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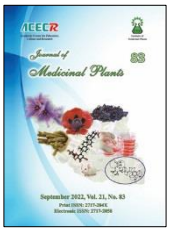

Research Article

Assessment of the antioxidant activity of the extrcat, cold press, and *n*-hexane oils and *in vitro* cytotoxic effects of *Capparis spinosa* L. seed on SH-SY5Y cancer cell lines

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ABSTRACT

Background: *Capparis spinosa* L. due to its anti-inflammatory and antioxidant properties plays a key role in preventing and treatment of cancer, also reduces the growth rate of cancer cells. This plant can be used in various ways in medicinal compounds, food, etc. as an extract, oil, or essential oil. **Objective:** In the current study, the rate of antioxidant activity extracts and oils (cold press and *n*-hexane oils) of *Capparis* seeds and also the effect of the seeds on the SH-SY5Y cell line have been investigated. **Methods:** First, extract, cold press, and *n*-hexane oils were prepared; then all reached concentrations of 50, 100, and 200 mg/ml, and their antioxidant capacity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. After that, the number of antioxidant compounds in the extract was measured, and finally, the toxicity of the extracts was evaluated by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method on SH-SY5Y cancer cells. **Results:** The IC_{50} data showed that the antioxidant activity of the *Capparis* seeds extracts was significantly ($P < 0.001$) increased compared to cold press and *n*-hexane. Antioxidant compounds analysis of *Capparis* seeds extract indicated high rate of total phenolic, flavonoid as rutin, and quercetin. MTT assay demonstrates that *Capparis* seeds extracts in a concentration of 1000 µg/ml decreased the viability of the SH-SY5Y cancer cell lines in comparison with hacked cells ($P < 0.001$). **Conclusion:** The seed extract of *Capparis* seeds at high concentrations, probably due to its high antioxidant content, inhibits the growth of SH-SY5Y cancer cells.

Abbreviations: CSS, *Capparis Spinosa* Seeds; DPPH, 2,2-Diphenyl-1-Picrylhydrazyl; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; TPC, Total Phenolic Contents

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1. Introduction

Since ancient times, medicinal plants have been employed as efficient and purely low risk therapeutic agents. Many of them are used for a variety of conditions and have multiple properties. They also tend to have fewer adverse effects than chemical medications [1, 2]. In the present time, medicinal plants and their compounds are considered to be an important range of bioactive elements like the phenolics and flavonoids, which are essential in the prevention and treatment of many diseases including cardiovascular, anticancer, and neurodegenerative problems [2-5]. One of the plants that has been used as an effective source of treatment since ancient times is the *Capparis spinosa* L. plant. is among the most important economical species in the Capparidaceae family which has a wide range of diversity (i.e., concerning 40–50 genera and 700–900 species [6]. Different parts of *Capparis spinosa* have been used as a plant in traditional medicine. Since ancient times, this plant has been used for the treatment of diseases such as liver, kidney, spleen, rheumatoid arthritis, gout and diabetes [7-11] due to the presence of a large number of flavonoids that have anti-inflammatory and antioxidant properties. It also plays a role in the treatment and prevention of many diseases, including cancer [12-16] and reducing the growth rate of cancer cells [17, 18].

The human neuroblastoma cell (SH-SY5Y) is also one of the cancer cells that is a subclone of the SK-N-H cell line [19, 20]. A juvenile tumor with a high prevalence and fatality rate is neuroblastoma. This nervous system tumor, which involves sympathetic cells generated from the neural crest, forms during the embryonic stage or in the early postnatal period. In additional to being the most prevalent malignant tumor in infants, it causes a significant percentage of pediatric cancer [21]. On the other hand, the effect of *Capparis* seed on SH-SY5Y Cell Lines has not been investigated so far. Therefore, we decided to test the effect of this plant on SH-SY5Y Cell Lines. On the other hand, the *Capparis* plant can be used in various ways in medicinal compounds, food, etc. In the form of extract, oil or essential oil, and even as a pickle (in combination with vinegar). However, since the number of ingredients and antioxidants activity in *Capparis spinosa* extract and seed oil may vary according to different studies. Therefore, in this study, we decided to compare free radical scavenging of *Capparis spinosa* extract, cold press and n-hexane oils and investigate the cytotoxic effect of the best one that possesses the highest amount of antioxidants on the SH-SY5Y cell line.

