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ORIGINAL ARTICLE

Anti-cancer Effects of *Nepeta Deflersiana* Extract (NDE) in Estrogen Positive and Negative Forms of Breast Cancer

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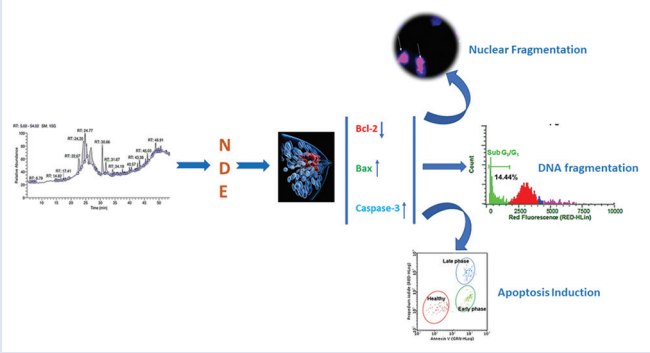
ABSTRACT

Aim: This study focused on efficacy of the ethanol extract gained from the aerial parts of *Nepeta deflersiana* (ND) on breast cancer (BC) cells. **Materials and Methods:** Shade-dried aerial parts of ND were extracted with ethanol using the Soxhlet extraction method (NDE). MTT assay was performed to assess the cytotoxicity of NDE in normal breast cells (MCF-10A), as well as estrogen receptor (ER) positive (MCF-7) and ER-negative (MDA-MB231) BC cells. Nuclear staining was performed using Hoechst/propidium iodide. Annexin V assays for apoptosis detection and cell cycle analysis were performed by flow cytometry. The effect of NDE on metastasis was analyzed by the transmigration assay. Western blot was used to detect the key signaling proteins. **Results:** NDE inhibited BC cell proliferation with GI_{50} values of 154.90 and 202.7 µg/ml for MCF-7 and MDA-MB231 cells, respectively. NDE was non-toxic to normal MCF 10A cells at concentrations up to 1000 µg/ml. NDE induced nuclear condensation/fragmentation in both types of BC cells near the GI_{50} doses values. An apparent increase in early and late apoptotic populations was confirmed by the flow cytometry. The extract also increased the sub-G_0/G_1 population in both types of BC cells. Dose-dependent increases in Caspase 3 and Bax levels were also recognized, as were NCE decreases in anti-apoptotic Bcl-2 levels in the same cell lines. **Conclusion:** NDE possessed excellent anticancer properties against ER-positive and negative BC cells, encouraging further research on the use of this natural product against BC.

Key words: Anti-cancer, apoptosis, breast cancer, flow cytometry, *Nepeta deflersiana*

SUMMARY

- *Nepeta deflersiana* extract (NDE), a bioactive plant extract, was tested for efficacy in estrogen positive and negative forms of breast cancer (BC). NDE was effective at controlling the proliferation and inducing apoptosis in both types of BC cells. The observed activities of this extract encourage further research on its effects against BC.



Abbreviations used: BC-Breast cancer; ER- Estrogen Receptor; GC-MS-Gas chromatography Mass spectroscopy; GI- Growth inhibition; *ND-Nepeta deflersiana*; NDE- *Nepeta deflersiana extract*; SDS-PAGE- Sodium dodecyl sulphate poly acrylamide gel electrophoresis

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INTRODUCTION

Notwithstanding recent advances in the medical field, breast cancer (BC) remains a major health concern and is, therefore, a highly relevant topic in the biomedical research. Globally, BC is the leading cancer diagnosis in women, and its incidence and associated mortality rates are continuously increasing.[1] Recent evidence suggests that BC is the main cause of cancer death in women aged <45 years.[1] The high heterogeneity of this disease results in the varying levels of aggressiveness and complex biological features.[2] Genetically, BCs are categorized into four main molecular subtypes, namely luminal-A, luminal-B, HER-2, and basal-like tumors.[3] Out of these subtypes, luminal-A and B are hormone receptor positive while the other two forms are negative.[3] In addition to their pathological features, these subtypes differ with regard to the outcomes of clinical treatments. Research indicates that receptor-negative cancers have a poor response to treatment and worse prognosis as compared to estrogen receptor-positive (ER [positive]) forms.[4] Therefore, the identification of new agents that are effective against both ER (positive) and ER (negative) BC would constitute a significant contribution to the field.

