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Optimization of betulinic and ursolic acids and phenolics extraction from endemic *Rosa pisiformis* using Box-Behnken design in relation to cytotoxic activities

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Despite its important pharmacological bioactivities, betulinic acid is still primarily obtained through extraction from heartwood and bark or synthesized synthetically, with less than 3% efficiency. Our endemic rose species, *Rosa pisiformis* (Christ.) D. Sosn., which is a new alternative source of betulinic acid and traditionally valued for its medicinal properties, was collected from its natural distribution in Gümüşhane province. The plant's organs, such as root, stem, leaf and fruit were air-dried and pulverized. The compounds were separately extracted using three different solvents (ethanol, dichloromethane and hexane) with an optimized Box-Behnken method. The amounts of quercetin, rutin, catechin, betulinic, ursolic and oleanolic acids in roots, stems, leaves, and fruits were determined using HPLC–DAD techniques with standard substances. Optimisation data revealed a 65% solvent ratio and five-times maceration with 75 ml of solvents. The highest amounts of catechin were found in the leaves (DCM) as 15.61 µg/ml. Stems were rich in rutin (28.96 µg/ml) and quercetin (39.90 µg/ml). Betulinic acid content was determined for the first time in stems (hexane, 11.84 µg/ml) and roots (9.32 µg/ml). Their cytotoxic activities against prostate and lung carcinoma cells were evaluated using ABTS-assay, revealing that stems exhibited the highest activity, followed by leaves and roots.

Keywords Box-Benchen, HPLC, *Rosa psiformis*, Secondary metabolites

Betulinic acid is a triterpenoid known to be isolated from different organs of various plants, including *Rosa* sp. This metabolite inhibits the growth of human melanoma[1] cells and the replication of the AIDS virus[2]. In addition, betulinic acid derivatives trigger apoptosis in human melanoma cells[3–5]. Despite its significant pharmacological potential, betulinic acid is primarily obtained through extraction from the bark or extracts of *Betula alba* and *Betula pendula*, or by synthetic processes that use betulin (alcoholic triterpene) isolated from the bark of these plants as an intermediate. Therefore, new research is required to identify natural resources capable of producing large quantities of products from easily regenerable parts (leaves, lateral branches, fruit) without adversely affecting plant growth. The aim of this study is to determine and compare the amounts of betulinic acid, oleanolic acid and ursolic acid, as well as the phenolic compounds (catechin, rutin, and quercetin) in the root, stem, leaves, and fruits of endemic *Rosa pisiformis* (Christ.) D. Sosn. (red rose). For this purpose, terpenes and phenolic compounds were isolated using three different solvent systems (hexane, dichloromethane and ethanol) through an extraction technique from the roots, stems, leaves, and fruits of the plant collected from the province of Gümüşhane, a natural distribution area. The structures were analyzed using HPLC–DAD techniques, and quantitative determinations were performed. The rose plant is well-known for its significant amount of vitamin C, particularly in its fruits and seeds, as well as its antioxidant and anti-inflammatory properties. In addition to these benefits, the presence of triterpene acids, especially betulinic acid with its anti-cancer effects, was determined in various organs (root, stem, leaf) besides fruit.

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Rosehip species, containing ascorbic acid, phenolic compounds and healthy fatty acids, have antioxidant properties and are used in the treatment of skin disorders, hepatotoxicity, kidney diseases, diarrhea, inflammation, arthritis, diabetes, hyperlipidemia, obesity and cancer[6–8].

Betulinic acid, identified in various *Rosa* species[9], particularly in *Rosa canina*[7,10], has gained significant attention in recent years due to its diverse biological and medicinal properties such as anti-bacterial, anti-malarial, anti-inflammatory, antihelmintic, antinociceptive, anti-HSV-I and anticancer effects[11–13]. Birch (*Betula* spp., Betulaceae) is the primary source of betulinic acid, a natural compound vwidespread in the plant kingdom. However, it is also found in species such as *Ziziphus* spp. (Rhamnaceae), *Syzygium* spp. (Myrtaceae), *Diospyros* spp. (Ebenaceae) and *Paeonia* spp. (Paeoniaceae) species[14].

Phytochemical studies[15], conducted on 11 rose species distributed in the Eastern, Southern and Western regions of Türkiye, reported the total phenolic contents, vitamin C levels, and antioxidant capacities of the fruits of *Rosa pisiformis*, *Rosa canina*, *Rosa villosa* and *Rosa dumalis* subsp. Antalyensis. Antibacterial and antioxidant activities were also determined.

In recent years, the optimization of compounds such as betulinic acid, oleanolic acid, catechin, etc., has been carried out using techniques such as microwave-assisted extraction, high-pressure liquid extraction, and response surface methodology with Box-Behnken design[16–21]. Gandhi et al.,[16] investigated the optimal conditions for extracting betulinic acid from the bark of *Dillenia indica* Linn. using microwave-assisted extraction and response surface methodology. Xiang et al.[17] investigated the extraction of phenolic-enriched compounds from olive leaves by evaluating the effects of temperature, solvent-to-solid ratio, and milling speed on extraction efficiency using response surface methodology and Box-Behnken design. Khoshsima et al.[18] aimed to optimize the ultrasound-assisted extraction of betulinic acid, oleanolic acid, and ursolic acid from Iranian jujube using response surface methodology using the Box-Behnken Design to model the effects of ultrasonic bath temperature, sonication time, and liquid-to-solid ratio on extraction efficiency. Wang et al.[19] used a green extraction method using high-efficiency hydrophobic deep eutectic solvents (HDES) to extract and recover the hydrophobic compound betulin from *Celtis sinensis* leaves. Naseem et al.[20] prepared choline chloride-based deep eutectic solvents (DES) using ethylene glycol and malic acid for extracting bioactive compounds from *Mentha arvensis* via ultrasound-assisted extraction, optimizing parameters through Box-Behnken design. Gao et al.[21] employed a green and efficient microwave and gravity-assisted solvent-free distillation followed by extraction method that utilizes in situ water from fresh *Melaleuca bracteata* leaves and optimized operational parameters through single-factor tests and Box-Behnken design to extract essential oil, oleanoic acid, 3-O-acetyloleanolic acid, and betulinic acid.

