Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Composition, Antioxidant Potential, Total Phenols and Flavonoids, and Cytotoxic Effects of the Aqueous Extract of *Bistorta officinalis* (Anjbar): An *In Vitro* Study

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ARTICLE INFO

Article History:

Received:2025/4/17

Accepted:2025/5/26

Keywords:

Lipid oxidation,

Bistorta officinalis,

Antioxidant activity,

Cytotoxicity,

Phenolic compounds,

Natural preservatives.

DOI: 10.22034/FSCT.22.165.164.

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ABSTRACT

Lipid oxidation has a considerable effect on the quality, safety, and nutritional value of food, resulting in undesirable flavors, nutrient depletion, and health concerns such as cardiovascular diseases and cancer. While synthetic preservatives like BHA and BHT are effective, they may carry health risks, leading to an increased interest in natural alternatives. Plant-based preservatives, especially those containing polyphenols, flavonoids, and essential oils, present safer and more sustainable solutions. This research explores the antioxidant and cytotoxic characteristics of the aqueous extract of *Bistorta officinalis* (Anjbar), a plant known for its traditional anti-inflammatory and antimicrobial uses. The extract, derived from dried roots, was analyzed for various phytochemicals, including alkaloids, saponins, flavonoids, and phenolics. The total phenolic content was measured at 76.65 mg GAE/g, while total flavonoids were at 40.38 mg QE/g. Antioxidant activity was evaluated using DPPH, ABTS, FRAP, and β-carotene bleaching assays, yielding IC_{50} values of 47.30 µg/mL (DPPH) and 29.20 µg/mL (ABTS), with a FRAP value of 8.69 mmol/g. Cytotoxicity assessments on HT-29 and HeLa cancer cell lines indicated a concentration-dependent decrease in cell viability, with IC_{50} values of 80.4 mg/mL and 70.92 mg/mL, respectively. The bioactive compounds within the extract, particularly phenolics and flavonoids, play a crucial role in its antioxidant and cytotoxic properties, highlighting its potential as a natural preservative and therapeutic agent. These results underscore the viability of *B. officinalis* as a natural alternative to synthetic antioxidants in both the food and pharmaceutical sectors.

1.Introduction

Lipid oxidation is a chemical process that significantly impacts the quality, safety, and nutritional value of food products, playing a crucial role in food spoilage. It leads to off-flavors, undesirable odors, and a reduction in essential nutrients, ultimately decreasing the shelf life of foods [1, 2]. Notably, the oxidation of polyunsaturated fatty acids generates reactive oxygen species and secondary oxidation products, such as aldehydes and ketones, which can be detrimental to human health when consumed in excessive amounts. These oxidative changes not only affect the sensory attributes of food but also carry health risks, as lipid oxidation products are linked to chronic diseases, including cardiovascular issues, cancer, and neurological disorders [2-4]. Consequently, managing lipid oxidation presents a significant challenge for the food industry, highlighting the need for effective strategies to mitigate its negative effects.

To address lipid oxidation and enhance the shelf life of food products, synthetic preservatives like Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), and tert-Butylhydroquinone (TBHQ) are commonly employed. These substances are effective in postponing oxidation due to their robust free radical scavenging properties [2, 4]. However, concerns regarding the potential health risks associated with synthetic preservatives have emerged. Research indicates that prolonged consumption of these synthetic antioxidants may lead to adverse health effects [5, 6]. As a result, there is a growing demand for safer, natural alternatives to synthetic preservatives.

Plant-based preservatives, particularly plant extracts, have garnered significant interest as natural substitutes for synthetic antioxidants. Plants are abundant in bioactive compounds like polyphenols, flavonoids, and essential oils that offer strong antioxidant, antimicrobial, and anti-inflammatory effects. These compounds can effectively curb lipid oxidation by scavenging free radicals, chelating metal ions, and disrupt oxidative chain reactions. Additionally, plant extracts are generally recognized as safe (GRAS) and are well-received by consumers due to their natural origins and perceived health benefits [7-14]. Utilizing plant preservatives not only overcomes the limitations of synthetic antioxidants but also aligns with the rising demand for clean-label and sustainable food products. Among the many plants investigated for their preservative capabilities, Anjbar (*Bistorta officinalis*) stands out. This perennial plant, native to Europe and Asia, has been traditionally utilized in folk medicine for its anti-inflammatory, antimicrobial, and antioxidant properties. It contains a diverse array of bioactive compounds, including tannins, flavonoids, and phenolic acids, which contribute to its robust antioxidant activity [15-18]. Given that no prior studies have explored the antioxidant activity of the aqueous extract of Anjbar, this study aimed to assess the levels of phenolic and flavonoid compounds and the antioxidant activity of this extract.

