Breast cancer is the most progressive cancer among women worldwide. The currently available chemotherapy agents induce severe unacceptable adverse effects in breast cancer patients. In this context, natural medicinal herbs are gaining importance to find non-toxic effective anticancer drugs. Solanum nigrum is one of the major traditional medicinal plants widely used in Ayurveda for the treatment of various diseases. This study investigated the anticancer effect of Solanum nigrum water extract (SNWE) against MCF-7 and triple-negative MDA-MB-231 breast cancer cell lines. SNWE significantly induced oxidative stress-mediated apoptotic cell death in a concentration-dependent manner. Real-time PCR results illustrated the upregulation of proapoptotic genes and downregulation of antiapoptotic genes after SNWE treatment in MCF-7 and MDA-MB-231 cell lines. Immunofluorescence analysis showed increased expressions of apoptotic markers like p53, Caspase3 and BAX by SNWE treatment. In conclusion, the findings of this study indicate the antiproliferative effect and apoptosis-inducing property of SNWE in both cell lines. Further studies are warranted on testing the anticancer activity of S. nigrum L. using animal models of cancer.
tentially regulate the cell signalling pathways like, PI3K/AKT/mTOR, MAPK and Wnt/β-catenin to inhibit invasion and metastasis (14). The herbal formulations isolated from medicinal plant species like Taraxacum officinale, Uncaria tomentosa, Astragalus membranaceus, Ocimum sanctum, Aegle marmelos, Allium sativum, Alpinia galangal, Artemisia annua L., and Brassica oleracea were especially useful in breast cancer treatments by inhibiting the signalling pathways responsible for tumor progression (15). Kushwaha et al (16) proved that phytochemicals isolated from B. frutescens regulate the notch signalling pathway to induce cell cycle arrest and apoptosis in triple-negative MDA-MB-231 cells and luminal A T47D breast cancer cells.

Solanum nigrum L. family of Solanaceae, commonly known as ‘Black nightshade’, is a widely used medicinal plant worldwide, especially in India and China (17). It has been reported that S. nigrum L. leaf extracts reduced oxidative stress and showed potential in quenching the formation of the free radicals in normal tissues (18). Further, studies illustrated that the water extract of S. nigrum L. decreased the cell growth of several types of cancer and enhanced the cytotoxicity of chemotherapeutic agents such as cisplatin, doxorubicin, and docetaxel (19,20). Various biologically active phytochemicals, such as alkaloids, polyphenols, polysaccharides, glycoproteins and some steroidal alkaloids have been isolated from S. nigrum L., and these compounds were reported to inhibit cell growth, migration and induce apoptosis in cancer cells (21,22). The aqueous extract of S. nigrum L. has been shown to induce apoptosis-mediated cell death and autophagy in breast cancer cells (20,23). The treatment with S. nigrum L. extracts effectively triggers cell cycle arrest leading to the destruction of breast cancer cells (24). Butt and co-workers proved that S. nigrum L. extracts inhibited the VEGF/VEGFR/AKT/mTOR signalling pathway in various cancer cell lines and reduced cancer progression (25). The medicinal compounds present in S. nigrum L. significantly induced apoptosis and altered the membrane polarization of mitochondria thereby decreasing the mRNA level of Bel-2, and increasing the expression of Bax, cleaved BCL-W, caspase3, and caspase7 in cholangiocarcinoma QBC939 cells (26). Degalactotigonin isolated from S. nigrum significantly inhibited EGF-induced phosphorylation of EGFR and arrested the cell cycle at G0/G1 phase in pancreatic cancer cells. The breast cancer cells having premalignant genes such as, VIM and CXCR4 were hypermethylated by S. nigrum extract through epigenetic modulations to prevent the cancer progression (27).

The network pharmacology on the chemical composition of Solanum nigrum fruit extract contains 170 compounds including steroidal alkaloids which are probably involved in several biological targets responsible for anticancer effects (28). The active phytochemicals present in S. nigrum extract could be the reason for regulating signaling pathways to reduce proliferation in the cancer cells. Moreover, nanoparticles synthesized from S. nigrum L. have been proven for their anticancer and antioxidant properties (29,30). Shi et al observed that steroidal alkaloids isolated from ripe berries of S. nigrum L. potentially inhibited tumor progression in non-small cell lung carcinoma (31). However, the apoptosis-inducing mechanism of aqueous extract of S. nigrum L. in breast cancer cells has not been adequately investigated. Therefore, in this study, we investigated the antiproliferative and oxidative-stress-mediated apoptosis-inducing property of S. nigrum L. water extract (SNWE) in triple-negative (MDA-MB-231) and luminal A (MCF-7) breast cancer cellular models.

