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Scientific Research

Identification of chemical compounds, antioxidant potential, total phenols and flavonoids, and the cytotoxic effect of dill on HT29 and HeLa cell lines

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ABSTRACT

Dill (*Anethum graveolens*) has been widely used for medicinal and therapeutic purposes since ancient times. Plants possess a large number of effective compounds with low toxicity. In this study, the *in vitro* antioxidant and anticancer effects of dill aqueous extract against HT29 and HeLa cancer cell lines were investigated. The antioxidant effect of dill aqueous extract was evaluated by DPPH radical scavenging, ABTS radical scavenging, ferric iron reduction capacity and beta-carotene bleaching inhibition assays. The extract contained 89.6 mg GAE/g total phenols and 25.49 mg QE/g total flavonoids. As observed by antioxidant activity assay, dill aqueous extract showed potent antioxidant activities that were comparable to the synthetic antioxidant dibutyl hydroxytoluene (BHT). Dill aqueous extract also showed concentration-dependent anticancer/cytotoxic potential against HT29 and HeLa cell lines, and the IC_{50} values for these cell lines were 84.59 and 74.95 mg/mL, respectively. Overall, the present study demonstrated that dill aqueous extract has potential for use for medical and nutritional purposes as an antioxidant and anticancer agent.

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1.Introduction

Oxidative damage refers to damage caused to biological systems by reactive oxygen species such as free radicals. This damage can contribute to a wide range of diseases and aging processes in organisms. In addition, oxidative deterioration of lipids is a serious problem because it reduces the shelf life of food products, reduces their nutritional value, and produces reactive products that can be toxic. Antioxidants are effective compounds for preventing lipid oxidation, and synthetic antioxidants are often added to foods due to their high efficacy and low cost. However, the safety of these antioxidants is an increasingly debated topic among the public. Synthetic antioxidants have potential negative effects on health due to their ability to accumulate in tissues and disrupt natural antioxidant systems [1-3]. Despite the fact that synthetic antioxidants dominate the market, the demand for natural antioxidants has increased in recent years and is expected to continue. This pattern can be explained by the growing consumer preference for organic and natural products that contain fewer additives and may have fewer side effects than synthetic ones [4].

Fruits, vegetables, spices, herbs, cereals and seeds are the main sources of plant antioxidants. The activity of extracts and essential oils obtained from these plant materials is mainly related to the presence of compounds with strong antioxidant activity, mainly polyphenols and terpenoids in their composition. Within each of them, anthocyanins, flavonols, and tannins or terpenes are usually the most abundant. Furthermore, it is important to note that the benefits attributed to plant extracts cannot be attributed to a single group of compounds, but rather to the multiple contributions of different bioactive compounds. Among terpenes, compounds such as camphor, camphene, carnosol, carvacrol, thymol, alpha-pinene, cymene, 1,8-cineole, limonene, gamma-terpinene, and terpinen-4-ol are the most common. Quercetin and kaempferol are the predominant flavonols, although the flavonols apigenin, isorhamnetin, luteolin, myristicin, and rutin have also been identified. In addition, phenolic acids such as caffeic, cinnamic, chlorogenic, ferulic, quinic, rosmarinic, and sinapic acids are among the known acids [5-8].

Dill (*Anethum graveolens*) belongs to the *Umbelliferae* family. It has been reported that the aqueous extract is equivalent to 1-4% of the whole dill plant and its main components include dicarvone (20-36%), dilapiol (15-20%), limonene (20-30%), trans-dihydrocarvone (7-10%), and thymol (5-7%) [9]. Dill aqueous extract has antioxidant activity due to the presence of dicarvone and limonene compounds and, by protecting cell membranes in liver tissues, leads to a decrease in the release of lactate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase enzymes into the bloodstream [10]. In addition, the presence of dilapiol in dill essential oil has made its antioxidant effect much stronger than the antioxidant activity of most synthetic antioxidants such as dibutyl hydroxytoluene and dibutyl hydroxyanisole [11].

