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In vitro chemo-protective effect of *Eisenia foetida* coelomic fluid against histone deacetylase inhibitor-induced oxidative toxicity in breast cancer cells

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ABSTRACT

Developing new drugs from natural products is important for therapeutic effects to minimise tissue toxicity of drugs used in cancer treatment. Eisenia foetida is a worm with a double transport system consisting of coelomic fluid (ECF) that can be used as alternative medicine. It is important to eliminate or reduce the high cytotoxicity of sodium butyrate (NaBu), a chemotherapeutic agent used in breast cancer treatment, for both neoplastic and normal cells. We aimed to evaluate the effect of ECF on the oxidative toxicity induced by NaBu in MCF-7 cells, changes in ROS production levels and expression of cell death and ROS-related genes. It was determined that the expression levels of *Bax* and *Bcl-2* genes remained unchanged, while the amount of ROS decreased significantly in MCF-7 cells exposed to NaBu with ECF. Thus, ECF may be a potential therapeutic molecule with fewer side effects in cancer treatment in the future.

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Breast cancer; chemo-protective effect; *Eisenia foetida*; ROS

Introduction

While 5–10% of cancer formation is caused by genetic reasons, 90–95% of tumour pathogenesis occurs due to environmental factors, alcohol/cigarette consumption, obesity, malnutrition, lack of physical activity, and unhealthy lifestyles such as high body mass index. (Schwingshackl et al. 2017; Seiler et al. 2018). It is important to determine cancer risk factors and treatment strategies to prevent cancer (Momenimovahed and Salehiniya 2019; Ozkan and Yuksel 2022). Modern medicine today focuses on natural nutrition for cancer prevention and treatment to alleviate cancer problems (Ezzati et al. 2020). Dietary elements exert anticancer properties by coping with tumour cells through proliferation modulation, including apoptosis and angiogenesis pathways (Wang et al. 2016). Breast cancer, one of the main causes of death due to the diversity of factors such as lifestyle, genetic factors and environment, is one of the malignant tumours that has been increasing among women in recent years. Breast cancer pathogenesis is a long process. Dietary habits and quality of life act as risk modifiers of carcinogenesis in cancer initiation and progression (Theodoratou et al. 2017). Unfortunately, drugs used in cancer treatment can often cause serious side effects. The chemotherapeutic drugs used during its treatment are known side effects such as nausea, vomiting, loss of appetite, muscle pain, alopecia and neurological disorders (Bray et al. 2018; Waks and Winer

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2019). Researchers have stated that it is important to develop new drugs from natural products to minimise tissue toxicity caused by drugs used in cancer treatment to maintain the therapeutic effect (Rizwan et al. 2019; Yuksel and Ozkan 2021). Given the frequency and severity of breast cancer, natural therapeutic targets with fewer side effects are needed to treat the disease.

Worms have been used in traditional medicine for a long time to treat disease while maintaining soil fertility and preventing soil pollution. (Mincarelli et al. 2016; Deng et al. 2019).Red California Worm (*Eisenia foetida*) is a ringworm class of earthworms. It has a double transport system consisting of coelomic fluid (ECF) moving in a packed coloboma and a simple, closed blood circulation system (Ozkan et al. 2022). ECF protects against both cellular and humoral immunity of the worm in the fight against pathogenic microorganisms by containing a large number of collagenocytes such as adipocytes, mucosides, circular cells and chloragogen cells. have biological functions (Augustine et al. 2019; Edwards et al. 2019; Fiołka et al. 2019; Shafi and Faleh 2019; Czerwonka et al. 2020). Researchers have stated that ECF can be used as an alternative drug due to its easy availability, low cost and minor side effects (Dajem et al. 2020). Considering the medical value, pharmacological importance and cellular effects of ECF, It can be said that the curative effect against the toxic effects of drugs used in cancer treatment is worth investigating.

