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https://doi.org/10.31925/farmacia.2026.5.11 **ORIGINAL ARTICLE**

ISOLATION OF ANTI-PROLIFERATIVE BIOACTIVE COMPOUNDS FROM *GYPSOPHILA ERIOCALYX* PLANT EXTRACTS BY ACTIVITY – GUIDED APPROACH

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Abstract

Endemic *Gypsophila eriocalyx* species have been traditionally used for several purposes. The aim of this study was to isolate anti-cancer compounds from *G. eriocalyx*. The aerial parts of *G. eriocalyx* were dried in the laboratory, and hexane, ethyl acetate, methanol, and methanol:water (70:30) extracts were obtained by maceration. The ethyl acetate extract exhibited the highest antiproliferative activity and was separated into subfractions by chromatographic methods. The chemical structure of the bioactive compound was elucidated by nuclear magnetic resonance (NMR) and Gas Chromatography-Mass Spectroscopy (GC/MS). Monolayer colony assay and 3D spheroid formation were applied to test the anti-cancer effect of subfraction 3.2 in prostate cancer cell PC3. As subfractionation of the ethyl acetate extract progressed to 3 and 3.2, the IC_{50} values decreased. Subfraction 3.2 showed a significant anti-proliferative effect in both 2D cells and spheroid cultures of PC3 cells. Spectral analyses revealed that the active compound isolated through step-wise fractionation of *G. eriocalyx* was 2,4-di-tert-butylphenol. 2,4-di-tert-butylphenol compound was obtained from the 3.2.2.2.2 coded fraction of the *G. eriocalyx* plant.

Rezumat

Scopul acestui studiu a fost izolarea unor compuși cu acțiune anticanceroasă din *G. eriocalyx*. Părțile aeriene ale plantei au fost uscate în laborator, iar prin macerare s-au obținut extracte în hexan, acetat de etil, metanol și metanol:apă (70:30). Extractul în acetat de etil a prezentat cea mai intensă activitate antiproliferativă și a fost separat în subfracțiuni prin metode cromatografice. Structura chimică a compusului bioactiv a fost identificată prin rezonanță magnetică nucleară (RMN) și cromatografie de gaze cuplată cu spectrometrie de masă (GC/MS). Pentru testarea efectului anticanceros al subfracțiunii 3.2 asupra celulelor de cancer de prostată PC3 s-au utilizat testul de formare a coloniilor în monostrat și testul de formare a sferoizilor 3D. Pe măsură ce subfracționarea extractului în acetat de etil a avansat către fracțiunile 3 și 3.2, valorile IC_{50} au scăzut. Subfracțiunea 3.2 a demonstrat un efect antiproliferativ semnificativ atât în culturile celulare 2D, cât și în cele sferoidale ale celulelor PC3. Analizele spectrale au evidențiat că respectivul compus activ, izolat prin fracționarea etapizată a plantei *G. eriocalyx*, a fost 2,4-di-terț-butilfenolul. Compusul 2,4-di-terț-butilfenol a fost obținut din fracțiunea codificată 3.2.2.2.2 a plantei *G. eriocalyx*.

Keywords: *Gypsophila eriocalyx*, anti-proliferative activity, 2,4-di-tert-butylphenol

Introduction

Plants are a rich source of secondary metabolites, making them an important resource for discovering therapeutic compounds in medicinal chemistry (1). A well-known example is paclitaxel, a plant-derived taxoid from the European yew tree *Taxus baccata*, which has been used clinically for many years to treat various cancers. Several widely used chemotherapeutic agents, such as vinblastine, etoposide and irinotecan, also have plant origins (2). Additionally, various phytochemicals, including resveratrol and quercetin, have been suggested for cancer prevention (3). Therefore, conducting phytochemical analyses of plants to identify similar compounds with therapeutic potential persists as an active area of research.

The genus *Gypsophila* L. belongs to the *Caryophyllaceae* family. This genus is represented by 65 species, 39 of which are endemic to Türkiye. *Gypsophila* species are economically valuable because of their high saponin glycoside content in their underground organs. *Gypsophila* species have been used for many years in Anatolia to prepare herbal cheeses, halva, ice cream, soap and liqueur (4). The underground parts of plants are also used as fire extinguishers, soap and

detergents, cleaning silk and delicate fabrics, pharmaceuticals, and gold bleaching. Additionally, *Gypsophila* species have medicinal value and are used as expectorants, diuretics, and acne relievers. *Gypsophila eriocalyx* is mainly distributed in Central Anatolia at an altitude of 600 - 1600 meters (5, 6).

The therapeutic landscape for prostate cancer has widened substantially in recent years. Management strategies for localized disease include active surveillance and radical prostatectomy, in addition to hormonal therapy. However, prostate cancer that no longer responds to hormonal treatment is called castrate-resistant prostate cancer (CRPC). In recent clinical practice, several new therapies have emerged, including the next-generation androgen receptor (AR) inhibitors such as Enzalutamide, Apalutamide, and Darolutamide. Additional treatment options for metastatic prostate cancer include a next-generation taxane, Cabazitaxel, as well as PARP inhibitors (7).

Despite this expansion of therapeutic options, concerns remain regarding treatment-related side effects, including quality of life and overall survival. As a result, prostate cancer is still considered a significant global health burden. Recent data indicate that its incidence continues to rise (8). Consequently, there is an ongoing need for new and safer therapeutic approaches.

In this study, the bioactive compounds were isolated from *G. eriocalyx* with multiple fractionations. In each fractionation step, antiproliferative activity measurements guided the selection of fractions for further isolation to obtain a pure, biologically active compound with antiproliferative activity.

Materials and Methods

Plant materials

The aerial parts of *Gypsophila eriocalyx* were collected from the natural habitat on 28.06.2017. The location is in Tuzluca, Igdır (Türkiye), which is between 40°02′58″ N and 43°39′39″ E and 1000 m above sea level. The voucher specimen was deposited at the Herbarium of the Biology Department of Erzincan Binali Yıldırım University (Voucher No. Korkmaz 4353), Türkiye. The plant material was identified by Assoc. Prof. Dr. Mustafa Korkmaz. The plant is a perennial with a woody caudex, numerous sterile shoots, and several erect branches. Stems reach 15 - 40 cm in height. The leaves are linear and fleshy, measuring 10 - 30 × 0.5 - 2.5 mm. Pedicels are up to 5 mm long. The petals are white, 3 - 4 mm in length, and the calyx is campanulate, 2 - 2.5 mm in diameter. The seeds bear flat tubercles.

