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Research Article

Stevia rebaudiana Methanolic Leaf Extract in Egypt: Phytochemical Analysis, Antioxidant, Antilipid Peroxidation, Antihemolytic, Antimetastatic, and Anticancer Properties

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The phenolic and flavonoid content of the *Stevia rebaudiana* hydromethanolic extract (SRHME) was examined, and phytochemical identification using GC-mass spectrometry was achieved. Also, the antioxidant, antihemolytic, and antilipid peroxidation capabilities of the extract were assessed. The extract's potential to induce apoptosis, inhibit cell proliferation, and reduce metastasis in SKVO3 cells was also checked. The findings of the GC-MS chromatogram demonstrated the existence of bioactive antioxidants and anticancer components in SRHME. Moreover, the extract demonstrated protection against cellular oxidative damage in human erythrocytes by preventing lipid peroxidation and hemolysis. Besides, SRHME demonstrated a selective cytotoxic effect with a strong IC_{50} value (17.5 μg/ml) on SKVO3 cells without any harmful effects on normal WI-38 cells using the MTT and LDH tests. Additionally, according to assays for wound healing and transwell chamber, the studied extract suppressed the ability of SKOV3 cells from migrating and invading, respectively. Also, the extract-treated SKVO3 cells showed rise in the percentage of apoptotic cells with a prominent comet nucleus, according to apoptotic assays in comparison to untreated cells. Furthermore, a flow cytometry analysis of SRHME-treated SKVO3 cells showed a halt in the *S* phase and an increase in sub-G1 apoptotic cells (25.44%). Also, the tested extract significantly decreased the levels of ROS in the treated cells, indicating that ROS was involved in the production of SKVO3 apoptosis. Lastly, SRHME strongly impacted the expression levels of proteins related to apoptosis, *S*-phase cell cycle arrest, and antimetastatic capacity in the treated SKVO3 cells.

1. Introduction

Various diseases can be treated using plant-based natural medications without the negative side effects associated with synthetic therapies [1], where the majority of phytoconstituents with substantial biological activity are secondary metabolites, such as flavonoids, carotenoids, and anthocyanins, as well as enzymes and proteins that the plant naturally produces through different growth phases [2].

Stevia rebaudiana (*S. rebaudiana)* is an annual natural herb that is used as a potent natural sweetener in a variety of foods, drinks, and pharmaceuticals [3], where *Stevia* leaf extract demonstrated greater sweetness than sucrose [4]. In addition, *Stevia* can be regarded as safe for human consumption because no harm has been linked to it throughout the years [5]. Also, *Stevia* contains bioactive metabolites, like polyphenols, which can be employed as nutraceuticals or food additives [6].

Stevia leaves contain a high concentration of phenolic compounds, including phenolic acids and flavonoids [7], where these compounds are distinguished by the presence of a hydroxyl group on their benzene ring, which is mainly accountable for their antioxidant action [8]. In addition, these compounds showed significant antibacterial, antitumor, anti-inflammatory, antihypertensive, and immunomodulatory effects both *in vitro* and in animal investigations

[7]. Moreover, some *Stevia* derivatives have been shown to be cytotoxic to cancer cells, including glioma, colon, breast, cervical, and pancreatic [9–11]. Cell cycle arrest caused by the control of essential proliferating proteins and stimulation of the apoptotic process are the two basic mechanisms underlying stevia's cytotoxicity against cancer cells [9].

Oxidative stress, which is the root cause of many human diseases, is caused by reactive oxygen molecules, which are created when there is an imbalance between the production and neutralisation of prooxidants. Because of the strong free radical scavenging potential of *Stevia* leaves [12], its phytoconstituents may protect human cells from oxidative stress by scavenging free radicals or by preventing their overproduction under challenging conditions [13].

Ovarian cancer is the seventh most frequent cancer among women around the globe and the primary cause of death from gynecologic diseases [14]. The ways of ovarian cancer treatment at this time are platinum-based chemotherapy and surgical therapy [15], but long-term usage of platinum-based chemotherapy commonly results in drug resistance, which raises the risk of death and relapse in ovarian cancer patients [16]. As a result, there is a pressing demand for herbal treatments that have effective therapeutic benefits against ovarian cancer [17].

Recent research has shown that consuming natural antioxidants is associated with a decreased risk of cancer and many chronic diseases, so investigating plant extracts' ability to act as antioxidants against both malignant and healthy cells is intriguing [18]. Although *Stevia* extracts and derivatives have antioxidant characteristics, only a few cancer cell lines were used to test their antiproliferative activities [19].

The impact of the methanol extract of *stevia* leaves of Egyptian origin on the development and spread of human ovarian cancer cells has not yet been reported. Hence, this study intends to assess the quantities of phenolic and flavonoid components in the prepared methanolic extract of *Stevia* leaves as well as confirm the existence of phytochemicals using GC-mass spectrometry. Moreover, we estimated the efficiency of *Stevia* extract as an antioxidant, antilipid peroxidation, and antihemolytic agent. Besides, we gauged the extract's potential influence on the suppression of the growth and metastasis of SKVO3 ovarian cancer cells with the clarification of the different routes for apoptosis induction.

2. Materials and Methods

2.1. Standards and Reagents. All substances were purchased from Sigma-Aldrich (USA), including all chemicals, reagents, solvents, buffers, and medium components.

2.2. Collection of Plant Materials and Processing. Plant material samples of fresh leaves of *S. rebaudiana* Egyptian cultivar were obtained from the Sugar Crops Research Institute (SCRI), Agricultural Research Centre (ARC), Egypt, with voucher specimen no. 2.9.2019.I. The leaves were collected, twice scrubbed with distilled water, dehydrated under free aeration at temperatures ranging from 35 to 43°C, pulverised with a mortar, and kept at −20°C until use.

2.3. Preparation of Hydromethanolic Extract of Stevia Leaves. *S. rebaudiana* hydromethanolic extract (SRHME) of plant leaves was prepared as reported earlier [20]. About 400 g of finely ground *Stevia* leaves was immersed in solution containing 80 methanol : 20 distilled water for 72 h with constant shaking, and the resulting mixture was filtered to remove the extract-containing solvent from the crushed leaves. Then, the obtained solvent was concentrated three times using a rotary evaporator. After being lyophilized in a freeze-dryer and solidified for 48 h, the extract was finally produced as a dried *S. rebaudiana* hydromethanolic extract (SRHME) that was kept at 4°C till usage.

2.4. Determining the Extract's Phytochemical Constituents

2.4.1. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis. The chromatographic analysis of the *Stevia* extract was performed using an Agilent Technologies 7890B GC system and 5977A mass selective detector. Before being injected onto GC-MS, the sample was treated with N, O-bis (trimethylsilyl) trifluoroacetamide, a silylation agent, for 2 h at 80°C. After the sample was injected, the column was maintained at 60°C for 5 min before being heated to 300°C using a 20°C/min heating ramp, with a 5.0 min hold in between. The MS scan range was (m/z): 50–550 atomic mass units (AMUs) under electron impact (EI) ionization (70 eV) and solvent delay (3.0 min). Helium served as the carrier gas at a pressure of 8.2 psi with a 1 μl injection in a capillary column (HP-5MS capillary; 30.0 m 0.25 mm ID 0.25 m film). Lastly, using mass fragmentations and the NIST mass spectral search program for NIST/EPA/NIH mass spectral library version 2.2, the sample constituents were detected.

2.4.2. Total Phenol Content Estimation. Gallic acid (GAE), a standard phenolic component, was used to assess the total amount of phenols in the *Stevia* leaf extract using the Folin–Ciocalteau procedure [21]. The extract was prepared in methanol at a concentration of 1 mg/ml, and the GAE stock solution was prepared in methanol at a concentration of 1 mg/ml. In addition, six working concentrations of GAE (ranging from 15.625 to 500 μg/ml) were used to construct the standard curve. The optical density of the samples was measured at 765 nm using methanol as a blank, and the total amount of phenol in the extract sample was calculated using the calibration curve of GAE by utilizing the formula:

$$\mathrm{TPC} = \frac{C\,\mathrm{x}V}{M}, \tag{1}$$

where TPC is the overall phenolic content that is expressed as mg GAE/g of the extract, C is a concentration of GAE in mg/ml that was deduced using the calibration curve, V is the quantity of the extract solution in ml, and M is the mass of the extract in g. All measurements were made in triplicate, and the findings were presented as the mean of three distinct experiments.

2.4.3. Total Flavonoid Content Estimation. Quercetin (QE), a standard flavonoid component, was used to measure the total amount of flavonoids in the *Stevia* leaf extract using a colorimetric aluminum chloride assay [22]. The extract was prepared in methanol at a concentration of 1 mg/ml, and the QE stock solution was prepared in methanol at a concentration of 1 mg/ml. Furthermore, six working concentrations of QE (ranging from 15.625 to 500 μg/ml) were used to create the standard curve. The optical density of the samples was measured at 415 nm using methanol as a blank, and the total amount of flavonoids in the extract sample was calculated using the calibration curve of QE by applying the formula:

$$C = \frac{(c \text{ x } V)}{m}, \tag{2}$$

where C is the total amount of flavonoid components that are expressed as mg of QE/g of the *Stevia* extract, c is the amount of QE that was obtained using the calibration curve (measured in mg/ml), V is the extract's volume (measured in ml), and m is the weight of the tested extract (measured in g). All measurements were carried out in triplicate, and the results were given as the average of three separate trials.

2.5. Analyzing SRHME's Biological Activity

2.5.1. In Vitro Analysis of the Extract's Antioxidant Capacity

(1) Radical Scavenging Ability for DPPH. A colorimetric approach was used to gauge the extract capability to remove DPPH free radicals [23]. Different concentrations of the extract were prepared in methanol (12.5–400 μg/ml), and various concentrations of ascorbic acid (positive control and standard solution) were prepared in methanol (12.5–400 μg/ml). Then, DPPH solution was added to each concentration of the extract and ascorbic acid. The absorbance at 517 nm was recorded, and the radical scavenging capacity (RSC) has been determined utilizing the formula:

$$\%\text{ DPPH RSC} = \left[\frac{(\text{Abs. of control} - \text{Abs. of sample or standa rd})}{\text{Abs. of control}}\right] \times 100, \tag{3}$$

where the control solution comprises all the components excluding the sample or ascorbic acid. Using a relationship between % DPPH and several concentrations of the extract, an IC_{50} value was calculated (the amount of the extract (in μg/ml) necessary to scavenge 50% of DPPH radicals). All data were conducted in triplicate, and the outcomes were presented as the mean of three separate investigations.

(2) Superoxide Radical Scavenging. The antioxidant potential of the obtained SRHME for removing superoxide radical was detected according to a previously described approach [24]. Various concentrations (12.5–400 μg/ml) of the extract and the ascorbic acid (positive control and standard solution) were prepared. The intensity of the amount of formazan produced was measured at 562 nm, and the capacity of the *Stevia* extract to remove the superoxide radical was calculated using the following equation:

$$\text{superoxide RSC \%} = \left[\frac{(\text{absorbance of control} - \text{absorbance of sample or standa rd})}{\text{absorbance of control}}\right] \times 100. \tag{4}$$

The control mixture contains all the components excluding the sample or reference ascorbic acid. Using a relationship between % superoxide RSC and several concentrations of the extract, an IC_{50} value was calculated (the amount of the extract (in μg/ml) necessary to scavenge 50% of superoxide radicals). All assessments were conducted in triplicate, and the results were shown as the mean of three independent trials.