Sodium butyrate (NaBu), which is used as a chemotherapeutic agent in the treatment of breast cancer, which is the most common cancer in women in the world, is a short-chain fatty acid found in humans, produced from dietary fibre (Yuksel et al. 2022). It inhibits proliferation in breast cancer cell lines and induces morphological changes, causing growth arrest, differentiation and apoptosis in cells. (Huang et al. 2017). In cancer treatment, necrotic cell death may disrupt membrane integrity and cause the release of mediators associated with inflammation and immunosuppression into the extracellular environment. This situation may pose a threat to the patient by promoting carcinogenesis. Therefore, it is very important to eliminate or reduce the high cytotoxicity of NaBu for both neoplastic and normal cells. Our study investigated the chemoprotective effect of coelomic fluid obtained from *Eisenia foetida* on the toxicity of NaBu, a histone deacetylase inhibitor, in breast cancer. For this purpose, the cytotoxic and chemoprotective effects of ECF on breast cancer cell viability were determined. To evaluate the effect of ECF on the oxidative toxicity induced by NaBu in MCF-7 cells, changes in ROS production levels and expression of cell death and ROS-related genes (*Bax*, *Bcl-2*, SOD and CAT) were detected.

Material and methods

Preparation of Eisenia foetida coelomic fluid (ECF)

Eisenia foetida worms were cultured and fed in cattle manure in an EkosolFarm CompostBox unit with 65% RH at 25°C in the dark, obtained from a commercial company (Ekosol Tarım ve Livestock A.Ş.) in Manisa, Turkey. Before obtaining ECF, worms were collected by cold shock method by transferring them to wet filter paper to empty their intestinal tract (Endharti et al. 2019). The ECF was then sterilised by filtration (0.22 mm diameter) and centrifuged at 1000 rpm for 5 minutes at 4°C. The protein concentration of the collected ECF was measured using the Qubit protein assay kit (Thermo Fisher Scientific) and stored at −20°C before experiments.

Cell culture conditions

MCF-7 breast cancer cells were used to determine the chemo-protective effect of ECF against NaBu-induced cell toxicity. MCF-7 cells were obtained from American Type Culture Collection (ATCC) commercially. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) cell culture medium with 0.1% penicillin and streptomycin, 10% FBS and cultured at 37°C with 5% CO_2.

Pre-Treatment with ECF and exposure to the NaBu

Firstly to determine the cytotoxic effects of ECF on MCF-7 cells, WST-1 assay was performed. For this purpose, an equal number of cells were seeded on 96 well plates ($1x10^4$ cells/well) and incubated for 24 h. After incubation, the cells were treated with different concentrations of ECF (1, 5, 10, 20, and 40 µg/mL) for 24 and 48 h at 37°C with 5% CO_2. After incubation time, WST-1 reagent (Biovision) was added to each well and incubated for 40 min. Absorbance was measured at 450 nm with a microplate reader (Thermo Fisher Scientific). Additionally, an equal number of cells were seeded on 96 well plates ($1x10^4$ cells/well) and incubated for 24 h. After incubation, the cells were treated with different concentrations of NaBu (0.5, 1, 2, 3, 4 and 8 nM) for 24 and 48 h at 37°C with 5% CO_2. After incubation, the cell viability was measured by WST-1 assay described below. In the pre-treatment procedure, MCF-7 cells were pre-treated with ECF (1, 5 and 10 µg/mL) for 24 h, followed by exposure to NaBu (2 and 8 nM). 24 h later, the cell viability was measured by the WST-1 assay described below and the selected concentrations of ECF and NaBu were used for further experiments.

Reactive oxygen species (ROS) microplate assay

To assess the chemo-protective effect of ECF on oxidative toxicity induced by NaBu in MCF-7 cells, the ROS generation levels were measured by ROS microplate assay. For this purpose, MCF-7 cells were seeded in 96 well plates (25×10^3 cells/well) and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (1, 5, and 10 µg/mL) for 24 h, followed by exposure to NaBu (2 nM) for another 24 h incubation. After incubation assay was conducted according to the DCFDA/H2DCFDA-Cellular ROS Assay Kit (Abcam) procedure. The absorbance of each well was measured at Ex/Em = 485/535 nm using a microplate reader (Benchmark Plus, USA), and ROS generation levels of each group were calculated as fold change.

Imaging of nucleus morphology

To assess the chemo-protective effect of ECF on oxidative toxicity induced by NaBu in MCF-7 cells, the nucleus morphology was visualised by 4′,6-diamidino-2-phenylindole (DAPI) staining. For this purpose, MCF-7 cells were seeded in 96 well plates (5×10^4 cells/well) and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (1, 5, and 10 µg/mL) for 24 h, followed by exposure to NaBu (2 nM) for another 24 h incubation. After incubation, the cells were stained with DAPI (Thermo Scientific) for 5 min and washed twice. After washing, the cells were visualised with a fluorescent microscope (Olympus) and captured.

