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Antioxidant Potentials, Protease Inhibitory, and Cytotoxic Activities of Various Isolated Extracts from *Salvia aegyptiaca*

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ABSTRACT

Background: Natural compounds can regulate the growth and progression of cancer cells with low toxicity to normal cell; therefore, these compounds are unique targets for cancer treatment. Recently, extracts from *Salvia* species have shown promising antiproliferative potential. This study aimed to isolate and characterize bioactive compounds from *S. aegyptiaca* and evaluate their antioxidant, cytotoxic, and protease-inhibitory activities.
Methods: In this study, various extracts of *S. aegyptiaca* were prepared, and several compounds, including ursolic acid, oleanolic acid, luteolin-7-O-glucoside, quercetin-3-O-rutoside, and rosmarinic acid, were isolated and characterized using different spectroscopic methods. Finally, the antioxidant activity, protease inhibitory activity, and cytotoxicity of the crude extract, multiple fractions, and isolated compounds were examined.
Results: According to the results obtained, rosmarinic acid demonstrated the highest antioxidant performance, as indicated by the following assays: DPPH (IC_{50}: 28.39 ± 0.75 µg/mL), ABTS (39.52 ± 0.72 µg/mL), FRAP (31.87 ± 0.67 µg/mL), NO scavenging (71.44 ± 1.04 µg/mL), and ORAC values (0.6 TE/mg). Furthermore, both cynaroside and rosmarinic acid exhibited the most potent antiproliferative effects against the Hep G2 cell line, with IC_{50} value of 34.4 ± 2.34 and 47.84 ± 5.87 µg/mL, respectively. The EtOAc fraction and rosmarinic acid also showed higher protease inhibitory activity, with IC_{50} of 17.6 ± 0.10 and 17.0 ± 0.30 µg/mL, respectively, as compared to other compounds.
Conclusion: Our findings suggest that the identified compounds may be responsible for the antiproliferative effects of *S. aegyptiaca*. Overall, *S. aegyptiaca* could serve as a valuable natural antioxidant and anticancer agent in both pharmaceutical and food industries. ***DOI: 10.61882/ibj.4567***

Keywords: Antioxidants, Salvia, Protease inhibitors, Hep G2 cell line

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List of Abbreviations:
AAPH: 2,2'-Azodiisobutyramidine dihydrochloride; **ABTS:** 2,2-azino-bis-3-ethylbenzothiazoline 6-sulfonate; **CAA:** cellular antioxidant activity; **$CHCl_3$:** chloroform; **DPPH:** 1,1-diphenyl-1-picrylhydrazyl; **EtOAc:** ethyl acetate; **FRAP:** ferric reducing antioxidant power; **LLE:** liquid-liquid extraction; **MeOH:** methanol; **n-BuOH:** n-butanol; **NMR:** nuclear magnetic resonance; **NO:** nitric oxide; **ORAC:** oxygen radical absorbance capacity; ***S.* aegyptiaca:** *Salvia aegyptiaca*; **SFr:** subfraction; **TCL:** thin layer chromatography; **TE:** Trolox equivalent; **TPTZ:** -4-6-tripyridyl-s-triazine

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INTRODUCTION

Cancer, a disease with a high mortality rate, poses a significant threat to human health. The urgency for more effective treatments is underscored by its severity. Medicinal plants, known for their potent anticancer and antiproliferative properties, represent a promising avenue for the discovery of cancer therapies. Most anticancer drugs, currently used in the clinical settings are natural products of plant origin[1]. Between 1981 and 2014, 52% of all approved molecules for cancer treatment were either natural products or their derivatives, including paclitaxel, docetaxel, vincristine, cabazitaxel, and romidepsin, highlighting the importance of plant sources in the research for effective cancer therapies[2].

A significant factor influencing cancer incidence is the presence of free radicals, which are generated through normal metabolic processes and external factors such as air pollution and ultraviolet radiation. Free radicals are reactive molecules that can efficiently interact with proteins, lipids, and nucleic acids. This interaction can result in cellar damage and increase in the risk of various diseases, including cancer, diabetes, heart and neurodegenerative diseases[3]. Antioxidants, particularly those derived from plant sources, offer a promising strategy for protecting cells against free radical damage. These natural antioxidants, known for their lower toxicity and potential health benefits, have garnered significant attention in recent researches[4,5].

Salvia, one of the largest and most valuable genera within the Lamiaceae family, is a treasure trove of biological properties. With over 1,000 species found worldwide, *Salvia* plants have been recognized for their diverse biological activities, including anticancer, antioxidant, antidiabetic, antimicrobial, anti-inflammatory, and neuroprotective properties[6-9]. Phytochemical analyses have revealed that *Salvia* species are rich in various chemical compounds, including phenolic acids, phenolic glycosides, flavonoids, anthocyanins, polysaccharides, terpenoids, and coumarins. This diversity of compounds contributes to the antioxidant and anticancer properties of Salvia plants, making them a fascinating subject for further study[7]. The antioxidant properties of *Salvia* extracts are primarily attributed to the presence of phenolic compounds[3]. Furthermore, *Salvia* species exhibit antibacterial activity and inhibitory effects on acetylcholinesterase, butyrylcholinesterase, α-glucosidase, β-glucosidase, β-glucuronidase, and tyrosinase[4].

Salvia aegyptiaca is a green dwarf plant belonging to the Tubiflorales class, Verbenales (Lamiaceae) phylum, Labiatae family, and Salvia genus. This species grows in various regions of the Arabian Peninsula, Egypt, Palestine, Iran, and Afghanistan. In Iran, it is primarily found in southern parts of the country, especially in Hormozgan Province. *S. aegyptiaca* has a rich history of application in traditional medicine, where it is valued for its sedative and antiseptic properties and its effectiveness in treating diarrhea, nervous disorders, vertigo, and tremors[5,7,8].

