Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(8): 180
https://doi.org/10.31083/j.fbl2808180

Original Research

The Role of Mitochondrial Dysfunction in Cytotoxic Effects of *Solanum nigrum* Water Extract on MCF-7 and MDA-MB-231 Breast Cancer Cells

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Academic Editor: Salvatore Nesci
Submitted: 5 March 2023 Revised: 18 July 2023 Accepted: 27 July 2023 Published: 28 August 2023

Abstract

Background: Recent studies suggest that numerous naturally occurring agents have the potential to kill cancer cells via mitochondrial dysfunction. *Solanum nigrum* is a herb widely used in alternative medical systems. This study aimed to investigate the cytotoxic effect of *Solanum nigrum* water extract (SNWE) against Michigan Cancer Foundation-7 (MCF-7) and MD Anderson-Metastatic Breast Cancer-231 (MDA-MB-231) cells. **Methods**: We used an MTT reduction assay for cytotoxicity analysis. To explore the mode of action, the cellular adenosine triphosphate (ATP) levels and mitochondrial membrane potential were analyzed using a colorimetric ATP assay and Rhodamine-123 fluorescent staining, respectively, during SNWE treatment for 72 h. **Results**: The cytotoxic effect was significant in both cell lines, with IC_{50} values of 4.26 µg/mL and 5.30 µg/mL in MCF-7 and MDA-MB-231 cells, respectively. The 24, 48, and 72 h treatments of 100 µg/mL SNWE showed 0.85 $\pm$ 0.07, 0.38 $\pm$ 0.1, and 0.20 $\pm$ 0.1 nM ATP in MCF-7 cells and 0.94 $\pm$ 0.07, 0.84 $\pm$ 0.2 and 0.46 $\pm$ 0.2 nM in MDA-MB-231 cells, respectively. The SNWE treatment altered the mitochondrial membrane potential ($\Delta\Psi$m) in a concentration-dependent manner in both the breast cancer cell lines, to 29.6 $\pm$ 4.1% in MCF-7 and 28.7 $\pm$ 4.17% in MDA-MB-231 cells, when compared with healthy mitochondria (100% $\Delta\Psi$m). **Conclusions**: The cytotoxic effects of *Solanum nigrum* against breast cancer cells are associated with energy metabolism. Additional studies are warranted to test the anticancer effect of *Solanum nigrum* using an animal model of breast cancer.

Keywords: *Solanum nigrum*; mitocans; mitochondrial dysfunction; mitochondrial membrane potential; energy metabolism; breast cancer

1. Introduction

Targeting mitochondria is emerging as a successful therapeutic approach for cancer because of the involvement of mitochondria in redox signaling, cellular metabolism, and cellular homeostasis [1]. The mitochondria are the site of adenosine triphosphate (ATP) synthesis from glucose and fatty acids through a series of biochemical reactions known as oxidative phosphorylation. The essential requirement for oxidative phosphorylation is the electron transport chain (ETC) that is associated with the production of reactive oxygen species (ROS), as well as adenosine triphosphate (ATP) synthesis [2]. Thus, an efficient redox balance during cellular ATP synthesis is necessary for cellular proliferation and viability. However, dysfunction of mitochondria has a significant negative impact on overall ROS generation and cellular energy metabolism, which ultimately adversely affects the fate of cells. Nevertheless, in cancer cells, the shifting of energy metabolism from the usual oxidative phosphorylation to active glycolysis impedes mitochondrial activity, leading to increased levels of ROS generation, which can ultimately be linked to numerous oncogenic signals [2].

Mitochondrial membrane potential (abbreviated to $\Delta\Psi$m) is a global indicator of mitochondrial function. The electrochemical gradient resulting from the imbalance of H+ outside and inside the inter-membrane space causes the development of mitochondrial membrane potential [3]. In a normal cell, mitochondrial membrane potential ranges between –108 and –159 mV, while optimum membrane potential for maximum ATP production fluctuates between –130 and –140 mV in almost all living creatures. Alteration by approximately 10% in the mitochondria membranes potentials results in, approximately, a 90% depletion in ATP synthesis and an increase in the generation of potentially toxic ROS by a similar magnitude [4]. Cancer cells are >50% more hyperpolarized across the inner mitochondrial membrane (ΨIM) than normal cells [5]. Furthermore, the invasiveness of cancer is associated with the increased hyperpolarization of ΨIM [6–8]. In cancerous cells, an increased mitochondrial membrane potential compared to cy-

