Gallic Acid Equivalents (GAE)/g of extract.

Total flavonoid content: The determination of total flavonoid content in the extracts was based on aluminium chloride method (Zhishen *et al*., 1999). A volume of 250 μL for each extract was mixed with 1.25 mL of distilled H_2O and 75 μL of 5% $NaNO_2$ solution. After 6 min, 150 μL of 10% $AlCl_3.H_2O$ solution was added and incubated at room temperature for another 5 min. Approximately 0.5 mL of 1 M NaOH solution was then added and the total volume was made up to 2.5 mL with distilled H_2O. Following thorough mixing of the solution, the absorbance against blank was measured at 510 nm. The total flavonoid content was calculated using a standard curve of catechin (0.06-1 mg mL^{-1}, y = 2.8396x, R^2 = 0.9973, y is the absorbance; x is the solution concentration). The results were expressed as mg Catechin Equivalents (CE)/g extract.

Determination of antioxidant activities

DPPH free radical-scavenging assay: The hydrogen atom or electron donation abilities of the corresponding extracts were measured based on a procedure described by Shimada *et al.* (1992). Each extract solution (200 μL) with various concentrations (6.25-100 μg mL) was loaded to each well of a 96-well plate. DPPH (1,1-diphenyl-2-picrylhydrazyl) in absolute methanol (50 μL, 1 mM) was added into wells. The solutions were incubated at room temperature for 30 min in the dark. The absorbance was measured against blank at 517 nm. DPPH solution and BHT (tert-butylated hydroxytoluene) were used as blank and positive control, respectively. All experiments were carried out in triplicate. The extract concentration that could scavenge 50% of the DPPH radicals (EC_{50}) was calculated from the plot of scavenging activity against the concentration of extract. The percentage inhibition of DPPH, were calculated according to the formula:

$$EC\ (\%) = \frac{A_0 - A_t}{A_0} \times 100$$

where, A_0 and A_t are the absorbance values of the blank sample and the test sample, at particular times, respectively. A lower EC_{50} value indicates greater antioxidant activity.

Estimation of reducing power: The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of the methanolic extracts (250 μL) were mixed with 250 μL of sodium phosphate buffer (0.2 M, pH 6.6) and 250 μL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 2.5 mL of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 650 rpm for 10 min. Supernatant obtained hereafter (100 μL) was immediately mixed with 100 μL of methanol and 25 μL of 0.1% of ferric chloride. The absorbance was measured after 10 min at 700 nm. Higher absorbance value indicates higher reducing power. The assay was carried out in triplicate and the results are expressed as Mean±SD. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used a positive control.

β-carotene/linoleic acid bleaching assay: The antioxidant activity of the extracts was determined according to the β-carotene bleaching method described by Tepe *et al.* (2005) with some modifications. A stock solution of β-carotene/linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL of chloroform (HPLC grade) and 25 μL linoleic acid and 200 mg tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 mL of aerated distilled water was added with vigorous shaking. About 2.5 mL of this reaction mixture were dispensed into test tubes and 350 μL portions of the extracts were added and the emulsion system was incubated in hot water bath at 50°C for 2 h. The absorbance of these solutions was measured at 470 nm using Shimazdu UV-120-01 spectrophotometer (Shimazdu, Kyoto, Japan) at initial time (t = 0) against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at 15 min intervals for 120 min. All samples were assayed in triplicate. The antioxidant activity was calculated in terms of percentage inhibition relative to the control. Antioxidative capacities of the extracts were compared with those of BHT and blank. The inhibition percentage (I%) of the samples were calculated using the following equation:

$$I\% = \frac{A\beta\text{-carotene after 1 h assay}}{A\text{initial }\beta\text{-carotene}} \times 100$$

where, Aβ-carotene after 1 h assay is the absorbance of β-carotene after 1 h assay remaining in the samples and Ainitial β-carotene is the absorbance of β-carotene at the beginning of the experiments.