2.5.2. Evaluation the Oxidative Damage of Human Erythrocytes. Production of peroxyl radicals in human erythrocytes [25], as a result of breakdown of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, a free radical generator), was used for determination of the preventive impact of the *Stevia* extract (SRHME) on cellular oxidative damage.

Blood was drawn from healthy volunteers, and then, after centrifuging at 5000 rpm for 10 min, the obtained erythrocytes were washed three times with phosphate buffer saline (PBS). Then, the final cell suspension (which had a 5% hematocrit) was kept at 4°C for use in the following tests.

(1) Hemolysis Test. Hemolysis is a sign of free radical damage to the red blood cell (RBC) membrane that could be neutralized by antioxidants. The tested extract's antihemolytic capability was analyzed using a modified version of the earlier spectrophotometric approach [26]. Cells (1% hematocrit) were incubated with different concentrations of the extract (50–400 μg/ml) over 30 min at 37°C. After centrifugation, the resulting erythrocytes were next incubated for 6 h with AAPH, and then, the obtained supernatant after centrifugation was used to determine the level of hemolysis by measuring the

absorbance of liberated hemoglobin from the cell at 540 nm in the tested extract and control sample (treated cells with AAPH only). As a reference antioxidant material, ascorbic acid was used. Lastly, the percentage of antihemolysis (% inhibition of hemolysis) was calculated using the following equation:

$$\%\ \text{of hemolysis inhibition} = \left[A_C - \frac{A_{\text{sample}}}{A_C}\right] \text{x } 100, \quad (5)$$

where A_C is a symbol for the control's absorbance (treated cells just with AAPH, no extract), and A_{sample} is the tested extract's absorbance. Furthermore, in order to compare the tested extract's IC_{50} concentration to that of ascorbic acid, the concentration needed to block hemolysis by 50% (IC_{50}) was computed.

In order to determine if and how much RBC oxidative damage was caused by the extract, the extract's hemolytic activity was assessed. The greatest concentration of the extract (400 μg/ml) was employed for this investigation, and the proportion of hemolysis was calculated using the following formula:

$$\%\ \text{hemolysis} = \frac{(A\ (\text{extract}) - A\ (-\text{sample}))}{(A\ (+\text{sample}) - \text{A}\ (-\text{sample}))}, \quad (6)$$

where A (+sample) is the optical density of the positive control (treated erythrocytes with AAPH only), A (−sample) is the optical density of the negative control (erythrocyte solution in PBS only), and A (extract) is the optical density of the erythrocyte containing the extract only. All values were measured in triplicate, and the outcomes are indeed the average of three attempts.

(2) Lipid Peroxidation Inhibition Using the TBARS Test. Lipid peroxidation is the term for the oxidative destruction of lipids through the generation of free radicals. The tested extract's ability to prevent lipid peroxidation was evaluated using the TBARS assay [27]. In this assay, lipid peroxidation products, primarily malondialdehyde (MDA), react with thiobarbituric acid (TBA) in the presence of trichloracetic acid to produce TBARS. In a nutshell, the treated erythrocytes with PBS (control) and the treated erythrocytes with various doses of the extract (25 to 250 μg/ml) were incubated with AAPH for 3 h and centrifuged after being exposed to TCA, and the obtained supernatant was incubated for 15 min at 100°C. Finally, the absorbance of the produced color was quantified at 535 nm, and the effectiveness of the *Stevia* extract or ascorbic acid (standard antioxidant) on inhibition of TBARS generation was calculated using the formula:

$$\%\ \text{of TBARS inhibition} = \left[1 - \left(\frac{\text{AT}}{\text{AC}}\right)\right] \times 100, \quad (7)$$

where AT denotes the optical density of the tested extract and AC denotes the optical density of the control (without extract). All values were assessed three times, and the findings represent the average of three trials.

2.5.3. Anticancer Properties

(1) Preparation and Maintenance of Cell Lines. SKOV3 cells, a human ovarian cancer cell line, and WI-38, a normal embryonic lung cell line, were both purchased by the American Type Culture Collection. These cells were grown in MEM, Minimum Essential Medium Eagle, augmented with fetal bovine serum and a concoction of two antibiotics (streptomycin and penicillin), and preserved at 37°C in a CO_2-filled incubator. After being trypsinized to obtain monolayer cells, they were subcultured in culture flasks for maintenance and usage in the ensuing assays.

(2) MTT-Based Cytotoxicity Assay. The toxicity of the tested SRHME toward SKOV3 cancer cells was assessed using the MTT assay, as described earlier [28, 29]. In a nutshell, the SKOV3 cells were exposed to various doses of the extract (12.5–100 μg/ml) over a period of 24 h at 37°C. Subsequently, an inverted microscope (Leica, DMI3000B) was used to check any visual toxicity signs, including granulation, cell rounding, partial or complete monolayer disruption, and shrinkage in both control and treated cells. The cells were then exposed to MTT solution to allow MTT degradation into formazan (MTT metabolic product), which is exactly proportional to the number of intact cells. Furthermore, the cells were treated with dimethyl sulfoxide in order to extract the formazan crystal. The optical density at 570 nm was recorded using a microplate reader (Bio-Rad, USA), and the percentage of SKOV3 cell viability was calculated to express the impact of the *Stevia* extract on cell proliferation using the following formula [30]:

$$\text{cell viability } \% = \frac{(\text{Abs. of tested sample} - \text{Abs. of blank})}{(\text{Abs. of control} - \text{Abs. of blank})} \text{ x } 100. \quad (8)$$

A dosage responsive curve was used to determine the required dose of the examined extract (IC_{50}) for 50% viability suppression over a 24 h incubation period.

Additionally, the influence of the extract on healthy embryonic lung cell (WI-38) viability was examined utilizing all the steps of the MTT technique at concentrations between 31 and 500 μg/ml of the extract in order to ascertain whether the *Stevia* extract was safe to use on living tissues or not. All values were evaluated three times, and the findings show the average of three attempts.

(3) Leakage Test for Lactate Dehydrogenase Detection (LDH). The cytotoxic influence of the SRHME against SKOV3 cells was further detected using a common lactate dehydrogenase (LDH) release assay [31], where spectrophotometric measurements of the LDH enzyme activities in the culture medium and cellular lysates of cells treated with the tested extract were made at 340 nm. The proportion of LDH leaking was evaluated using the following equation:

$$\%\ \text{of LDH activity} = \frac{\text{the supernatant activity of LDH}}{\text{the total activity of LDH}} \text{ x } 100, \quad (9)$$

where the sum of the LDH activities in the cell lysate and supernatant is expressed as the total LDH activity. All data were tested three times, and the results reflect the mean of the three trials.

(4) Annexin V/PI Analysis for Apoptosis Gauging. The annexin V/PI assay relies on phosphatidylserine (PS) migrating from the core of the plasma membrane to the outer surface of apoptotic cells. As a result, the proportions of cells that undergo apoptosis and necrosis in both the extract-treated and untreated SKOV3 cells for 24 h were evaluated using aforementioned Annexin V-FITC detection kit I (BD Biosciences), as before stated [32]. In a nutshell, the SKOV3 cells were exposed to the extract for only 24 h. Then, the extract-treated and untreated SKOV3 cells were trypsinized, centrifuged, washed with PBS, and then combined with the Annexin V binding buffer (1x) before being exposed to Annexin V-FITC and propidium iodide dyes. Lastly, employing a flow cytometer (BD Biosciences), the findings are depicted as the percentage of apoptosis for both early and late apoptotic cells.

(5) Fluorescence Microscopy-Based Morphological Study. The morphological apoptotic nuclear alterations for both the control and extract-treated SKOV3 cells were studied using the acridine orange/ethidium bromide stain (AO/EB) procedure [33]. In a nutshell, after treatment of SKOV3 cells with the *Stevia* extract, the cells were washed with PBS, fixed in formaldehyde for roughly 20 min, and stained with AO/EB stain for 2 min. Lastly, the nuclear morphologies of both the control and treated SKOV3 cells were scrutinized employing fluorescence microscopy (Carl Zeiss), and the proportions of cell death were reported. All values were examined three times, and the results reflect the average of the three evaluations.

In order to test the influence of the *Stevia* extract on the nuclear morphology of healthy tissues and ascertain whether the safety of the extract on living tissues has been compromised or not, all these steps of the AO/EB assay were also repeated in the presence of healthy WI-38 cells rather than SKOV3 cells.

(6) In Vitro Comet Assay (DNA Fragmentation Test). A comet test was used for examining the induction of genotoxicity in the *Stevia*extract-treated cells. With fragmentation, defective DNA is pulled to an electric field, and strand breaks will separate from healthy cellular DNA to form a structure resembling a comet tail that can be seen under a fluorescent microscope. This assay entails numerous processes, many of which have already been discussed [32], where 100 photos of randomly shaped comets were acquired for each slide utilizing a computerized image analysis system, which examined these images and determined the comet parameters using TriTek Comet Score™ software (TriTek Corp., VA, USA). Two widely used indicators, the tail DNA and tail moment, were constructed to be used in finding the data. The Olive tail moment (OTM), also referred to as a tail moment parameter, is thought to be the best measurement that has been employed to quantify DNA damage. It relies on DNA movement and DNA quantity in the tail, and its value was calculated using the following equation:

$$\text{OTM} = \text{quantity of tail moment} \times \text{quantity of tail DNA di vide d by 100.} \tag{10}$$

In order to test the extract's genotoxicity against healthy tissues, all these phases of the comet assay were performed in the presence of healthy WI-38 cells rather than SKOV3 cells.

(7) Cell Migration (Wound Healing) Assay. Malignant tumors can invade nearby healthy tissues, proliferate uncontrollably, followed by circulatory system distribution throughout the body. So using the wound healing assay [34], it was determined how the *Stevia* extract affected the ability of ovarian cancer cells to migrate. In brief, SKVO3 cells were grown in a well plate at 37°C in an incubator until they reached 90% confluence as a monolayer. A pipette tip was then used to create an artificial scratch through the well that was parallel to the cell layers. At 0 h, the scratch was imaged using a light microscope following three phosphate buffer saline washes with the floating cells. The scratched cells were then cultured in fresh media containing the *Stevia* extract, and the control group of untreated scratched cells was incubated in new media containing sterile saline. After a 24 h incubation period, the migration of both the control and treated scraped cells into the wound was once more photographed. Finally, the ImageJ program was used to calculate the migration rate of the examined cells based on each cell's scratch width at 0 and 24 h, using the following formula:

$$\text{cell migration ratio \%} = \frac{(\text{average scratch area at 0 h} - \text{average scratch area at time t})}{\text{average scratch area at 0 h}} \times 100. \tag{11}$$

(8) In Vitro Cell Invasion Test. Using a transwell chamber, the ability of cells to invade was tested as described previously [35]. Using 100 μl of diluted Matrigel (BD Biosciences, USA), the upper chamber of the transwell chamber was covered dropwise into each well of the 24-well plate and solidified in the incubator for 2 h. At the end of incubation, SKVO3 cells at a density of 5×10^4 cells in the serum-free DMEM medium containing the extract were cultured per well in an upper chamber, whereas the medium containing 10% fetal bovine serum was placed in the bottom chamber,

which acted as a chemoattractant, and then, the cells were incubated for 24 h. The cells that had intruded through the lower membrane surface were then fixed with methanol for 20 min at room temperature after the remaining cells on the upper membrane surface had been removed using a cotton swab. The cells were then rinsed with PBS after the fixative was removed, stained for 20 min with crystal violet solution, and then viewed under a light microscope. The tagged cells were then treated with acetic acid to remove their color, and an ELISA reader was used to detect the absorbance of the resultant mixture at 540 nm.