Plants and their constituents have been used in the traditional medicine since time immemorial. Several of the bioactive compounds present in these plants, such as tannins, flavonoids, alkaloids, and phenolic compounds, have therapeutic value.[5] The medicinal properties of individual plant species may vary depending on the concentrations of these active ingredients. Medicinal plants have attracted increasing global research attention due to their efficacy and low toxicity, and potential applicability to the development of alternative products to expensive synthetic medicines.[6] *Nepeta deflersiana* (ND) is an aromatic perennial herb belonging to the Labiate family chiefly found throughout

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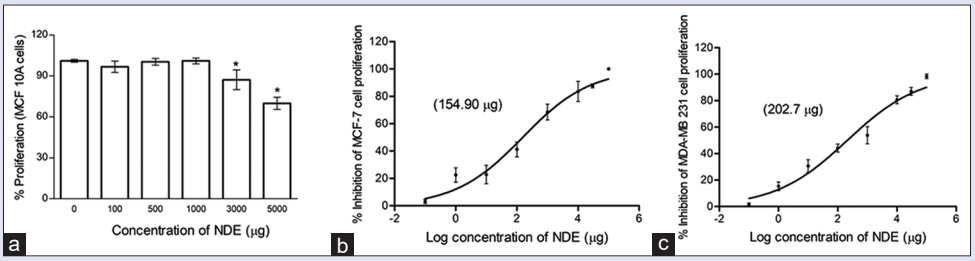

Figure 1: (a) Non-toxic dose limit of NDE on proliferation of non-cancerous, normal breast cells. **P* and #8804; 0.05 considered significant. Percentage proliferation inhibitions of NDE in cancerous MCF-7 (b) and MDA-MB 231 (c) cells depicting the GI_{50} values. The results are representative of three individual experiments performed in triplicate. NDE: *Nepeta deflersiana* extract

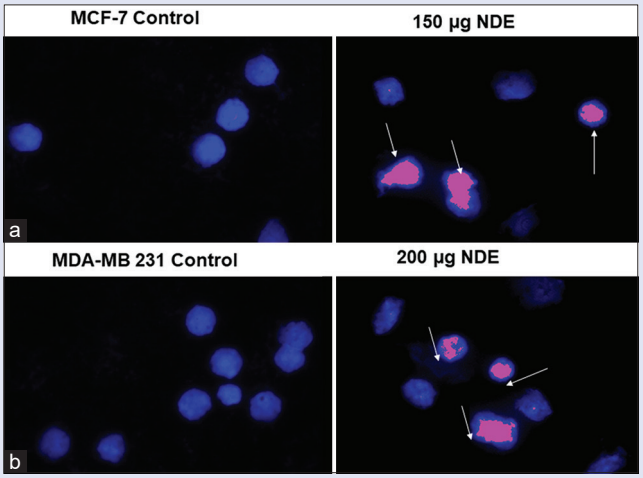

Figure 2: Ability of NDE to induce nuclear fragmentation in (a) MCF-7 and (b) MDA-MB 231 breast cancer cell lines. Hoechst 333258 and propedium iodide are shown in blue and pink pseudo colors, respectively. NDE: *Nepeta deflersiana* extract

Central and Southern Europe, North America, Africa, and Asia.[7] This herb has been used in the traditional medicine as a treatment for rheumatic disorders, an antiseptic for wounds, and a carminative.[8] Recently, this plant was evaluated for its effect on lung cancer cells.[9] The current work focuses on exploring the anticancer efficacy of ND ethanol extract (NDE) against receptor-positive and receptor-negative BCs.

MATERIALS AND METHODS

Materials

All reagents and chemicals were obtained from Sigma (St. Louis, MO, USA). The cell lines used in this work were purchased from the American Type Culture Collection (ATCC, Virginia, USA). Trans-endothelial migration assay kits and Guava cell cycle reagent were used (Millipore Co., Burlington, Massachusetts, USA). Apoptosis kits were purchased from Invitrogen eBioscience Ltd (CA, USA). All reagents used for Western blotting were obtained from Santacruz Biotechnology (Santa Cruz, CA, USA).

Preparation of extracts

The aerial parts of ND were harvested, dried, powdered, and dissolved in ethanol for Soxhlet extraction. Crude extracts were evaporated at RT and re-dissolved in ethanol at a concentration of 2 g/ml.

Cell culture

MDAMB237 and MCF-7 cells were cultured in Leibovitz and DMEM full growth media, respectively. Human vein umbilical endothelial cells (HUVEC) were grown in F-12K media-containing heparin and endothelial cell growth supplement (0.1 and 0.05 mg/ml, respectively). Experimental procedures were performed when cells grew to 80% confluency.

