Pharmacogn. Commn. 2021;11(2):88-94.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogcommn.org

Original Article

Anti-proliferative Activity of *Hibiscus sabdariffa* L. Calyx Flavonoid Extracts on Cervical Cancer Cells using Flow Cytometry

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ABSTRACT

Background: Impairment of the normal progression of the cell cycle affects the growth and development of the daughter cells, which may result in cancer cell proliferation. Herein, we investigate the effect of flavonoid fractions of *Hibiscus sabdariffa* L. calyx on cell cycle progression in cervical cancer cells. **Methods:** Flavonoids (bound and free), lipids and aqueous fractions were extracted from a methanolic extract of *H. sabdariffa* calyx using ethyl acetate, diethyl ether and petroleum ether. Each of these fractions were used to treat HeLa cervical cancer cells and monitored using a flow cytometer. **Results:** The cells exposed to the extract and its fractions had higher percentages of inactive cells (no cell division) at the quiescent (G_0) and gap one phases (G_1) of the cell cycle in comparison to control cells. The highest values were obtained from cells treated with the bound and free flavonoid fractions. **Conclusion:** The results of this study suggest that HeLa cervical cancer cell proliferation is significantly inhibited by *H. sabdariffa* flavonoid fractions. Daily consumption of beverages consisting of *H. sabdariffa* may therefore prevent the early stages of cervical cancer and inhibit the progression of the disease once it is established.

Key words: Flavonoids, HeLa, Cervical cancer, Roselle, Flow cytometer, Cell cycle inhibition, Anti-proliferative extract.

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DOI: 10.5530/pc.2021.2.18

INTRODUCTION

Cervical cancer is caused by the cells in the linings in the cervix of women developing abnormalities. Squamous cell carcinoma is the most common form of the disease, accounting for approximately 70% of all cases. Adenocarcinomas are also relatively frequent but are more difficult to diagnose as they start higher in the cervix. Whilst there are many causes of cervical cancer, the most frequent etiology of this disease involves human papillomavirus (HPV) transmission from the carrier to the non-carrier during sexual activity.[1] According to the World Health Organization (WHO, 2018), cervical cancer was recorded as the fourth largest cause of cancer among middle-aged women globally, mostly affecting women in under-resourced countries.[2] In higher income countries, pap smear (cytology-based) testing remains the most widely used screening method for early detection among women. However, this method is relatively difficult to administer in low income countries due to the high costs required for clinical testing and treatment.[3]

Although several widely adopted practical alternatives including combinational visual effects using acetic acid and rapid human papillomavirus (HPV) detections have been beneficial in some low-income countries, even these screening methods are relatively expensive to those populations and therefore their uptake remains limited.[3] Consequently, the annual worldwide burden on cervical cancer is a triage beset by: (i) primary healthcare facilities; (ii) affordability and accessibilities to services; and (iii) perception and practises in different geographical locations. Notably, HPV vaccinations, which were launched for adolescent girls over a decade ago,[4] have now been deployed to, and are routinely used in, approximately 100 countries (WHO, 2019).[5] Despite the major milestones that have been achieved in the effective deployment of these vaccines, developing countries remain beset by high costs, difficulties with implementation, geographical isolation, as well as ethnic and socio-cultural issues. Due to these difficulties, it is estimated that more than 80% of women diagnosed with cervical cancer live in low- and middle-income countries.[6]

According to folklore medicine, therapeutic herbal plant treatments for diseases predate modern medicine by hundreds, or even thousands of years in some cultures. Furthermore, complementary and alternative medicine continues to attract attention, and indeed there has been a noteworthy increase in their uptake in some societies during the last decade.[7] Some plants have been reported to have inherent anti-cancer properties, and often the toxicity levels have been suggested to be lower than in synthetic anti-cancer drugs.[7,8] It is estimated that 80% of developing countries rely on the use of herbal plants to meet their primary health care needs.[7] Additionally, the regular consumption of vegetables and fruits (particularly from high antioxidant plants) have been suggested to have prophylactic effects against many chronic diseases, including the prevention of certain cancer types.[9]