While it was found that *Rosa pisiformis* fruits had the highest α-tocopherol (17.60 mg/g) content and a fructose ratio of 17.20 mg/g in fruits[22], there is currently no literature data available on the amounts of betulinic acid, ursolic acid and phenolics. Therefore, in the present study, we aimed to quantify betulinic acid, ursolic acid, and phenolics in various organs such as roots, stems, leaves, and fruits of our endemic species using the Box-Benchen Method with a focus on their cytotoxic activities.

Experimental section

Plant material

Plant materials (root, stem, leaf, and fruit) of *Rosa psiformis* (Christ.) D. Sosn., were collected from Gümüşhane (Akçakale, Gümüşhane-Turkey; coordinates 40°26′15.9″N, 39°30′46.8″E) and air-dried at room temperature (25 °C) for two weeks. A voucher specimen with code number of 44010 is deposited at Ege University Herbarium Center. After drying, the plant materials were ground and stored at room temperature (25 °C) until extraction.

Chemicals and standards

Ethanol (HPLC grade), dichloromethane, hexane, and standards of betulinic acid, oleanolic acid and ursolic acid and catechin, rutin and quercetin phenolics were sourced from (Sigma-Aldrich, Germany).

Cell lines for anticancer activity

Human prostate cancer cell line PC3 (PC-3) and A549 human lung carcinoma epithelia cells were treated with DMEM High Glucose (Capricorn CP 40–1309), 10% Fetal Bovine Serum (FBS, A0500-3010, Cegrogen Biotech, Almanya), 0.5% Gentamisin 10 mg/mL (A2712 Merck, Almanya), Sodyum pirüvat 100 mM (L0473 Merck, Almanya). Negative Control consisted of 1% Dimethylsulfoxide (DMSO) in nutrient medium while the blank was the cultured prepared with cell culture medium without test substance.

Methods

Extraction and Box-Behnken method

In brief, each of roots, stems, leaves, and fruits, collected from Gümüşhane, the natural distribution area of Rosa pisiformis subsp. pisiformis, was subjected to extraction using three solvents (hexane, dichloromethane, and ethanol) at three concentrations (65%, 80%, and 95%), maceration durations (1, 3, and 5 days), and solvent volumes (50, 75, and 100 mL). In this way, a total of 180 experiments were conducted, with 45 experimental designs for each organ (root, stem, leaf, and fruit). Each extract was filtered and evaporated to dryness using a rotary evaporator.

A Box-Behnken[45] factorial trial design was conducted for each solvent and drug to optimize the extraction process[23]. The Box-Behnken statistical experimental design is a model developed to investigate the relationship between independent variables and response functions, as well as to predict optimal conditions. This method requires fewer experiments compared to other response surface methodology designs and demonstrates efficiency at intermediate levels that have not been experimentally explored. In this study, a three-factor, three-level Box-

Behnken statistical design was applied to optimize the selected independent variables during the extraction process. Design Expert 13.0 (https://www.statease.com/software/design-expert/) was utilized to facilitate the analysis and generate the figures. The independent variables selected for optimization were solvent volume (X1), solvent concentration (X2), and maceration time (X3). The range of these variables was determined based on preliminary experiments, and each variable was coded at three levels: -1, 0, and 1, representing low, medium, and high values. The experimental design consisted of 15 trials with 12 different combinations of coded levels and three central points (0,0,0), where all independent variables were set at their medium levels. These trials were repeated with three different solvents. This method establishes a mathematical relationship between the response function (Y) and the independent variables (X) using a second-order polynomial equation: $Y = b0 + b1X1 + b2X2 + b3X3 + b12X1X2 + b13X1X3 + b23X2X3 + b11X12 + b22X22 + b33X32$. Here, Y represents the yield, b0 is the constant, b1, b2, and b3 are the linear coefficients, b12, b13, and b23 are the cross-product coefficients, and b11, b22, and b33 are the quadratic coefficients.

The extraction process was carried out by maceration of 1 g drug material in a 300 ml percolator, repeated one, three and five times. The drug/solvent ratio (DSR) (65%, 80%, 95%), types of solvent (STG) (ethanol, dichloromethane, hexane) and solvent contents (50, 75, 100) were evaluated for the root, stem, leaf and fruit (Table 1). Dichloromethane and hexane solutions were prepared by dilution with ethanol. Each factor was tested in 15 trial designs, with low, medium, and high levels (−1, 1, and 0) evaluated in triplicate. The coded value was calculated as follows: Coded value = [Actual value − 0.5 × (high value + low value)]/0.5 × (high value – low value).