(9) Examining the Variant Phases of the Cell Cycle. Cell division phases were identified using a cell cycle test, which involves usage of DNA content of the tested cells. Using a flow cytometry kit (Abcam, catalog ab139418) as described before [29, 32], the percentages of the untreated and extract-treated cells through the various stages of the cell cycle were assessed. In a 6-well culture plate, SKOV3 cells were placed, incubated overnight, and then cultured for 24 h. Following that, these cells were exposed to the extract for 24 h. The cells were then collected, trypsinized, and preserved overnight in cold ethanol. These cells were then exposed to propidium iodide mixture for 30 min in the dark at 37°C. The final step was estimating the proportion of cells for each cell cycle phase using DNA measurement employing flow cytometry. The results were shown in the form of a histogram.

(10) ELISA Method for Quantifying Reactive Oxygen Species (ROS). The effectiveness of the tested *Stevia* extract on ROS production within SKOV3-treated cells was assessed via a human ROS ELISA kit (MyBioSource, USA) in accordance with the manufacturer's recommendations. In a nutshell, the control and *Stevia* extract-treated SKOV3 cells were trypsinized and centrifuged, and the resulting supernatant was added to the appropriate wells in a 96-well microplate reader that had already been coated with an anti-ROS antibody. After that, about 100 μl of a working solution containing biotinylated recognition Ab (1x) was added to the tested plate. Once the wells were cleaned, 100 μl of the conjugate avidin-horseradish peroxidase (1x) was poured and left for 30 min at 37°C. In addition, once it was washed five times, 90 μl of the substrate had been poured into the wells of the plate for fifteen min in the darkness. In order to generate a blue-colored complex (enzyme-substrate reaction), the assayed plate was carefully cleaned and dried, and about 90 μl of the substrate was applied to every well for fifteen min at 37°C. Then, each well got 50 μl of stop solution in the end, producing the yellow-colored solution. At 450 nm, the absorbance of yellow color has been recorded using a microplate reader. Based on the standard curve, which displays standard dosages versus the intensity of the color, the ROS level in the extract-treated cells was obtained. All values were assessed three times, and the outcomes reflect the average of the three trials.

(11) Quantitative Real-Time PCR Technique (qRT-PCR). To investigate the possible mechanisms that are related to the influence of the *Stevia* extract on cell cycle arrest, apoptosis, and metastasis (migration and invasion) within the treated SKVO3 cancer cells, the mRNA expression levels of the following genes were checked: cell cycle-related genes (*P*21, *CDK*2, and *cyclin A*2), apoptosis-associated genes (*BCL*-2, *BAX*, and *caspase*-3), and antimetastatic-connected genes (migration and invasion-related genes, *MMP*-2 and *MMP*-9). In a nutshell, Thermo Fisher Scientific's RNA purification kit (catalog #K0731) was applied in accordance with the manufacturer's specification guidance to separate the total RNA from both the untreated and *Stevia*-treated SKOV3 cells. Following that, using a spectrophotometer to quantify the ratio of A260/A280, the amounts of the extracted RNA for all tested samples were determined. First-strand cDNA was then created from the obtained RNA samples using the Thermo Fisher Scientific RevertAid cDNA synthesis kit (catalog #K1621) in accordance with the manufacturer's guidelines. The next step was real-time PCR amplification employing a Thermo Fisher Scientific Maxima SYBR Green qPCR kit (catalog #K0221) through an Applied Biosystems StepOnePlusTM device employing particular primers (Invitrogen) for the investigated genes, as stipulated in Table 1. Then, using a comparative threshold cycle approach, the relative quantification (RQ) of the used genes was computed, compared to their differential expression in the untreated ones, and standardized to a housekeeping gene called *GAPDH*. Every experiment was performed in triplicate, and the results were averaged over three separate experiments.

(12) ELISA Testing. The protein expression levels of the investigated genes were measured in *Stevia*extract-treated SKVO3 cells using an ELISA method.

The ELISA method was employed using BAX ELISA kit (ab199080), cleaved caspase-3 ELISA kit (ab220655), BCL-2 ELISA kit (ab272102), P21 ELISA kit (ab214658), MMP-2 ELISA kit (ab267813), MMP-9 ELISA kit (ab246539), and GAPDH ELISA kit (ab176642), which were supplied via Abcam (Cambridge, UK). All of these kits were established on the high sensitivity of the single 90-minute sandwich enzyme assay, which employs trapping antibodies coupled to an affinity tag that is also recognized with an anti-tag antibody that has been precoated on the testing ELISA plate. After centrifuging the cultured SKVO3 cells containing the tested extract, the resulted pellets were thrown away, and the remaining supernatant was added to a 96-well ELISA plate with an equal amount of an antibody cocktail solution, followed by incubation for 1 h at ambient temperature. A tetramethylbenzidine substrate solution was applied to every well for ten min only after the plate had been properly rinsed in wash buffer (1x). The plate was then preserved in a dark place. With the aid of an automatic microplate reader, the intensity at 450 nm was then quantified just after the addition of a stop solution.

Similarly, the CDK2 ELISA kit (catalog no: MBS2020144) and cyclin *A*2 ELISA kit (catalog no: MBS7237496) were supplied by MyBioSource (USA) for measuring the protein expression levels of CDK2 and cyclin *A*2, respectively, as follows: These kits implemented sandwich ELISA double-antibody methodology,

Table 1: The primer sequences of the studied genes with their GenBank accession numbers.

Gene	Forward primer	Reverse primer	Accession number
Cyclin A2	TGGAAAGCAAACAGTAAACAGCC	GGGCATCTTCACGCTCTATTT	NM_001237.5
CDK2	CCAGGAGTTACTTCTATGCCTGA	TTCATCCAGGGGAGGTACAAC	NM_052827.4
P21	CGATGGAACTTCGACTTTGTCA	GCACAAGGGTACAAGACAGTG	NM_001374511.1
MMP-9	CGCCAGTCCACCCTTGTG	CAGCTGCCTGTCGGTGAGA	NM_004994.3
MMP-2	CGTCTGTCCCAGGATGACATC	ATGTCAGGAGAGGCCCCATA	NM_001302510.2
BCL-2	CATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTACTCAGTCA	NM_000633.3
BAX	GATCCAGGATCGAGCAGA	AAGTAGAAGAGGGCAACCAC	NM_001291428.2
Caspase-3	CAGAACTGGACTGTGGCATTGAG	GGATGAACCAGGAGCCATCCT	NM_004346.4
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC	NM_001357943.2

including 96-well plates that have already been covered with anti-human cyclin A2 and anti-human CDK2. The wells were then wiped with washing buffer after inserting the standard, test sample (supernatant of SKVO3 cells treated with the extract) and detecting the antibody containing biotin-conjugated. The wells were next incubated with horseradish peroxidase streptavidin, and afterwards, the HRP substrate was put on them to elicit the formation of a blue color, which then transformed into yellow after halting the reaction with the application of an acidic solution. Consequently, the amount of CDK1 and cyclin-*A2* proteins in the tested samples that were recovered in testing plates is directly correlated with the intensity of the yellow color. All measurements were tested three times, and the findings reflect the average of the three evaluations.

2.6. Statistical Analysis. All assays were conducted in triplicate, and the outcomes were presented as the mean ± SD. One-way ANOVA was employed to check the variations among means. SPSS 17.0 software was utilized for all statistical analyses. Levels of $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ were considered statistically significant.

3. Results and Discussion

3.1. Discovering Phytochemical Components Using GC-MS. The total ion chromatogram of the studied SRHME revealed the existence of 29 distinct physiologically active compounds with varying retention times (Figure 1). The biological activity of these components, along with their percentages of the peak area and retention time, is shown in Table 2. Therefore, the existence of these components may be ascribed to the current findings about the antioxidant and anticancer effectiveness of the examined *Stevia* extract (SRHME).

3.2. Estimation of Total Phenolic and Flavonoids. The primary antioxidants in medicinal herbs are phenolic and flavonoid compounds. Many of these plant secondary metabolites are recognized to be physiologically active substances and to have a variety of pharmacological activities such as anticancer, antifungal, antimicrobial, and antioxidant and may help in the prevention of chronic diseases [64]. *Stevia* leaf extracts' polyphenols and flavonoids can shield cells from oxidative damage, lowering the possibility that oxidative stress would result in the growth of tumors [17].

The current obtained findings showed that, based on the standard calibration plots for GAE and QE, respectively, the total phenolic and flavonoid content in the studied *Stevia* extract was 115 ± 2.8 mg of GAE and 76.8 ± 1.8 mg of QE per g of the extract, respectively.

Due to their redox characteristics, phenolic compounds can scavenge singlet oxygen, free radicals, and superoxide radicals [65]. This radical scavenging action is attributable to hydroxyl groups substituting the aromatic ring in polyphenolic compounds [66]. Plant secondary metabolites called flavonoids, such as flavones, flavanols, and condensed tannins, have antioxidant properties that rely on the existence of free OH groups, particularly 3-OH. Additionally, earlier research demonstrated that plant flavonoids serve as antioxidants both *in vivo* and *in vitro* [67, 68]. Additionally, previous research showed that the methanol extract of *Stevia* leaves had significant amounts of phenols and flavonoids [69].

3.3. Antioxidant Effects. One of the most commonly used techniques for evaluating the antioxidant potential of plant samples is the free radical DPPH inhibition analysis [70]. Likewise, one of the strongest reactive oxygen species among free radicals is superoxide anion that causes the production of singlet oxygen and toxic hydroxyl radicals, which both promote harmful oxidative stress to cellular components [71, 72]. As a result, in the current work, the *Stevia* extract's antioxidant activity was assessed in terms of its capacity to scavenge DPPH and superoxide radicals. The examined extract's free radical DPPH and superoxide anion scavenging potential (Figure S1) was assessed at various concentrations (12.5–400 μg/ml) in comparison to the standard ascorbic acid. At a concentration of 12.5 μg/ml, the extract's scavenging activities % for DPPH and superoxide radicals were 41.4 ± 2.8 μg/ml and 30.4 ± 1.6 μg/ml, respectively, while at a concentration of 400 μg/ml, the activities were 96.5 ± 2.5 μg/ml and 95.6 ± 2.3 μg/ml, respectively. Additionally, Table 3 shows the extract's scavenging activity for DPPH and superoxide radicals in comparison to the reference ascorbic acid in terms of IC_{50}, where the extract's scavenging activity outperformed that of the reference ascorbic acid. According to a prior study [73], the methanolic leaf extract of *S. rebaudiana* also demonstrated a higher substantial antioxidant capacity.