Real-Time polymerase chain reaction (RT-PCR) analysis

RT-PCR was conducted to explore the changes in the expression of apoptosis-related genes (*Bax, Bcl-2, SOD* and *CAT*). For this purpose, MCF-7 cells were seeded in T_{25} flasks (1×10^6 cells/flask) and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (1, 5, and 10 µg/mL) for 24 h, followed by exposure to NaBu (2 nM) for another 24 h incubation. After incubation, total RNA was isolated from the cells using Trizol Reagent (Thermo Fisher) according to the manufacturer's instructions. The concentration of isolated total RNA was measured by a Qubit 3 Fluorometer (Thermo Fisher Scientific), and cDNA was synthesised with a cDNA Reverse Transcription Kit (Thermo Fisher Scientific). After cDNA conversion, the relative gene expression levels of *Bax, Bcl-2, SOD* and *CAT* were analysed by using CFX Connect Real-Time PCR Detection System from Bio-Rad. Additionally, β-actin was used as an endogenous reference gene.

Statistical analysis

Statistical analyses were evaluated with GraphPad version 6.0 (La Jolla, CA) with a statistically significant level of $p < .05$. One-way ANOVA analysis with variance Tukey's test was used for multiple comparisons. For analysing the gene expression results, the software of Qiagen (https://www.qiagen.com/tr/shop/genes-andpathways/data-analysis-center-overviewpage/other-real-time-pcrprobes-orprimersdataanalysis-center/) was used.

Results

Cytotoxic and chemo-protective effects of ECF on cell viability

The cytotoxic effect of ECF and NaBu alone on MCF-7 cells and cell viability values obtained when the oxidative damage model was performed with NaBu in ECF pre-treated cells were determined by WST-1 assay, as shown in Figure 1. The results showed that after the cells were treated with 1, 5, 10, 20 and 40 µg/mL ECF, the cell viability rate of MCF-7 cells was decreased in a dose-dependent manner for 24 and 48 h and cell viability was more inhibited after 48 h treatment. However, 20 and 40 µg/mL ECF concentrations significantly decreased the viability of MCF-7 cells compared to the non-treated control group for 24 h ($p < .05$). Therefore, we selected 1, 5, and 10 µg/mL ECF concentrations as the optimal treatment concentration of ECF treatment (Figure 1a).