Materials and Methods

Preparation of water extract of S. nigrum

The S. nigrum L. plants were collected from the South Indian regions and air-dried for one week to make them free of moisture. The leaves of S. nigrum L. were powdered using an electrical grinder. The powdered leaves (50 g) were soaked in distilled water (250 ml) and the mixture was kept in a boiling water bath for one hour. Then the solution was filtered through Whatman No. 1 filter paper followed by 0.45 µm pore size syringe filters. After that, the solution was evaporated by keeping in a shaking incubator (60°C) for about 24 hours. Finally, the S. nigrum L. water extract (SNWE) was weighed and dissolved in dimethyl sulfoxide (DMSO) for in vitro experiments. The reconstituted SNWE was stored at -20°C for further use.

Cell lines maintenance and growth conditions

Breast carcinoma cell lines, MDA-MB-231 (Triple-negative) and MCF-7 (Luminal A) cells, were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in the DMEM containing 10% fetal bovine serum (FBS), 50 units/ml penicillin-streptomycin at 37°C in a 5% CO₂ incubator.

Cytotoxicity assay

The breast cancer cells were seeded in a 96-well plate at a density of 10^4 cells/well and incubated at 37°C in 5% CO₂ atmosphere. Then, the cells were treated with serial dilutions of SNWE at concentrations of 0-100 µg/ml after 72 h of treatment incubation, 100 µl of MTT solution (1 mg/ml) was added and the color was allowed to develop by 4 h incubation. Finally, the formazan crystals were dissolved by adding 100 µl of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, USA) and the cell survival curves were generated.

Intracellular ROS measurement

The fluorescent probe 2,7-diacetyl dichlorofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS generation in MDA-MB-231 and MCF-7 cells (32). In brief, the MDA-MB-231 and MCF-7 cells (1×10⁶ cells per well) were seeded in 6 well plates, respectively. After the 72 h treatment, experimental cells were stained with DCFH-DA (1 µg/ml) for 30 minutes under dark conditions. Then, the cells were washed 3 times at 5 minutes intervals with PBS to remove excess dye staining. Images were acquired using a fluorescence microscope (FluoCell Imaging Station, Life Technologies, USA), and the fluorescence intensity was measured with the excitation at 485 ± 10 nm and emission at 530 ± 12.5 nm, using a microplate reader (Molecular Devices, USA).

Apoptosis assay

Acridine orange/ethidium bromide (AO/EtBr) dual staining assay protocol was used to detect the morphological changes during apoptosis (33). The MCF-7 and MDA-MB-231 cells (1×10⁶ cells per well) were seeded in 6 well
showed that SNWE treatment significantly increased intracellular ROS production in both the breast cancer cell lines. We observed that 0.1 mg/ml of SNWE treatment induced 92.7 ± 2.4% ROS levels in MCF-7 and 80.4 ± 5.09% ROS in MDA-MB-231 cells, respectively. In addition, the microscopic fluorescence images clearly showed increased DCF-fluorescence in SNWE-treated groups as compared to the control group; paclitaxel was considered a positive control (Fig. 2).

### Effect of SNWE on apoptotic morphological changes in breast cancer cells

The AO/EtBr dual staining was used to study the apoptotic morphological changes in cancer cells. We found that 0.1 mg/ml of SNWE significantly increased the apoptotic cellular populations in MCF-7 and MDA-MB-231 breast cancer cell lines (Fig. 3). The SNWE treatment induced plates, respectively. After 72 hours of SNWE treatment, both the cells were stained with AO/EtBr in the 1:1 ratio for 30 minutes. Then, the cells were washed with PBS three times. The images were acquired with a fluorescence microscope (Fluid Cell Imaging Station, Life Technologies, USA).

### Gene expression analysis

The total cellular mRNA was isolated using the Qia-gen mini RNAeasy kit following the manufacturer's instructions, and mRNA levels were quantified by Nano-drop Spectrophotometer (Thermo Scientific, USA). The custom-based pathway-focused gene expression profiling was analyzed by real-time RT-PCR array. Ten prominent apoptotic genes expression at the mRNA level in the SNWE-treated breast cancer cell lines were analyzed using the CFX-96 BIO-RAD real-time System. The designed PCR plates have apoptosis pathway genes and a house-keeping gene panel to normalize array data (HK1). The fold changes of gene expression analysis were plotted as heat map with clustergrams.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0.0 software package. The data are expressed as mean ± standard error of the mean (SEM) and the statistical significance (p≤ 0.05) of the group difference was analyzed using one-way ANOVA and post-hoc Duncan test to compare with control.

### Results

#### Effect of SNWE against MCF-7 and MDA-MB-231 breast cancer cell viability

We observed that SNWE induced cellular cytotoxicity in both luminal-A MCF-7 and triple-negative MDA-MB-231 breast cancer cells in a concentration-dependant manner (Fig. 1). The antiproliferative effect of SNWE was more prominent in MCF-7 cells as compared to MDA-MB-231 cells because of their absence of hormonal receptors. The IC50 values were found to be 4.26 µg/ml in MCF-7 and 5.30 µg/ml in MDA-MB-231 cells, respectively.

