Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Anticancer Potential of Steviol in MCF-7 Human Breast Cancer Cells

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Submitted: 26-01-2017 Revised: 21-02-2017 Published: 19-07-2017

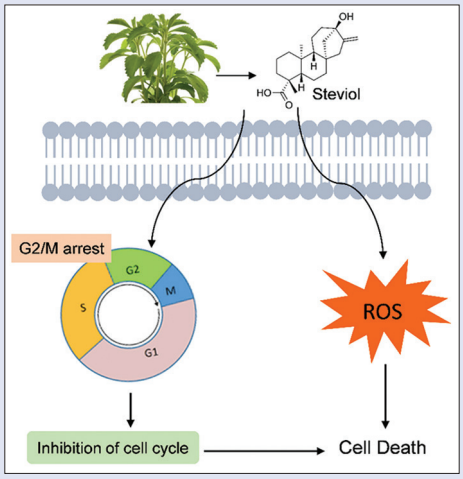
ABSTRACT

Objective: This study aimed to investigate the cytotoxicity, apoptosis induction, and mechanism of action of steviol on human breast cancer cells (Michigan Cancer Foundation-7 [MCF-7]). **Materials and Methods:** Sulforhodamine-B assay was performed to analyze cytotoxic potential of Steviol whereas flow cytometer was used to analyze cell cycle, apoptosis, and reactive oxygen species generation. **Results:** Studying the viability of cells confirms the IC_{50} of Steviol in MCF-7 cells which was 185 μM. The data obtained from fluorescence-activated cell sorter analysis reveal Steviol-mediated G2/M-phase arrest ($P < 0.05$) in addition to the presence of evident sub-G0/G1 peak ($P < 0.05$) in the MCF-7 cells, signifying the ongoing apoptosis. **Conclusion:** Thus, results suggest that induction of apoptosis in MCF-7 cells was due to dose-dependent effect of Steviol. Our first *in vitro* findings indicate Steviol as a promising candidate for the treatment of breast cancer.

Key words: Apoptosis, Michigan Cancer Foundation-7, *Stevia rebaudiana*, Steviol

SUMMARY

- Steviol remarkably inhibited the growth MCF-7 HBCCs in a dose dependent manner
- It abolishes cell cycle progression by arresting cells at G2/M phase
- Steviol induces the cells to undergo apoptosis
- Steviol induces the cells to generate reactive oxygen species (ROS).



Abbreviations used: MCF-7: Michigan Cancer Foundation-7; SRB: Sulforhodamine-B assay; FACS: Fluorescence-activated cell sorter; ROS: Reactive oxygen species; DNA: Deoxyribonucleic acid.

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DOI: 10.4103/pm.pm_29_17

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INTRODUCTION

Worldwide, the majority of cancer deaths in women is due to breast cancer.[1] It also leads to a significant morbidity and mortality among women, and the outcome of disease is mainly affected by metastasis.[2] There are multiple factors involved in the pathogenesis of breast cancer, namely, life style, genetic susceptibility, and hormonal and environmental factors. In general, for *in vitro* studies, the human breast adenocarcinoma cell line (Michigan Cancer Foundation-7 [MCF-7]) is most widely used. Recent studies suggest that chemo- and radio-therapies are receiving more importance in inducing apoptosis in neoplastic cells by working effectively against most cancer types, but many tumors do not respond to these drug therapies and limit the successful outcomes.

Furthermore, in most cases, these drugs lack the ability to distinguish between normal and cancerous cells and they fail in activating the apoptotic pathways.[3] Therefore, the main emphasis should be laid on searching valuable natural alternative medicinal therapy which may increase the efficiency of complete and safe treatment for breast cancer.

Literatures suggest that approximately 74% of novel anticancer compounds are either natural products or derivatives of natural products.[4,5] Majority of drugs used in the treatment of cancer, whether artificial chemicals or natural products, inhibit the synthesis of new genetic material and cause irreversible damage to deoxyribonucleic acid (DNA) and its precursors. Basic research investigations confirm that, at molecular levels, several dietary chemopreventive compounds are activated.[6,7] A variety of plants and their derived bioactive compounds possess antiproliferative and anticarcinogenic effects toward breast cancer cells.

