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RESEARCH

Flavonoids from *Cirsium italicum* (Savi) DC. reduce reactive oxygen species and modulate histone deacetylase activity in triple-negative breast cancer cells

Cirsium italicum (Savi) DC.'den elde edilen flavonoidler üçlü negatif meme kanseri hücrelerinde reaktif oksijen türlerini azaltır ve histon deasetilaz aktivitesini modüle eder

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Abstract

Purpose: The present study aims to investigate the potential anti-cancer activities of flavonoid compounds from *Cirsium italicum* (Savi) DC. plants in breast cancer cells.

Materials and Methods: MDA-MB-231 triple-negative breast cancer (TNBC) cells were used to demonstrate reactive oxygen species (ROS) production effects and histone deacetylase (HDAC) inhibitory activity of flavonoids [5,3',4'-trihydroxy-7-methoxy isoflavone (Santal) **(1)**, 5,5",7"-trihydroxy-3,7-dimethoxy-4',4"'-*O*-biflavone **(2)**, 6-2′-dihydroxy-5-methoxy isoflavonone-4′-*O*-α-D-glucopyranoside **(3)**, (8,3',4',5'-tetrahydroxy-7-*O*-α-glycosyl isoflavone)-6-8"-(4"'-hydroxy-7"-*O*-α-glycosyl isoflavone) biflavone **(4)**] isolated from *C. italicum*.

Results: Significant differences in intracellular ROS levels were observed in MDA-MB-231 breast cancer cells treated with the compounds at concentrations of 0.06, 0.08, and 0.1 mg/ml. Compound **2** at 0.06 mg/ml, and compounds **2** and **4** at 0.08 and 0.1 mg/ml, induced a significant decrease in intracellular ROS levels compared to control cells. At a concentration of 0.06 mg/ml, compound **2** showed a mean value of 0.003±0.004. At 0.08 mg/ml, the mean values were 0.006±0.018 for compound **2** and 0.013±0.013 for compound **4**. At 0.1 mg/ml, compound **2** and compound **4** exhibited mean values of 0.014±0.017 and 0.014±0.007, respectively. Even though all flavonoids

Öz

Amaç: Bu çalışma, Cirsium italicum (Savi) DC. bitkisinden elde edilen flavonoid bileşiklerin meme kanseri hücrelerindeki potansiyel anti-kanser aktivitelerini araştırmayı amaçlamaktadır.

Gereç ve Yöntem: MDA-MB-231 üçlü negatif meme kanseri hücreleri, Cirsium italicum'dan izole edilen flavonoidler [5,3',4'-trihidroksi-7-metoksi izoflavon (Santal) (1), 5,5",7"-trihidroksi-3,7-dimetoksi-4',4"'-O-biflavon (2), 6-2′-dihidroksi-5-metoksi izoflavonon-4′-O-α-D-glukopiranozid (3), (8,3',4',5'-tetrahidroksi-7-O-α-glikozil izoflavon)-6-8"-(4"'-hidroksi-7"-O-α-glikozil izoflavon) biflavon (4)]; reaktif oksijen türleri (ROS) üretim etkilerini ve histon deasetilaz (HDAC) inhibitör aktivitesini göstermek için kullanıldı.

Bulgular: 0.06, 0.08 ve 0.1 konsantrasyonlarında uygulanan bileşiklerle muamele edilen MDA-MB-231 meme kanseri hücrelerinde, hücre içi ROS düzeylerinde anlamlı farklılıklar gözlemlendi. 0.06 mg/ml konsantrasyonunda uygulanan bileşik 2 ile, 0.08 ve 0.1 mg/ml konsantrasyonlarında uygulanan bileşik 2 ve 4'ün, kontrol hücrelerine kıyasla hücre içi ROS düzeylerinde anlamlı bir azalmaya yol açtığı gözlemlendi. 0.06 mg/ml konsantrasyonunda, bileşik 2'nin ortalama değeri 0.003±0.004 olarak ölçüldü. 0.08 mg/ml konsantrasyonunda, ortalama değerler bileşik 2 için 0.006±0.018 ve bileşik 4 için 0.013±0.013 olarak belirlendi.