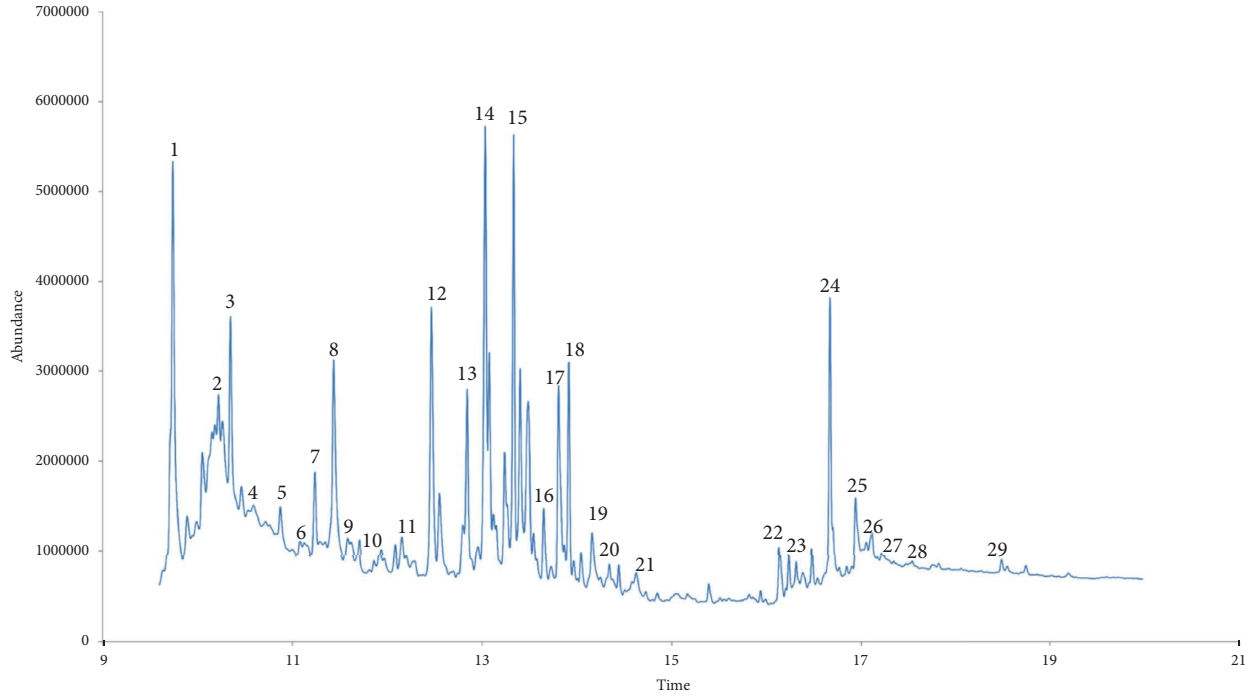

Figure 1: GC-MS chromatogram of the methanol extract of *Stevia* leaves (SRHME) for measurement of phytochemicals. Table 2 contains a list of all compounds associated with the numbers depicted in this figure.

Recently, the antioxidant activities of *S. rebaudiana* have been linked to flavonoid and phenolic substances [74]. The ability of most phenolic compounds, including phenolic acids, to scavenge free radicals relies on the presence of methoxy (-OCH3) and hydroxyl (-OH) groups in their structures [75]. Besides, according to the earlier findings [76], flavonoids are the most efficient antioxidants because they can effectively scavenge superoxide anions. Additionally, the majority of oxidizing substances, such as singlet oxygen and other free radicals linked to a number of illnesses, are effectively destroyed by flavonoids [77]. Moreover, flavonoids upregulate and protect antioxidant defenses by scavenging reactive species, chelating trace elements involved in the creation of free radicals, and suppressing reactive oxygen generation [78]. Similar to this, phenolic provide plants higher tolerance for oxidative stress. Consequently, the antioxidant activity that was found in the tested *Stevia* extract could be linked to the significant phenolic and flavonoid content. Therefore, the use of crude extracts of phenolic-rich fruits, herbs, vegetables, cereals, and other plant components in food is increasing as a result of their antioxidant qualities and health advantages [79].

3.4. Protective Activity toward Cellular Oxidative Damage

3.4.1. Antihemolytic Effectiveness. The circumstances of oxidative stress were replicated through adding AAPH to extracted human red blood cells in order to verify the antihemolytic effectiveness of the studied SRHME, where both free radicals created by redox processes and high quantities of polyunsaturated fatty acids were assumed to be the main targets of erythrocytes [80].

This contact between the polyunsaturated fatty acids of the RBC membrane and free radicals that are generated by AAPH results in membrane rupture and hemolysis, which are identified by a steady decline in optical density at 450 nm [78]. So the obtained findings reveled that SRHME had significant antioxidant capacity that shielded red blood cells from hemolysis. As presented in Figure S2, the outcomes exhibited that the extract significantly increased inhibition of hemolysis in a concentration-dependent mode, where the extract had a greater antihemolytic potency than that of the standard antioxidant ascorbic acid. Moreover, the calculated IC_{50} value of the examined extract (25 μg/ml), following 5 h of incubation that had been deduced from the dose-response curve, was more efficient than the IC_{50} value of ascorbic acid (27.2 μg/ml).

Multiple studies have emphasized the use of polyphenols and medicinal plants to protect against oxidative stress damage, particularly in red blood cells [81], where polyphenols may inhibit hemolysis and thus increase red blood cell half-life [82]. Polyphenols' protective properties, on the one hand, are based on their ability to boost cellular antioxidant defense by activating the Nrf2 cascade or by activating enzymatic activity such as superoxide dismutase, catalase, and glutathione peroxidase [83]. A protective impact against oxidative stress is provided by polyphenols, on the other hand, because of their ability to act as free radical scavengers and electron donors [84]. As a result, the obtained findings point to a strong relationship between the studied *Stevia* extract high phenolic content and its antihemolytic efficiency. Additionally, a hemolysis test for the effect of the extract against erythrocytes in the absence of AAPH revealed that

Table 2: Bioactive compounds identified in the methanol extract of *Stevia* leaves (SRHME) using GC-MS analysis.

Peak no	Retention time (RT)	Area %	Name of the compound	Biological activity
1	9.7414	5.7629	Butanoic acid, 2-methyl-3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	Antioxidant and anti-inflammatory activity [36]
2	10.2163	13.0684	Isoquinoline,1-[(3,5-dihydroxy)benzyl]-N-formyl-1,2,3,4,5,6-hexahydro-	Anticancer [37]
3	10.3422	5.8358	Hexasiloxane, tetradecamethyl-	Antimicrobial [38]
4	10.5825	5.273	Phenanthrene, 4-methyl-	Antitumor and antimicrobial [39]
5	10.8686	2.6019	Pyrido[3,2-d]pyrimidine-2,4(1H,3H)-dione, 1,3-dimethyl-	Antitumor [40]
6	11.0917	1.4064	n-Tridecanoic acid, pentamethyldisilanyl ester	Antimicrobial activities and anticancerous activity [41]
7	11.2291	2.4031	2,5-Dihydroxybenzoic acid, 3TMS derivative	Antipyretic analgesic, antirheumatism, and antimicrobial activity [42]
8	11.4293	4.0799	Salicylic acid, 2TMS derivative	Antioxidant activity [43]
9	11.5895	2.0524	Acetic acid, 2-[2 [(trimethylsilyl)oxy]ethoxy]-, trimethylsilyl ester	Antibacterial [44]
10	11.9271	1.4945	Levulinic acid, TMS derivative	Antioxidant and anti-inflammatory activities [45]
11	12.1617	2.4581	Pentanedioic acid, 2TMS derivative	Anti-inflammatory [46]
12	12.4707	5.4965	Quinoline	Anticancer activity [47]
13	12.8427	3.1798	Erythritol, 4TMS derivative	Sweet antioxidant [48]
14	13.0372	9.2649	Glutarimide, N-(2-ethylphenyl)-	Anticancer and antibacterial [49]
15	13.3348	12.8264	Quininic acid (5TMS)	Antioxidant, antimutagenic, and anti-inflammatory [50]
16	13.6552	1.427	D-(+)-Glucuronic acid gamma-lactone, tris(trimethylsilyl) ether, methyloxime	Antibacterial [51]
17	13.8154	3.0038	Myo-inositol, 6TMS derivative	Anticancer effects [52, 53]
18	13.9184	2.9602	D-Glucose, 5TMS derivative	Antibacterial [54]
19	14.1702	1.4725	Palmitic acid, TMS derivative	Anticancer and antioxidant [55]
20	14.3418	1.1128	2-Oxopentanoic acid, TMS derivative	Antibacterial [56]
21	14.6222	1.8005	Caffeic acid, 3TMS derivative	Antioxidant and anticarcinogenic [57]
22	16.1442	0.6277	D-(+)-Xylose, 4TMS derivative	Antibacterial [54]
23	16.2358	1.4261	Thymol-beta-d-glucopyranoside, tetrakis(O-trimethylsilyl)-	Antibacterial [58]
24	16.6764	4.2388	6-Hydroxyflavone-beta-D-glucoside, tetra(trimethylsilyl)-	Anticancer and antioxidant [59]
25	16.951	2.08	Maltose, 8TMS derivative, isomer 2	Antibacterial [54]
26	17.1112	1.6449	1H-Indole-2,3-dione,1-(tert butyldimethylsilyl)-5-ethyl-, 3-[O-(tert-butyldimethylsilyl)oxime	Antioxidant activity and anticancer activity [60]
27	17.2257	1.1946	Levoglucosan, 3TMS derivative	Anticancer and antitumor activity [61]
28	17.5289	0.4946	Azelaic acid, 2TBDMS derivative	Antibacterial agents [62]
29	18.496	0.2325	9-Tetradecen-1-ol, (E)-, TMS derivative	Antifungal and antibacterial [63]

Table 3: *In vitro* antioxidant activity of *Stevia* extract using different antioxidant methods.

Antioxidant tests	IC_{50}(μg/ml)	
	Stevia extract	Ascorbic acid
DPPH assay	31.5	59
Superoxide radical assay	70.1	98.2

the extract under investigation had a 6.8% hemolytic activity against the utilized erythrocytes, confirming the biocompatibility of the extract against living tissues.

