



## Antioxidant Potential and the Effects of the Aqueous Extract of *Spondias cytherea* Fruit Pulp on B16-F10 Melanoma Cell Cycle

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### Authors' contributions

This work was carried out in collaboration among all authors. Authors TBC and MRS participated in the design of the study. Authors FNY, KMO, TG, KN and NJ collected the data. Authors TBC, MRS and FNY contributed to the manuscript design. All the authors contributed to the review and approved the final version.

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### ABSTRACT

Consumption of diets rich in fruits and vegetables bring substantial health benefits. *Spondias cytherea* is a plant belonging to the family of Anacardiaceae, the fruits are highly consumed in Cameroon and commonly known as "kassimango". In this work, the antioxidant and cytotoxic activities of the aqueous extract of *Spondias cytherea* pulp was investigated. The antioxidant capacity was evaluated by DPPH (2,2-diphenyl-1-picryl-hydrazyl) ferric reducing antioxidant power (FRAP) and metal chelating power assays. The Total phenolic and flavonoid contents were determined by Folin-ciocalteu test and aluminum chloride colorimetric test respectively. Selenium, and Zinc contents were evaluated. The effect of *S. cytherea* fruit aqueous extract on cell cycle of B16-F10 skin cancer cells was investigated using propidium iodide staining. The results revealed that the extract was efficient in reducing  $Fe^{3+}$  into  $Fe^{2+}$  and its ion chelating power increased with

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the extract concentration. The IC<sub>50</sub> value for DPPH radical was found to be 96.28 µg/ mL. Total phenolic and flavonoid contents of *Spondias cytherea* fruits were 649.45 ± 0.71 mg GAE/100 g and 86.69 ± 0.02 mg CE/100 g of dry matter respectively. As far as the tested minerals are concerned, *S. cytherea* fruit presented 4.48 mg/100 g of zinc and 0.86 µg/100 g of selenium. It was observed that *S. cytherea* fruit aqueous extract inhibit the proliferation of B16-F10 cancer cells by inducing the cell cycle arrest at Sub G0 phase (apoptotic phase), G0/G1 (proliferation phase) and G2/M phase (mitosis phase). These results indicated that *Spondias cytherea* fruit have antioxidant and can be used in the management of cancer pending on further researches.

**Keywords:** Antioxidant; anticancer; fruit pulp *Spondias cytherea*.

## 1. INTRODUCTION

Oxidative stress is defined as an imbalance between production of free radicals and reactive oxygen species (ROS). Under a prolonged continuous stress, significant damage may occur to cell structure and functions; this may induce mutations and neoplastic transformations leading to diseases such as cancer. Cancer incidence is increasing all over the world. In Cameroon, 15 769 new cases of cancer have been diagnosed in 2018 and 10 533 deaths have been registered [1]. Cancer initiation and progression have been associated to oxidative stress through the increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation [2]. The potential advantageous effects of antioxidants in protecting against disease have been used as an argument for recommending increasing intakes of micronutrients such as vitamins (C, E), minerals (Selenium, Zinc) [3]. It has been shown that intake of controlled diets rich in fruits and vegetables significantly increases the antioxidant capacity of plasma, due to the presence of plant bioactive non-nutrient called phytochemicals [4,5]. Diets rich in phytochemicals such as polyphenols, flavonoids and anthocyanin can provide protection against various diseases and one-third of all cancer deaths are preventable by changing dietary habits [6]. *Spondias cytherea* or golden apple, commonly known as "Kassimango" belongs to the Anacardiaceae family which includes several important tropical fruit trees such as mango and cashew. Basically, it is mainly used as a fruit, but other parts of the plant are also used in various medical treatments. Astringent skin can be used as a treatment for diarrhea, as well as to improve eyesight and eye infections. The fruit can also be used to treat itching, internal ulcers, sore throats and inflammation of the skin [7]. In addition, it polysaccharides have eliciting activity on peritoneal macrophages, while plant leaves have anti-diabetic effects [8]. Earlier studies of our laboratory team on this *S. cytherea* showed that