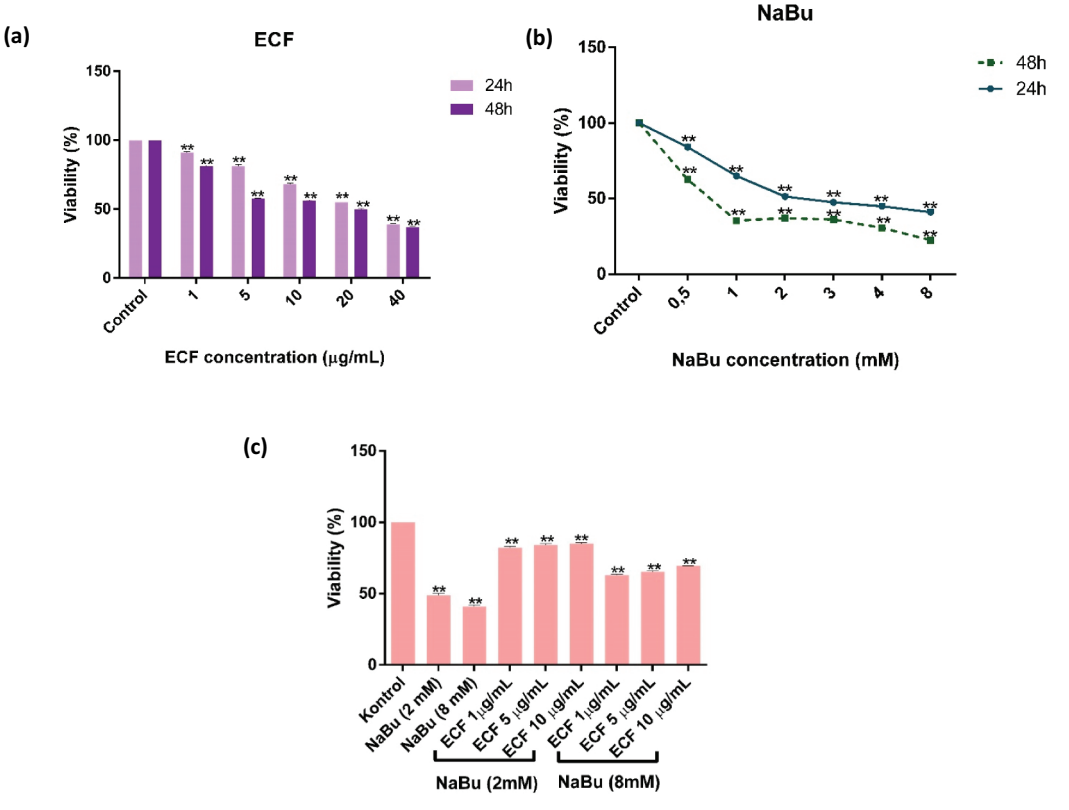


Figure 1. Direct and chemo-protective effects of ECF on cell viability on MCF-7 cells. (A) the effect of different concentrations of (1, 10, and 20 µg/ml) ECF on the viability of MCF-7 cells. (B) the effect of different concentrations of (0.5, 1, 2, 3, 4 and 8 mM) NaBu on the viability of MCF-7 cells. (C) the viability rate of MCF-7 cells pre-treated with ECF for 24 h at different concentrations (1, 5, and 10 µg/ml) followed by exposure to NaBu (2 and 8 mM) for another 24h incubation (**$p < .05$).

Additionally, NaBu treatment (0.5, 1, 2, 3, 4 and 8 nM) decreased the viability of HUVEC cells in a dose-dependent manner. The cell viability was found to be about 40%–51% in the 2 and 8 nM NaBu treatment groups for 24 h, and we concluded that this concentration could produce sufficient oxidative injury to the cells in this study ($p < .05$) (Figure 1b). To evaluate the effects of ECF on oxidative injury induced by NaBu in MCF-7 cells, ECF (1, 5, and 10 µg/mL) was pre-treated for 24 h before the 2 and 8 mM NaBu treatment. The cell viability was decreased in the 8 mM NaBu treatment group compared with the 2 mM NaBu treatment. On the other hand, the viability was significantly higher in ECF pre-treated cells than in the 2 mM NaBu treatment group ($p < .05$) (Figure 1c). Consequently, these results showed that ECF protects the viability of MCF-7 cells against NaBu-induced oxidative toxicity.

The effects of ECF on ROS level on MCF-7 cells

ROS levels were determined to determine the chemo-protective effect of ECF against NaBu-induced oxidative toxicity in MCF-7 cells. The results showed that the ROS level increased about 4.7-fold after being treated with NaBu (2 mM) ($p < .01$) (Figure 2). After pre-treatment with (1, 5, and 10 µg/mL) ECF followed by exposure to NaBu (2 mM), the ROS levels were markedly decreased compared with NaBu alone treatment group in a dose-dependent manner. Therefore the results demonstrated that pre-treatment of ECF decreased ROS production of MCF-7 cells against NaBu-induced oxidative toxicity.

The effects of ECF on nucleus morphology on MCF-7 cells

To evaluate the effect of ECF on NaBu-induced toxicity inMCF-7 cells, DAPI staining was conducted (Figure 3). According to our DAPI staining results, nuclear blebbing and condensations were increased after treatment with NaBu (2 mM) compared with the control group. On the other hand, pre-treatment with (1, 5, and 10 µg/mL) ECF followed by exposure to NaBu (2 mM)