Extraction of plant materials

The aerial parts of the *G. eriocalyx* plant (309 g), collected from nature, were dried in the laboratory at room temperature in the shade using a pressing method. The dried plant sample was powdered in a grinding mill. The powdered plant sample was transferred to a brown bottle and initially covered with hexane solvent (600 mL). With intermittent shaking, the plant sample was macerated in the hexane solvent for 24 hours, with the solvent being changed. Then, the plant sample in hexane was filtered through a funnel lined with filter paper. This process was repeated three times. After filtration, the solvents from the resulting macerates were removed on a rotary evaporator to dryness. In the rotary evaporator operation, the heating mantle temperature was set to 45°C. The same procedures were repeated for ethyl acetate, methanol, and methanol:water (70:30) solvents. The extraction processes were carried out sequentially. As a result, hexane (3.5 g), ethyl acetate (5.5 g), methanol (11.0 g), and methanol:water (70:30) (10.5 g) crude extracts were obtained, respectively. The yields of hexane, ethyl acetate, methanol, and methanol:water (70:30) extracts were 1.1%, 1.8%, 3.6%, and 3.4%, respectively.

Isolation of active compound

The crude extracts obtained were evaluated for antiproliferative activity against the PC3 cell line. As a result of the activity test, it was determined that the ethyl acetate extract had the highest activity. Ethyl acetate extract (5.5 g) was separated into fractions by column chromatography. 200 g of silica gel 60 (70 - 230 mesh) was packed into a 600 mm × 30 mm column. Dichloromethane, ethyl acetate, and methanol were used as eluents of increasing polarity for the mobile phase in column chromatography. The mobile phase system is given in Table I. Fractions were compared by TLC using CH_2Cl_2:ethyl acetate (5:1) as the mobile phase, and $CeSO_4$ was sprayed to visualize spots on TLC plates. Fractions containing similar bands on the chromatograms were combined to obtain the final seven fractions. The antiproliferative activity of the seven fractions was investigated, and fraction three was determined to have the highest activity. Fraction 3 (1.8 g) was separated into two subfractions with the help of molecular sieve chromatography (Sephadex LH-20). 80 g of Sephadex LH-20 was applied to a 300 mm x 10 mm column. The mobile phase was petroleum ether:$CHCl_3$:methanol (7:4:1). It was determined that of the two sub-fractions, fraction coded 3.2 (1.2 g) had the highest activity against the PC3 cell line. The compounds in subfraction number 3.2 were attempted to be separated by preparative thin-layer chromatography. In preparative thin-layer chromatography, silica gel 60 F254 type plates with a layer thickness of 210 - 270 µm were used. The mobile phase was $CHCl_3$:Ethyl acetate (8:1). Bands were cut separately into small pieces from the aluminium plate and transferred to Erlenmeyer flasks. Enough mobile phase was added to cover the subfractions transferred to the Erlenmeyer flasks, and the flasks were placed in an ultrasonic bath for 1 hour. Then, they were left in the solvent overnight,

and vacuum filtration was performed the next day in 10 replicates. After vacuum filtration, the solvents from the subfractions were removed on a rotary evaporator at 45°C, and TLC was performed using different mobile-phase systems to assess purity. After preparative thin-layer chromatography, three subfractions were obtained. Fraction coded 3.2.2 (0.8 g) had the highest activity against the PC3 cell line. The subfraction coded 3.2.2 was attempted to be separated by preparative thin-layer chromatography again. The mobile phase was $CHCl_3$:Ethyl acetate (3:1). Three subfractions were obtained. Fraction coded 3.2.2.2 (0.12 g) had the highest activity against the PC3 cell line. The subfraction coded 3.2.2.2 was attempted to be separated by thin-layer chromatography again. The mobile phase was $CHCl_3$. The pure compound was isolated from fraction coded 3.2.2.2.2 (0.03 g).

Table I

Mobile phase systems used in column chromatography

Mobile phase system	Volume	%	Elution time (minute)
CH_2Cl_2	300 mL	100	0 - 30
CH_2Cl_2:Ethyl acetate	300 mL	80 + 20	30 - 60
CH_2Cl_2:Ethyl acetate	300 mL	60 + 40	60 - 90
CH_2Cl_2:Ethyl acetate	300 mL	40 + 60	90 - 120
CH_2Cl_2:Ethyl acetate	300 mL	20 + 80	120 - 150
Ethyl acetate	300 mL	100	150 - 180
Ethyl acetate:MeOH	300 mL	90 + 10	180 - 210
Ethyl acetate:MeOH	300 mL	50 + 50	210 - 240
Ethyl acetate:MeOH	300 mL	10 + 90	240 - 270
MeOH	300 mL	100	270 - 300

Identification of active compound

The chemical structure of the bioactive compound was elucidated by spectral methods such as nuclear magnetic resonance (NMR) (^{1}H-NMR and APT) and Gas Chromatography-Mass Spectroscopy (GC/MS). NMR experiments were performed in $CDCl_3$ using TMS as the internal standard on a Bruker Avance NEO 500 MHz spectrometer. The GC-MS analysis was employed with an Agilent 5975C Inert XL EI/CI MSD system in EI mode. The bioactive compound was analysed by GC-MS using a non-polar column, HP-5MS (5% phenyl, 95% methyl polysiloxane; 30 m × 0.25 mm, 0.25 m film thickness). The oven temperature was programmed as follows: isothermal at 60°C for 1 min, then increased to 246°C at 3°C/min, and subsequently kept isothermal for 30 min. The carrier gas was helium, with a flow rate of 0.9 mL/min. The bioactive compound was injected (1 µL) in split mode. The ion source temperature was set to 230°C, and the mass range was set to 35 - 420 *m/z*. The inlet temperature of the transfer line and injection block of the mass spectrometer was set to 250°C, and sample injection was performed at a 1/50 split ratio. The identification of the compounds was performed by comparing the relative retention indices of the *n*-alkane series with the literature and by comparing mass spectra (NIST17 Mass Spectra Library). The relative percentages of the separated compounds were calculated from peak integrations in MS chromatograms. The retention index formula is below.

$$Ix = 100n + 100(t_x - t_n)/(t_{n+1} - t_n),$$

where, t_n and t_{n+1} are retention times of the reference *n*-alkane hydrocarbons eluting immediately before and after chemical compound "X"; t_x is the retention time of compound "X". A portion of the analyses was conducted after the GC system was upgraded with a splitter, enabling simultaneous GC–MS and GC–FID detection. Due to the altered flow path, absolute retention times showed slight variations between instrument configurations. Consequently, compound identification relied primarily on relative retention indices calculated separately for each configuration using a C8–C40 *n*-alkane series-together with EI mass spectral matching.

Cell lines and culture conditions

The human prostate cancer cell lines PC3, LNCaP (DSMZ (German Collection of Microorganisms and Cell Cultures GmbH), Germany), and the human prostate epithelial cell line RWPE-1 (ATCC, USA) were cultured in an RPMI-1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin (P/S); human fibroblast cell line CCD1072Sk (ATCC, USA) was cultured in DMEM medium supplemented with 10% FBS, 1% P/S at 37ºC incubator with 5% CO_2.