2. Materials and Methods

The plant samples, *Capparis spinosa*, were collected from Pars Abad Moghan, Ardebil, Iran and identified in the Herbarium of Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (voucher no. 3969 TMRC).

After preparation and identification, the seeds were separated and dried, hydroalcoholic extract (ethanol 70 %), cold press oil, and n-hexane oil were prepared by the researcher. Then all three types of extracts and oils reached concentrations of 50, 100, and 200 mg/ml, and their antioxidant activity was evaluated by DPPH method. Then the number of antioxidant compounds of total phenolic, total flavonoid, rutin, BHT in the

hydroalcoholic extract was measured and finally, the toxicity of the plant was evaluated by the MTT assay on cancer cells.

2.1. Extraction method

Percolation was used as method of extraction. For this purpose, CSS is ground and weighed the digital was weighed at 500 g and placed inside the percolator and 70 % ethanol was used as a solvent (at a temperature of 25-27 °C). Extract which was obtained in this way, indicated a dark color and a strong odor. This extract was considered a pure extract. At the end of the percolation stage, the solvent by the device Rotary Evaporator (RV; 05B, IKA, Germany) with a temperature of 45-50 °C separated and vacuumed at 45 °C (53 OT, Iran). It was dried until it reached a constant weight. These steps were repeated three times (It was repeated every 24 hours) and then it was kept in a dark and closed containers at a temperature of 4 °C [22].

2.2. Extraction of Capparis spinosa seed oil sample

The extraction of *Capparis spinosa* seed oil was done through the method provided by Talhaoui et al. with moderate changes. About 4 g of oil was diluted in 50 ml of n-hexane and extracted 3 times with a sparkling part of 20 ml of 60 % (vol/vol) absolute methanol (99.9 % pure) solution by shaking in a funnel separator for 10 min. Then, it was centrifuged at 5000 rpm for 10 min (4,472 g). The extraction process was repeated two times and centrifuged. The extracts were combined with absolute methanol solution, and then the solvent was evaporated to dryness in a vacuum rotary evaporator at 37 °C. Finally, the mix was filtered for HPLC analysis [23].

2.3. Preparation of n-hexane oil

In this method in order to obtain oil, the ground grain of *Capparis* seed was placed in the cartridge of the automatic Soxhlet extractor (Buchi Switzerland, 811-B) in the biochemistry laboratory at the school of pharmacy of Zanjan university of medical sciences, Iran. N-hexane was used as the solvent. Then 50 g of copper seed powder was furthered to 200 ml of the solvent in separate containers at room temperature and the mixture was stirred once a day for 6 days. Then the mixture was filtered using a vacuum filter. In the end, after modulating the solvent's temperature and pressure, extracted oil was separated using a rotary under vacuum (Switzerland, 215/210-R, Buchi).

2.4. Preparation of cold press oil

A cold press machine (VN26954) was used to prepare oil in this way. To perform this method, first clean machine and then CSSE was entered into the machine to remove oil (The oil was prepared pure). After oiling, the machine was cleaned again (oiling was done at room temperature and two days before the DPPH test).

2.5. DPPH radical scavenging activity

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. Experiments were carried out with a slight modification [24, 25]. After reduction of the radicals, there is a decrease in absorbance at 517 nm, 2 ml of methanol solution or water-methanol extracts were placed in test tubes and 2 ml of 1 mM DPPH solution was added. The tubes were then covered with parafilm and again placed in a dark container for 30 min. Absorbance at 517 nm

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was measured with a spectrophotometer UV-vis (Jasco V-530) and compared to an ascorbic acid calibration curve. This procedure was repeated for the extracts and both oils in all three concentrations of 50, 100, and 200 at 0, 10, 20, and 30 minutes in triplicate. The percentage inhibition of the DPPH radical was calculated using the following formula:

$$I\,\% = \frac{A_0 - A}{A_0} \times 100$$

where I = DPPH inhibition (%), A_0 = absorbance of control sample (t = 0 h) and A = absorbance of a tested sample at the end of the reaction (t = 30 min) [26].

2.6. Total phenolics contents

Total phenolics contents of *Capparis spinosa* seed extract (TPC) were determined by the Folin-Ciocalteu method using pyrogallol as the standard material [27]. The expressed value for the extract is mg.

25 µl of the extract was introduced into test tubes followed by 125 µl Folin-Ciocalteu's reagent (diluted 10 times in water). The solution was then kept at a dark room for 1 min and then 100 µl sodium carbonate 7.5 % w/v was added, then 96 well plates seeded. The plate was covered with aluminum foil and the solution's absorbance was read after two hours at 760 nm by ELISA (this protocol was repeated eight times and averaged between 6 close replicates).

