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Apoptotic Effects of Urtica dioica extract and Boric Acid in MCF-7 Breast Cancer Cell Line: A Study

Urtica dioica ve Borik Asidin MCF-7 Meme Kanseri Hücre Hattındaki Apoptoz Etkileri: Bir Çalışma

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Abstract: This study investigates the effects of *Urtica dioica* (nettle), Boric acid, and their combinations on MCF-7 human breast cancer cells. The goal is to explore how these substances impact apoptosis (programmed cell death) and anti-apoptotic pathways in a breast cancer model while assessing the protective effects of *Urtica dioica* and Boric acid.The MTT assay was employed to measure cell viability, and IC50 doses were determined. Protein levels of BAX, p53, Cytochrome C, and Calpain were quantified using the ELISA method. Additionally, phytochemical analyses were conducted using HPLC.Results indicated a statistically significant increase in p53 levels between the control group and those treated with *Urtica dioica* and Boric acid ($p<0.001$). Cytochrome C levels also showed a significant increase in the *Urtica dioica* group, with Boric acid demonstrating a meaningful increase ($p<0.001$, $p<0.01$). HPLC analysis of *Urtica dioica* extract identified caffeic acid as one of the critical phytochemicals.The findings suggest that combining *Urtica dioica* and Boric acid has limited effects on apoptosis in breast cancer cells, although positive impacts on p53 and Cytochrome C were noted. These results provide a foundation for considering *Urtica dioica* and Boric acid in breast cancer treatment. However, further clinical and animal studies are needed to evaluate these substances' efficacy, safety, and appropriate dosages. Future research will deepen our understanding of *Urtica dioica* and Boric acid's roles in breast cancer therapy and help develop new treatment strategies.
Keywords: Breast cancer, MCF-7, *Urtica dioica*, Boric acid, Apoptosis

Özet: Bu çalışmanın amacı, *Urtica dioica* (ısırgan), borik asit ve bunların kombinasyonlarının MCF-7 insan meme kanseri hücreleri üzerindeki etkilerini değerlendirmektir. İki maddenin apoptozu (programlı hücre ölümü) ve anti-apoptotik yolları nasıl etkilediğini araştırmak ve *Urtica dioica* ile borik asidin koruyucu etkilerini belirlemektir.Hücre canlılığını değerlendirmek için MTT testi kullanılmış ve hücreler için IC50 dozları belirlenmiştir. Ayrıca, hücrelerde BAX, p53, Sitokrom C ve Kalpain protein düzeyleri ELISA yöntemi ile ölçülmüştür. Fito-kimyasal analizler ise HPLC yöntemi ile gerçekleştirilmiştir.P53 konsantrasyon ölçümleri, kontrol grubu ile *Urtica dioica* ve borik asit ile tedavi edilen gruplar arasında istatistiksel olarak anlamlı bir artış göstermiştir ($p<0.001$). Sitokrom C konsantrasyon ölçümleri, kontrol grubu ile *Urtica dioica* arasında çok anlamlı bir artış, borik asit için ise anlamlı bir artış göstermiştir ($p<0.001$, $p<0.01$). Ayrıca, *Urtica dioica* ekstresi HPLC ile analiz edilerek, kafeik asit fito-kimyasallardan biri olarak belirlemektir. Bu araştırma, *Urtica dioica* ve borik asidin kombinasyonunun meme kanseri hücrelerinde apoptoz üzerindeki etkilerinin sınırlı olduğunu göstermektedir. Bu bileşenlerin p53 ve Sitokrom C üzerindeki olumlu etkileri gözlemlenmiştir. Bu bulgular, *Urtica dioica* ve borik asidin meme kanseri tedavisinde potansiyel kullanımına zemin hazırlamaktadır. Ancak, bu maddelerin etkinliğini, güvenliğini ve uygun dozlarını belirlemek için daha fazla klinik ve hayvan çalışmasına ihtiyaç vardır. Gelecek araştırmalar, *Urtica dioica* ve borik asidin meme kanseri tedavisindeki rollerini anlamamıza katkı sağlayacak ve yeni tedavi stratejilerinin geliştirilmesine yardımcı olacaktır.
Anahtar Kelimeler: Meme kanseri, MCF-7, Isırgan Otu, Borik asit, Apoptoz

Ethics Committee Approval: Since no cell culture experiments live animal or human subjects were used no ethics committee approval was required. Our study was conducted in accordance with scientific reserarch standards.

Informed Consent: The authors declared that informed consent form was signed by the participants.

