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Suppressive Potential of *Rosmarinus officinalis* L. Extract against Triple-Negative and Luminal A Breast Cancer

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ABSTRACT

Rosemary is an aromatic plant with ancient and modern applications as a spice and herbal remedy. Due to the strong antioxidant potential of rosemary, the present study investigated the anti-proliferative and pro-apoptotic characteristics of rosemary on luminal A and triple-negative breast cancer cells. The effect of rosemary extract on the WNT10B and β-Catenin genes was also evaluated. The WNT10B and β-Catenin expression were measured by real-time PCR. The outcomes of the MTT assay and AnnexinV/PI flow cytometry assay showed that exposure of MCF-7 and MDA-MB-231 cells to rosemary reduced cell viability in a dose-time-dependent routine and promoted apoptosis in breast cancer cells. It was revealed that the extract could exert cytotoxic and apoptotic effects by downregulation of WNT10B and β-Catenin. Our results suggest rosemary as a promising complementary herbal medicine for breast cancers, without the adverse effects of chemotherapy drugs.

Keywords: *Rosmarinus officinalis*, luminal A, triple-negative breast cancer, anti-proliferative, pro-apoptotic, Wnt/ β-catenin

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Introduction

Breast cancer incidences have experienced an increase lately by 1.28-fold. This occurred in 27 years beginning in 1990. In addition to that, according to the global burden of the disease (GBD) (1, 2), there has been a projection that between the years 2020 and 2050, there will be an annual growth in the number of global incidences of BC (3). The terminal ductal-lobular unit is the main source of breast cancers (4). Breast cancers are classified into heterogeneous subtypes falling into two realms namely molecular profiles and gene signatures which entail Luminal A, Luminal B, HER2 overexpression, and Triple-negative breast cancers (TNBC) (4) Luminal A (ER+/PR+/HER2-/lowKi-67) being the most frequent molecular subclass of breast cancer, makes up for up to almost two-thirds of cases (60%-70%). The greatest favorable prognosis, low invasive potential, and positive response to endocrine therapy are its main categorizations. Despite this, cancer relapse may occur 5 years following its initial therapy (5). What makes it a vital medication for the luminal A subtype is Endocrine treatment, however, it falls under a limited efficacy by de novo and acquired tumor resistance (6). Approximately 10-20% of all breast carcinoma patients have triple-negative breast cancer (ER-/PR-/HER2-). Increased proliferation, earlier relapse, higher metastatic potency, and lower rates of survival are what TNBC demonstrates (7). When it comes to a mere alternative for TNBC, the role of systematic therapy of Chemotherapy is more highlighted, nevertheless, the success of conventional chemotherapy is below what is expected (8).

For the purpose of normal development and the maintenance of homeostasis, what is critical is apoptosis. In breast cancer, not only dysregulation of apoptosis is rampant but also plays a key role in resistance to therapy (9). A critical regulator of proliferation, differentiation as well as migration of cells is the Wnt signaling pathway which plays a pivotal role in the development of embryo and tissue homeostasis, but what has been indicated in a wide range of cancers, including breast cancer is the dysregulation of Wnt signaling. A key component of the pathway is the stabilization and nuclear translocation of β-catenin (10).

Being an indispensible part of Wnt signaling pathway, β-catenin is accountable for transporting Wnt signals to the nucleus and activating specific gene expression. The development of the embryo and maintenance of adult tissue in which β-catenin is involved has led to the concept that it has a wide range of interaction counterparts ensuring strong Wnt signaling (11).

When Wnt signaling is not present, what is targeted for degradation by a destruction complex involving APC (adenomatous polyposis coli) and glycogen synthase kinase 3β (GSK-3β) is cytoplasmic β-catenin. The destruction complex is prohibited parallel with Wnt ligands binding to their receptors, prompting the accumulation and nuclear translocation of β-catenin (12). When β-catenin is in the nucleus, it collaborates with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to trigger the expression of Wnt target genes involved in the proliferation, survival, and metastasis of cells (13).

