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RESEARCH ARTICLE

Effect of the Ethanolic Extract of *Eryngium billardieri* on the Viability of the Triple-Negative Breast Cancer Cell Line (MDA-MB-231) and *CDKN2A*, *MDM2*, and *HER2* Expression

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ABSTRACT

Objective: Currently, treating triple-negative breast cancer (TNBC) poses a significant challenge. *Eryngium billardierei* is widely employed as a medicinal plant, particularly for the treatment of cancers. However, there is a notable shortage of research investigating the effects of *E. billardierei* on TNBC. This study seeks to evaluate the anticancer capabilities of the ethanolic extract of *E. billardierei* concerning TNBC, as well as its impact on the expression of *CDKN2A, MDM2*, and *HER2*.

Materials and Methods: The MTT assay, N-acetyl-L-cysteine (NAC) assay, AO/EtBr dual staining, and flow cytometry were utilized to assess the anticancer potential of the extract of *E. billardierei* against the MDA-MB-231 cell line. In addition, the impact of *E. billardierei* extract on the expression of *CDKN2A, MDM2*, and *HER2* was examined using real-time PCR.

Results: The results conclusively demonstrated that the extract of *E. billardierei* exhibited a dose-dependent suppression of the growth of MDA-MB-231 cells. Moreover, treatment with the extract induced apoptosis in cancer cells. The inhibitory effect on growth was attributed to the generation of ROS, leading to a significant upregulation ($P<0.01$) of *CDKN2A* expression and a downregulation ($P<0.05$) of *MDM2* expression. Conversely, the expression of HER2 remained unaffected following extract treatment.

Conclusion: The outcomes of this study provide a foundation for considering the use of *E. billardierei* extract in the treatment of TNBC. However, additional research is warranted to investigate the mechanisms of *E. billardierei* extract across a broader spectrum of TNBC cell lines.

Keywords: *Eryngium billardierei*, Triple-negative breast cancer, *CDKN2A, MDM2, HER2*

INTRODUCTION

Breast cancer is a widespread disease that affects numerous women globally. It is a complex disease comprising various subtypes, each yielding distinct responses to clinical interventions. Among the several subtypes of breast cancer, triple-negative breast cancer (TNBC) stands out as one of the most challenging cancers to treat. Triple-negative is a categorization in immunohistochemistry for breast cancers that do not express human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and estrogen receptor (ER). These receptors play a pivotal role in the proliferation and development of cancer cells. HER2 is a membrane receptor of tyrosine kinases (RTKs) and is encoded by the *HER2* oncogene located on chromosome 17. In the majority of cases, aberrations in *HER2* expression, whether at the protein or gene level, have been associated with an unfavourable prognosis in both lymph node-positive and lymph node-negative breast malignancy.[1] TNBC is also characterised by overexpression of P-cadherin, fascin, caveolin 1 and 2, epidermal growth factor receptor (EGFR), and alpha-beta crystallin.[2]

A tumour suppressor protein frequently inactivated in cancer is p16INK4a, which is encoded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene. The *CDKN2A* gene (8.5 kb full length) includes three exons and two introns. Inactivation of $p16^{INK4a}$ has been documented as a potential contributor to cancer progression. Frequently, this inactivation is observed through promoter hypermethylation, homozygous deletion, or point mutations and has been identified in numerous cancers. Hence, managing abnormalities in CDKN2A regulation has potential benefits for cancer therapy.[3] CDKN2A is capable of ac-

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tivating the p53 tumour surveillance pathway by inhibiting and interacting with the p53 antagonist and murine double minute 2 (MDM2).[4] The MDM2 homologue protein is encoded by the proto-oncogene gene *MDM2*. The human *MDM2* gene is on the long arm of chromosome 12, specifically at the 13-14 position (12q13-14). The overexpression of MDM2 can be identified in several malignancies like colorectal, esophagogastric, lung, liver, and breast cancers.[5]

Nowadays, although recent methods of breast cancer therapy, such as surgery, radiation therapy, and chemotherapy, have made substantial developments in improving patient outcomes, there is growing interest in discovering complementary or alternative treatment options.[2] Recently, several researches focussed on the potential of plant-based compounds in breast cancer treatment. Countless natural plants contain functional components that can act as anticancer agents. Moreover, when compared with synthetic substances, natural products have been recognised as cost-effective, relatively non-toxic, and suitable for ingestion.[6] *Eryngium*, belonging to the Umbelliferae family, comprises 274 species, nine of which are native to Iran. Remarkably, *E. billardierei* is widely utilised as a medicinal plant globally for treating several ailments.