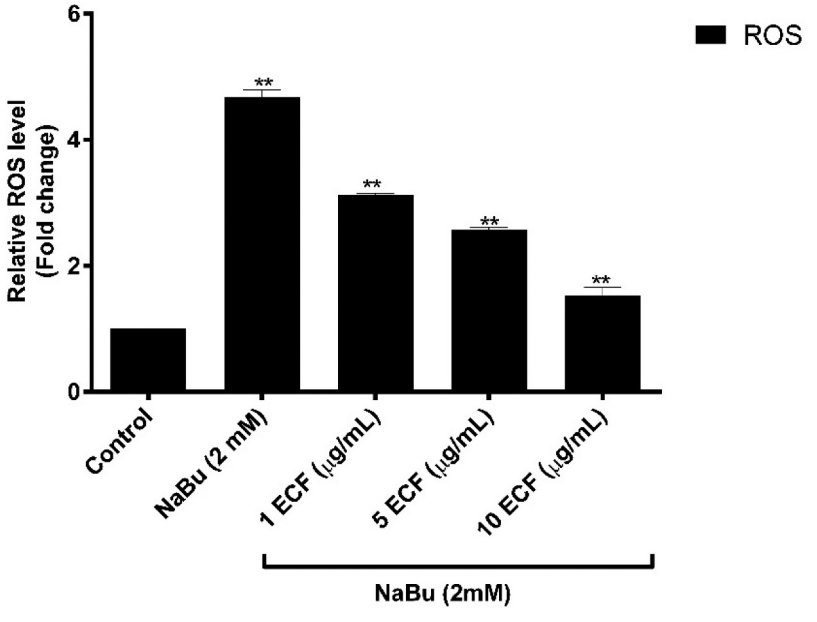


Figure 2. ECF decreased ROS production in MCF-7 cells after NaBu-induced toxicity. The cells were pre-treated with 1, 5 and 10 µg/ml ECF and followed by exposure to NaBu (2 mM) for another 24h incubation. The control group was not treated with ECF or/ and NaBu *(**$p < .05$)*.

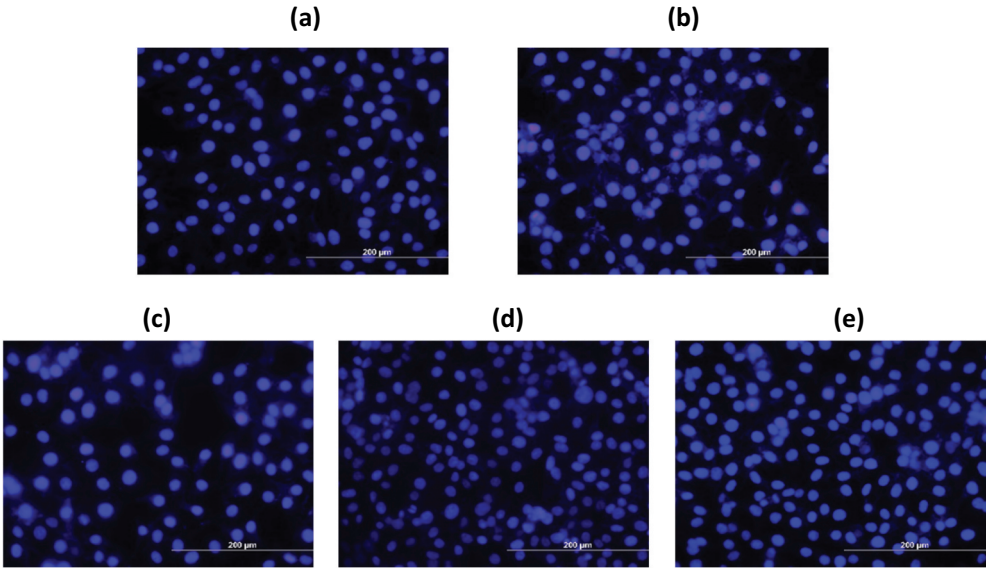


Figure 3. The effect of ECF on nucleus morphology in MCF-7 cells after NaBu-induced toxicity was determined by DAPI staining. The cells were pre-treated with 1, 5, and 10 µg/ml ECF and followed by exposure to NaBu (2 mM) for another 24h incubation. The control group was not treated with ECF or/and NaBu [(a) Control, (b) 2 mM NaBu, (c) 1 µg/ml ECF+ 2 mM NaBu, (d) 5 µg/ml ECF+ 2 mM NaBu, and (e) 10 µg/ml ECF+ 2 mM NaBu].

decreased morphological changes in the nucleus and improved cell morphology, similar to the control group (Figure 3). Therefore, the results were consistent with the viability and ROS analysis results.

The effect of ECF on gene expression level in MCF-7 cells

To further evaluate the chemo-protective effect of ECF on cell death and oxidative damage, RT-PCR analysis was conducted, and the expression levels of *Bax, Bcl-2, SOD* and *CAT* genes were analysed (Figure 4). As shown in Figure 4, the mRNA level of *Bax* increased, and *Bcl-2, SOD* and *CAT* decreased after NaBu alone treatment ($p < .05$). However, pre-treatment of MCF-7 cells with ECF followed by exposure to NaBu (2 mM) increased the mRNA levels of *SOD* and *CAT* compared with the NaBu alone treatment group without changing apoptotic cell death-related gene expression levels (*Bax* and *Bcl-2*) ($p < .01$). These results showed that ECF exhibited a chemo-protective effect on NaBu-induced oxidative toxicity in MCF-7 cells.