Hibiscus *sabdariffa* L. (Malvaceae) is an annual shrub widely cultivated as a food and beverage, for cosmetics, and for use in the health industry.[10] Different parts of the plant including the leaves, seeds, stem and calyces are used as food, flavouring agent, and as a herbal beverage.[11] Tea processed from the calyces is non-toxic and is regarded as relatively safe for consumption, even at high concentrations.[12] Beverages sourced from the calyces of *H. sabdariffa* have been used effectively in traditional medicine for the treatment of numerous ailments and diseases, including the treatment of cough, abscesses, debility, dyspepsia, dysuria, fever, hangover, heart ailments, neurosis, scurvy, strangury, hypertension and cancer.[13] Calyces of *H. sabdariffa* have been reported to contain phytochemicals that include alkaloids, anthocyanins, phenols, saponins, tannins and flavonoids.[14,15] The active constituents of *H. sabdariffa* have been used for the treatment of some cancers and other diseases,[14] particularly in the prevention of diseases associated with oxidative stress. Several studies have been published on the metabolic and hepatoprotective activities of the anthocyanin-rich calyces of *H. sabdariffa* examined in rats.[16,17] However, the metabolic and hepatoprotective activities of isolated *H. sabdariffa* flavonoids, as well as

crude extracts also containing other active components, remain to be rigorously examined at the cellular level. Interestingly, flavonoids abound in most plants, and the bioactivity of the some of these compounds have been identified to be beneficial to human health.[17,18]

Several epidemiological studies have indicated that the consumption of dietary flavonoids can suppress the onset and progression of some cancers.[19] Case studies from Shanghai, Hawaii and Uruguay show that flavonoids reduce the incidence of esophagus, oral cavity, larynx and pharynx cancer by up to 70% in human subjects.[18] In a different study, it was observed that the mortality rate of consumers of high levels of soy isoflavones was relatively low.[17] Flavonoids can prevent cancer progression via the inhibition of several mechanisms, including invasion, metastasis, angiogenesis, apoptosis and cell cycle arrest.[20] In this study, we determined the phytochemical constituents of crude extracts from the dried calyx of *H. sabdariffa* using quantitative and qualitative methods.[21] We also determined the effect of lipid, aqueous, free and bound flavonoids fractions from the dried calyx of *H. sabdariffa* on the cell cycle of cervical cancer cell lines *in vitro*.[22]

MATERIALS AND METHODS

Labio Scientific Centre (Lagos, Nigeria) supplied the analytical grade petroleum ether, ethyl acetate, diethyl ether, methanol, dimethyl sulfoxide (DMSO) solvents used in this study. Jena Bioscience, Germany supplied the propidium iodide and ribonuclease (RNase). Sigma Aldrich (St. Louis, MO, USA) supplied the tissue culture grade penicillin/streptomycin 100X solution, Eagle's minimum essential medium (EMEM), filter sterilised trypsin-EDTA solution 10X, ammonia solution, acetic anhydride, sulphuric acid, chloroform, Folin–Ciocâlteu reagent (FCR), phenol, isobetyl alcohol, rutin, tannic acid and Folin-Denis' reagents. All reagents were analytical grade unless otherwise specified.

Plant extraction process

Dried calyces of *H. sabdariffa* were purchased from a local food store at a registered local market in Lagos, Nigeria. Authentication of the specimen was carried out at the Department of Botany, University of Lagos, Nigeria, with a voucher specimen (LUH No-6300) deposited in the herbarium.

Preparation of methanolic extract of *H. sabdariffa*

The *H. sabdariffa* calyces were air-dried in the shade for 21 days at room temperature. The air-dried calyces were ground to a coarse powder using a mesh size of 1 mm. The ground material (3.2 kg) was soaked in 20 L methanol and allowed to stand at room temperature with continuous agitation for 5 days. The suspension was subsequently filtered through sterile muslin cloth and the filtrate was transferred to a rotary evaporator and concentrated at 3000 rev/min at 40°C to remove the solvent. The concentrate recovered from the rotary evaporator was completely dried (until there was no further change in mass following additional drying time) for 72 hr at 40°C to ensure that all residual methanol was removed.