#### Effect of SNWE on intracellular ROS generation in breast cancer cells

The DCFH-DA dye was used to measure the intracellular ROS generation in MCF-7 and MDA-MB-231 breast cancer cells (Fig. 2). The spectrofluorometric reading
78.4±2.5% and 58.9±4.2% of apoptotic cell populations in MCF-7 and MDA-MB-231 cells, respectively. The positive control paclitaxel group showed both acridine orange and ethidium bromide staining. Whereas, the SNWE treatment groups showed concentration-dependent increase of apoptosis incidence in both MCF-7 and MDA-MB-231 breast cancer cell lines. Conversely, the untreated control cells showed no apoptotic cell population and stained only with the acridine orange (Fig. 3).

Effect of SNWE on gene expression analysis

The gene expression pattern of major genes involved in apoptosis such as BAX, p53, BAD, BAM, CASP3, CASP8, CASP9, BCL-XL, BCL2, and BCL-W was analyzed in both MCF-7 and MDA-MB-231 cell lines (Fig. 4). Treatment with SNWE (0.1 mg/ml) induced the mRNA level expressions of proapoptotic genes such as BAX, p53, BAD, BAM, CASP3, CASP8, and CASP9 and downregulated the gene expression of antiapoptotic markers such as BCL-XL, BCL2, and BCL-W. The fold changes in gene expression analysis clearly indicated that SNWE treatment regulates apoptotic signalling pathways in both MCF-7 and MDA-MB-231 cell lines to induce cytotoxicity.

Effect of SNWE on apoptotic markers by immunocytochemistry

To clarify whether SNWE treatment decreased the cell viability by activation of programmed cell death in MCF-7 and MDA-MB-231 breast cancer cells, BAX, p53, and caspase 3 were investigated by immunofluorescence analysis. The BAX, caspase-3 and p53 are the essential biomarkers for caspase dependant apoptosis. The results showed significant increases in BAX, Caspase-3 and p53 apoptotic markers in both MCF-7 and MDA-MB-231 cell lines (Fig. 5).
cells in the dose-dependent treatment of SNWE (12.5, 25.0 and 50.0 µg/ml) (Fig. 5). These results suggest that SNWE mainly activated the caspase-dependent apoptotic pathway, which has resulted in the cytotoxicity of the two tested breast cancer cells.

Discussion

The rising incidence of breast cancer and the adverse effects of anticancer chemotherapy are the major concerns of oncologists and cancer researchers. Traditional plant-based medicines offer treatment modalities for cancer because they actively suppress early and prolonged stages of tumorigenesis by blocking cell cycle arrest and promoting apoptosis (34). Solanum nigrum L. has been traditionally used for the treatment and prevention of various chronic diseases. Several bioactive medicinal compounds present in Solanum nigrum L. behave as cytotoxic agents in the acidic cancer cell environment. In this study, we tested the cytotoxicity of Solanum nigrum L. water extract (SNWE) in MCF-7 and MDA-MB-231 cells (Fig. 1). The IC50 values of SNWE in MCF-7 and MDA-MB-231 cells were found to be 4.26 µg/ml and 5.30 µg/ml, respectively. Several studies have proved the anticancer properties of Solanum nigrum L., including induction of apoptosis and cytotoxicity in numerous cancer types, such as breast, bladder, lung, colorectal and neuroblastoma cells (35). Ling et al (24) proved that Solanum nigrum L. aqueous extract showed cytotoxicity against MCF-7 breast cancer cells and exhibited no cytotoxic activity to normal breast MC-F12A cells. The withanolides, a steroidal lactone present in Solanum nigrum L., depletes ATP levels to induce anti-proliferative activity in three breast cancer cells, SKBR3, MCF-7, and MDA-MB-231 cells (36). Recently, thirteen bioactive steroidal alkaloids were isolated from S. nigrum L. and nine of them displayed better cytotoxicity in different types of cell lines such as HepG2, MCF-7, H1299, HeLa and HCT116, because of the open E-ring in their unusual structure (37).

The intracellular reactive oxygen species (ROS) generation leads to oxidative stress-mediated apoptotic cell death (38). We observed that the SNWE treatment induced ROS levels and subsequently caused apoptotic cell death in luminal-A MCF-7 and triple-negative MDA-MB-231 cells (Fig. 2). Aqueous extract of Solanum nigrum has been reported to markedly increase ROS generations and trigger mitochondrial apoptotic pathway in SCC-4 oral squamous cancer cells (39). Solanine, a steroidal glycoalkaloid found in S. nigrum L., induced ROS generation and activated the P38 pathway to suppress prostate cancer progression (40). Conversely, S. nigrum extracts restored oxidative status exposed by glutamate in rat astroglial primary culture cells which showed the antioxidant property of these extracts in particular cell types (18). The ROS generation by phytochemicals present in the SNWE might probably have caused oxidative DNA damage, leading to apoptosis. Collectively, the present data suggest that SNWE induces apoptotic cell death through ROS formation.