Anti-proliferative effect test

The cells were seeded into 96-well plates at the respective cell numbers: 6 x 10^3/well for PC3; 12 x 10^3/well for LNCaP, RWPE-1 and CCD1072Sk. After 24 hours of incubation, the cells were treated with different concentrations of plant extracts (25 - 500 µg/mL for EA extract, 25 - 250 µg/mL for SF3, 25 - 100 µg/mL for SF3.2). DMSO was used as a vehicle control. The relative amount of DMSO was always kept below 1%. After 48 hours of treatment, cell viability was determined using two different methods: crystal violet (CV) staining and CVDK8 (Ecotech, Türkiye). For CV staining, the cells were briefly washed with PBS, fixed, and stained with 0.5% crystal violet in ethanol for 30 minutes. Methanol was added to release the crystal violet by incubating for

20 minutes at RT while shaking, and the absorbance was measured at 590 nm. For CVDK8, 10% CVDK8 reagent (10 µL) was added to each well, and the wells were incubated at 37°C for 1 - 4 hours. The absorbance of the plates was measured at 450 nm using a microplate reader.

Clonogenic assay

PC3 cells were seeded into 6-well plates at 2 x 10^5 cells/well in 2 mL of RPMI. The plates were incubated at 37°C, 5% CO_2 for 24 hours, and the cells were treated with respective IC_{50} values: 29.5 µg/mL for SF3.2, then incubated at 37°C, 5% CO_2 for another 24 hours. The cells were trypsinized and seeded into new 6-well plates at a density of 3 x 10^2 cells *per* well in 2 mL of medium. On the 7th day, the cells were treated again. On the 14th day, the colonies were washed with PBS, fixed, stained with 0.5% crystal violet in ethanol, and counted using ImageJ.

3D spheroid formation and viability assay

PC3 cells were seeded into 96-well U-bottom non-adherent plate with 2x10^3 cells/well in 200 µL. The plates were incubated at 37°C, 5% CO_2 for 48 hours. Half of the media was removed from each well without touching the spheroids. Then, spheroids were treated with the respective IC_{50} value: 29.5 µg/mL for SF3.2, with 100 µL fresh medium. The treatment was repeated two more times at 48-hour intervals. At the end of the incubation, 10% CVDK8 (10 µL) reagent was added to each well. The plates were incubated for 1 - 5 hours and measured by a microplate reader at 450 nm. The percent viability was calculated considering control groups as 100%. The images were taken by an inverted microscope.

Statistical analysis

All viability analyses were performed with three technical replicates *per* experiment and three biological replicates. The colony formation assay was performed with two technical replicates *per* experiment and three biological replicates. The IC_{50} values for each extract were calculated using GraphPad Prism 8.0.2. Non-linear regression with [Inhibitor] vs. normalized response-Variable slope Least squares fit method was applied. Statistical significance was determined using ANOVA followed by Tukey's multiple-comparison test when there were more than two groups and Student's t-test when two groups were used, using GraphPad Prism 8.0.2. $p < 0.05$ was accepted as significant.

Results and Discussion

Anti-proliferative effect of Gypsophila eriocalyx subfractions

The extracts obtained from the maceration of *Gypsophila eriocalyx* with hexane, ethyl acetate, methanol, and methanol:water (7:3 v/v) solvents were applied to PC3 cells at respective concentrations of 100, 250, and 500 µg/mL. The anti-proliferative activity results showed that among these four extracts, ethyl acetate (EA) was the most effective, with dose-dependent inhibition of PC3 cell viability (Figure 1). The IC_{50} value for the EA extract in PC3 cells was 106.7 µg/mL.

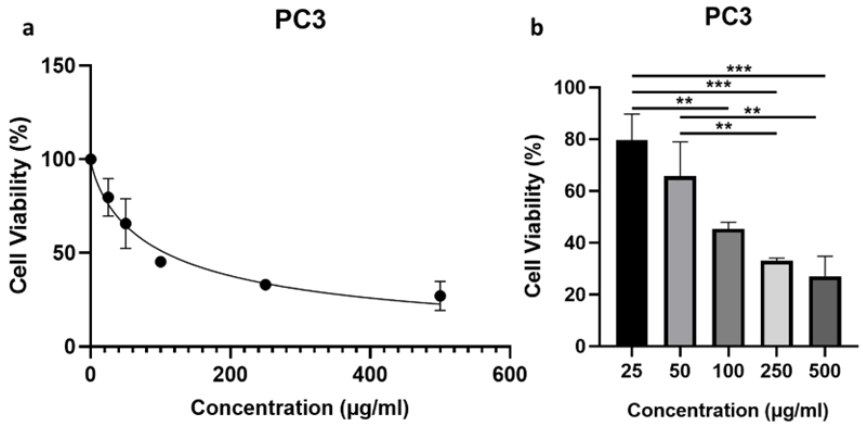


Figure 1.

EA extracts exert dose-dependent tumour-suppressive activity in cultured PC3 cells

a. IC_{50} graph of EA extract **b.** Dose-dependent cell viability of EA extract

%Viability was calculated using GraphPad comparing treatment groups to control. All experiments were conducted in triplicate (technical replicates) and independently repeated at least 3 times. *p < 0.05, **p < 0.01, ***p < 0.001 by ANOVA

The EA extract was fractionated using column chromatography, yielding seven subfractions labelled SF1 through SF7. These subfractions were initially tested in PC3 cells. SF5 and SF7, which precipitated, and the inactive SF1 were excluded from further evaluation. The antiproliferative activities of SF3, SF4, and SF6 were then assessed in both cancerous PC3 and normal RWPE-1 cells. Among these, SF6 demonstrated the highest IC_{50} value in PC3 cells (391.3 µg/mL), whereas SF3 and SF4 exhibited much lower IC_{50} values (51.73 and 49.63 µg/mL, respectively) (Table II).

Table II

IC_{50} values of different subfractions in PC3 and RWPE1 cells

Extract code	PC3 IC_{50} (µg/mL)	RWPE-1 IC_{50} (µg/mL)
SF3	51.73	481.2
SF4	49.63	290.9
SF6	391.3	~258

RWPE-1 normal prostate epithelial cells demonstrated reduced sensitivity to SF3 compared with PC3 cells (Figure 2). Although SF3 and SF4 had comparable IC_{50} values, SF3 was chosen for further studies due to its more consistent and pronounced dose-dependent anti-proliferative effects.