Properly characterized as a triple-negative breast cancer cell, MDA-MB-231 is exceedingly invasive and metastatic. Studies have demonstrated that these cells show dysregulated Wnt/β-catenin signaling, instigating their aggressive phenotype. As cell proliferation, migration, and invasion increase, aberrant activation of the Wnt/β-catenin pathway is noticeable (14). The core concentration of research has been on

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differentiating the particular alterations of molecules that drive Wnt/β-catenin signaling in MDA-MB-231 cells and how breast cancer will progress in its wake. With the dysregulated Wnt/β-catenin pathway in MDA-MB-231 cells being targeted, implicit remedial strategies for prohibiting their aggressiveness and reducing metastasis may be offered (15).

The correlation between β-catenin, Wnt signaling, and MDA-MB-231 breast cancer cells triggers a pivotal realm of research to better understand the mechanisms of molecules involved in the progression of breast cancer.

To illustrate the correlation between traditional Wnt/β-catenin signaling and new β-catenin activation mechanisms, a map has been presented (16). When it comes to embryonic development and adult tissue maintenance, there is a group of nineteen different proteins namely Wnts which play vital roles through activating specific target genes. This pathway is affected by several protein interactions and compartments (17). Although understanding the pathway has been met with breakthroughs, there still exists numerous numbers of unanswered questions from discrepancies between canonical and non-canonical pathways to the various proffered interactions of β-catenin, and even differences within the Wnt protein family in Wnt-mediated gene transcription (11).

One of the pivotal parts of the Wnt signaling pathway is nuclear beta-catenin, which has a principal role in the development of various human tumors. When the Wnt ligand is not present, beta-catenin as a protein complex undergoes phosphorylation and degradation. Yet, by the time Wnt binds to its receptors, it prohibits this process and lets beta-catenin go into the nucleus and have specific genes activated (18). The regulation of the Wnt/beta-catenin pathway happens through various key components, such as the LRP receptor, Dickkopf (DKK) and Kremen proteins, GSK3beta, and adenomatous polyposis coli (APC), among others, which help to curb beta-catenin's activity and inhibit its oncogenic effects in cancer (19).

The Wnt/β-catenin signaling pathway promoted cancer cells to keep a balance between quiescence, proliferation, and regeneration and plasticity maintenance, proliferation, metastasis, and chemotherapy resistance in luminal and Triple-negative breast cancer cells (20).

Because of their chemical compounds, lower number of deleterious effects, decreased toxicity, and less amount of cost, the extracts and bioactive compounds from herbal remedies have been used in the therapy of adjuvant breast cancer. In addition, they can regulate the signals of hormones, inhibit proliferation, and angiogenesis, and improve chemotherapy and radiotherapy responses (21). *Rosmarinus officinalis* L., rosemary, an evergreen herb that is mainly grown in the Mediterranean is a member of the Lamiaceae family that can still be harvested around the world. It is used in various fields such as a flavor in dishes, a natural preservative in food production, and traditional and alternative herbal therapies (22).

Derived from rosemary, the phytocompounds are accountable for their therapeutic power which contains the following classes: phenolic diterpenes, phenolic acids, flavonoids, and triterpenes (23). In breast cancer cells, the anti-neoplastic elements of rosemary have been exhibited. Furthermore, by the regulation of cellular antioxidant systems, it applied antitumor effects and impeded signaling pathways that coordinate with tumor initiation, invasion, and drug resistance (24). FDA has confirmed the safety of its extract for human health. There has been a wider efficacy in its complete extract compared to each of its discovered bioactive ingredients, distinctively because of the synergistic effect of the combination of the bioactive

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composites, and feasibly other anti-malignancy components with little-known potential (25). In this paper, we evaluated the anti-proliferative, and apoptosis-inducing characteristics of rosemary on luminal and triple-negative breast cancer cells in the light of pursuing our previous research on herbal derivatives that hinder the progression of breast cancer via interfering with the wnt/β-catenin pathway (4, 26-28). Therefore, the effect of rosemary on the Wnt/β-catenin target genes (*WNT10B* and *β-Catenin*) was evaluated. It is noteworthy that the effect of rosemary on the mentioned genes has not been studied before and is completely novel.