Fig. 1. Preparation of *Solanum nigrum* water extract (SNWE). The leaves of *Solanum nigrum* plants were air-dried for one week to remove the moisture content. The dried leaves were soaked in distilled water and incubated in a boiling water bath for 1 h. The mixture was then filtered through Whatman filter paper and syringe filters and incubated in a shaker incubator for 24 h at 60 °C. The final aqueous extract of *Solanum nigrum* was dissolved in 0.1% dimethyl sulfoxide (DMSO) for experimental use.

tosol also makes mitochondria a potential selective target as mitochondria allow selective agents to pass across the double membrane. The outer membrane of the mitochondria is associated with signals related to cell survival and death [9]. The porosity of the mitochondrial membrane increases during the activation of proapoptotic proteins in the state of oxidative stress, enabling the extensive escape of cytochrome c to the cytosol and thereby leading to cellular damage [10].

Many recent reports showed that herbal extracts and active isolated compounds can cause the disruption of mitochondrial membrane potential in different cancer cell lines [11–13]. Solanum nigrum (black nightshade) is a commonly used medicinal herb in alternative systems of herbal medicine. The crude extract from Solanum nigrum has shown anticancer potential in different types of cancer including breast cancer, cervical cancer, endometrial cancer, colorectal cancer, and human melanoma [14–17]. The Solanum nigrum water extract from leaves is significantly

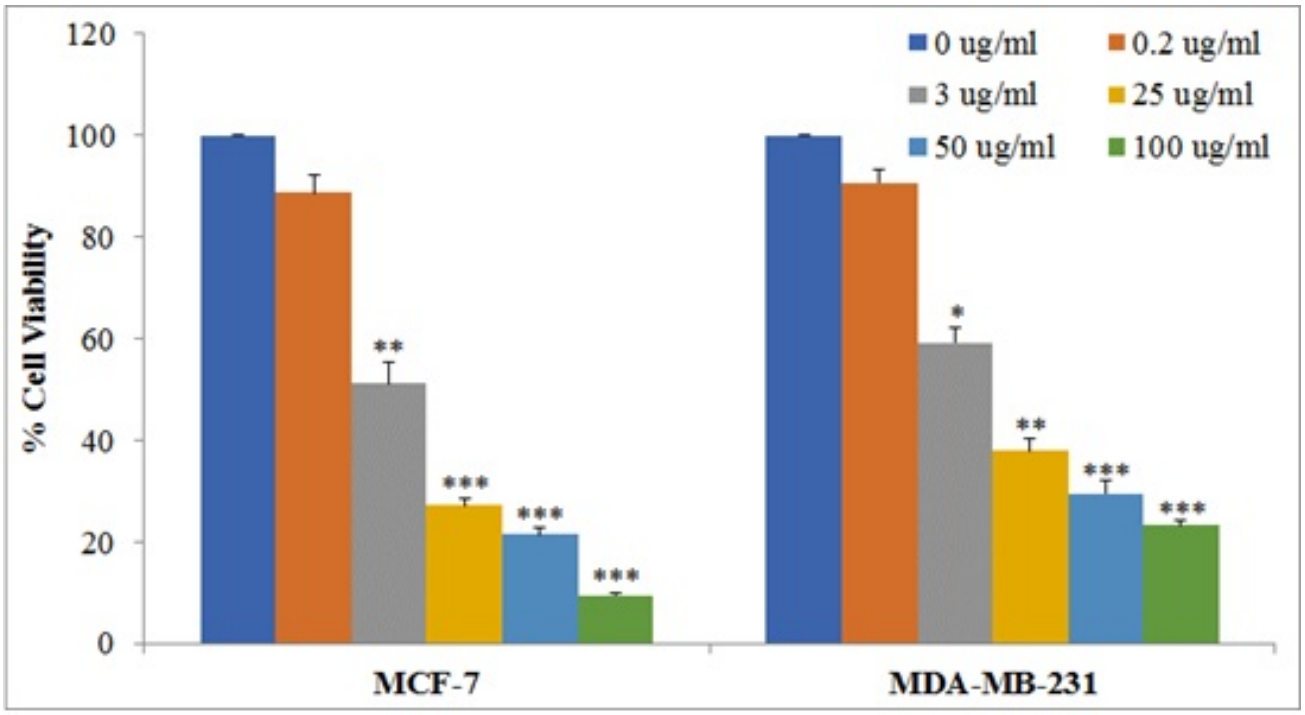

Fig. 2. Effect of SNWE on cytotoxicity in MCF-7 and MDA-MB-231 cells. Cells (10,000 cells/well) were treated with increasing concentrations of SNWE ranging from 0.20–100 µg/mL for 72 h, and then assessed for cell viability using MTT assay. Data are presented as mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control group.