2. Materials and Methods

2.1. Extract preparation

The aqueous extract of Anjbar was prepared following the modified method of Salehi et al. (2022) [18]. Fresh Anjbar roots were harvested from the Sahand highlands in Azerbaijan, chopped into smaller segments, and then dried in the shade. Subsequently, the dried roots were ground using a grinder; 300 g of the resulting powder was then steeped in 1200 ml of distilled water. After allowing the mixture to sit at room temperature for 72 hours, the solution was filtered and centrifuged for 20 minutes. The supernatant was dried at 37 °C, and the resulting semi-solid mass was stored in the refrigerator for future use [18].

2.2. Alkaloids

Five ml of 1% HCl was added to 0.25 g of the Tarragon extract and boiled for 5 minutes. The volume was then adjusted back to the original level, and the resulting acidic solution was filtered using filter paper. The filtrate was alkalized with an appropriate amount of 10% ammonia and extracted with ethyl ether. The ether solution was evaporated until dry, after which 5 ml of 1% HCl was added. The acidic solution was then divided into three portions; one served as the blank while Mayer and Bosshardt indicators were added to the other two. The formation of a brown precipitate with the Bosshardt indicator and a white-yellowish precipitate with the Mayer indicator indicates the presence of alkaloids [19].

2.3. Saponins

In a test tube, 5 ml of the aqueous extract was mixed thoroughly with 5 ml of distilled water and gently heated. The development of stable foam served as an indicator for the presence of saponins [20].

2.4. Flavonoids

To 1 ml of the aqueous extract, 1 ml of a 10% lead acetate solution was added. The appearance of orange, red, crimson or magenta color was considered a positive indication for the presence of flavonoids [19].

2.5. Phenolics

1 ml of ferric chloride solution was mixed with 2 ml of the Anjbar extract. The appearance of a blue or green color indicates a positive result [19].

2.6. Total phenolics content

The total phenolic content in the Anjbar extract was assessed using the Folin-Ciocalteu reagent, following the method of Javanmardi et al. (2003). In a reaction mixture, 50 µl of the sample was combined with 2.5 ml of a 1/10 dilution of Folin-Ciocalteu's reagent and 2 ml of 7.5% (w/v) Na_2CO_3. This mixture was incubated at 45 °C for 15 minutes. Absorbance was then measured at 765 nm for all samples, and the results were reported as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw) [21].

2.7. Total flavonoids content

The total flavonoid content of the extract was estimated using the aluminum chloride method. One ml of the extract was combined with 1 ml of 2% methanolic aluminum chloride. The mixture was then allowed to stand at room temperature in the dark for 15 minutes before measuring the absorbance at 430 nm. The results were expressed as milligrams of quercetin equivalent (QE) per gram of dry weight of the plant powder (mg QE/g), with methanol used as the blank [19].

2.8. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The method described by Yeganegi et al. (2018) was utilized to assess the antioxidant activity of the extract, with some modifications [22]. One ml of the extract (ranging from 10 to 500 µg/mL) was mixed with 1 ml of a 0.2 mM DPPH solution in methanol. The mixture was then kept in the dark at 24 °C for 30 minutes, after which the absorbance of both the extract and a blank sample (containing all components except the extract) was measured at 517 nm. Antioxidant activity was calculated as bellow:

$$\text{Scavenging effect (\%)} = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{sample} and A_{blank} represent the absorbance of the extract and the blank sample, respectively. The antioxidant activity of the extract was compared to that of synthetic antioxidant BHT, using the IC_{50} value. The IC_{50} value indicates the concentration of the sample required to inhibit 50% of free radicals and is determined using the slope of the radical scavenging activity curve [22].

2.9. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity

First, a 7 mM aqueous solution of ABTS was prepared and subsequently diluted with potassium persulfate to achieve a concentration of 2.45 mM. This solution was then stored in the dark for 16 hours before being diluted with methanol to reach an absorbance of 0.7 at a wavelength of 734 nm. Finally, 300 µL of the extract was combined with 3.9 mL of the ABTS radical solution, and the absorbance was measured after storing the mixture for 5 minutes. The free radical scavenging activity of the extract was assessed using the formula described in Section 2.8 and was reported as IC_{50} [23].