Moisture content determination

The moisture content of the extract was quantified using a Mettler Toledo moisture analyser. The initial weight of sample analysed was 0.831 g and the final weight was 0.82 g. The total moisture content was determined to be 0.61 %.

Fractionation of the *H. sabdariffa* extract

The *H. sabdariffa* extract was fractionated by liquid partitioning in petroleum ether, diethyl ether and ethyl acetate following standard methods.[23] Compounds were separated based on their differential solubility in a two-solvent system. A mass of 20 g of the solid extract was dissolved in 50 mL of distilled water in a beaker and transferred into a 500 mL separating funnel. A volume of 150 mL petroleum ether was added into the funnel to remove the extract and the resulting immiscible mixture was shaken vigorously and allowed to stand for 30 min. The mixture was subsequently separated into aqueous and organic layers and the two layers were collected separately. The aqueous layer was re-extracted twice with 150 mL petroleum ether to ensure complete removal of the lipid components. The aqueous layers were pooled together for further fractionation, while the organic layers containing the lipid components were evaporated to dryness at 50°C. The aqueous layer was further partitioned using diethyl ether to remove the free flavonoids, or with ethyl acetate to fractionate the bound flavonoids. The organic fractions were evaporated to dryness separately at 50°C and weighed to determine the yield of extract. The dried ethyl acetate fraction was hydrolyzed with 7% (v/v) H_2SO_4 for 24 hr, filtered, and re-extracted thrice with ethyl acetate. The ethyl acetate layers were neutralized by washing with distilled water and subsequently re-dried at 50°C.[24] The extracts were subsequently resuspended in deionised water for biological screening.

Qualitative phytochemical screening

Chemical tests were carried out on crude extracts (dried and resuspended in sterile deionised water) following standard procedures to identify phytochemical constituents.[22] Briefly:

(1) **Tannins;** 0.5 g of the extract was dissolved in 20 mL of distilled water in a test tube. A few drops of 0.1% ferric chloride were added and checked for the development of a brownish green or a blue-black colouration.

(2) **Phlobatannins;** The plant extract was boiled in 1 % hydrochloric acid and a deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

(3) **Alkaloids;** A mass of 2.5 g of the ethanolic extract was evaporated to dryness and the residue was heated in a boiling water bath with 10 % (v/v) HCl (5 mL). After cooling, the mixture was filtered, and the filtrate was divided into two equal portions. One portion was treated with a few drops of Mayer's reagent.[25] The samples were then observed for the presence of cream precipitate.

(4) **Saponins;** A mass of 0.5 g of plant extract was dissolved in 20 mL of distilled water in a test tube and shaken vigorously to form a stable froth. The froth was mixed with three drops of olive oil and then shaken vigorously. The formation of an emulsion signifies the presence of saponin.

(5) **Flavonoids;** The extract was heated with 1 mL of dilute ammonia solution. A yellow coloration indicated the presence of flavonoids.

(6) **Steroids;** A 2 mL volume of acetic anhydride was added to 0.5 g of extract in 2 mL concentrated H_2SO_4. A colour change from violet to blue or green indicates the presence of steroids.

(7) **Terpenoids (Salkowski test);**[22] The plant extract (2 mL) was mixed with 2 mL of chloroform and 3 mL of concentrated H_2SO_4 was carefully added to form a layer. A reddish-brown coloration at the interface indicated the presence of terpenoids.

(8) **Cardiac glycosides;** A mass of 0.5 g of the plant extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was overlayed with 1 mL of concentrated sulphuric acid. A brown ring at the interface identifies the deoxy-sugar characteristics of cardiac glycosides. A violet ring may also appear below the brown ring while in the acetic layer.