it IC50 value on B16-F10 melanoma cancer cells was 13.37 mg/ml, it was also established that the aqueous fruit extract reduce the expression of CD133 on melanoma cells, decreases the proliferation of melanoma cancer cells by downregulating COX-2 thus reducing tumor inflammation and tumor size on mice models [9]. Generally, it is said that constant consumption of plant-derived phytochemicals from vegetables, fruits, tea and herbs can help transform the balance into a controlled antioxidant state. Currently, the potential toxicity of synthetic antioxidants as a supplement compared to natural antioxidants has been criticized [10]. The aim of this study was to evaluate the antioxidant potential of the aqueous extract of the fruit pulp of *Spondias cytherea* on the cell cycle of the B16-F10 melanoma or skin cancer cell line.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Vegetable material

Fruits of *S. cytherea* were collected in September 2016 in Dschang market place, Menoua Division, West Region, Cameroon. The plant was identified at National Herbarium of Cameroon and registered under the number 21249/SRF-Cameroon.

#### 2.1.2 Cancer cell line

Melanoma (skin cancer) cancer cell line (B16-F10) was obtained from the Cell Bank of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO<sub>2</sub> incubator at 37°C. The medium was renewed every three days.

### 2.1.3 Chemicals and reagents

DPPH (2, 2 diphenyl-1-picryl hydrazil radical), vitamin C (ascorbic acid), Folin–Ciocalteu phenol reagent, gallic acid (3,4,5-trihydroxybenzoic) and sulfuric acid were obtained from Fluka Chemie (Buchs, Switzerland). Potassium acetate (CH<sub>3</sub>CO<sub>2</sub>K), Sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) and trypan blue were supplied by Merck (Darmstadt, Germany). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Company (St. Louis, MO, USA),

## 2.2 Methods

### 2.2.1 Preparation of fruits extract

The peels and seeds of the fruits were removed, and chopped fruits were then shade dried in a laboratory oven at 45°C for five days followed by grinding into fine particles. The fruit powder (100 g) was soaked in 500 ml of water and left for 24 h with a magnetic stirrer at room temperature. The mixture was then filtered with Whatman paper N°4, and the crude extract was obtained after evaporation in the oven at 45°C for three days.

### 2.2.2 DPPH radical scavenging assay

The radical scavenging activity of *Spondias cytherea* extract was evaluated spectrophotometrically using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical [11]. The extract (5000 µg/mL) was twofold serially diluted with methanol. One hundred microliters of the diluted extract were mixed with 900 µL of 0.3 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution, to give final extract concentrations of 31.25, 62.5, 125, 250 and 500 µg/mL respectively. After 30 min incubation in the dark at room temperature, the optical densities were measured at 517 nm using UV/Visible light spectrophotometer (Jenway, model 1605). Ascorbic acid (Vitamin C) was used as control. Each assay was done in triplicate. The radical scavenging activity (RSA, in %) was calculated as follows:

$$\text{RSA} = \frac{[(\text{Absorbance of DPPH} - \text{Absorbance of sample}) / \text{Absorbance of DPPH}] \times 100}{}$$

Curves were constructed by plotting percentage of inhibition against extract logarithmic values of concentrations. The equation of this curve was used to calculate the IC<sub>50</sub> corresponding to the sample concentration that reduced 50% of the

initial DPPH<sup>•</sup> absorbance. A smaller IC<sub>50</sub> value corresponds to a higher antioxidant activity.

### 2.2.3 Ferric reducing antioxidant power (FRAP) assay

Determination of iron to reduce antioxidant strength (FRAP)

The reduced capacity of *Spondias cytherea* fruit extract was previously measured by the method described by Oyaizu with minor changes [12,13]. For 1 ml of extract at different concentrations (31,25, 62, 5, 125, 250 and 500 / g / ml), mixed with 2.5 ml of phosphate buffer and 2.5 ml of potassium ferricyanide (1%), catechin was used as a control, the mixture was kept at 50°C for 20 minutes, then 2.5 ml of trichloroacetic acid 10% (TCA) was added and centrifuged at 3000 rpm for 10 minutes. From each tube, 0.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferrous chloride. Absorption was measured at 700 nm using visible UV. The absorbance was measured under similar conditions. Increase the absorption of the effect mixture indicates higher reduction capacity of the tested extracts [14].