Stevioside (triglucosylated Steviol), the predominant *ent*-kaurene diterpene glycoside obtained from the leaves of *Stevia rebaudiana*

Cite this article as: Gupta E, Kaushik S, Purwar S, Sharma R, Balapure AK, Sundaram S. Anticancer potential of steviol in MCF-7 human breast cancer cells. Phcog Mag 2017;13:345-50.

Bertoni (Asteraceae family), is an effective noncaloric natural sweetener (around 200–250 times sweeter than sucrose) used in the diet in many countries in Asia and South America.[8] A study by Geuns *et al.*[9] on biotransformation of stevioside reports that, in the large intestine, bacterial flora of the cecum or colon degrade stevioside into free Steviol which is further transformed in the liver into its glucuronide derivative and excreted from the body through urine. Hutapea *et al.*[10] studied the *in vitro* digestibility of stevioside by a variety of digestive enzymes and reported that intestinal microflora hydrolyzed the stevioside to further compounds Steviol and Steviol-16, 17 alpha-epoxide. Finally, Steviol 16, 17 alpha-epoxide was subsequently transformed back into Steviol and excreted as Steviol glucuronide in urine. Studies suggest that stevioside and its related compounds (Steviol and isosteviol) offer therapeutic properties, which include antihypertensive,[11] antitumor,[12] antihyperglycemic,[13] anticancerous,[14] and anti-inflammatory.[15]

It is widely known that apoptosis or a process of programmed cell death is generally indicated by cytosol condensation, marginalized chromatin, protein fragmentation, degradation of DNA, and finally smaller units of apoptotic bodies are formed by the breakdown of cells.[16] A well-regulated process of apoptosis is characterized by one of the two pathways. When a cell senses any stress, it kills itself in intrinsic pathway whereas killing of cells occurs due to signals received from other cells in extrinsic pathway. Both the pathways activate caspases (protein digestive enzymes) which induce cell death and participate in the initiation and execution of apoptosis. Extreme apoptosis leads to atrophy whereas insufficient amount causes cancer (uncontrolled proliferation of cells). Studies report that the essential role of free radicals or reactive oxygen species (ROS) is vice versa in cancer and apoptosis.

Evidences suggest that ROS induces apoptosis and they regulate apoptotic cell death by activating the enzyme caspase which releases mitochondrial cytochrome c. The free radicals inside the cells were created by the ROS which are primarily the by-products of normal cellular metabolism. They initiate carcinogenesis and impose oxidative destruction to proteins, DNA, and lipids,[17] and as a result, they produce highly mutagenic oxidative DNA product (8-oxo-2'-deoxyguanosine). ROS regulates the initiation of apoptotic signaling and produces carcinogenesis by depolarizing mitochondrial membrane and interfering the signal cascade systems such as activated protein-1, nuclear transcription factor c-Jun kinase, kappa B, mitogen-activated protein kinases, and phospholipase A_2.[18,19] Although the mechanism involved in ROS is still controversial regarding its role during apoptosis, till now, there is no information reported on the effect of Steviol on human breast cancer. Therefore, the present investigation was carried out to study the role of Steviol on human breast cancer cell line (MCF-7).

Our results showed that Steviol dose dependently inhibits MCF-7 cell growth and arrests cell cycle arrest at G2/M phase. This study indicates the antineoplastic action of Steviol in breast cancer.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle medium (DMEM), streptomycin sulfate, penicillin G, gentamicin sulfate, N-[2-hydroxyethyl] piperazine-N9-2-ethanesulfonic acid (HEPES), sulforhodamine-B (SRB), phosphate-buffered saline (PBS; pH 7.4), and trichloroacetic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. Dichlorofluorescin diacetate (DCFDA) was purchased from Merck–Calbiochem. Fetal bovine serum (FBS) was procured from GIBCO BRL Laboratories, New York, USA. In addition, all the chemicals used were of analytical grade.

Cell culture

Human breast cancer cell lines (MCF-7) were purchased from the National Centre for Cell Sciences (Pune, India). These cells are regularly cultured according to the procedure described by Shagufta *et al.* and Gupta *et al.*[20,21] For culturing the MCF-7 cells, DMEM (pH 7.4) was used which contains streptomycin (100 U/mL), penicillin (200 mg/mL), and gentamicin (50 mg/mL) supplemented with 10 mM HEPES and 10% FBS at 37°C at a humidified atmosphere of 5% CO_2 incubator in T-25 tissue culture flask (TPP, Switzerland). Before performing the experiments, the cells were trypsinized which were obtained from a confluent flask and further cultured for 4 days. For the initial 2 days, DMEM phenol red free was used which contains 10% charcoal-stripped FBS to preculture the cells.[22] Subsequently, the cells were exposed to ligand (Steviol) for 48 h. The number of cells required for analyzing cytotoxicity and the exposure of cells with different concentrations of ligands has been described individually below.

