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Anti-Epithelial-Mesenchymal Transition Property of *Arnica montana* Attributed to Mitochondria-Mediated Apoptosis in Triple-Negative Breast Cancer

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Abstract: Triple-negative breast cancer (TNBC) has a poor prognosis due to its highly invasive and metastatic properties. Hormonal therapies are ineffective because of the absence of hormone receptors in TNBCs; treatment modalities primarily depend on aggressive chemotherapy regimens and surgery. These procedures have a high degree of systemic toxicity and lead to drug resistance. Therefore, novel drugs with less or no toxicity are the need of the hour. *Arnica montana* has potent anticancer properties against hormone-dependent breast cancer. In the current study, we explored the anti-epithelial-to-mesenchymal transition (EMT) ability of *Arn* in TNBC. Cell invasion and migration assays were conducted on the TNBC cell line, MDA MB231, using *Arnica montana*. The expression of major genes playing a role in apoptosis and EMT were examined through real-time PCR. *Arnica montana* displayed a reduction in cell viability, invasion, and migration of MDA MB231 cells. Additionally, gene expression analysis showed that there was upregulation of apoptotic genes, Caspase 3 & Caspase 9, and epithelial marker E-cadherin along with downregulation of mesenchymal markers N-cadherin and Vimentin and anti-apoptotic marker Bcl-2. *Arnica montana* inhibited the progression of metastasis and EMT under *in vitro* conditions. These results lay down the foundation for future studies in animal models to elucidate its potential therapeutic role in TNBCs.

Keywords: TNBC; *Arnica montana*; homeopathy; EMT; apoptosis; gene expression analysis.

1. Introduction

According to World Health Organization, cancer is a worldwide killer, with about 10 million deaths in 2020 [1]. Among all, breast cancer is now the most common cancer [2]. They may be broadly classified into hormone-dependent and hormone-independent, depending upon the presence and absence of the hormone receptors (HR) on the breast cells [3]. The triple-negative breast cancers (TNBC) do not have any of the hormone receptors like estrogen receptors (ER), progesterone receptors (PR), or human epidermal growth factor receptor 2 (HER2) and possess highly invasive and metastatic properties and thus have poor prognosis [4]. TNBCs account for 15-20% of all breast cancers. Since there are no hormone receptors in TNBCs, hormonal therapies are ineffective; hence, treatment modalities primarily depend on aggressive chemotherapy regimens and surgery [5–7]. Harsh chemotherapies have been documented to adversely impact the survival of normal cells developing high systemic toxicity

and resistance [8, 9]. Therefore, new treatment strategies with anti-tumor properties vis-à-vis not toxic to the normal cells are required.

In cancers, the epithelial cells tend to transform into mesenchymal phenotype, known as epithelial-to-mesenchymal transition (EMT) [10]. This EMT facilitates the invasion and migration of cancer cells to distant places [11, 12]. Anticancer therapies need to reverse this EMT to arrest metastasis [13, 14]. Apoptosis is a process of cell death that is programmed to maintain homeostasis [15]. Alteration in this process may lead to uncontrolled growth and proliferation of cells leading to cancers [16]. Therefore, the regulation of apoptosis is an important aspect of any anticancer therapy [13, 17].

Arnica montana (*Arn*) is a plant-origin medicine that exhibits antiseptic, anti-inflammatory, antibacterial, antifungal, and anti-tumor properties [18–20]. This medicine is widely used in homeopathy for pathological conditions with pain, swelling, and stiffness due to traumas [21, 22]. Ethanolic extract of *Arn* has exhibited potent cytotoxic effects on MCF 7 breast cancer cells in earlier studies done by our group [23]. Therefore, in this study, we investigated the cytotoxic effects of serially diluted and agitated *Arn* in the TNBC cell line, MDA MB231.

2. Materials and Methods

2.1. Cell line used.

TNBC cell line, MDA-MB231, used in the study, was procured from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, USA) containing 10% Fetal Bovine Serum (FBS) (Gibco, Life Technology Ltd., Paisley, UK) and 1% Penicillin-Streptomycin (PS) (Gibco, Life Technology Ltd., Paisley, UK) in a humidified incubator with 5% CO_2 and 37ºC temperature.