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inhibited HDAC activity, this inhibition was not statistically significant.
Conclusion: Among the flavonoids isolated from *C. italicum*, compounds **2** and **4** showed potential anticancer effects in breast cancer cells, potentially through modulation of intracellular ROS levels.

Keywords: Asteraceae; breast cancer; *Cirsium italicum*; flavonoid; histone deacetylase; reactive oxygen species

0.1 mg/ml konsantrasyonunda ise, bileşik 2 ve bileşik 4 sırasıyla 0.014±0.017 ve 0.014±0.007 değerlerini gösterdi. Her ne kadar tüm flavonoidler HDAC aktivitesini inhibe etmiş olsa da, bu inhibisyon istatistiksel olarak anlamlı bulunmadı.
Sonuç: C. italicum bitkisinden izole edilen flavonoidler arasında, bileşik 2 ve 4 hücre içi ROS düzeylerini modüle etme yoluyla meme kanseri hücrelerinde potansiyel antikanser etkiler gösterdi.

Anahtar kelimeler: Asteraceae; meme kanseri; Cirsium italicum; flavonoid; histon deasetilaz; reaktif oksijen türleri

INTRODUCTION

Cancer is the second leading cause of death worldwide. One in eight men and one in eleven women die from cancer[1]. Worldwide, the total number of cancer patients who have survived within five years of a cancer diagnosis (5-year prevalence) is estimated to be 50.6 million. Among women, breast cancer ranks first in both incidence and mortality across many countries[2].

Breast cancer originates from breast tissue, and—as in other cancers—its development involves genetic and epigenetic alterations in healthy cells. Recent studies revealed that the diagnosis of breast cancer is lower in countries with a Mediterranean diet[3]. Researchers have suggested that this may be due to the presence of flavonoids in vegetables and fruits. According to epidemiological studies, significant positive effects of flavonoids have been observed in cancer chemoprevention and chemotherapy[4].

Flavonoids and other dietary polyphenol antioxidants are found in plants as bioactive molecules. As secondary plant metabolites, polyphenols are effective free radical scavengers, and flavonoids represent the largest and most structurally diverse group among plant phenolics. Flavonoids are polyphenols containing large heterogeneous groups occurring in benzo-γ-pyrone derivatives. They are very potent anti-tumor agents; their antioxidant and anti-proliferative functions can stimulate apoptosis, cell differentiation, and cell cycle[5].

Both *in vitro* and *in vivo* studies indicate that flavonoids can modulate histone deacetylase (HDAC) activity. Over the past two decades, interest in flavonoids has significantly increased in the context of traditional medicine, and extensive research has demonstrated that flavonoids possess diverse biochemical and pharmacological properties[6].

The ε-amino group acetylation at lysine residues plays an important role in gene expression[7, 8]. Acetylation is the attachment of acetyl groups to lysine residues on histone proteins, a process regulated by enzymes such as histone acetyltransferase (HAT) and HDAC1. N-terminal ε-amino group acetylation and deacetylation of lysine residues are regulated by HAT and histone deacetylase, respectively.

An imbalance in the activities of HAT and HDAC enzymes is responsible for the development and progression of various cancers. Histone deacetylase inhibitors (HDACi) increase the level of histone-acetylated lysine residues, thereby re-initiating the expression of suppressed regulatory genes in cancer cells. Therefore, HDAC inhibitors are used as anti-cancer agents. The opposing activities of HATs and HDACs are essential for the epigenetic regulation of gene expression[9].

Mutations that mimic acetylation can impair deacetylase activity, leading to disruption of mitochondrial function or alterations in mitochondrial morphology[10]. The synthesis of enzymes necessary for mitochondrial metabolism is suppressed. Simultaneously, the suppression of antioxidant defense enzymes leads to increased levels of free radicals. As a result of the overproduction of free radicals, cells cannot effectively neutralize these reactive species, leading to a condition known as oxidative stress. Hence, cell membranes and other macromolecules, such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA), can be severely damaged.

Reactive oxygen species (ROS) initiate the chemical chain reactions that lead to the formation of cytotoxic and mutagenic malondialdehyde (MDA). MDA reacts with bases in the DNA structure and has a mutagenic effect. When hydroxyl radicals are generated in close proximity to DNA, they can induce oxidative damage to DNA bases, potentially

leading to strand breaks or base modifications. This may also interfere with normal cellular processes by altering the transcription of target genes[11, 12].