Earlier studies have shown that extracts derived from both the aerial and root components of *E. billardierei* exhibit antibacterial, anti-inflammatory, antinociceptive, and antioxidant impacts.[7,8] Several research studies have shown that *E. billardieri* has cytotoxic effects on diverse cancerous cell lines. Roshanravan et al. demonstrated that n-hexane and dichloromethane extracts of *E. billardieri* significantly induced apoptosis in pancreatic cancer cells by incrementing Bax and reducing cyclin D1 mRNA expression.[7] The cytotoxic effects of *E. billardieri* on hepatocellular carcinoma (HepG-2), breast adenocarcinoma (MCF-7), lung carcinoma (A-549), and colorectal adenocarcinoma (HT-29) cell lines were also confirmed.[9] In a recent study by Hasanbeiglu et al., it was detected that non-terpenoid compounds present in *E. billardierei* could be accountable for the cytotoxic impacts observed in the aerial part extracts of this plant on MCF-7 and musculus skin melanoma (B16) cell lines.[10]

Although previous studies have emphasised the anticancer impacts of *Eryngium* species in certain cancerous cell lines,[7, 9-11] research specifically addressing the impact of *E. billardierei* on TNBC remains limited. Therefore, the objective of this study was to assess the anticancer potential of the ethanolic extract of *E. billardierei* against the MDA-MB-231 cell line and to explore its impact on *CDKN2A, MDM2*, and *HER2* expression. The findings from this study could offer a foundation for considering the use of *E. billardierei* for treating TNBC.

MATERIALS AND METHODS

Materials

The TNBC cell line MDA-MB-231 (ATCC® HTB-26™, USA) was purchased from the Iranian Biological Resource Centre (IBRC), Iran. The cells were stored in a 5% CO_2 humidified atmosphere incubator at 37 °C. MTT (Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and acridine orange (AO) were acquired from Sigma-Aldrich Co., USA. The Annexin FITC kit was purchased from IQ Product, Netherlands. Dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), and N-acetylcysteine (NAC) were obtained from Merck, Germany. The fetal bovine serum (FBS), streptomycin, penicillin, and RPMI-1640 medium were prepared from BioIdea, Iran. The plant materials were acquired from a local market in Tehran, Iran. The voucher specimen (No. IAUH-12161) was authenticated by the Avicenna Herbarium, Azad University, Iran. An easy cDNA synthesis kit was purchased from Parstous Co., Iran. RNX-Plus was obtained from Sinaclon Co., Iran. From Ampliqon. (Odense, Denmark), the SYBR Green real-time Master Mix kit was obtained.

Preparation of the Ethanolic Extract

The extraction procedure was modified on the basis of methodologies outlined in prior research.[12] In summary, the petal and sepal portions of *E. billardieri* were dried in an oven and ground into a fine powder using a mechanical grinder (Retsch, Germany). Subsequently, 50 g of plant powder was macerated in 500 mL of ethanol (70% v/v), covered with aluminium foil, and placed in a shaker at 25 °C. After 72 h, the extract was filtered through Whatman filter papers and then centrifuged at 3500 rpm for 20 min. Condensation was performed using a rotary evaporator (Heidolph, Germany). The supernatant was then dried at 37 °C, resulting in a semisolid mass stored at 4 °C for further analyses.

Samples Preparation

MDA-MB-231 cells in the log phase were trypsinize with trypsin-EDTA and seeded in 96-well plates. Afterward, the cells were washed with PBS buffer, and 10^5 cells/well were seeded into 96-well plates, and after that 180 µL growth medium was added to each well. The cells were randomly divided into three groups, consisting of one control group and two experimental groups. The experimental groups were exposed to varying concentrations (10, 20, 30, 40, 50, 75, 100, 250, 500, and 1000 µg/mL) of the ethanolic extract of *E. billardierei* for 24 and 48 h.

