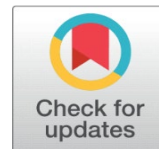


COMPARATIVE EFFECTS OF BORIC ACID AND RESVERATROL ON MCF-7 BREAST CANCER CELLS METASTATIC BEHAVIOUR



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ABSTRACT

Motivation/Background: The metastasis of tumor cells consists of steps such as epithelial-mesenchymal transition, transendothelial migration and the formation of metastatic colonization. E-Cadherin and vimentin are main proteins associated with EMT, whereas MMP-9 is associated with migration.

Method: We aimed to investigate effects boric acid and resveratrol comparatively on metastatic behaviors on MCF-7. 30 μ M boric acid and 50 μ M resveratrol were administered to BA, BA+RES, and RES groups 48hours. Cells stained immunocytochemically by Anti-E-Cadherin, Anti-Vimentin, and Anti-MMP-9 antibodies and H-Score analysis carried out and migration analyzed by woundhealing, morphologically.

Results and Conclusions: It's been observed that boric acid doesn't affect the EMT capability of the MCF-7 cells in terms of E-Cadherin and vimentin expression; whereas it's affected migration both by decreasing the MMP-9 expression and also by inhibiting migration.

Received 01 December 2021

Accepted 26 December 2021

Published 31 January 2022

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DOI

[10.29121/granthaalayah.v10.i1.2022.4460](https://doi.org/10.29121/granthaalayah.v10.i1.2022.4460)

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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Keywords: Boric Acid, MCF-7 Breast Cancer, Epithelial-Mesenchymal Transition, Metastasis, Resveratrol.

1. INTRODUCTION

Breast cancer is a type of cancer which has the highest mortality rates and is the most common cancer in women [Global Cancer Observatory \(2021\)](#). There are novel therapeutic approaches being developed in breast cancer treatment nowadays [Mahjoubin-Tehran et al. \(2020\)](#). Despite this, there is still need for effective methods in terms of cancer treatment. In general, deaths based on cancer is mainly caused by metastasis [Dillekås et al. \(2019\)](#). [Banfalvi \(2012\)](#) believes that the development of breast cancer is mainly metastasis rather than the primary tumor and states that the main reason of death caused by breast cancer is tumor invasion and metastasis [Banfalvi \(2012\)](#). 10-15% of patients usually have metastasis during the three years following the first treatment. Metastasis includes stages such as local invasion (Epithelial-Mesenchymal Transition (EMT)), intravasation, survival in the circulation, extravasation (transendothelial migration (TEM/diapedesis)), the development of micrometastasis and the development of metastatic colonization [Reymond et al. \(2013\)](#). Metastasis in cancer cells is a complex process directed by a great number of biochemical and molecular factors. Although this process and the mechanisms of action are not completely enlightened, the chance to eliminate the disease will arise if the cancer cells can be stopped in the invasion stage.



Invasion occurs when cell-cell and cell-matrix interactions are damaged. Cadherin, one of the cell-cell adhesion proteins, takes the leading role in this process. While N-Cadherin shows an increase in the gastrulation period, is highly found in mesenchymal cells and is the cadherin which is accepted as the marker of Epithelial-Mesenchymal Transition (EMT); in metastatic breast cancer, the decrease in E-Cadherin expression is the hallmark of invasion [Özkara et al. \(2020\)](#). Studies on breast, pancreas, lungs, and bladder cancer cells have shown that cells that have normal E-Cadherin expression display epithelioid phenotype and are not invasive; whereas cells that have low-expression of E-Cadherin display fibroblast-like phenotype and are invasive [Farahani et al. \(2014\)](#). Moreover, proteins that are essential for extracellular matrix (ECM) degradation and tumor invasion such as matrix metalloproteinase (MMP) -1 and MMP-9 play an important role in metastasis and consequently in the course of the disease. Some studies have shown an over-expression of MMP-9 in breast cancer cells and have reported that it is effective in cancer metastasis and invasion [Jones et al. \(1999\)](#), [Westermarck and Kähäri \(1999\)](#).