### 2.2.4 Chelation power on ferrous (Fe<sup>2+</sup>) ions

The chelating effect on ferrous ions of the prepared extract was estimated by standard methods with slight modifications [15]. Briefly, 200 µl of each test sample (1 mg / ml) was withdrawn and made up to 3 ml with methanol. 740 µl of methanol was added to 20 µL of 2 mM FeCl<sub>2</sub>. The reaction was started by adding 40 µl of 5 mM ferrozine to the mixture, which was then allowed to stand at room temperature for 10 minutes, and the optic density of the mixture was measured at 562 nm. As a reference, methanol without sample was used and as blank methanol was used without iron solution. The positive control was represented by a solution of a standard antioxidant, catechin whose absorbance was measured under the same conditions as the samples. Increased absorbance of the reaction mixture indicates higher reduction capacity of the tested extracts.

### 2.2.5 Determination of the total phenolics content

The total phenol content was determined using the Folin-Ciocalteu substrate [16]. The reaction mixture contained 20 µl concentrate (5,000 µg / ml), 1380 µl of distilled water, 200 µl of Folin-

Ciocalteu 2N substrate and 400  $\mu$ l of 20% sodium carbonate solution. The mixture was incubated at 40°C for 20 minutes and the absorbance at 760 nm was measured. The standard curve was represented by gallic acid as a standard. The tests were duplicated and the results were expressed in milligrams of acid equivalents per 100 grams of dry weight (mg GAE / 100 g DW).

#### 2.2.6 Determination of total flavonoids content (TFC)

The amount of flavonoids is determined by aluminum chloride [17]. Briefly, 100  $\mu$ l (5000  $\mu$ g / ml) was extracted with 1.49 ml of dissolved water and 30  $\mu$ l of 5% NaNO<sub>2</sub> solution. After 5 minutes at room temperature, 30  $\mu$ l of a 10% aluminum chloride solution (AlCl<sub>3</sub>, H<sub>2</sub>O) was added, after 6 minutes of incubation with the addition of 200  $\mu$ l of sodium hydroxide (0.1 M) and 240  $\mu$ l of dissolved water. The solution was mixed well and the weight was measured at 510 nm using a UV-Visible spectrophotometer (Jenway, model 1605). The total amount of flavonoids was calculated using the simple catechin formula. Results are expressed as milligrams of catechin equivalents (mg CE) per 100 grams of dry weight.

#### 2.2.7 Determination of zinc and selenium contents

*Spondias cytherea* was digested using nitric/sulphuric acid (1:1 v/v) mixtures, prior to mineral content determination. The atomic absorption spectrophotometric method 975.03B, was used to determine zinc content and the selenium content was determined using the atomic absorption spectrophotometer [18]. The atomic absorption spectrophotometer (AAS) was calibrated against zinc and selenium standard solution. Mineral concentrations of each sample were quantified using the standard curves. These curves were obtained after the calibration of the instrument was performed using a series of standards for each corresponding pure mineral of zinc and selenium [19].

#### 2.2.8 Cell cycle phase distribution

B16-F10 cells (cells 5 x 10<sup>5</sup> / well) were treated with *S. cytherea* aqueous extract at different concentrations 0, 2, 5, 10 and 15 mg / ml for 24 hours chosen according to the IC<sub>50</sub> value obtained earlier on B16-F10 skin cancer cell lines [9] (at 37°C and 5% CO<sub>2</sub>). The specimen was

then removed and the cells were washed with PBS and extracted with 5mM EDTA / PBS. After completion (10 min; 1200 rpm), cells were incubated at 70% ice- cold ethanol for 2 h. Pellet was suspended in 250  $\mu$ l of PBS containing propidium iodine (PI-25  $\mu$ g / ml) and RNAase (40 /g / ml) and then placed at a temperature of 37°C in the dark for 30 min. Finally, the cells were immediately examined with a flow cytometer FACS Calibur (Becton Dickinson, USA). Data were collected in a listing position for about 10,000 events and displayed on histogram where the cell numbers were (counts) was plotted against the relative fluorescence intensity of PI (FL-2;  $\lambda$ em: 585 nm; red fluorescence). The resulting DNA distributions were analyzed by Modfit 2.0 (Verity Software House Inc., Topsham, ME) for the proportions of cells in Sub G0 phase, G0/G1, S phase, and G2/M phases of the cell cycle [20].