In this study, we employed the LLE method, along with various mixtures of organic solvents to fractionate the air-dried aerial parts of *S. aegyptiaca*. We subsequently used ^{1}H-NMR and ^{13}C-NMR spectroscopy to identify the chemical structures of five isolated compounds (**1-5**). The antioxidant potential of these compounds was assessed using DPPH, ABTS, FRAP, ORAC, and NO assays. Furthermore, we investigated the protease inhibitory activities and cytotoxic effects of the extracted compounds against the Hep G2 cell line.

MATERIALS AND METHODS

Chemicals

DPPH, quercetin, ABTS, and TPTZ were acquired from Sigma-Aldrich, Germany. The solvents used in the present study, including MeOH, $CHCl_3$, EtOAc, n-BuOH, and petroleum ether, were commercially purchased from Merck Chemical Co. (Germany). All chemicals and reagents were applied without any further purification. TLC sheets (20 × 20 cm; pre-coated silica gel 60 F_{254}; Merck) were used for analytical TLC, and spot detection was performed using an anisaldehyde/sulfuric acid reagent, followed by heating. Column chromatography was conducted with silica gel (70-230 and 230-400 mesh; Merck). For all assays, we used standard 96-well microplates.

Plant material

S. aegyptiaca aerial parts were randomly collected from Geno Mountain (Hormozgan, Iran) in March 2017. The voucher sample was deposited in the Medicinal Processing Research Center, Shiraz, Iran, herbarium under code MPPRC-93-2.

Extraction and isolation procedures of *S. aegyptiaca*

The air-dried aerial parts of *S. aegyptiaca* (3.4 kg) were powdered and macerated (drenching) in 70% hydroethanolic solvent (24 L × 48 h × 3). Afterwards, ethanol was removed under vacuum using a rotary evaporator to achieve 0.9 kg of dark green gummy extract. Subsequently, the crude extract (400 g) was dissolved in 1,000 mL of distilled water. The crude extract was successively fractionated using the LLE method and various solvents, including petroleum ether,

$CHCl_3$, EtOAc, and n-BuOH, to obtain the compounds **1-5**. The mixture of compounds **1** and **2** was obtained after washing with acetone. Then, 6 g of $CHCl_3$ fraction was subjected to an open silica gel (200 g; 70-230 mesh) column chromatography (3.5 cm × 40 cm) and eluted with a gradient system of *n*-hexane-EtOAc-MeOH to obtain 85 SFrs. Having developed the TLC of SFrs and checked them under UV light at 254 and 365 nm by using a vanillin/sulfuric acid reagent, the similar SFrs were collected with the final six fractions (Fr_A-Fr_F). Fr_B (411 mg) was re-chromatographed on a flash silica column (2 cm × 20 cm) and washed with $CHCl_3$-acetone (6:4), in which 35 SFrs were obtained. SFrs 24-31 afforded compound **3** (17 mg). Fr_E (1310 mg) was submitted to another flash silica column (2 cm × 25 cm) and eluted with EtOAc-acetone (70:30), in which 55 SFrs were obtained. SFrs 18-27 and SFrs 30-36 yielded compounds **4** (13 mg) and **5** (15 mg).

NMR spectroscopy

The ^{1}H-NMR and ^{13}C-NMR spectra of isolated compounds **1-5** were recorded on a Bruker BioSpin GmbH 300 spectrometer (Germany). The ^{1}H-NMR and ^{13}C-NMR spectra of the isolated compounds 1-5 were recorded on a Bruker BioSpin GmbH 300 MHz NMR spectrometer (Germany), equipped with a 5 mm multinuclear probe. The ^{1}H-NMR spectra were obtained at a frequency of 300 MHz, while the ^{13}C-NMR spectra were recorded at a frequency of 100 MHz. The spectra were acquired with the standard acquisition parameters for each nucleus at room temperature. $CDCl_3$ was used as the solvent, and tetramethylsilane as the internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz.

DPPH radical scavenging activity assay

The DPPH assay was carried out using a previously described method[3]. Briefly, 200 µL of methanolic DPPH solution was mixed with 20 µL of tested samples at various concentrations (6.25-3200 µg⁄mL) in a 96-well microplate. Then, the microplate was placed in a dark condition at 37 °C for 30 min, and after that, the absorbance was measured at 517 nm with a microplate reader (Biotek, USA). In this assay, quercetin was used as the standard. The blank wells contained 20 µL of extracts and 200 µL of MeOH, and the control wells contained 20 µL of MeOH and 200 µL of DPPH solution. The experiments were repeated in triplicate for each sample. The percentage of DPPH scavenging activity of each extract was calculated using the following formula: % inhibition = 100 – (absorption $_{sample}$ – [absorption $_{control}$/absorption $_{blank}$]) ×100.

ABTS assay

The ABTS radical cation ($ABTS^{+\bullet}$) was generated by mixing 5 mL of ABTS diammonium salt aqueous solution with 5 mL of ammonium persulfate solution. The obtained mixture was kept in the dark at room temperature for 16 hours before use. The $ABTS^{+\bullet}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. Then, 200 µL of $ABTS^{+\bullet}$ radical cation solution was added to 20 µL of different concentrations of samples in a 96-well microplate. Finally, the absorbance was determined at 734 nm after 15 minutes.

FRAP assay

The FRAP capacity of fractions and extracted compounds **1-5** to reduce Fe^{3+} to Fe^{2+} was assessed based on the Benzie method[5]. The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM; pH 3.6), TPTZ (10 mM) in 40 mM of HCl, and 20 mM of $FeCl_3.6H_2O$ in a ratio of 10:1:1 (v/v). The FRAP reagent was placed in the dark at 37 °C for 10 minutes before use. The experiment was conducted in a 96-well plate. Briefly, 20 µL of different concentrations of each extract (6.25-3200 µg⁄mL) was added to 180 µL of freshly prepared and pre-warmed (37 °C) FRAP solution in test wells. Control wells contained 20 µL of MeOH and 180 µL of FRAP solution. The absorbance of samples was measured at 593 nm using a microplate reader (Biotek), and the following formula calculated the FRAP values: FRAP value = (absorption $_{control}$-[absorption $_{sample}$/absorption $_{control}$]) × 100.