Materials and methods

Preparation of rosemary extract

The fresh aerial parts of rosemary were gathered. The herb identifications were confirmed with the aid of authentic specimens deposited at the Herbarium of the Research Centre of Islamic Azad University. The dry leaves were transformed into powder using a blender. With the aim of extraction, 100gr of dry rosemary powder was added into a glass container, including 96% ethanol. The glass container was placed in our laboratory for 4-5 days at room temperature. Subsequently, the mixture was filtered, and the solvent was removed by applying a rotary evaporator device. Finally, the extract was kept at 20-25˚C for 24 hours to evaporate any remaining solvent.

Cell culture

The MCF-7 cell line, a luminal human breast cancer cell, was procured from the Stem Cell Research Center in Tehran, Iran. Meanwhile, the MDA-MB-231 cell line, a human triple-negative breast cancer cell, was sourced from the national cell bank of the Pasteur Institute, also located in Tehran, Iran. Both cell lines were cultured in DMEM high in Glucose from Gibco, UK, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and were incubated in a 5% CO2 atmosphere at 37 ˚C.

Cell viability assay

The impact of rosemary extract on the survival of MDA-MB-231 and MCF-7 cells was assessed through an MTT assay. 5×10^3 cells of each cell line were seeded in 96-well plates. The following day, the cells were exposed to different concentrations of rosemary extract (2, 5, 10, 15, 20, 25, 30, 40, 50, and 60 µg/ml for MDA-MB-231 cells and 5, 10, 20, 30, 40, 45, 50, 60, 70, 80 µg/mL for MCF-7 cells) for 24 and 48 hours. Subsequently, 200 mL of MTT (Sigma-Aldrich) solution (5 mg/mL in PBS) was added to each well and incubated for 4h at 37 ˚C. After removal of the solution, 100 mL of dimethyl sulfoxide (DMSO) was added, and the plates were agitated for 15 min. The absorbance of each well was measured at 570 nm using a microplate reader.

Apoptosis analysis

The Annexin-V-FLUOS Staining Kit (Roche) was applied for the apoptosis assay. MBA-MD-231 and MCF-7 cells were seeded in 6-well plates. The cell lines were exposed to doses of the rosemary extract for 24h (MCF-7 cells were added 20, 40, and 80 µg/ml, and MDA-MB-231 cells were treated with 15, 23, and 30 µg/ml). The following day breast cancer cells were collected and centrifuged. Afterwards, they were pipetted in 1000 µL of PBS at standard temperature and transferred to microtubes, where 20 µL of PI and

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Annexin V, were added to the control and treated specimens. The cancerous cells were analyzed instantly by flow cytometry.

Real-time PCR

The RNA was isolated from breast cancer cells treated with rosemary extract using TRIzol®. MCF-7 cells were treated with 40 µg/ml of extract, while MDA-MB-231 cells were treated with 23µg/ml for 24 hours. The quality and quantity of the extracted RNA were assessed using NanoDrop ND-1000 and agarose gel electrophoresis. Subsequently, complementary DNA was synthesized with the PrimeScriptTM RT Reagent Kit. Primers for *WNT10B*, *β-Catenin* (*CTNNB1*), and glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*) genes were designed using Oligo7 software. Table 1 displays the list of the primers used in the investigation. Quantitative PCR amplification was performed with a light cycler instrument, and the achieved threshold cycle (29) values were used for subsequent assessments based on the comparative Ct method. The expression levels of *WNT10B* and *CTNNB1* genes were normalized to *GAPDH*, resulting in the ΔCt value. The expression level of each target mRNA was measured using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Statistical analysis

Three independent evaluations of each test were carried out using the data analysis application GraphPad Prism v.10. (GraphPad Prism Software, Inc., San Diego, CA, USA). Student's *t*-test was applied to compare the mean values of each group to those of the control group. A p-value of less than 5 percent ($p<0.05$) was accepted as statistically significant. $p<0.05$, $p<0.01$ and $p<0.001$ are represented by *, **, and ***, respectively.