2.10. Ferric reducing antioxidant power (FRAP)

A solution was prepared by combining 2.5 mL of 0.2 M phosphate buffer with 2.5 mL of 1% potassium ferricyanide, which was then mixed with the extract. This mixture was incubated at 50°C for 20 minutes, after which 2.5 mL of 10% trichloroacetic acid was added to halt the reaction. The solution was then centrifuged at 1000 g for 10 minutes. Following this, 2.5 mL of supernatant was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% chloride. After storing for 30 minutes, the absorbance was measured at 700 nm. The ferric iron reducing power of the extract was expressed in terms of ascorbic acid equivalent (mmol/g) [24].

2.11. β-Carotene bleaching inhibition

In the beta-carotene bleaching inhibition test, a spectrophotometric method was employed. Specifically, the absorbance of the solution was measured at 490 nm after 120 minutes of incubation (A_{120}), compared to the control sample at both time zero (C_0) and after 120 minutes (C_{120}). The inhibitory effect was then calculated using the following formula [25]:

$$\text{Inhibitory effect (\%)} = [(A_{120}-C_{120})/(C_0-A_{120})] \times 100$$

2.12. Cytotoxic effect

The MTT assay was conducted to evaluate the cytotoxicity of the extract against HT29 and HeLa cell lines. The cells were grown in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, then incubated at 37°C in an environment with 95% humidity and 5% carbon dioxide. A total of 100,000 cells were added to each well, along with various concentrations of the extract (0, 10, 25, 50, 100, and 200 mg/mL), DMEM medium, and 200 µL of fetal bovine serum. After 24 hours of incubation, cell proliferation was assessed using the MTT assay: 30 µL of a 5 mg/mL MTT solution was added to each well, and the plates were incubated in a carbon dioxide incubator for 3 hours. The absorbance at 570 nm was then measured using an ELISA reader (ELX 808, Bio Tek Instruments, USA). Cell viability curves were created based on the control cells [26].

2.3. Statistical analysis

The experiments were conducted three times, and the results were analyzed using Minitab software (version 16) with Tukey's test at a significance level of 5%.

3. Results and Discussion

Table 1 details the phytochemical constituents and their concentrations in the Anjbar (*B. officinalis*) extract. This information is vital for understanding the extract's chemical makeup and its potential bioactivity, which can be utilized in various pharmacological and therapeutic settings. Alkaloids were detected, appearing yellow or brown, indicating their presence in low concentrations (+). These compounds are noted for their wide-ranging

pharmacological effects, which include anti-inflammatory actions [27]. The Shinoda test produced a red solution, implying that flavonoids are present in moderately high concentrations (++). Flavonoids are powerful antioxidants known to possess anti-cancer, anti-viral, and anti-inflammatory properties [7, 28]. Saponins, also present in low concentrations (+), were identified by stable foam formation and are acknowledged for their immune-boosting and cholesterol-lowering effects [29]. The Ferric chloride test yielded a greenish-blue color, indicating a high concentration (+++) of phenolic compounds. These phenolics are celebrated for their antioxidant abilities, which help alleviate oxidative stress and its related diseases [22]. Experiments investigating phenolic and flavonoid compounds confirmed their substantial presence in the aqueous extract, with phenolic compounds being the most abundant.

Table 1. Phytochemical constituents of *Bistorta officinalis* aqueous extract.

Chemical	Verification method	Observation	Occurrence
Alkaloids	Mayer and Bosshardt	Formation of a yellow or brown color	+
Flavonoids	Shinoda test	Red solution	++
Saponins	Froth test	Formation of a stable foam	+
Phenolics	Ferric chloride	Green-bluish	+++

+ present in small concentrations; ++ present in moderately high concentrations; +++ present in high concentrations.