NO radical scavenging

The Griess-Ilosvay reaction using sodium nitroprusside was utilized to determine the NO radical scavenging activity of the various fractions of crude extract and isolated compounds from *S. aegyptiaca*. In general, 50 µL of sodium nitroprusside (10 mmol) and 50 µL of MeOH were mixed and added to 50 µL of the *S. aegyptiaca* species solution. The blank containing 50 µL of extract, 50 µL of sodium nitroprusside and 50 µL of MeOH was considered as control wells. The microplate was then incubated at 25 °C for 150 min. Subsequently, 100 µL of Griess reagent was added to each well, except for blanks, and incubated at room temperature for 5 min. Finally, the absorption was read at 542 nm. The following formula was used to achieve the relative inhibition ratio for each compound[5,6]: inhibition ratio = (absorption$_{sample}$-[Absorption $_{blank}$/absorption $_{control}$]) × 100.

ORAC assay

The oxygen radical scavenging efficacy of the crude extract, various fractions, and isolated compounds from

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S. aegyptiaca. were measured using the ORAC assay. Briefly, a mixture of 20 µL of blank, Trolox standard, or samples in potassium phosphate buffer (75 mM) was prepared and added to a 96-well microplate in triplicates. A 200 µL of 0.96 µM fluorescein in working buffer was added to each well and incubated at 37 °C for 20 min. Then, the fluorescein solution of AAPH was added, and the fluorescence decay at 538 nm was measured with excitation at 485 nm every 4.5 min for 2.5 h using the polar star omega device. The ORAC values were defined as TE[4,5].

CAA assay

The Hep G2 cells were seeded at a density of 6×10^4 cells/well on a 96-well microplate overnight. Then, the growth medium was discarded, and each well was washed with PBS in triplicate. The wells were treated with 100 µL of treatment medium containing different concentrations of *S. aegyptiaca* extracts plus 25 µM of DCFH-DA and incubated at 37 °C. After incubation, the liquid was removed, and the wells were washed with 100 µL of PBS. Finally, an AAPH (free radical initiator) solution was applied to the cells, and the microplate was placed in a polar star omega device at 37 °C. The emission at 538 nm was read after excitation at 485 nm every 5 min for one hour. The IC_{50} values for all samples and quercetin as a standard drug were determined at concentrations for scavenging 50% of AAPH radicals[5].

Cell lines and MTT cytotoxic assay

The growth inhibitory effect of the crude extract, various fractions, and isolated compounds from *S. aegyptiaca* on the hepatocellular carcinoma (Hep G2; the Pasteur Institute of Iran) cell line was evaluated using the MTT calorimetry method. Briefly, Hep G2 cells with a density of 10,000 cells/well were seeded in a 96-well microplate in a 5% CO_2 incubator at 37 °C. After incubation for 24 h, cells were treated with crude extract and obtained fractions of *S. aegyptiaca* at 12.5-200 µg/mL concentrations. Subsequently, after 24 h, the medium was aspirated, and then 150 µL of MTT solution was added to each well, followed by incubation in a 5% CO_2 incubator at 37 °C for 4 hours. The produced formazan crystals were solubilized in DMSO. The optical density of each well was read using an ELISA reader at a wavelength of 570 nm[7]. The IC_{50} values were obtained by plotting the percentage of 50% growth inhibition versus concentration. The data are presented as the mean ± SD for each analysis.

Determination of protease inhibition

Different concentrations of *S. aegyptiaca* (1-300 µg/mL) were prepared. In brief, 200 µL of phosphate buffer (50 mM) was combined with 50 µL of each concentration of the samples to evaluate the inhibitory activity against the protease enzyme. Subsequently, 200 µL of protease and 400 µL of casein (1%) as a substrate were added to the mixture. It was then allowed to incubate at 25 ºC for 10 minutes. 800 µL of trichloroacetic acid (10%) was added to the mixture as the stopper of the reaction. After a 21-minute interval, the mixture was centrifuged (Peco, Iran) at 895 ×g for 5 minutes. A UV-Vis spectrophotometer (PG instrument T90, England) was used to read the absorbance of the supernatant at 280 nm[8].

Statistical analysis

The obtained values were statistically analyzed using SPSS and Excel 2013 software. The IC_{50} values were expressed as mean ± SD, the mean of three replicates.

RESULTS

Spectroscopic data

The chemical structures of five extracted compounds from *S. aegyptiaca*, including ursolic acid (**1**), oleanolic acid (**2**), luteolin-7-O-glucoside (**3**), quercetin-3-O-rutoside (**4**), and rosmarinic acid (**5**) (Fig. 1), were confirmed using NMR spectroscopy. The ' ^{1}H-NMR and ^{13}C-NMR spectra showed signals corresponding to their structures.

Compound **1** (*Ursolic acid*): ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 12.07 (1H, brs, 28-COOH), 5.15 (1H, m, H-12), 4.33 (1H, d, J = 3.9 Hz, OH-3), 2.99 (1H, brs, H-3), 2.10 (1H, d, J = 10.5 Hz, H-18), 1.03 (3H, s, Me-27), 0.91 (3H, m, Me-30), 0.89 (3H, s, Me-23), 0.86 (3H, s, Me-25), 0.81 (3H, d, J = 6.5 Hz, Me-29), 0.74 (3H, s, Me-26), 0.67 (3H, s, Me-24). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_C 178.66 (C-28), 138.23 (C-13), 124.62 (C-12), 76.86 (C-3), 54.82 (C-5), 52.40 (C-18), 47.09 (C-9), 46.86 (C-17), 41.75 (C-14), 38.55 (C-4), 38.43 (C-20 & C-8), 38.26 (C-10), 36.58 (C-1), 32.89 (C-21), 32.74 (C-7), 30.46 (C-19), 30.22 (C-22), 28.31 (C-23), 27.53 (C-15), 27.02 (C-2), 23.85 (C-16), 23.41 (C-27), 22.95 (C-11), 21.14 (C-30), 18.06 (C-6), 17.08 (C-29), 16.95 (C-26), 16.15 (C-24), 15.28 (C-25)[3, 4].