### 2.3 Statistical Analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses  $\pm$  standard deviation. The statistical analysis was carried out using one way analysis of variance (ANOVA) to study the statistic difference. The levels of significance, considered at P < 0.05, were determined by Neumann keuls using Graph pad prism 5. Graph pad prism 5 was used to plot graph.

## 3. RESULTS

### 3.1 Antioxidant Activity of *Spondias cytherea* Aqueous Fruit Extract

#### 3.1.1 DPPH radical scavenging capacity

The DPPH radical scavenging activity of the *S. cytherea* aqueous extract is shown in Table 1. The aqueous extract of *S. cytherea* scavenged free DPPH radical with an IC<sub>50</sub> of 96.28  $\pm$  0.24  $\mu$ g / ml showing low antiradical activity. These results show that ascorbic acid as the benchmark antioxidant remains the most effective antioxidant with an IC<sub>50</sub> of 6.60  $\pm$  0.03  $\mu$ g / ml.

#### 3.2 Ferric Reducing Antioxidant Power

The results of ferric reducing antioxidant power (FRAP) of *S. cytherea* aqueous extract at different concentrations, determined through the reduction of Fe<sup>3+</sup> (i.e Fe<sup>3+</sup>  $\rightarrow$  Fe<sup>2+</sup>), are shown in Fig. 1. It was observed from the results that

catechin (standard antioxidant) has the greatest reducing activity. The absorbance increased with the concentration of the extract as well as of the catechin used as control (Fig. 1). We can therefore said that the aqueous extract of *S. cytherea* has the ability to reduce iron, but it is still lesser than that of catechin.

### 3.3 Iron Chelating Activity

The results of the metal chelating activity revealed an increase of absorbance with *Spondias cytherea* aqueous extract which reflects the increased of the ion chelating power (Fig. 2). Still, the iron chelating power of *S. cytherea* aqueous extract fruit pulp was lower than that of catechin consider as a standard antioxidant.

### 3.4 Total Phenolic and Flavonoids Contents

The phenolic content of *S. cytherea* fruit pulp is shown in Table 2. Phenolic content of the plant material, calculated as the equivalent of gallic acid per gram of dry matter, was  $649.45 \pm 0.71$  mg. GAE / g. The total flavonoid content of *S. cytherea*, calculated as milligrams of catechin equivalent per gram dry weight (mgCE / g), was found to be  $86.69 \pm 0.02$  mg CE/g. These results show that the *S. cytherea* fruit pulp has a high concentration of phenolic compounds.

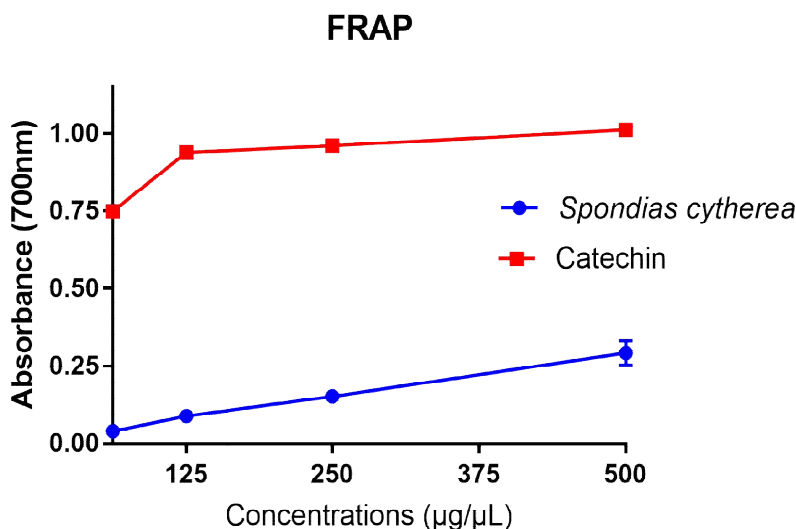
### 3.5 Zinc and Selenium Content

Analysis showed that, the aqueous fruit extract of *S. cytherea* contains 4.48 mg of zinc in 100 g of