Analysis of cell growth and cytotoxicity

To determine toxic, cytostatic, and proliferative effect(s) of Steviol in MCF-7 cells, SRB assay was done according to a procedure given by Shagufta *et al.*[23] For treatment, the dose of Steviol should be below IC_{50} value (i.e., sublethal) where the number of live cells should be more than 50% and the viability of cells was analyzed to achieve IC_{50} value. In brief, under inverted-phase contrast microscope, the MCF-7 cells were counted by a hemocytometer, and in a 96-well plate, 10^4 cells/well were plated in phenol red-free DMEM (200 µl) and left for 48 h in CO_2 incubator at 37°C. The used medium was changed with fresh medium, and a different concentration of Steviol (10–500 µM) was added in triplicate. After completing the incubation with the drug (Steviol), SRB assay was performed.

Cellular morphological alteration studies

For the cellular morphological analysis, 2×10^6 cell was plated in 6-well plates in phenol red-free medium for 48 h. Thereafter, cells were exposed for 48 h with 10–250 µM Steviol. Afterward, microscopic analysis was conducted under inverted phase-contrast microscope.

Analysis of cell cycle kinetics and apoptosis by flow cytometry

For analyzing the cell cycle distribution and apoptosis, 0.2×10^6 MCF-7 cells were seeded in 6-well plates and incubated for 48 h in a phenol red-free DMEM medium supplemented with 10% charcoal-stripped FBS. The adherent cells were exposed to 10–250 µM Steviol and kept for 48 h in a 5% CO_2 incubator. Subsequently, the cells were trypsinized and washed with ice-cold PBS. Seventy percent chilled ethanol was used to fix the cells for 1 h at 4°C. Cells were rewashed with chilled PBS twice and resuspended in 500 µl of PBS containing 100 µg/ml RNase and 40 µg/ml of propidium iodide. Becton–Dickinson fluorescence-activated cell sorter (FACS) analysis was used to perform flow cytometric analysis using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Analysis of intracellular reactive oxygen species generation

Generation of ROS was assessed using fluorescent probe 2',7'-DCFDA staining. MCF-7 cells at a concentration of 0.2×10^6 cells/well were seeded in 6-well plates and exposed to Steviol (10–250 µM) for 24 h. Thereafter, the cells were washed with ice-cold PBS twice and incubated with 10 µM DCFDA at 37°C for 30 min in dark. Rewashing of the cells was performed twice with ice-cold PBS. Subsequently, the cells

were trypsinized and fluorescent intensity was measured through flow cytometry on FACS using CellQuest software (DCFDA Ex 480 nm, Em 530 nm).

Statistical analysis

The obtained results were expressed as mean ± standard deviation. To perform statistical analysis, Bonferroni's multiple comparison tests and one-way analysis of variance were used. $P < 0.05$ was considered statistically significant.

RESULTS

Steviol induces cytotoxicity in human breast cancer cells (MCF-7)

This study was started by investigating the effect of Steviol on viability of cells, SRB assay [Figure 1] was conducted in human breast cancer cell type (MCF-7). The result demonstrates that Steviol induces dose-dependent reduction in viability of MCF-7 cells. 10 μM of Steviol shows insignificant antiproliferative activity; however, an abrupt decline was noticed in MCF-7 cells treated with 25–500 μM. The IC_{50} value of Steviol in MCF-7 cells was found to be 185 μM. The microscopic analysis confirms the cytotoxicity results [Figure 2] where dose-dependent alteration in morphology of MCF-7 cells as compared to control was observed.