2.2. Medicine used.

Ethanolic extract of *Arn*, also known as *Arn* Mother Tincture (*Arn* MT), including serially diluted and agitated *Arn* 6C potency (diluted 10^{-12} times), 30C potency (diluted 10^{-60} times), and 200C potency (diluted 10^{-400} times) were procured from Dr. Willmar Schwabe India Pvt. Ltd. that was manufactured as per Homoeopathic Pharmacopeia of India. To rule out the possibilities of toxicity due to 90% alcohol in the medicines used, we considered using 90% v/v alcohol as the solvent control (SC) in all the experiments. Doxorubicin (Sigma-Aldrich, St. Louis, USA) was used as the positive control.

2.3. Apoptosis analysis using flow cytometry.

Applying the theory that AnnexinV-FITC stains the apoptotic cells while PI stains the late apoptotic or necrotic cells, dual staining with AnnexinV/PI was carried out to analyze apoptosis using flow cytometry. Briefly, $2x10^5$ cells were seeded in 60mm culture dishes and treated with the medicines and SC for 96 hours. The cells were harvested and washed with ice-cold PBS. $1x10^6$ cells were counted and resuspended in 1X binding buffer, and 5µl each of AnnexinV-FITC and PI was added to the samples and incubated for 10 minutes per the manufacturer's protocol (BD AnnexinV-FITC Apoptosis Detection Kit, BD Biosciences,

Mountain View, CA, USA). Then they were analyzed by Accuri C6 flow cytometer (BD Biosciences, USA).

2.4. Clonogenic assay.

The clonogenic assay is an *in vitro* colony-forming assay to assess the ability of a single cell to form a colony that can determine the effectiveness of an anti-proliferative agent. MDA MB231 cells (100 cells/well) were seeded in six-well plates. After initial treatment for 96 hours, the treated media was removed, and fresh media (without treatment) was added every 3rd day and kept for 10 days. After 10 days, when the colonies were formed, the plates were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, USA), and 0.5% crystal violet (Sigma-Aldrich, St. Louis, USA) was used to stain the colonies. The dishes were rinsed under running water and air-dried overnight. The colonies were then photographed and counted using Image J software.

2.5. Wound healing assay.

A wound healing assay is done to assess the migration property of adherent cells *in vitro*. Briefly,1x10^5 cells were seeded in each well of a six-well plate, and scratches were made by a 200µL pipette tip when 80% confluence had been attained by the cells in the culture. This wound healing assay is a useful guide to measure cell migration by interacting the cells with extracellular matrix and cell-cell interactions. After treatment, the gaps were photographed under a phase-contrast microscope (EVOS EL Core) initially at 0 hours and then at 48 hours. The images were quantitatively analyzed with Image J software. The distance between the wounds of the treated samples was calculated (µm) and compared with that of the SC.

2.6. Gene expression analysis.

The total RNA was extracted by TRIzol (Invitrogen, United States) following treatment for 96 hours. Precipitation was done by chloroform and isopropanol. DNase (Qiagen) was used to eliminate impurities of DNA from isolated mRNA, and Nanodrop (Thermo Fisher Scientific) was used to check the purity. Synthesis of cDNA was done with Verso cDNA Synthesis Kit (Thermo Fisher Scientific), as per the manufacturer's protocol, by reverse transcription. To quantify the relative mRNA expression levels of selected genes, real-time PCR was carried out with the StepOnePlus Real-Time PCR System (Applied Bioscience) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Primer sequences were obtained from the National Centre for Biotechnology Information (NCBI) database (National Library of Medicine, United States). The sequences for forward and reverse primers used for specific amplifications are given in Table 1. GAPDH was used as the reference gene. Real-time qPCR was performed for 40 cycles under the following conditions: initial denaturation at 95°C for 3 minutes, denaturing at 95°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and the melt curve was recorded at 55 to 95°C (in 0.5°C increments). The 2^-^{ddCt} method was used to calculate the relative fold change in gene expression.

Table 1. Primer sequences of Bcl-2, Caspase 3, Caspase 9, E-cadherin, N-cadherin, Vimentin, and GAPDH genes.