The results of the total phenol and flavonoid content of the aqueous extract of Anjbar are presented in **Figure 1**. According to the results, the extract contained 76.65 mg GAE/g total phenols and 40.38 mg QE/g total flavonoids. In a study, HPLC was used to identify five major phenolic compounds in *B. officinalis*, including galloylglucoside isomer, catechin, and chlorogenic acid, while also quantifying total phenols and extractable tannins [30]. In a separate study, a total of 31 polyphenols were detected in the roots of *B. officinalis* extract, comprising 20 benzoyl derivatives and 11 caffeoyl derivatives, with 26 trace elements newly identified in the herb [31]. Additionally, Yang et al. [32] applied an online microextraction technique for the rapid analysis of antioxidants in *B. officinalis*, finding key phenols like neochlorogenic acid, caffeic acid, gallic acid, procyanidin B2, and chlorogenic acid. To assess the chemical components and cytotoxicity of *B. officinalis*, a methanol-water extract was analyzed, leading to the identification of several phenols—including protocatechuic acid, gallic acid, p-hydroxybenzoic acid, vanillic acid, chlorogenic acid, syringic acid, 4-methyl catechol, catechol, and syringol—across 13 fractions obtained via conventional preparative high-pressure liquid chromatography [33]. The ethanolic extract of the aerial parts of *B. officinalis* underwent qualitative (HP)TLC analysis, which identified flavonoid derivatives as the primary compounds present, including kaempferol, quercetin, and luteolin [34].

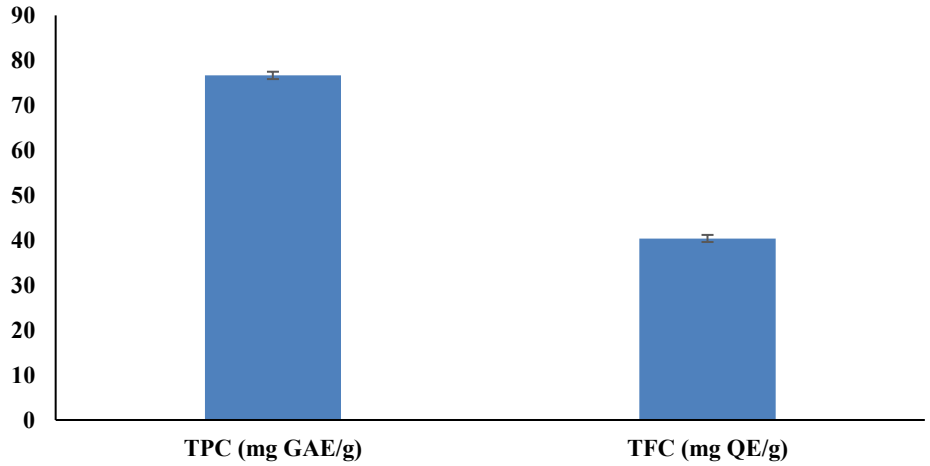

Figure 1. Total phenolics content (TPC) and total flavonoids content (TFC) of *Bistorta officinalis* aqueous extract.

Figure 2 illustrates the antioxidant potential of the extract, assessed through two common assays: DPPH and ABTS. These methods are frequently employed to evaluate the free radical scavenging ability of compounds, a crucial indicator of antioxidant activity. The results are benchmarked against BHT (butylated hydroxytoluene), a synthetic antioxidant that serves as a standard in such studies.

In the DPPH assay, the extract's capacity to donate hydrogen atoms or electrons to stabilize the DPPH radical is measured, with lower absorbance at 517 nm indicating greater antioxidant activity. The ABTS assay assesses the extract's ability to quench the ABTS radical cation, determined by a reduction in absorbance at 734 nm. The results revealed that the extract exhibited IC_{50} values of 47.30 µg/ml in the DPPH assay and 29.20 µg/ml in the ABTS assay, both significantly higher than BHT's IC_{50} of 9.65 µg/ml, indicating that the extract was less effective in neutralizing free radicals.

Additionally, the antioxidant activity of the extract was measured using FRAP and beta-carotene bleaching inhibition methods, yielding values of 8.69±0.34 mmol/g and 55.65±0.63%, respectively. The antioxidant activity of the *B. officinalis* aqueous extract can be attributed to its phenolic compounds, flavonoids, and other phytochemicals known for their ability to scavenge free radicals. These compounds effectively donate hydrogen atoms or electrons to free radicals, neutralizing them and preventing oxidative damage.