Steviol induces G2/M-phase arrest and apoptosis in MCF-7 cells

It was already specified that cell proliferation was decreased by Steviol and it also induces death of MCF-7 cells; by flow cytometry, its outcome on cell cycle distribution was analyzed. Following treatment with Steviol at concentrations of 10–250 μM for 48 h, accumulation of cells occur significantly in the G2/M phase at a concentration of 250 μM ($P < 0.05$) which was compared to untreated cells [Figure 3]. The cells gradually increase in G2/M phase indicating that the conditions of cells were neither in replicating nor in resting phase. It was perceived that cells were in a process of death. Moreover, finding of hypodiploid (apoptotic) sub-G0/G1 population of cells confirms its role in inducing apoptosis.

In treatment groups, apoptosis was high compared to controls. Significant apoptosis was obtained even at lower dose, i.e., 10 μM Steviol. Interestingly, approximately 26% of cells had undergone apoptosis when treated with 250 μM Steviol for 48 h [Table 1], which was highly significant ($P < 0.05$) than control cells.

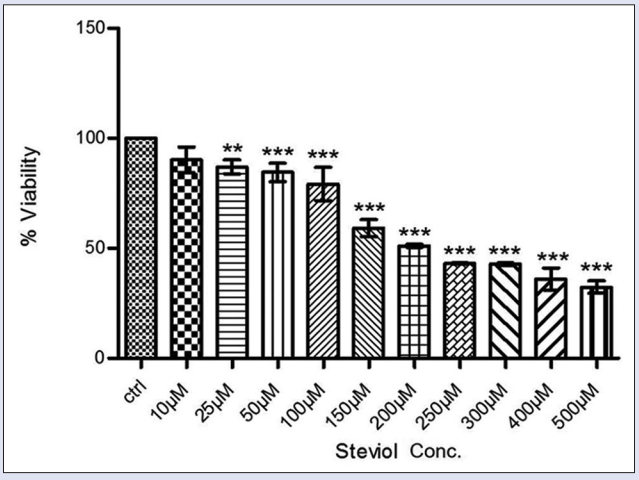

Figure 1: Dose-dependent cytotoxicity evaluation of Steviol in cancerous (MCF-7) cell lines versus controls (untreated MCF-7 cells). Analyzed data represent mean cell viability ± standard error in each concentration of Steviol. Data shown are Mean ± S.D. of three similar experiments, each performed in triplicate. **P<0.01; ***P<0.001 when compared to control

Decline in reactive oxygen species generation by Steviol in MCF-7 cells

For measuring the ROS levels within the cells, DCFDA (a ROS-sensitive fluorometric probe) was used. Treatment with Steviol at a concentration of 100 μM onward shows a significant decline in ROS generation after 24 h [Figure 4] when compared to the control. Thus, it was suggested that Steviol has a certain association with the underlying mechanism of apoptosis by impairing mitochondrial function.

DISCUSSION

Worldwide, cancer is a well-known health issue with 1 million new cases every year, the most common malignancy in women is breast cancer which comprises around 18% of all female cancers. Medicinal plants and its isolated bioactive compounds are recognized as an alternative source of nontoxic, inexpensive, and efficacious medications to synthetic chemotherapeutic compounds. Currently, researchers are making enormous efforts to synthesize anticancer drugs from plant sources, provided that they kill the cancer cells devoid of unnecessary damage to the normal cells.

The outcome of this study illustrates the cytotoxic or anticancerous effects of Steviol from the plant *S. rebaudiana* against cultured human breast cancer MCF-7 cells, and its probable mechanisms of action include suppression of cell viability, cell cycle arrest, and induction of apoptosis in cancer cells. Steviol inhibits the proliferation of MCF-7 cells in a dose-dependent manner. Till now, no study has reported on the effect of Steviol on human breast cancer (*in vitro* or *in vivo*), so in this aspect, this work has its own originality.

Study on cell growth and cytotoxicity [Figure 1] and cellular morphological analysis [Figure 2] shows that, in MCF-7 cells, a dose-dependent decline was induced by Steviol in the surviving cell percentage when compared to parallel controls. This action of Steviol toward breast cancer cells might be credited to differences in the levels of specific tissue and cytochrome P450 (CYP) isoform expression patterns which mediate cell-specific cytotoxicity.[24] Inducible and constitutive enzymes come under a multigene family of CYPs' which influences tumors' response to anticancer drugs. The reason is this enzyme system can either activate or detoxify many anticancer agents.