Compound **2** (*Oleanolic acid*): ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 12.06 (1H, brs, 28-COOH), 5.15 (1H, m, H-12), 2.99 (1H, brs, H-3), 2.73 (1H, d, J = 13.7, 4.6 Hz, H-18), 1.90 (1H, m, H16a), 1.82 (2H, m, H-11a & H-11b), 1.09 (3H, s, Me-27), 0.91 (3H, m, Me-30), 0.89 (3H, s, Me-23), 0.86 (3H, s, Me-25), 0.85 (3H, s, Me-29), 0.74 (3H, s, Me-26), 0.67 (3H, s, Me-24). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_C 178.36 (C-28), 143.87 (C-13), 121.56 (C-12), 76.86 (C-3), 54.52 (C-5), 40.83 (C-18),

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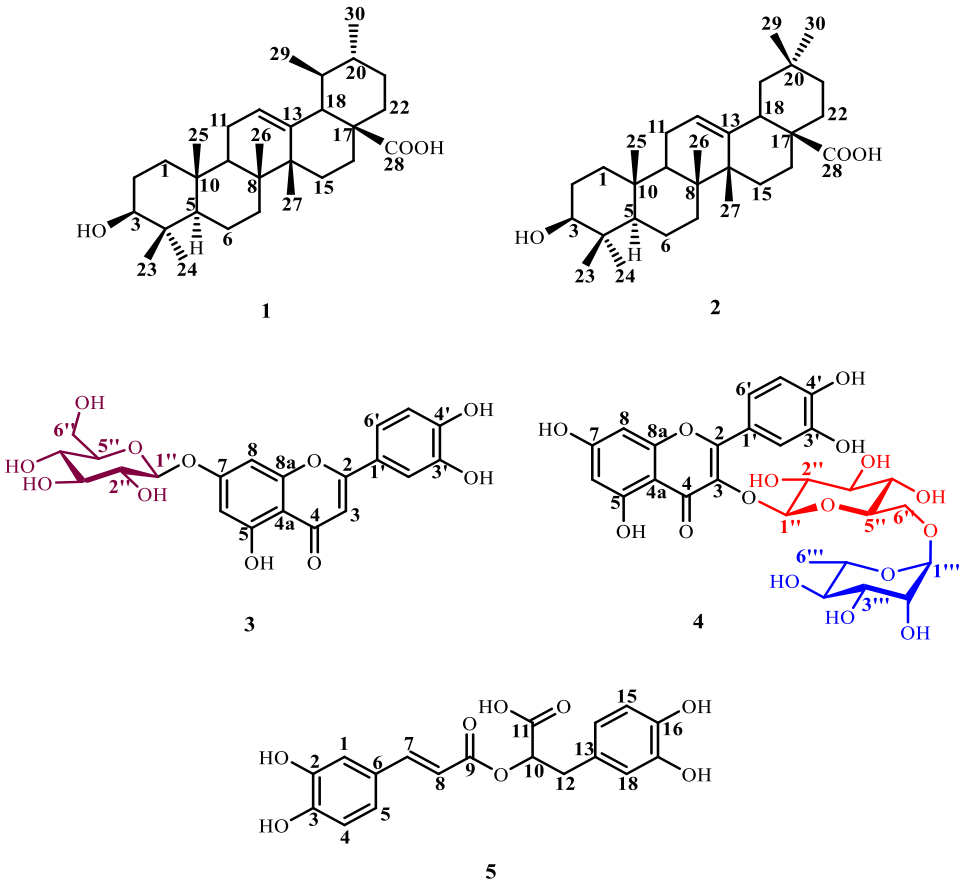

Fig. 1. Chemical structures of the isolated compounds from *S. aegyptiaca* extracts. Ursolic acid (**1**), oleanolic acid (**2**), luteolin-7-O-glucoside (**3**), quercetin-3-O-rutoside (**4**), and rosmarinic acid (**5**).

46.86 (C-9), 45.71 (C-17), 45.49 (C-19), 41.35 (C-14), 38.26 (C-8), 38.09 (C-4), 36.58 (C-1), 33.35 (C-10), 32.89 (C-21), 32.45 (C-29), 32.13 (C-7), 30.46 (C-22), 30.22 (C-20), 28.28 (C-23), 27.24 (C-15), 27.02 (C-2), 25.65 (C-27), 23.41 (C-30), 22.95 (C-16), 22.64 (C-11), 18.05 (C-6), 16.88 (C-26), 16.09 (C-24), 15.16 (C-25)[4,5].

Compound **3** (*Luteolin-7-O-glucoside*): ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 12.99, (1H, s, 5-OH), 7.45 (1H, dd, J = 8.3, 2.2 Hz, H-6'), 7.42 (1H, d, J = 2.0 Hz, H-2'), 6.90 (1H, d, J = 8.3 Hz, H-5'), 6.79 (1H, d, J = 2.1 Hz, H-8), 6.76 (1H, s, H-3), 6.45 (1H, d, J = 2.0 Hz, H-6), 5.08 (1H, d, J = 7.1 Hz, H-1''), 3.71 (1H, brd, J = 10.1 Hz, H-6''a), 3.15-3.49 (5H, m, H2'', H-3'', H-4'', H-5'', H-6''b). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_C 181.98 (C-4), 164.53 (C-2), 163.00 (C-7), 161.20 (C-5), 157.01 (C-8a), 150.01 (C-4'), 145.85 (C-3'), 121.42 (C-1'), 119.25 (C-6'), 116.04 (C-5'), 113.60 (C-2'), 105.39 (C-4a), 103.22 (C-3), 99.90 (C-1''), 99.58 (C-6), 94.77 (C-8), 77.21 (C-5''), 76.43 (C-3''), 73.16 (C-2''), 69.57 (C-4''), 60.65 (C-6'')[6,7].