Cell ViabilityAssay

After treating MDA-MB-231 cells in both control and experimental groups for 24 and 48 h, 0.5 mg/mL MTT (20 µL) was

added to each well of 96-well plates. Subsequently, the cells were incubated for 3 h in a CO_2 incubator at 37 °C. Following incubation, the formed insoluble formazan was dissolved in 100 µL of DMSO and thoroughly mixed. The optical density (OD) of each well at 570 nm was measured against a reagent blank using an ELISA reader (Model 680, Bio-Rad Inc., USA). Each experiment was replicated three times.

Measurement of Reactive Oxygen Species (ROS) Generation

The influence of the *E. billardieri* extract on ROS generation, both in the absence and presence of NAC, was investigated. NAC powder was dissolved in RPMI 1640, and subsequently, MDA-MB-231 cells were pretreated with 5 mM NAC for 3 h in a CO_2 incubator at 37 °C. Following this pre-treatment, the cells were exposed to the *E. billardieri* extract. After a 48-h treatment of the cells, 0.5 mg/mL MTT (20 µL) was introduced into each well of 96-well plates, and cell viability was examined.

Flow Cytometry Analysis

A total of 10^6 cells/well were seeded into 6-well plates for 48 h and subsequently treated with *E. billardieri* extract. Following treatment, the cells were collected by centrifugation at 1000 g for 5 min and washed twice with PBS buffer (0.01 M, pH 7.4). Subsequently, the cells were suspended in 100 µL of Annexin V binding buffer. The cells underwent double staining with a 5 µL solution of PI (Propidium iodide) and a 5 µL solution of FITC-labelled Annexin V. Samples were then incubated for 30 min in the dark at 25 °C and later analysed via flow cytometry applying BD FACSCalibur™, BD Biosciences Inc., USA.

AO/EtBr Dual Staining

MDA-MB-231 cells, cultivated in 6-well plates at a density of 10^4 cells/well, were treated with *E. billardieri* extract. The cells were then washed three times with PBS, and 9 µL of the cell suspension was stained with 1 µL of a dye mixture (100 mg/mL EtBr and 100 mg/mL AO in PBS). The stained cell suspension (10 µL) was applied to a clean microscope slide and promptly observed using an Axoscope 2 plus fluorescence microscope (Zeiss, Germany).

Extraction of RNA

Total RNA was extracted from MDA-MB-231 cells treated with the ethanolic extract of *E. billardierei* using an RNA isolation kit. The extracted RNA was immediately frozen in liquid nitrogen and stored at 75 °C. The purity of purified RNA was determined by measuring absorbances at wavelengths of 260 and 280 nm using a UV spectrophotometer. The ratio of absorbances was calculated to assess total RNA purity. Furthermore, agarose gel electrophoresis (1.2% w/v) of the extracted RNA (15 µL) was conducted in 1X TBE buffer at 80 V for 30 min. The gel was stained with a solution of EtBr and visualised under UV illumination.

cDNA Synthesis and Real-Time Quantitative PCR

The expression of *CDKN2A, MDM2*, and *HER2* genes was evaluated in both control and experimental samples. A total of 1 µg of RNA was utilised for cDNA synthesis, and real-time PCR was conducted using the SYBR Green real-time Master Mix kit. The PCR conditions included an initial denaturation at 95 ºC for 2 min, followed by 40 cycles of denaturation at 95 ºC for 15-20 sec, and annealing at 55-65 ºC for 60 s. The relative expression was calculated by applying the REST 2009 software (version 2.0.13). Gene-specific primers were designed using Primer Express 3.0 software and confirmed for specificity through a BLAST search. Each primer was synthesised by Yekta Tajhiz, Iran. Two negative controls were included in each PCR reaction, one without RT (reverse transcription) treatment (minus-RT) and one without cDNA template. From the same RNA, the experiments were repeated with a cDNA template synthesised three times. For each sample, the mRNA levels represent the mean value of data obtained from three independent real-time PCR trials. Lastly, alterations in fold expression were analysed using the comparative CT method,[13] with normalisation performed through the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene serving as the housekeeping gene (Eq. 1). The comparative CT method makes various assumptions, including that the efficiency of the PCR is close to 1 and that the PCR efficiency of the interest gene (here are *CDKN2A, HER2*, and *MDM2*) is similar to the internal control gene.