Gene	Forward primer	Reverse primer
Bcl-2	5'GTGGCCTTCTTTGAGTTCGGT3'	5'GTGCCGGTTCAGGTACTCAGT3'
Caspase 3	5'CAAAGAGGAAGCACCAGAACCC3'	5'GGACTTGGGAAGCATAAGCGA3'

Gene	Forward primer	Reverse primer
Caspase 9	5'AGGCCCAAACCAAGGGTAAA3'	5'GAGGAGACAGGGAAGGCAAC3'
E-cadherin	5'GAACAGCACGTACACAGCCCT3'	5'GCAGAAGTGTCCCTGTTCCAG3'
N-cadherin	5'GACGGTTCGCCATCCAGAC3'	5'TCGATTGGTTTGACCACGG3'
Vimentin	5'CGGGAGAAATTGCAGGAGGA3'	5'AAGGTCAAGACGTGCCAGAG3'
GAPDH	5'AGCCACATCGCTCAGACA3'	5'TGGACTCCACGACGTACT3'

2.7. Statistical analysis.

The data were obtained from three independent experiments performed in triplicates and presented as the mean ± standard deviation (SD). Statistical analysis used student's t-test as well as ordinary one-way analysis of variance (ANOVA) with Dunnett's tests for multiple inter-group comparisons, using GraphPad Prism 8 software. A p-value of <0.05 was taken as statistically significant.

3. Results and Discussion

Hormone-independent TNBCs are very aggressive and have a poor prognosis, and their treatment options are harsh [24]. Therefore, new therapeutic agents are needed to have a potent cytotoxic effect against the breast cancer cells vis-à-vis non-toxic to normal cells to reduce adverse drug effects. Numerous studies have shown the anticancer effects of phytochemicals that are used for cancer prevention and therapy [15, 16]. A number of such phytochemicals are used in homeopathy after serially diluting and agitating the mother solution [25, 26]. Earlier studies reported the use of essential oil from rhizomes and roots of Arnica to induce apoptosis, necrosis, and autophagy in glioblastoma multiforme and anaplastic astrocytoma cells [27]. Zitek *et al*. reported the anticancer activity of *Arn* in melanoma cells [19]. We have earlier reported the apoptotic and anti-migratory effect of the ethanolic extract of *Arn* in hormone-dependent breast cancer cells [23]. In the current study, we used *Arn*, to elucidate its apoptosis and anti-EMT potential in TNBC cells.

3.1. Arnica montana displayed apoptosis in MDA MB231 cells.

We evaluated the potential of *Arn* in hormone-independent breast cancer (MDA MB231) cells. Our findings showed apoptosis in the *Arn*-treated samples compared to SC and doxorubicin when analyzed by flow cytometry after dual staining with AnnexinV/PI. The dot-plot graphs showed the live cells (lower left quadrant), early apoptotic cells (lower right quadrant), late apoptotic cells (upper right quadrant), and necrosis (upper left quadrant). The cell population is represented as percentages of three independent experiments. MDA MB231 cells treated with Arn MT, 6C, 30C and 200C revealed increased apoptosis, 25.08±0.98, 17.60±0.23, 14.27±0.20 and 25.00±0.92 respectively, as compared to SC, 12.54±1.07 (Figure 1a). Figure 1b gives the graphical quantification of the flow cytometry data.

3.2. Arnica montana inhibited colony formation in MDA MB231 cells.

To assess the inhibitory effect after the treatment with different potencies of *Arn*, clonogenic assay was done and we found that *Arn* could inhibit the ability of a single TNBC cell to form discrete colonies as compared to SC and doxorubicin treated cells. The representative images revealed the number of colonies in MDA MB231 cells when visualized under a microscope (Figure 2.a.) after crystal violet staining. When quantified, both doxorubicin and *Arn* showed the reduced formation of the number of colonies, doxorubicin

(12±1), *Arn* MT (16±1), *Arn* 6C (44±4), *Arn* 30C (58±4) and *Arn* 200C (63±1) compared with the SC (71±2). (Figure 2.b.).