Dill is of great importance in the pharmaceutical and food industries. However, there is limited information about the chemical composition and biological properties of its aqueous extract. In this study, the chemical compounds present in dill leaf extract and its antioxidant and anticancer properties were investigated in detail.

2. Materials and Methods

2. 1. Dill aqueous extract preparation

Dill leaves were obtained from Ahvaz and were confirmed by our colleague in the Phytomedicine Department of Khuzestan University of Agricultural Sciences and Natural Resources. For aqueous extraction, 20 g of dried dill leaves were mixed with 200 mL of water and boiled for 10

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min. After cooling, the mixture was filtered and the resulting extract was concentrated to a final volume of 100 mL [12].

2.2. Chemical composition

Identification of the extracted extract components was carried out by injecting 2 µl of dill extract into a gas chromatography device connected to a mass spectrometer (TRACE MS, uest-FinniganThermoQ). The column used was DB-5 with a length of 30 m, an internal diameter of 0.25 mm and a stationary phase thickness of 0.25 µm, which was connected to a mass spectrometer (Quadropole). The column temperature was increased from 40 to 250 °C at a rate of 2.5 °C/min. Helium was used at a rate of 1.1 ml/min and an ionization energy of 70 electron volts. Identification of the extract components was done with the help of the normal spectrum of alkanes (C_8-C_{24}) and also obtaining the inhibition index and comparing it with the reported Kovats retention index of the components in the NIST 05 software and the mass spectrum of each component with the device library (Wiley7n.1) [13].

2.3. Total phenol content (TPC)

For this purpose, 20 µl of the extract (with a concentration of 10 g/l), 2 ml of distilled water and 100 µl of Folin-Ciocalteu reagent were mixed. After 3 minutes of incubation, 300 µl of sodium bicarbonate solution was added to it and the mixture was stirred for 2 hours. The absorbance was measured using a spectrophotometer (Sigma3, 30k) at a wavelength of 765 nm. A standard curve was created with gallic acid concentrations ranging from 0 to 500 mg/L and total phenol content was expressed as mg gallic acid equivalent per gram of extract (mg GAE/g) [14].

2.4. Total flavonoid content (TFC)

To measure total flavonoid content, 1 mL of extract was mixed with 1 mL of 2% methanolic aluminum chloride and kept in the dark for 15 min at room temperature. The absorbance of the sample was measured at 430 nm and a standard curve was created using quercetin. Total flavonoid content was reported as micrograms of quercetin equivalent per gram of dry weight of extract (mg QE/g) [14].

2.5. Antioxidant activity

The antioxidant effect of dill aqueous extract was investigated based on DPPH and ABTS free radical scavenging methods, ferric iron reducing power, and beta-carotene bleaching inhibition.

In the DPPH radical scavenging test, 50 µL of the extract or control was mixed with 5 mL of ethanolic DPPH solution (0.12 mM) and the resulting solution was kept at room temperature in the dark for 30 min. The absorbance of the solution was finally recorded at a wavelength of 517 nm. In the ABTS test, first an aqueous solution of ABTS (7 mM) was prepared and then potassium persulfate was added to it until its concentration reached 2.45 mM. Subsequently, the solution was kept in the dark for 16 h and then diluted with methanol until an absorbance of 0.7 at a wavelength of 734 nm was reached. Finally, 300 µL of the extract was mixed with 3.9 mL of ABTS radical solution and its absorbance was recorded after 5 min of storage. The free radical scavenging activity of the extract was calculated according to the following formula [15]:

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

The free radical scavenging activity of the extract was finally reported in terms of IC_{50} (µg/mL).