The DSR factor was evaluated at levels of 2 and 6, using 10% of the powdered drug; the STG was evaluated at levels of 65%, 80%, and 95%. The solvent was divided into three equal parts (250 ml). The weighed drog was left to macerate in the solvent for 24 h. After opening the percolator, the extract was transferred to a covered container, and a fresh portion of solvent was added, allowing a total of 72 h (3 × 24 h) of static maceration. All three portions were combined, homogenized, filtered through quantitative filter paper, and evaporated to dryness using a rotary evaporator. The percentage yield was then calculated, and subsequent experiments for the quantification of oleanolic, ursolic, and betulinic acids were performed. Maceration trials were carried out in triplicate, and the results were reported as the mean ± standard error. Data were statistically analyzed using a t-test with a significance level of $p < 0.05$.

Validation of the Method: The method for the analysis of oleanolic, ursolic, and betulinic acids was validated for selectivity, linearity, range, precision, accuracy, quantification limits, detection, and robustness parameters. Selectivity was assessed by comparing the retention times of the peaks in the sample with those obtained from oleanolic, ursolic, and betulinic acid standards. UV scanning at 215 nm confirmed the purity of the peaks. The areas corresponding to the standard and plant drug concentration curves were used for linearity analysis. The linear correlation coefficient (r), coefficient of determination (r2), and the linear equation y = ax + b was calculated for statistical tests.

Selection of the extraction solvent is a critical aspect of plant analysis. The optimal solvent must ensure quantitative extraction of the target analytes while being sufficiently selective to avoid excessive extraction of other compound groups that may interfere with the analytical process. Using solvents of varying polarities (methanol, dichloromethane, hexane), we ensured the best yields and optimal solvents for the extraction of quercetin, rutin, catechin, and betulinic, ursolic, and oleanolic acids.

HPLC analysis of compounds

Root, stem, leaf, and fruit samples (1 g) were extracted by shaking with 30 mL of hexane, dichloromethane, and methanol, followed by centrifugation at 10,000 xg for 5 min. A 2 mL aliquot from this extract was evaporated in an evaporation flask and reconstituted with 10 mL of methanol. The resulting extract was filtered using a 0.2 mm PVDF membrane filter and diluted with methanol prior to HPLC analysis. The calibration curve was established using betulinic acid, oleanolic acid, ursolic acid, and phenolic compounds (quercetin, catechin, and rutin) in methanol at concentrations ranging from 10 ng/mL to 1000 ng/mL. HPLC analyses were be conducted using Agilent 1260 Infinity II machine with an Agilent Eclipse XDB-C18 column (5 µm, 4.6 × 150 mm) through outsourced services at EGE MATAL. The limits of detection (LOD) and quantification (LOQ) for different compounds analyzed by HPLC are as follows: for ursolic acid, the LOD is 0.06 µg/mL and the LOQ is 0.19 µg/mL; for catechin, the LOD is 0.09 µg/mL and the LOQ is 0.29 µg/mL; for quercetin, the LOD is 3.18 µg/mL and the LOQ is 10.61 µg/mL; for rutin, the LOD is 4.38 µg/mL and the LOQ is 14.61 µg/mL; and for betulinic acid, the LOD is 5.06 µg/mL and the LOQ is 16.87 µg/mL.

Tri terpenes

The HPLC method was conducted using an Agilent 1260 Infinity II device equipped with an Agilent Eclipse XDB-C18 column (5 µm, 4.6 × 150 mm). The mobile phase consisted of 50% acetonitrile and 50% ethanol. The

	Factor Level			Dependent variables
Independet variables	−1	0	1	
Solvent (V)	50	75	100	Total content (mg)
Maceration Time (day)	1	3	5	
Solvent Percantage (%)	65	80	95	

Table 1. Extraction variables selected for Box-Bohnken Design (BBD) optimization.

detection wavelength was set to 210 nm, with the oven temperature maintained at 25 °C. The flow rate was 0.5 mL/min, and the injection volume was 20 µL.

Phenolics

Phenolics were detected using the HPLC method with the following conditions: an Agilent 1260 Infinity II device, equipped with an Agilent Eclipse XDB-C18 column (5 µm, 4.6×150 mm). The mobile phase consisted of water containing 0.2% phosphoric acid (80%) and acetonitrile (20%). The detection wavelength was set to 210 nm, with the column oven temperature maintained at 25 °C. The flow rate was 1 mL/min, and the injection volume was 20 µL.

Anticancer activity study

The root, stem, leaf, and fruit drogs (100 g) were subjected to extraction using the cold extraction technique with hexane, dichloromethane, and methanol solvents, following the Box-Behnken method as previously described. The extracts obtained from each sample with the three different solvent systems were filtered using a 0.2 mm PVDF membrane filter and was sent to EGE-MATAL center (İzmir, Türkiye) for cytotoxicity analysis.

The cytotoxicity test was performed using the MTT assay on A549 and PC3 cell lines. The optimized plant material was applied to the cells for 24 h in an environment of 5.0% CO2 and 95% humidity at 37 °C. The cells were seeded at a concentration of 1×10^5 cells/mL in 96-well microplates. The cytotoxic effect was evaluated by measuring the absorbance at 570 nm using a spectrophotometer, following the MTT assay protocol (ISO 10993-5:2009).