$$\text{Fold change} = 2^{-\Delta\Delta C_T} = \left[(C_T\text{gene of interest} - C_T GAPDH)\right. \quad (1)$$
$$\left.\text{sample with extract} - (C_T\text{gene of interest} - C_T GAPDH)\right]$$

Primers for CDKN2A, HER2, MDM2, and GAPDH were as follows: CDKN2A, forward 5'-CTCGTGCTGATGCTACTGAGGA-3' and reverse 5'-GGTCGGCGCAGTTGGGCTCC-3'; HER2, forward 5'-TGTGACTGCCTGTCCCTACAA-3' and reverse 5'-CCAGACCATAGCACACTCGG-3'; MDM2, forward 5'-TAATTGAAGGGTCATGCCTAAGTGT-3' and reverse 5'-GCTGCCTCCGACTTAACTGC-3'; and GAPDH, forward 5'-GGAAGGTGAAGGTCGGAGTCA-3' and reverse 5'-TCATTGATGGCAACAATATCCACT-3'.

Statistical Analysis

Significant differences were assessed using the t-test in GraphPad Prism Software (Version 8.4.3, GraphPad Software Inc., USA). FlowJo software (Version 7.6.1.) was used for the analysis of flow cytometry data. All data are presented as the mean

± standard deviation (SD). The symbol * indicates a significant difference, where * and # signify $P<0.05$ and ** and ## indicate $P<0.01$.

RESULTS

UV-Vis Spectroscopy Results

The ethanolic extraction of *E. billardierei* was subjected to UV-Vis spectroscopy, which revealed a prominent absorption band with a maximum wavelength (λmax) of approximately 266 nm, as illustrated in Figure 1. In line with the existing literature, it is noteworthy that the spectra of flavonoids and phenolic acids regularly demonstrate distinct λmax values around 270 or 340 nm.[14] In addition, phenolic acids characterised by the benzoic acid carbon framework display their λmax within the range of 200–290 nm.[15] Consequently, the extract from *E. billardierei* showed significant levels of total flavonoids and phenolics.

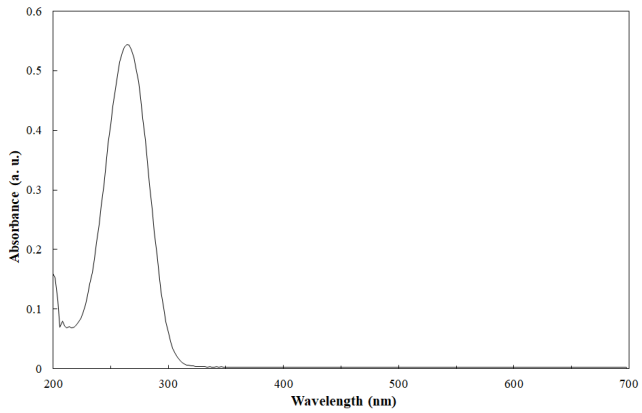

Figure 1. The UV-Vis spectrum of the *E. billardierei* ethanolic extract.

Growth Rates of MDA-MB-231 Cells

Because cell cultures serve as prominent biological systems for examining the effect of drugs or compounds on cell proliferation rates, the initial step involved utilising the MTT assay to measure the influence of the *E. billardierei* extract on the growth of MDA-MB-231 cells. The MTT assay results confirmed that the *E. billardierei* extract displayed a dose-response suppression effect on the growth of MDA-MB-231 cells (Figure 2). Hence, the IC50 (50% inhibition concentration) of *E. billardierei* extract after 24 and 48 h of incubation were determined to be 400.3 and 104.9 µg/mL. The type of solvent used for extracting plants is important. Polar solvents such as ethanol and water are employed in polar compound extraction. Ethanol, in particular, is non-toxic at low concentrations and possesses self-preservative properties when its concentration exceeds 20%.[16] In addition, prior studies have proposed that the ethanolic extract of certain plants may have a potential therapeutic impact on TNBC.[17-19] Therefore, in this study, we utilised the ethanolic extract of *E. billardierei*. Hence, after cells were cultured, the EtOH solution was added, and the proliferation effects of the solvent alone were determined. As shown in Figure 2, EtOH had no impact on the viability of the cells. A previous study also confirmed that EtOH at a lower concentration is a good solvent choice because it has low toxicity in the MDA-MB-231 cell line.[20] Hence, it can be concluded that the dose-dependent inhibitory effects observed after the treatment of cells with *E. billardierei* extract are a result of the plant extract contents.