Both *in vivo* and *in vitro* studies have shown that resveratrol (RES), which is a polyphenol naturally found in grapes and red wine, has anti-cancer properties and delays carcinogenesis [Baur and Sinclair \(2006\)](#), [Signorelli and Ghidoni \(2005\)](#). [Chen et al. \(2019\)](#) and [Xu et al. \(2017\)](#) have shown in their *in vitro* studies that in breast cancer and gastric cancer cells, resveratrol changes protein expressions that play a role in mesenchymal transition such as E-Cadherin and Vimentin [Chen et al. \(2019\)](#), [Xu et al. \(2017\)](#). Likewise, in the year of 2021, [Chang et al. \(2021\)](#) have reported that the application of 50 μM Resveratrol decreases the expression of MMP-9 protein in cancer cells [Chang et al. \(2021\)](#). Boron, a trace element for organisms, has high affinity to oxygen and nature is filled with plenty of borates such as in the forms of boric acid and borax [Devirian and Volpe \(2003\)](#). Boric acid, which has a soluble boron form, can easily be taken into the human body by food and drinks. Following the intake of a boric acid in diet, it is absorbed by the gastrointestinal system which is then passed on to the blood circulation and it plays a role in several physiological and biochemical events due to its antioxidant or anti-cancer properties [Barranco et al. \(2007\)](#), [Pawa and Ali \(2006\)](#). Most cancer studies that are carried out with BA have focused on the aspect of its cytotoxic effect [Aydin et al. \(2021\)](#), [Hacioglu et al. \(2020\)](#). Nonetheless, in literature there are not quite enough studies on boric acid's effect on the EMT capabilities of cancer cells. In this study, we have examined *in vitro* boric acid's effect on MCF-7 breast cancer cells in terms of cell migration, metastasis, and morphology in comparison to resveratrol.

2. MATERIALS AND METHODS

2.1. PREPARATIONS OF CHEMICAL SOLUTIONS

Resveratrol (TCI-R0071, TCI AMERICA) was solved into 100 μM of medium [Leon-Galicia et al. \(2018\)](#), [Schmidt et al. \(2020\)](#) as a IC_{50} dose which was determined according to the published reports [Leon-Galicia et al. \(2018\)](#), [Schmidt et al. \(2020\)](#). MTT test was done for boric acid and solved into an IC_{50} dose of 30 μM in a medium preheated to 37 °C was used.

2.2. CELL CULTURES

MCF-7 breast cancer cells were used for the study (ATCC, USA). Cells were cultured in RPMI-1640 medium including 10% Fetal bovine serum (FBS), 1% L-Glutamin and 1% antibiotics (penicillin-streptomycin) and incubated at 5% CO_2 density and 37 °C during the experimental period of 2 days. When the cells reached

the confluency number of 80%, they were divided into 4 groups as follows: Boric acid (BA), boric acid+resveratrol (BA+RES), resveratrol (RES) and control (CONT) groups and the cells were treated by the effective substances (boric acid and resveratrol) for 48 hours.

2.3. DETERMINATION OF CELL VIABILITY USING MTT ASSAY

To determine the cytotoxic effect and IC_{50} dose of boric acid on cells, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was carried out. For resveratrol, the IC_{50} dose was used in accordance to the literature [Leon-Galicia et al. \(2018\)](#), [Schmidt et al. \(2020\)](#). MCF-7 cells were cultured in 96-well plates with a density of 2×10^4 cells per well and following the cells' adherence, different concentrations of boric acid (10, 30, 50 and 100 μ M) were exposed to cells for 48 hours. At the end of 48 hours, MTT (Sigma-Aldrich) solution prepared in accordance with manufacturer's instructions was added to each well as 20 μ L/well and incubated at 37 °C for 4 hours. Following 4 hour of incubation, MTT was removed and 200 μ L of dimethyl sulfoxide (DMSO) was added to each well. Then they were incubated on the shaker in a dark room for 5 minutes and the absorbance values were measured at a wavelength range of 570-690 nm with a microplate reader (Shimadzu UV-1601). Three wells were used for each concentration and each measurement was repeated seven times.

2.4. ANALYSIS OF CELL MORPHOLOGY

To analyze cell morphology, the cells were visualized at the time points of 0th (zero) and 48th hours following the treatments of the substances under the phase-contrast microscope (ZEISS Primo Vert iLED).