Results and discussion

HPLC analysis of flavonoids and tri terpenes

The optimised dichlorometane and ethanol extracts of root, stem, fruits, and hexane extracts of fruit and leaf, contained the phenolics catechin, rutin and quercetin. However, rutin was not determined in the stem and roots of *R. pisiformis* subsp. p*isiformis* (Fig. 1 and Supplementary Fig. S1-S4 online). Betulinic and ursolic acids were the terpenes found in the stems of *R. pisiformis* subsp. *pisiformis* (Table 2). Oleonolic acid was not identified in any of the plant organs.

Cytotoxicity results

Cytotoxic activity was not determined only in the DCM stem extract of *R. pisiformis* subsp. *Pisiformis,* which was rich in catechin (3.56 µg/mg), rutin (28.96 µg/mg), and quercetin (39.90 µg/mg). However, cytotoxic activity was detected in the hexane extracts of root, which were rich in catechin (9.43 µg/mg), quercetin (2.32 µg/mg), betulinic acid (9.72 µg/mg) and ursolic acid (45.90 µg/mg), as well as in the stem, which contained catechin (0.07 µg/mg), quercetin (2.94 µg/mg), betulinic acid (11.84 µg/mg), and ursolic acid (206.40 µg/mg). Additionally, DCM leaf extract, which was rich in catechin (15.61 µg/mg), rutin (12.80 µg/mg) and quercetin (20.70 µg/mg), showed cytotoxic activity based on the absorbances readings at 570 nm (Fig. 2).

Box-Benchen experimental design

The Box-Behnken design was employed to assess the effects of operational parameters such as maceration time, solvent volume, and solvent concentration on extraction yields (Supplementary Fig. S5 -S21 online). Table 2 presents the independent variables and their corresponding values for the Box-Behnken design. The results of the Box-Behnken statistical design, presented in Table 3, show the experimental outcomes under various conditions determined by the low, medium, and high levels of independent variables. The near-identical results obtained at the central point indicate the reproducibility of the data. The correlation between observed and predicted results was also calculated, which is crucial for verifying the method's applicability. The experiment running was showed in a raw with its responses [total content (mg) (Y1) and fenolic, terpenoid and flavonoid content (Y2)]. The reliability of the model fit was evaluated through the coefficient of variance (CV%), coefficient of determination (R2) and adjusted coefficient of determination (R2a). The modal capacity was determined by these tests to give the maximum mg and phenolic and terpenoid content. By examining the total content amount, the highest dry matter yield was obtained with 65% solvent concentration, 75 mL solvent volume, and 5 days of maceration (Table 3).

The optimum total content was found to be 51.26 mg/g in the fruit extracted with ethanol, 40.93 mg/g in the stem extracted with dichloromethane, and 44.28 mg/g in the fruit extracted with hexane. The results were obtained from a five-day maceration using 65% ratio and a 75 ml volume of solvent (Table 3 and Supplementary Fig. S5 - S16 online).

For each response, the sequential model sum of squares and lack-of-fit tests were performed for quadratic, cubic, 2-factor interaction, and linear models. While factor B2 had a directly proportional effect, C2 had an inversely proportional effect and was highly significant. A study was conducted to optimize the extraction process of phenolic and terpenoid compounds from the aerial parts of *Rosa psiformis*. The experiment involved four models (quadratic, cubic, 2-factor interaction, and linear) to determine the maximum yield, as well as phenolic and terpenoid content. The effects of the factors and their interactions on each response were evaluated using analysis of variance. The results indicated that maceration time had the highest effect on increasing the total content, while the solvent and plant had a significant effect on the total phenolic content obtained.

The model was validated through model accuracy and multiple response optimization. The maximum total content was achieved with 75 ml of 65% solvent over five days. The maximum phenolic content was found to be 206.40 µg/mg ursolic acid and 72.42 µg/mg phenolics (dichloromethane), and 11.84 µg/mg betulinic acid (hexane) in stem. The actual results were in close agreement with the predicted values, confirming the adequacy of the response model (Supplementary Table S1 and Table 4 and Supplementary Fig. S17 – S21 online).