Various studies have proved that phytochemical-rich extracts isolated from Solanum species induce apoptosis by regulating several signaling pathways (41-43). The AO/ EtBr dual staining method shows evidence of SNWE on the induction of apoptosis. The SNWE treatment showed apoptotic morphological changes in both the breast cancer cells compared with untreated control cells (Fig. 3). Similarly, S. nigrum L. ethanolic fruit extract induced apoptosis and cell cycle arrest in the MCF-7 breast cancer cells (44). The complex mechanism of apoptosis is regulated by various pro-apoptotic and anti-apoptotic genes. It is activated by internal signals such as DNA damage, oxidative stress or loss of mitochondrial membrane potential (45). In our study, we noticed the upregulation of pro-apoptotic genes like BAX, P53, BAD, BAM, CASP3, and CASP9 and downregulation of anti-apoptotic genes such as BCL-XL, BCL2, and BCL-W in SNWE treated breast cancer cells (Fig. 4). These findings clearly indicate the role of SNWE in the induction of caspase-dependent mitochondrial pathway of apoptosis. Previously it has been found that aqueous extract of Solanum nigrum L. induces cell death in endometrial carcinoma cells via activation of caspase-3 dependent apoptotic pathway (46). Solanum nigrum L. bioactive polysaccharides inhibit tumor growth by activating caspase-3 and downregulation of Bel-2 in H22-bearing mice (47). The ethyl acetate fraction of Solanum nigrum L. has been shown to activate apoptotic cell death in HT29, MCF-7, and A2780 cells in a dose-dependent manner (48).

To further investigate the apoptosis-mediated cell death by SNWE treatment we examined the role of BCL-2 family members including BAX, Caspase3 and p53. These BCL-2 family members play an important role in inducing the intrinsic pathway of apoptosis (49). In normal conditions, BAX is localized in the cytosol, when the apoptosis is induced it undergoes a conformational change and is translocated to mitochondria to release further apoptotic factors. The results of the immunofluorescence assay showed SNWE treatment induced concentration-dependent overexpression of p53 and BAX in both the tested breast cancer cells (Fig. 5). The SNWE treatment induces the cascade reaction by activating caspase 3 to trigger the intrinsic pathway of apoptosis. Our study clearly demonstrates that SNWE augments caspase-dependent activation of apoptosis by activating its proapoptotic effects in the tested breast cancer cells. Recently, Li et al showed that Solanum nigrum L. methanolic extract induced the expression of apoptotic proteins like BAX, and cleaved caspase3 (50). Ding et al isolated six steroidal glycoalkaloids by alkaline precipitation and acid extraction methods and those compounds exhibited decreased BCL-2 and increased BAX expression which induced caspase3-dependent apoptotic activation (51,52). The aqueous extract of Solanum nigrum L. induced apoptosis in colorectal cancer cells in combination with anti-cancer drugs (46).

In this study, we noticed that the SNWE treatment showed better antiproliferative activity, intracellular ROS generation and apoptosis-inducing properties in luminal A MCF-7 cells than in triple-negative MDA-MB-231 cells. Although both the cell lines were of epithelial origin, they were different in their genetic make-up. It has been known that the MCF-7 is an ER+ breast cancer cell line and it responds well to conventional anticancer therapeutic agents. In contrast, the MDA-MB-231 is a triple-negative breast cancer cell line that is hard to treat by conventional anti-cancer treatments. Our results suggested that SNWE treatment can trigger caspase-dependent apoptotic cell death in both MCF-7 and MDA-MB-231 breast cancer cells.

There are five stages of breast cancer and the disease is highly treatable in its early stages. It is anticipated that tra-
ditional medicines such as *S. nigrum* L. can play an important role in the management of early-stage disease without appreciable side effects. This study confirms that *S. nigrum* L. water extract (SNWE) significantly induces cytotoxicity by ROS induction, apoptotic morphological changes and nuclear alterations in MCF-7 and MDA-MB-231 cell lines. Further, the mRNA level gene expression analysis and immunofluorescence assay of apoptotic markers confirm the apoptosis-inducing capacity of SNWE in two tested breast cancer cells. We noticed that the SNWE treatment caused significant anticancer potential in luminal A-type cancer cells than the triple-negative breast cancer cells. Our findings suggest that SNWE could be used as a lead candidate for anticancer drug development. Further in-vivo studies need to be carried out to investigate the anticancer effects of SNWE using animal models of cancer.

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**References**