2.5. IMMUNOCYTOCHEMICAL METHODS

At the end of 48-hour treatments, the cells in each group were washed with PBS once and fixed with 4% paraformaldehyde for 30 minutes. After fixation 3% hydrogen peroxide (H_2O_2) was applied for 5 minutes followed by washing 3 times with PBS each taking five minutes again. Then it was incubated in 0,1% Triton-X 100 for permeabilization for 15 minutes. After the washing, an hour of protein blockage and then the incubation with anti-E Cadherin (1/200, ab76055, abcam), anti-Vimentin (1/200, ab8978, abcam) and anti- MMP-9 antibodies (1/200, ab76002, abcam) were carried out overnight at +4 °C. Following the incubations, the secondary antibodies were applied, and immunoreactivity was visualized using DAB (3,3'Diaminobenzidine) chromogen. Finally, covering medium was added and the pictures were taken under light microscope (NIKON Eclipse E600) using the Image Analysis Program (NIS elements, Japan). Following immunohistochemical staining, H-Score assessment which is a semiquantitative method was performed for statistical analysis. H-score: for each group, 500 cells were counted for each region. Based on the intensity of the cellular staining; using the formula of the total cell number stained being as (%) X (staining intensity 0, 1,2,3,4,5) H-score were evaluated between 0 – 500 [Numata et al. \(2012\)](#).

2.6. WOUND HEALING ASSAY

Once MCF-7 breast cancer cells (ATCC) were 100% confluent, the treatments were carried out to the designated groups and a wound field were generated in the middle by making a gap with the help of a 10 μ L-sterile pippete tip. To compare the migration tendencies of the cells towards the wound field to close it and their

velocities during these migrations, the pictures were taken from each group using a phase-contrast microscope for twelve-hour intervals and evaluated comparatively Zhang et al. (2019).

2.7. STATISTICAL ANALYSIS

H-score assessment data were analyzed by using binary comparison test (Kruskal Wallis test) using SPSS 20.0.(Table 1).

2.8. ETHICS COMMITTEE APPROVAL

The cells used in the study are commercially available, so ethics committee approval is not required.

3. RESULTS

3.1. ANALYSIS OF THE EFFECT OF BORIC ACID ON CELL VIABILITY USING MTT ASSAY

To decide the IC₅₀ dose of boric acid in MCF-7 cells, the level of viability was determined using MTT assay. Boric acid doses of 10, 30, 50 ve 100 µM to the MCF-7 cells were administered for 48 hours. At the end of this 48-hour period, it was observed that 48-hour administration of 100 µM dose of boric acid abolished cell viability completely. Viability in MCF-7 cell groups decreased by approximately 81%, 54% and 27% at 10, 30 and 50 µM doses of boric acid, respectively. Therefore, the IC₅₀ dose of boric acid for MCF-7 cells were determined as 30 µM and administered to the study groups in other stages (Figure 1). No dose determination tests were done for resveratrol since sufficient studies were done previously.

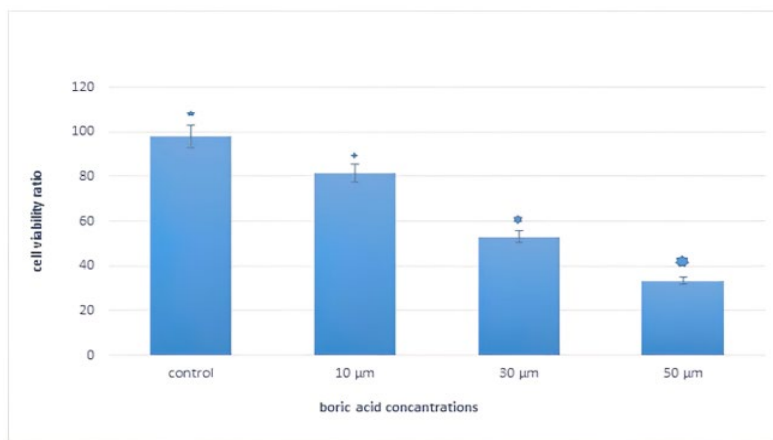


Figure 1 MTT analyzes of the effects of boric acid administration on cell viability in MCF-7 cells