cytotoxic in breast cancer cell lines due to apoptosis and epithelial-mesenchymal transition [18]. The leaf extract of Solanum nigrum possesses antioxidant [19] and anti-inflammatory [20] properties that can efficiently cope with oxidative stress and inflammation, the primary hallmarks of cancer. Furthermore, the leaf extract of Solanum nigrum also proved capable of promoting mitochondrial fission and, hence, reducing the normal function of mitochondria in breast cancer cell lines [18]. These reports suggest that Solanum nigrum works mechanistically by targeting mitochondria membrane potential and hampering ATP generation in breast cancer. We, therefore, attempted to investigate the effect of Solanum nigrum on mitochondria membrane potential and ATP generation in breast cancer cells.

2. Materials and Methods

2.1 Preparation of Solanum nigrum Water Extract

The *Solanum nigrum* plants were collected from regions in South India and air-dried for 7 days to remove the moisture content (Fig. 1). The leaf of *Solanum nigrum* was powdered, and 50 g of the powdered plant material was immersed in 250 mL of distilled water. The mixture was transferred into a 500 mL conical flask, plugged with sterile cotton, and then incubated in a boiling water bath for 60 minutes. Next, the solution was filtered through Whatman filter paper and syringe filters (Fig. 1). The solution was then incubated in a shaking incubator for 24 h at 60 °C. Finally, a clear *Solanum nigrum* water extract (SNWE) was obtained and stored in the refrigerator at –20 °C for later use. The required amount of SNWE was weighed and a stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) [21]. The final concentration of DMSO in the solution was 0.1%.

2.2 Cell Line Maintenance and Growth Conditions

Breast carcinoma cell lines including MDA-MB-231 (Triple-negative) and MCF-7 (Luminal A) were obtained from National Centre for Cell Science (NCCS), Pune, MH, India. Both the cell lines were authenticated using the AmpFISTR Identifier Plus PCR amplification Kit from Applied Biosystems (Waltham, MA, USA) and sixteen short tandem repeats (STR) loci were amplified using this kit. The commercial supplier stated that no mycoplasma contaminations were detected in these cell lines. The cells were grown in Dulbecco's modified Eagle medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and 50 units/mL of penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. The study protocol was approved by the Institutional Review Board of King Saud University, Riyadh, Saudi Arabia (Approval No. KSU-SE-22-18, dated 24/03/2022). The identity of the cell line used in the experiment was confirmed by PCR and was negative for mycoplasma contamination.