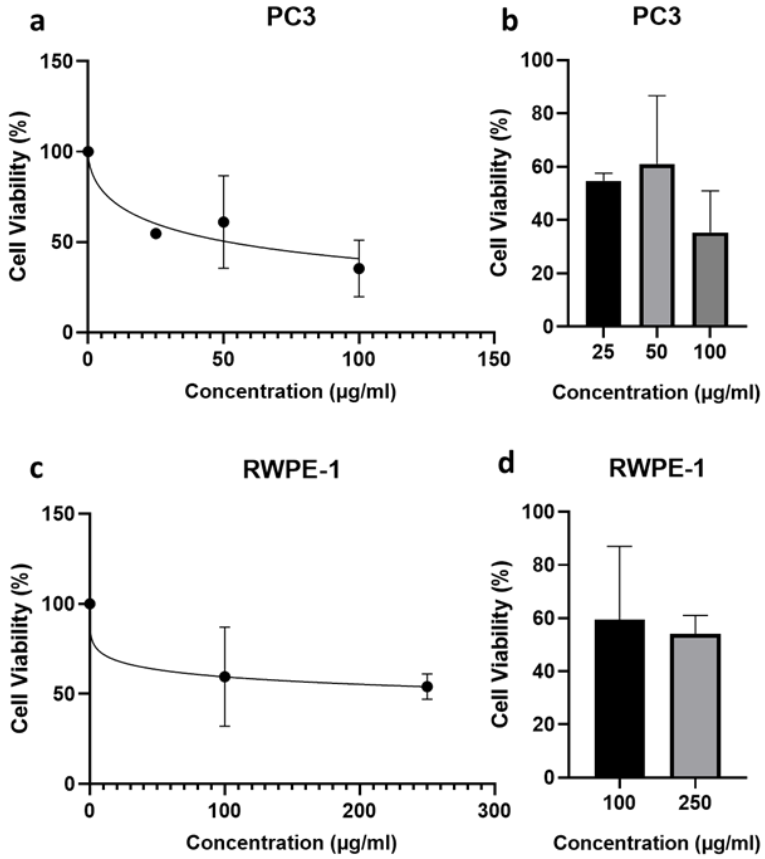


Figure 2.

SF3 extracts exert dose-dependent antiproliferative activity in cultured PC3 and RWPE-1 cells
a. IC_{50} graph of SF3 extract on PC3 cells. **b.** Dose-dependent cell viability of SF3 extract on PC3 cells.
c. IC_{50} graph of SF3 extract on RWPE-1 cells. **d.** Dose-dependent cell viability of SF3 extract on RWPE-1 cells
%Viability was calculated using GraphPad comparing treatment groups to control. All experiments were conducted in triplicate (technical replicates) and repeated at least three times

Molecular sieve chromatography of the SF3 extract produced two subfractions, separating larger molecules labelled as "1" and smaller molecules labelled as "2" based on their elution order from the column. The SF3.2 fraction was tested in the prostate carcinoma cell lines PC3 and LNCaP, and its effects on cell viability were also assessed in CCD1072Sk, a normal human skin fibroblast cell line. The SF3.2 extract exhibited IC_{50} values of 29.5 µg/mL in PC3 cells (Figure 3a and Figure 3b), 27.95 µg/mL in LNCaP cells (Figure 3c and Figure 3d), and 98.61 µg/mL in CCD1072Sk cells (Figure 3e and Figure 3f). SF4.2 was additionally tested in PC3, LNCaP and CCD1072Sk cells for comparison to SF3.2 (Table III). Fraction 3.2 was selected for further experiments because it showed greater differential cytotoxicity between prostate cancer cells and normal fibroblasts, suggesting a more favourable selectivity profile.

Table III

Comparison of IC_{50} values of 3.2 and 4.2 in different cell lines

Cell Line / Extract code	PC3 IC_{50} (µg/mL)	LNCaP IC_{50} (µg/mL)	CCD1072Sk IC_{50} (µg/mL)
SF3.2	29.5	27.95	98.61
SF4.2	8.451	26.68	39.26

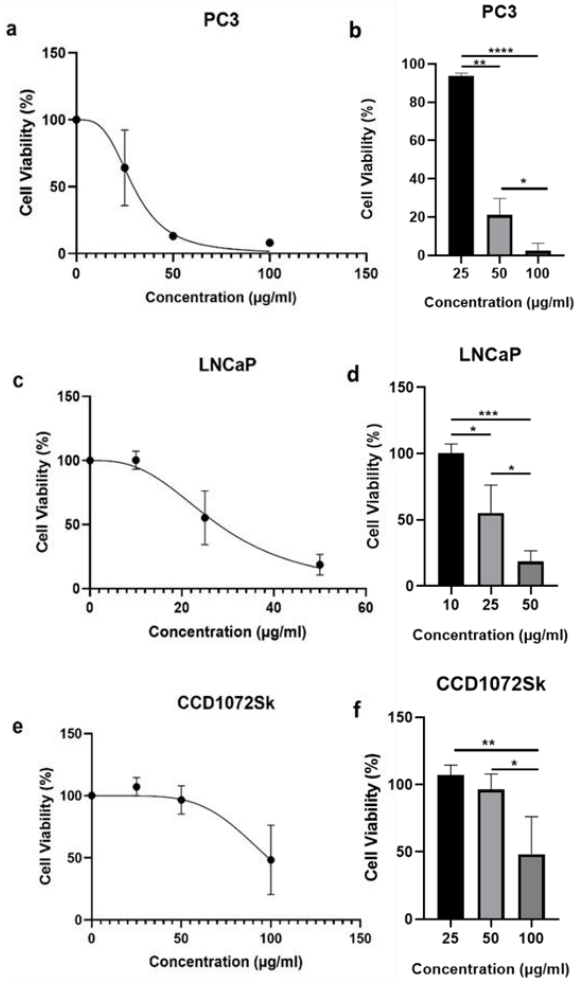


Figure 3.

SF3.2 extracts exert dose-dependent anti-proliferative activity in cultured PC3, LNCaP, and CCD1072Sk cells. **a.** IC_{50} graph of SF3.2 extract on PC3 cells **b.** Dose-dependent cell viability of SF3.2 extract on PC3 cells. **c.** IC_{50} graph of SF3.2 extract on LNCaP cells. **d.** Dose-dependent cell viability of SF3.2 extract on LNCaP cells. **e.** IC_{50} graph of SF3.2 extract on CCD1072Sk cells. **f.** Dose-dependent cell viability of SF3.2 extract on CCD1072Sk cells

Viability was calculated using GraphPad by comparing treatment groups to the control. All experiments were conducted in triplicate (technical replicates) and repeated at least three times. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by ANOVA

Clonogenic assay

A 2D clonogenic assay was performed to evaluate the autonomous growth ability of cancer cells. To assess the impact of SF3.2 on this property, PC3 cells were treated with SF3.2 at its IC_{50} concentration, and colony numbers were assessed. The results showed that SF3.2 significantly reduced colony formation, with the treated group exhibiting only 53% of the colonies compared to the control group (Figure 4).