Compound **4** *(Quercetin-3-O-rutoside)*: ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 12.60, (1H, s, 5-OH), 7.54 (1H, dd, J = 7.4, 1.9 Hz, H-6'), 7.52 (1H, d, J = 1.9 Hz, H-2'), 6.84 (1H, d, J = 8.7 Hz, H-5'), 6.38 (1H, d, J = 1.9 Hz, H-8), 6.19 (1H, d, J = 1.9 Hz, H-6), 5.34 (1H, d, J = 7.1 Hz, H-1''), 4.37 (1H, brs, H-1'''), 3.70 (1H, brd, J = 9.8 Hz, H-6''a), 3.00-3.72 (9H, m, sugar H-2'', H-3'', H-4'', H-5'', H-6''b, H-2''', H-3''', H-4''', H-5'''), 0.98 (1H, d, J = 6.1 Hz, H-6'''). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_C 177.42 (C-4), 164.19 (C-7), 161.27 (C-5), 156.67 (C-2), 156.48 (C-8a), 148.48 (C-4'), 144.82 (C-3'), 133.34 (C-3), 121.65 (C-6'), 121.22 (C-1'), 116.31 (C-2'), 115.28 (C-5'), 104.00 (C-8), 101.22 (C-1''), 100.82 (C-1'''), 98.75 (C-4a), 93.66 (C-6), 76.48 (C-3''), 75.95 (C-5''), 74.12 (C-2''), 71.88 (C-4'''), 70.60 (C-3'''), 70.43 (C-2'''), 70.04 (C-4''), 68.32 (C-5'''), 67.06 (C-6''), 17.81 (C-6''')[8].

Compound **5** *(Rosmarinic acid):* ^{1}H-NMR (300 MHz, DMSO-d_6): δ_H 7.38 (1H, d, J = 15.8 Hz, H-7), 7.05 (1H, d, J = 2.0 Hz, H-1), 6.94 (1H, dd, J = 8.2, 2.0 Hz, H-5), 6.75 (1H, d, J = 8.1 Hz, H-4), 6.67 (1H, d, J = 2.0 Hz,

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H-18), 6.61 (1H, d, J = 8.0 Hz, H-15), 6.50 (1H, dd, J = 8.0, 2.0 Hz, H-14), 6.20 (1H, d, J = 15.9 Hz, H-8), 4.89 (1H, d, J = 9.9, 3.2 Hz, H-10), 3.02 (1H, dd, J = 14.4, 3.1 Hz, H-12a), 2.77 (1H, dd, J = 14.3, 10.0 Hz, H-12b). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_C 172.82 (C-11), 166.31 (C-9), 148.64 (C-3), 145.91 (C-2), 145.00 (C-17), 144.68 (C-7), 143.71 (C-16), 129.55 (C-13), 125.57 (C-6), 121.09 (C-5), 119.82 (C-14), 116.70 (C-4), 116.03 (C-8), 115.43 (C-18), 115.06 (C-15), 114.62 (C-1), 75.54 (C-10), 37.11 (C-12)[9].

Results of antioxidant activity

According to the studies, oxidative stress arising from disruptions in the production of reactive oxygen species during metabolic processes, involves in many pathologies, including cancer, cardiovascular diseases, neurodegenerative diseases, and skin disorders[10,11]. The antioxidant properties of plant products with less toxicity are an essential strategy for protecting organisms against the harmful effects of free radicals[12,13]. The antioxidant capacity of plants is attributed to their phenolic compounds, which can easily donate electrons and hydrogen atoms due to their highly conjugated systems and aromatic structures[14-16]. Thus, the five extracted compounds **1-5** were evaluated for their antioxidant capacity using the DPPH, ABTS, FRAP, ORAC, and NO assays. The obtained antioxidant results are expressed as IC_{50} values (µg/mL) and represented in Table 1. As demonstrated in this table, the significant antioxidant activity of *S. aegyptiaca* extracts is impressive and piques further scientific interest.

Results of DPPH scavenging activity

The DPPH assay is one of the most popular and well-established spectrophotometric methods commonly used to measure the free radical scavenging activity of plant extracts and isolated pure compounds. In this assay, a stable free radical, DPPH, is reduced by antioxidant agents. The antioxidant activity was measured based on the color change from purple to yellow and the corresponding absorption changes occurring at a wavelength of 517 nm[1,17,18]. The DPPH radical scavenging activities of the fractions and extracted compounds **1-5**, as well as quercetin as a standard compound, were measured, and the obtained results are shown in Table 2. According to the results, cynaroside, rutin, and rosmarinic acid exhibited the highest radical scavenging activities with IC_{50} values of 36.37 ± 1.07, 33.48 ± 0.52, and 28.39 ± 0.75 µg/mL, respectively, which were comparable to quercetin with an IC_{50} value of 26.51 ± 0.06 µg/mL. Meanwhile, ursolic and oleanolic acids showed no significant scavenging activities (IC_{50} > 2,000 µg/mL). The crude extract with an IC_{50} value of 72.68 ± 0.77 µg/mL exhibited higher scavenging activity than the fractions. In general, the scavenging activities of the fractions were moderate. It was observed that the EtOAc and n-BuOH fractions with IC_{50} values of 121.33 ± 1.77 and 136.12 ± 1.45 µg/mL, respectively, exhibited higher DPPH scavenging activities compared to that of the $CHCl_3$ fraction with IC_{50} value of 252.14 ± 1.62 µg/mL. The petroleum ether fraction showed weak scavenging activity (IC_{50} = 1036.38 ± 3.48). The order of DPPH scavenging activity of fractions was as follows: crude extract > EtOAc > n-BuOH > $CHCl_3$ > petroleum ether (Table 1).