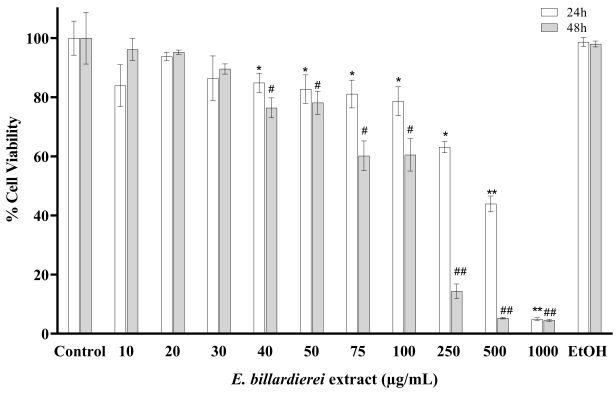

Figure 2. MTT assay of MDA-MB-231 cells after treatment with diverse concentrations of *E. billardierei* ethanolic extract after 24 and 48 h. Values are mean ± standard deviation; * $P<0.05$ vs. control, ** $P<0.01$ vs. control, # $P<0.05$ vs. control, ## $P<0.01$ vs. control.

As can be observed, after 48 h, the IC50 decreased significantly. The doubling time of MDA-MB-231 is ca. 25-35 hours[21], thus, our results indicated that the effect of *E. billardieri* can be dependent on cell doubling time. Consequently, a concentration of 104.9 µg/mL (lower IC_{50}) of the extract and 48-h treatment was chosen for further studies.

Measurement of ROS Levels

To investigate whether oxidative stress plays a role in the antiproliferative effects of the *E. billardierei* extract, cells were treated with the extract and NAC. As shown in Figure 3, compared with the control, pretreatment with NAC abolished *E. billardierei* extract–induced cytotoxicity in MDA-MB-231 cells significantly. This result implied that *E. billardierei* extract could produce ROS in cells, which could inhibit cell proliferation.

Flow Cytometry Results

The determination of necrotic and apoptotic cell values involved the measurement of membrane integrity and phosphatidylserine externalisation. This was accomplished through double staining with Annexin V and PI and analysed via flow cytometry. As shown in Figure 4 and Table 1, the amount of

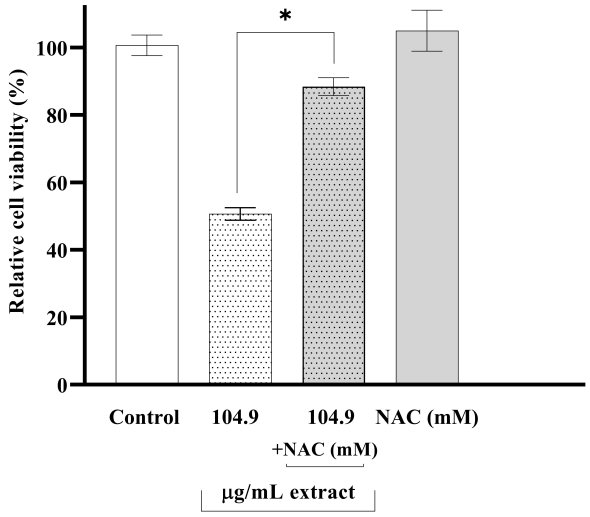

Figure 3. Measurement of ROS generation. MDA-MB-231 cells were pre-treated with NAC and then treated with *E. billardierei* ethanolic extract after 48 h. Values are mean ± standard deviation; * indicates $P<0.05$.

necrotic cells was higher than the control cell ($P<0.05$). In addition, the proportions of total cell apoptosis (late apoptotic cells+early apoptotic cells) were enhanced significantly after treatment with *E. billardierei* extract ($P<0.05$) compared with the control group.