3.2. CELL MORPHOLOGY

MCF-7 breast cancer cells were incubated for 2 days. When the cells reached 80% of confluency, they were divided into groups and the doses determined as a result of MTT assays of boric acid (BA), resveratrol (RES) and boric acid + resveratrol (BA+RES) together were administered for 48 hours. Before treatment, all MCF-7 cells in all study groups and the control group were observed to have an epitheloid morphology except for a few of them having a round structure as well as the fact that their cell membrane boundaries were visible the structure of the nucleus and cytoplasm were normal (Figure 2A). After the treatment for 48 hours, it was

noticed that cell morphology in BA, RES and BA+RES groups was round shaped like apoptotic cells as well as having nuclear condensation and noticeable morphologic changes in the cytoplasm such as vacuolization. These cells with apoptotic properties were observed to be higher in number in BA and BA+RES groups in comparison to CONT and RES groups. However, vacuolization and nuclear condensation increased in cells in the RES group while no noticeable changes in cell morphology was seen (Figure 2), Gharagozloo et al. (2015).

Table 1 Immunocytochemical analyses of E-Cadherin, Vimentin and MMP-9 in control, Boric acid, Boric acid+Resveratrol and Resveratrol groups were given mean \pm SD values

ANTIBODY	GROUPS	$\bar{x} \pm SD$
E-CADHERIN	BA	496 \pm 4,35 ^{ac} (497)
	BA+RES	1103 \pm 3,71 ^b (1103)
	RES	1467 \pm 2,73 ^{bc} (1467)
	CONT	738 \pm 2,30 ^a (739)
VIMENTIN	BA	1714 \pm 2,73 ^{bc} (1714)
	BA+RES	1984 \pm 2,38 ^b (1984)
	RES	1074 \pm 2,86 ^a (1074)
	CONT	1348 \pm 3,56 ^{ac} (1350)
MMP-9	BA	984 \pm 2,73 ^{ac} (984)
	BA+RES	923 \pm 3,03 ^a (923)
	RES	1173 \pm 3,36 ^{bc} (1173)
	CONT	1365 \pm 3,05 ^b (1366)

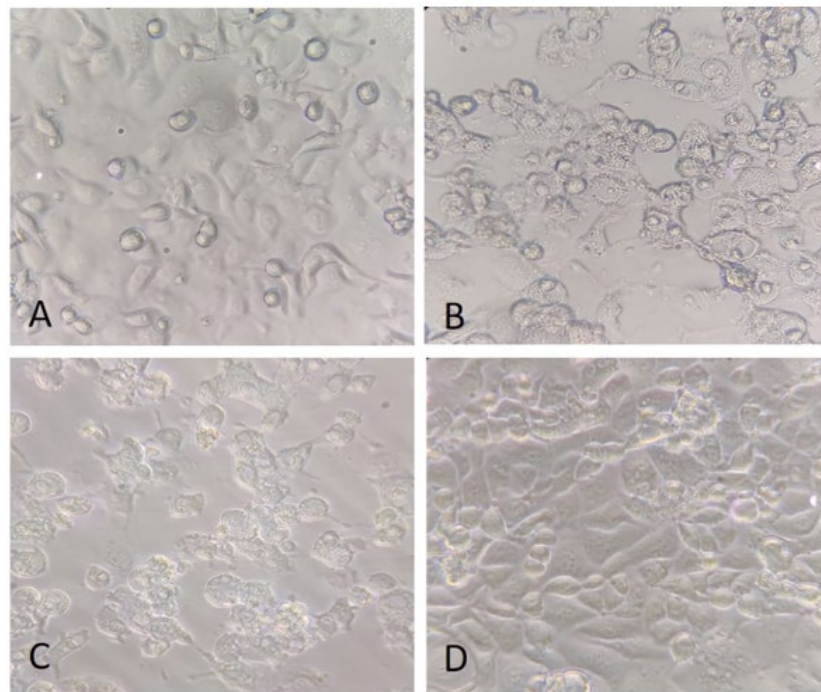


Figure 2 Morphology of MCF-7 cells in various groups (All magnifications are x400.). Morphological changes seen after 48 hours of application of Boric acid, Boric acid+Resveratrol and resveratrol to the cells. A) Control group; B) Boric acid; C) Boric acid+ resveratrol; D) Resveratrol (400x magnification).