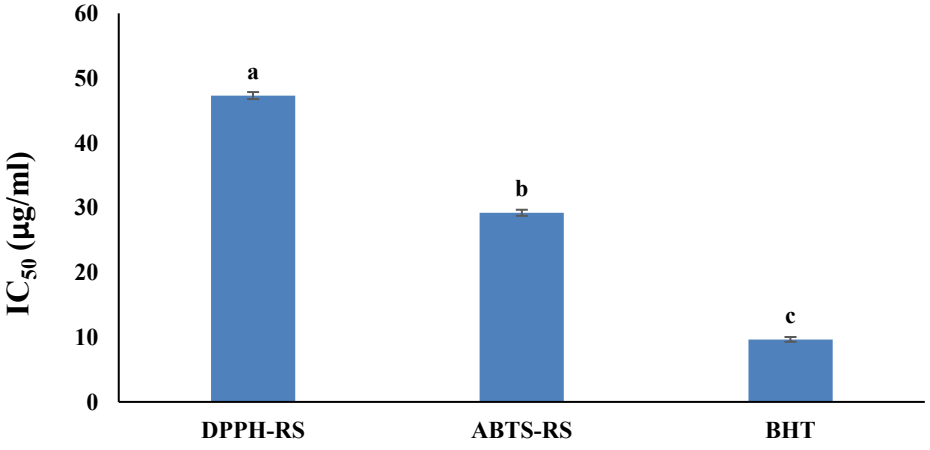

Figure 2. Antioxidant activity of *Bistorta officinalis* aqueous extract.

Javid et al. (2024) performed a partwise analysis to evaluate the antioxidant activity of *B. amplexicaulis*. Their findings demonstrated that both the aboveground parts (including leaf, stem, and inflorescence) and belowground parts (the rhizome) exhibited radical scavenging activity, with the belowground parts showing notably higher scavenging potential. Specifically, the ethyl acetate extracts from the rhizomes recorded the highest inhibition percentages in DPPH, NO−, and OH− radical scavenging assays [35]. In another study, the ethyl acetate fraction of B. *amplexicaulis* was found to possess the strongest antioxidant potential, with an IC50 value of 5.76 ± 0.03 µg/ml for DPPH, an IC50 of 0.74 ± 0.1 µg/ml for ABTS, and a total antioxidant capacity of 72.55 ± 0.098 ascorbic acid equivalents [36]. Batool et al. (2015) used a DNA plasmid protection assay along with a DPPH radical scavenging experiment to assess the antioxidant activity of *B. amplexicaulis*, identifying active fractions through HPLC. The study evaluated the antioxidant potential of crude methanolic extracts from the shoot, rhizome, and leaf, finding that the leaf extract had the strongest antioxidant activity. The crude methanolic leaf extract exhibited maximum scavenging activity, displaying an IC_{50} value of 1.03 µg/ml, while the other fractions had IC_{50} values ranging from 1.03 to 58.2 µg/ml [37]. Munir et al. (2014) explored the antioxidant potential of B. officinalis rhizome extracts using methanolic and ethanolic solvents, finding that the ethanolic extract exhibited superior antioxidant potential compared to the methanolic extract. The antioxidant properties were analyzed using DPPH, FRAP, and ABTS methods, and the study also investigated the correlation between antioxidant activity and phenolic content. The results indicated that the plant demonstrates strong scavenging activities with a positive correlation between total phenolic content and antioxidant activity [38]. Additionally, Pirvu et al. (2017) assessed the antioxidant capacity of the ethanolic extract from the aerial parts of B. officinalis using chemiluminescence tests, revealing a strong antioxidant capacity that surpassed that of rutin and gallic acid, which were used as reference compounds [34].

Figure 3 illustrates the cytotoxic effects of the aqueous extract of B. officinalis on two human cancer cell lines: HT-29 (colon adenocarcinoma) and HeLa (cervical adenocarcinoma), as assessed through cell viability assays. The results show a concentration-dependent decline in cell viability for both cell lines, indicating significant cytotoxic activity of the extract. At the control concentration of 0 mg/ml, HT-29 cells had a viability of 98.62%. As the concentration of the extract increased, cell viability decreased progressively: to 80.65% at 10 mg/ml, 65.8% at 25 mg/ml, 56.3% at 50 mg/ml, 24.3% at 100 mg/ml, and 13.25% at 200 mg/ml.