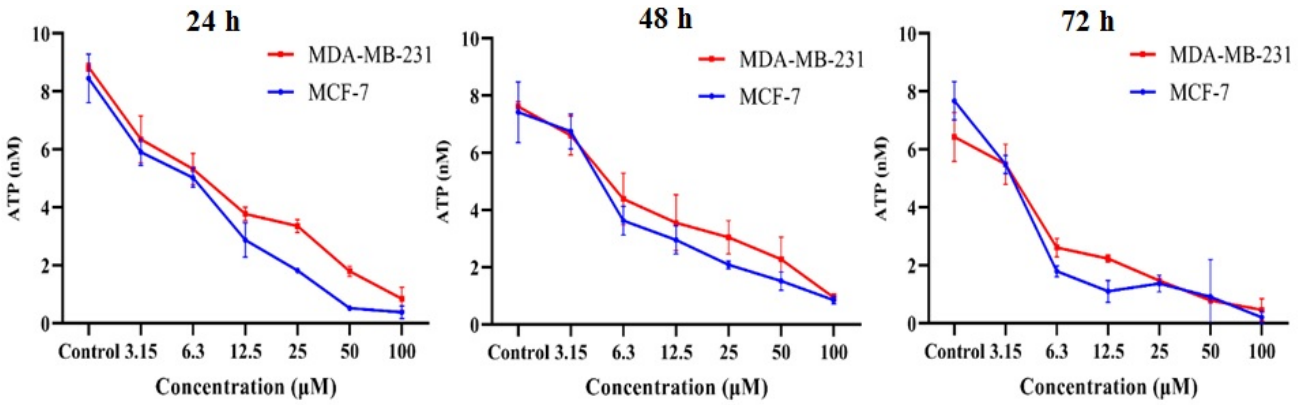

Fig. 3. Effect of SNWE on adenosine triphosphate (ATP) levels in the breast cancer cells at different time intervals (24 h, 48 h, and 72 h). There was a concentration-dependent decrease in cellular ATP levels after SNWE treatment. The data are presented as mean ± SEM.

2.3 Cytotoxicity Assay

The MCF-7 and MDA-MB-231 cells were seeded in a 96-well plate at a density of 10,000 cells/well and incubated for 24 h at 37 °C in 5% CO_2 atmospheric conditions. Then, cells were treated with SNWE at different concentrations, ranging from 0 (control) to 100 µg/mL. After 72 h of SNWE treatment, 100 µL of MTT solution (1 µg/mL) was added to micro-plate wells, and the color was allowed to develop in the incubator for an additional 4 h. Subsequently, 100 µL of DMSO was added to each well for dissolving the formazan crystals. The absorbance was recorded at 570 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). Percentage cell viability was calculated, and cell-survival curves were plotted [22].

2.4 Intracellular ATP Measurement

The intracellular ATP measurement is the most sensitive method available for evaluating cell viability [23]. The MCF-7 and MDA-MB-231 breast cancer cells were seeded in 96-well plates and allowed to reach confluence, upon which different concentrations of SNWE treatment were added to the cells. The cellular ATP levels were determined after the treatment of SNWE for 24 h, 48 h, and 72 h using a colorimetric ATP assay kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. To calculate the unknown concentrations of test values, a standard curve was plotted with the concentration ranges from 0–12 nmoL and analyzed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA) software.

2.5 Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential was analyzed using Rhodamine-123 fluorescent staining [24]. The MCF-7 and MDA-MB-231 cells (1×10^6 cells per well) were seeded in 6-well plates. After 72 h of treatment with SNWE, the cells were stained with Rhodamine-123 for 30 minutes. Then, the cells were washed with PBS three times to remove excessive staining. Fluorescent measurements were taken with excitation at 450 ± 10 nm and emission at 490 ± 10 nm, using a multimode plate reader (Molecular Devices, USA). Images were acquired with a fluorescence microscope (Floid Cell Imaging Station, Life Technologies, Carlsbad, CA, USA).

2.6 Statistical Analysis

We conducted one-way ANOVA (analysis of variance) on the resulting data using the GraphPad Prism 9.0.0 software package. The results are reported as mean ± standard error of the mean (SEM). We used Duncan's multiple range test to establish statistically significant differences between groups. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1 Effect of SNWE on Cell Viability

The MCF-7 and MDA-MB-231 cells were treated with different concentrations of SNWE. We observed that SNWE induces cytotoxicity in both the breast cancer cell lines in a concentration-dependent manner (Fig. 2). We noticed that the antiproliferative effect of SNWE was more prominent on MCF-7 cell lines than on MDA-MB-231 cells. The IC_{50} values were found to be 4.26 µg/mL in MCF-7 and 5.3 µg/mL in MDA-MB-231 cells.

3.2 Effect of SNWE on Intracellular ATP Levels

The high energy metabolism rate in cancer cells results in the production of significant amounts of ATP. As shown in Fig. 3, the untreated control group had a high ATP concentration which was significantly reduced by SNWE treatment in both MCF-7 and MDA-MB-231 cells. Furthermore, we observed that the cells showed decreased ATP concentration in a concentration-dependent manner that was directly proportional to the decrease in the viability of cells (Fig. 3). The incubation of 24 h, 48 h, and 72 h