(9) **Reducing sugars;** A 0.5 mL volume of plant extract was mixed with 5 to 8 drops of Fehling's solution and boiled for 2 min.[26] A brick red precipitate indicates the presence of reducing sugar.

Quantitative phytochemical screening

Quantitative analysis of the extract of *H. sabdariffa* calyx were performed to confirm the presence of some active constituents, employing standard conventional protocols.[27]

(1) **Total phenolic content**; The amount of phenol in the aqueous extract was quantified using the Folin-Ciocalteu method with some modifications. A volume of 2.5 mL of 10% Folin-Ciocalteu reagent and 2 mL of 2 % Na_2CO_3 solution was added to 1 mL of plant extract. The resulting mixtures were incubated for 15 min at room temperature. The absorbance of the sample was measured at 765 nm. Tannic acid was used as a standard in the quantification of phenols as reported previously.[28]

(2) **Total flavonoid content;** A modified standard colorimetric method using aluminium chloride was used to determine the flavonoid content of the extract. A 1 mL of sample plant extract was mixed with 3 mL of methanol. Subsequently, 0.2 mL of 10 % aluminium chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water were added and allowed to stand at room temperature for 30 min. The absorbance was measured at 420 nm using a Camspec M106 spectrophotometer against quercetin standard.[29]

(3) **Alkaloid content;** Alkaline precipitation was quantified using standard gravimetric methods.[30] A mass of 1 g of the extract was dispersed into a solution of 10% acetic acid in ethanol in a ratio of 1:10 (10%). The mixture was allowed to stand for 4 hr and then filtered. The filtrate was evaporated to one quarter of its original volume and treated dropwise with the addition of concentrated aqueous NH_4OH until the alkaloid precipitated. The precipitated alkaloid was filtered in a weighted filter paper, washed with 1 % ammonia solution, and dried in the oven at 800°C. The alkaloid content was calculated and expressed as percentage of the weight of sample analyzed.

(4) **Tannin content***;* A mass of 0.2 g of the extract was weighed and added to 20 mL of 50 % methanol. The mixture was covered with paraffin and placed in a water bath at 77- 80°C for 1hr and stirred with a glass rod to prevent clumping. The extract was filtered through two layers of Whatman No.1 filter paper rinsed with 50 % methanol and made up to the 100 mL mark with distilled water. A 1mL volume of the resulting sample extract, 20 mL distilled water, 2.5 mL Folin-Denis reagent and 10mL of 17% Na_2CO_3 were added and mixed thoroughly. The mixture was made up to 50 mL mark with distilled water and allowed to stand at room temperature for 20min. A bluish-green coloration indicates the presence of tannins. The absorbance was measured and compared with a standard curve of standard tannic acid solution using a Camspec M106 spectrophotometer at a wavelength of 760 nm.[31]

(5) **Saponin content;** A volume of 100 mL isobutyl alcohol was added to 1g of the extract. The mixture was filtered through a Whatman No.1 filter paper and 20 mL of 40 % saturated solution of magnesium carbonate was added. The resulting mixture was filtered using Whatman No 1 filter paper to obtain a clear, colorless solution. A volume of 1 mL of the colorless solution was pipetted into 50 mL volumetric flask and 2 mL of 5% $FeCl_3$ solution was added and made up to 50 mL with distilled water. The solution was allowed to stand for 30 min. A blood red coloration indicated presence of saponins. The absorbance was read and compared to a saponin standard solution[32] using a Camspec M106 spectrophotometer at a wavelength of 380 nm.