$ABTS^{+\bullet}$ scavenging activity findings

The antioxidant capacity of the isolated compounds **1-5** and fractions was assessed through ABTS assay. In this assay, rosmarinic acid showed the highest activity with an IC_{50} value of 39.52 ± 0.72 µg/mL, followed by rutin (IC_{50} = 87.31 ± 0.68 µg/mL) and cynaroside

Table 1. Antioxidant activities of *S. aegyptiaca* extracts and fractions

Extracts and compounds	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP IC_{50} (µg/mL)	NO scavenging ability percentage (200 µg/mL)	ORAC TE
Crude extract	72.68 ± 0.77	78.71 ± 0.56	84.10 ± 0.84	41.8 ± 0.018	0.41
Petroleum ether Fr	1036.38 ± 3.48	585.77 ± 3.59	339.15 ± 4.87	52.01 ± 0.029	0.17
$CHCl_3$ Fr	252.14 ± 1.62	236.44 ± 2.48	245.52 ± 3.23	36.89 ±.52	0.25
EtOAc Fr	121.33 ± 1.77	158.51 ± 1.41	185.63±1.69	59.37 ± 0.81	0.77
n-BuOH Fr	136.12 ± 1.45	143.13 ± 1.64	175.38 ± 1.64	48.77 ± 0.67	0.51
Ursolic acid	>2000	>2000	298.51 ± 1.76	55.52 ± 0.43	0.31
Oleanolic acid	>2000	>2000	198.86 ± 1.49	48.82 ± 0.91	0.46
Cynaroside	36.37 ± 1.07	112.92 ± 1.10	46.88 ± 0.83	67.7 ± 0.02	0.95
Rutin	33.48 ± 0.52	87.31 ± 0.68	42.62 ± 0.86	81.30 ± 0.37	0.83
Rosmarinic acid	28.39 ± 0.75	39.52 ± 0.72	31.87 ± 0.67	71.44 ± 1.04	0.60
Quercetin[s]	26.51 ± 0.06	26.35 ± 0.03	18.53 ± 0.12	89.96 ± 0.01	1.06

Each value is the mean ± SD of triplicate analysis.

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Table 2. In vitro cytotoxic and protease inhibitory activity (IC_{50}, µg/mL) of extracts and compounds isolated from *S. aegyptiaca* extracts

Extracts and compound	Hep G2 cells IC_{50} (µg/mL)	CAA IC_{50} (µg/mL)	Protease inhibition IC_{50} (µg/mL)
Crude extract	>200	70.45 ± 1.10	23.3 ± 0.5
Petroleum ether Fr	>200	>200	28.1 ± 0.2
$CHCl_3$Fr	123.45 ± 8.85	123.69 ± 0.24	228.0 ± 0.4
EtOAc Fr	41.47 ± 2.12	126.15 ± 2.38	17.6 ± 0.1
n-BuOH Fr	89.38 ± 7.34	>200	26.2 ± 0.3
Ursolic acid	>200	183.81 ± 9.25	24.5 ± 0.1
Oleanolic acid	123.43 ± 8.84	>200	28.0 ± 0.2
Cynaroside	34.4 ± 2.34	174.41 ± 2.5	23.8 ± 0.1
Rutin	63.91 ± 0.96	>200	22.4 ± 0.1
Rosmarinic acid	47.84 ± 5.87	171.33	17.0 ± 0.3
Quercetin[s]	ND	38.94 ± 0.87	ND
Cisplatin	12.65 ± 0.78	ND	ND
Iodoacetamide[b]	ND	ND	10.6 ± 0.5

ND: not detected; [s]control for CAA test, a positive control for cytotoxicity against Hep G2 cell lines; [b]positive control for anti-protease activity.

IC_{50} = 112.92 ± 1.10 µg/mL). On the contrary, similar to the DPPH assay, ursolic and oleanolic acids exhibited no significant ABTS activity (IC_{50} > 2000 µg/mL). Furthermore, the crude extract (IC_{50} = 78.71 ± 0.56 µg/mL) showed higher $ABTS^{+\bullet}$ scavenging activity than the other fractions. Among the fractions, the n-BuOH fraction had the highest $ABTS^{+\bullet}$ scavenging activity (IC_{50} = 143.13 ± 1.64 µg/mL), while the lowest values were observed for petroleum ether and $CHCl_3$ fractions (Table 1).

FRAP scavenging activity findings

Cynaroside, rutin, and rosmarinic acid showed good antioxidant activity, as observed in the DPPH radical scavenging assay. Rosmarinic acid exhibited the best FRAP activity with an IC_{50} value of 31.87 ± 0.67 µg/mL, which is relatively comparable to quercetin. Rutin indicated an IC_{50} value of 42.62 ± 0.86 µg/mL, followed by cynaroside with IC_{50} = 46.88 ± 0.83 µg/mL. On the other hand, among the extracts, ursolic acid (IC_{50} = 298.51 ± 1.76 µg/mL) and oleanolic acid (IC_{50} = 198.86 ± 1.49 µg/mL) exhibited moderate FRAP activity. Furthermore, the crude extract displayed significant antioxidant potential with an IC_{50} value of 84.10 ± 0.84 µg/mL, while the others showed the IC_{50} values ranging from 175.38 to 339.15 µg/mL. Differences in FRAP activities could be related to various phenolic content of the extracted compounds (Table 1).

Radical scavenging activity outcomes

The scavenging efficiency of crude extract, fractions, and isolated compounds (**1-5**) from the aerial parts of *S. aegyptiaca* was evaluated and compared with quercetin as a standard agent. The results clearly showed that the activity of rutin was comparable to that of quercetin at a concentration of 200 µg/mL. Rosmarinic acid and cynaroside could scavenge NO radicals with 71.44 ± 0.37 % and 67.7 ± 0.02% at 200 µg/mL concentration, respectively. However, ursolic and oleanolic acids inhibited NO inhibition with 55.52% and 48.82%, respectively. The order of NO-scavenging capacity of the fractions is ethyl acetate > petroleum ether > n-butanol > chloroform; therefore, the EtOAc fraction had the highest NO scavenging percent with 59.37 ± 0.37 % among the fractions (Table 1). These NO scavenging outputs support the antioxidant and anti-inflammatory activities of extracts of *S. aegyptiaca.* The phytochemical analysis of *S. aegyptiaca* showed the presence of rutin, cynaroside, and rosmarinic acid in the EtOAc fraction, which are associated with NO scavenging action in biological systems[19].