MTT cell proliferation assay

The assay was performed according to a previous protocol with minor changes.[10] First, 5×10^3 cells were seeded in 96 well plates and incubated overnight to adhere to the plate. Then, the cells were incubated with NDE for a period of 72 h and reincubated with 5 mg/ml MTT for 3.5 h. After terminating the reaction using DMSO, the color developed was measured at 560 nm, and readings were analyzed with the GraphPad Prism software.

Nuclear morphological analysis

The assay was carried out as described by Belloc *et al*. Both types of BC cells at a concentration of 100,000/Petri dish were cultivated for 48 h followed by NDE treatment. On the following day, the cells were washed with phosphate-buffered saline and added to propidium iodide and Hoechst 333258 reagents, each at a concentration of 100 mg/ml. Changes in nuclear morphology were captured by fluorescent microscopy.

Annexin V assay

Annexin assay was performed according to the manufacturer's instructions. Shortly, 0.5×10^6 of the BC cells were treated with NDE for a period of 72 h. After cell washing, 0.25 µg/ml Annexin V was added to the samples. After 15 min, propidium iodide (0.5 µg/ml) was added. Normal cells and those in early and late apoptosis were identified using flow cytometry and e-positive cells were analyzed with GraphPad Prism.

Analysis of the cell cycle

Cell cycles in both types of BC cells were evaluated using a Guava® cell cycle assay kit as per the manufacturer's instructions. Prior to this procedure, 0.5×10^6 of both cells were plated into each well in 6-well plates, treated with NDE, and incubated for 72 h. The percentage of cell cycle phase was evaluated under flow cytometry, and the percentage of cells in the desired phase was presented.

Trans-endothelial cell migration assay

Migration of the BC cells was analyzed using a commercial kit as described by the manufacturer's instructions. After starving in serum-free media, both types of cancer cells were added to migration

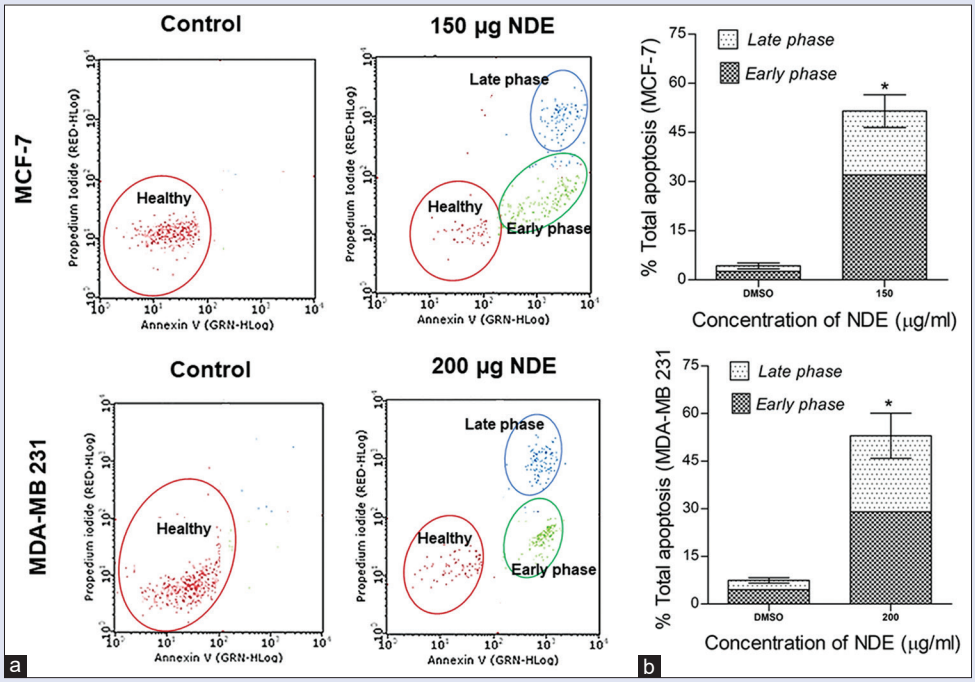

Figure 3: Flow cytometric analysis of the apoptotic cells, based on Annexin V staining in (a) MCF-7 and (b) MDA-MB 231 cells following NDE treatments. All experiments were performed thrice, and representative graphs are shown. The bar chart represents the dose-dependent increase of total apoptotic cells in breast cancer cell lines after treatment with different doses of ICY-5. The results are expressed as mean ± standard deviation. *P* and #8804; 0.05 compared to * control cells. NDE: *Nepeta deflersiana* extract