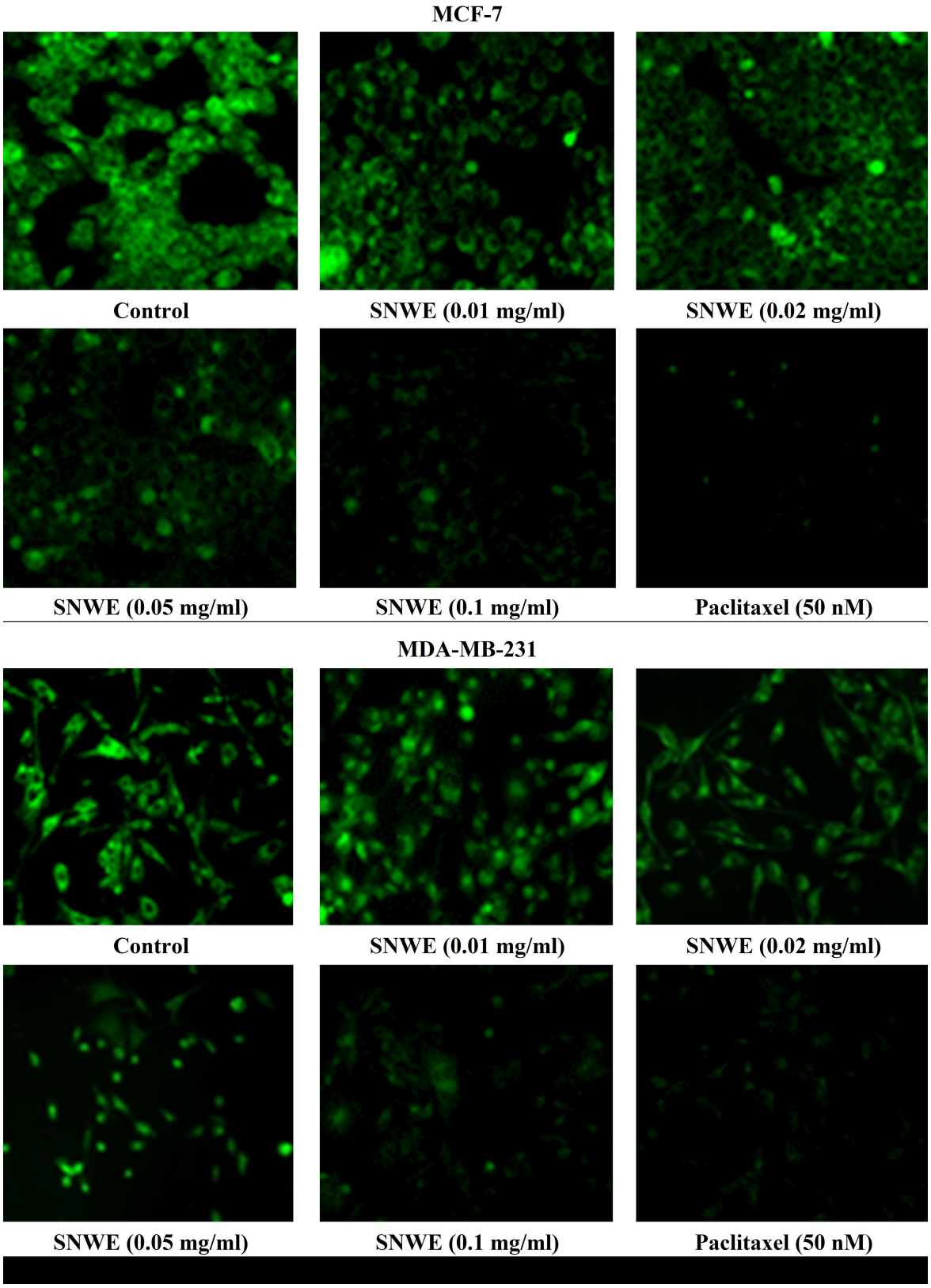

Fig. 4. Effect of different concentrations of SNWE on Rhodamine-123 fluorescence dye retention in MCF-7 (upper panel) and MDA-MB-231 cells (lower panel). Control cells showed a massive accumulation of Rhodamine-123 which was gradually decreased after exposure to different concentrations of SNWE. The anticancer drug, paclitaxel, was used as a positive control (Original magnification, 20×).