Results

Effect of rosemary extract on proliferation of MDA-MB-231 and MCF-7 cells

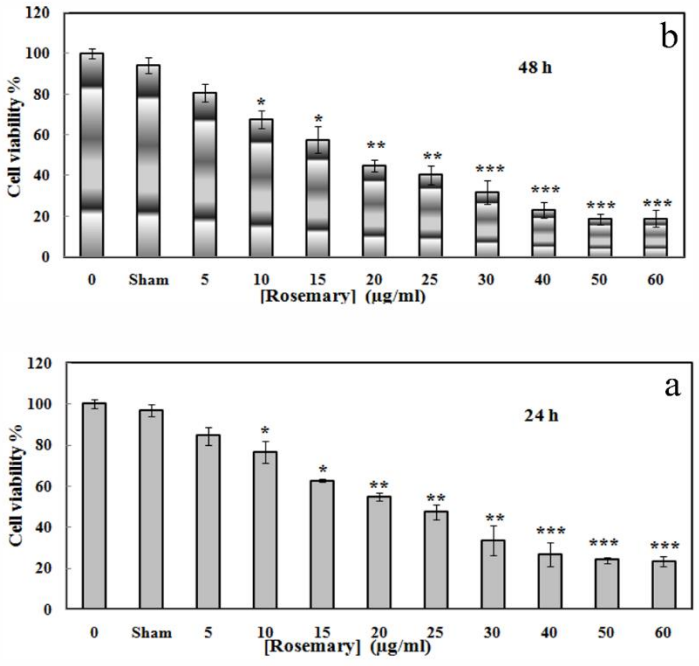

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Fig. 1. Effects of the different doses of rosemary extract (0-60µg/ml) on the proliferation of MDA-MB-231 cells: (A) 24h treatment, (B) 48h treatment. The results are displayed as a percentage of viability compared to control and are displayed as mean±SD from three independent experiments. Significance was set at *p<0.05; **p<0.01; ***p<0.00.1.

MTT assay demonstrated that treating MDA-MB-231 and MCF-7 cells with various doses of rosemary extract significantly decreased cell survival (Figures 1 and 2). The IC50 values of rosemary extract for MDA-MB-231 cells were 23 and 18 µg/mL for 24 and 48 hours, respectively. The corresponding IC50 values for MCF-7 cells were 40 and 28 µg/mL for 24 and 48 hours, respectively. It is noteworthy that the control group was treated with ethanol.