Preparation of fractions for cell culture medium

Stock solutions of each fraction (lipid fraction, free flavonoid fraction, bound flavonoid fraction and aqueous fraction) were prepared by dissolving 0.2 g of each fraction in 1 mL of dimethyl sulphoxide (10 % DMSO). The mixture was stirred in a crucible until a fine paste was obtained and made up to 10 mL with PBS (phosphate buffer saline). The resulting solution was used to prepare the culture medium with a concentration of 200 µg/mL.[33]

Cell culture methods

Cervical cancer cell lines (HeLa cells) obtained from the American Type Culture Collection (ATCC; USA) and were used for the cellular assays. The cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution and maintained in an incubator with a humified atmosphere at a temperature of 37°C and 5% CO_2. The medium was aspirated when the cells reached 70% – 80% confluence. Subsequently, the cells were seeded into a six well plate. A concentration of 200 µg/mL of each flavonoid fraction previously prepared from the stock solution (section 2.7) was added to the cultured cells in the plate wells, along with 5 mL of fresh culture medium. The cells were incubated for 24 hr at 37°C. The media was subsequently aspirated, the cells were harvested by washing twice with cold PBS to remove residual medium, and then trypsinized by adding 1.5 mL trypsin and centrifuged at 200g for 5 min. The supernatant was removed, leaving the cell pellet.[34]

Flow cytometry analysis

The cell pellet was fixed with 70 % (v/v) ethanol for 20 min and chilled at 4°C for 20 min. The cells were then centrifuged at 200 g for 5 min and the ethanol supernatant discarded. The pellets were then re-suspended in 500 µL of reagent buffer. Subsequently, 500 µL of ribonuclease at a final concentration of 200 µg/mL was added to the cells and incubated for 30 min. Propidium iodide (100 µg/mL) was added to the cells and the resulting mixture was incubated for 30 min at room temperature in the dark.[34] The propidium iodide-stained cells were analyzed with a PARTEC flow cytometer using the FL3 channel and the cell cycle analysis of the data obtained was performed with Flowmax software to obtain the percentage of cells in G_0/G_1, S phase and G_2/M phases of the cell cycle using previously reported methods.[34]

Statistical analysis

Data are expressed as the mean ± SEM of repeated experiments and one-way ANOVA was used to calculate statistical significance between control and treated groups ($p<0.01$ deemed to be statistically significant).

RESULTS AND DISCUSSION

The cell cycle refers to the sequence of events by which a cell duplicates its DNA, synthesizes other cellular constituents, and eventually divides into two daughter cells. This sequence of events is: (1) the mitotic (M) phase, (2) gap 1 phase (G_1), (3) gap 2 phase (G_2), and (4) the synthesis phase (S). Some cells may also undergo the quiescent (G_0) phase where they do not divide but are metabolically active. Several studies have suggested that inducing cell cycle arrest at any of the major phases may prevent proliferation of cancer cells.[35]

Several scientific discoveries on the medicinal nature of plants have attributed the prevention and treatment of chronic diseases to the phytochemical compounds present in plants. *Hibiscus sabdariffa* is an example of plant known for its beneficial role in the treatment and prevention of some diseases.[36] In the present study, phytochemical analysis of the methanolic extract of the calyx revealed the presence of flavonoids, tannins, phlobatannins, alkaloids, phytosterols, polyphenolics, cardiac glycosides and terpenoids. Table 1 shows the result of the qualitative and quantitative analyses. The results are consistent with previous studies[37,38] that also reported the presence of these phytochemical classes in the methanolic extract of the calyx.

Quantitative screening of the methanolic extract (Table 1) of the calyx of *H sabdariffa* showed that the major phytochemical components are

flavonoids, alkaloids, tannins, and phenols, in decreasing order of relative abundance, respectively. This analysis agrees with the work of Nkumah[15] which showed that *H. sabdariffa* is rich in flavonoids. Similarly, Alaga *et al.*[39] also reported that flavonoid is the most abundant phytochemical class in ethanolic extracts of *H. sabdariffa* calyx.