Authorship Contributions: AA and HK planned the experiments. AA, HK, GG and AK carried out the experiments. AA and HK performed the data analysis. AA and HK wrote the manuscript. HK, thesis advisor.

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1. Introduction

Cancer is a disease characterized by the uncontrolled proliferation of cells due to various factors, including DNA damage (1,2). Breast cancer, a multifactorial malignancy, can spread without symptoms (3). Hormonal factors, particularly estrogen, are pivotal in its development (4). The MCF-7 cell line has been widely utilized in breast cancer research (5). Apoptosis is a crucial mechanism for maintaining cellular homeostasis and eliminating damaged cells. Proteins such as P53 (Tumor Protein P53), BAX (Bcl-2 Associated X Protein), Cytochrome C, and Calpain play significant roles in this process. P53 is a tumor suppressor protein that regulates cellular stress responses to DNA damage, triggering apoptotic cell death. The activation of P53 can initiate DNA repair mechanisms or halt the cell cycle to promote apoptosis (25). BAX, a pro-apoptotic protein activated by P53, increases mitochondrial membrane permeability, leading to the release of Cytochrome C into the cytoplasm, which subsequently activates caspases and induces apoptosis. Cytochrome C is a key regulator of the mitochondrial apoptosis pathway (26). Once released into the cytoplasm, it forms the apoptosome complex, leading to Caspase-9 activation and cell death. Calpain-1 (CAPN-1), also referred to as Calcium-activated neutral protease 1, is a protease that plays a crucial role in caspase-independent cell death mechanisms, cytoskeletal remodeling, and apoptotic regulation. CAPN-1 is associated with tumor progression, making it a potential target for anti-cancer therapies. It facilitates apoptotic morphological changes by degrading cytoskeletal proteins (33).

Urtica dioica, also known as stinging nettle, has been used for centuries for its medicinal properties. Its bioactive compounds, including polyphenols, exhibit potent cytotoxicity and anticancer activity (5,6). *Urtica dioica*, or nettle, grows wild in North Africa, North America, Europe, and Asia (7). Various forms of this plant, such as dried powder and infusions, are significant in phytotherapy (1). It contains valuable compounds, including biochemicals like formic acid and histamine, flavonoids, tannins, and sterols (8). Research has isolated numerous compounds from nettle, demonstrating its anti-proliferative and antimicrobial properties, making it helpful in treating infectious diseases (9). Nettle may serve as an adjuvant therapeutic agent in cancer treatment by inhibiting cell growth and inducing apoptosis through modulation of key regulatory enzymes (10,11).

Boron (B), a metalloid in group 3A of the periodic table, has an atomic number of 5 and an atomic weight of 10.81 g/mol (12). It was used as early as 2000 BC in Babylon and currently accounts for 72% of the world's boron reserves in Turkey (13). Boric acid (H_3BO_3), the most common soluble form of boron, is a colorless, odorless crystal (14). Boric acid (BA), derived from minerals such as kernite and borax, is absorbed predominantly through the respiratory and gastrointestinal tracts (15). Boron influences metabolic processes and immune regulation (16), and its chemical properties support its potential in cancer treatment (17). Boric acid has demonstrated antiproliferative effects across various cancers (18), indicating its promise for breast cancer therapies.

This study evaluates the combined effects of *Urtica dioica* (nettle) and Boric acid on MCF-7 human breast cancer cells. The research investigates how these two substances together influence apoptosis (programmed cell death) and anti-apoptotic pathways. Additionally, the protective effects of the combination of *Urtica dioica* and Boric acid are assessed. The findings contribute to the scientific community by highlighting the potential applications of this combination in breast cancer treatment. Future research will expand these results through animal and clinical trials to further elucidate the synergistic roles of *Urtica dioica* and Boric acid in breast cancer therapy. In this context, additional investigations are necessary to determine this combined treatment's efficacy, safety, and appropriate dosages.

2. Materials and Methods

Cell Culture and Viability Assessment

Plant Material and Extraction Methods

The *Urtica dioica* L. (nettle) samples were collected in July 2023 during their full maturation stage from a natural habitat in Istanbul, Türkiye (Aktar Diyarı), while Boric acid was sourced from Sigma-Aldrich (B6768). Extractions were performed following European Pharmacopoeia standards using aqueous ethanol (%70). The obtained extract was analyzed by High-performance liquid chromatography (HPLC) to determine the concentrations of active compounds. HPLC analyses were performed using an Agilent 1100 system with a C18 column to quantify active compounds (Fig 1-2).