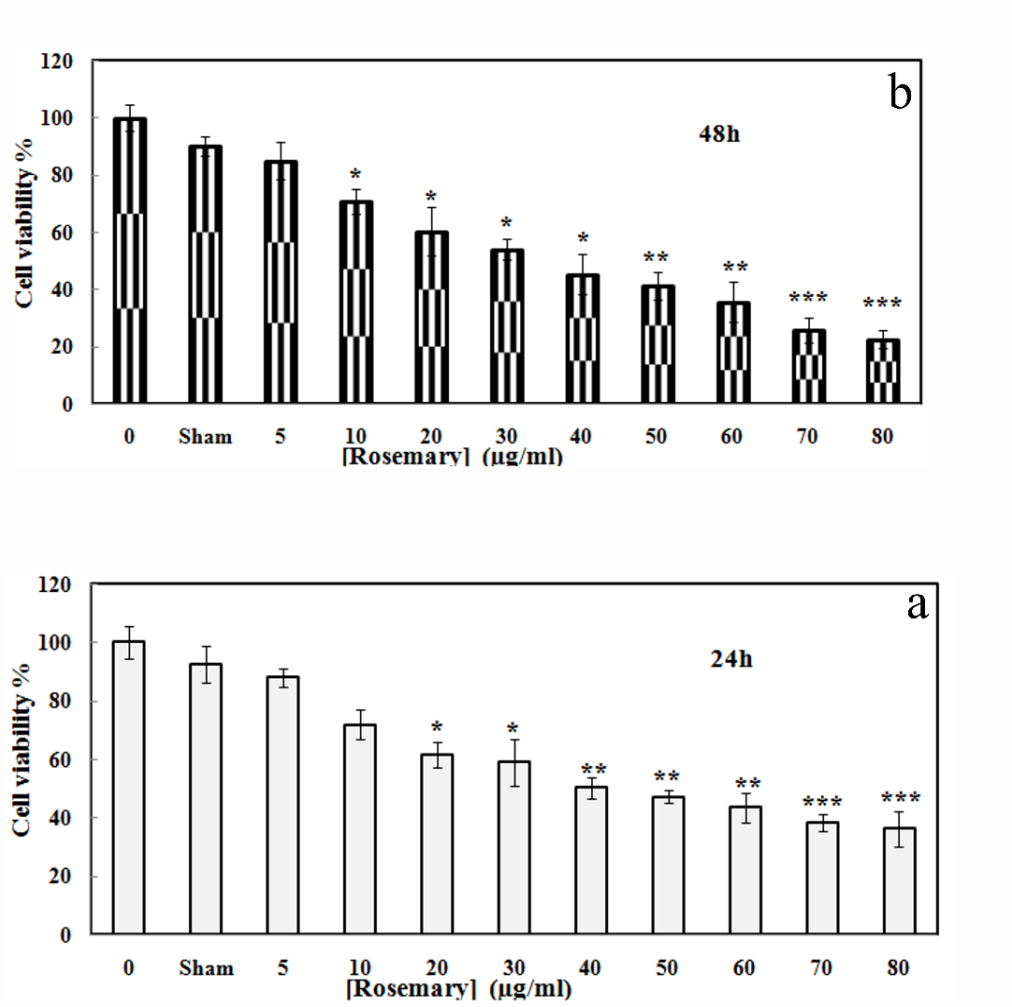

Fig. 2. Effects of the different concentrations of rosemary extract (0-80µg/ml) on the proliferation of MCF-7 cells: (A) 24 h treatment, (B) 48 h treatment. The results are displayed as a percentage of viability compared to control and are displayed as mean±SD from three independent experiments. Significance was reported at *p<0.05; **p<0.01; ***p<0.00.1.

Effect of rosemary extract on the induction of apoptosis in MDA-MB-231 and MCF-7 cells

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The quantitative analysis of apoptosis was conducted using the Annexin V/PI flow cytometry assay. After treating the MDA-MB-231 cells with 15, 23, and 30 µg/mL of rosemary extract for 24 h, the apoptosis rate was assessed at 35.2%, 49.6%, and 39.6% respectively (Figure 3A, B, C, and D).

As Figures 4 A, B, C, and D display, exposure of MDA-MB-231 cells with 20, 40, and 80 mg/mL of rosemary extract for 24 h, induced apoptosis in 15.3%, 44.6%, and 32% of the cells respectively.