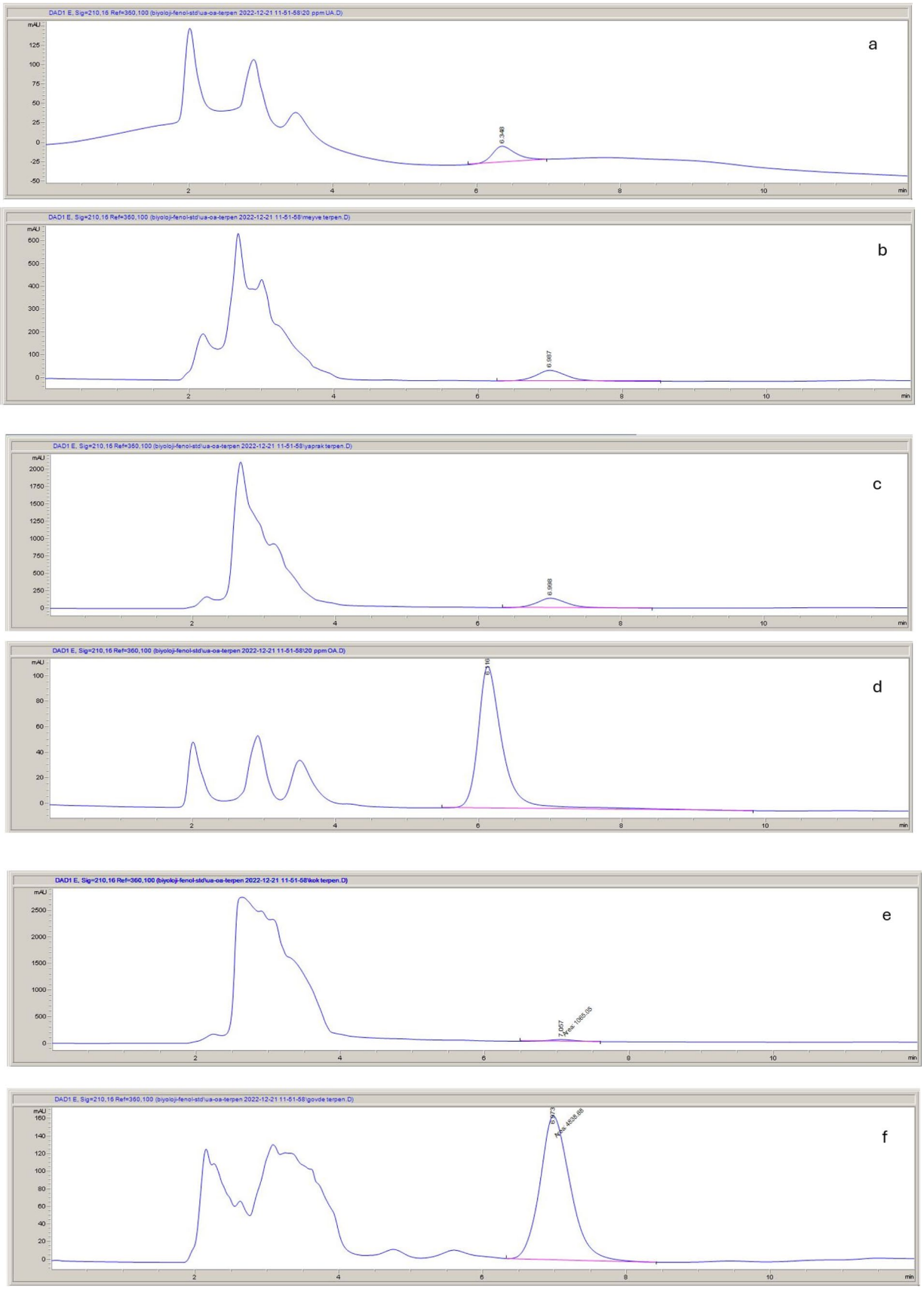

Fig. 1. HPLC chromatogram of *R.pisiformis* ethanolic, dichloromethane and hexane extracts. HPLC chromatograms were original outputs and obtained using an Agilent 1260 Infinity II machine with an Agilent Eclipse XDB-C18 column. (**a**) Ursolic acid, (**b**) fruit (hexane), (**c**) leaf (hexane), (**d**) oleonolic acid, (**e**) root (hexane), (**f**) stem (hexane).

Solvent extracts	Catechin (µg/mg)	Rutin (µg/mg)	Quercetin (µg/mg)	Betulinic acid (µg/mg)	Ursolic acid (µg/mg)
DCM Fruit	3.65	0.93	23.97	ni	ni
DCM Leaf	**15.61**	12.80	20.70	ni	ni
DCM Stem	3.56	**28.96**	**39.90**	ni	ni
DCM Root	13.87	18.99	14.24	ni	ni
ETOH Fruit	1.30	1.09	9.71	ni	ni
ETOH Leaf	6.46	19.50	7.41	ni	ni
ETOH Stem	7.13	10.05	11.51	ni	ni
ETOH Root	1.57	0.30	3.45	ni	ni
Hexane Fruit	0.94	0.66	2.71	ni	59.10
Hexane Leaf	1.45	0.18	2.95	ni	171.50
Hexane Stem	0.07	ni	2.94	**11.84**	**206.40**
Hexane Root	9.43	ni	2.32	9.72	45.40

Table 2. Phenolics (catechin, rutin, quercetin), Betulinic and Ursolic acids content of *R. pisiformis* subsp. *pisiformis* extracts. DCM: dichloromethane, EtOH: ethanol, ni: not identified.

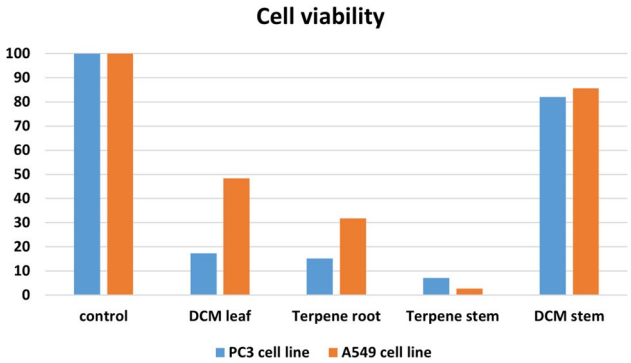

Fig. 2. Cell viability results. Control, DCM Leaf, Root (hexane), Stem (hexane), DCM Stem.