Urtica dioica extract was prepared as a stock solution at a concentration of 10 mM/mL. For experimental use, this stock solution was diluted to final concentrations ranging from 10 to 800 times, and added to the culture medium in a concentration not exceeding 10%. In addition, micromolar (µM) concentrations were calculated based on the estimated amount of caffeic acid identified in the extract via HPLC, in order to assess the biological activity of this specific compound.

HPLC Analysis

Phytochemical analyses were performed using Agilent 1200 HPLC system (Agilent Technologies, USA) with a C18 column (4.6×250 mm, 5 µm, Agilent, USA) at 40°C. The mobile phase consisted of:

Phase A: Acetonitrile: Water: Formic acid (10:89:1)

Phase B: Acetonitrile: Water: Formic acid (89:10:1)

A gradient elution (0–35 min, B% 15–100) was applied at a flow rate of 0.7 mL/min, with an injection volume of 20 µL following Karadağ et al. (2019). Phenolic compounds were identified by comparing their retention times and mass spectra with standards (19).

HPLC confirmed that caffeic acid is a major phenolic compound in *Urtica dioica* extract, and the µM concentrations in biological assays were calculated accordingly. While this study evaluates the overall bioactivity of the extract, caffeic acid quantification was included to provide insight into its potential contribution.

Cell Culture, Experimental Groups, Viability Assessment, and Biochemical Analysis

MCF-7 human breast cancer cells (ATCC, USA) were cultured in low-glucose DMEM (Dulbecco's Modified Eagle Medium, Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and incubated at 37°C in a 5% CO_2 atmosphere. Cells were frozen in a cryopreservation solution for storage and quickly thawed before use. The experimental groups included MCF-7 cells alone, cells treated with *Urtica*, cells treated with Boric acid, and cells treated with a combination of both.

MCF-7 cells were cultured and harvested via trypsinization, followed by centrifugation to separate the cell pellet from the medium. The cells were washed with Phosphate-buffered saline (PBS) and resuspended for freezing in a cryopreservation solution. Cell viability was assessed using the Trypan Blue exclusion (Sigma-Aldrich, USA) method to determine cell concentration. Subsequently, an MTT assay was performed to establish the IC50 values of the treatments.

Biochemical Assays and ELISA Kits

Protein levels of p53, BAX, Cytochrome C, and Calpain were quantified using commercial ELISA kits (Elabscience Biotechnology Inc., Houston, USA; Cat. No: E-EL-H0910 for p53, E-EL- H0562 for BAX, E-EL- H0056 for Cytochrome C, and E-EL-H5553 for Calpain). The assays were performed following the manufacturer's instructions to ensure precise and reproducible measurements. Absorbance values were recorded at **450 nm** using a **Multi-Mode Microplate Reader (BioTek Instruments, USA)**, and data analysis was conducted using **Gen5 software**. The results were calculated based on standard curves provided by the kit (Fig. 3).

Statistical Analysis

Statistical analyses were conducted to evaluate the differences between experimental groups. The IC_{50} values were determined using a nonlinear regression model (log[inhibitor] vs. normalized response) with GraphPad Prism 9 software. Data points were fitted to a sigmoidal dose-response curve, and the IC_{50} values were calculated based on the concentration that reduced cell viability by 50%. Data distribution was assessed for normality using the Shapiro-Wilk test, confirming that the data followed a normal distribution. Group comparisons were performed using one-way ANOVA, followed by Tukey's post-hoc test to identify significant differences between groups.

All experiments were conducted in triplicate (n=3) to ensure the reliability of the results. The number of data points per experimental condition was at least three independent measurements. IC50 values and all quantitative results were expressed as mean±standard deviation (SD). Statistical significance was determined at $p<0.05$, and all results were supported by detailed variance analyses and standard deviation measures.