Previous research work has revealed the anticancer properties of flavonoids, with diverse mechanisms of action, including inhibition of cell growth and proliferation by arresting the cell cycle, induction of apoptosis and differentiation, or a combination of these mechanisms as suggested by Raffa *et al.*[40] Notably, flavonoids including quercetin induce cell cycle arrest at G_1/S or G_2/M phase in multiple cancer cell lines, including some cervical cancer cell lines.[41]

Consequently, we tested the cell cycle inhibitory effect of the lipid, bound flavonoids, free flavonoids, and aqueous fractions that were obtained from the methanolic *H. sabdariffa* calyx extract. Each of these fractions were screened to investigate the effect of flavonoid components on the cell cycle of HeLa cervical cancer cells. Analysis of the cell cycle phases using the FL3 channel on flow cytometry revealed a higher percentage of cells at the G_0/G_1 phase interface, and a lower percentage of cells in the S phase, or in the G_2/M phases when the cells were exposed to the bound flavonoid fraction, compared with the untreated control cells (Figure 1A and 1B). The effects of the free flavonoid, lipid and aqueous fractions on cell cycle distribution of HeLa cells are reported in Figures 1C, 1D and 1E respectively. The result from each of the responses after 24 h exposure suggests that the flavonoid containing extracts suppress cell proliferation by inducing cell cycle arrest at the G_0/G_1 phase. This is in accordance with the work of Tavsan and Kayali[42] and Song *et al.*[43] who both reported that flavonoids induce cell cycle arrest at G_0/G_1 phase. Indeed, luteolin induces cell cycle arrest at the G_0/G_1 interface in colon cancer cells,[42] while the flavonoid extract (casticin) from *Vitex rotundifolia* L induces cell cycle arrest at G_0/G_1 phase in gall bladder cancer.[43] It is likely that the *H. sabdariffa* flavonoid components contribute to the cell cycle arrest reported in our study, although this remains to be verified.

Numerous studies have been published examining the mechanism of action of flavonoids in regulating the cell cycle of cervical cancer cells. Cyclin dependent kinases (CDKs) and cyclins, which represents a family of cell progression proteins, play a significant role in the cell cycle regulation.[44] Different cyclins and CDKs regulate cell progression through each phase of the cell cycle. Of particular interest, CDK2, CDK4, and cyclin D are important regulators of G_0/G_1 phase progression.[45]

At the end of G_1 phase, CDK4 is activated by dephosphorylation of its threonine 172 residue, which enables it to bind with cyclin D, with CDK 6 activated by binding to cyclin D.[46] Formation of either cyclin D-CDK 4 complex or cyclin D-CDK6 complex is important for the transition from the G_1 phase to the S phase of the cell cycle.[46] It was also observed that the activation of cyclin-CDK complex (cyclin D-CDK6 and cyclin D-CDK4) can be inactivated by binding to cyclin dependent kinase inhibitors (CDI) that are specific to the G_1 phase of the cell cycle. Two types of CDI associated with G_1 phase are the CIP/KIP family, which includes $p21^{cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ and the INK4 family (which includes $p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$). Binding of any of these CDIs to the activated cyclin-CDK complex would inactivate it, thereby stopping cell cycle progression from the G1 to S phase.[47]

Given the results from this study, we deduced that the inhibition of a cell

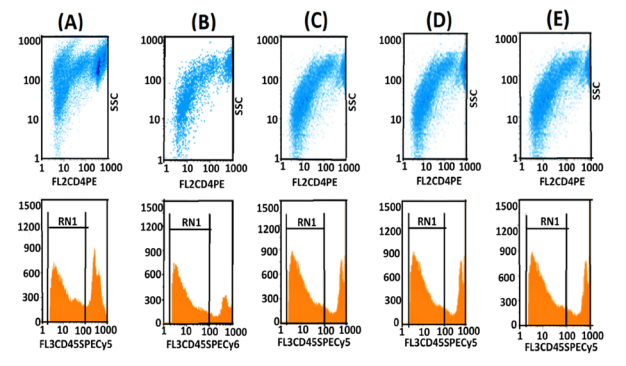
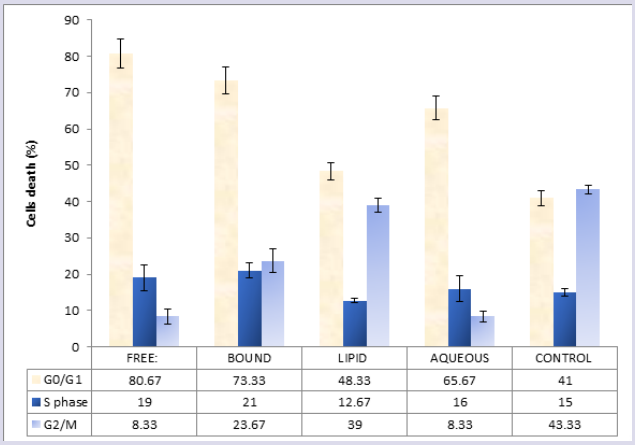