2.7. Determination of total flavonoid content

Total flavonoid content in extracts and fractions of *M. calabura* L. was determined by visible spectrophotometry using the aluminum chloride ($AlCl_3$) method. This method refers to Saleem and Ahmad with some modifications [13, 14]. The flavonoid content was calculated as a quercetin equivalent.

2.8. Rutin and quercetin contents

HPLC method was used for the quantification of rutin and quercetin in the CSSE [28].

Test solution: 1 g of the seed extract was placed in a 5 ml conical flask and 4ml methanol was added to the sample. The mixture was treated in an ultrasonic bath for 30 min and then it was adjusted with methanol to 5 ml. A portion of this solution was filtered through a membrane filter.

HPLC condition was as follows: AQC18, ACE, 4.6 × 250 mm, 5 µm column (Normal phase), formic acid 1 % in water: methanol with gradient mode (Table 1) as a mobile phase, flow rate 0.6 ml/min in wavelength of 257 nm, column temperature 25 °C, injection volume 100 µl.

2.9. Cell culture and transfection

The target cells used in this experiment were HEK293 (prepared from Pasteur Institute of Iran) as a normal cell and the tumorigenic neuroblastoma cells (SH-SY5Y) (prepared from Pasteur Institute of Iran) as a cancer cell line. The normal HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) which was supplemented with 10 % fetal calf serum (FBS) (JR Scientific) and 1 % antibiotics (100 U penicillin G, 100 U streptomycin), and maintained in a 100 percent humidified atmosphere with at 37 °C and 5 % CO_2. The cancer cell line SH-SY5Y was supplemented in DMEM/f12 medium.

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When the cells reached 80 % confluency, HEK293 cells were trypsinized and diluted appropriately with fresh complete medium, and 2.4×10^4 cells/well (~100 µl) were seeded with 2 ml of complete culture medium in 96-well plates then transferred to the incubator for 24, 48, 72 h. In summary, cells with 10^3 cells density in each well were selected and seeded in 96-well plates, then they incubated for 24 h. They were detached using 0.5g/l trypsin and 0.2g/l EDTA and transferred to new culture flasks. Eventually, after removing the old culture media, 100 µl of FBS-free medium containing 20 µl of MTT solution (5 mg/ml) was added to each well. To measure cell viability using MTT two 96-cluster well culture plates were independently accumulated and all treatments was accomplished in triplicate. By adding DMSO (100 µl/well) followed 4 h incubation in 30 min, a plate reader (Bio-Rad 680, Hercules, CA) at wavelengths of 570-630 nm was used to measure the absorbance.

2.10. Cytotoxicity evaluation by MTT assay

MTT assay is a colorimetric assay for determination of cell viability and proliferation. This assay measures cell viability in terms of reductive activity as enzymatic conversion of the MTT to water insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells by NAD(P)H-dependent cellular oxidoreductase enzymes [29].

In this study, MTT assay was performed to evaluate the cytotoxicity and viability of SH-SY5Y and HEK293 cells. Cells were seeded at a density of 10^3 cells per well in 96-well plates and finally dried at 37 °C under 5% CO_2 for 2 h. kept in an incubator to maintain normal morphology. Then, cells were treated with different concentrations (7.6, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml) of *Capparis* extract treatment groups and untreated cells, which were considered as control cells. were implemented. Three 96-well culture plates were separately prepared to evaluate cells in 24 h, 48 h, and 72 h. After removing the old culture media, 100 µl of FBS-free medium containing 20 µl of MTT solution (5 mg/Ml; MTT was dissolved in DMSO) was added to each well. Then DMSO (100 µl/well) was added after 4 h incubation, and finally the absorbance was measured by a plate reader (Bio-Rad 680, Hercules, CA) at wavelengths of 570 nm following 30 min. The relative percentage of cell survival was calculated using the following formula and all treatments were performed in triplicate [18]:

Cell viability % = (average OD of treated group/average OD of the control group) × 100.

2.11. Statistical analysis

Statistical analysis was performed by using SPSS (Version 25, IBM Corp., and Armonk, NY, USA). Results were expressed as Mean ± SEM and all statistical comparisons were made using a one-way ANOVA test followed by Tukey's post hoc analysis. A P-value less than 0.05 was considered to be significant.