Table 1: Cell cycle analysis of Steviol-treated MCF-7 cells

Treatment	MCF-7 cells			
	Apoptosis		Cell cycle	
	Sub-G0/G1	G0/G1	S	G2/M
Control	0.92±1.30	54.34±0.56	45.31±1.03	0.36±0.44
Steviol (μM)				
10	5.53±1.13	52.93±0.63	46.53±0.28	0.29±0.007
50	8.13±1.04	55.20±1.04	43.59±0.31	1.20±0.73
100	12.84±0.60	58.09±0.04	39.60±3.18	2.77±2.50
200	24.26±0.52	55.43±0.43	41.41±3.97	5.14±1.67
250	26.89±0.75	55.68±0.96	38.65±2.15	5.67±1.17*

*P<0.05. Each value represents mean±SD of each performed in triplicate. SD: Standard deviation; MCF: Michigan Cancer Foundation-7

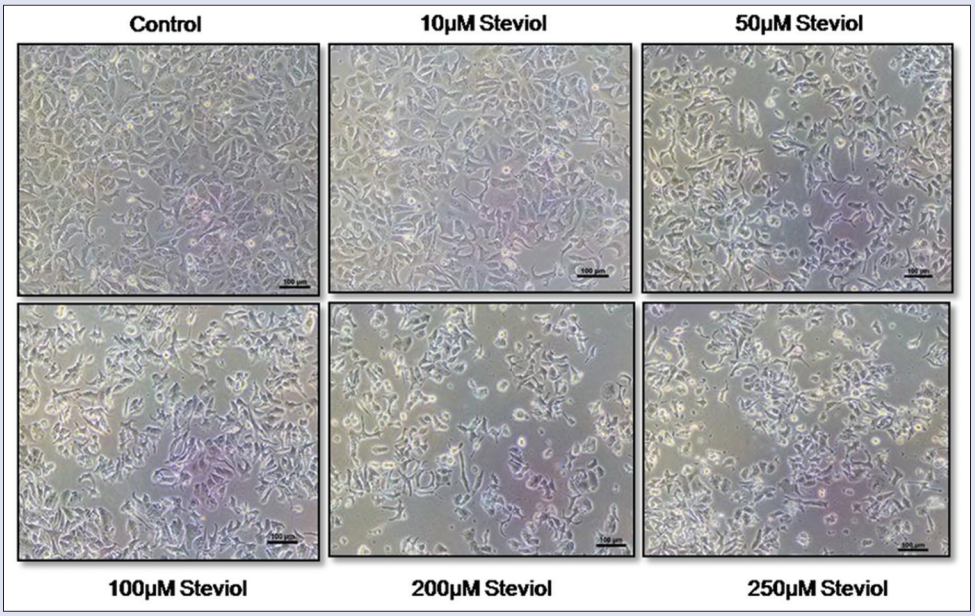

Figure 2: Morphological analysis (using phase-contrast microscope at ×100 magnification) of Steviol-treated MCF-7 cells. Different doses of Steviol decrease the survival rate of MCF-7 cells. MCF-7: Michigan Cancer Foundation-7