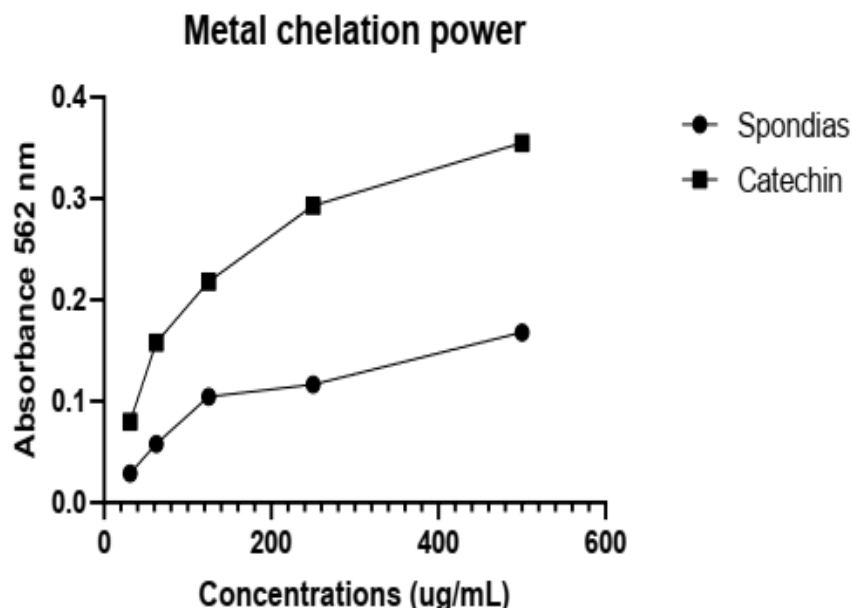
**Table 1. Variation of inhibition percentage of DPPH radical with the concentration of *Spondias cytherea* aqueous extract**

Concentration (µg/ml)	<i>Spondias cytherea</i> Inhibition Percentage	Vitamin C
31,25	$33.81 \pm 0.00^a$	$94.46 \pm 0.22$
62,5	$45.51 \pm 0.01^b$	$94.77 \pm 0.39$
125	$53.84 \pm 0.02^c$	$95.69 \pm 0.13$
250	$57.53 \pm 0.05^d$	$97.37 \pm 0.19$
500	$60.02 \pm 0.04^e$	$97.79 \pm 0.36$
IC50	$96.28 \pm 0.24$ µg/ml*	$6.60 \pm 0.03$ µg/ml

Values with letters (a, b, c, d and e) are significantly different



**Fig. 1. Reducing power of *Spondias cytherea* aqueous extract at different concentrations. Each value represents means  $\pm$  SD (n=3). High absorbance at 700 nm indicates high reducing power**



**Fig. 2.** Ion chelating power of *Spondias cytherea* aqueous extract at different concentrations. Each value represents means  $\pm$  SD (n=3). High absorbance at 700 nm indicates high reducing power

dry matter (4.48 mg/100g D.W.) and 0.86  $\mu$ g of selenium in 100 g dry matter (0.86  $\mu$ g / 100 g. D.W.) The results are presented on Table 2.