In the ferric iron reducing power test, a solution containing 2.5 mL of 0.2 M phosphate buffer and 2.5 mL of 1% potassium ferricyanide was prepared and mixed with the extract. The resulting solution was kept at 50°C for 20 minutes, and then 2.5 mL of trichloroacetic acid (10%) was added to stop the reaction. The

[DOI: 10.22034/FSCT.22.163.231]

solution was centrifuged at 1000 *g* for 10 minutes and then 2.5 ml of supernatant was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% chloride and after 30 minutes of storage, its absorbance was read at 700 nm. The reducing power of ferric iron of the extract was reported in terms of ascorbic acid equivalent (mmol/g) [14].

In the beta-carotene bleaching prevention test, the spectrophotometric method was used. Briefly, the absorbance of the solution after 120 minutes of incubation (A_{120}) was examined at 490 nm against the control sample at times zero (C_0) and 120 minutes (C_{120}). The inhibitory effect was calculated according to the following formula [16]:

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

In all tests, dibutyl hydroxytoluene (BHT) was considered as a control and to compare the antioxidant power of the extract.

2.6. Cytotoxicity

MTT method was used to investigate the cytotoxicity of the extract against HT29 and Hela cell lines. Cells were cultured in DMEM medium with 10% fetal bovine serum and penicillin/streptomycin and then incubated at 37°C, 95% humidity, and 5% carbon dioxide. Cells (100,000 cells) were added to the wells and different concentrations of extract (0, 10, 25, 50, 100, and 200 mg/ml), DMEM culture medium and 200 µl of fetal bovine serum were added to it. Each well was measured for cell proliferation using the MTT method after 24 h of incubation as follows: 30 µl of MTT solution with a concentration of 5 mg/ml was added to each well and the plates were kept in a carbon dioxide incubator for 3 h. The absorbance of the medium at a wavelength of 570 nm was recorded using an ELISA reader (ELX 808, Bio Tek Instruments, USA). A cell viability curve was drawn using control cells [17].

2.7. Statistical analysis

The experiments were performed in triplicate. The results were analyzed with Minitab software (version 16) using the Tukey test at a significance level of 95%.

3. Results and Discussion

The chemical compositions of dill aqueous extract are given in Figure 1. According to the figure, 12 chemical compounds were identified in dill extract, which constituted 98.09% of the extract compounds. Limonene (35.2%), alpha-phellandrene (27.56%), and carvone (16.2%) were the most abundant compounds identified in the extract, respectively. In addition, dill aqueous extract contained 89.6 mg GAE/g total phenols and 25.49 mg QE/g total flavonoids (Figure 2).

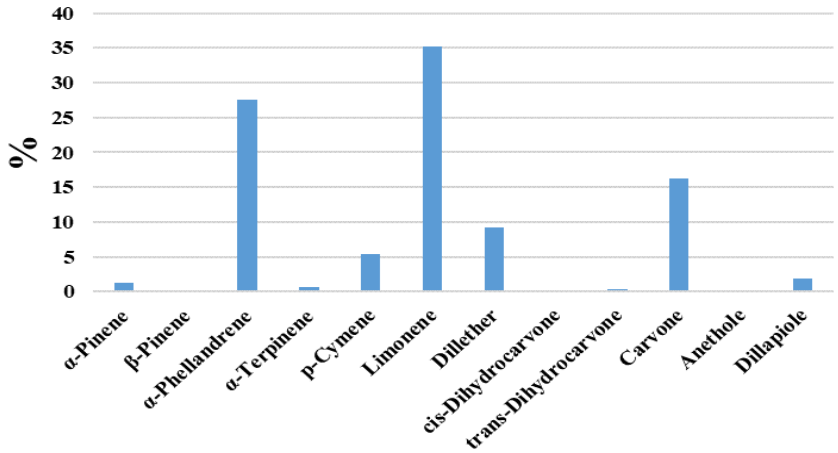

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Figure 1. The chemical composition of *Anethum graveolens* extract. Compounds with concentrations below 0.1% are not listed in the figure.