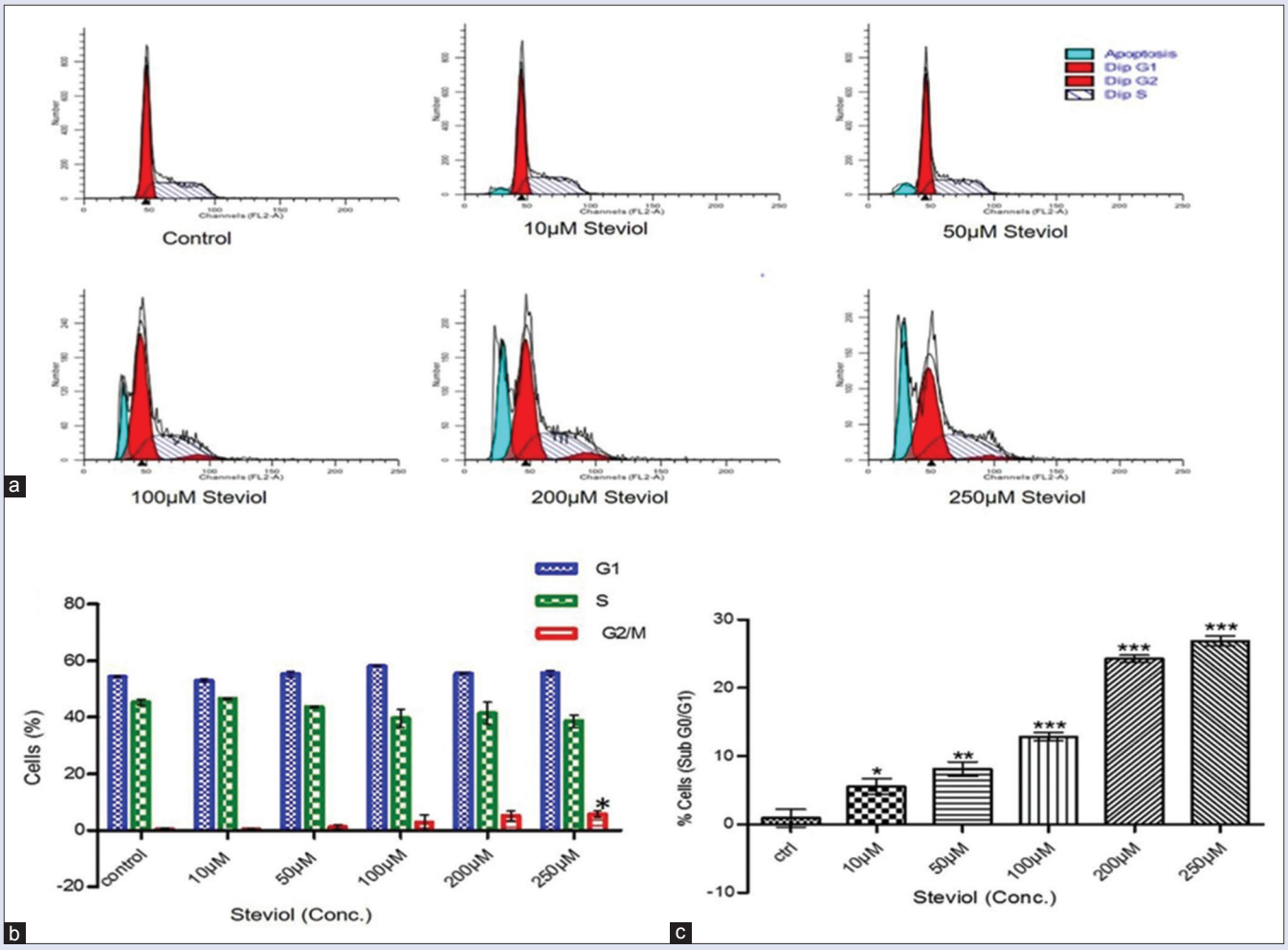

Figure 3: Cell cycle analysis of Steviol-treated MCF-7 cells. Results indicate that Steviol decreased cell proliferation and induced apoptosis in MCF-7 cells. (a) Cell cycle analysis by flow cytometry. (b) Graphical representation of different phases of cell cycle (G1, S, G2/M). (c) Graphical representation of cell population accumulated in sub-G0/G1 phase. MCF-7: Michigan Cancer Foundation-7