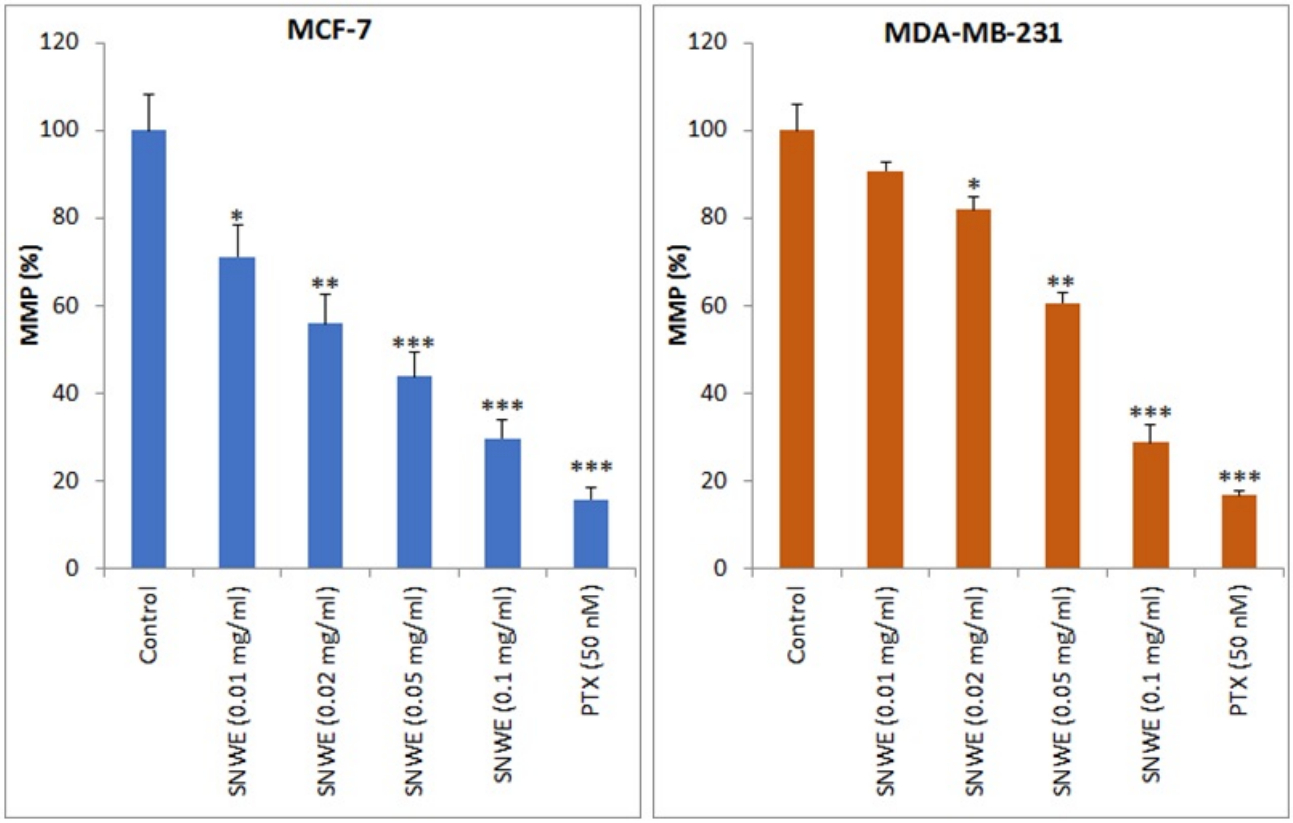

Fig. 5. Effect of SNWE on the loss of mitochondrial membrane potential (MMP) in MCF-7 (left panel) and MDA-MB-231 cells (right panel). SNWE treatment significantly decreased mitochondrial membrane potential in a concentration-dependent manner. Bar graphs show MMP in terms of % fluorescence intensity of Rhodamine-123 at excitation and emission wavelengths of 450 nm and 490 nm, respectively. Paclitaxel (PTX) was used as a positive control. Values are mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control group.

treatment of 100 µg/mL SNWE showed 0.85 ± 0.07, 0.38 ± 0.1 and 0.20 ± 0.1 nM ATP in MCF-7 cells and 0.94 ± 0.07, 0.84 ± 0.2 and 0.46 ± 0.2 nM in MDA-MB-231 cells, respectively.

3.3 Effect of SNWE on Mitochondrial Membrane Potential (ΔΨm)

Rhodamine-123 (Rh-123) is a membrane-permeable cationic dye that stains the mitochondria in living cells. There was significant retention of Rh-123 fluorescent dye in untreated MCF-7 and MDA-MB-231 cells, indicating functional mitochondria in these cells (Fig. 4). However, this green fluorescence was gradually reduced with increasing concentrations of SNWE exposure in both the cancer cell types (Fig. 4).