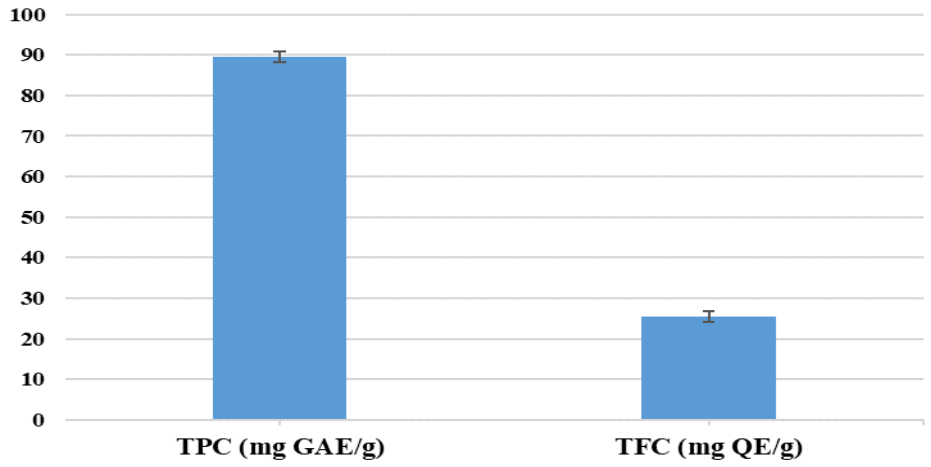

Figure 2. Total phenol content (TPC) and total flavonoids content (TFC) of *Anethum graveolens* extract.

The antioxidant activity results of dill aqueous extract and synthetic antioxidant BHT are reported in Figure 3. The aqueous extract had antioxidant activities of 16.85 µg/mL, 13.53 µg/mL, 7.85 mmol/g and 76.95% based on DPPH radical scavenging, ABTS radical scavenging, ferric iron reducing power and beta-carotene bleaching inhibition methods, respectively. While these values for synthetic antioxidant BHT were 7.85 µg/mL, 6.9 µg/mL, 0.41 mmol/g and 90.25%, respectively. The results show that the antioxidant activity of aqueous extract is weaker but comparable to BHT.

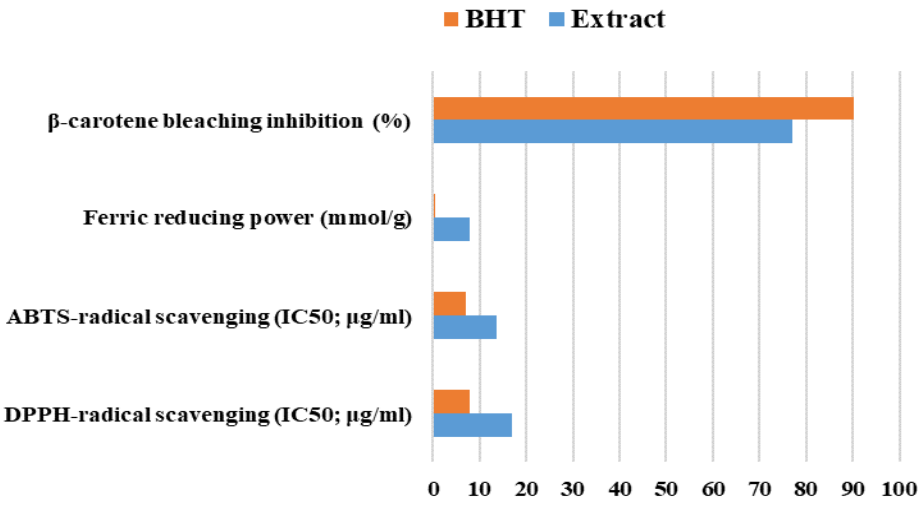

Figure 3. Antioxidant activity of *Anethum graveolens* extract.