Figure 1: Flow cytometry analysis of the cell cycle distribution in HeLa cells at 200 µg/mL for 24 h. In the control, the cells were incubated without any dose of the prepared fractions (A) Control for the G_0/G_1 = 41.00 ± 1.00%; S phase= 15.00 ± 1.00%; G_2/M = 43.33 ± 1.16 %. (B) Bound flavonoid fraction: G_0/G_1= 73.33 ± 2.08 %; S phase = 21.00 ± 2.00%; G_2/M = 23.67 ± 3.22% (C) Lipid fraction: G_0/G_1= 48.33 ± 1.53%; S phase = 12.67 ± 0.58% and G_2/M = 39.00 ± 2.00 % (D) Free flavonoid fraction: G_0/G_1= 80.67 ± 2.08%; S phase= 19.00 ± 3.60%; G_2/M = 8.33 ± 2.08% (E) Aqueous fraction: G_0/G_1= 65.67 ± 4.07%; S phase = 16.00 ±3.61%; G_2/M = 8.33 ± 1.53%. The side-scattered light (SSC) is proportional to the cell granularity and measures the HeLa cells' physical properties in the flow cytometry.

Table 1: Phytochemical constituents of the crude methanolic extract of *Hibiscus sabdariffa* calyx.

Observed(N)	Test	Aqueous extract	Fractions	Absorbance	Yield (µg/g)
1	Tannins	+		0.10	2.15 ± 0.00
2	Phlobatannins	+			
3	Alkaloids	+		0.77	3.86 ± 0.05
4	Steroids	+			
5	Flavonoids	+		0.03	6.56 ±1.01
6	Phenols	+		0.003	1.66 ± 0.01
7	Cardiac glycosides	+			
8	Terpenoids	+			
9	Saponin	-			
10	Reducing sugars	+			

Values are means of triplicate determination (n = 3) ± standard deviation (SD); + indicates that the component was present; - indicates that the component was absent



	FREE:	BOUND	LIPID	AQUEOUS	CONTROL
G0/G1	80.67	73.33	48.33	65.67	41
S phase	19	21	12.67	16	15
G2/M	8.33	23.67	39	8.33	43.33

Figure 2: Analysis of the cell cycle distribution of HeLa cells. Values are expressed as the percentage of the cell population in the G_0/G_1, S, and G_2/M phase of cell cycle. Cells were treated with the extract fractions (200 µg/mL), except for the deionised water control. They were incubated for 24 hr. Each value represents the mean of three experiments; bars ± SD (P<0.05 was considered significant).

cycle arrest by the flavonoid containing fractions at G_0/G_1 phase may be due to their ability to down regulate cyclin D, CDK4 and/or CDK6 protein, although this is yet to be verified. Furthermore, the progression from the G_1 phase to the S phase of the cell cycle may be attributed to the inactivation of the activated cyclin D-CDK4 and cyclin D-CDK6 complexes by CDIs in those fractions. Such inactivation can also prevent cell progression from the G_1 phase to the S phase of the cell cycle.[47]

Whilst the four fractions were able to induce cell cycle arrest at the G_0/G_1 phase, exposure to the bound and free flavonoid *H. sabdariffa* fractions induced a substantially higher percentage of cells to remain in the G_0 phase compared to untreated control cells (Figures 1). A quantification of the responses of each of the *H. sabdariffa* fractions (as well as the control cells) on HeLa cells is shown in Figure 2. Notably, the flavonoid containing fractions were substantially more effective in inducing cell cycle arrest in HeLa cells, thereby inhibiting the proliferation of this cervical cancer cell line.