Discussion

In the world population approaching eight billion[24], the fruits of rose plants (*Rosa* sp) are rich functional foods containing polyphenols (flavonoids, proanthocyanidins, catechins), triterpene acids, essential fatty acids, galactolipids, folate, vitamins A, C, and E, and minerals (Ca, Mg, K, S, Si, Se, Mn, and Fe)[25]. The amount of ascorbic acid, which is beneficial for Alzheimer's and cancer diseases, is 1200 mg/l for *R. rugosa* and 600 mg/l for *R. canina* fruits[26]. The main bioactive compounds isolated from this genus are flavonoids (e.g. kaempferol, quercetin, apigenin), triterpenoids (e.g. ursolic acid, tormentic acid, euscaphic acid, betulinic acid), and phytosterols (e.g. β-sitosterol)[27–34]. Triterpenes in rose fruits were determined as oleonolic acid, betulinic acid and ursolic acid[35].

R. canina L. fruit powders showed an immunomodulatory effect on human monocytic Mono Mac 6 cells. Triterpenic acid mixture (oleanolic, ursolic and betulinic acid) inhibited the release of IL-6 and TNF-α[36]. Botanical pentacyclic triterpenes have antioxidant, anti-tumor, anti-microbial and anti-inflammatory effects[37]. The mechanism of these bioactivities is believed to be due to the regulation of the immune system. For instance, treatment with a combination of natural compounds yields better responses than treatment with synthetic drugs alone. An example is the combination of escin, isolated from the seeds of *Aesculus hippocastanum* L., with corticosterone, which reduces the synthesis of inflammatory markers such as tumor necrosis factor alpha (TNF-α) by 24.43%, interleukin 1-beta (IL-1β) by 46.9%, and nitric oxide (NO) by 31.8%[38]. Another study demonstrated that a mixture of oleanolic, ursolic, and betulinic acid obtained from *Rosa canina* L. has a more effective immunomodulatory effect than any single compound[35]. Additionally, it has been proven that a standard powder prepared from rosehip seeds and peels improves cell aging and reduces skin wrinkles by accumulating in the membranes of erythrocyte cells[39].

Ursolic, oleanolic, and betulinic acids are biologically active compounds in rosehip, acting as immune system modulators[11,13,40,41]. Among the triterpenic acids, betulinic acid was found in concentrations ranging from 36 to 772 mg/kg, oleanolic acid from 66 to 1723 mg/kg, and ursolic acid from 37 to 2531 mg/kg, as determined by negative ionization model. The amount of triterpenic acids varies between genotypes, species, and sections. The highest triterpene value, 4600 mg/kg, was observed in *R. spinosissima*. *R. pulverulenta*, which contains the lowest levels of carotenoids, tocopherols, and flavonoids, has moderate triterpene acid content, with betulinic acid at 213 mg/kg, oleanolic acid at 1045 mg/kg, and ursolic acid at 452 mg/kg[42]. Our results showed 11.84 µg/mg betulinic acid in the stem of *R. pisiformis* subsp. *pisiformis*, 9.34 µg/mg in the roots, 206.40 µg/mg ursolic