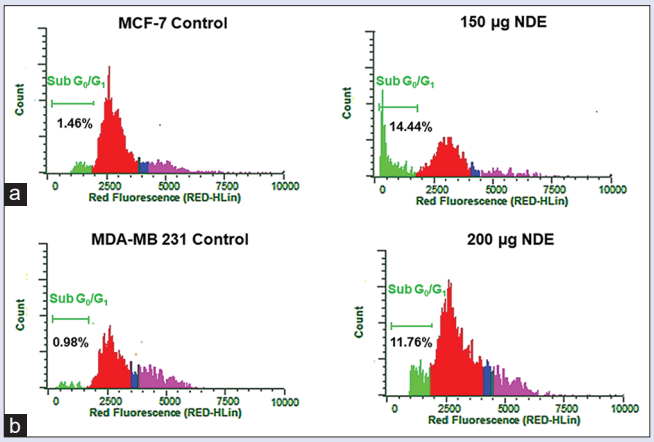

Figure 4: Analysis of cell cycle changes after different concentrations of NDE treatments in (a) MCF-7 and (b) MDA-MB 231 cells at 72 h. Representative histograms from several repeats of the experiment are shown. Results expressed as mean ± standard deviation. NDE: *Nepeta deflersiana* extract

inserts with HUVEC-covered membrane at a density of 100,000 cells per insert. After NDE treatment, these inserts were placed in new wells with 50 ng/ml hepatocyte growth factor (HGF) and serum-free media. The BC cells were left to migrate from the insert to the lower part of the well for 12 h. After swabbing, the inserts were stained, eluted, and assayed at 540 nM. The percent migration of treated cells was then compared to control cells.

Western blot

The assay was performed as reported elsewhere.[11] After 48 h of treatment with NDE, the cells were lysed, and protein concentrations were determined. Samples containing 20–40 µg protein were analyzed by SDS-PAGE. Then, proteins were transferred from SDS-PAGE gel to a nitrocellulose membrane. The membrane was incubated with primary and secondary antibodies, and then, the blots were developed and processed using Image J (ver. 1.46, NIH) software. β-actin was diluted to 1:5000.

Statistical analysis

GraphPad Prism 6.0 (La Jolla, USA) was used for the statistical evaluations. The results are expressed as mean ± standard deviation. Student's *t*-tests were used for the analysis. Statistical significance was defined as * $P \leq 0.05$ or ** $P \leq 0.005$.

RESULTS

Nepeta deflersiana extract controlled the growth of breast cancer cells

Prior to analyzing the effects of NDE on BC cells, the effects of nonlethal concentrations of NDE were evaluated in noncancerous, normal MCF-10A cells. The proliferation of these cells was unaltered by NDE at concentrations up to 1000 µg/ml [Figure 1a]. The proliferation of both ER (positive)-MCF-7 and ER (negative)-MDAMB-231 was dose dependently inhibited by NDE with respective GI_{50} values of 154.90 µg/ml [Figure 1b] and 202.70 µg/ml [Figure 1c]. The closest GI_{50} value for each cell line was used in subsequent assays.

Nepeta deflersiana extract caused nuclear fragmentation in breast cancer cells

The changes in the nucleus of BC cells when treated NDE were further examined. After treatment with propidium iodide and Hoechst 333258, both cell types were screened for the morphological changes. Evidence of nuclear condensation and fragmentation was observed in 150 µg/ml

NDE-treated MCF-7 cells [Figure 2a]. Similar effects were also observed in 200 µg/ml NDE treated MDA-MB 231 cells.

Nepeta deflersiana extract induced apoptosis in breast cancer cells

To assess anti-proliferative efficacy and investigate the nuclear damage caused by NDE in BC cells, the Annexin V assay was used. A significant increase in both early/late apoptotic populations was observed in both BC cell types [Figure 3]. Treatment with 150 µg/ml NDE caused the percentage of early and late apoptotic cells to increase to 51.5% and 32.0% from 4.25% to 2.60%, respectively, in MCF-7 cells (untreated cells) [Figure 3a]. In MDA-MB 231 cells, 200 µg/ml NDE treatment led the percentage of early and late apoptotic cells to increase to 53.21% and 29.31% from 7.45% to 4.55%, respectively, in the control cells [Figure 3b].