3.3. EFFECTS OF BORIC ACID AND RESVERATROL ON EMT

To analyze the effect of boric acid and combined Boric acid + Resveratrol on epithelial-mesenchymal transition (EMT) proteins, boric acid, combined boric acid + resveratrol and resveratrol treatments were done in MCF-7 cells for 48 hours. The control group was considered as the group with no treatments at all. After treatments, the changes in Vimentin and E-Cadherin protein expressions were analyzed immunocytochemically and H-Score analysis were carried out (Figure 3). E-Cadherin immunoreactivity was found to be higher in the RES group ($p = 0,04$) compared to the CONT group, while E-Cadherin protein expression levels in BA and BA+RES groups were observed to be increased slightly in comparison to the CONT group ($p > 0,005$). H-Score analysis revealed that Vimentin immunoreactivity was observed to be increased in the BA+RES group showing a higher protein expression compared to the control group ($p = 0,008$). However, the change in the intensity of immunoreactivity in RES and BA groups was not significantly different compared to the control group ($p > 0.05$).

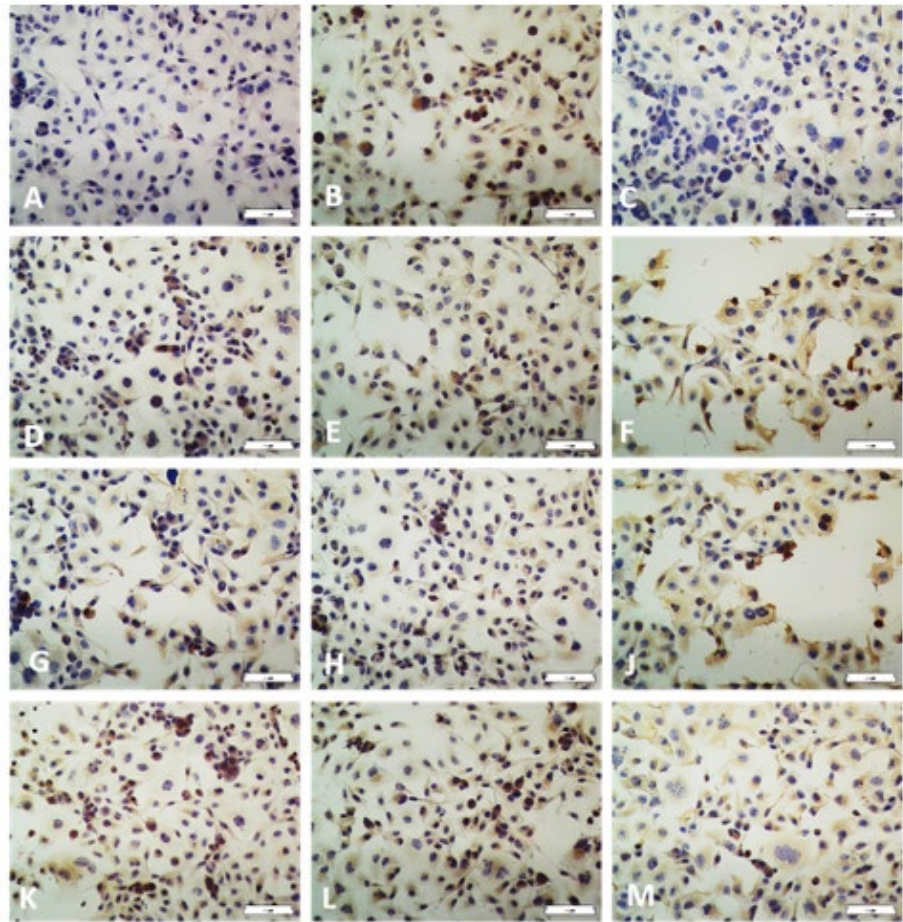


Figure 3 Immunohistochemical clearances of E-Cadherin, Vimentin and MMP-9 immunoreactivities. (Scale bar 100 μ m). The vertical column groups and the horizontal row indicate the antibodies used. E-Cadherin stain: A) Control group; D) Boric acid; G) Boric acid+ resveratrol; K) Resveratrol. MMP-9 stain: B) Control group; E) Boric acid; H) Boric acid+ resveratrol; L) Resveratrol. Vimentin stain: C) Control group; F) Boric acid; J) Boric acid+ resveratrol M) Resveratrol (200x magnification).