A total of 250 species of the genus *Cirsium* (Asteraceae) have been identified worldwide. *Cirsium* (thistle) has long been used in traditional folk medicine, and the leaves and stems of many species, which are also edible, are consumed in teas, soups, and salads[13]. Recent pharmacological studies indicate that *Cirsium* species with tumor-inhibiting properties possess hepatoprotective effects and may serve as antidiabetic and antioxidant agents in therapeutic applications[14-17].

Previous phytochemical studies on this plant have led to the isolation of various flavonoids and phenolic acids[18]. This research project aims to investigate the anti-cancer effects of flavonoids derived from the plant species *C. italicum* in breast cancer cells. Four of these compounds were isolated for the first time from *C. italicum*, and three of them were reported for the first time from any natural source[18]. However, the anticancer activity of these compounds has not yet been investigated in the MDA-MB-231 human triple-negative breast cancer cell line.

To the best of our knowledge, this is the first study to evaluate the effect of *C. italicum*-derived flavonoids on intracellular ROS levels and HDAC activity in TNBC cells. While previous studies have examined natural compounds with antioxidants or epigenetic activity separately, our study offers new insight into the potential dual role of these flavonoids. We hypothesize that flavonoids extracted from *C. italicum* significantly reduce intracellular ROS levels, thereby exerting potential anticancer effects in TNBC cells.

MATERIALS AND METHODS

Cell culture

Triple-negative breast cancer (TNBC) cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC). MDA-MB-231 cells in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% inactivated Fetal Bovine Serum (FBS) and 100 μg/ml Gentamicin, humidified with 5% CO_2 and 95% air, incubated at 37°C.

Procedure

This study was approved by the Tekirdağ Namık Kemal University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee (Approval number: 2019.71.05.03; approval date: 21.05.2019).

The study was conducted at the Medical Biology and Genetics Research Laboratory, Department of Medical Biology, Faculty of Medicine, Tekirdağ Namık Kemal University. The laboratory adheres to standard protocols to ensure the reliability, reproducibility, and integrity of the data collected. All experimental procedures were performed under the supervision of qualified and experienced researchers.

Plant and Flavonoids

C. italicum is a wild plant species in the Trakya region, which was collected in July 2017. The plants were taxonomically identified by Trakya University, Department of Biology (NGBB 6807). The whole plant was dried and cut into small pieces. The plant was extracted by maceration with 80% methanol for three days, repeated four times. After solvent evaporation using a rotary evaporator, the crude extract was obtained. A small amount of water was added to the resulting crude extract, which was back-extracted with n-hexane, dichloromethane, ethyl acetate (EtOAc), and n-butanol in polarity order. To obtain each crude extract, the solvents were evaporated.

Based on the antioxidant activity data, the ethyl acetate extract exhibited the highest activity among the tested extracts[18]. Therefore, isolation studies were carried out on the ethyl acetate extract. The EtOAc extract of *C. italicum* was purified by different chromatographic methods, such as a combination of various column chromatographic techniques, including reverse-phase recycling high-performance liquid chromatography (HPLC).

The structures of compounds [5,3',4'-trihydroxy-7-methoxy isoflavonone (Santal) **(1)**, 5,5",7"-trihydroxy-3,7-dimethoxy-4',4"'-*O*-biflavone **(2)**, 6-2′-dihydroxy-5-methoxy isoflavonone-4′-*O*-α -D-glucopyranoside **(3)**, (8,3',4',5'-tetrahydroxy-7-*O*-α-glycosyl isoflavone)-6-8"-(4"'-hydroxy-7"-*O*-α-glycosyl isoflavone) biflavone **(4)**] isolated from the plant *C. italicum* are shown in Figure 1. These compounds were elucidated by extensive one-dimensional and two-dimensional nuclear magnetic resonance (1D and 2D NMR), and mass spectrometry (MS) data analyses[18].