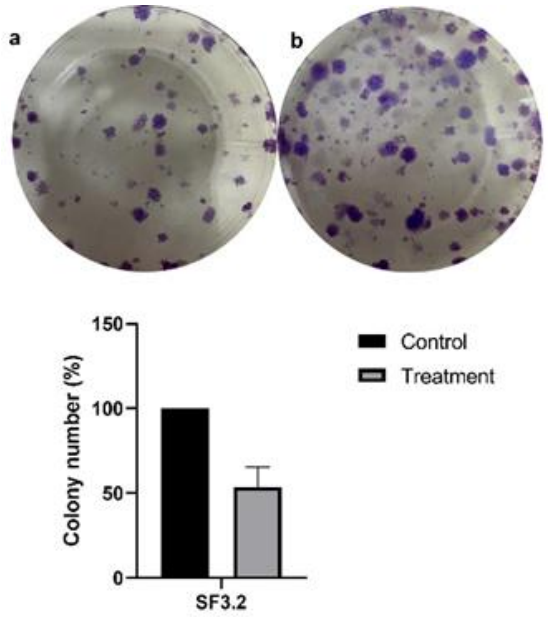


Figure 4.

SF3.2 extracts exert suppression on colony formation of PC3 cells**: a.** SF3.2-treated PC3 cells; **b.** DMSO control group; **c.** %Colony number of SF3.2 extract on PC3 cells

All experiments were conducted in duplicate (technical replicates) and repeated independently at least three times

3D spheroid formation and viability assay

3D spheroid culture is used to better mimic cancer cells *in vivo*. To evaluate the effect of SF3.2, a 3D spheroid formation assay was conducted in PC3 cells using the IC_{50} concentration. Images were captured with an inverted microscope, showing smaller, less compact spheroids. Treatment with SF3.2 markedly reduced spheroid formation, with cell viability in the 3D spheroids decreasing to 54.33% compared to the control group, consistent with the 2D culture (Figure 5).

Determination of the chemical structure of the active compound by spectroscopic methods

After several fractionations of SF3.2, an active compound was obtained from the 3.2.2.2.2 coded fraction. The IC_{50} values of subfractions 3.2.2 and 3.2.2.2 in PC3 cells guiding this selection were given in Table IV.

Table IV

IC_{50} values in PC3 for fractions 3.2.2 and 3.2.2.2

Extract code	PC3 IC_{50} (µg/mL)	Yield (g)
3.2.2	40.13	0.8
3.2.2.2	31.82	0.12

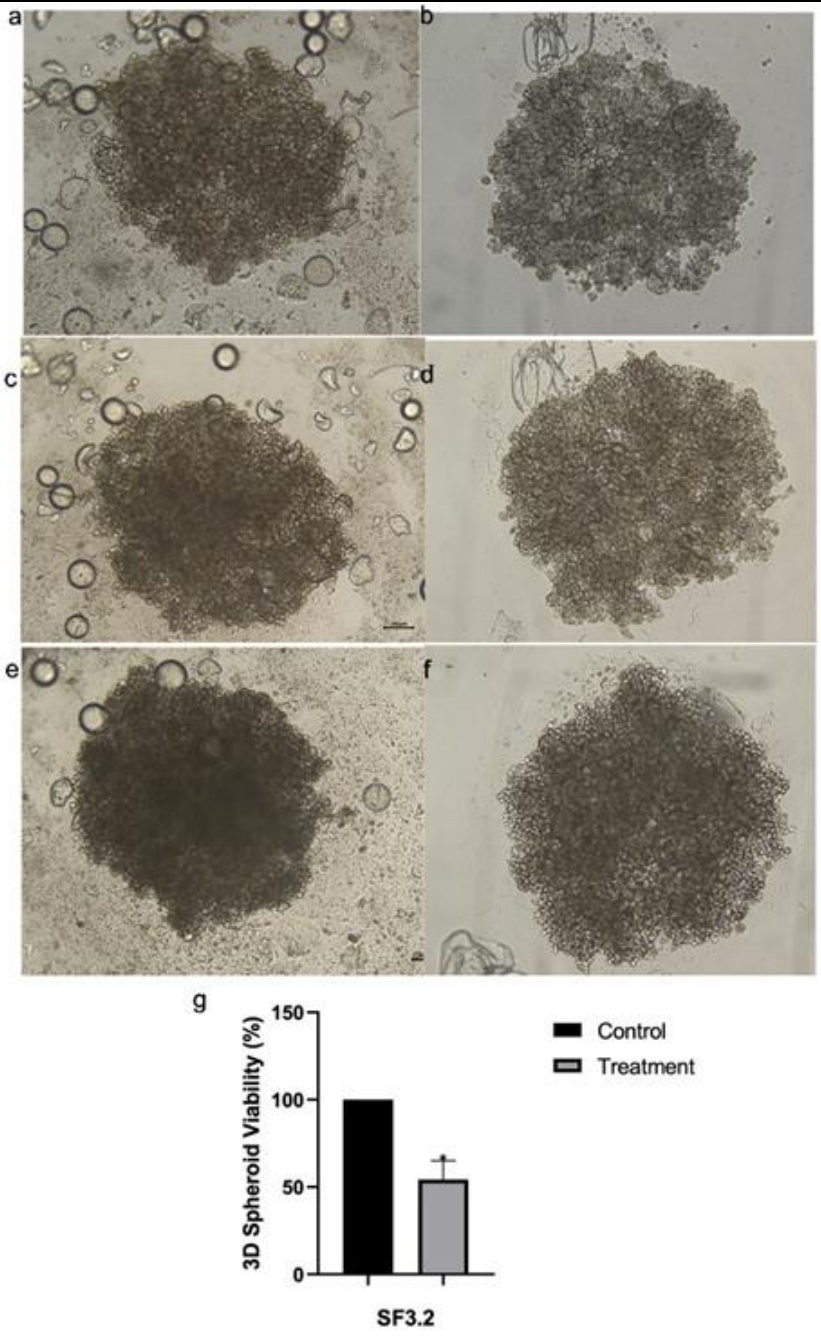


Figure 5.

SF3.2 extracts exert suppression on 3D spheroid formation of PC3 cells**. a.** SF3.2-treated PC3 cells for 48 hours. **b.** DMSO control group. **c.** SF3.2-treated PC3 cells for 96 hours. **d.** DMSO control group. **e.** SF3.2-treated PC3 cells for 144 hours. **f.** DMSO control group. **g.** %Spheroid viability of SF3.2 extract on PC3 cells
All experiments were conducted in triplicate (technical replicates) and repeated independently at least three times

As a result of the GC-MS analysis, the retention time of the 2,4-di-tert-butylphenol (2,4-DTBP) compound was determined to be 29.432 min, and the relative retention index was 1517. The chemical structure was identified by comparing the mass spectra of the original and known compounds in the NIST17 commercial library using the GC-MS device. Additionally, 2,4-DTBP was purchased commercially and subjected to GC-MS analysis. The analysis results were compared to confirm the accuracy of the isolated compound. The retention time of commercial 2,4-DTBP was 29.592 min, and the relative retention index was 1518. Chemical composition of fraction 3.2.2 was determined by GC-MS. Compounds were identified by comparison of relative retention indices with literature values and by mass spectral matching against the NIST17 library. Relative percentages were calculated by peak area normalization without response factor correction. The chemical composition of 3.2.2 is given in Table V.