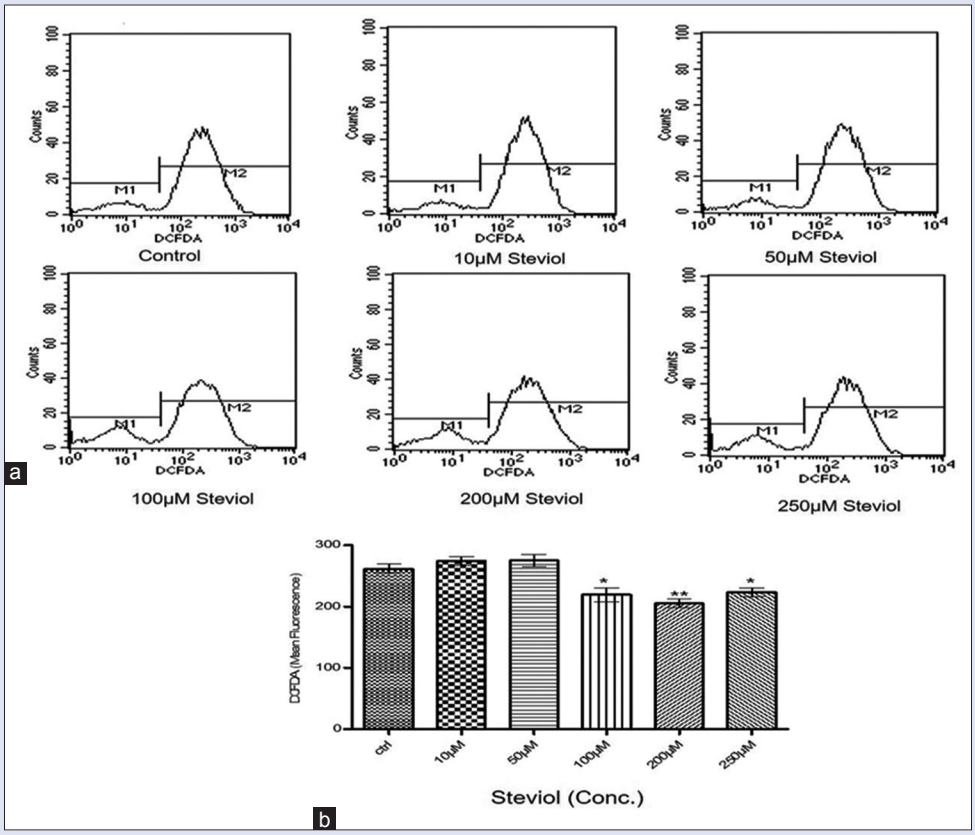

Figure 4: (a) MCF-7 cells (0.2×10^6) were exposed in 6-well plates and exposed with 10–250 μM Steviol for 24 h. Cells were incubated with 10 μM DCFDA for 30 min in dark. Reactive oxygen species generation was detected through flow cytometry. The result signifi es the deviation in curve from the control to the treated samples, (b) Graphical representation of Flow data

An essential factor of tumor cell survival is resistance to apoptosis. The advancement of drug resistance (reduced effectiveness of a drug) in cancer cells is the main reason for the unsuccessful cancer treatment. Therefore, new drugs are immediately required which are effective against tumor cells and do not induce toxicity in normal cells.

By studying the fundamental mechanisms, it was observed that, in MCF-7 cells, apoptosis was induced by Steviol through DNA damage which correlates with the fact that, in a sub-G0/G1 phase, a significant amount of cell population was found [Figure 3] and it also causes specific cell-type G2/M growth arrest in MCF-7 cells. Results indicate the genotoxic effect of Steviol, which damages the DNA by inducing some proteins.[25] Moreover, an increase in the number of apoptotic cells was noticed.

According to previously reported studies, leaves of *S. rebaudiana* contain a significant amount of total phenolic compounds and have a strong antioxidant activity in normal tissues.[26] Mostly, very invasive or metastatic cancer cells retain a balance between proliferation and apoptosis by involving a critical level of oxidative stress. Higher ROS is generally accompanied with the activation of oncogene, which is an early event of malignant transformation, thus the usage of antioxidant supplements is receiving higher attention. Studies have shown that downregulation of ROS is also responsible for the induction of apoptosis.[28,29]

In this study, a sharp decrease in the levels of ROS after 100 μM Steviol doses was observed, which indicates the involvement of ROS in Steviol-mediated induction of apoptosis [Figure 4].

CONCLUSION

From the results, it can be concluded that the naturally occurring pure compound such as Steviol isolated from the leaves of *S. rebaudiana* induces apoptosis and cell cycle disruption and effectively reduces the number of human breast cancer cells (MCF-7). Moreover, Steviol has antitumor property and involves signaling pathways with ROS and mediated by G2/M arrest. There is a requirement of understanding the regulating pathways or molecular mechanism of action of Steviol to establish its therapeutic applications which is safe for human consumption.

Acknowledgements

The authors thank the Director of CDRI for giving permission to carry out the research work at CDRI. The authors also thank Dr. Anil K. Balapure and Dr. Ramesh Sharma for their guidance during the experiments with cell culture techniques. Financial support from the Department of Science and Technology, New Delhi, India, under a Women Scientist Project Scheme (WOS-A), vide letter no. SR/WOS-A/LS-668/2012, is deeply acknowledged.

Financial support and sponsorship

This study was financially supported by the Department of Science and Technology, New Delhi, India, under a Women Scientist Project Scheme (WOS-A), vide letter no. SR/WOS-A/LS-668/2012.

Conflicts of interest

There are no conflicts of interest.

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