HDAC activity measurement

HDAC activity in MDA-MB-231 cells treated with or without flavonoids was measured using a colorimetric HDAC Assay/Drug Discovery Kit (Enzo), following the manufacturer's instructions. Briefly, $2.8x10^5$ cells were seeded into each well of a 96-well microtiter plate 24 hours prior to flavonoid treatment and incubated at 37°C. Assay buffer or diluted trichostatin A (TSA) was added to the appropriate wells of the microtiter plate. TSA, an inhibitor of HDAC activity, was used as a positive control during the analysis. Except for the "no enzyme control" wells, compounds **1**, **2**, **3**, and **4** were added to the wells at concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml. The diluted Color-de-Lys™ substrate and the flavonoid samples were equilibrated at 37°C in the plate.

A diluted substrate (25 µl) was added to each well and HDAC enzyme reactions were initiated by mixing well. HDAC reactions were stopped by adding Color-de-Lys™ developer (50 µl). The microtiter plate was incubated at 37°C for 15 minutes. The plate was read in a microtiter plate reader at 405 nm. After the standard curve was generated, it was used for standard validation across all experiments. Each treatment was performed in triplicate on each plate.

ROS measurement

The generation of ROS in the MDA-MB-231 cell line, with and without flavonoid treatment, was investigated using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; H2-DCF, DCF) (Invitrogen) according to the manufacturer's protocol. Briefly, $1x10^4$ cells were seeded into each well of a 96-well microtiter plate and incubated at 37°C. Following cell culture, MDA-MB-231 cells were treated with compounds **1**, **2**, **3**, and **4** at concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml.

After 24 hours of incubation, the cells were treated with 10 µM H2DCFDA, followed by 1 hour of incubation at 37°C in a 5% CO_2 incubator. Following PBS washing, the cells were exposed to 300 µM H_2O_2 for 1 hour to induce intracellular oxidation. Cells not treated with H_2O_2 were considered the negative control, whereas cells treated with 300 µM H_2O_2 for 1 hour were considered the positive control. The fluorescence intensity was measured at 405 nm excitation and 490 nm emission using a spectrofluorometer. Each treatment was performed in triplicate on each plate.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (SPSS Inc., Chicago, IL, USA). Descriptive statistics were calculated for each variable. Results were presented as mean ± standard deviation (SD) in tables and as mean ± standard error of the mean (SEM) in graphs. Analysis of variance (ANOVA) was used to compare the means of ROS levels and HDAC activity across different concentrations within each flavonoid group. Dunnett's post hoc test was applied to compare each treatment group with the control group. A p-value of <0.05 was considered statistically significant.

RESULTS

As shown in Table 1, relative intracellular ROS generation in MDA-MB-231 breast cancer cells significantly differed following treatment with compounds, identified as natural flavonoids isolated from *C. italicum,* at concentrations of 0.06, 0.08, and 0.1 mg/ml, with p-values of 0.045, 0.035, and 0.015, respectively. In this context, a significant decrease in intracellular ROS levels compared to control cells was observed in cells treated with compound **2** at a concentration of 0.06 mg/ml, and in cells treated with compounds **2** and **4** at concentrations of 0.08 and 0.1 mg/ml. No significant differences in ROS levels were observed for compounds **1**-**4** at concentrations of 0.02 and 0.04 mg/ml.

Figure 2 illustrates the relative ROS generation in cells treated with compounds **1**-**4** at concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml.

Table 2 showed that HDAC inhibitory activity in MDA-MB-231 breast cancer cells did not significantly differ following treatment with compounds at concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml, with p-values of 0.690, 0.858, 0.881, 0.951, and 0.925, respectively.