HeLa cells, likewise, exhibited a viability of 97.8% at 0 mg/ml, which dropped to 79.85% at 10 mg/ml, 59.5% at 25 mg/ml, 47.3% at 50 mg/ml, 19.6% at 100 mg/ml, and 11.95% at 200 mg/ml. The calculated IC_{50} values were 80.4 mg/ml for HT-29 cells and 70.92 mg/ml for HeLa cells. These IC_{50} values indicate the concentration of extract necessary to decrease cell viability by 50%, with the lower IC_{50} for HeLa cells suggesting a slightly higher sensitivity to the extract compared to HT-29 cells.

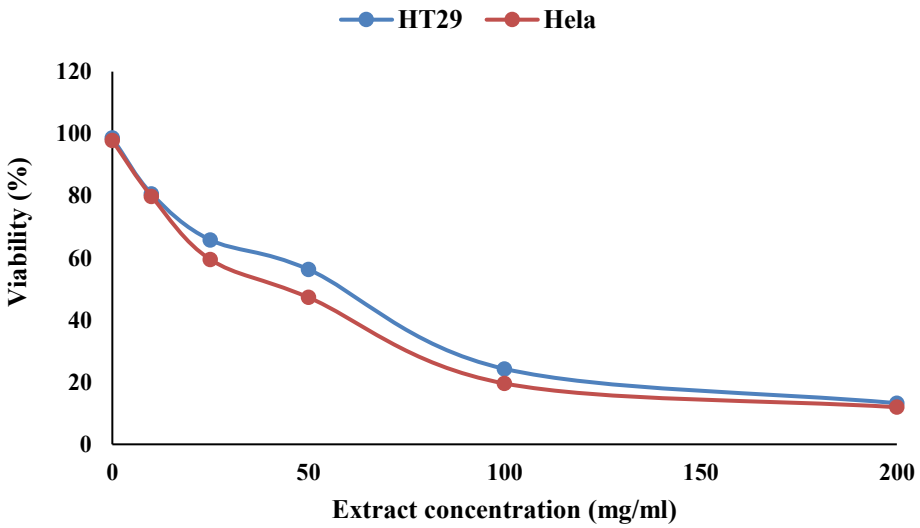

Figure 3. Cytotoxic effect of *Bistorta officinalis* aqueous extract against HT-29 and Hela cell lines.

Pirvu et al. (2017) performed a cytotoxicity test on ethanolic extracts from the aerial parts of *B. officinalis* and discovered that concentrations above 25 µg GAE/mL exhibited significant toxicity to mouse embryonic fibroblast NIH3T3 cells [34]. A study by Pillai Manoharan et al. (2007) assessed the cytotoxic activity of chloroform and hexane fractions, along with their sub-fractions derived from rhizomes, against various cancer cell lines. This research revealed that these fractions and some sub-fractions of *B. officinalis* exhibited moderate to high activity against HL60, P388, and LL2 cancer cell lines [39]. Intisar et al. (2013) isolated anticancer fatty acids and phenolic compounds from several fractions of a methanol-water extract of *B. officinalis*, finding that eleven out of thirteen fractions demonstrated good to strong cytotoxicity against the HCCLM3 cancer cell line, highlighting the significant bioactivity of *B. officinalis* [33]. The cytotoxic effects of the *B. officinalis* extract may be attributed to several mechanisms associated with its bioactive compounds. Plant extracts are known to induce apoptosis in cancer cells via the activation of both intrinsic (mitochondrial) and extrinsic (death receptor) pathways. This process may involve the upregulation of pro-apoptotic proteins and the downregulation of anti-apoptotic proteins. Additionally, many plant-derived compounds generate reactive oxygen species (ROS) within cancer cells, leading to oxidative stress and eventual cell death. This imbalance between ROS production and the cell's antioxidant defenses can result in DNA damage, lipid peroxidation, and protein oxidation. Furthermore, bioactive compounds can disrupt the cell cycle, causing cell arrest at specific phases and inhibiting proliferation, often through the modulation of cyclins and cyclin-dependent kinases [40, 41].

4. Conclusion

The research emphasizes the promise of *B. officinalis* (Anjbar) as a natural source of antioxidants and bioactive compounds, presenting an appealing alternative to synthetic preservatives in the food sector. The aqueous extract of *B. officinalis* revealed substantial amounts of phenolic and flavonoid compounds, which enhance its strong antioxidant properties, demonstrated through DPPH, ABTS, FRAP, and beta-carotene bleaching assays. While its antioxidant effectiveness was lower than that of synthetic BHT, its natural origin and safety profile render it a suitable

choice for clean-label food products. Furthermore, the extract displayed significant cytotoxic effects on HT-29 and HeLa cancer cell lines, indicating its potential role in cancer treatment. The presence of alkaloids, saponins, and phenolics further highlights its pharmacological potential. These results advocate for the utilization of *B. officinalis* as a sustainable, natural preservative and therapeutic agent, aligning with the increasing demand for safer, plant-based options in both the food and pharmaceutical sectors.