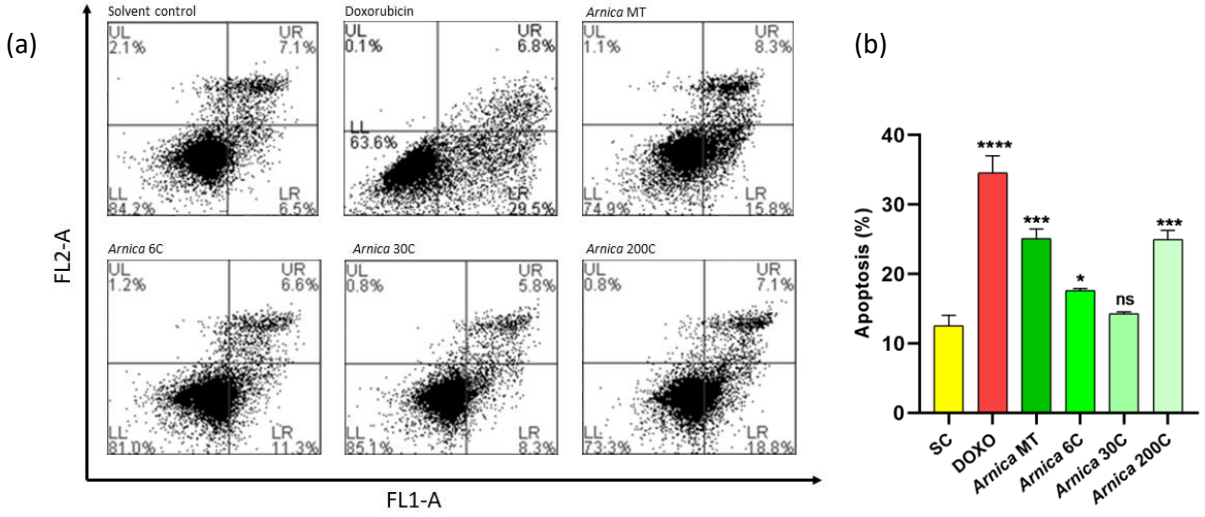

Figure 1. *Arnica montana* induces apoptosis in MDA MB231 cells. (**a**) Flowcytometric assessment of apoptosis by Annexin V-FITC/PI staining assay. LL-Lower Left quadrant (Live cells); LR-Lower right quadrant (Early apoptotic cells); UR-Upper right quadrant (Late apoptotic cells); UL-Upper left quadrant (Necrotic cells). The images are representative of three independent experiments. (**b**) Graphical quantification of flow-cytometry data. Data are presented as mean ± standard deviation (n=3). *p ≤ 0.05, ***p ≤ 0.001 and ****p ≤ 0.0001, indicates significant difference in percentage of apoptosis. (One-way ANOVA).

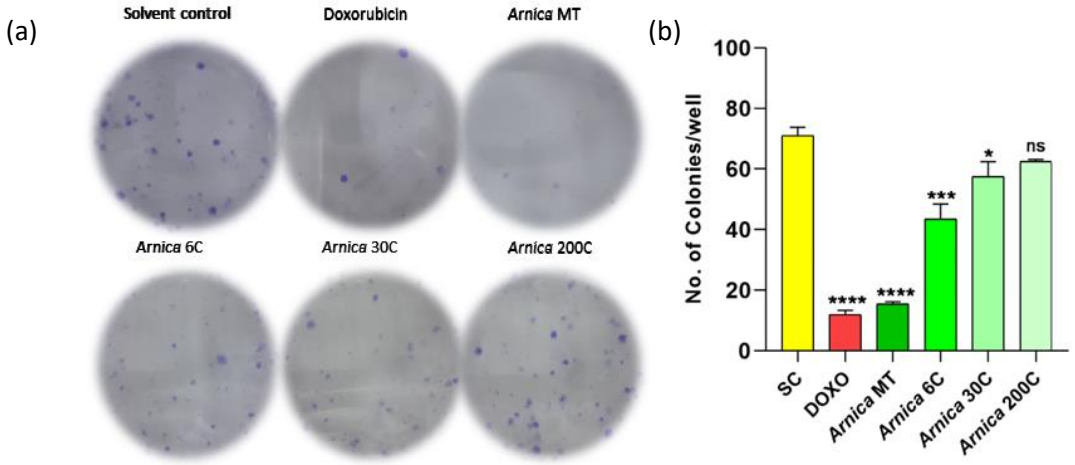

Figure 2. *Arnica montana* inhibited colony formation in MDA MB231 cells. (**a**)Representative images of colony formation, stained with crystal violet. Magnification: x10; scale bar: 100 µM; (**b**) Quantification of the number of colonies in each well. Data are presented as mean ± standard deviation (n=3). (* p < 0.05, *** p < 0.001, **** p < 0.0001. (One-way ANOVA).

3.3. Arnica montana inhibited migration in MDA MB231 cells.

Cancer cells migrate to distant sites, which leads to a poor prognosis of the disease. Our study showed that *Arn* MT, *Arn* 6C, *Arn* 30C, and *Arn* 200C inhibited migration of the TNBC cells into the cell-free area by 25±1%, 51.5±1.5 %, 61±2 %, and 60±2 %, respectively, in the scratch assay as compared to SC (106±6 %). Positive control, doxorubicin also inhibited migration by 51.5±1.5 %. (Figure 3.a.).