Cell cycle of breast cancer cells was altered by *Nepeta deflersiana* extract treatments

NDE modified the cell cycle in BC cell lines by increasing sub-G_0/G_1 cell populations [Figure 4]. Treatment with 150 µg/ml NDE increased the percentage of sub-$G_0/G_{1\ cells}$ from 1.46% to 14.44% in MCF-7 cell samples [Figure 4a]. Similarly, 200 µg/ml NDE treatment increased the proportion of sub-$G_0/G_{1\ cells}$ in MDA-MB 231 samples from 0.98% to 11.76% [Figure 4b].

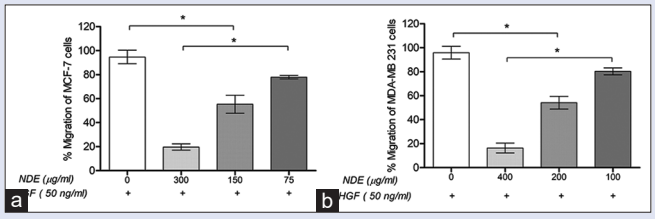

Figure 5: Dose dependent anti-migration efficacy of NDE on migration of (a) MCF-7 and (b) MDA-MB 231 cells across the HUVEC membrane under influence of 50 ng/ml HGF which served as chemo-attractant. Results expressed as mean ± standard deviation from three individual experiments in duplicates and *P and #8804; 0.05 was considered statistically significant. NDE: *Nepeta deflersiana* extract

Effects of *Nepeta deflersiana* extract on metastatic events in breast cancer cells

To evaluate whether NDE was involved in the metastasis of BC cells, a migration assay was used. BC cells were treated with NDE at concentrations near the GI_{25}, $GI_{50,}$ and GI_{100} values. Dose-dependent effects on migration inhibition were observed after NDE treatment in both BC cell types [Figure 5a and b].

Nepeta deflersiana extract and apoptosis in breast cancer cells

In order to validate the observed bio-efficacy of NDE in BC cells, the effects of NDE on key apoptotic signaling proteins were studied. NDE significantly increased the expression of caspase-3 and Bax proteins and decreased Bcl-2 expression in both cell types [Figure 6].

DISCUSSION

Plant products are often researched for their biomedical applications to various diseases. Although some plants are widely used in traditional folk medicine, there is still a need to identify and document their properties. The high availability, low toxicity, and reduced side effects of natural compounds constitute the advantages over synthetic compounds. BC is considered one of the deadliest types of cancer, particularly in female populations. The search for novel compounds that can treat this condition is therefore of crucial importance. Hence, this study focused on efficacy of NDE on both ER (positive) and ER (negative) BC cells. The results of this investigation indicated that NDE has potent inhibitory effects on both types of BC cell. The fact that the MCF-10A cells tolerated a much higher dose of NDE indicating the safety of this extract for normal cells. Research shows that compounds from natural sources could be therapeutically beneficial,[12] and it is likely that one or more active compounds present in NDE [Figure 7] were responsible for the findings observed in this study.

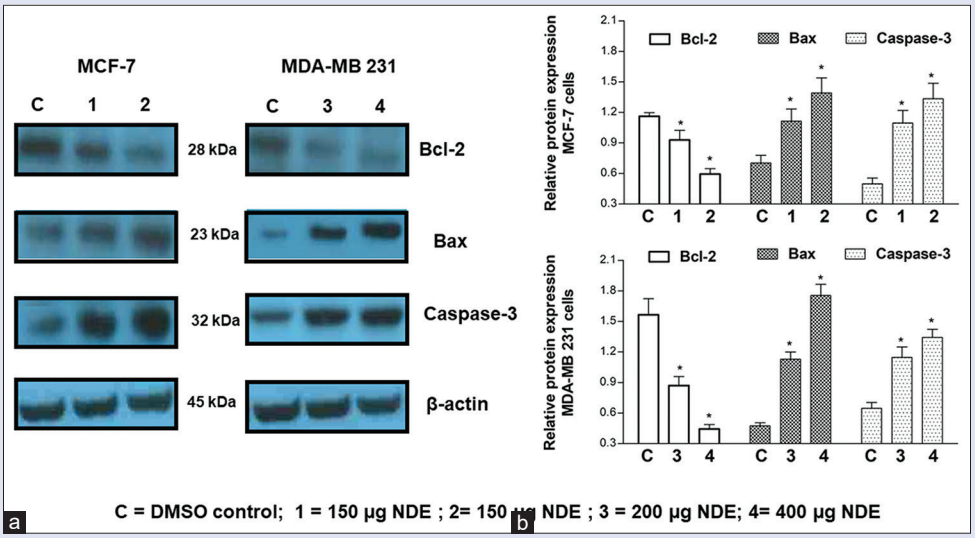

Figure 6: The effect of NDE treatment on caspase-3, Bcl-2 and Bax expressions in MCF-7 and MDA-MB 231 cells. (a) Representative blots are from three individual experiments are shown. And #946; actin was used as loading control. (b) Western blot quantifications are expressed as mean ± standard deviation, based on relative protein expression. The results were statistically significant with *P and #8804; 0.05 or **P and #8804; 0.005 with respect to controls. NDE: *Nepeta deflersiana* extract