Std	Run	Factor 1: Solvent (mL)	Factor 2: Maceration Time (day)	Factor 3 : Solvent Percantage(%)	R1 + SD (total content "mg/g") (ethanol/water)				R2 + SD (total content "mg/g") (Dichloromethane/ethanol)				R3 + SD (total content "mg/g") (hexane/ethanol)			
					Stem	Root	Leaf	Fruit	Stem	Root	Leaf	Fruit	Stem	Root	Leaf	Fruit
4	1	100	5	80	22.78 ± 1.94	24 ± 2.15	37.2 ± 2.89	44.5 ± 3.21	35 ± 3.37	35 ± 2.55	17.86 ± 1.87	32.5 ± 1.88	34.25 ± 2.46	15 ± 1.82	20.8 ± 1.73	43.5 ± 1.78
14	2	75	3	80	19.41 ± 1.94	20.12 ± 2.15	33.85 ± 2.89	42.2 ± 3.21	31 ± 3.37	33.85 ± 2.55	15.06 ± 1.87	30.7 ± 1.88	28.56 ± 2.46	13.2 ± 1.82	19 ± 1.73	41.8 ± 1.78
6	3	100	3	65	23.06 ± 1.94	23.98 ± 2.15	38.2 ± 2.89	45 ± 3.21	36 ± 3.37	35.26 ± 2.55	18 ± 1.87	33 ± 1.88	31 ± 2.46	15.5 ± 1.82	21 ± 1.73	43.92 ± 1.78
5	4	50	3	65	23.02 ± 1.94	25.03 ± 2.15	36.86 ± 2.89	44.1 ± 3.21	34.36 ± 3.37	34 ± 2.55	17 ± 1.87	32 ± 1.88	30 ± 2.46	14.89 ± 1.82	20.2 ± 1.73	43 ± 1.78
11	5	75	1	95	19.2 ± 1.94	20.12 ± 2.15	33.42 ± 2.89	41 ± 3.21	30 ± 3.37	31 ± 2.55	14.42 ± 1.87	29.5 ± 1.88	28 ± 2.46	12.68 ± 1.82	18.75 ± 1.73	40 ± 1.78
15	6	75	3	80	19.64 ± 1.94	20.56 ± 2.15	34 ± 2.89	42 ± 3.21	31 ± 3.37	33.12 ± 2.55	15.5 ± 1.87	30.5 ± 1.88	29.02 ± 2.46	13.01 ± 1.82	19.1 ± 1.73	41.75 ± 1.78
7	7	50	3	95	19.63 ± 1.94	19.92 ± 2.15	33 ± 2.89	40 ± 3.21	30 ± 3.37	31.25 ± 2.55	14.78 ± 1.87	30.42 ± 1.88	28.03 ± 2.46	12.44 ± 1.82	18.7 ± 1.73	40.5 ± 1.78
13	8	75	3	80	19.98 ± 1.94	21 ± 2.15	34.68 ± 2.89	42.4 ± 3.21	32.25 ± 3.37	31.85 ± 2.55	16 ± 1.87	31 ± 1.88	28 ± 2.46	13.44 ± 1.82	19.38 ± 1.73	42 ± 1.78
10	9	75	5	65	24.2 ± 1.94	25.87 ± 2.15	42.88 ± 2.89	51.26 ± 3.21	40.93 ± 3.37	40.11 ± 2.55	20.95 ± 1.87	36.38 ± 1.88	36.02 ± 2.46	18.03 ± 1.82	23.4 ± 1.73	44.28 ± 1.78
9	10	75	1	65	19.25 ± 1.94	20 ± 2.15	33.5 ± 2.89	39.68 ± 3.21	30 ± 3.37	31.69 ± 2.55	14.87 ± 1.87	30.1 ± 1.88	28.63 ± 2.46	12.5 ± 1.82	18.65 ± 1.73	39.98 ± 1.78
3	11	50	5	80	23.56 ± 1.94	24.02 ± 2.15	38.66 ± 2.89	45.15 ± 3.21	36.25 ± 3.37	35.56 ± 2.55	18.21 ± 1.87	33.2 ± 1.88	30.22 ± 2.46	15.03 ± 1.82	21.1 ± 1.73	40 ± 1.78
2	12	100	1	80	19.5 ± 1.94	20 ± 2.15	33.65 ± 2.89	39.75 ± 3.21	30.25 ± 3.37	31 ± 2.55	15 ± 1.87	30.15 ± 1.88	27.96 ± 2.46	11.02 ± 1.82	18.8 ± 1.73	40.2 ± 1.78
1	13	50	1	80	18.23 ± 1.94	19.89 ± 2.15	31.5 ± 2.89	37.69 ± 3.21	27.65 ± 3.37	30 ± 2.55	13.89 ± 1.87	28.56 ± 1.88	27.36 ± 2.46	10.98 ± 1.82	15.4 ± 1.73	38 ± 1.78
12	14	75	5	95	20.36 ± 1.94	23.1 ± 2.15	35 ± 2.89	43.5 ± 3.21	33.25 ± 3.37	32.69 ± 2.55	16.85 ± 1.87	31.42 ± 1.88	29.32 ± 2.46	14.24 ± 1.82	19.85 ± 1.73	42.85 ± 1.78
8	15	100	3	95	20.03 ± 1.94	21.2 ± 2.15	34.8 ± 2.89	42.95 ± 3.21	32.87 ± 3.37	32.25 ± 2.55	16.25 ± 1.87	31.1 ± 1.88	28.35 ± 2.46	13.63 ± 1.82	19.5 ± 1.73	42.2 ± 1.78

Table 3. The Box-Behnken response surface design and corresponding response values.

p-values shading	Intercept	A	B	C	AB	AC	BC	A^2	B^2	C^2
Dichloromethane (stem)	31.42	0.73	3.44	− 1.90	− 0.96	0.31	− 1.92	0.32	0.55	1.57
p-values		0.03	< 0.0001	0.00	0.04	0.41	0.00	0.42	0.18	0.01
Ethanol (stem)	19.68	0.12	1.84	− 1.29	− 0.51	0.09	− 0.95	1.01	0.33	0.75
p-values		0.60	0.00	0.00	0.14	0.77	0.02	0.02	0.33	0.06
Hexane (stem)	28.53	0.74	2.23	− 1.49	0.86	− 0.17	− 1.52	0.14	1.28	0.68
p-values		0.04	0.00	0.00	0.07	0.67	0.01	0.74	0.02	0.14
Dichloromethane (root)	32.94	0.34	2.46	− 1.73	− 0.39	− 0.065	− 1.68	− 0.37	0.32	0.62
p-values		0.29	0.00	0.00	0.37	0.88	0.01	0.42	0.48	0.20
Ethanol (root)	20.56	0.04	2.12	− 1.32	− 0.03	0.58	− 0.72	0.84	0.58	1.13
p-values		0.90	0.00	0.01	0.94	0.24	0.16	0.13	0.26	0.06
Hexane (root)	13.22	0.23	1.89	− 0.99	− 0.02	0.145	− 0.99	− 0.23	0.02	1.13
p-values		0.14	< 0.0001	0.00	0.93	0.46	0.00	0.28	0.92	0.00
Dichloromethane (leaf)	16.31	0.40	1.96	− 1065.00						
p-values		0.20	< 0.0001	0.00						
Ethanol (leaf)	34.18	0.48	2.71	− 1.90	− 0.90	0.115	− 1.95	0.30	0.78	1.24
p-values		0.04	< 0.0001	0.00	0.01	0.66	0.00	0.30	0.03	0.00
Hexane (leaf)	19.16	0.59	1.69	− 0.81	− 0.925	0.00	− 0.91	− 0.22	0.09	0.91
p-values		0.01	< 0.0001	0.00	0.01	1.00	0.01	0.33	0.69	0.01
Dichloromethane (fruit)	30.73	0.32	1.90	− 1.13	− 0.57	− 0.08	− 1.09	0.07	0.29	0.82
p-values		0.09	< 0.0001	0.00	0.05	0.73	0.00	0.75	0.25	0.01
Ethanol (fruit)	42.20	0.66	3.29	− 1.57	− 0.68	0.51	− 2.27	− 0.64	0.21	1.45
p-values		0.01	< 0.0001	0.00	0.04	0.10	0.00	0.06	0.46	0.00
Hexane (fruit)	41.85	1.04	1.56	− 0.70	0.325	0.195	− 0.36	− 0.40	− 1.03	0.95
p-values		0.01	0.00	0.04	0.41	0.61	0.36	0.34	0.04	0.05