Table V

Chemical composition of 3.2.2 fraction

RT	RRI	RRI Lit.	Compound	%	Reference
17.649	1389	1391	1-Tetradecene	3.2	9
20.994	1524	1521	2,4-Di-tert-butylphenol	43.3	10
22.560	1591	1590	1-Hexadecene	5.7	11
22.727	1598	1591	Diethyl Phthalate	2.1	12
24.814	1689	1675	Dodecyl acrylate	2.1	13
26.952	1788	1790	1-Octadecene	5.2	14
29.676	1922	1929	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	2.3	15
30.050	1941	1943	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	1.0	16
30.771	1978	1970	*n*-Hexadecanoic acid	8.9	17
30.954	1988	1994	1-eicosene	3.1	18
31.284	2005		Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, ethyl ester	2.2	
34.596	2186	2190	1-Docosene	1.7	14
34.922	2204	2211	1-Octadecanol acetate	5.6	19
37.580	2362		2,2'-Biphenol, 3,3',5,5'-tetrakis(1,1-dimethylethyl)-	3.1	
40.883	2542	2550	Bis(2-ethylhexyl) phthalate	10.5	20

RT: Retention time (minute); RRI: Relative retention index; RRI Lit.: Relative retention index in the literature

The subfraction coded 3.2.2 was attempted to be separated by preparative thin-layer chromatography again. Three subfractions were obtained. Fraction coded 3.2.2.2 (0.12 g) had the highest activity against the PC3 cell line. Subfraction 3.2.2.2 was determined to be impure by GC-MS analysis. Therefore, the subfraction coded 3.2.2.2 was attempted to be separated by thin-layer chromatography again. The mobile phase was $CHCl_3$. The pure compound was isolated from fraction coded 3.2.2.2.2 (0.03 g).

The chemical structure of 2,4-DTBP was confirmed by NMR spectroscopy (^{1}H-NMR and APT). In the ^{1}H-NMR spectrum, the peak observed at δ 7.30 (1H, d, J = 2.4 Hz) belongs to the H-3 proton, and this proton interacts with the H-5 (1H, dd, J = 8.2; 2.4 Hz) proton at δ 7.07. In addition, the H-5 proton interacts with the H-6 (1H, d, J = 8.2 Hz) proton located at δ 6.60. Six methyl group peaks were observed at δ 1.29 (9H, s, $3CH_3$) and 1.41 (9H, s, $3CH_3$), respectively. Two singlets are observed, each integrating for nine protons and representing three equivalent methyl groups. The APT (Attached Proton Test) experiment was used to distinguish quaternary carbon, methine (CH), and methyl (CH_3) signals by their characteristic phase behaviour. In the APT spectrum, six peaks were observed between 151.7 and 115.9 ppm in the aromatic region. Among these carbons, three resonances (135.1, 142.9, and 151.7 ppm) were identified as quaternary carbons, consistent with the presence of aromatic substitution. The quaternary carbon peak to which the hydroxyl (-OH) group is attached was seen at 151.7 ppm; the quaternary carbons to which the tert-butyl groups are attached were seen at 142.9 and 135.1 ppm. The peaks at 32.0 and 37.0 ppm in the APT spectrum correspond to quaternary carbons. As a result of the NMR spectrum, the presence of twenty-one protons was detected, and it was determined that eighteen of these protons belonged to the six methyl groups in the tert-butyl structure, and the remaining three protons belonged to the peaks in the aromatic region (Table VI). The chemical structure of the compound was supported by a literature review (21, 22).

Besides 2,4-DTBP, the most abundantly found compounds in SF 3.2.2 were Bis(2-ethylhexyl) phthalate (DEHP) (10.5%), *n*-Hexadecanoic acid (Palmitic acid) (8.9%), 1-Hexadecene (5.7%) and 1-Octadecanol acetate (5.6%).

Table VI

^{1}H NMR (500 MHz, $CDCl_3$, δ) and APT (125 MHz, $CDCl_3$, δ) of 2,4-di-tert-butylphenol

C	Carbon type	APT (ppm)	^{1}H-NMR (ppm)
1	C	151.7	-
2	C	142.9	-
3	CH	124.2	7.30 d (1H, J = 2.4 Hz)
4	C	135.1	-
5	CH	123.5	7.07 dd (1H, J = 8.2; 2.4 Hz)
6	CH	115.9	6.60 d (1H, J = 8.2 Hz)
7	C (C2)	37.0	-
8	C (C4)	32.0	-
9	$3CH_3$ (C7)	29.7	1.29 s (9H)
10	$3CH_3$ (C8)	31.6	1.41 s (9H)

This study explored the endemic species *G. eriocalyx* as a potential source of novel anticancer compounds. Although several species in the genus *Gypsophila*, including *G. eriocalyx*, have been extensively investigated for their bioactive properties due to their abundance of secondary metabolites such as saponins and triterpenoids, studies specifically examining *G. eriocalyx* extracts remain limited (23-25). Previous research has demonstrated that water and methanol extracts of *G. eriocalyx* inhibited the proliferation of liver (HepG2), colon (HT-29), and breast (MCF7) cancer cell lines, with IC_{50} values of 0.269, 0.170, and 0.381 mg/mL for water extracts, and 0.856, 0.644 and 0.814 mg/mL for methanol extracts, respectively (26). Another study reported that water, methanol, and ethanol extracts of *G. eriocalyx* exerted dose- and time-dependent anticancer effects in the MDA-MB-231 breast cancer cell line, with ethanol extracts showing the strongest antiproliferative activity (IC_{50} values of 0.249 mg/mL at 24 h and 0.217 mg/mL at 48 h) (27). The anticancer potential of *G. eriocalyx* has also been investigated in other cancer models. In neuroblastoma, the methanolic extract of *G. eriocalyx* exhibited cytotoxic activity against SH-SY5 cells, with an IC_{50} of 0.1 mg/mL. In addition to reducing cell viability, the extract induced apoptosis through mitochondrial dysfunction, suggesting activation of the intrinsic apoptotic pathway (28). Similarly, Azbazdar *et al*. (29) demonstrated that the methanolic root extract of *G. eriocalyx* exerted potent anticancer effects against melanoma cells. The extract significantly decreased cell viability, with IC_{50} values of 21.62 µg/mL and 4.6 µg/mL in Malme-3M and SK-MEL-28 cells, respectively. Beyond its cytotoxic effects, the extract promoted apoptosis and suppressed both migratory capacity and clonogenic potential, indicating its ability to interfere with multiple hallmarks of cancer (29). In comparison, the IC_{50} value of the EA extract in PC3 cells, observed in our study at 106.7 µg/mL, was consistent with previously reported values. Since cytotoxic potency is influenced by multiple factors, including cell type, exposure duration, extraction procedures, and assay methodology, no direct comparison between these studies and ours is possible. Our results suggested that the EA extract contains potent anticancer constituents.