Discussion

Cancer patients not only tolerate the biological effects of cancer but also endure the high cost, toxicity, drug resistance, and regulatory limitations of treatment (Seiler et al. 2018). Therefore, it is very important to progress a safe, tolerable and effective treatment. For this purpose, our study investigated the chemoprotective effect of coelomic fluid obtained from *Eisenia foetida* on the toxicity of NaBu, a histone deacetylase inhibitor, in breast cancer. The cytotoxic and chemoprotective effects of ECF on breast cancer cell viability were determined. Also, to evaluate the effect of ECF on the oxidative toxicity induced by NaBu in MCF-7 cells, ROS production levels, cell death and changes in the expression of ROS-related genes (*Bax, Bcl-2, SOD* and *CAT*) were determined.

The ECF contains a large number of collagenocytes such as amoeocytes, mucosides, circular cells and chloragogen cells and contains various bioactive compounds that play a very important role in

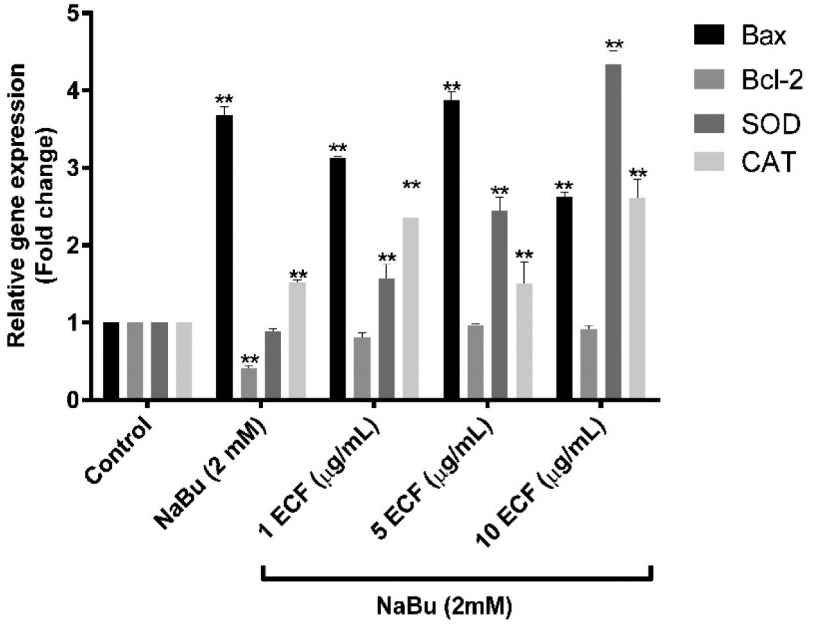


Figure 4. The effect of ECF on *Bax, Bcl-2, SOD* and *CAT* gene expression levels in MCF-7 cells after NaBu-induced toxicity. The cells were pre-treated with 1, 5 and 10 µg/ml ECF and followed by exposure to NaBu (2 mM) for another 24h incubation. The control group was not treated with ECF or/and NaBu (**$p < .05$).

the worm's cellular and humoral immunity in the fight against pathogenic microorganisms. Researchers have reported that these compounds in ECF have various biological functions such as anticancer, hemolytic, cytotoxic, hemagglutinating and proteolytic activities (Dinesh et al. 2013; Fiołka et al. 2019; Luparello et al. 2019; Ozkan et al. 2022). In our study, the cell viability of MCF-7 cells treated with only ECF was significantly reduced over 24 and 48 hours compared to the control group in a dose-dependent manner. It can be thought that cells that are increasingly damaged in the early days of exposure to ECF extracts begin to decrease in DNA replication activity during this time period, resulting in a slowing of the specific cycling phase, if not a halt, compared to controls (Chao et al. 2017; Luparello et al. 2019). In addition, Lysenin, Lumbrokinase and translationally controlled tumour proteins in ECF have a toxic effect on cultured cells such as colon and breast cancer cells by inducing hemolysis (Kobayashi et al. 2006; Ozkan et al. 2022). These proteins may be associated with the anticancer activity of ECF in MCF-7 cells. The data in our study supports the results of the researchers' study. However, considering the importance of ECF in terms of its medicinal value and its features regarding its use, its effect on cancer cells has the potential to be investigated.