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3. Results

3.1. Antioxidant activity of Capparis spinosa seed (DPPH assay)

In this study, the radical scavenging activity of the extract, cold press oil, and n-hexane oil of CSS was via DPPH method compared at concentrations of (50, 100, and 200 mg/ml) the order of absorption in ethanol extract solution was the highest, and then cold press oil and n-hexane oil were higher, respectively (32.83 g of extract and 30 ml of oil were obtained from every 500 g of seeds).

Analysis of the IC_{50} data showed the increased mean of the cold press oil with a concentration of 50, 100, and 200 mg/ml compared to both oils with $P < 0.001$. It should be noted that the values obtained are the average of three replications (Fig. 1).

The results of the amount of antioxidant compounds of CSSE have been demonstrated in Table 1.

Quantitative analysis of the extracted quercetin and rutin was performed using HPLC chromatogram (A).

The amount of quercetin extracted from CSSE was 34.00 ± 1.28 (B): The amount of quercetin extracted from CSSE is 4.00 ± 1.13 (Fig. 2).

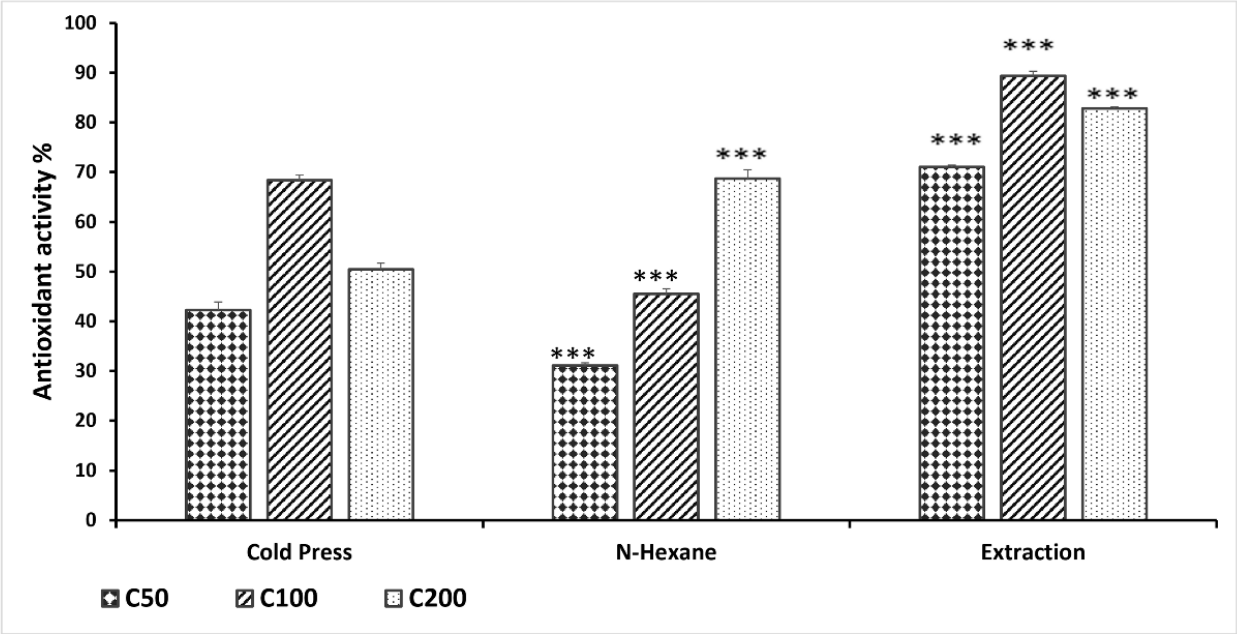

Fig. 1. Comparison and evaluation of the free radical scavenging percentage of N-hexane oil, cold press, and hydroalcoholic extract of CSSE by method DPPH: In this diagram, concentrations of 50, 100, and 200 (C50, C100, C200) in all three samples were compared. $P < 0.001$ was indicated by ***.