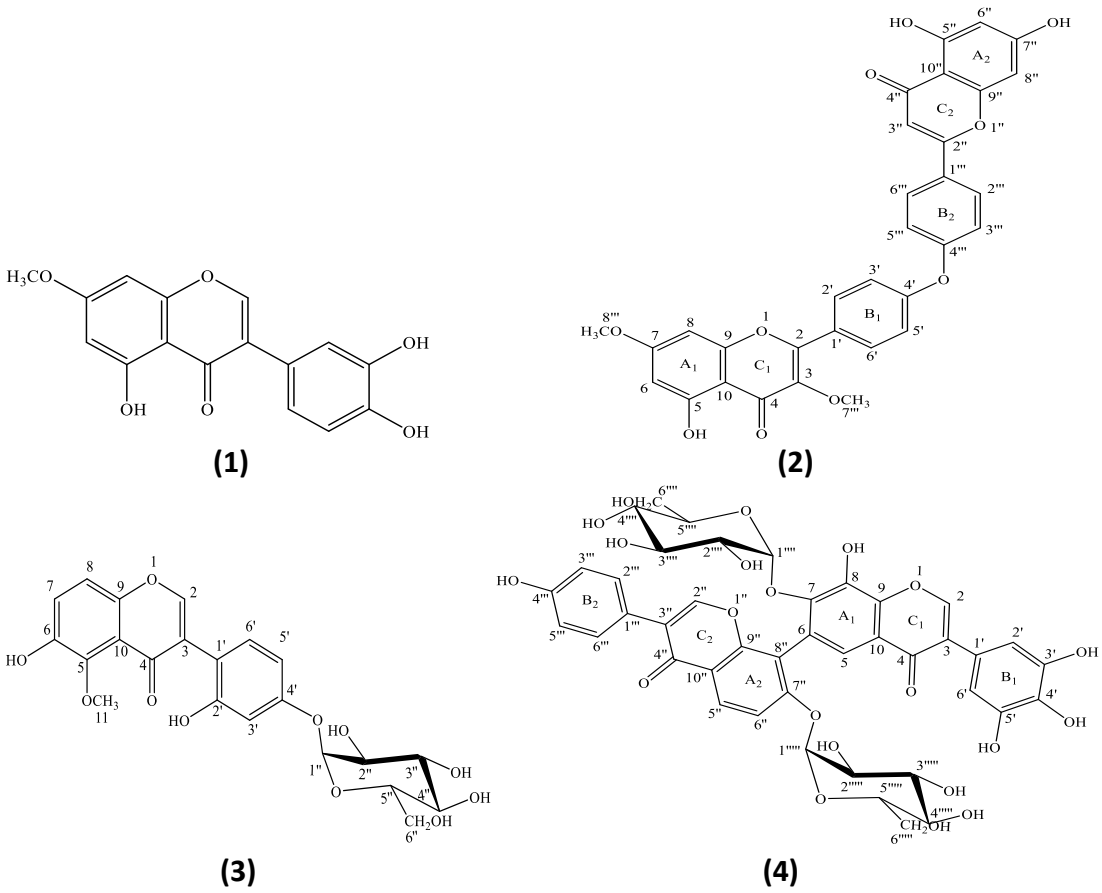

Figure 1. Structures of isolated compounds 1 - 4.

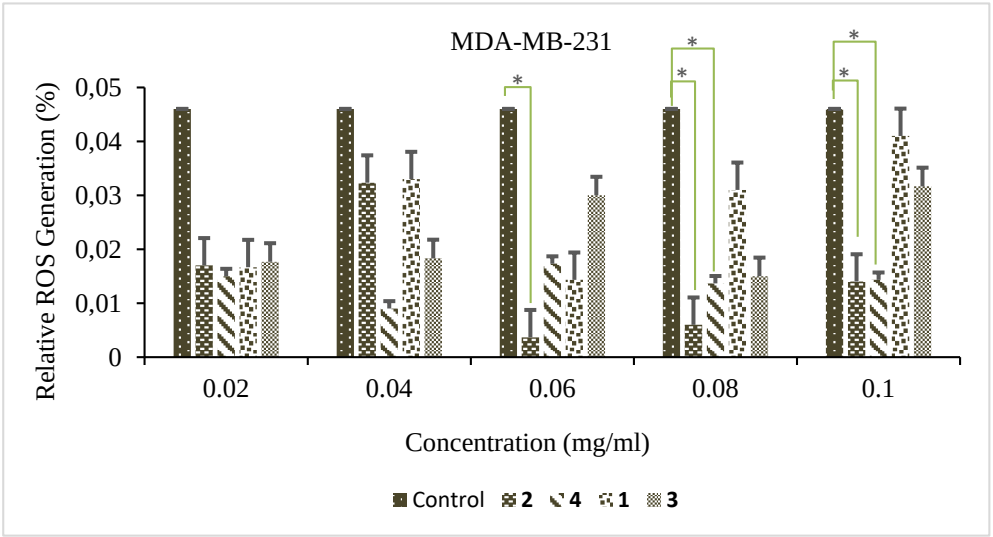

Figure 2. Effects of compounds 1-4 on relative ROS generation in MDA-MB-231 breast cancer cells.