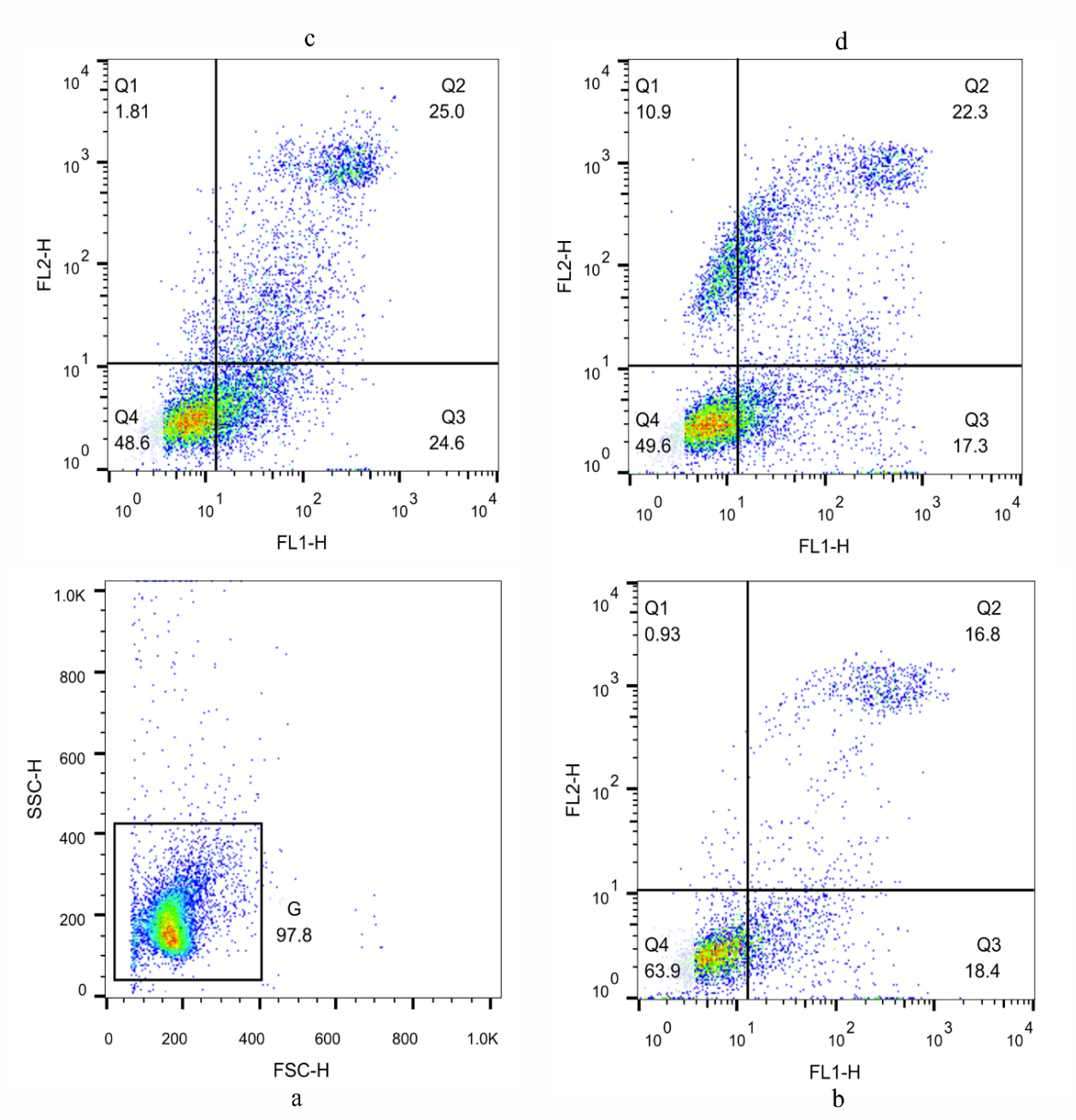

Fig. 3. The apoptosis-inducing effects of rosemary extract on MDA-MB-231cells measured by Annexin V/PI flow cytometry assay: (A) control, (B) 15 µg/mL, (C) 23µg/mL, (D) 30 µg/mL.

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Effects of rosemary extract on gene expression in MDA-MB-231 and MCF 7 cells

23 µg/ml of rosemary extract dramatically downregulated the *WNT10B* and *β-Catenin* expressions in MDA-MB-231 cells, i.e., 0.74 % and 0.53% in comparison with the control. Treatment of MCF-7 cells with 40 µg/ml rosemary extract attenuated the expressions of *WNT10B* and *β-Catenin* by 0.55% and 0.36% relative *to the control* (Figure 5 A and B).