Table 1. The amount of antioxidant compounds of *Capparis spinosa* seed extract

Total phenolic (µg/mg)	Total flavonoid (µg/mg)	Rutin (µg/mg)	Quercetin (µg/mg)
25.12 ± 1.19	13.92 ± 1.77	4.00 ± 1.13	34.00 ± 1.28

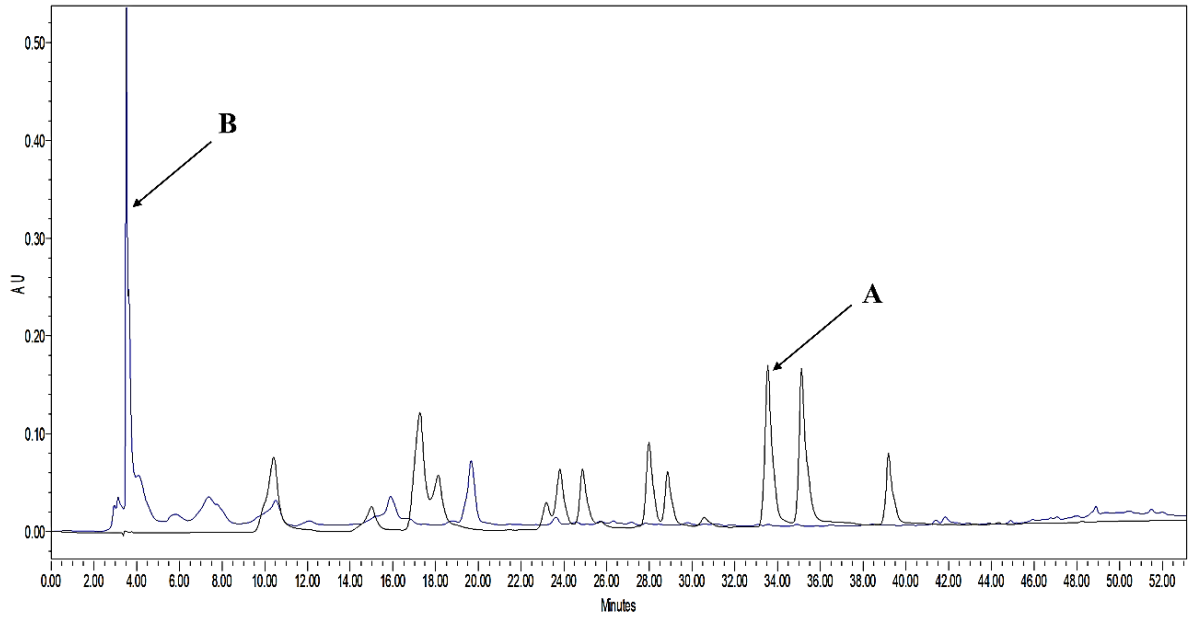

Fig. 2. (A): The chromatogram of standard solution of quercetin (µg/ml). **(B):** The chromatogram of rutin standard solution (µg/ml) of CSSE.

3.2. Determination of Capparis spinosa extract Cytotoxicity

The MTT assay was performed on SH-SY5Y cancer cell line and the results are expressed in Fig. 3. The results show that CSSE at the concentration of 1000 µg/ml, Cell viability percentage of SH-SY5Y cancer cells at, 48, and 72 hours after treatment compared to normal HEK cells significantly ($P < 0.001$) reduced. Normal cells compared to cancer cells have been affected by various CSSE concentrations and their viability decreased at 48 and 72 h after treatment with 1000 ug/ml of CSSE, but the result was not significant.