5. Acknowledgements

The authors would like to express their sincere gratitude to the Vice-chancellor for Research and Technology of Agricultural Sciences and Natural Resources University of Khuzestan for supporting this study as a project number 1403.53.

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سایت مجله: www.fsct.modares.ac.ir

مقاله علمی_پژوهشی

ترکیبات، پتانسیل آنتی اکسیدانی، فنل و فلاونوئید کل و اثر سمیت سلولی عصاره آبی انجبار: مطالعه در شرایط آزمایشگاهی

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۴/۱/۲۸

تاریخ پذیرش: ۱۴۰۴/۳/۵

کلمات کلیدی:

اکسیداسیون لیپید،

انجبار،

فعالیت آنتی‌اکسیدانی،

سمیت سلولی،

ترکیبات فنولی،

نگهدارنده‌های طبیعی.

DOI: 10.22034/FSCT.22.165.164.

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چکیده

اکسیداسیون لیپید تأثیر قابل‌توجهی بر کیفیت، ایمنی و ارزش غذایی مواد غذایی دارد و در نتیجه طعم نامطلوب، کاهش مواد مغذی و نگرانی‌های بهداشتی مانند بیماری‌های قلبی- عروقی و سرطان را به همراه دارد. درحالی‌که نگهدارنده های مصنوعی مانند BHA و BHT مؤثر هستند، ممکن است خطراتی برای سلامتی داشته باشند که منجر به افزایش علاقه به جایگزین‌های طبیعی می‌شود. نگهدارنده های گیاهی، به‌ویژه آن‌هایی که حاوی پلی‌فنول‌ها، فلاونوئیدها و اسانس‌ها هستند، راه‌حل‌های ایمن‌تر و پایدارتری ارائه می‌دهند. در این مطالعه، ویژگی‌های آنتی‌اکسیدانی و سیتوتوکسیک عصاره آبی گیاه انجبار (*Bistorta officinalis*)، گیاهی که به‌خاطر کاربردهای سنتی ضدالتهابی و ضد میکروبی آن شناخته شده است، مورد بررسی قرار گرفت. عصاره به دست آمده از ریشه‌های خشک شده، برای فیتوکمیکال‌های مختلف از جمله آلکالوئیدها، ساپونین‌ها، فلاونوئیدها و فنول ها مورد تجزیه و تحلیل قرار گرفت. محتوای فنولی کل برابر با ۷۶/۶۵ mg GAE/g بدست آمد، درحالی‌که محتوای فلاونوئید کل برابر با ۴۰/۳۸ mg QE/g بود. فعالیت آنتی‌اکسیدانی با استفاده از روش‌های DPPH، ABTS، FRAP و رنگبری بتا-کاروتن مورد ارزیابی قرار گرفت و مقادیر IC50 بدست آمده برابر با ۴۰/۳۰ میکروگرم در میلی‌لیتر (DPPH) و ۲۹/۲۰ میکروگرم در میلی‌لیتر (ABTS) با مقدار ۸/۶۹ mmol/g برای آزمون FRAP بود. ارزیابی سمیت سلولی بر روی رده‌های سلولی سرطانی HT-29 و HeLa نشان دهنده اثر کاهشی وابسته به غلظت در زنده ماندن سلولی با مقادیر IC_{50} به ترتیب ۸۰/۴ میلی‌گرم در میلی‌لیتر و ۷۰/۹۲ میلی‌گرم در میلی‌لیتر بود. ترکیبات زیست فعال موجود در عصاره، به‌ویژه فنولیک ها و فلاونوئیدها، نقش مهمی در خواص آنتی‌اکسیدانی و سیتوتوکسیک آن دارند و پتانسیل آن را به عنوان یک نگهدارنده طبیعی و عامل درمانی برجسته می‌نمایند. این نتایج بر کارایی انجبار به عنوان یک جایگزین طبیعی برای آنتی‌اکسیدان‌های مصنوعی در هر دو بخش غذا و دارو تأکید می‌کند.