3. Results

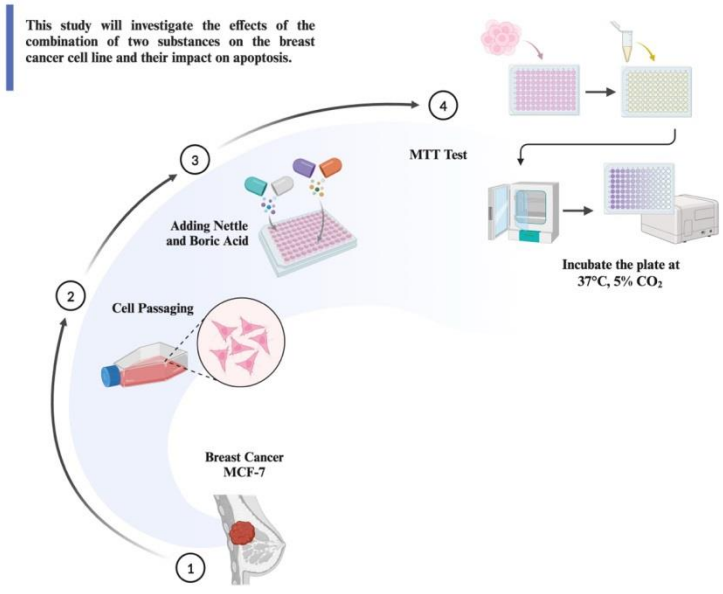
Figure 2 shows the phenolic compounds found on *Urtica dioica* extract. For data analysis, the positive identification of each compound was based on retention time and mass information of library score standards. It can be reported that the extract contains caffeic acid according to the standards. The analysis revealed caffeic acid (1) as the one of the flavonoid component. Furthermore, Fig 1 depicts the HPLC

chromatogram obtained of caffeic acid standard. Herein, caffeic acid (R.T.: 5.84) were detected in the *Urtica dioica* extract according to HPLC results (Fig. 2). These compounds were determined by comparing them with the known standards (Fig. 1). Hence, the biological activity of the extract could be linked to both identified flavonoid and un-identified compounds.

The viability of MCF-7 cells was assessed at 24 and 48 hours following treatment with *Urtica dioica* and Boric acid. The IC50 values determined through bio-statistical analyses were 45.32±2.15µg/mL and 24.62±1.87µg/mL for *Urtica dioica* at 24 and 48 hours, respectively, with a selected dose of 50 µg/mL. For Boric acid, the IC50 values were 55.90±3.42 µM and 36.91±2.74 µM at the same time points, with a selected dose of 50 µM. Notably, the combination of *Urtica dioica* and Boric acid (I6.25+B50) demonstrated the highest difference from the control group, yielding an IC50 value of 72.93±3.08 at 24 hours. All experiments were performed in triplicate, and results were presented as mean±standard deviation (SD).

To better visualize the effects of different concentrations of *Urtica dioica* and Boric Acid on cell viability, dose-response curves are presented in Figure 4. The cell viability percentages at 24h and 48h are illustrated in Figure 5, showing the concentration-dependent effects of *Urtica dioica* and Boric Acid on MCF-7 cells.

Biochemical assessments revealed significant changes in protein levels. P53 levels showed a statistically significant increase with both *Urtica dioica* and Boric acid treatments compared to the control ($p<0.001$). Conversely, BAX levels decreased significantly ($p<0.001$), while the combination treatment did not show a significant difference compared to the control ($p>0.05$). Cytochrome C levels increased significantly with both *Urtica dioica* and Boric acid ($p<0.001$ and $p<0.01$, respectively), while the combination did not differ significantly from the control ($p>0.05$). Lastly, Calpain-1 (CAPN-1) levels exhibited a significant decrease for both treatments compared to the control ($p<0.01$), and the combination treatment showed a very significant reduction ($p<0.001$) (Fig 4).



Graph Abstract. This figure was created with BioRender.com. Agreement NO: FY288ANX9N.

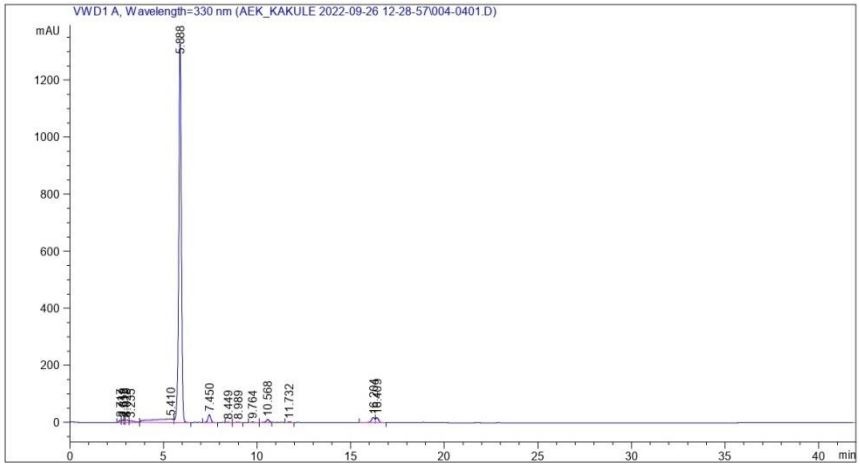


Fig 1. Caffeic acid (0.5 mg/mL) HPLC chromatogram (R.T.: 5.88)