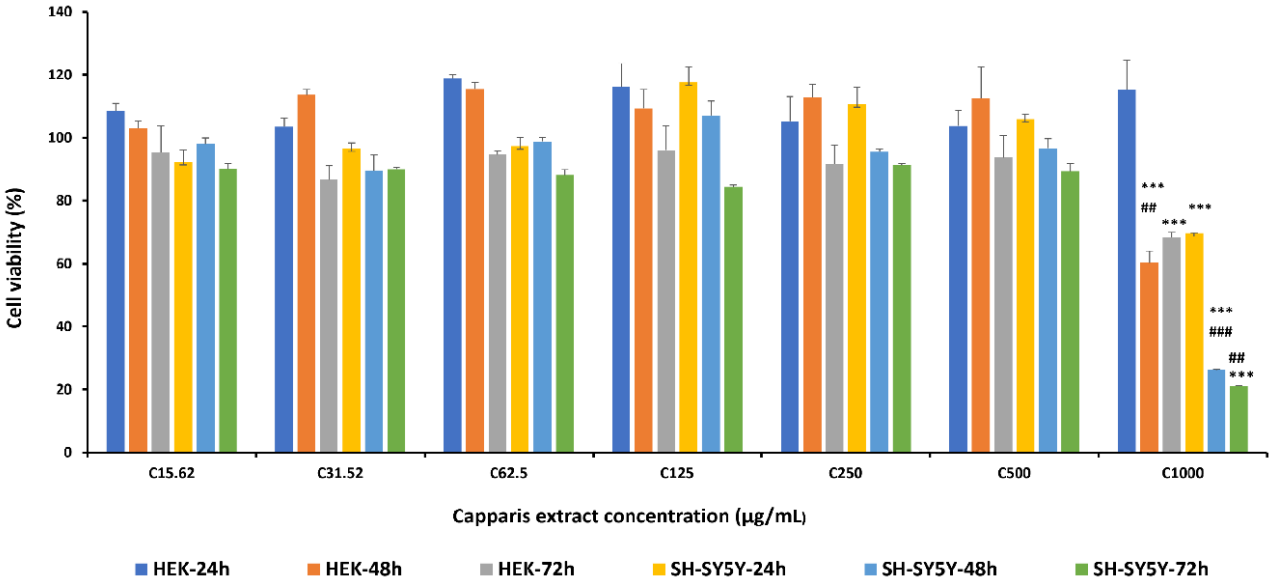

Fig. 3. Cell viability percentage determined by MTT assay in concentrations of 15.62, 31.52, 62.5, 125, 250, 500, and 1000: $^{***}P < 0.001$; SH-SY5Y Cell viability percentage at 24, 48 and 72 h compared to normal HEK cell. $^{##}P < 0.01$; $^{###}P < 0.001$; cell viability percentage in 48 h compared to 24 h and 72 h compared to 48 h.

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4. Discussion

In this study, the DPPH assay method was used to investigate and compare the free radical inhibition rate of CSS extract, cold press oil, and hexane in concentrations of 50, 100, and 200 mg. According to the studies of this method is more specific than similar tests (ABTS) in this area [30]. The results of this assay indicated that antioxidant activity is significantly higher in the extract compared to the two types of oils. Other studies have used fresh CSSE extract; 72.78 % [31], hexane; 29.32 % and Cold press; 16.41 % [23]. Based on these results, CSSE was used to continue our study due to antioxidant activity (free radical scavenging power) of the extract, which was more effective than the two types of oils.

Next, we examined the number of antioxidant compounds in CSSE (phenol and total flavonoids, rutin, and quercetin). The results showed the amount of total phenol content was 25.12 ± 1.19 µg/mg and total flavonoid was 13.92 ± 1.77 µg/mg. The values presented indicate that CSS have high antioxidant power. As it have been shown in N Tlili & et al; in 2015, total phenolics increased from 1.31 to 8.14 mg GAE / g DR [32], and total flavonoids; total flavonoid content ranged from 4.71 mg QE/g. On the other hand, the content of total phenolics is; 0.19 ± 0.01 %, rutin; 3.10 ± 0.03 mg/100 g fruits, quercetin; mg/100 g fruits [33], Total flavonoids; 39.96 ± 10.94 mg/100 g fresh weight. Another study in 2019 showed that (CSSE) increases the activities of superoxide dismutase, catalase, and glutathione peroxidase significantly. Therefore, it can be said that CSSE is rich in antioxidant compounds [34, 35].

The results of the present study in the investigation of the cytotoxic effect of CSSE showed that CSSE at a concentration of 1000 µg/ml, Cell viability percentage of the SH-SY5Y cancer cells at 24, 48, and 72 hours compared to normal hacked cells significantly reduced. Therefore, CSSE at a concentration of 1000 µg/ml significantly killed cancer cells, however, lower in concentrations, this effect was not seen. This means that the lethal effect of CSSE is concentration-dependent. In other words, cell survival percentage decreased significantly at 48 and 72 hours compared to 24 hours which indicates time-dependent effects. Research by M. Shaikh and others. In 2017, when investigating the cytotoxic effect of Capparis L. on the human hepatocellular carcinoma cell line PLC/PRF/5, it was shown. The growth of PLC/PRF/5 cells was significantly dependent to the concentration. Nevertheless, the addition of 620-5000 µg/ml extract caused a significant increase in cytotoxicity. The growth inhibitory effect of CSE was highest at 5000 µg/ml. The extract at 150 µg/ml has no cytotoxic effect on the cells [36]. Other studies also showed the inhibitory effect of *Capparis* on HepG2 (liver), HT-29 (colon), MCF-7 (breast), and Hela (cervix) cells, *C. spinosa* has the highest inhibitory effects on liver cancer cell lines [37, 38].