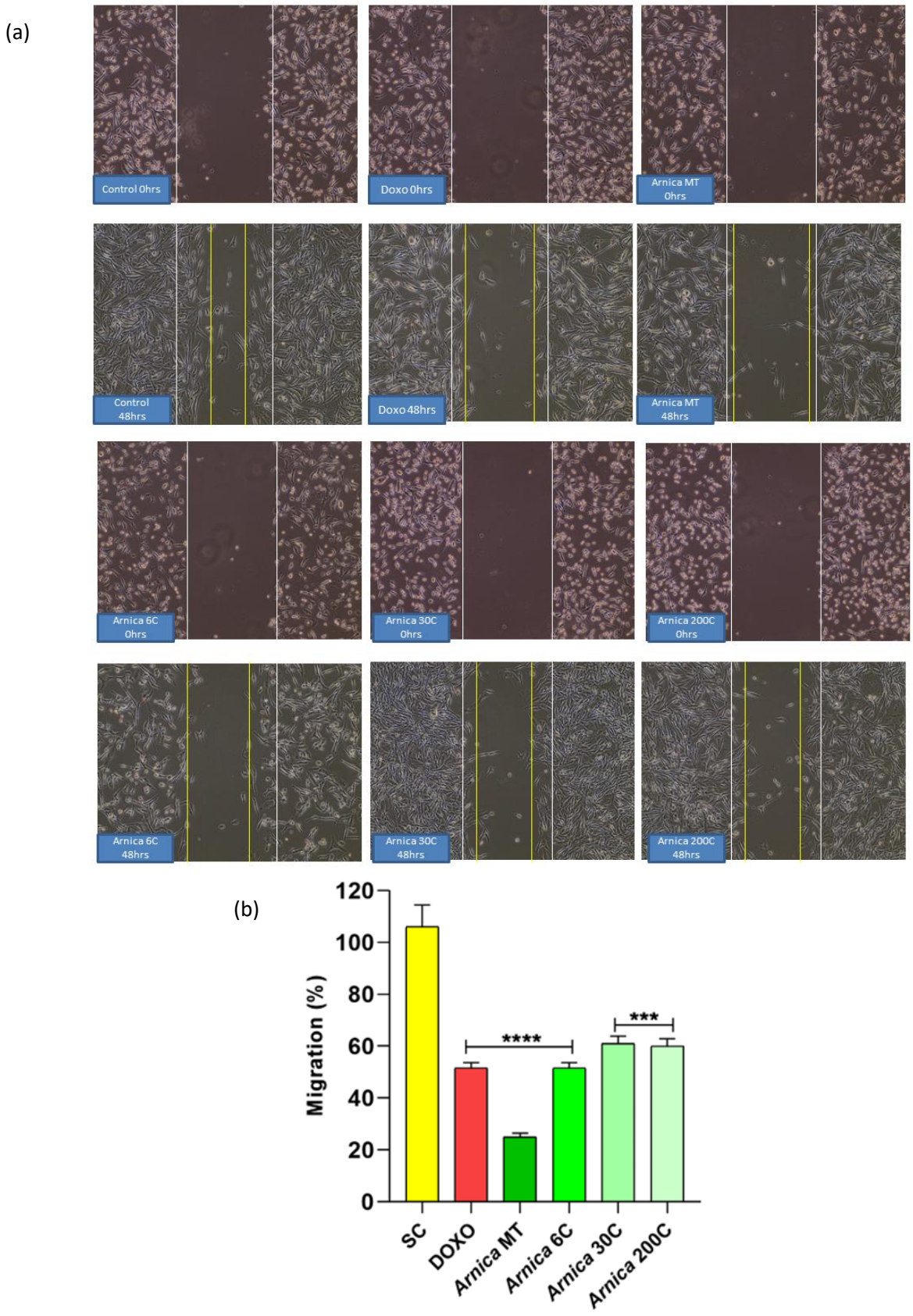

Figure 3. *Arnica montana* inhibits the migration of MDA MB231 cells. (**a**) Representative images of three separate experiments show the migration of MDA MB231 cells visualized under the microscope in the wound healing assay. The white lines depict the initial scratch made in the confluent dishes. The yellow lines depict the distance between the gap after 48 hours. Magnification: x10 scale bar: 100 μM; (**b**) Quantification of the results by subtracting the distance between the initial wound and the gap at the end of the treatment period and normalizing to solvent control. ***$p \leq 0.001$ and ****$p \leq 0.0001$ indicates a significant difference in the percentage of migration. (One-way ANOVA).