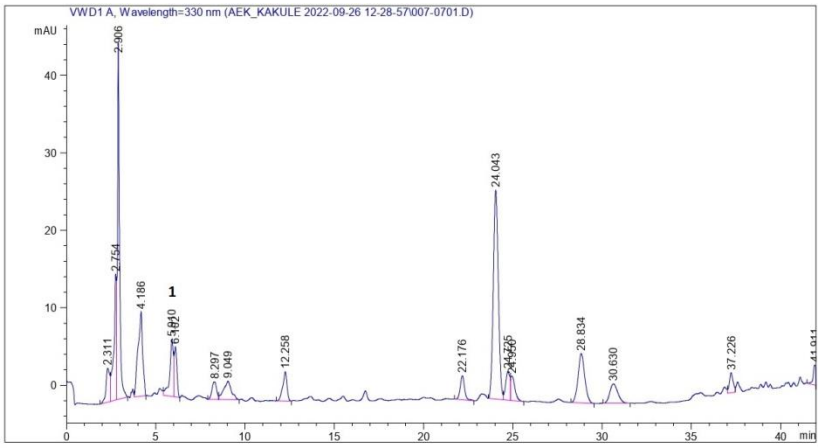


Fig 2. *Urtica* extract (10 µg/mL) HPLC chromatogram (1. Caffeic acid R.T.: 5.84)

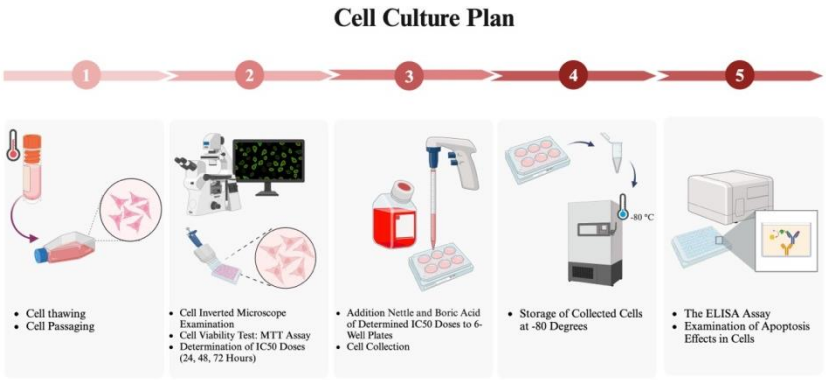


Fig 3. Experimental plan. This figure was created with BioRender.com. Agreement NO: QD288ANXLA

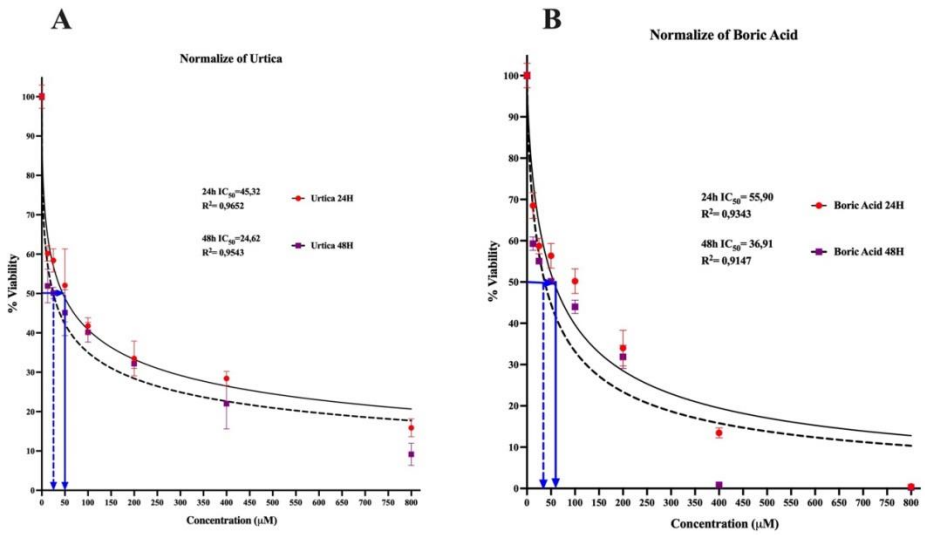


Fig 4 . Dose-response curve for *Urtica dioica* and Boric Acid on MCF-7 cell viability. The viability of MCF-7 cells was assessed at 24 and 48 hours following treatment with increasing concentrations of *Urtica dioica* (**A**) and Boric Acid (**B**). IC_{50} values were calculated for each treatment condition. Data are represented as mean±standard deviation from three independent experiments.