CONCLUSION

This study demonstrates that bound and free flavonoid containing fractions prepared from a methanolic *H sabdariffa* extract were effective in preventing proliferation of cervical cancer cells by inducing cell cycle arrest at the G_0/G_1 phase. Whilst the phytochemical composition of these fractions was not comprehensively evaluated, it is likely that their flavonoid components may contribute (at least in part) to the cell cycle inhibitory activity reported herein. However, our phytochemical evaluation studies also revealed a relative abundance of alkaloids, tannins and other polyphenols in these extracts and these are likely to also contribute to the bioactivities reported herein. Further studies to identify and determine the contribution of all components is required. However, from the results of our study, it is likely that regular consumption of beverages made from the calyx of *H. sabdariffa* may be effective in preventing the proliferation of cervical cancer cells, although this is yet to be verified *in vivo*. It is anticipated that these findings enhance awareness of relevant health and food regulatory agencies on the consumption of beverages made from the calyx of *Hibiscus sabdariffa*. Furthermore, it is hoped that this study will highlight the benefits of this important species and focus further studies in this field.

ACKNOWLEDGEMENT

L.A. acknowledges the College of Medicine, Idi-Araba, and University of Lagos for support towards this research. We are grateful to PZ Cussons, Lagos Nigeria for the use of the Mettler Toledo moisture analyser.

ABBREVIATIONS

CDKs: Cyclin dependent kinases; **CDI:** cyclin dependent kinase inhibitors (CDI); **KIP:** kinase inhibiting protein; **M:** mitotic phase; **G_1:** gap 1 phase; **G_2:** Gap 2 phase, **S:** synthesis phase; **G_0:** Quiescent phase; **HPV:** human papillomavirus; **FCR:** Folin–Ciocâlteu reagent; **WHO:** world health organization; **ATCC:** American Type Culture Collection; **EMEM:** Eagle's minimum essential medium; **FBS:** fetal bovine serum; **PBS:** phosphate buffer saline; **DMSO:** dimethyl sulphoxide.

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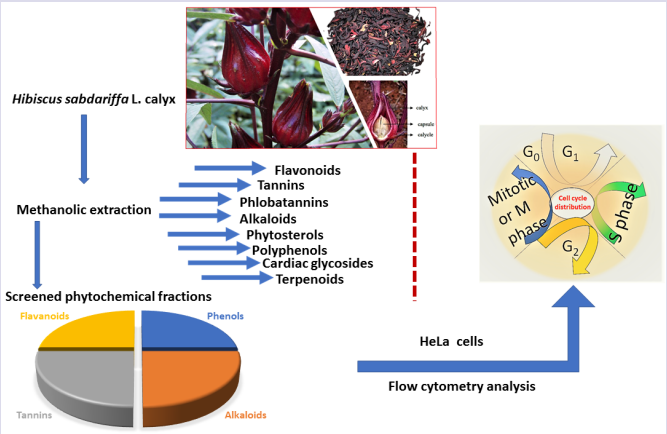
PICTORIAL ABSTRACT



SUMMARY

- *Hibiscus sabdariffa* L. calyx were screened for the avility to inhibit cervical cancer cells.
- The phytochemical screened dosed applied to HeLa cells included: bound flavonoid, lipid, free flavonoid and aqueous fractions.
- The antiproliferative activity of the phytochemical screened factions were quantified using flow cytometry method.

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Dr. Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including Aloe vera, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens*, *Pittosporum phylliraeoides*, *Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 250 scientific publications in a variety of peer reviewed journals.