3.4. EFFECTS OF BORIC ACID AND RESVERATROL ON CELL INVASION

To investigate the effect of boric acid and resveratrol on cell invasion, the intensity of MMP-9 protein expressions was analyzed immunocytochemically between the groups and H-Score analyses were done (Figure 3). MMP-9 immunoreactivity levels were found to be decreased in BA and BA+RES groups, compared to the control group (respectively $p = 0,008$ $p = 0,000$), while no change was recorded in the RES group ($p = 0,181$).

3.5. EFFECT OF BORIC ACID AND RESVERATROL ON CELL MIGRATION

MCF-7 cells were cultured in a 24-well plate and waited until they became confluent, a wound field was generated in the middle of each well and the administrations were done for BA, BA + RES and RES groups. No administration was performed for the control group. To analyze cell migration tendency, the groups were photographed from the wound fields at the time points of 0, 12, 24, 36 and 48 hours. A complete wound healing was observed at the time point of 36 hours in the control and RES groups and velocity and organization of wound healing was the same in the RES group as the control group. In BA and BA+RES groups, the wound field was not healed until the time point of 48 hours and the cell migration to the wound field ceased, besides no healing was observed in the wound field (Figure 4).

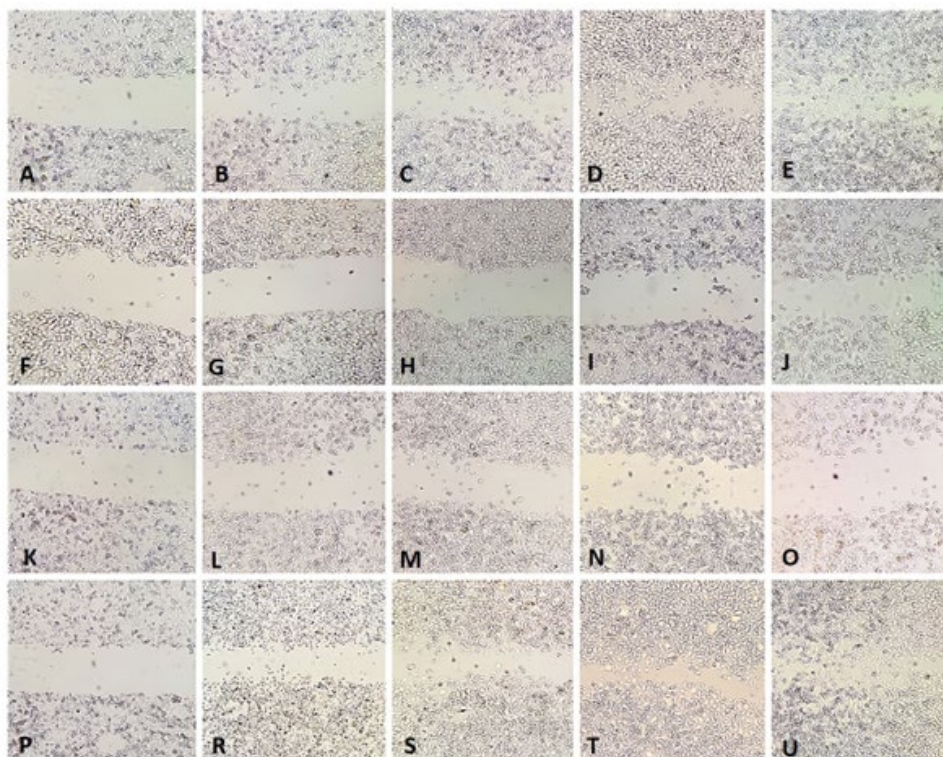


Figure 4 Evaluation of cell migration by wound healing assay (Scale bar 200 μm). Specifies vertical column groups, and horizontal row specifies application times. Control groups; A, B, C, D, E. Boric acid groups; F, G, H, I, J. Boric acid+ resveratrol; K, L, M, N, O. Resveratrol; P, R, S, T, U. 0. Hours; A, F, K, P. 12. Hours; B, G, L, R. 24. Hours; C, H, M, S. 36. Hours; D, I, N, T. 48. Hours; E, J, O, U