### 3.6 Cancer Cell Cycle Analysis

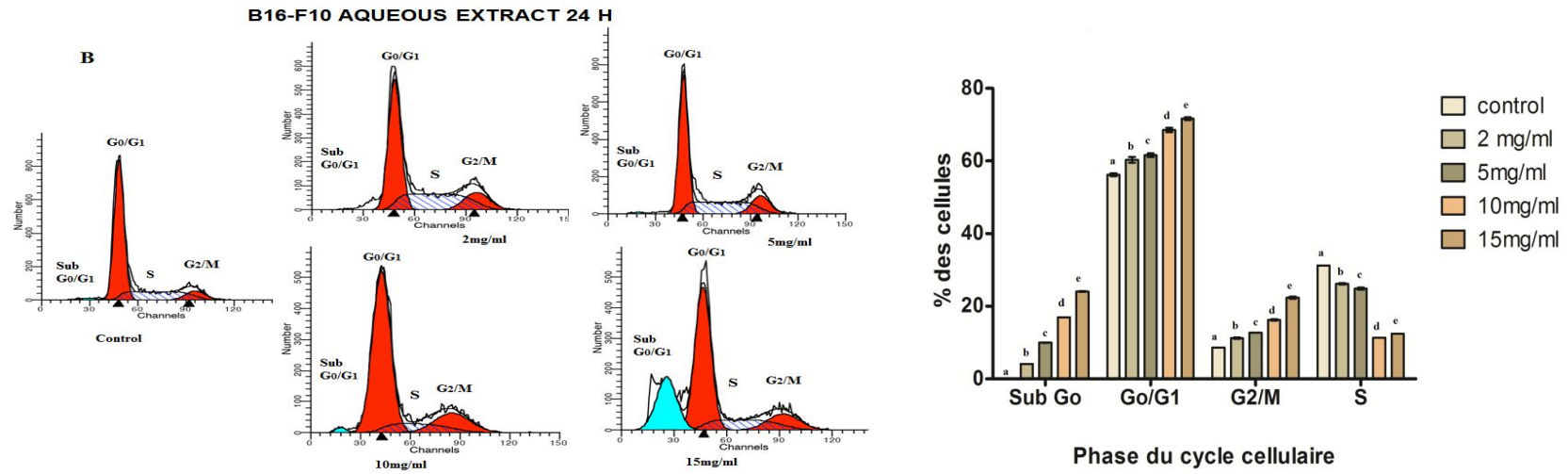
The aqueous extract of *S. cytherea* induced, regardless of the concentration, a significant accumulation of the number of murine B16-F10 melanoma cells at the proliferation phase (G0 / G), an accumulation with increasing the concentration of the aqueous extract of *S. cytherea* in the mitotic phase (G2 / M) and in the apoptosis Sub Go phase, reflecting the cell cycle arrest at these phases (Fig. 3).

## 4. DISCUSSION

Diets consisting of fruits and vegetables rich in phenolic compounds, have been linked with reduced morbidity and mortality brought by chronic diseases [21,22]. Many chronic diseases are caused by the inability of the cell to regulate the redox state due to injuries both internally and externally caused by free radicals. Hence, it has been said that altering redox state of cell, may shift the cell back to its normal state or induce apoptosis in aberrant carcinoma cells [22]. The antioxidant activity of

*Spondias cytherea* was assessed using DPPH based on an assessment of the radical uptake activity of the sample [23]. The ability of *Spondias cytherea* to scavenge DPPH radicals showed an IC50 of 96.28  $\mu$ g / mL. This was lower compared to 1.91  $\mu$ g / mL methanol extract of the same fruit [24].

The aqueous extract of *Spondias cytherea* can be considered as a low antioxidant according to the classification of Souiri and collaborators [25]. The antioxidant ability of *Spondias cytherea* aqueous fruit extract can be associated to its total phenol content. Results obtained showed that, *Spondias cytherea* fruit contained 649.45  $\pm$  0.71 mg GAE of total phenolic content and 86.69  $\pm$  0.02 mg CE/100g of total flavonoids content. However, previous studies showed that the methanolic extract of *Spondias cytherea* fruit total phenolic contents of 659.74  $\pm$  0.97 GAE/g of dry weight and total flavonoids content of 225.6  $\pm$  0.88 mg QE/g of dry weight (Islam et al., 2013). The phenolic (including flavonoids) compounds of plants majorly act as primary antioxidants, they have high redox potentials which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [26]. The difference between flavonoids contents can be associated to the solvent of extraction.