3.4. Arnica montana modulates gene expression in MDA MB231 cells.

Cytotoxic medicines induce apoptosis by initiating synchronous or subsequent activation of death receptor systems, disrupting mitochondrial function, proteolysis of caspases, DNA damage, and ROS generation [28–33]. Downregulation of Bcl-2 is an indication of mitochondrial-mediated apoptosis [34]. In our study, the mRNA transcripts of Bcl-2 were downregulated in *Arn* MT (0.53±0.09), *Arn* 6C (0.55±0.06), *Arn* 30C (0.4±0.02) as compared to the SC. Due to the downregulation of Bcl-2, caspase activities are initiated. The cytochrome C is released from the mitochondrial inner membrane, and an apoptosome converts pro-caspase 9 to caspase 9, which activates Caspase 3 [35]. Finally, Caspase 3 is responsible for the apoptosis of cancer cells [36]. We have seen that there was upregulation of initiator Caspase 9 in *Arn* MT (1.48±0.13), *Arn* 6C (1.4±0.12), *Arn* 30C (3.15±0.51), and *Arn* 200C (2.07±0.42) along with upregulation of effector Caspases 3 in *Arn* MT (1.48±0.13), *Arn* 6C (1.4±0.12), *Arn* 30C (3.15±0.51) and *Arn* 200C (2.07±0.42) against SC (Figure 4-7). Thus, these findings suggest that *Arn* in different potencies could induce apoptosis in the TNBC cells.

Epithelial-to-mesenchymal transition (EMT) contributes to tumor progression, cancer cell invasion, and therapy resistance [37, 38]. In our study, the epithelial marker, E-cadherin, was upregulated after treatment with *Arn* MT (1.2±0.08), *Arn* 6C (2.34±0.23), *Arn* 30C (1.77±0.15) and *Arn* 200C (2.1±0.21). Simultaneously, there was decreased expression of the mesenchymal marker, N-cadherin, when the relative expressions of the N-cadherin gene after treatments with *Arn* MT (0.53±0.03), *Arn* 30C (0.64±0.03) and *Arn* 200C (0.67±0.09) was assessed. We also found a relatively decreased expression of another mesenchymal marker of EMT, Vimentin, in *Arn* MT (0.75±0.01) and *Arn* 30C (0.71±0.11).

However, we did not find any changes in gene expression after treatment with *Arn* 6C for the mesenchymal markers and *Arn* 200C for Bcl-2 and Vimentin. Figure 4-7

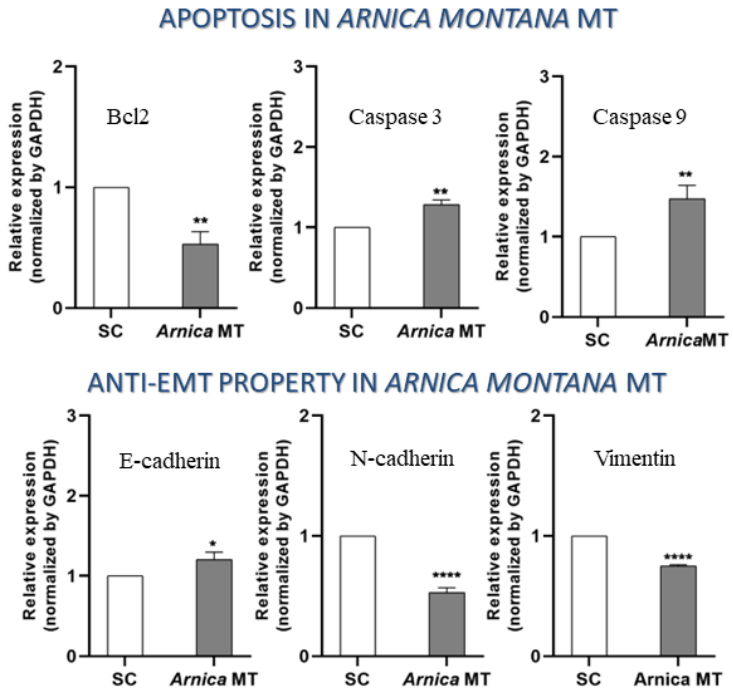

Figure 4. Response to *Arn* MT in TNBC cells. Bcl2, Caspase 3, Caspase 9, E-cadherin, N-cadherin, and Vimentin mRNA detected by real-time PCR. Results are representative of three independent experiments. Values are mean±SD. * $p < 0.05$, ** $p \leq 0.01$ and **** $p \leq 0.0001$ versus SC.