4. DISCUSSION AND CONCLUSIONS

Depending on the stage of the disease, the patient's properties and general health, cancer treatment might include several options such as surgery, radiation therapy, hormonal therapy, chemotherapy, goal-directed therapy, or alternative medicine. Alternative medicine is among the methods that aims to improve the immune system and support the main treatment, yet its reliability and efficiency is not proven by controlled experiments. Up to this day, several anti-cancer substances used in alternative medicine have been determined, produced, and tested; and 70% of the anti-cancer medicine that is being used is obtained from natural resources [Karikas \(2010\)](#). There are also studies regarding boron not having a carcinogenic effect or rather it is accelerating carcinogenesis [Yalcin and Abudayyak \(2020\)](#). It has been shown that many boron products and its compounds have cytotoxic effects on rats and humans, as well as boron's effects on the migration, invasion and epithelial-mesenchymal transition capabilities of cancer cells have not yet been able to be clarified. In this study we present, the IC₅₀ dose, which is accepted as the toxic dose of applying boric acid to MCF-7 breast cancer cells, has been investigated and identified as 30 µM. The identified dose has been used in establishing the treatment groups and boron's effect on the migration, invasion and EMT tendencies of MCF-7 cells has been compared to resveratrol, in which several scientific studies report that it decreases the migration, invasion and epithelial-mesenchymal transition tendencies of cells.

It is known that E-Cadherin is a protein that is closely involved in the invasion and metastasis of human malignant tumor cells and the inactivation of it plays an important role in the development of the tumor [Lee et al. \(2015\)](#). It has been observed that with the loss of E-Cadherin, EMT is induced and in malignant differentiated carcinomas of different cancers, the E-Cadherin expression is either decreased or completely gone [Graff et al. \(1995\)](#). In our study, there was a significant increase in the RES group when the E-Cadherin immunoreactivity of cells were compared to the control group. However, the fact that there was no significant increase in BA and BA+ RES groups, compared to the CONT group, has shown that BA does not change the E-Cadherin expression in MCF-7 cells on its own and that RES might have a negative effect on the repressive property of E-Cadherin protein expression. It has been shown in our study and the other studies in literature that resveratrol treatment on MCF-7 cells decreases E-Cadherin expressions [Shi et al. \(2013\)](#), [Sun et al. \(2019\)](#). In spite of this, our study is among the lead studies that show the negative effect on E-Cadherin protein expressions that BA has on cancer cells.

Vimentin, a member of the intermediate filament protein family, is highly expressed in normal mesenchymal cells and is known to maintain cellular integrity and provides resistance against stress. An increase of Vimentin expression has been reported in various epithelial cancers including prostate cancer, gastrointestinal tumors, central nervous system tumors, breast cancer, malignant melanoma, lung cancer and other cancer types. The over-expression of Vimentin in cancer is well associated with increased tumor growth, invasion, and poor prognosis [Satelli and Li \(2011\)](#). In various studies, increased E-Cadherin expression level and an increase in Vimentin expression in MCF-7 cells were associated with inhibition of poor prognosis and decreased EMT [Wang et al. \(2020\)](#). In our study, along with the similarity between the RES-CONT groups, a slight increase was observed in the BA group when the BA-CONT groups were compared ($p > 0,05$). Nonetheless, when the BA+RES group was compared to the CONT group, it was observed that there was an increase in Vimentin expression and this increase gave statistically significant p -

value ($p = 0,008$). This result of our study makes us think that administration of resveratrol or boric acid alone to MCF-7 cells for 48 hours, was not effective in the change of Vimentin expression with the applied doses. In literature, there are studies that report the decrease of Vimentin expression subsequent to the resveratrol treatment. In 2011, Vergara et al., have reported that treatment of resveratrol for 24 hours at the dose of 25 μM of MCF-7 cells led to a decrease in Vimentin expression, whereas, in 2013, Tsai et al., did not observe any differences between resveratrol (RES) and control groups when applied at the dose of 20 μM for 24 hours in MCF-7 cells [Tsai et al. \(2013\)](#) , [Vergara et al. \(2011\)](#). Our results support the finding by Tsai et al. It is thought that the difference between our finding and the study of Vergara et al. may be caused by the different doses and times that were applied. According to our result, while BA and RES alone did not change Vimentin expression at these durations and doses, it is thought that when BA+RES is applied together, it may cause Vimentin expression which is associated with poor prognosis.