3.4.2. Influence of the Stevia Extract on TBARS Development. The impact of the studied SRHME on the TBARS generation in erythrocytes exposed to AAPH compared to vitamin C is displayed in Figure S3. With an increasing concentration of the investigated extract (25–250 μg/ml), the inhibition % of TBARS generation increased significantly, reaching 95 ± 1.3 μg/ml and 38.8 ± 2.2 μg/ml at 250 μg/ml and 25 μg/ml, respectively. Interestingly, the dose-response curve demonstrated that the IC_{50} value of the investigated extract (25.3 μg/ml) was more potent than that of vitamin C (47.3 μg/ml). Polyphenols, as previously established, protect against lipid peroxidation and cell membrane damage caused by oxidative stress [85, 86]. Consequently, the obtained results indicated that the higher polyphenol content of the tested extract serves as a more effective lipid peroxidation inhibitor.

3.5. Efficiency of SRHME on Proliferation of SKOV3 Cells

3.5.1. MTT and LDH Cytotoxicity Assays. A developing approach to inhibit, impede, delay, and treat malignant tumors is cancer chemoprevention utilizing natural phytochemical substances [87]. Thus, the harmful impact of the studied SRHME on the proliferation of SKOV3 cells was established utilizing MTT and LDH measurements. The outcomes demonstrated that the percentage of viable SKOV3 cells significantly decreased in a concentration-dependent pattern after 24 h of exposure to the examined extract using the MTT test (Figure 2(a)). This finding may emphasize the extract's potential anticancer capabilities, which are indicated by its lower IC_{50} dose (17.5 μg/ml) derived from the relationship between the extract dose and its effect on cell viability. In contrast to control cells, which usually possess firmly adherent cells to the surface and develop an epithelial monolayer, the extract-treated SKOV3 cells had highly morphological apoptotic variations like rounded cells, cell shrinkage, and a progressive decrease in the number of cells with continuing to increase the extract concentration (Figure 3). The IC_{50} value of the produced SRHME was stronger than the previously disclosed IC_{50} value of the standard methotrexate drug against SKOV-3 cell lines [88].

It is well established that polyphenolic substances reduce human mutagenesis and carcinogenesis [89]. Furthermore, the *Stevia* plant extract has been demonstrated to prevent several cancer cell lines from proliferating, possibly as a result of its antioxidant properties [90]. Therefore, the increased polyphenol content of the investigated extract may be responsible for its anticancer effectiveness. Additionally, *Stevia* may assist in lowering blood sugar, depriving tumor cells of a crucial source of energy. Consequently, tumor models *in vivo* and cancer cells showed cytotoxic and antiproliferative activities by *Stevia* plant derivatives [20]. The anticancer effectiveness of the methanol *Stevia* extract against MCF7 cells was previously reported, with an IC_{50} value of 228 μg/ml [91]. Phenols are recommended as key dietary components in the protection of cancer and heart disorders due to their direct participation in antioxidative function [92]. Therefore, the higher phenol content in the studied extract may be responsible for its greater anticancer potential.

The function of the LDH enzyme was monitored experimentally to provide further verification of the extract's toxicity on the survival of SKOV3 cells. The outcomes revealed that the amount of LDH released in the extract-treated SKOV3 cells increased significantly (89 ± 1.5), compared with the percentage of leakage in untreated control ones (8.6 ± 2.3).

Additionally, the MTT test for the impact of the extract against WI-38 cells revealed that the tested extract's various concentrations (31.25–500 μg/ml) did not show any harmful effect on the viability of healthy WI-38 cells. The results are shown in Figure 2(b), where the viability percentage at the maximum extract concentration (500 μg/ml) was 97.12 ± 1.7.

3.5.2. Estimation of SKOV3 Cells' Apoptosis Generation. A variety of assays, including annexin V/PI, AO/EB, and comet, were used to determine whether the tested SRHME caused the SKVO3 cells to undergo apoptosis or not.

In the beginning, flow cytometry-derived annexin V/PI for the extract-treated cells compared to untreated ones is plotted in Figure 4, where the numbers of early and late apoptotic cells increased in the extract-treated SKOV3 cells in contrast to the control cells. Metformin, as anticancer medicine, decreased SKOV3 development and accelerated apoptosis as shown by the annexin V/PI test, according to the previous study [93].

Additionally, in order to estimate the changes in nuclear morphology of apoptotic cells and calculate their percentages in SKOV3 cells treated with the *Stevia* extract, a quantitative AO/EB approach that is specifically designed for nuclear labelling of apoptotic cells was applied. As depicted in Figure 5, when compared to green viable control cells (Figure 5(a)) that did not exhibit any significant apoptosis, the extract-treated SKOV3 cells were colored green, yellow-green, and orange, indicating the presence of viable, early, and late apoptotic cells, respectively (Figure 5(b)). Besides, treated cells had a significantly higher percentage of apoptotic cells (86 ± 2.1) than control cells (10 ± 1.3). Moreover, as shown in Figures 5(c) and 5(d), the AO/EB test for the impact of the extract on normal WI-38 cells revealed that the extract-treatedWI-38 cells had a green color that was

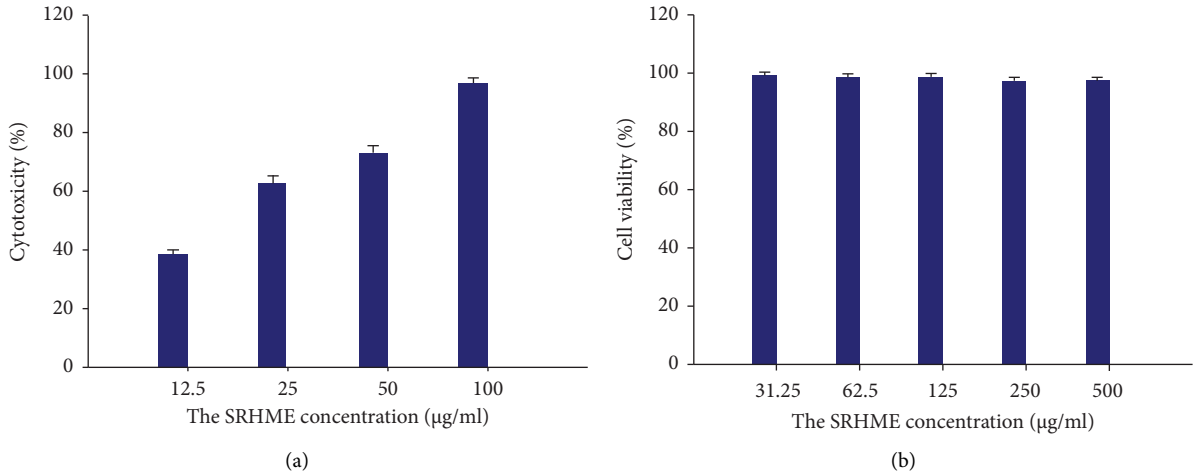

Figure 2: The correlation between the SKVO3 cytotoxicity (a) and WI-38 cells' viability (b) following treatment with various doses of *Stevia* extract (SRHME) in comparison to untreated cells.

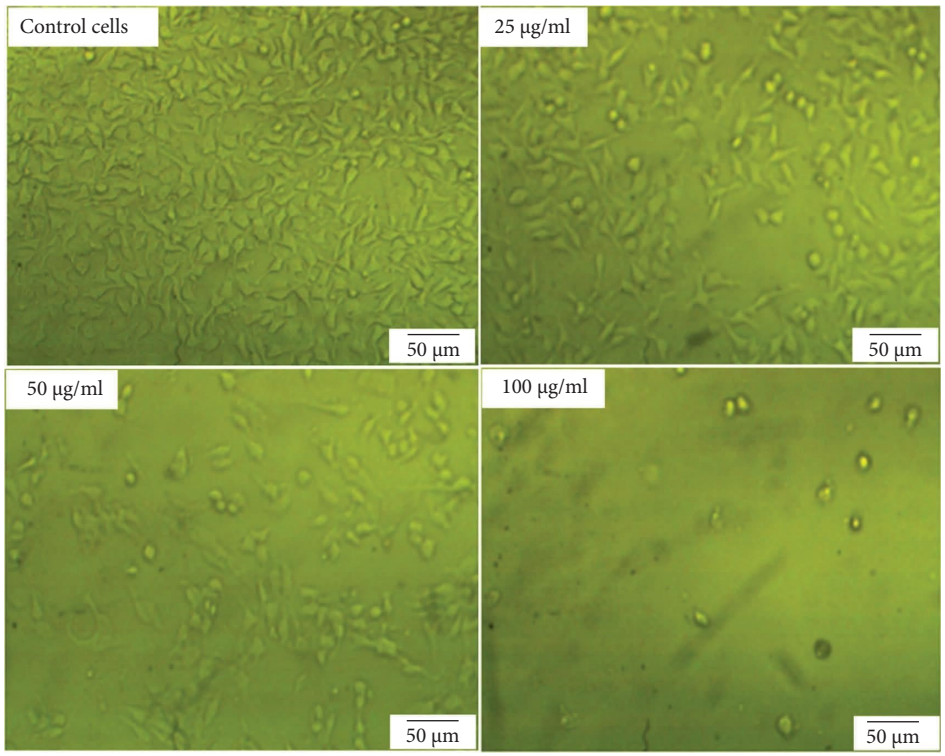

Figure 3: The morphological alterations in SKVO3 cells after 24 h treatment with various concentrations (25–100 μg/ml) of *Stevia* extract (SRHME) compared to normal control cells.

similar to the green color seen in untreated cells and lacked any signs of apoptosis. Earlier investigations suggested that methotrexate therapy increased apoptosis as judged by AO/EB check in SKOV-3 cells [88].

Comet technology is also performed to recognize DNA damage caused by the onset of apoptosis in treated cells. According to Table 4, there was a substantial difference in OTM among the tested extract-treated cells and the control ones. Moreover, the images obtained employing fluorescence microscopy (Figure 6) showed that the control cells had preserved nuclei (Figure 6(a)), while in treated cells (Figure 6(b)), a comet-like structure was observed with an increment in the tail length, suggesting that shrinkage in nuclear density might have been caused by a disproportionate buildup of DNA in the tail. Cleaved caspase-3 (activated caspase-3), as previously described, plays a crucial role in the apoptosis-mediated cell death process that results in DNA fragmentation and nuclear condensation [94]. In order to validate *caspase*-3 critical function in DNA