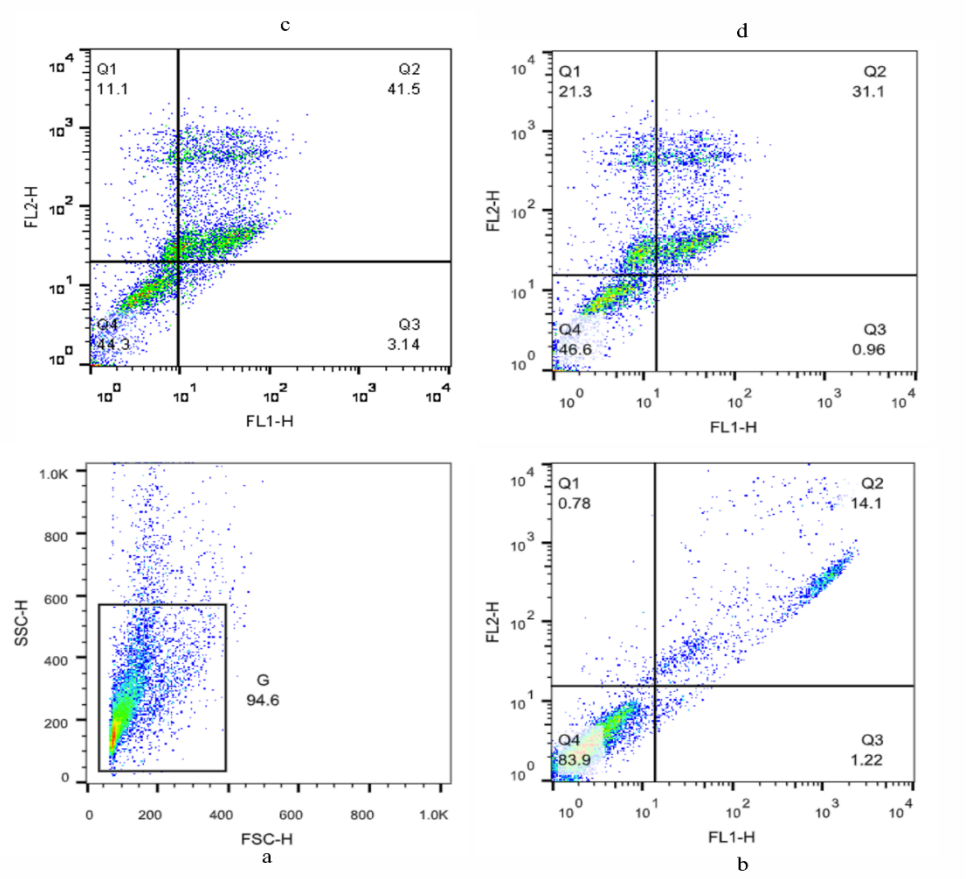

Fig. 4. The apoptosis-inducing effects of rosemary extract on MCF-7 cells measured by Annexin V/PI flow cytometry assay: (A) control, (B) 20 µg/mL, (C) 40 µg/mL, (D) 80 µg/mL.

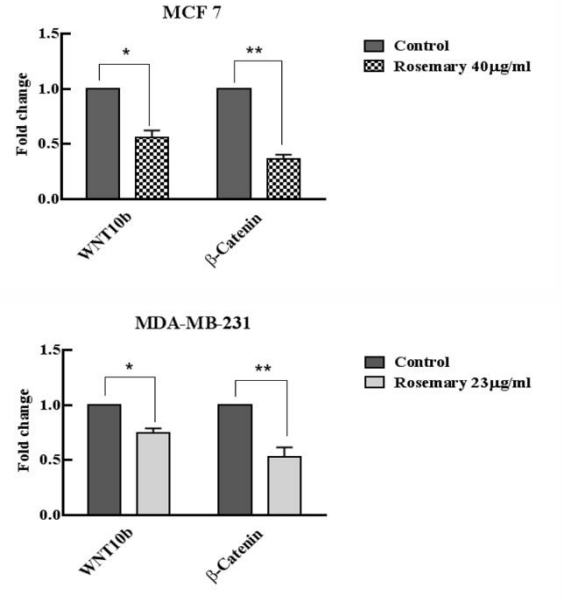

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Fig. 5. Effect of rosemary extract on the expression of WNT10B and β-catenin genes in breast cancer cells, assessed by Real-time PCR:(A) treatment of MDA-MB-231 with 23µg/ml of extract, (B) treatment of MCF-7 with 40 µg/ml of extract. The Data are shown as the fold change compared with *GAPDH* based on the Comparative Ct ($2^{-\Delta(\Delta Ct)}$) method, presented as mean±SD. Significance was set at *p<0.05;**p<0.01.