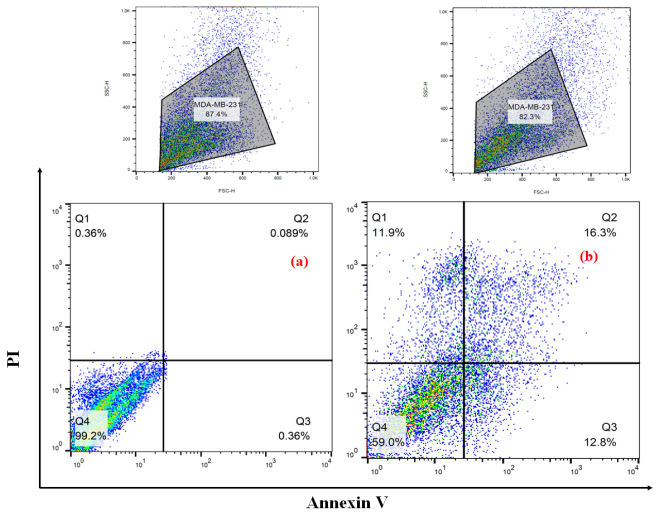

Figure 4. Flow cytometry analyses of MDA-MB-231 cells: (a) control cells and (b) cells treated with 104.9 µg/mL of *E. billardierei* ethanolic extract. Cells were gated on forward (FSC) vs. side scatter (SSC) to select the cell population (The upper graphs). Harvested cells were stained with Annexin V-FITC (x-axis) and PI (y-axis).

AO/EtBr Double-Staining Results

For a comprehensive understanding of the effects of the *E. billardierei* extract, the morphology of MDA-MB-231 cells was examined using the AO/EtBr double staining assay. As shown in Figure 5, no significant change and apoptosis were detected in the control group. In AO/EtBr staining, living cells display a normal green nucleus.[22] After 48 h of treatment with the extract, early-stage apoptotic cells, characterised by crescent-shaped or granular yellow-green AO nuclear staining, were recognised. In addition, late-stage apoptotic cells, distinguished by concentrated and asymmetrically localised orange nuclear EtBr staining, were observed following a 48-h treatment with the ethanolic extract of *E. billardierei*. Note that an enhancement in volume and the presence of uneven orange-red fluorescence at the periphery were monitored in necrotic cells. However, it is important to mention that the number of necrotic cells was insignificant. These results indicate that the *E. billardierei* extract can induce nuclear fragmentation and chromatin condensation in MDA-MB-231 cells.

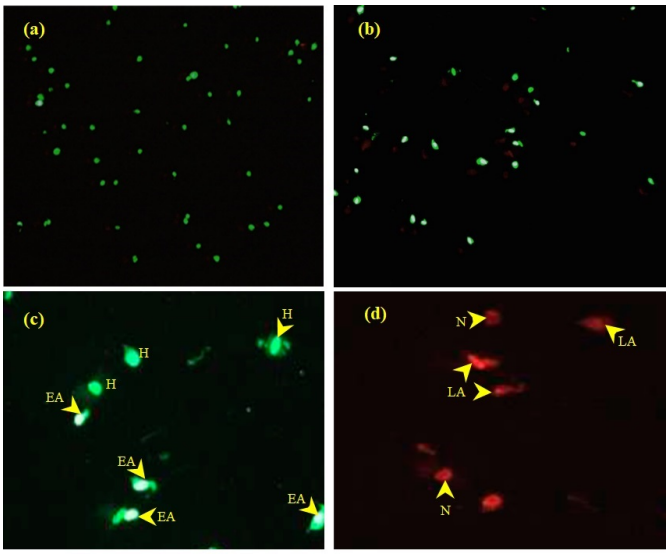

Figure 5. Apoptosis analysis by AO/EtBr: (a) Negative control group (H); (b) cells treated with *E. billardierei* extract (×10). (c) Nuclei showed yellow-green fluorescence by AO staining (×40), and (d) showed orange fluorescence by EtBr (×40). H: live cell, EA: early apoptotic cells, LA: late apoptotic cells, and N: necrotic cell.

Results about *CDKN2A, MDM2*, and *HER2* Gene Expression

Real-time qPCR was employed to assess the influence of the *E. billardierei* extract on *CDKN2A* and *HER2* expression in MDA-MB-231 cells. As shown in Figure 6, compared with the control group, after 48 h of treatment with *E. billardierei* extract, the expression of *HER2* did not alter. However, the expression of *CDKN2A* increased slightly notably ($P<0.01$) after treatment with *E. billardierei* extract. Conversely, the expression of *MDM2* was reduced notably ($P<0.05$) after treatment with *E. billardierei* extract. In summary, the statistical analysis revealed a significant correlation between the expression of *CDKN2A* and *MDM2* following treatment with the *E. billardierei* extract for 48 h.