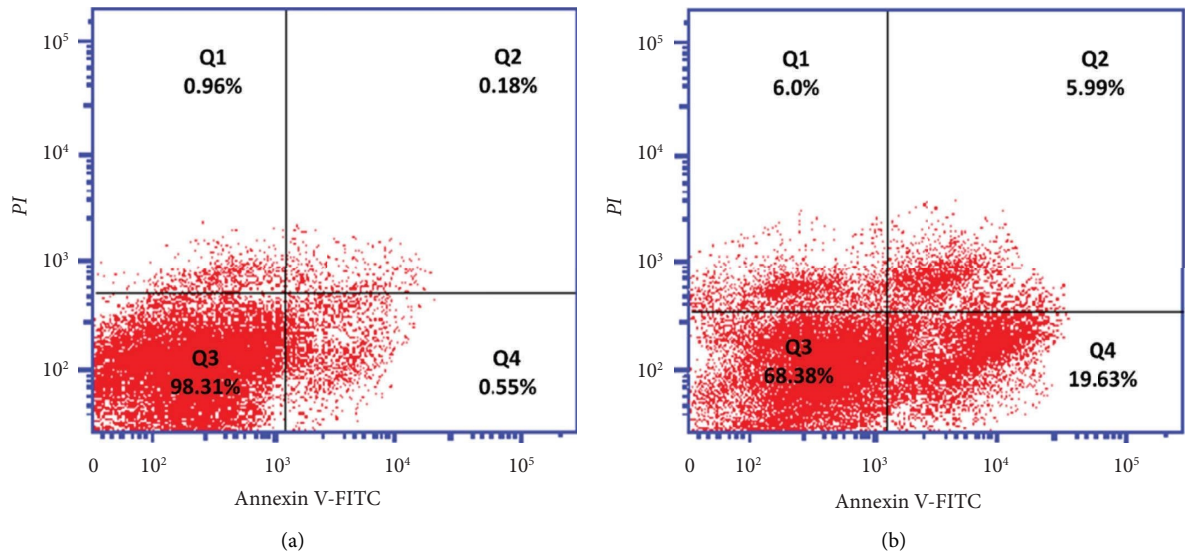

Figure 4: Using flow cytometry, the apoptotic pattern of SKVO3 cells was assessed, displaying Q1 (An^-, PI^+) % of necrotic cells, Q2 (An^+, PI^+) % of late apoptotic cells, Q3 (An^-, PI^-) % of viable cells, and Q4 (An^+, PI^-) % of early apoptotic cells for treated SKVO3 cells with an IC_{50} dose (17.5 μg/ml) of SRHME (b) in comparison to untreated SKVO3 cells (a).

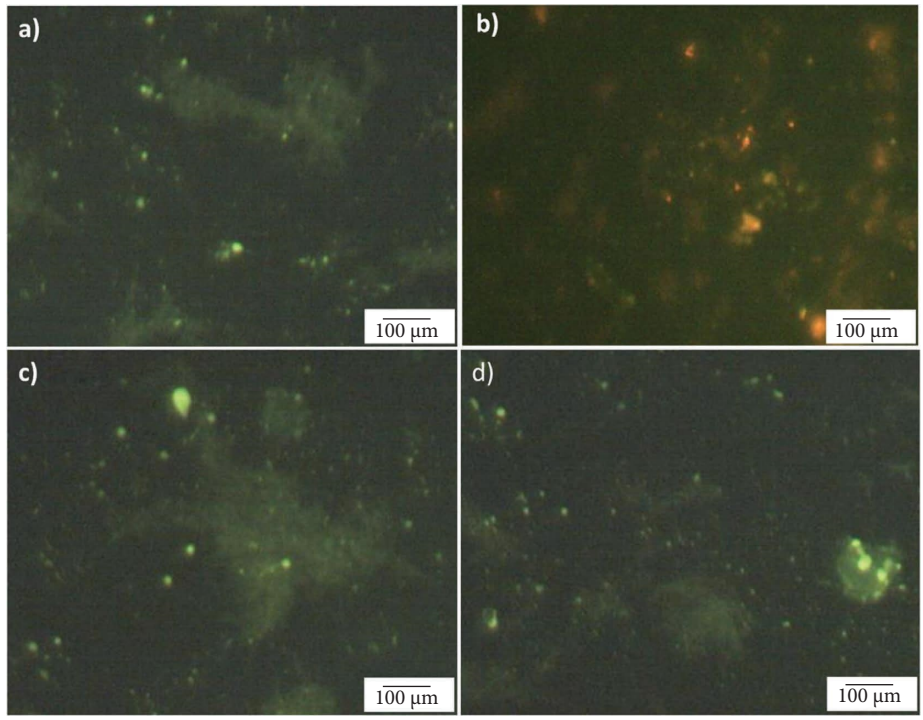

Figure 5: Using fluorescence microscopy and AO/EB labelling, the nuclear morphological alterations in treated SKVO3 cells with an IC_{50} dose (17.5 μg/ml) of SRHME (b) in comparison to untreated SKVO3 cells (a) and treated normal WI-38 cells with an IC_{50} dose (17.5 μg/ml) of SRHME (d) in comparison to untreated WI-38 cells (c) have been noticed.

fragmentation and apoptosis execution in treated SKOV3 cells, the influence of the extract on the level of *caspase*-3 expression was measured. The obtained finding exhibited that the treated cells had a high expression level of *caspase*-3 compared to that of control cells. Prior studies using Annexin V-FITC/PI flow cytometry demonstrated that steviol therapy produced an apoptotic mechanism in gastrointestinal cancer cells [95].

As well, the comet assay findings for the influence of the extract on DNA of normal WI-38 cells revealed that the OTM values of the cells treated with the tested extract and the control cells did not differ significantly from one another

Table 4: The comet assay parameters were used to compare the DNA damage between SRHME-treated SKVO3 cells and control SKVO3 cells as well as between SRHME-treatedWI-38 cells and control WI-38 cells.

The tested cells	Tail length (px)	%DNA in tail	Tail moment	Olive tail moment
Control SKVO3 cells	$5 \pm 0.2^{***}$	$5.238196 \pm 0.53^{***}$	$0.352847 \pm 0.02^{***}$	$0.562865 \pm 0.03^{***}$
Treated SKVO3 cells with the *Stevia* extract (SRHME)	$11.66667^{***} \pm 0.51$	$8.643875 \pm 0.13^{***}$	$1.664525 \pm 0.05^{***}$	$2.155456 \pm 0.09^{***}$
Control WI-38 cells	6.555556 ± 1.2	8.461906 ± 1.6	0.490939 ± 0.02	0.933713 ± 0.02
Treated WI-38 cells with the *Stevia* extract (SRHME)	7 ± 1.4	8.3806 ± 2.1	0.532099 ± 0.04	1.143319 ± 0.05

Data from three separate tests are presented as the mean ± standard deviation. $^{***}p < 0.001$.

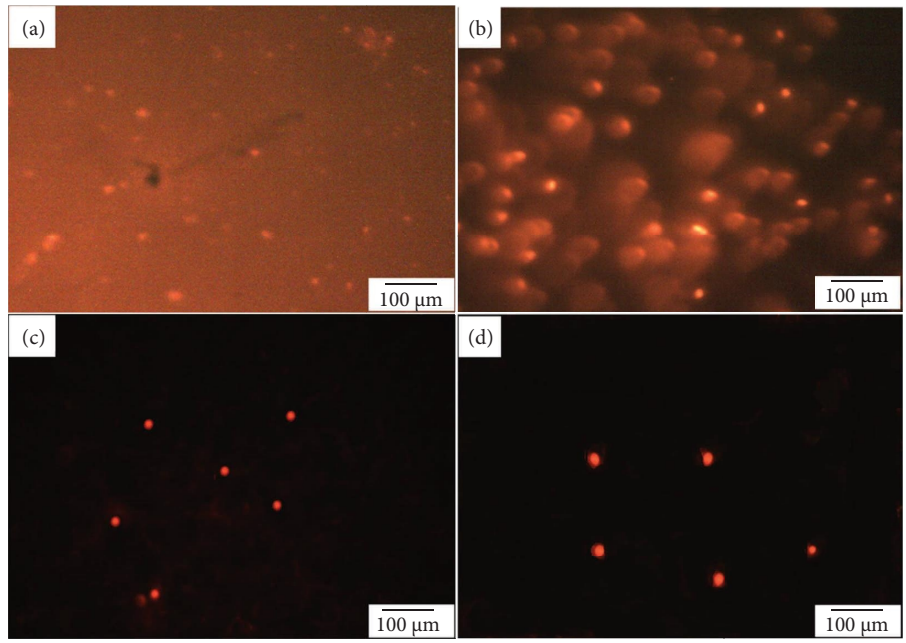

Figure 6: Fluorescence microscopy images of the comet nucleus in treated SKVO3 cells with an IC_{50} dose (17.5 μg/ml) of SRHME (b) in comparison to an intact nucleus in untreated SKVO3 cells (a) and in treated WI-38 cells with an IC_{50} dose (17.5 μg/ml) of SRHME (d) in comparison to untreated WI-38 control cells (c).

(Table 4). Additionally, the comet fluorescence result in the extract-treatedWI-38 cells revealed an undamaged intact nucleus (Figure 6(c)) without any impact on DNA, identical to the intact nucleus of control cells (Figure 6(d)), demonstrating that the extract had no genotoxic effects on healthy living tissues. Initial findings revealed a substantial increase in the production of OTM in methotrexate-treated SKOV3 cells [88].

3.5.3. Effectiveness of Stevia Extract (SRHME) in Preventing SKOV3 Cell Metastasis. There have been no prior investigations on how *S. rebaudiana* leaf extracts affect the movement of various cancer cells. Consequently, in the current inquiry, the antimetastatic potential of the produced *Stevia* extract was examined against the employed ovarian cancer cells. The primary factor in cancer-related deaths is metastasis, a complex process that is brought about by a number of interconnected processes, such as cancer cell invasion, migration, and adhesion. The growth of multiple tumors is a response of the invasion of malignant cells into target tissues [96]. Therefore, it has been hypothesized that one way to inhibit the spread of cancer is to interfere with the adherence and motility of tumor cells as well as their invasion into target organs [97].

Moreover, the majority of antimetastatic medications in use today work by causing tumors to shrink through apoptosis [98]. Tumor shrinkage, on the other hand, is not an antimetastatic mechanism and is rarely sustained [99]. As a result, there is a demand for medicines that can prevent metastasis without also inhibiting apoptosis. Therefore, in the current study, a sublethal concentration of the *Stevia* extract was chosen for wound healing migration and transwell invasion experiments to see if they might suppress the metastasis of SKVO3 cells without causing apoptosis. Thus, the movement of scratched SKVO3 cells into the wound over the course of 24 h was monitored. In contrast to the migration rate of untreated SKVO3 cells (92%), the investigated SRHME dramatically reduced the migration of SKVO3 cells into wounds to 42%, as shown in Figure 7. This result was comparable to that of the earlier investigation, which showed that prostate cancer cells' survival and migration were suppressed by the methanolic root extract of *Stevia pilosa* and *Stevia eupatoria* [100].