Cancer cells frequently excrete MMPs and serine protease that are able to degrade extracellular matrix proteins. MMP-2 and MMP-9 are highly expressed in malignant tumors and make cell migration easier by playing a role in the degrading the extracellular matrix which is an important component of basal membrane that leads to cancer metastasis. In an *in vivo* study in rats treated with fucoidan in 2019, He et al., associated with the decrease of MMP-9 expression, with increased apoptosis of breast cancer cells and decreased EMT capabilities [Xue et al. \(2019\)](#). It has been observed in our study that the MMP-9 immunoreactivities of RES and CONT groups were similar ($p = 0,181$). In the year of 2021, [Chang et al. \(2021\)](#) have reported that applying 50 μM of Resveratrol for 24 hours causes a significant decrease in the MMP-9 expression of cancer cells [Chang et al. \(2021\)](#). Likewise, in the year of 2016, Park et al., have shown that there was a significant decrease in the MMP-9 expressions of breast cancer cells when treated with 10 μM of resveratrol, that were loaded to pieces of gold, for 24 hours [Park et al. \(2016\)](#). In our study, the applied 50 μM of resveratrol for 48 hours did not change the MMP-9 expression. Although the used dose was the same as the one used by Chang et al., it did not have the same effect probably due to different cell lines being used. In a similar manner, Park et al., applied resveratrol in a lower dose and time, but they applied it indirectly in the form of gold-coated nanoparticles. It is thought that the difference may be due to the fact that nanoparticles enter the cell more easily and effectively. We found that BA significantly reduced MMP-9 immunoreactivity when the BA group was compared to the CONT group ($p = 0,008$). Compared to the CONT group ($p = 0,000$), it has been observed that there was also a significant decrease in MMP-9 in the BA+RES group and this decrease was caused by BA. It is thought that applying 30 μM dose of BA for 48 hours to the MCF-7 cells may repress cell invasion by decreasing MMP-9 expression. Our wound healing experiment results also support the decrease in MMP-9 protein expression. When looked at the results of this experiment, for CONT and RES groups, the wound area had completely healed, and the cells had completed the migration to the wound field at the end of 48 hours. However, in the BA and BA+RES groups, no cell migration was recorded throughout 48 hours and at the end of the 48th hour and the wound field remained as it was at the 0th hour. According to these results, when BA alone or with Resveratrol is applied to MCF-7 cells *in vitro*, it seems to inhibit cell migration.

The effect of BA and RES, both on their own and combined, on the morphologic characteristics of MCF-7 cells was evaluated with phase-contrast microscope both before and after treatment. The negative effects of boric acid on cell morphology were seen in both the BA group and BA+RES group. Similar to the changes in mitotic cells, there was an increase of spheric shape and nuclear condensation in cells. When

it comes to the RES group, besides the amount of increase in nuclear condensation, the distribution of sphere-shaped cells was similar to the CONT group. In their study carried out in 2020, Josifovska et al., have shown with electron microscopic methods that there was an increase in the number of autophagic vacuoles in resveratrol treated cancer cells [Josifovska et al. \(2020\)](#). In the study we present, there was also an increase in intracellular vacuolization for both the RES group and BA and BA+RES groups. As shown in Josifovska et al. study, this increase is thought to be caused by the cells' autophagic or apoptotic differences. The effects boric acid has on cell autophagy and apoptosis must be evaluated further with advanced molecular methods.

In conclusion, it has been observed that BA does not affect the EMT capabilities of MCF-7 cells in terms of E-Cadherin and Vimentin protein expressions, whereas it does affect cell migration both by decreasing MMP-9 protein expression as well as inhibiting cell migration in vitro. Our study can be accepted as a lead study in terms of the effects boric acid has on cell migration and EMT.

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