The results of the cytotoxic activity of dill aqueous extract against HT29 and Hela cancer cell lines are presented in Figure 4. Increasing the extract concentration significantly reduced the

[DOI: 10.22034/FSCT.22.163.231]

viability of cancer cells. The percentage of HT29 cell viability decreased from 99.77% in the absence of the extract to 16.2% in the presence of 200 mg/mL of the extract. These values for the Hela cell line were 96.85% and 14.75%, respectively. In addition, the IC_{50} values for the HT29 and Hela cell lines were calculated to be 84.59% and 74.95 mg/mL, respectively.

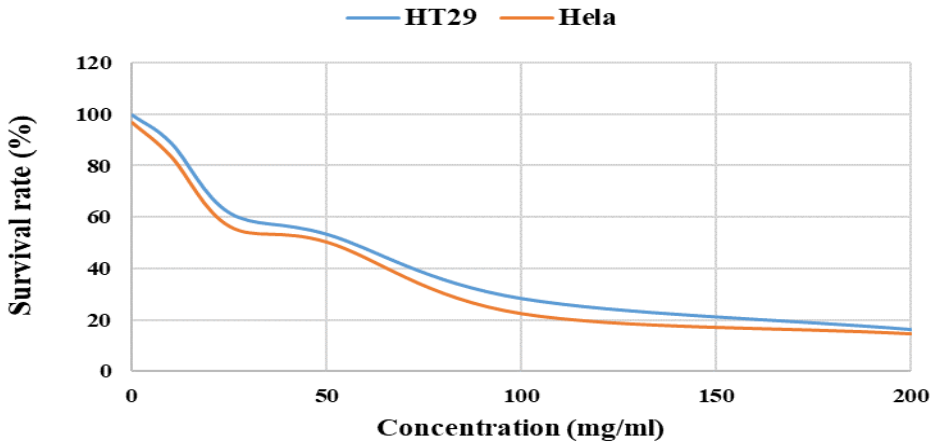

Figure 4. Cytotoxicity of *Anethum graveolens* extract against HT29 and Hela cell lines.

Similar results have been reported by other researchers in scientific literature. Hadi et al. (2024) investigated the chemical composition and antioxidant activity of essential oils and extracts obtained from dill seeds collected in southern Morocco [18]. The results showed that the essential oil extracted by water distillation contained E-anethole (38.13%), estragole (29.32%), fenchone (17.21%) and α-pinene (7.37%). The ethanolic extract recorded the highest levels of phenolic compounds and flavonoids with 52.65 mg GAE/g and 35.58 mg QE/g, respectively, followed by the aqueous extract with 39.72 mg GAE/g total phenols and 13.79 mg QE/g total flavonoids. Furthermore, researchers have shown that dill essential oils and extracts have a strong capacity to scavenge harmful free radicals, control the production of reactive oxygen species and oxidative stress [18]. Kaur et al. [19] determined through DPPH method that Indian dill essential oil, which is mainly composed of carvone (41.15%), limonene (23.11%), camphor (9.25%), and dihydrocarvone (3.75%), showed low antioxidant activity (0.65 mg/mL) compared to standard ascorbic acid (0.04 mg/mL). These authors attributed this antioxidant power to the presence of polar compounds [19]. Similarly, Osanloo et al. [20] observed low antioxidant activity of Iranian dill essential oil, the main components of which were alpha-phellandrene (26.75%), p-cymene (24.81%), carvone (10.77%), dill ether (9.78%), and cis-sabinol (3.61%) [20]. A study conducted in Serbia showed that dill essential oil, consisting of limonene (45.24%) and carvone (45.90%), had higher anti-radical activity than aniseed, which was mainly composed of anethole (96.4%) [21]. Stanojević et al. [22] reported that dill essential oil from Serbia, which is mainly composed of carvone (85.9%), limonene (5.1%), and cis-dihydrocarvone (3%), showed 79.62% scavenging of DPPH radicals at a concentration of 29 mg/mL for 60 min [22]. Basavegowda et al. [23] reported the potent antioxidant activities of methanolic extract of dill seeds from India. They showed IC_{50} values of 19, 28, and 36 µg/mL by three radical scavenging assays: DPPH, hydroxyl, and nitric oxide, respectively [23]. Furthermore, Al-Oqail et al. [24], using DPPH and hydrogen peroxide radical scavenging