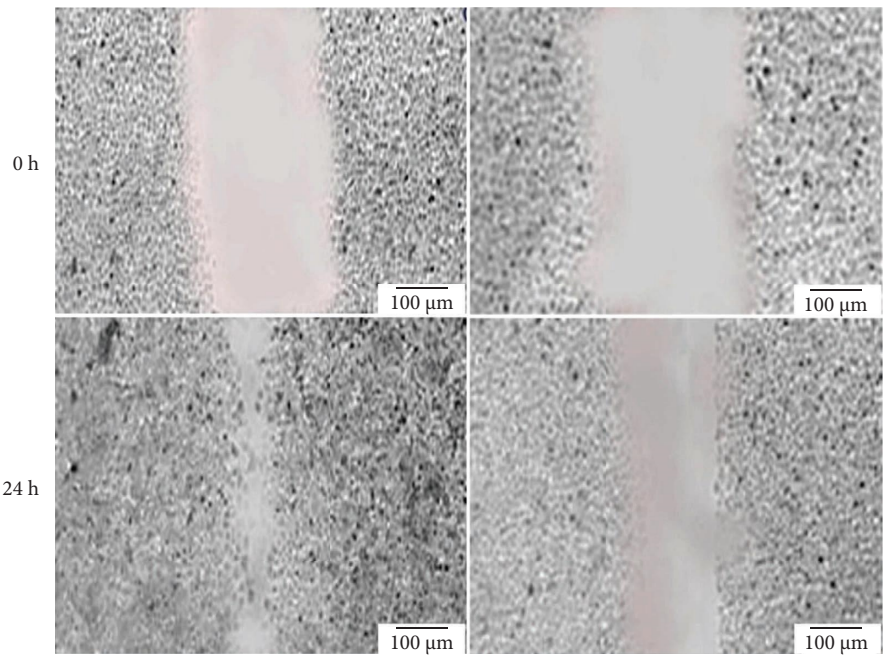

Figure 7: The sublethal concentration of the SRHME (10 μg/ml) inhibits SKVO3 cells from migrating, as demonstrated by a wound healing assay. After scratching the cells, the migration of control cells (a) and treated cells (b) to the wound area after 24 h was imaged.

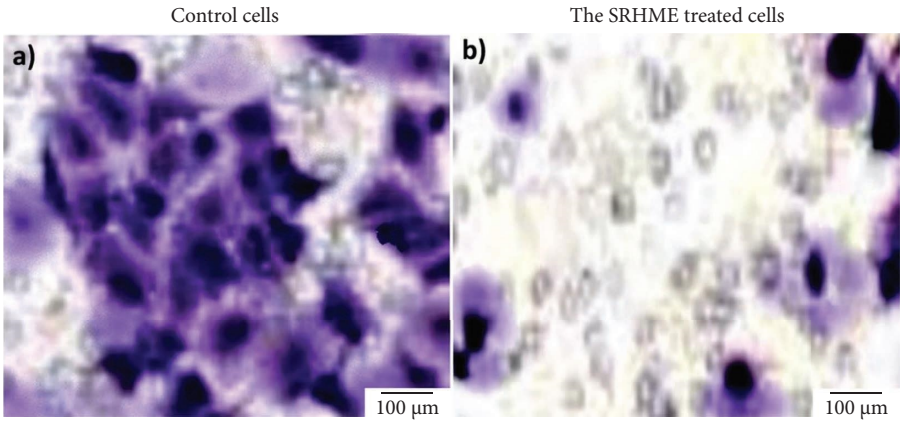

Figure 8: Transwell chamber assay demonstrates that the sublethal concentration of the SRHME (10 μg/ml) prevents the invasion of SKOV3 cells. Control cell (a) and treated cell (b) images of invasion were taken.

Additionally, as shown in Figure 8, the number of SKVO3 cells that invaded Matrigel and entered the lower chamber decreased dramatically to 25% after being treated with the *Stevia* extract. This was in contrast to the percentage of invaded cells in untreated ones (94%). Metformin, as anticancer therapy, has previously been proven to decrease the invasion and migration of SKOV3 cells [93].

Matrix metalloproteinases (MMPs), most notably MMP-2 and MMP-9, are known to regulate tumor growth and metastasis [101]. Overexpression of MMPs has been shown to disrupt the basement membrane, a vital step in the tumor invasion and dissemination of cancer [102]. So much data has accumulated that it is possible to use MMP inhibition as an early target in the fight against cancer spread. As a result, based on the antimetastatic effects seen in this study, the influence of the tested extract on the expression levels of MMP-2 and MMP-9 genes was gauged using qRT-PCR, and their protein expression levels were confirmed using ELISA techniques. The results revealed that the *Stevia* extract significantly downregulated the expression of MMP-2 and MMP-9, confirming their role in the inhibition of SKVO3 cells metastasis.

3.5.4. The Hypothesized Apoptotic Mechanisms of the Stevia Extract against SKVO3 Cells. Cell cycle arrest caused by the control of essential proliferating proteins and stimulation of the apoptotic process are the two basic mechanisms underlying *Stevia's* cytotoxicity [20]. As a result, in this study, the following potential apoptotic

pathways in SKVO3 cells that had been exposed to the tested SRHME were studied:

(1) Cell Cycle Analysis. Inhibiting the progress of the cell cycle is one of the key tactics for suppressing cancer growth [103]. So using flow cytometry, it was possible to analyze the findings for the impact of the studied extract on the regulation of the SKVO3 cell cycle stages. As noted in Figures 9(a)–9(c), the control cells did not display any signs related to apoptosis (Figure 9(a)), while the *Stevia* extract produced a higher significant percentage of apoptotic cells in the sub-G1 and *S* phases in treated SKVO3 cells (Figure 9(b)), proving that it triggers apoptosis. This might be brought on by interfering with DNA synthesis, which would stop cell phase progression and trigger apoptosis. Additionally, a common histogram that contrasts control cells with cells treated with the extract indicates the percentages of the cell population inside each stage of the SKVO3 cell cycle (Figure 9(c)). In earlier research [104], steviol, a *Stevia* derivative, was demonstrated to have prevented the G2/M phase transition in MCF-7 cells. Amentoflavone, as medication for cancer therapy, has been shown to cause the *S* phase and G2 cell cycle arrest in SKOV3 cells via modulating the expression of cell cycle-associated proteins [105].

Cell cycle arrest at G1-S or/and G2-M transition caused by cytotoxic agents is primarily caused by DNA damage or its effect on CDK inhibitor proteins (P21 and P27) [92]. These proteins control the activities of certain complexes between cyclins and cyclin-dependent kinases (CDKs) that play an important role in cell cycle transition from one phase to the other. Delay in S-phase advancement, for instance, may be caused by blockage of CDK2/cyclin A complex activity [106]. So in the current study, the expression levels of P21, CDK2, and cyclin A were assessed in the extract-treated cells through Q-RT-PCR and ELISA techniques to check if the arresting S phase that was confirmed in the above obtained cell cycle results was through the pathway of inhibiting the activity of the CDK2/cyclin A complex or another way. The obtained outcomes showed that the extract increased the expression of P21 while decreasing the expression of CDK2 and cyclin *A* in treated SKOV3 cells, confirming their functions in stopping the cell at the *S* phase.

(2) Effect of SRHME on ROS Generation in SKVO3 Cells. Almost all cancer cells have high levels of ROS due to increased metabolic activity, and these ROS play specialized roles in the formation of cancer cells [107]. It has been suggested that ROS plays a role in angiogenesis, cell cycle progression, cell survival, and cell proliferation. Since earlier studies have demonstrated that down regulation of ROS is also responsible for the production of apoptosis [108], so inhibition of ROS may be a valuable technique in the treatment of cancer [109]. As shown in Figure 10, the SRHME caused a considerable reduction in ROS levels in treated cells compared with control cells. This suggests that generation of apoptosis in the *Stevia*extract-treated cells may be related to the impact of the extract on reduction of ROS concentration. Similarly, earlier research suggested that steviol (one of the *Stevia* derivatives) reduced ROS in MCF-7 cells, which thereafter underwent apoptosis [104].

In the malignant transformation that increases primary tumor propensity to spread, ROS was reported to play a substantial role. Additionally, greater ROS concentrations have been demonstrated to trigger signaling pathways that support cancer metastasis [110], where ROS activates the gelatinolytic activity of MMPs by oxidizing the cysteine residue in the propeptide domain, which increases cellular movement [111]. As a result, the extract's antimetastatic ability that was observed in this study may be associated with its impact on ROS reduction in treated SKVO3 cells. These findings might lend credence to the extract's inhibitory action on SKVO3 metastasis and proliferation.

(3) Quantification Analysis Using qRT-PCR and ELISA Assays. According to the reports [109], the family of BCL-2 proteins regulates apoptosis by acting as either proapoptotic controllers (BAX, BAK, and BAD) or antiapoptotic controllers (BCL-2 and BCL-XL). Additionally, *BAX* overexpression causes mitochondrial permeabilization, which boosts *caspase*-3 activation and the release of cytochrome c into the cytoplasm [94]. As a result, *caspase*-3 activation results in DNA damage, the execution of apoptosis, and a reduction in the level of *BCL*-2 gene expression [112]. Thus, in the present study, the impact of the *Stevia* extract on the mRNA expression levels of genes related to apoptosis (*BCL*-2, *BAX*, and *caspase*-3) was evaluated, and the results revealed that the expression levels of *BAX* and *caspase*-3 considerably elevated, along with a substantial reduction in *BCL*-2 (Figure 11). These results imply that induction of apoptosis in the extract-treated SKOV3 cells may have occurred through the mitochondrial pathway. Prior research suggests that methotrexate impacted the mRNA expression levels of *BAX* and *BCL*-2 in treated SKOV-3 cells [88].

Additionally, it is thought that lowering MMP expression levels is a necessary first step in preventing cancer metastasis [113]. So the extract impact on *MMP*-2 and *MMP*-9 mRNA expression in SKVO3-treated cells was checked to see if the antimetastatic action that was seen in the current study was associated with a decrease in *MMP* expression or not.

According to Figure 11, the *Stevia* extract reduced the expression of *MMP*-2 and *MMP*-9 when compared to control cells, indicating that it can prevent SKVO3 cells from migrating and invading by lowering their levels of expression.