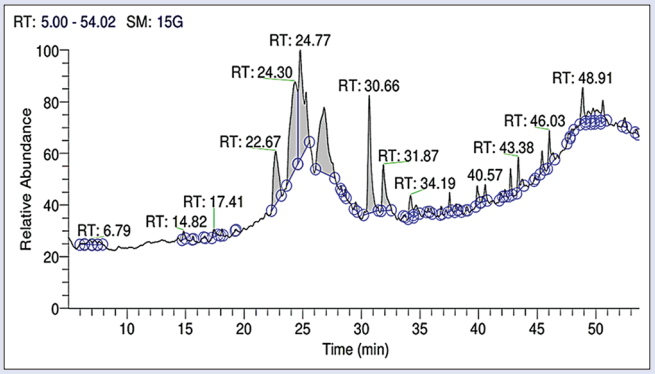

Figure 7: GC-MS chromatogram of ethanol extract of *Nepeta deflersiana* with respective retention factors for active compounds; 2-Hexenol, Cis linalool oxide, 4 (8)-P-menthene, 2-cyclohexen, methyl perillate, 2,5-cyclohexadiene-1,4-dione, selina-3,7 (11)-diene, 1,2,3-propanetriol, 1-acetate, 4-ethyl phenol, 4-ethyl-1,2-benzenediol, 3-(1-methyl-1H -pyrrol-2-yl)-Pyridine(nicotyrine),1,2-benzenedicarboxylic acid and cycloundecane

Several studies indicate that cancer cells are vulnerable to apoptosis when challenged by drugs. It has also been shown that morphological alterations in the nucleus of cancer cells are at the basis of apoptosis.[13,14] In the current investigation, morphological evaluation after staining was used to explore nuclear condensation and DNA fragmentation in MCF-7 and MDA-MB321 cells treated with NDE. The results were suggestive of apoptosis in BC cells, which was confirmed by the increase in Annexin V-positive cells reflecting a higher number of early and late apoptotic cells after NDE treatment.[15] When cancer cells are confronted by active drugs, the regulation of the cell cycle determines the point at which cell death begins.[16] The results of the current study demonstrated an increment in the sub-G_0/G_1 phase. Considering the critical output of apoptosis, where cellular DNA is digested by endonuclease, the increase in the sub-G_0/G_1 population can be seen as further evidence of the effects of NDE on the cell cycle.[16] Therefore, these results support the anti-proliferative and apoptotic effects of NDE. These findings were further corroborated by the dose-dependently effects of NDE on the HGF-induced migration of BC cells, which indicates the anti-metastatic effects of NDE.

Reports indicate that apoptosis can begin through the mitochondrial-dependent intrinsic pathway or the surface receptor-mediated extrinsic pathway.[17] The former involves Bcl-2/Bax proteins and caspase activation.[17] The results of this study showed an increase in the level of pro-apoptotic Bax protein and a decrease in anti-apoptotic Bcl-2 protein after NDE treatments in both BC cell types. This could be due to the attenuation of the mitochondrial potential in MCF-7 and MDA-MB 231 cells, which leads to bio-energy loss and culminates in apoptosis.[18] Hence, it is possible that NDE lowered the anti/pro-apoptotic protein ratio, enhancing apoptosis in BC cells. This hypothesis is supported by the increase in caspase-3 expression which is associated with the mitochondrial intrinsic pathway and may be responsible for the bio-efficacy of the extract in controlling BC cell proliferation.[19] However, alternative hypotheses should not be discarded without further research.

CONCLUSION

In summary, NDE was effective at controlling the proliferation of estrogen positive and negative forms of BC by influencing the activity of mitochondrial (intrinsic) pathway proteins. The observed anticancer and anti-metastatic efficacy of this extract encourages further research to explore bio-active compounds and validate their use in the treatment of different cancer types.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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