Values are expressed as mean ± standard error of the mean (SEM). * $p < 0.005$.

Table 1. Effects of compounds 1 - 4 isolated from *C. italicum* on ROS generation in MDA-MB-231 breast cancer cells.

Concentration (mg/ml)	Compounds	Mean±SD	p value
0.02	Control	0.046±0.005	0.238
	3	0.016±0.017	
	2	0.017±0.021	
	1	0.017±0.018	
	4	0.015±0.021	
0.04	Control	0.046±0.005	0.084
	3	0.033±0.011	
	2	0.032±0.016	
	1	0.018±0.021	
	4	0.009±0.014	
0.06	Control	0.046±0.005	0.045
	3	0.014±0.022	
	2	0.003±0.004b1	
	1	0.030±0.013	
	4	0.017±0.020	
0.08	Control	0.046±0.005	0.035
	3	0.031±0.009	
	2	0.006±0.018b1	
	1	0.015±0.018	
	4	0.013±0.013d1	
0.1	Control	0.046±0.005	0.015
	3	0.041±0.008	
	2	0.014±0.017b1	
	1	0.031±0.012	
	4	0.014±0.007d1	

[a]Control-**3**; [b]Control-**2**; [c]Control-**1**; [d]Control-**4**; [1]p<0.05; [2]p<0.01; [3]p<0.001; SD = Standard deviation.

Table 2. HDAC inhibitory activities of compounds 1 - 4 isolated from *C. italicum* in MDA-MB-231 breast cancer cells.

Concentration (mg/ml)	Compounds	Mean±SD	p value
0.02	Control	34.858±38.189	0.690
	3	14.742±2.911	
	2	18.553±4.165	
	1	22.365±1.680	
	4	22.365±3.966	
0.04	Control	34.858±38.189	0.858
	3	20.036±2.864	
	2	25.786±7.408	
	1	22.577±2.230	
	4	23.212±2.404	
0.06	Control	34.858±38.189	0.881
	3	21.730±1.270	
	2	26.812±5.041	
	1	22.788±3.829	
	4	23.424±1.598	
0.08	Control	34.858±38.189	0.951
	3	25.330±2.041	
	2	28.929±6.792	
	1	28.294±0.733	
	4	24.482±2.231	
0.1	Control	34.858±38.189	0.925
	3	38.505±20.555	
	2	35.493±4.934	
	1	29.988±6.446	
	4	25.118±3.002	

SD = Standard deviation.

DISCUSSION

Despite recent advances in cancer therapies, treatment resistance or adverse side effects remain major challenges, often leading to treatment failure. The use of natural compounds holds potential as a stand-alone treatment strategy for cancer patients or in combination with conventional therapeutic agents. Evidence suggests that combination therapies are generally more effective than monotherapies in overcoming resistance and improving patient outcomes[19,20]. The current study investigated the effects of compounds **1**, **2**, **3**, and **4** isolated from *Cirsium italicum* Savi DC. (Asteraceae) on TNBC cells. A key finding of our study is that compounds **2** and **4** significantly reduced ROS generation in breast cancer cells *in vitro*. In addition, all flavonoids exhibited HDAC inhibitory activity; however, this inhibition was not statistically significant. The present study suggests for the first time that various flavonoid concentrations from *C. italicum* can reduce ROS production and also promote HDAC inhibition.