Chemical analysis revealed that the SF3.2.2 subfraction of the EA extract contained DEHP, Palmitic acid and 2,4-DTBP as major constituents.

DEHP is well identified as an endocrine disruptor, yet its potential anticancer effects remain controversial. While some studies report cytotoxic effects of DEHP, others have noted that it can promote proliferation in cancer cells. In prostate cancer cell lines PC3 and DU-145, DEHP was found to induce cytotoxicity at lower doses compared to other phthalates (30).

Palmitic acid (*n*-hexadecanoic acid), a common fatty acid present in various *Gypsophila* species, has well-established anticancer properties (31, 32). It has demonstrated inhibitory effects on prostate cancer in both *in vitro* and *in vivo* settings, and its mechanism of action is primarily through modulation of the PI3K/Akt signalling pathway (33).

The most abundant bioactive compound in the extract, 2,4-DTBP, is both an antioxidant and a marine-derived metabolite. To date, in the literature, Inanir *et al*. (27) are the only study to examine the chemical composition of various *G. eriocalyx* extracts; however, they did not report the presence of 2,4-DTBP (15). This discrepancy may be attributed to differences in plant collection sites or harvesting seasons. Additionally, Inanir *et al*. (27) highlighted that different extraction methods rarely yield the same compounds. Accordingly, the EA extraction performed in our study produced a distinct set of chemical components.

2,4-DTBP is a lipophilic phenol known for its toxic effects across a wide range of species. It is produced by at least 169 species, including dicots, bacteria, animals, and fungi. This phenolic compound has been reported to retain antioxidant, anti-inflammatory, antibacterial, antiviral, and antifungal properties (34). A previous study on *G. turcica* reported 2,4-DTBP as a component of its essential oil (32), implying that *Gypsophila* species are a source of this compound.

Several studies have investigated the production of 2,4-DTBP from various plant sources and examined its cytotoxic activity against different cancer types, with a range of IC_{50} values (35, 36). However, to our knowledge, no studies have yet investigated the role of 2,4-DTBP in prostate cancer. In a cytotoxicity study on the human colorectal cancer cell line HCT116, increasing concentrations of 2,4-DTBP resulted in an IC_{50} value of 57.044 ± 0.32 µM (37). Another study evaluated 2,4-DTBP isolated from *Plumbago zeylanica* against the epithelial monkey kidney cell line Vero and the human breast cancer cell line MCF-7. While its effect on Vero cells was minimal, it exhibited significant cytotoxicity against MCF-7 cells, with an IC_{50} of 5 µg/mL and a clear concentration-dependent effect (22).

A limitation of the present study is that, although bioassay-guided fractionation resulted in a progressive increase in cytotoxic potency from the crude ethyl acetate (EA) extract to the SF3 and SF3.2 fractions (IC_{50}: 106.7, 51.73, and 29.5 µg/mL, respectively), the complete chemical compositions of these fractions have not yet been fully elucidated. Subsequent purification and compound identification analyses suggested 2,4-di-tert-butylphenol (2,4-DTBP) as a potential bioactive constituent. However, SF3 and SF3.2 remain partially purified fractions and likely contain additional bioactive compounds that contribute to the observed anticancer activity, either individually or through synergistic interactions. Therefore, comprehensive phytochemical characterization of these

fractions is warranted to identify the compounds responsible for their biological effects. Furthermore, given the identification of 2,4-DTBP as a candidate bioactive molecule, its anticancer activity and underlying molecular mechanisms should be investigated independently in prostate cancer models to clarify its specific contribution to the overall activity of the fractions.

Conclusions

To the best of our knowledge, this is the first study to report the effects of *G. eriocalyx* ethyl acetate (EA) extracts on prostate cancer. This study highlights *G. eriocalyx* as a promising source of anticancer bioactive compounds. It is the first report to examine both the anticancer activity and the chemical composition of the EA extract of *G. eriocalyx*. The *G. eriocalyx*-derived 2,4-DTBP warrants further investigation as a potential active compound and drug candidate for prostate cancer.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the authors used Paperpal and ChatGPT for English proofreading to enhance its readability. The author(s) reviewed and revised all outputs from these tools and take(s) full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

References

1. Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov. 2021;20(3):200-216.
2. Fridlender M, Kapulnik Y, Koltai H. Plant derived substances with anti-cancer activity: from folklore to practice. Front Plant Sci. 2015;6:799.
3. Lee K, Bode AM, Dong Z. Molecular targets of phytochemicals for cancer prevention. Nat Rev Cancer. 2011;11(3):211-226.
4. Korkmaz M, Özçelik H. Economic importance of Gypsophila L., Ankyropetalum Fenzl and Saponaria L. (Caryophyllaceae) taxa of Turkey. Afr J Biotechnol. 2011;10(47):9533-9541.
5. Kaya AS, Karagüzel Ö, Aydınşakir K, Kazaz S, Özçelik A. Usage possibilities of some Gypsophila species naturally grown in Türkiye as ornamental plants. Horticult Stud (HortiS). 2012;29(1):37-47.
6. Özçelik H, Özgökçe F. Taxonomic contributions to genus Gypsophila in Turkey and a new taxon from Erzurum: G. venusta subsp. staminea. Anatolian J Bot. 2021;5(1):6-18.
7. Rebello RJ, Oing C, Knudsen KE, Loeb S, Johnson DC, Reiter RE, et al. Prostate cancer. Nat Rev Dis Primers. 2021;7(1):9.
8. James N, Tannock I, N'Dow J, Feng F. The Lancet Commission on prostate cancer: planning for the surge in cases. Lancet. 2024;403(10437):1683-1722.
9. Vujisić LJ, Vučković I, Tešević V, Đoković D, Ristić MS, Janaćković P, et al. Comparative examination of the essential oils of Anthemis ruthenica and A. arvensis wild-growing in Serbia. Flavour Fragr J. 2006;21(3):458-461.
10. Xu LL, Han T, Wu JZ, Zhang QY, Zhang H, Huang BK, et al. Comparative research of chemical constituents, antifungal and antitumor properties of ether extracts of Panax ginseng and its endophytic fungus. Phytomedicine. 2009;16(6-7):609-616.
11. Skaltsa HD, Demetzos C, Lazari D, Sokovic M. Essential oil analysis and antimicrobial activity of eight Stachys species from Greece. Phytochemistry. 2003;64(3):743-752.
12. Kotowska U, Żalikowski M, Isidorov VA. HS-SPME/GC-MS analysis of volatile and semi-volatile organic compounds emitted from municipal sewage sludge. Environ Monit Assess. 2012;184(5):2893-2907.
13. Horna A, Táborský J, Dufka O, Matoušek P, Churáček J. Chromatography of monomers: II. Glass capillary gas chromatography of C1-C18 alkyl esters of acrylic and methacrylic acids. J Chromatogr A. 1985;325(2):367-378.
14. Adams RP, Morris JA, Pandey RN, Schwarzbach AE. Cryptic speciation between Juniperus deltoides and Juniperus oxycedrus (Cupressaceae) in the Mediterranean. Biochem Syst Ecol. 2005;33(8):771-787.
15. Lalel HJ, Singh Z, Tan SC. Glycosidically-bound aroma volatile compounds in the skin and pulp of 'Kensington Pride' mango fruit at different stages of maturity. Postharvest Biol Technol. 2003;29(2):205-218.
16. Andriamaharavo NR. Retention data [Internet]. Gaithersburg (MD): NIST Mass Spectrometry Data Center; 2014.
17. da Silva MHL, Andrade EHA, Zoghbi MDGB, Luz AIR, da Silva JD, Maia JGS. The essential oils of Lantana camara L. occurring in North Brazil. Flavour Fragr J. 1999;14(4):208-210.