The SNWE treatment altered the mitochondrial membrane potential (ΔΨm) in a concentration-dependent manner in both the breast cancer cell lines (Fig. 5). We observed that 0.1 mg/mL of SNWE treatment decreased ΔΨm to 9.6 ± 4.1% in MCF-7 and 28.7 ± 4.17% in MDA-MB-231 cells when compared with untreated cells. The non-treated control cells displayed high fluorescence due to the accumulation of Rh-123 in the healthy mitochondria (100% ΔΨm).

4. Discussion

Solanum nigrum is used in various medicinal systems due to its numerous valuable pharmacological properties [25–28]. Our results showed that *Solanum nigrum* water extract (SNWE) inhibited the growth of both estrogen receptor-positive as well as estrogen receptor-negative breast cancer cell lines (MCF-7 and MDA-MB-231, respectively) in a concentration-dependent manner (Fig. 2). Several studies showed the cytotoxic effect of *Solanum nigrum* water extract in breast cancer cell line AU565; cervical cancer cell line U14; hepatoma cell line HepG2; endometrial cancer cell lines HEC1A, HEC1B, and KLE; and colorectal cancer cell lines DLD-1 and HT-29 [14–16,29,30]. Furthermore, SNWE induces apoptosis and autophagy in cancer cells [31,32]. Similarly, lower concentrations of SNWE caused autophagy and no apoptosis, whereas high concentrations inhibited p-Akt and resulted in apoptosis- and autophagy-induced cellular damage [16]. Moreover, adjuvant therapy with SNWE can also augment the cellular toxicity of known cancer drugs such as docetaxel, doxorubicin, and cisplatin [32–34].

In our study, we found that SNWE significantly diminished intracellular ATP levels in a dose-dependent manner (Fig. 3). The intracellular ATP levels are markers for cell viability and can be used to evaluate the efficacy of anticancer drugs in cancer cells. Various concentrations of SNWE significantly reduced the ATP levels in the breast cancer cells in a dose-dependent manner, while the untreated control cells showed higher ATP levels. These results reveal the failure of the mitochondria to produce ATP for cellular proliferation in both MCF-7 and MDA-MB-231 breast cancer cells. Similarly, Hernández *et al*. [35] observed that *Petiveria alliacea L* leaf extracts reduced the cellular ATP concentrations in breast cancer cells and breast cancer murine models.

Our results show that SNWE treatment significantly disrupts mitochondrial membrane potential (MMP) in breast cancer cells (Fig. 5). Mitochondria are the central regulators of the intrinsic apoptosis pathway as they maintain the balance between pro-apoptotic and anti-apoptotic events while controlling programmed cell death [36]. The recent literature proposes targeting mitochondria as an attractive strategy to combat cancer [37]. The various concentrations of SNWE caused the dose-dependent reduction in oxidative phosphorylation and electron transport through the disruption of MMP, which leads to the induction of caspase-dependent apoptosis. Rhodamine-123 is a fluorescent chemical probe widely used for its ability to accumulate in the mitochondria and thus acts as an indicator for mitochondria membrane potential [38]. High retention of rhodamine-123 fluorescence was detected in both untreated MCF-7 and MDA-MB-231 cells (Fig. 4). Further, the fluorescence was significantly reduced after SNWE treatment in both types of breast cancer cells. This mechanism of action suggests that SNWE induces mitochondrial damage and thereby leads to ATP depletion that, in turn, causes cell death. Several other evidence-based studies have shown that a significant number of naturally occurring agents and herbs, including withanone from *Withania somniferum*, can induce mitochondria-mediated cancer cell death by elevating oxidative stress, disturbing the cellular redox system, reducing ATP levels, and causing loss of mitochondrial membrane potential [39,40]. The concept of mitochondria-targeted anticancer agents is attracting increased attention and, in the literature, such agents have been termed "mitocans", mitochondria-targeting anticancer drugs, with various modes of action [41]. Naturally occurring products act as mitocans by targeting the mitochondrial membrane potential [42].