Eventually, *P*21 has the potential to modify CDK activity, which results in its suppression of all cyclin-CDK complexes (cyclin E-CDK2, cyclin B-CDK1, cyclin D-CDK4, and cyclin A-CDK2) that play a crucial role in regulating the progression of the cell cycle stages [114]. On the one hand, *P*21 gene overexpression might cause cell cycle abnormalities and prevent cell growth [115]. A blockage in the activity of the CDK2/cyclin A complex, on the other hand, may be the source of the arrest in the S-phase that was seen in this work. Accordingly, the expression levels of *cyclin A*, *CDK*2, and *P*21 in the extract-treated cells was checked, and the obtained findings showed that the extract significantly decreased *cyclin A* and *CDK*2 expression levels while significantly increasing

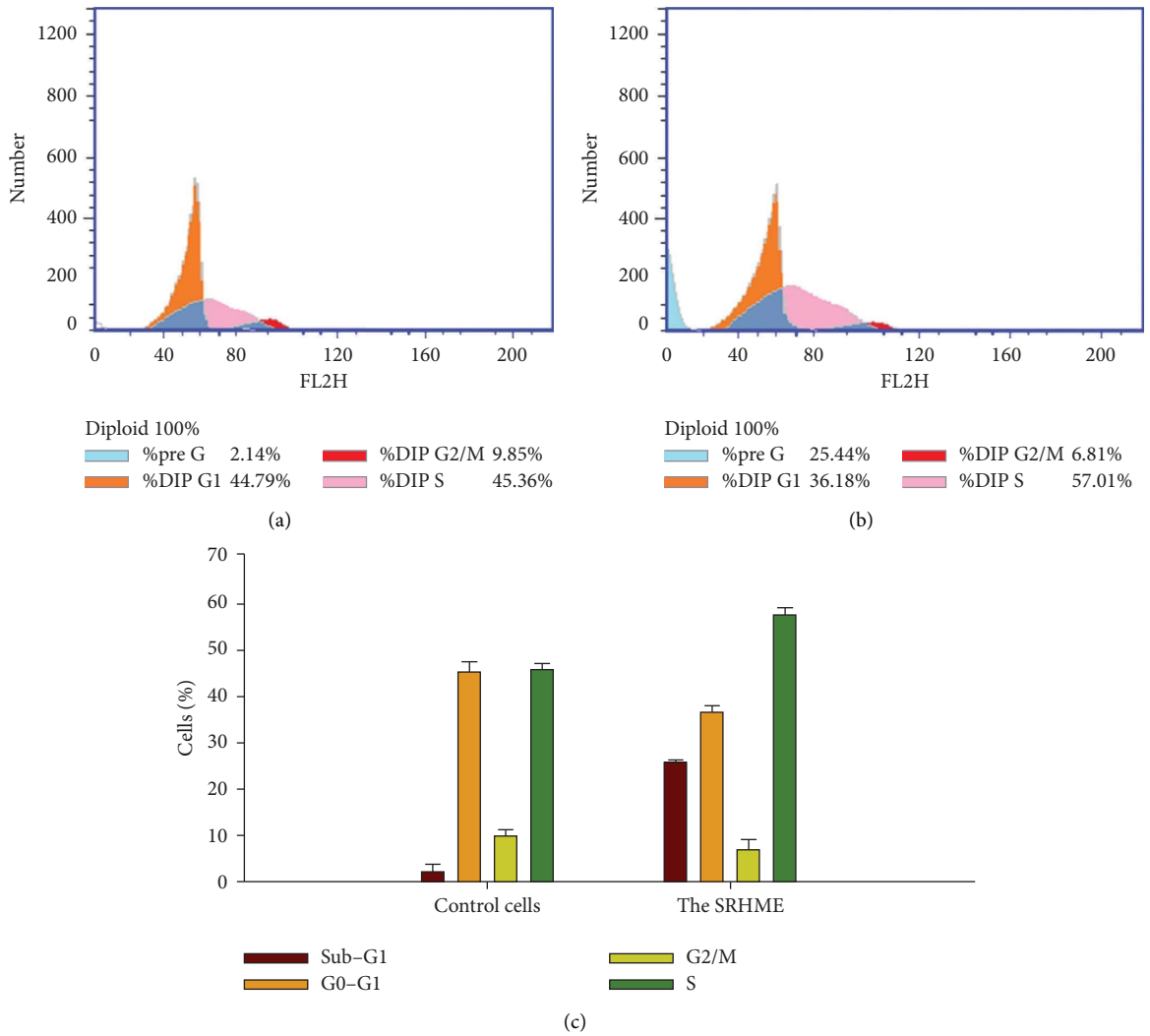

FIGURE 9: Flow cytometric examination of the distribution of the cell cycle at various stages in control cells (a) and in SKVO3 cells that had been treated with an IC_{50} dose (17.5 μg/ml) of *Stevia* extract (SRHME) (b) An illustration of a histogram that displays the proportions of cells in each stage of the cell cycle in untreated and SRHME-treated cells (c).

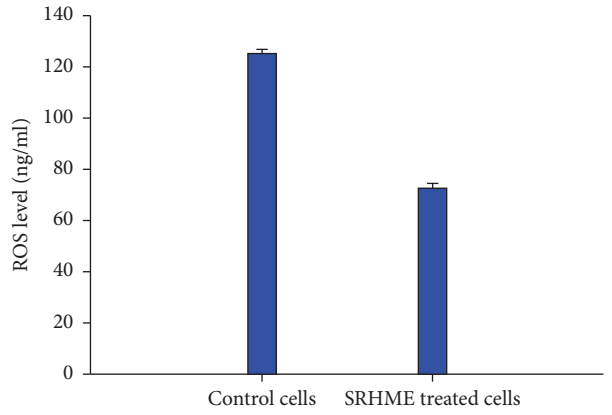

FIGURE 10: The amount of reactive oxygen species (ROS) inside the treated SKVO3 cells with an IC_{50} dose (17.5 μg/ml) of *Stevia* extract (SRHME) when compared to untreated cells. Each value displayed was the average of three different studies' means and standard deviations; $p < 0.001$.

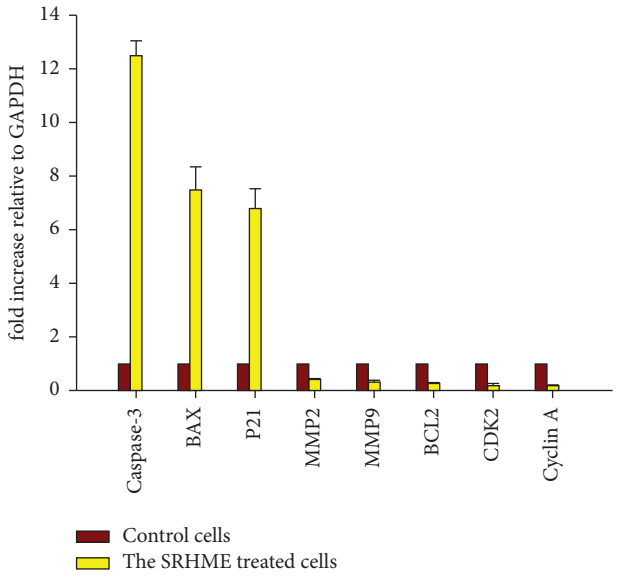

Figure 11: Using qRT-PCR, the effects of the IC_{50} dose (17.5 μg/ml) of *Stevia* extract (SRHME) were assessed on the expression levels of *caspase*-3, *BAX*, *P*21, *MMP*-2, *MMP*-9, *BCL*-2, *CDK*2, and *cyclin A* in SKVO3-treated cells in comparison to control cells. The findings are shown as the mean ± standard deviation of three different runs; $p < 0.001$.

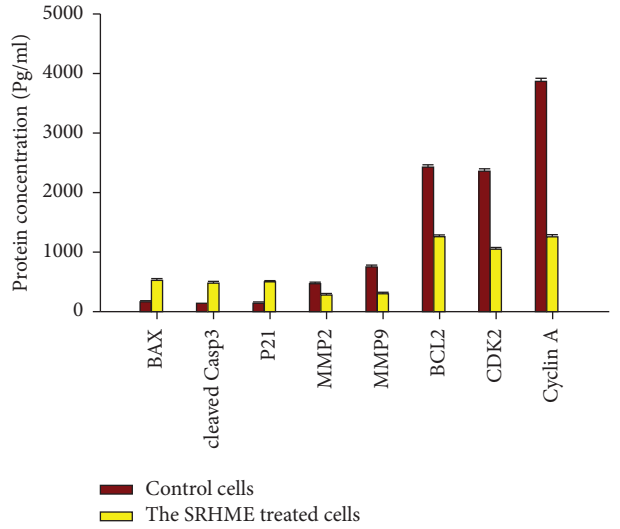

Figure 12: The effect of the IC_{50} dose (17.5 μg/ml) of *Stevia* extract (SRHME) on the protein content of BAX, BCL-2, cleaved caspase-3, cyclin *A* CDK2, P21, MMP-2, and MMP-9 in SKVO3-treated cells compared to that of control cells using the ELISA method. The results are shown as the average standard deviation over three separate experiments; $p < 0.001$.

*P*21 levels (Figure 11), implying that by controlling proteins connected to the *S* phase, the extract triggered *S*-arrest in the treated SKOV3 cells.

In order to confirm the levels of mRNA expression of all studied genes, the proteins expressed by these genes were examined in the extract-treated cells using the ELISA test. The resulting data are reported in Figure 12, which affirmed the upregulated levels of P21, BAX, and cleaved caspase-3 proteins and downregulated levels of BCL-2, CDK2, cyclin *A*2, MMP-2, and MMP-9 proteins in treated cells versus untreated ones. Thus, these results revealed that the tested *Stevia* extract induced significantly higher variation in apoptosis, *S*-phase cell cycle arresting, and antimetastatic-related proteins within SKOV3-treated cells compared to control cells, which is in line with the findings of the aforementioned qRT-PCR.

4. Conclusions

In the current study, a relationship was proposed between the bioactive phytochemical components (antioxidant and anticancer constituents) that were identified by GC-MS analysis in the prepared hydromethanolic extract of *Stevia* leaves (SRHME) and the extract's potent effects on the viability and metastasis of the tested SKVO3 cancer cells. Additionally, compared to ascorbic acid, the produced SRHME displayed much higher quantities of phenols and flavonoids, as well as stronger antioxidant effectiveness in regards to O_2 and DPPH radical scavenging. As a result, the tested SRHME demonstrated more effective IC_{50} values for inhibiting hemolysis and lipid peroxidation in the used human erythrocytes (25 μg/ml, 25.3 μg/ml, respectively) than the standard ascorbic acid (27.2 μg/ml, 47.3 μg/ml, respectively). The SRHME, on the other hand, had a superior effect on SKVO3 cells in terms of producing cytotoxicity with a strong IC_{50} value (17.5 μg/ml), apoptosis induction, stopping the cell cycle at the *S* phase with higher % of apoptotic cells in sub-G1, reducing levels of ROS, and preventing the possibility of metastasis, compared to control cells. Moreover, the extract had an impact on the proteins that were related to apoptosis, cell cycle arresting, and antimetastatic capacity within SKOV3-treated cells, compared to control cells. Thus, according to these findings, *Stevia* extract (SRHME) leaf, which possesses strong antioxidant properties, may be employed as a successful natural remedy for the treatment of human ovarian cancer. However, to support such results, *in vivo* research using an animal model is required.

Data Availability

The data presented in this study are available within the article and supplementary material.

Additional Points

Stevia rebaudiana (*Stevia*), a naturally occurring medicinal plant, has a variety of bioactive compounds in its leaves that give rise to many pharmacological and biological capabilities, including anticarcinogenic and antioxidant activities. Therefore, the current work sets out to evaluate the relationship between the antioxidant activity of the dried leaves of SRHME and its effectiveness

against the proliferation and metastasis of SKVO3 ovarian cancer. According to the obtained findings, SRHME has strong antioxidant activities that supported its usage as a potential natural treatment for human ovarian cancer; nevertheless, more long-term therapy, intensive animal tests, and clinical investigations are needed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

The published version of the manuscript has been read and approved by all authors.

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Supplementary Materials

The following supporting information: Figure S1: the antioxidant properties of different doses of *Stevia* extract in comparison to those of vitamin C against the DPPH radicles (a) and superoxide radicles (b). Figure S2: the influence of various doses of *Stevia* extract (SRHME) compared with those of vitamin C for the suppression of hemolysis in erythrocytes. Figure S3: the impact of various *Stevia* extract (SRHME) dosages on erythrocytes' ability to produce TBARS in contrast to vitamin C. (*Supplementary Materials*)

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