18. Radulović NS, Blagojević PD, Palić RM, Zlatković BK, Stevanović BM. Volatiles from vegetative organs of the palaeoendemic resurrection plants Ramonda serbica Panč. and Ramonda nathaliae Panč. et Petrov. J Serb Chem Soc. 2009;74(1):35-44.
19. Fokialakis N, Melliou E, Magiatis P, Harvala C, Mitaku S. Composition of the steam volatiles of six Euphorbia spp. from Greece. Flavour Fragr J. 2003;18(1):39-42.
20. Xian Q, Chen H, Zou H, Yin D. Allelopathic activity of volatile substance from submerged macrophytes on Microcystin aeruginosa. Acta Ecol Sin. 2006;26(11):3549-3554.
21. Chawawisit K, Bhoopong P, Phupong W, Lertcanawanichakul M. 2,4-Di-tert-butylphenol, the bioactive compound produced by Streptomyces sp. KB1. J Appl Pharm Sci. 2015;5(Suppl 3):7-12.
22. Kavisri M, Malathy BR, Lavanya G, Seema S, Christy HJ, Anand DA, et al. Molecular structure and bioactivities of 2,4-di-tert-butylphenol extracted from Plumbago zeylanica, investigated using HPLC and NMR. Biomass Convers Biorefin. 2023;14(19):23793-23803.
23. Gaidi G, Correia M, Chauffert B, Beltramo JL, Wagner H, Lacaille-Dubois MA. Saponins-mediated potentiation of cisplatin accumulation and cytotoxicity in human colon cancer cells. Planta Med. 2002;68(1):70-72.
24. Kikuchi Y, Sasa H, Kita T, Hirata J, Tode T, Nagata I. Inhibition of human ovarian cancer cell proliferation in vitro by ginsenoside Rh2 and adjuvant effects to cisplatin in vivo. Anticancer Drugs. 1991;2(1):63-67.
25. Zhang W, Luo JG, Zhang C, Kong LY. Different apoptotic effects of triterpenoid saponin-rich Gypsophila oldhamiana root extract on human hepatoma SMMC-7721 and normal human hepatic L02 cells. Biol Pharm Bull. 2013;36(7):1080-1087.
26. Altay A, Tohma H, Durmaz L, Taslimi P, Korkmaz M, Gulcin I, et al. Preliminary phytochemical analysis and evaluation of in vitro antioxidant, antiproliferative, antidiabetic, and anticholinergics effects of endemic Gypsophila taxa from Turkey. J Food Biochem. 2019;43(7):e12908.
27. Inanir M, Uçar E, Tüzün B, Eruygur N, Ataş M, Akpulat HA. The pharmacological properties of Gypsophila eriocalyx: the endemic medicinal plant of northern central Turkey. Int J Biol Macromol. 2024;266(Pt 2):130943.
28. Dolanbay SN. Saponin rich Gypsophila eriocalyx Boiss methanolic extract (GEME) exhibit anticancerogenic effect against neuroblastoma cancer cell line (SH-SY5Y). Cukurova Med J. 2024;49(1):31-39.
29. Azbazdar Y, Helvacıoglu S, Ozhan G. Gypsophila eriocalyx roots inhibit proliferation, migration, and TGF-β signaling in melanoma cells. Turk J Biochem. 2025;50(1):134-143.
30. Kismali G, Yurdakok-Dikmen B, Kuzukiran O, Arslan P, Filazi A. Phthalate induced toxicity in prostate cancer cell lines and effects of alpha lipoic acid. Bratisl Lek Listy. 2017;118(8):460-466.
31. Servi H, Keskin BE, Çelik S, Budak Ü, Kırmızıtaş FC, Bektaş EB. Essential oil composition and fatty acid profile of two endemic Gypsophila species from Turkey. Am J Essent Oil Nat Prod. 2017;5(3):16-20.
32. Servi H, Keskin BE, Yılancıoğlu K, Çelik S. Essential oil composition and antibacterial activities of Gypsophila species. Int J Second Metab. 2019;6(1):20-27.
33. Zhu S, Jiao W, Xu Y, Hou L, Li H, Shao J, et al. Palmitic acid inhibits prostate cancer cell proliferation and metastasis by suppressing the PI3K/Akt pathway. Life Sci. 2021;269:119062.
34. Zhao F, Wang P, Lucardi RD, Su Z, Li S. Natural sources and bioactivities of 2,4-di-tert-butylphenol and its analogs. Toxins (Basel). 2020;12(1):35.
35. Kaari M, Joseph J, Manikkam R, Kalyanasundaram R, Sivaraj A, Anbalmani S, et al. A Novel Finding: 2,4-Di-tert-butylphenol from Streptomyces bacillaris ANS2 Effective Against Mycobacterium tuberculosis and Cancer Cell Lines. Appl Biochem Biotechnol. 2023;195(11):6572-6585.
36. Song YW, Lim Y, Cho SK. 2,4-di-tert-butylphenol, a potential HDAC6 inhibitor, induces senescence and mitotic catastrophe in human gastric adenocarcinoma AGS cells. Biochim Biophys Acta Mol Cell Res. 2018;1865(5):675-683.
37. Saha P, Hegde M, Chakraborty K, Singha A, Mukerjee N, Ghosh D, et al. Targeted inhibition of colorectal cancer proliferation: the dual-modulatory role of 2,4-DTBP on anti-apoptotic Bcl-2 and Survivin proteins. J Cell Mol Med. 2024;28(7):e18150.