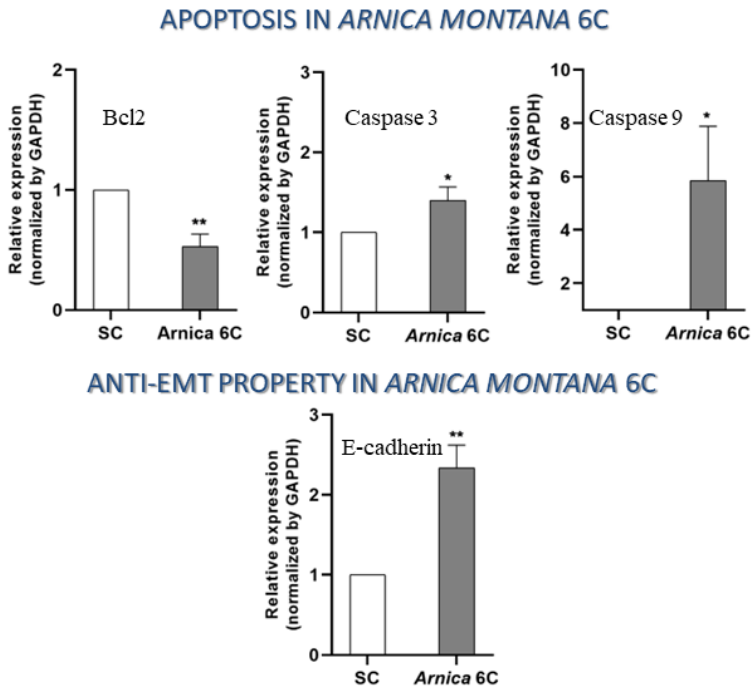

Figure 5. Response to *Arn* 6C in TNBC cells. Bcl2, Caspase 3, Caspase 9, and E-cadherin mRNA detected by real-time PCR. Results are representative of three independent experiments. Values are mean±SD. * $p < 0.05$ and ** $p \leq 0.01$ versus SC.

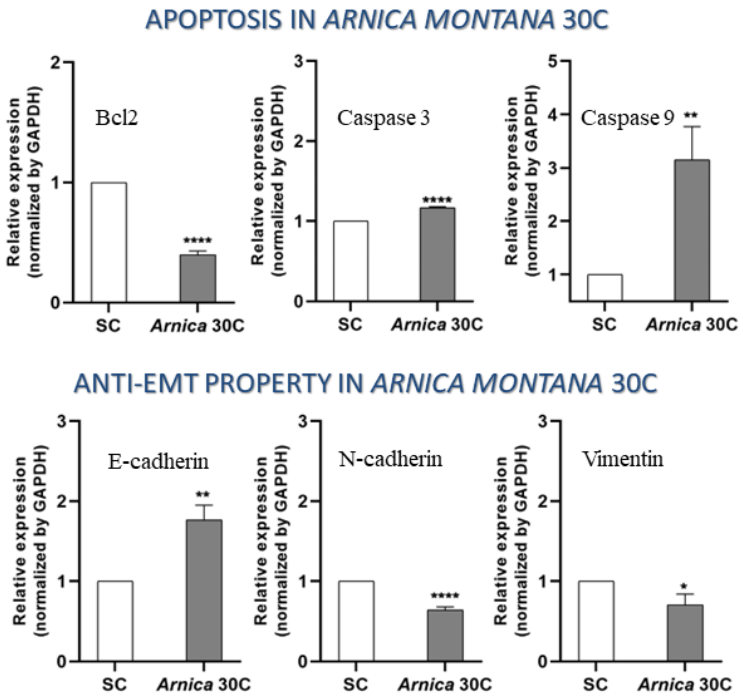

Figure 6. Response to *Arn* 30C in TNBC cells. Bcl2, Caspase 3, Caspase 9, E-cadherin, N-cadherin, and Vimentin mRNA detected by real-time PCR. Results are representative of three independent experiments. Values are mean±SD. * $p < 0.05$, ** $p \leq 0.01$ and **** $p \leq 0.0001$ versus SC.