Mechanistically, several bioactive ingredients found in *Solanum nigrum*, including uttroside B, solanine, solamargine, and physalins, were tested for their anticancer effects in both *in-vitro* and *in-vivo* cancer models [43]. αSolanine is reported to be involved in the regulation of oncogenic miRNA-21 and tumor suppressor miRNA-138 in prostate cancer [43]. The active components of *Solanum nigrum* also produced inhibitory actions on various cellular pathways, such as matrix metalloproteinases (MMPs), VEGF/VEGFR, JAK-STAT, and PI3K/AKT in various cancer types [43]. α-Solanine was shown to suppress cancer metastasis via the expression of MMPs and inhibition of epithelial-mesenchymal transition [44]. It can also downregulate the expression of vascular endothelial growth factor (VEGF) through the ERK1/2-HIF-1α and STAT3 signaling pathways in cancer cells [45]. Molecular docking analysis showed that phytoconstituents of *Solanum nigrum*, including solanidine, solasodine, and solamargine, have positive drug-likeness values, suggesting the therapeutic potential of these compounds as future anticancer drugs [46]. Moreover, the glycoalkaloids and saponins found in *Solanum nigrum* did not show any general toxicity, tumorigenicity, or irritant effects [46]. These findings suggest that *Solanum nigrum* is a non-toxic medicinal herb that contains numerous bioactive compounds with pharmacological potentials. Moyo *et al*. [47] studied the impact of boiling and *in-vitro* human digestion of predominant compounds of *S. nigrum* including, myricetin, quercetin-3-O-arabinoside, 3,4-dicaffeoylquinic acid, 3-caffeoylquinic acid, and rutin. The study showed improvement in phenolics after boiling but reduction following *in-vitro* digestion, without affecting their bioactivity, particularly in preventing oxidative stress in Caco-2 cells [47]. Thus, the heating steps used for the preparation of SNWE in our study would have no detrimental effect on the stability of bioactive compounds, particularly, those with antioxidant activity.

Reprogramming of energy metabolism is one of the emerging hallmarks of cancers because uncontrolled cell division demands an increase in fuel and biosynthetic precursors by adjusting energy metabolism in cancerous cells. Our findings showed that SNWE significantly interferes with energy metabolism in cancer cells by disrupting mitochondrial membrane potential that results in the depletion of cellular ATP levels. However, the involvement of other potential targets behind the anticancer effects of SNWE may not be ruled out. For instance, solanine, the main ingredient of *Solanum nigrum*, showed antitumor ability against different tumors by targeting different proteins [48].

Thus, the use of SNWE alone or as an adjuvant with low doses of known cancer drugs could serve as an economical therapeutic modality with fewer side effects.

5. Conclusions

The SNWE treatment induced the loss of mitochondrial membrane potential, which resulted in reduced cellular ATP levels and subsequent apoptotic cell death in the MCF-7 and MDA-MB-231 breast cancer cells. Since this study was conducted in breast cancer cells, further studies are required to confirm the implications of our findings for the treatment of human breast cancer. In the early stages, breast cancer is treatable with high success rates. Pharmacotherapies based on traditional medicine are not only cost-

effective but mostly free from side effects. Further studies are warranted to explore the therapeutic and prophylactic anticancer effects of *Solanum nigrum* using animal models of cancer.

Availability of Data and Materials

Datasets used and/or analyzed for this study are available from the corresponding author upon appropriate request.

Author Contributions

Conceptualization, HAK; methodology, HAK, NRP, AAA, SHA, BSA, AAKH; formal analysis, NRP, AAA, BSA, AAKH; investigation, HAK, NRP, AAA, SHA; resources, HAK, SHA, NRP; data curation, HAK, NRP, AAA, BSA, AAKH; original draft preparation, HAK, AAA; writing, review and editing, HAK, AAA, NRP; supervision, HAK, AAA; project administration, HAK; funding acquisition, HAK. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The study protocol was approved by the Institutional Review Board of King Saud University, Riyadh, Saudi Arabia (Approval No. KSU-SE-22-18, dated 24/03/2022). This study was conducted in rats and did not involve human subjects.

Acknowledgment

We acknowledge the technical support from the laboratory staff.

Funding

This research was funded by the National Plan for Science, Technology, and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award Number (2-17-03-001-0044).

Conflict of Interest

The authors declare no conflict of interest. HAK is serving as one of the Guest Editors and Editorial Board Members of this journal. We declare that HAK had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to SN.

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