**Fig. 3. Effet de *S. cytherea* aqueous fruit extract on B16-F10 cancer cell line after 24 hours of treatment**

*B16-F10* cells were treated with different doses (0, 2, 5, 10 and 15 mg/mL) of aqueous extract of *S. cytherea* fruit pulp for 24h; followed by incubation in PBS containing propidium iodide (PI-25  $\mu$ g/mL) and RNAase (40  $\mu$ g/mL). Each value represents means  $\pm$  SD (n=3). Histograms with different letters (a,b,c,d,e) are significantly different  $p < 0.05$

**Table 2. Quantitative analysis of some selected minerals and phytochemicals of the fruit pulp of *S. cytherea***

Phenols (mg GAE/g D.W)	Flavonoïds (mg CE/g D.W)	Zinc (mg/100 g D.W)	Selenium (µg/100 g D.W)
649.452 ± 0.071	86.69 ± 0,02	4.48 ± 0.31	0.86 ± 0.02

There are many ways to assess the antioxidant capacity of plant extracts or pure chemicals. However, due to the complex reaction aspects of plant extracts; one procedure cannot explain all possible mechanisms of antioxidant properties. Therefore, two other in vitro antioxidant assays were used to validate this study. FRAP is an electron transfer based total antioxidant assay, also called redox linked colorimetric method. The results obtained showed that ferric reducing power increases with the concentration of the extract, indicating that *Spondias cytherea* aqueous extract has ferric reducing antioxidant activity. Iron is crucial for life but an excess of iron is toxic. Induction of oxidative stress through the increase in ROS production is considered as the main mechanism for toxicity of ion metals including iron [27]. There is no physiological mechanism for the removal of iron from the organism. Its elimination from the body is the first priority in some instances such as iron poisoning and iron overload [28]. In situation of excess iron, cancer cells rapidly proliferate because these cells have higher requirement for iron than normal cells [29].

The significant cell cycle arrest of B16-F10 cells in the Sub G0 phase suggest that the aqueous extract of *S. cytherea* acts by specific mechanisms on the cell cycle inducing apoptosis of B16-F10 cells. In fact, anticancer substances eliminate rapidly proliferating cancer cells by inducing apoptosis [30]. The aqueous extract from the pulp of *S. cytherea* stimulates cell cycle arrest of B16-F10 cells in the G0 / G1 and G2 / M phases. Indeed, stopping the cell cycle at specific checkpoints and initiating apoptosis are mechanisms widely used in the treatment of cancer with cytotoxic agents [31]. Cell cycle checkpoints protect dividing cells from DNA damage and ensure genomic integrity. The anticancer effect of *S. cytherea* fruit aqueous extract could be explained by its phenolic and flavonoid contents as well as selenium and zinc contents. Several observations have suggested that natural flavonoids have growth inhibitory effects on various kinds of cancer cells mediated by different molecular targets and acting through diverse metabolic pathways [32].

*Spondias cytherea* zinc content was found to be 4.48 mg/100 g of DW which is higher than the amount in *Mangifera indica* endocarpe (1.57±0.20 to 2.18±0.08 mg/100) from the same Anarcadiaceae family [33]. The results obtained is comparable with those of *Rhus coriaria* belonging to the family of anarcadiaceae like *S. cytherea*, which had a selenium content (1.1 ± 0.2 µg/ 100 g) not significantly different from *Spondias cytherea* (0.86 µg/100 g) selenium contents [34].

Selenium (Se) is a micronutrient with an important antioxidant action. It functions as an antimutagenic agent, preventing the malignant transformation of normal cells. This protective effect of selenium was primarily associated with its presence in the glutathione peroxidase and thioredoxin reductase, enzymes that are known to protect DNA and other cellular components from oxidative damages [35]. A strong correlation between the antiradical activity and the presence of phenolic compounds, flavonoids and zinc has been observed [36]. Evidence indicates that increase consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with aging [37,38].

## 5. CONCLUSION

The present study shows that *Spondias cytherea* fruit extract has remarkable antioxidant and induced B16-F10 cell cycle arrest. Thus, *Spondias cytherea* fruit could be exploited as a valuable source of antioxidant agent and for prevention of cancer. Further studies need to be conducted to evaluate the insight mechanisms of the anticancer activity and also to identify and to isolate active principles in this extract.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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