Cytotoxicity screening

Vero cell line: Vero cells (ATCC, CCL-81) are mammalian epithelial cell line established from the kidney of the normal adult African green monkey (*Cercopithecus aethiops*) on March 27th, 1962, by Yasummura and Kawakita at the Chiba University, Japan American Public Health Association, 1992. Vero cells were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 raM), penicillin (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}). The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

MTT assay: The plant extracts were tested for *in vitro* cytotoxicity, using Vero cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with slight modifications (Mosmann, 1983). Briefly, after being harvested from culture flasks, the cells were seeded at 1×10^6 cells in each well of 96-well plate containing 100 μL of fresh growth medium per well and cells were permitted to adhere for 24 h. The cells were treated with the extracts which were serially diluted with growth medium to obtain various concentration (100, 50, 25, 12.5 and 6.25 μg mL^{-1}). 100 μL of each concentration was added to each well. After 24 h of treatment, the medium was aspirated and the cells were washed once with sterile Phosphate Buffered Saline (PBS). Subsequently, 5 mg mL^{-1} of MTT in PBS was added to each well and the plate was incubated at 37°C in 5% CO_2 for 2 to 4 h, until a purple precipitate was clearly visible under a microscope. The medium was discarded and 200 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the dark blue crystals of formazan salt and the plates shaken for 5 min. After incubation at 37°C for 10 min, the absorbance was measured at 540 nm using a Bio-TEK Microplate Scanning Spectrophotometer. The result was expressed as a percentage relative to solvent treated control incubations and the IC_{50} values were calculated using non-linear regression analysis (percent survival vs. concentration).

Statistical analysis: The results were analyzed using the Statistical Package for Social Sciences (SPSS) version 10.0 for windows. All the data are expressed as Mean±SD (n = 3).

RESULTS

Extraction yields and antioxidant contents: The highest extraction yield was found in *S. polyanthum* leaf extract (11.9%) followed by *Z. mauritiana* leaf extract (10.34%) and stem bark extract (8.51%) on dry weight basis, respectively. Phenolic compounds were found to be the major antioxidant components of all extracts *S. polyanthum* leaf extract showed the highest total phenolic contents (333.75±1.92 mg GAE g^{-1}) followed by *Z. mauritiana* leaf and stem bark extracts which were (283.5±0.54) and (253.35±1.51) GAE g^{-1}. *Z. mauritiana* extracts had the highest flavonoid contents (111.5±0.71 mg CE g^{-1} leaf extract and 80.3±2.11 mg CE g^{-1} stem bark extract, respectively), whereas, *S. polyanthum* leaf extract showed the lowest flavonoid contents (65.2±1.83 mg CE g^{-1}).

Antioxidant activity: DPPH free radical-scavenging assay, reducing power and β-Carotene/linoleic acid bleaching assay.

The results as illustrated in Fig. 1 indicate that all the investigated extracts potentially scavenged the free radicals in a concentration-dependent manner. *Z. mauritiana* (leaf and stem bark) and *S. polyanthum* leaf extracts were able to reduce the stable, purple-colored radical DPPH to yellow colored DPPH-H reaching 50% of reduction with EC_{50} values of 21.4±0.15, 20.09±0.19, 20.9±0.26 μg mL^{-1}, respectively (Table 1). The reducing power of *Z. mauritiana* and *S. polyanthum* methanolic extracts are shown in Fig. 2. Based on

Table 1: Antioxidant activities of *Z. mauritiana* and *S. polyanthum* extracts

Antioxidant tests	*Z. mauritiana* leaf	Stem bark	*S. polyanthum* leaf	BHT	Ascorbic acid
EC_{50} of DPPH free radical-scavenging[a] activity (μg sample mL^{-1})	21.40±0.15	20.09±0.19	20.90±0.26	18.50±0.19	ND
EC_{50} of reducing power (μg sample mL^{-1})[b]	20.62±0.34	111.11±0.55	77.55±0.76	ND	20.69±0.49
Inhibition % of β-carotene-linoleic acid bleaching assay	85.14±3.21	80.86±2.83	91.43±2.52	92.69±3.15	ND