[DOI: 10.22034/FSCT.22.163.231]

assays, have shown that the methanolic extract of dill seeds has significant antioxidant capacity with IC_{50} values of 225 and 126.3 µg/mL, respectively. These researchers showed that dill extract exhibited dose-dependent anticancer/cytotoxic potential against MCF-7, A-549 and HeLa cell lines, and IC_{50} values for these cell lines were 104, 122 and 156 µg/mL, respectively [24]. The anticancer effects of dill extract may be due to the presence of phytochemicals in it. Also, the relationship between cytotoxicity and antioxidant activities has been confirmed [20-30]. Consequently, the antioxidant activities of dill extract may contribute to its cytotoxic/anticancer activities.

4. Conclusion

The present study showed that dill extract can be used for medicinal and nutritional purposes. Dill aqueous extract showed significant antioxidant effects as observed by DPPH radical scavenging, ABTS radical scavenging, ferric iron reduction capacity and beta-carotene bleaching inhibition. As can be seen from this study, dill extract may be used as a good source of natural antioxidants through potential nutritional supplements. Dill aqueous extract also showed strong concentration-dependent anticancer/cytotoxic potential against HT29 and HeLa cell lines. However, further studies are needed to investigate the biological and anticancer activities *in vivo* to investigate the potential beneficial use of dill aqueous extract in the food and pharmaceutical industries.

5.Acknowledgements

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مقاله علمی_پژوهشی

شناسایی ترکیبات شیمیایی، پتانسیل آنتی اکسیدانی، فنل و فلاونوئید کل و اثر سمیت سلولی شوید بر رده های سلولی HT29 و HeLa

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چکیده

شوید (*Anethum graveolens*) از دیرباز به‌طور گسترده برای اهداف دارویی و درمانی مورد استفاده قرار گرفته است. گیاهان دارای تعداد زیادی از ترکیبات مؤثر با سمیت کمتر هستند. در این مطالعه، اثرات آنتی‌اکسیدانی و ضد سرطانی در شرایط آزمایشگاهی عصاره آبی شوید در برابر رده‌های سلولی سرطانی HT29 و HeLa مورد بررسی قرار گرفت. اثر آنتی‌اکسیدانی عصاره آبی شوید توسط روش‌های مهار رادیکال DPPH، مهار رادیکال ABTS، ظرفیت احیا آهن فریک و جلوگیری از رنگبری بتا-کاروتن مورد ارزیابی قرار گرفت. عصاره حاوی ۸۹/۶ mg GAE/g فنول کل و ۲۵/۴۹ mg QE/g فلاونوئید کل بود. همان‌طور که توسط سنجش فعالیت آنتی‌اکسیدانی مشاهده شد، عصاره آبی شوید فعالیت‌های آنتی‌اکسیدانی قوی نشان داد که قابل مقایسه با آنتی‌اکسیدانی سنتزی دی‌بوتیل هیدروکسی تولوئن (BHT) بود. عصاره آبی شوید همچنین پتانسیل ضد سرطان/سیتوتوکسیک وابسته به غلظت را در برابر رده‌های سلولی HT29 و HeLa نشان داد و مقادیر IC50 برای این رده‌های سلولی به ترتیب برابر با ۸۴/۵۹ و ۷۴/۹۵ میلی‌گرم در میلی‌لیتر بود. بطور کلی، مطالعه حاضر نشان داد که عصاره آبی شوید قادر به استفاده برای اهداف پزشکی و غذایی به‌عنوان یک عامل آنتی‌اکسیدان و ضد سرطان می‌باشد.

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