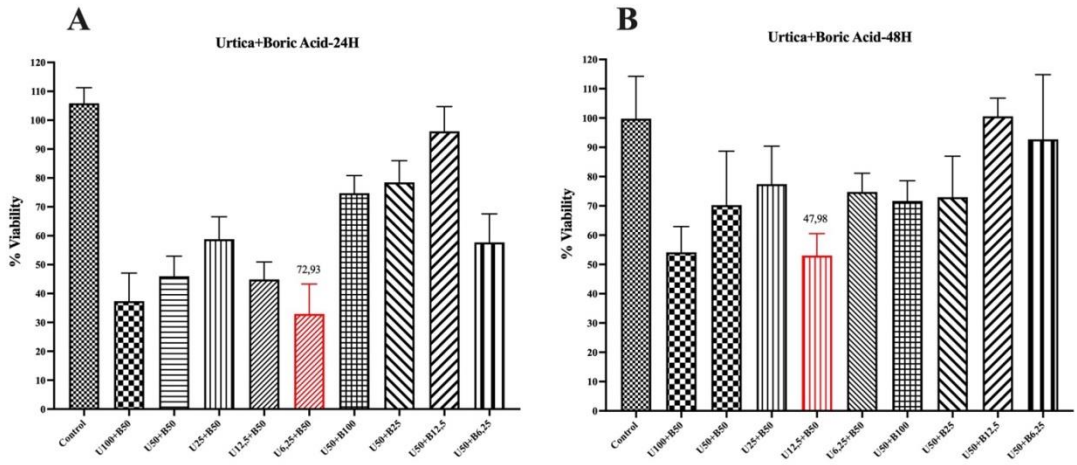


Fig 5. Effects of *Urtica dioica* and Boric Acid combinations on MCF-7 cell viability at 24h and 48h. (**A**) Cell viability percentages after 24 hours of treatment with different *Urtica dioica* (U) and Boric Acid (B) concentrations. (**B**) Cell viability percentages after 48 hours of treatment with different *Urtica dioica* (U) and Boric Acid (B) concentrations. Data are presented as mean±standard deviation from three independent experiments.

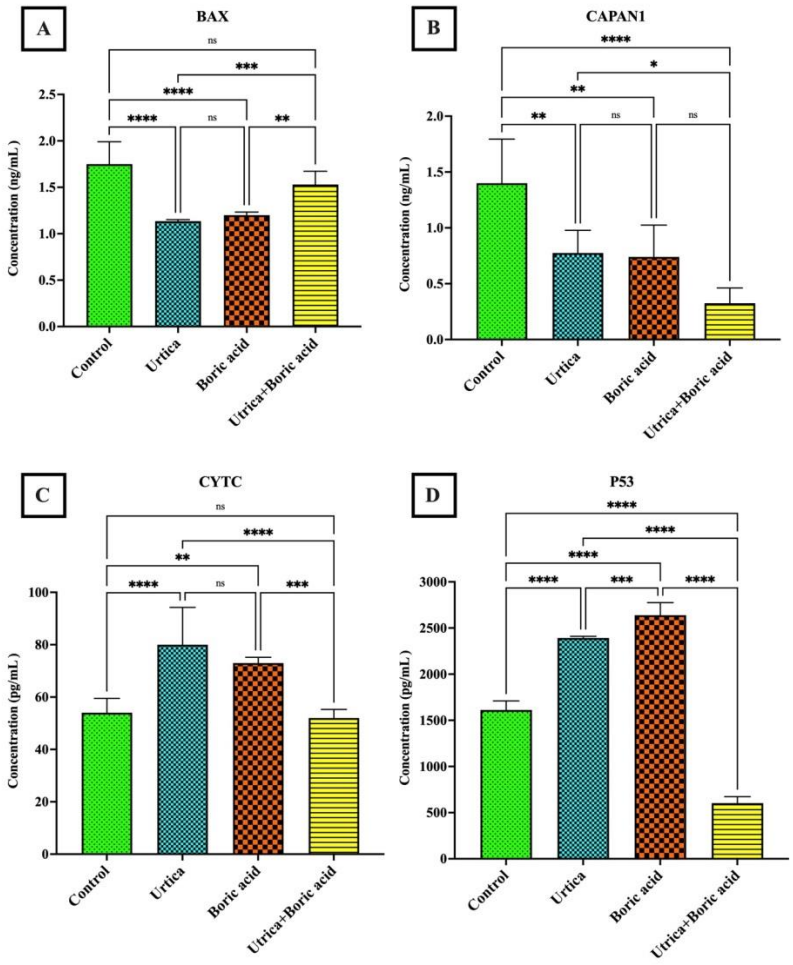


Fig 6. Concentration levels of **A.** BAX, **B.** CAPN1, **C.** CYTC, and **D.** p53 in the control group, as well as in the *Urtica*, Boric acid, and *Urtica*+Boric acid treatments.