ORAC test results

This assay is widely used to assess the antioxidant capacity of a compound. It measures the ability of a sample to neutralize oxygen free radicals, which are reactive molecules associated with oxidative stress and various diseases. In the ORAC test, a fluorescent probe sensitive to oxidation is used in the presence of free radicals generated by thermal reaction. As antioxidants in the sample react with the radicals, the fluorescence decay is monitored, providing a quantitative measure of the antioxidant capacity. The higher the ORAC value, the greater the antioxidant potential of the tested compound, indicating its ability to protect against oxidative damage. Cynaroside (0.95 TE) and rutin (0.83 TE) showed significant ORAC effects, followed by rosmarinic acid (0.60 TE). Furthermore, the ORAC

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activity of the EtOAc fraction (0.77 TE) was higher than that of the crude extract and other fractions (Table 1).

CAA Results

The CCA assay, a key component of our study, measures the antioxidant activity in the cell culture. It is based on the interaction between the tested extract and complex enzymatic reactions in a biological system, representing a more relevant physiological/biological importance[20]. The outcomes of CCA values for the *S. aegyptiaca* fractions and isolated compounds are illustrated in Table 2. Interestingly, the most potent CCAs were observed for the crude extract. The order of the CAA activities is as follows: chloroform > ethyl acetate > petroleum ether and n-BuOH fractions. The IC_{50} values for all tested compounds were not remarkable. The IC_{50} values of all sub-fractions were higher than 170 µg/mL.

Evaluation of cytotoxic activity

The cytotoxic activity of five isolated compounds (**1-5**) from *S. Aegyptiaca* and different fractions is depicted in Figure 2. Remarkably, the crude extract showed no cytotoxic effect on the Hep G2 cell line, nor did the petroleum ether fraction. Among the various fractions, distinct differences in cytotoxic potential were observed. The EtOAc fraction demonstrated the highest cytotoxic activity with IC_{50} = 41.47 ± 2.12 µg/mL. At the same time, moderate growth inhibition was obtained for the n-BuOH and $CHCl_3$ fractions. The different cytotoxicity suggests the presence of various compounds in their fractions. On the other hand, a comparative study of the isolated compounds revealed that cynaroside was the most cytotoxic compound with IC_{50} value of 34.4 ± 2.34 µg/mL, and also, rosmarinic acid and rutin exhibited moderate activity with IC_{50} of 47.84 ± 5.87 and 63.91 ± 0.96 µg/mL, respectively. Oleanolic acid showed a low antiproliferative effect on the Hep G2 cells, and ursolic acid demonstrated negligible cytotoxicity on these cells, with an IC_{50} value greater than 200 µg/mL.

Protease inhibitory activity

The protease inhibitory activity assay is essential for evaluating the potential therapeutic applications of the compounds. Protease inhibitors work by binding to the catalytic site of the protease, thereby preventing the cell from replicating. Table 2 demonstrates no significant differences in the protease inhibition values across all fractions and isolated compounds of *S. aegyptiaca* and its fractions, except for the $CHCl_3$ fraction. Notably, the EtOAc fraction (IC_{50} value of 17.6 ± 0.1 µg/mL) exhibited higher activity than the other fractions, while the CHCl3 fraction showed minimal protease inhibition. Interestingly, rosmarinic acid, rutin, and cynaroside—isolated from the active ethyl acetate sub-fractions—had IC_{50} values of 17.0 ± 0.3, 22.4 ± 0.1, and 23.8 ± 0.1 µg/mL, respectively. Furthermore, ursolic acid and oleanolic acid demonstrated promising protease inhibition, with IC_{50} values of 24.5 ± 0.1 and 28.0 ± 0.2 µg/mL, respectively.

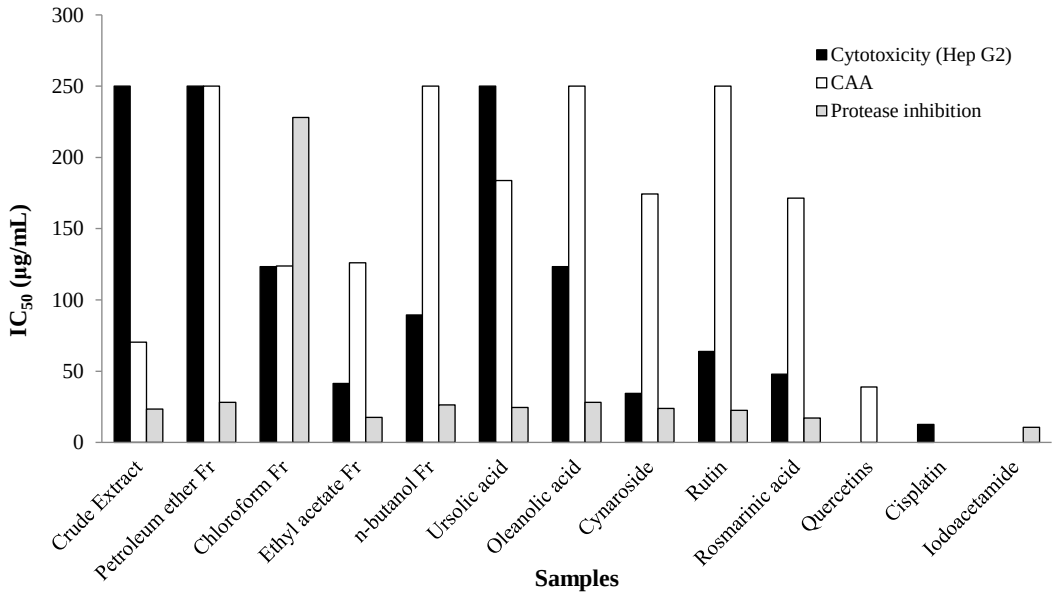

Fig. 2. In vitro cytotoxicity, cellular antioxidant (CCA), and protease inhibitory activity of fractions and isolated compounds from *S. aegyptiaca* (IC_{50}, µg/mL). Each data represent mean ± SD in triplicates.

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DISCUSSION

Despite significant advancements in the treatment and control of cancer, this disease remains the second leading cause of mortality and morbidity worldwide. The lack of selectivity and the undesirable side effects of available drugs have prompted ongoing research in this field. Today, treatments derived from natural products have been shown to reduce various side effects. Although only a limited number of plant-derived medicines is currently used in cancer therapy, numerous studies demonstrate the promising potential of natural compounds in vitro, which needs further examination in human trials.