Values are Mean±SD of triplicate determinations, EC_{50} is the concentration required to inhibit radical formation by 50%, [b]EC_{50} is the concentration for which the absorbance at 700 nm is 0.5, ND: Not determined

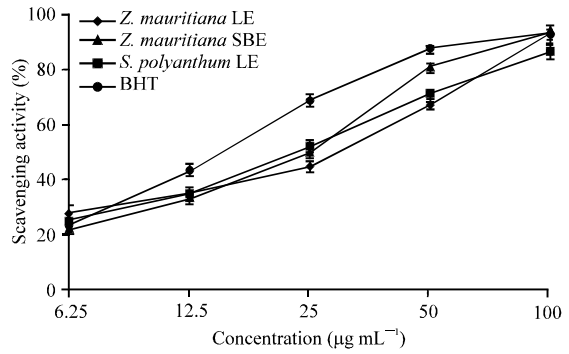

Fig. 1: DPPH radical scavenging activity of *Z. mauritiana* leaf extract (LE), stem bark extract (SBE), *S. polyanthum* leaf extract (LE) and BHT, values are Mean±SD of triplicate determinations

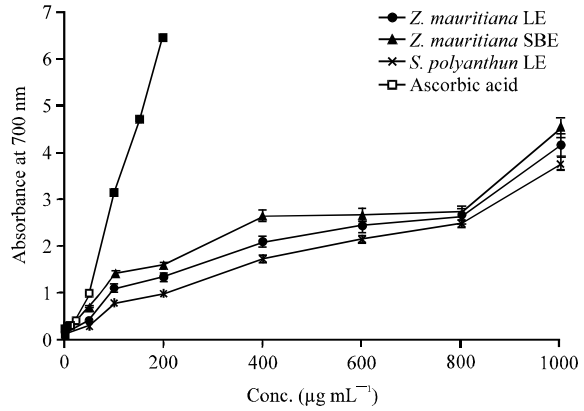

Fig. 2: Reducing power of *Z. mauritiana* leaf extract (LE), stem bark extract (SBE), *S. polyanthum* leaf extract (LE) and ascorbic acid, values are Mean±SD of triplicate determinations

the results, it can be seen that only *Z. mauritiana* leaf extract (EC_{50} = 20.62±0.34 μg mL^{-1}) expressed stronger reducing power effect than ascorbic acid (EC_{50} = 20.69±0.49 μg mL^{-1}). *Z. mauritiana* stem bark extract and *S. polyanthum* leaf extract exhibited weaker reducing power ability with EC_{50} values of 111.11±0.55 and 77.55±0.76 μg mL^{-1}, respectively. The β-carotene bleaching rates of the extracts are shown in Fig. 3. Oxidation of linoleic acid was effectively inhibited by

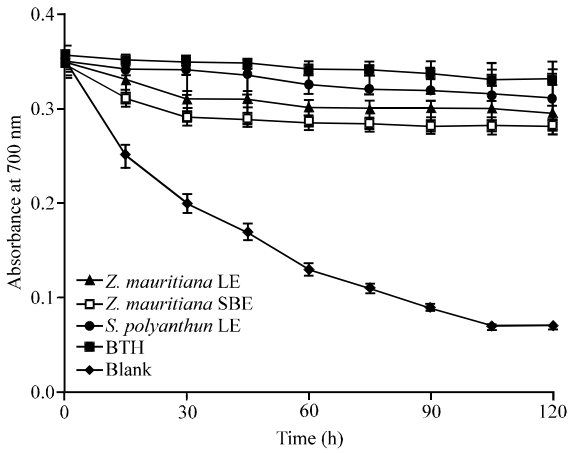

Fig. 3: Absorbance change of β-carotene at 470 nm in the presence of *Z. mauritiana* leaf extract (LE), stem bark extract (SBE), *S. polyanthum* leaf extract (LE) and ascorbic acid, values are Mean±SD of triplicate determinations