NaBu is a short-chain fatty acid that acts as an inhibitor of histone deacetylase that plays an important role in the regulation of gene expression and chromatin structure, is involved in inducing apoptosis in many cancers, DNA double-strand break repair, oxidative stress inhibition, and various mechanisms, including gene expression and has important roles in cellular processes (Li et al. 2015; Salimi et al. 2017; Yuksel et al. 2022). Therefore, it is widely used in the clinical treatment of many tumours. In our study, we concluded that NaBu treatment decreased the viability of MCF-7 cells in a dose-dependent manner, and especially 24 hours of 2 and 8 nM NaBu treatment could produce sufficient oxidative damage to the cells. Additionally, we aimed to reduce this oxidative damage by treating ECF. As a result of our studies, the amounts of ROS were significantly lower in cells pre-treated with ECF than in the 2 mM NaBu treatment group. These results conclude that ECF can protect against NaBu-induced oxidative toxicity in MCF-7 cells. In addition, we think that the significant decrease in

ROS levels compared to the control group without ECF may indicate that pre-treatment of ECF protects MCF-7 cells against ROS production against oxidative toxicity caused by NaBu.

The protein encoded by *Bax* belongs to the *Bcl*-2 protein family and functions as an apoptotic activator (Lessene et al. 2008). The *Bax* gene is an apoptosis inducer and overexpresses *Bcl-2* in cancerous tissues. If proapoptotic proteins are present in a cell, the cell is more prone to apoptosis. If antiapoptotic proteins are abundant, they are less prone to apoptosis (Zhang et al. 2000). *Bax* is proapoptotic, while *Bcl-2* is antiapoptotic. (Opferman and Kothari 2018; Alam et al. 2019). Therefore, the intracellular *Bcl-2*/*Bax* ratio is extremely important in determining whether the cell will go to apoptosis. If *Bax* is high, the cell will go to apoptosis; if *Bcl-2* is high, apoptosis will be inhibited. The data we obtained determined that the mRNA level of *Bax* increased, and the expression levels of *Bcl-2* decreased after only NaBu treatment. We can say that the expression levels of *Bax* and *Bcl-2* genes related to apoptotic cell death remained unchanged in MCF-7 cells exposed to NaBu after pre-treatment with ECF, indicating that ECF has a chemoprotective effect in MCF-7 cells.

Long-term release of reactive oxygen species (ROS) caused DNA damage and increased mutation rate. This can induce oncogenic transformation in cancer-related genes and cause changes in DNA transcription and replication, activation of signal transduction pathways, and genomic instability (Ozkan and Yuksel 2022). Cells have evolved various antioxidant mechanisms to counterbalance ROS-induced oxidative damage to maintain their genomic stability. These mechanisms include enzymatic scavengers such as superoxide dismutase (SOD) and catalase (CAT), antioxidant enzymes involved in cell defence, DNA repair enzymes, and cellular mechanisms of genomic control. Cancer cells show an increase in reactive oxygen species (ROS) due to oncogenic stimulation, increased metabolic activity and mitochondrial damage (Srinivas et al. 2019; Perillo et al. 2020; Yuksel and Ozkan 2021). When we look at the gene expression data of SOD and CAT enzymes, which catalyse the oxidative stress product, superoxide, and hydrogen peroxide, it was determined that they decreased only in MCF-7 cells treated with NaBu. On the other hand, detecting an increase in related genes in cells treated with ECF seems remarkable. The reason for this increase in cancer cells may be a result of the therapeutic efficacy of ECF, which has biological functions such as anticancer.

Conclusion

The effects of various components as an alternative treatment strategy should be investigated due to various disadvantages such as toxic effects, serious side effects, and lack of cell specificity of current treatment methods used in cancer treatment. Because a treatment method that can be a definitive solution for many types of cancer has not been fully developed and the disease is on the rise, studies in this direction are increasingly continuing and keeping up to date. A more detailed examination of the potential therapeutic property of ECF in cancer treatment and, thus, the fact that new data will be obtained for the first time to fill the gap in the literature reveals the importance and originality of our study. In addition, the results to be obtained will support the acquisition of data that can be used for the pharmacological development of ECF in the future, and it can move toward becoming a potential therapeutic molecule with fewer side effects in cancer treatment. Thus, data could help support the clinical trial of epigenetic treatments for breast cancer.

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