The conceivable mechanism of action of Capparis extract to inhibit SH-SY5Y cancer cells is the induction of cell apoptosis by up-regulating the activity of caspase8/9 and down-

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regulating Bcl-2, which is involved in the extrinsic and intrinsic pathways of Apoptosis or Chrom, CSE by inhibiting the expression of Cip1/p21. initiates the cell cycle [39].

5. Conclusion

Based on our results, it has been shown that CSSE prevents SH-SY5Y cancer cell proliferation. One of the possible mechanisms of this effect could be the high antioxidant amount of CSSE.

Author contributions

N. Kh conceived of the presented idea and supervised the project. L. Gh developed the theory, performed the computations, and participated in writing the manuscript. H. S assisted in performing the practical section of the project. H. R edited the manuscript with input from all authors. S. S helped supervise the project and guided in the scientific section of the project.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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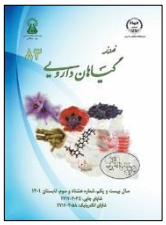

مقاله تحقیقاتی

بررسی فعالیت آنتی‌اکسیدانی عصاره، روغن‌های حاصل از پرس سرد و هگزان نرمال و اثر سیتوتوکسیک دانه کبر روی رده سلول‌های سرطانی SH-SY5Y در شرایط آزمایشگاهی

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اطلاعات مقاله

گل‌واژگان:
سمیت سلولی
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کبر
آنتی‌اکسیدان
SH-SY5Y
DPPH
MTT

چکیده

مقدمه: گیاه کبر (*Capparis spinosa*) به دلیل خواص ضد التهابی و آنتی‌اکسیدانی خود نقش اساسی در پیشگیری از سرطان و همچنین کاهش سرعت رشد سلول‌های سرطانی دارد. این گیاه را می‌توان به روش‌های مختلف در ترکیبات دارویی، غذایی و ... به عنوان عصاره، روغن و یا اسانس استفاده کرد. **هدف:** در مطالعه حاضر فعالیت آنتی‌اکسیدانی عصاره و روغن‌های (روغن‌های پرس سرد و اِن-هگزان) دانه کبر و همچنین تأثیر دانه بر روی رده سلولی سرطانی SH-SY5Y بررسی شده است. **روش بررسی:** ابتدا عصاره و روغن‌های پرس سرد و اِن‌هگزان تهیه شد. سپس آنها به غلظت‌های ۵۰، ۱۰۰ و ۲۰۰ میلی‌گرم بر میلی‌لیتر رسیدند و ظرفیت آنتی‌اکسیدانی آنها با روش ۲، ۲-دی فنیل-۱-پیکریل هیدرازیل (DPPH) ارزیابی شد. سپس تعداد ترکیبات آنتی‌اکسیدانی موجود در عصاره اندازه‌گیری شد و در نهایت سمیت عصاره‌ها به روش MTT بر روی سلول‌های سرطانی SH-SY5Y ارزیابی شد. **نتایج:** داده‌های IC_{50} نشان داد که فعالیت آنتی‌اکسیدانی عصاره دانه کبر به ترتیب نسبت به روغن‌های پرس سرد و هگزان به طور معنی‌داری ($P < ۰/۰۰۱$) افزایش یافته است. تجزیه و تحلیل ترکیبات آنتی‌اکسیدانی عصاره دانه کبر نشان داد که عصاره حاوی میزان بالایی از فنول کل، فلاونوئید، روتین و کوئرستین است. سنجش MTT نیز نشان می‌دهد که CSSE در غلظت ۱۰۰۰ میکروگرم بر میلی‌لیتر به طور قابل‌توجهی ($P < ۰/۰۰۱$) زنده‌مانی رده‌های سلولی سرطانی SH-SY5Y را در مقایسه با سلول‌های هک کاهش داد. **نتیجه‌گیری:** عصاره دانه کبر احتمالا به دلیل محتوای آنتی‌اکسیدانی بالا از رشد سلول‌های سرطانی SH-SY5Y جلوگیری می‌کند.

مخففها: CSS، دانه کاپاریس اسپینوزا؛ DPPH، ۲و۲ دی فنیل-۱-پیکریل هیدرازیل؛ DMEM، محیط کشت پایه؛ FBS، سرم جنین گاوی؛ TPC، محتوی فنولی تام

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