S. polyanthum leaf extract (91.43±2.52%), followed by *Z. mauritiana* leaf extract (85.14±3.21%) and stem bark extract (80.86±2.83%), respectively (Table 1). The inhibiting activity of synthetic BHT was measured at 92.69±3.15%. Among the studied extracts, *S. polyanthum* exhibited greatest inhibition of β-carotene bleaching rates that is close to the synthetic antioxidant reagent BHT.

Cytotoxicity effect of plant extracts: The *in vitro* cytotoxic affect of plant extracts were measured against Vero cell line. As shown in Fig. 4, the IC_{50} values for *Z. mauritiana* leaf and stem bark extracts followed by *S. polyanthum* leaf extract were 59.78, 61.47 and 53.5 μg mL^{-1}, respectively.

DISCUSSION

Medicinal plants have curative properties due to presence of various phytotherapeuticals such as phenolic compounds and flavonoids which are distributed in different parts of the plants with varying composition. Phenolic compounds are known to be the most important class of phytochemicals in plant food sources. They can

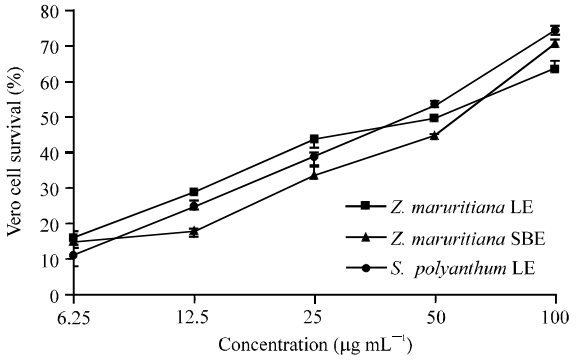

Fig. 4: *In vitro* cytotoxic activity of *Z. mauritiana* leaf extract (LE), stem bark extract (SBE) and *S. polyanthum* leaf extract (LE) against Vero cells. The values for % of cell survival were calculated by comparison with the vehicle treated control cells, values are Mean±SD of triplicate determinations