Natural products and their extracts have recently gained attention as anti-proliferative agents due to their accessibility, applicability, and reduced cytotoxicity. The natural products exert their effects by modulating the cancer microenvironment and influencing various signaling pathways, including cell death pathways (apoptosis and autophagy) and embryonic developmental pathways (Notch, Wnt, and Hedgehog pathways)[21]. Different species of *Salvia* and their phytochemicals have shown potential applications in preventing and treating cancer with minimal side effects on normal cells. Due to its phenolic compounds, *S. aegyptiaca* is a valuable natural antioxidant with health-promoting properties. Several studies have reported that the DPPH radical scavenging activity and antioxidant performance of S. aegyptiaca are noteworthy[22]. In this regard, we focused on identifying the primary components of *S. aegyptiaca* and evaluating their antioxidant, protease inhibitory, and cytotoxic properties. Initially, the biologically active substances were isolated from *S. aegyptiaca,* and sub-fractions were characterized using different spectroscopic methods, including ^{1}H-NMR and ^{13}C-NMR. Ursolic acid and oleanolic acid were identified in the $CHCl_3$sub-fraction. Phytochemical investigations revealed that the EtOAc sub-fraction contains flavonoids and phenolic acid compounds, including cynaroside, rutin, and rosmarinic acid. Many *Salvia* species are known to be rich in phenolic compounds, particularly rosmarinic acid, which confirmed our findings[22]. Antioxidant activity of the crude extract, various fractions, and isolated compounds was evaluated using six complementary tests: DPPH, ABTS, FRAP, NO radical scavenging, ORAC, and CCA.

According to the results of DPPH, ABTS, FRAP assays, cynaroside, rutin, and rosmarinic acid exhibited the highest radical scavenging activities. The DPPH scavenging activities of antioxidants are closely related to their chemical structures. Studies have shown that the presence of phenolic hydroxyl groups, with ability to donate their hydrogen or electrons to the DPPH radical, enhances the efficiency of antioxidants. Therefore, the superior DPPH scavenging activities of cynaroside, rutin, and rosmarinic acid were attributed to more phenolic hydroxyl groups in their structures. Our findings confirmed a substantial correlation between phenolic content and reducing power, which is associated with the reduction potential of plants through donating electrons or hydrogen atoms. This process leads to the termination of chain reactions by converting free radicals or reactive oxygen species into stable forms[23]. The high antioxidant activity of crude extract, ethyl acetate, and n-BuOH fractions may be attributed to many polyphenolic compounds or a high concentration of bioactive compounds in these samples. The NO scavenging data revealed that the EtOAc sub-fraction and its constitutes, especially cynaroside, rutin, and rosmarinic acid, exhibited significant antioxidant activity. The ORAC values (TE) for the EtOAc fraction and its components were 0.77, 0.95, 0.83, and 0.6 compared to quercetin, demonstrating the ability of *S. aegyptiaca* to scavenge alkoxyl and alkyl peroxyl radicals. Additionally, the CAA test indicated the capacity of these compounds to inhibit peroxyl radicals within cells. The results showed that all compounds exhibited substantial antioxidant activity. Notably, *S. aegyptiaca* contains both hydrophobic and hydrophilic constituents, with water-soluble phenolic acids responsible for the antioxidant activities observed in the EtOAc extract. These findings support and confirm other antioxidant tests. Natural compound inhibitors are bioactive molecules derived from natural sources such as plants, fungi, bacteria, or marine organisms, which can inhibit specific biological targets like enzymes, receptors, or signaling pathway. They are widely studied for their therapeutic potential in diseases such as cancer, diabetes, infections, and inflammation[24]. The findings indicate that *S. aegyptiaca* could inhibit the protease enzyme via its promising constituents, such as rosmarinic acid, rutin, and cynaroside. In general, the EtOAc extract contains rosmarinic acid, rutin, and cynaroside, along with semipolar analogs, including phenolic and flavonoid-like compounds. The enhanced anticancer activity of these constituents may be associated with their intracellular antioxidant activities. Chen and co-workers showed that rosmarinic acid induces apoptosis in SGC-7901 and Hep G2 cells through the mitochondrial pathway and exhibits anti-tumor effects[25]. Moreover, some studies have reported that cynaroside can inhibit the MET/AKT/mTOR signaling pathway, significantly regulate biological processes such as cell proliferation, apoptosis, autophagy, invasion, and tumorigenesis[26,27]. Lastly, rutin is an effective radical inhibitor and displays

chemopreventive activity across various cancer cell lines[28]. As previously reported, flavonoids and phenolic compounds are considered potent anticancer agents due to their hydroxyl group[29]. Taken together, *S. aegyptiaca* represents a significant source of natural anticancer agents.

CONCLUSION

The present study investigated the antioxidant activity and cytotoxicity of the crude extract, various fractions, and five isolated compounds—ursolic acid, oleanolic acid, luteolin-7-O-glucoside, quercetin-3-O-rutoside, and rosmarinic acid— extracted from *S. aegyptiaca*. Our findings from antioxidant assays, including DPPH, ABTS, FRAP, ORAC, and NO, revealed the highest radical scavenging activities for cynaroside, rutin, and rosmarinic acid, which can be attributed to the presence of phenolic hydroxyl groups in their structures. These compelling results position *S. aegyptiaca* as a promising source of antioxidant and anticancer agents.

DECLARATIONS

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Ethical approval
Not applicable.

Consent to participate
Not applicable.

Consent for publication
All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions
AH: acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, and administrative, technical, and material support; MM: study concept and design, administrative, technical, and material support, and study supervision; ZS: study concept and design, administrative, technical, and material support, and study supervision; SM: analysis and interpretation of data, administrative, technical, and material support; SHG: analysis and interpretation of data, drafting of the manuscript, and statistical analysis; MN: acquisition of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content; ZZ: acquisition of data and statistical analysis.

Data availability
All data is provided in the manuscript.

Competing interests
The authors declare that they have no competing interests.

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Supplementary information
The online version does not contain supplementary material.

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