Discussion

Our outcomes illustrated the dose and time-dependent cytotoxicity of rosemary on MCF-7 and MDA-MB-231 cells. It should be noted that rosemary exhibited an anti-proliferative effect on MCF-7 cells at higher concentrations compared to MDA-MB-231 cells. Luminal A cells are more similar to normal mammary cells compared to TNBC cells. This can be attributed to the fact that Luminal A cells have low histological grade, modest nuclear pleomorphism, and low mitotic activity. Whereas, MDA-MB-231 cells are characterized by aggressive borders, significant nuclear pleomorphism, and numerous mitoses (2, 30, 31). This finding is in line with a study on crude ethanolic rosemary extract, which revealed that rosemary extract possessed anti-proliferative effects due to its anti-oxidant potential on human mammary adenocarcinoma. Furthermore, the extract acts more efficiently on MDA-MB-231 cells compared to MCF-7 cells (32). Our study affirmed the cell viability results since treatment of MCF7 and MDA-MB-231 cells with 40 and 23 µg/mL of rosemary (respectively corresponding to IC_{50} doses of MCF-7 and MDA-MB-231 cells) for 24 h accelerated apoptosis in about half of the malignant breast cells. It is worth noting that concentrations more than IC_{50} values promote necrosis in breast cancerous cell lines. These results are in contrast to a previous investigation by Gonz´alez-Vallinas *et al*., which assayed apoptosis induction by PARP1 cleavage analysis and claimed that supercritical fluid rosemary extract mediated death induction in MDA-MB-231 and MCF-7 cells by a distinct sort of cell death, whereof PARP1 cleavage was not detected in cancerous cells (33). Conversely, Jaglanian and Tsiani indicated that the medication of the TNBC cells (MDA-MB-231 cells) with rosemary extract resulted in a notable increase in cleaved PARP, hence the extract promoted the apoptotic pathway in MDA-MB-231 cells (34).

Aberrant expression of Wnt ligands has been associated with cancer stemness potential, tumorigenesis, and metastasis (35). WNT10B was originally regarded as an oncogene in the mammary gland (36). The elevated levels of WNT10B significantly enhanced the ability of breast cancer cells to proliferate, form colonies, and preserve their stem cell characteristics. Also, its over-expression increased carcinogenicity *in vivo* (37). The impact of rosemary extract on the WNT10B expression level has not been studied previously. Our data revealed the potential of rosemary to notably lessen the expression of WNT10B in MDA-MB-231 and MCF-7 cells. A previous study demonstrated a bilateral correlation between WNT10B and β-catenin, as WNT10B up regulated β-catenin/Tcf activity, and conversely, WNT10B expression was associated with β-catenin/Tcf activity (38). β-catenin is the most crucial hallmark for Wnt/ β-catenin signaling. Thus, it is considered a novel prognostic biomarker in breast cancer patients (39). β-catenin was expressed comparably in MDA-MB-231 and MCF-7 cell lines, which regulates cell migration, colony formation, and stem cell

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efficacy in vitro and tumor burden mice models of breast cancer (40, 41). In the present study, 24 h exposure of MDA-MB-231 and MCF-7 cells to rosemary extract showed a decrease in the expression of β-catenin.

The present investigation firmly establishes the anti-proliferative and pro-apoplectic potential of rosemary extract on human Luminal A cells and TNBC cells. It is illustrated that rosemary represented anti-survival and apoptosis-inducing effects on MCF-7 and MDA-MB-231 cell lines. However, the extract prompted higher cytotoxicity on MDA-MB-231 cells than on MCF-7 cells. Besides, it is demonstrated that the anti-tumor effects of rosemary can occur by impeding the wnt/β-catenin signaling pathway. Considering that rosemary extract has been approved as a safe compound for human health by the FDA and its anti-proliferative and pro-apoptotic potency on breast cancer cells, rosemary is proposed as an auspicious complementary herbal medicine for Luminal A and TNBC patients.

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