4. Discussion

Breast cancer is the most prevalent cancer among women and ranks as the second leading cause of cancer-related death globally. Consequently, extensive research is being conducted on various drugs and compounds for its treatment. Plants, including *Urtica dioica*, have shown promise as adjunctive, complementary, or alternative sources for cancer prevention and treatment due to their bioactive properties (6). *Urtica dioica* exhibits biological activities that may induce or inhibit critical cellular processes and activate apoptotic pathways. Previous studies have reported its anti-proliferative and apoptotic effects on various human cancers, with significant cell death observed in MCF-7 cells (20,21).

Boric acid is recognized for its potent antioxidant properties and potential to mitigate cancer treatment side effects by reducing reactive oxygen species levels (22). Research has demonstrated that Boric acid can induce cytotoxic effects in breast cancer cell lines, including MDA-MB-231 (23), significantly reducing cell viability in other cancer types (24). In this study, the apoptotic effects of *Urtica dioica* and Boric acid on MCF-7 cells were investigated using markers such as p53, BAX, Cytochrome C, and CAPN-1, indicating their potential as therapeutic agents in breast cancer treatment.

P53 is a crucial tumor suppressor gene that responds to DNA damage and coordinates various cellular stress responses, including apoptosis and autophagy (25). Previous studies have demonstrated that *Urtica dioica* enhances p53 activity, potentially contributing to its anti-tumor effects in various cancer models (20,21). In this study, both *Urtica dioica* and Boric acid were found to increase p53 levels, suggesting their potential as therapeutic agents. However, the combination of *Urtica dioica* and Boric acid decreased p53 levels, indicating a possible negative impact on apoptosis. This finding suggests that while individual treatments may enhance p53-mediated apoptosis, their combination could be counterproductive, warranting caution in clinical applications. The results align with existing

literature that supports the role of *Urtica dioica* and Boric acid in cancer therapy, highlighting their potential benefits when used separately. However, the observed reduction in p53 levels with combined treatment calls for further investigation into the mechanisms underlying these interactions and their implications for cancer treatment strategies.

BAX is a crucial regulator of the intrinsic apoptotic pathway, facilitating mitochondrial membrane permeabilization in response to apoptotic stimuli (26). This studies have shown that *Urtica dioica* can enhance BAX expression, promoting apoptosis in various cancer cell lines, including acute myeloid leukemia and colon cancer (27,28). Similarly, Boric acid has been reported to increase BAX expression in lung cancer cells (29) and suppress cell viability in a time- and dose-dependent manner (30). Contrary to existing literature, our findings revealed that *Urtica dioica* and Boric acid decreased BAX levels, resulting in a lack of apoptotic effect. This suggests that the mechanisms underlying their actions may differ from previously reported studies. Given the critical role of BAX in understanding the targeted effects of cancer therapies, further research into the modulation of BAX levels is essential for advancing new cancer treatment strategies.

Cytochrome C is a mitochondrial protein that, upon release into the cytoplasm during apoptosis, activates a cascade of events leading to cell death (31). Previous studies, such as Hacıoğlu et al. (2020), have shown that Boric acid significantly increases Cytochrome C levels, effectively initiating apoptotic pathways in cancer cells (32). However, other research has indicated no significant change in Cytochrome C levels with Boric acid treatment in breast cancer cells (23). In our study, *Urtica dioica* and Boric acid increased Cytochrome C concentrations, suggesting potential apoptotic effects. Conversely, the combination of *Urtica dioica* and Boric acid did not exhibit an apoptotic effect, indicating that their interaction may hinder the apoptotic process. Identifying compounds that modulate Cytochrome C levels can contribute to establishing new therapeutic targets in cancer treatment, highlighting the need for further investigation into their mechanisms of action.