be classified into two main groups: Polyphenols and flavonoids (Escarpa and Gonzales, 2000). According to Velioglu *et al.* (1998), there are well established correlation existed between antioxidant activity of plant extracts with the content of their phenolic compounds. Phenolics are usually associated with the inhibition of oxidative stress related diseases such as mutagenesis and carcinogenesis in humans (Tanaka *et al.*, 1998). Flavonoids are polyphenolic compounds, commonly found in fruits, vegetables and beverages. Besides being potential antioxidant compound, flavonoid also exhibit potential pharmacological activities such as anticarcinogenic, antithrombotic, antimutagenic and antimicrobial activities (Cook and Samman, 1996; Gill *et al.*, 2011). The *in vitro* antioxidant activities of *Z. mauritiana* and *S. polyanthum* extracts were investigated on the basis of their scavenging activities of the stable DPPH free radical. DPPH is a stable free radical containing an odd electron in its structure and widely used for detection of the radical scavenging activity in chemical analysis. The method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction (Shon *et al.*, 2003). Comparison of the DPPH scavenging activity of the assessed extracts with those expressed by BHT (18.5±0.19 μg mL^{-1}) confirmed that all of the examined extracts expressed strong antioxidant effects. These potent antioxidant activities can be attributed to the abundance of phenolic and flavonoid contents in these plants. Phenolic and flavonoid act as an efficient free radical scavengers by donating their alcoholic hydrogen or one of their delocalized electrons (Madsen *et al.*, 1996). In reducing power assay, the presence of radicals causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. This resulted in the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of each compound. The ability of the assessed extracts to inhibit the bleaching of β-carotene was measured and compared with that of the positive control, BHT. β-carotene undergoes rapid discoloration in the absence of an antioxidant. Consequently, the oxidation loses its chromophore and bleaches the characteristic orange colour, which can be monitored spectrophotometrically. The presence of different antioxidants can hinder β-carotene bleaching by neutralizing the linoleic-free radical and other free radicals formed in the system. As illustrated in Fig. 3, the absorbance values of β-carotene in the absence of extracts decrease due to the oxidation of β-carotene and linoleic acid. The high absorbance values indicated that the extracts possessed strong antioxidant activity. The antioxidant activity shown by the extracts may be due to the presence of various phenolic acid and flavonoids. Phenolic compounds are considered to be the most important plant constituents. They represent one of the major groups of compounds acting as a primary antioxidant mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals (Zin *et al.*, 2002). In addition, phenolic compounds possess ideal structural properties to donate hydrogen atoms to free radicals (Kanatt *et al.*, 2007). As stated by Anokwuru *et al.* (2011), polyphenols are the major contributors of radical-scavenging activity in plants. Several studies have reported on the relationships between phenolic content and antioxidant activity whereby some authors found no such correlation (Demiray *et al.*, 2009). In this study, the findings do not show any relationship between antioxidant activity and total phenolic contents. For example, the stem bark extract of *Z. mauritiana* had the lowest total phenolic content whereas its antioxidant capacity on DPPH-free radical scavenging activity was higher than *S. polyanthum* leaf extract which had the highest total phenolic content. The leaf extract of *Z. mauritiana* had remarkable reducing power ability where the EC_{50} value which equally comparable to that of ascorbic acid (positive control) although, the total phenolic content was lower than *S. polyanthum.* Different reports found from the literature recorded a wide degree of variation between different phenolic compounds in their effectiveness as antioxidant (Robards *et al.*, 1999). The low relationship confirms that phenolic compounds are not the only contributor to the antioxidant activities of these extracts. The type and

quantity of phenolic compounds and the presence of non-phenolic antioxidants may also contribute to the antioxidant activity of the extracts. Flavonoids are polyphenolic compounds which generally have more hydroxyl groups (Rivas-Arreola *et al*., 2010). The ortho-substitution with electron-donating alkyl or methoxy groups of flavonoid, increases the stability of the free radical and hence, its antioxidant potential (Rice-Evans *et al*., 1995). In food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides (Das and Pereira, 1990). The findings of this study indicate that each studied extract had a different antioxidant activity, contributed by different antioxidant components, such as phenols, flavonoids or β-carotene. The high antioxidant activity of *S. polyanthum* might be due to its phenolic and β-carotene contents; for *Z. mauritiana* leaf and stem bark extracts, the high total flavonoid contents corresponded with the high antioxidant activity. In order to investigate the toxicity effects of extracts on the growth of normal mammalian cells, Vero cells were treated with various concentrations of the extracts for 1 day and the viable cells were measured by the MTT method. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells (Mosmann, 1983). Based on US NCI plant screening program, a crude extract is generally considered to have in vitro cytotoxic activity if the IC_{50} value (concentration that causes a 50% cell kill) is less than 20 μg mL^{-1}, while it is less than 4 μg mL^{-1} for pure compounds (Boik, 2001). This finding therefore supports the common belief that ethnopharmacological selection of plant species is always safe and harmless to consume thus remains as a valuable criterion in drug discovery.

CONCLUSIONS

In summary, results presented in this study confirmed that the methanolic extracts of *Z. mauritiana* and *S. polyanthum* possess strong antioxidant properties. This finding is useful in pharmaceutical and food industry as the plant extracts can be used as natural antioxidant. Based on the remarkable antioxidant activities exhibited by these plants, an extensive study is currently underway particularly in isolating and characterizing the active principles that contribute to the bioactivities thus maximize the therapeutic efficacy of these plant extracts.

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