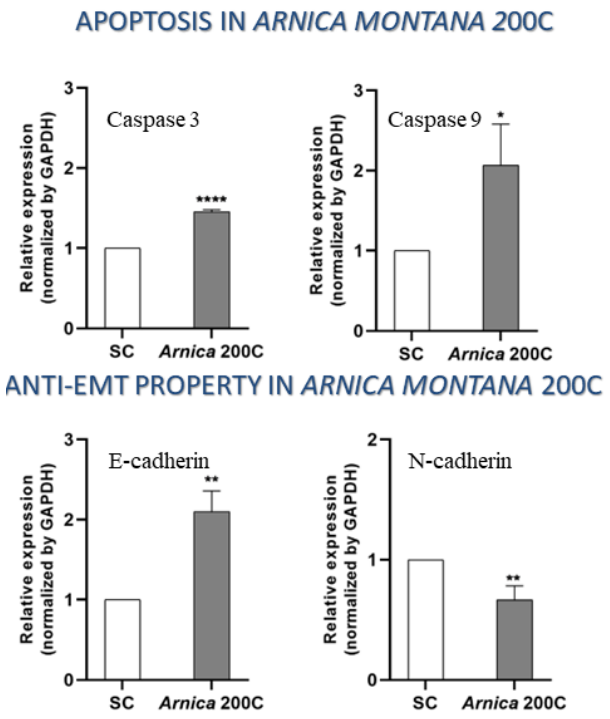

Figure 7. Response to *Arn* 200C in TNBC cells. Caspase 3, Caspase 9, E-cadherin, and N-cadherin mRNA detected by real-time PCR. Results are representative of three independent experiments. Values are mean±SD. * $p < 0.05$, ** $p \leq 0.01$ and **** $p \leq 0.0001$ versus SC.

4. Conclusions

Our data suggested that *Arnica montana* in various serially diluted and agitated potencies could inhibit the MDA MB231 cell proliferation and migration by altering the EMT pathway. It could also induce apoptosis via a mitochondria-mediated pathway, indicating that *Arnica montana* may serve as a new option in cancer therapy of TNBC (Figure 8).

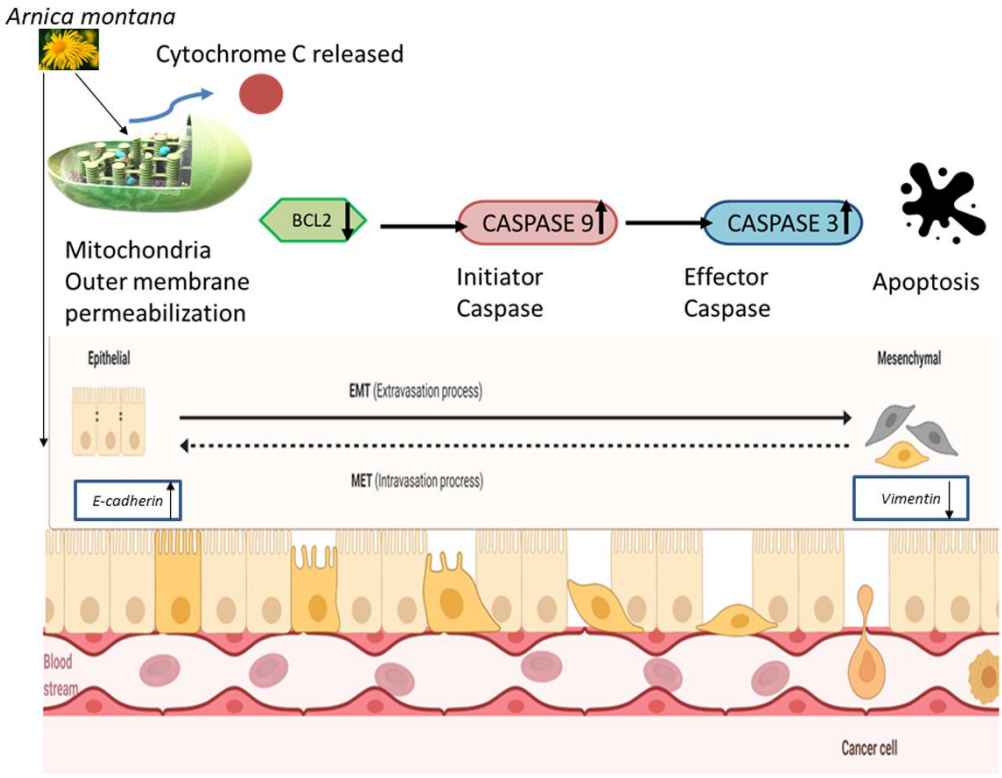

Figure 8. Graphical representation of the probable mechanism of action of *Arnica montana* on TNBC cells.

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Conflicts of Interest

The authors declare no conflict of interest.

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