Table 1. Relative number (in %) of living, early and late apoptotic, and necrotic cells in the control and sample treated with *E. billardierei* extract (Mean±SEM, n=3, * P<0.05, and ** P<0.01).

Sample	Live cells (Q4: $PI^-/FITC^-$)	Early apoptotic cells (Q3: $PI^-/FITC^+$)	Late apoptotic cells (Q2: $PI^+/FITC^+$)	Necrotic cells (Q1: $PI^+/FITC^-$)
Control	99.2±0.20	0.36±0.42	0.089±0.15	0.36±0.55
Treated with the *E. billardierei* extract	59.0±2.38**	12.8±0.55*	16.3±2.70*	11.9±1.01*

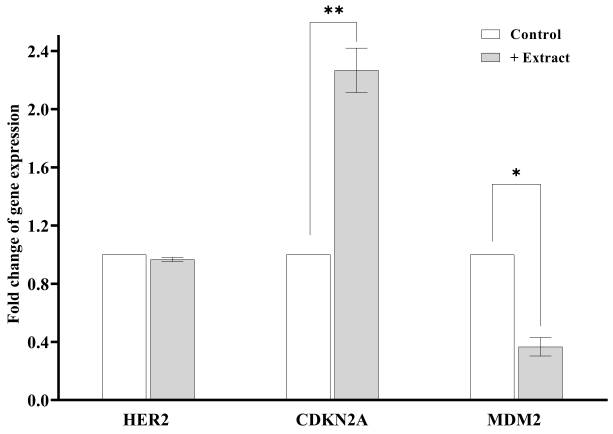

Figure 6. Fold changes in the gene expression levels of *HER2, CDKN2A*, and *MDM2* in cells treated with 104.9 µg/mL of *E. billardierei* extract after 48 h compared with the control groups. The fold change in gene expression was calculated according to the comparative CT method as described in the Materials and Methods section. Values are mean ± standard deviation; *indicates $P<0.05$ and ** indicates $P<0.01$.

DISCUSSION

TNBC represents the most aggressive subtype of breast cancer, constituting ~15 to 20% of all breast cancers globally. Recently, various research focussed on the potential of plant-based compounds in breast cancer treatment. The current study implies that the proliferation of MDA-MB-231 cells could be inhibited by *E. billardierei* extract in a time- and dose-dependent manner. Previous studies have confirmed the anticancer effect of *Eryngium* species, but the IC_{50} value in different cells was different depending on the type of solvent and time of treatment. For example, Paşayeva *et al*. found the cytotoxic effects of *E. billardieri* extract with IC_{50}=99.50 µg/mL) on the MCF7 cell line.[23] Results of Hasanbeiglu et al. confirmed that the dichloromethane extract of *E. billardieri* could inhibit MCF7 growth with IC_{50} = 110.30 and 31.37 µg/mL after 24- and 48-h treatment, respectively.[10] The preceding study further confirmed that the methanolic extract of *E. thyrsoideum* inhibited the growth of MDA-MB-231 cells, yielding IC_{50} values of 78.46 and 47.51 µg/mL after 24- and 48-h treatment, respectively.[17]

Flow cytometry analysis revealed that the treatment of cells with *E. billardierei* extract induced apoptosis, pushing MDA-MB-231 cells into both early and late apoptosis stages. The flow cytometry results of Hasanbeiglu et al. also showed that *E. billardieri* extract caused apoptosis in MCF7 cells.[10] The fluorescence microscopic image also proved that the majority of the *E. billardieri* extract-treated cells were at the apoptosis stage (early and late stage). In addition, as cells in the necrotic stage display an orange colouration with normal nuclear morphology and no condensed chromatin, our data implied that the predominant response of MDA-MB-231 cells to *E. billardierei* extract treatment was late apoptosis. These data are in agreement with the flow cytometry results.