Mechanisms regulating ROS and the use of HDAC inhibitors are considered important in cancer therapy. Accumulation of ROS within cells can induce apoptosis or promote carcinogenesis. Elevated ROS levels contribute to carcinogenesis by inducing genetic mutations and transforming proto-oncogenes into oncogenes[21]. Furthermore, elevated ROS generation has been shown to promote metastasis, angiogenesis, and tumor progression[22]. ROS overproduction can activate hypoxia-inducible factor (HIF) signaling pathways, leading to the initiation and proliferation of cancer cells[23]. Certain flavonoids are known to exert anticancer properties primarily through their antioxidant effects[23]. A study reported that heteroside flavonoids decreased ROS production and contributed to the reduction of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) activities in murine osteosarcoma[24]. Generally, cancer cells exhibit higher ROS levels compared to normal cells. Although ROS is often considered a tumor suppressor in cancer, its effects vary depending on factors such as stimuli and cell type[25]. Several flavonoids possess significant antioxidant properties by reducing ROS formation[26, 27]. A further study demonstrated that alpinetin reduced ROS production, which inhibited cell growth and activated mitochondrial apoptosis, resulting in decreased breast cancer progression[25]. Consistent with these findings, kaempferol has been suggested to inhibit ROS production and reduce tumor metastasis by blocking the ROS-PAD4 pathway[28]. Kim et al. demonstrated that sea buckthorn leaf extract decreased ROS production and activated the ROS-mediated mitochondrial pathway through upregulation of Bax expression in glioma cells[29].

Moreover, numerous studies have proposed that increased ROS production leads to programmed cell death in cancer cells. Accordingly, certain flavonoids, acting as prooxidants, exert anti-cancer effects by inducing oxidative stress and apoptosis through activation of the mitochondrial apoptotic pathway and by elevating ROS levels[30,31]. It has been suggested that 2-Hydroxycinnamaldehyde (HCA), a phenolic natural compound, induces programmed cell death through ROS generation in human cancer cells[32]. A recent study reported that 4,4'-dimethoxychalcone (DMC) increased intracellular ROS levels and inhibited the spread of the cancer cell[33]. Another study reported that neo-hesperidin-induced ROS generation activated both apoptotic and autophagic death pathways in human osteosarcoma cells[34].

Alterations in epigenetic mechanisms, including aberrant histone acetylation, can dysregulate gene expression, leading to cancer initiation and progression. Among these, histone deacetylation is considered one of the most critical epigenetic modifications involved in the spread of human cancer cells. HDAC enzymes play an essential role in histone deacetylation. Histone deacetylation is a reversible process that can be inhibited by HDAC inhibitors.

Several flavonoids have been evaluated for their HDAC inhibitory activity. Although an association between flavonoids derived from *C. italicum* and HDAC inhibition with potential anti-cancer effect has been observed, it remains unclear. Among the flavonoids evaluated, Apigenin was identified as the most potent HDAC inhibitor[35]. A recent study demonstrated that apigenin contributed to HDAC inhibition by promoting apoptosis and inducing cell cycle arrest in non-small cell lung cancer cells[35]. Another study suggested that prenylflavonoids 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN) enhanced histone acetylation by inhibiting HDAC activity in melanoma cells[36]. Berger et al. reported that kaempferol negatively modulated HDAC activity and could function as an HDAC inhibitor in human hepatoma and colon cancer cells[37]. Gao et al. demonstrated that resveratrol acted as a potent HDAC inhibitor in MDA-MB-231 breast

cancer cells[38]. In addition to these studies, flavone has also been shown to enhance histone acetylation through its HDAC inhibitory activity[39].

This study has several limitations that should be acknowledged. First, the study was conducted exclusively *in vitro* using a TNBC cell line. While this cell line is widely used for studying aggressive breast cancers, the results may not be generalizable to other subtypes of breast cancer or to *in vivo* conditions. Second, the flavonoids were applied at five concentrations for a single time point. A more comprehensive dose- and time-response analysis is needed to fully understand the effects of the flavonoids. Third, although ROS generation and HDAC activity were assessed as key indicators of the anticancer potential of the flavonoids, the underlying molecular mechanisms and downstream signaling pathways were not explored. Additional studies, including transcriptomic or proteomic analyses, would provide a deeper understanding of how these flavonoids exert their effects.

In conclusion, our findings demonstrate that flavonoid compounds **2** and **4** from *C. italicum* significantly reduce intracellular ROS levels in TNBC cells, suggesting that potential as anticancer agents in this aggressive cancer subtype. These findings provide novel insight into the therapeutic potential of flavonoids from *C. italicum* in cancer treatment. Our results highlight the need for further research into *C. italicum*-derived bioactive compounds in oncology.

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Peer-review: Externally peer-reviewed.
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