Calpains are calcium-activated cysteine proteases that play crucial roles in various cellular processes, including transcription, survival, proliferation, apoptosis, migration, and invasion, making them potential anti-cancer targets (33). Previous studies have indicated that calpains regulate multiple intracellular proteins and are involved in cytoskeletal remodeling and apoptosis (34). Our findings demonstrate that both *Urtica dioica* and Boric acid significantly reduce calpain protein concentrations in breast cancer cells, with the combination of both compounds having a more pronounced effect than either agent alone. This suggests that the *Urtica dioica* and Boric acid combination may enhance therapeutic strategies by further inhibiting calpain activity in breast cancer treatment. Given the established role of calpains in cancer progression and therapy response, additional research is warranted to explore the potential of these compounds in therapeutic applications for breast cancer.

In this study, MCF-7 breast cancer cells were used as the primary model, with the control group consisting of untreated MCF-7 cells to ensure baseline comparisons within the same cellular environment. While normal cell lines were not included, the use of a cancer-specific control group allows for accurate assessment of the apoptotic effects of *Urtica dioica* and Boric acid under comparable experimental conditions. Future research should aim to evaluate the selectivity of these compounds by comparing their effects on both malignant and non-malignant breast epithelial cells to better understand their therapeutic potential and safety profile.

Our findings indicate that the combination of *Urtica dioica* and Boric acid positively affects apoptotic markers such as p53 and Cytochrome C in MCF-7 cells. However, despite the known role of BAX as a pro-apoptotic factor induced by p53, our results did not show a statistically significant difference in BAX levels between the combination treatment group and the control.

This may be attributed to the complex regulation of BAX expression, as previous studies suggest that its activation is not solely dependent on p53 but can also be modulated by additional intracellular signaling pathways. Furthermore, it is possible that post-translational modifications or mitochondrial translocation dynamics could influence the detectable BAX protein levels in the experimental conditions used.

In contrast, CAPAN-1 levels exhibited a decreasing trend following combination treatment, which aligns with literature findings indicating that this protein plays a role in inhibiting apoptosis. The observed reduction in CAPAN-1 expression further supports the apoptotic potential of *Urtica dioica* and Boric

acid, emphasizing the need for further investigation into their mechanistic effects on apoptotic pathways.

5. Conclusion

Cancer is characterized by the uncontrolled growth of abnormal cells that can invade and spread throughout the body. This study aimed to investigate the anti-cancer effects of *Urtica dioica* and Boric acid on MCF-7 breast cancer cells, focusing on their potential to induce apoptosis. The results demonstrated that both compounds significantly increased p53 and Cytochrome C levels, suggesting their role in promoting apoptotic pathways. However, the combination of *Urtica dioica* and Boric acid decreased p53 levels, indicating a possible antagonistic effect that warrants further investigation.

This study suggests that the apoptotic effects of *Urtica dioica* and Boric Acid may vary depending on concentration, exposure time, and interaction with other cellular factors. While both compounds individually showed apoptotic potential by increasing p53 and Cytochrome C, their combination did not always exhibit the same level of apoptotic activation. These results indicate that their mechanism of action is complex and may involve additional regulatory pathways beyond traditional apoptotic markers. Therefore, further studies are needed to explore their apoptotic pathways in more detail and to determine their potential as adjunctive agents in cancer therapy. These findings provide a foundation for future research on the selective and dose-dependent effects of *Urtica dioica* and Boric Acid in breast cancer treatment.

HPLC analysis identified caffeic acid as a major phenolic compound in *Urtica dioica* extract, suggesting that it may contribute to the apoptotic effects observed in this study. Caffeic acid has been reported to induce apoptosis by modulating mitochondrial membrane potential and activating caspase-dependent pathways in various cancer models. Previous studies have demonstrated that caffeic acid enhances p53 activation, increases BAX expression, and promotes cytochrome C release, all of which are critical apoptotic markers examined in this study.

Although the current study did not specifically isolate caffeic acid, its presence in the extract suggests that it may play a role in the observed biological activity. Future studies should focus on investigating the independent effects of caffeic acid and its potential synergistic interactions with other bioactive compounds in *Urtica dioica* extract. This will provide further insight into the molecular mechanisms underlying its anti-cancer properties.

Future studies should focus on elucidating the mechanisms behind their effects and evaluating the clinical efficacy of their combination in cancer treatment strategies. Although the current study focused on IC50 concentrations, future research should explore the effects of lower concentrations to better understand the apoptotic and cytotoxic responses at sub-IC50 levels. Investigating lower doses could provide further insight into dose-dependent cellular responses and optimize treatment strategies for cancer therapy.

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