Control of intracellular ROS levels is important for maintaining cellular homeostasis. At low levels, ROS function as signalling molecules, whereas at elevated levels, they could induce cell damage and lead to cell death. The results revealed that *E. billardieri* extract inhibited proliferation and promoted the ROS-mediated endogenous apoptotic pathway in MDA-MB-231 cells. Oxidative stress has the potential to disrupt the equilibrium between ROS production and radical scavenging, ultimately triggering cellular apoptosis.[24, 25] Thus, our findings imply that ROS play a role as effectors in the *E. billardierei* extract-induced inhibition of MDA-MB-231 cell viability. Elevated ROS levels may activate several signalling molecules in tumour cells, leading to cell cycle arrest and apoptosis.[24]

After 48 h of treatment with *E. billardierei* extract, *HER2* expression was not affected. It has been demonstrated that HER2 can inhibit p53 through two indirect pathways, both of which are mediated by protein kinase B (PKB), also known as AKT. The initial mechanism entails AKT-mediated suppression of the Alternate Reading Frame Protein (ARF). Typically, ARF interacts with and inhibits MDM2, preventing its interaction with p53. In cells overexpressing *HER2*, a decrease in *ARF* expression was detected in an AKT-dependent manner, leading to heightened MDM2 activity and diminished p53 levels. In addition, it was detected that AKT or HER2 activation decreased the expression and nuclear localisation of p53. As a result, a hypothesis was developed suggesting that AKT directly phosphorylates the MDM2 protein, guiding it to the nucleus.

Once in the nucleus, MDM2 can ubiquitinate p53, facilitating its degradation.[26] In addition, our data revealed that the *CDKN2A* and *MDM2* expression was pregulated and downregulated, respectively, following *E. billardierei* extract treatment. As previously mentioned, MDM2 serves as the primary cellular antagonist of p53, which restrains the growth-suppressive function of p53 in unstressed cells. Clinical and preclinical data on MDM2 inhibitors in the context of human cancer treatment have shown that targeting MDM2 could present an innovative approach to improving the efficacy of tumour therapeutics. MDM2 inhibitors exert their effects by obstructing the binding between p53 and MDM2 through competitive binding to region I of the MDM2 protein. This disruption results in increased p53 levels and activation of the p53 signalling pathway. Accordingly, MDM2 inhibitors can foster cell apoptosis and impede cell growth.[5] In contrast, an augmented expression of CDKN2A can trigger growth arrest, demonstrating one of the distinctive features of premature senescence.[27] CDKN2A can also facilitate the degradation of MDM2 and physically obstruct the interaction between MDM2 and p53. This process results in the stabilisation of p53.[4] An earlier study showed that the extract of *E. billardierei* could upregulate *Bax* and *p53* expression in MCF7 cells[10], which is in agreement with our present data.

In previous studies, it has been confirmed that the ethanolic extract of *E. billardierei* contains significant total flavonoids and phenolics.[8,28] Flavonoids exhibit a dual role in maintaining ROS homeostasis. They act as antioxidants under normal condition. However, in cancer cells, they have significant prooxidant properties. Flavonoids can restrain the proliferation of tumour cells by inhibiting several pathways, including PKB, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), phosphatidylinositide 3-kinases (PI3K), and the epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MAPK) pathway.[29] Several flavones have also been found to regulate the cell cycle at different points.[30] Phenolic compounds extracted from plant sources have been shown to have inhibitory effects on the proliferation of tumour cells, including breast cancer cells.[31] Thus, it could be concluded that flavonoids and phenolics play a significant role in the anticancer properties of *E. billardierei* extract. Nonetheless, this study is solely a preliminary *in vitro* investigation. Subsequent studies, particularly *in vivo* experiments, are necessary to validate the potential low toxicity of the *E. billardierei* extract.

CONCLUSION

In summary, the current study demonstrated a robust growth-inhibitory effect of *E. billardierei* extract on human TNBC MDA-MB-231 cells. Moreover, treatment with *E. billardierei* extract was found to induce apoptosis in cancer cells. Following a 48-h treatment with *E. billardierei* extract, no significant effect on *HER2* expression was observed. The growth-inhibitory effect was associated with the generation of ROS, influencing the upregulation of *CDKN2A* and the downregulation of *MDM2* expression. These findings suggest that *E. billardierei* extract holds promise for development into an innovative and effective anticancer drug against TNBC. However, further research and validation, including *in vivo* studies, are warranted to verify its potential therapeutic application.

Ethics Committee Approval: Ethics committee approval is not required for the study.

Peer Review: Externally peer-reviewed.

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