Table 4. P-Values and estimated regression Coefficients of the two studied responses. *Statistically significant: $p < 0.05$.

acid in the stem, 171.50 µg/mg in the leaves, 59.10 µg/mg in the fruit, and 45.40 µg/mg in the roots, which are significantly higher than the 662–1078 mg/kg of betulinic acid in the fruit of *Rosa canina* reported by Schwager et al.[43] in their study on the biological activities of rose hip powder.

Kerasioti et al.[12] conducted a study to quantify the bioactive compounds present in extracts from the dried fruits of *Rosa canina* and *Rosa semprevirens*. In their study, Kerasioti et al. found that *Rosa canina* had significantly higher levels of (+)-Catechin (134.75 µg/g) and Rutin (25.64 µg/g) compared to *Rosa semprevirens* (25.48 µg/g and 2.62 µg/g, respectively). In contrast, our study revealed that *Rosa pisiformis* had lower catechin content (3.65 to 15.61 µg/mg) but higher Rutin concentration, particularly in the DCM Stem (28.96 µg/mg). Moreover, quercetin levels were much higher in *Rosa pisiformis*, reaching up to 39.90 µg/mg in the DCM Stem, while Kerasioti et al.[12] reported lower levels for both *Rosa canina* (0.67 µg/g) and *Rosa semprevirens* (0.19 µg/g). Additionally, while betulinic acid was detected in *Rosa pisiformis* (11.84 µg/mg in Hexane Stem and 9.72 µg/mg in Hexane Root), it was present only at low levels in *Rosa canina* (0.47 µg/g). Ursolic acid was significantly more abundant in *Rosa pisiformis*, reaching up to 206.40 µg/mg, while it was undetected in *Rosa semprevirens* and found at low levels in *Rosa canina*.

Our catechin content (3.65 to 15.61 µg/mg) is comparable to that reported by Peña[8] for *R. canina and R. rubiginosa* (14.3–45.7 mg/g), while being significantly higher than the range reported by Elmastaş et al.[44] (0.225–0.472 mg/g) for *Rosa dumalis, R. canina*, and *R. villosa;* by Jiménez et al.[45] (1.90 µg/g to 237.00 µg/g) for *R. pouzinii*, *R. corymbifera*, *R. glauca* and *R. canina*; by Demir et al.[46] (7.18 µg/g to 50.46 µg/g) for *R. canina*, *R. dumalis*, *R. gallica*, *R. dumalis*, and *R. hirtissima*; by Nađpal et al.[47] (2.37–7.83 µg/g) for *Rosa canina* and *Rosa arvensis*; by Butkevičiūtė et al.[48] (26.30–522.48 µg/g) for the Rosa subcanina species.

The variations in bioactive compound content and concentrations in rosehip fruits may result from several factors, primarily species differences and geographical collection areas. Genetic diversity within the Rosa species contributes to qualitative and quantitative differences in total phenolic and flavonoid content, while environmental factors such as light, temperature, and soil nutrients play critical roles[46,48,49]. These differences indicate that Rosa pisiformis may be a more potent source of certain bioactive compounds compared to the other species studied.

Some medically important secondary metabolites with antibacterial, anticancer, astringent, depurative and anti-inflammatory activities include polysaccharides, flavonoids, steroids, tannins, laevigatins (E, F, G), triterpenoids, 11α-hydroxytormentic acid, 2α-methoxyursolic acid, 6-methoxy-β-glucopyranosyl ester, tormentic acid and 5α-diol 3-O-β-d-glucopyranosides were determined[50].

In addition to flavonoids, triterpenoids eucaphic acid, nigaikigoside, betulinic acid, kajiikigoside, rubusid, tomentic acid and rosamutin were obtained from *Rosa laevigata* roots[51]. The presence of ursan-type triterpenes in the root of *Rosa cymosa* Tratt, used in Chinese herbal medicine, was determined qualitatively and quantitatively

by HPLC and TLC[52]. Two new triterpenes, 19-oxo-18,19-seco-ursan-type lactone saponin and oleanin-type triterpenoid with anti-inflammatory effects, were obtained from ethylacetate extracts of *Rosa laevigata* leaves[53]. *Rosa rugosa* var. *plena* and *R. woodsii* leaves were found to be rich in sesquiterpenes[54]. It has been reported that flavonoids, carotenoids and fatty acids in *Rosa canina* pseudo fruits have anti-inflammatory activity[55]. In the fruits of *Rosa pseudofructus cum/sine fructibus* and *Rosa canina* L., triterpenoid acids including ursolic, oleanolic and betulinic acids were detected in hexane and dichloromethane extracts, and these acids inhibited cyclooxygenases[11]. Fruits of *R. pisiformis* subsp. *pisiformis* were found to be rich in ursolic acid (59.1 µg/mg, and also contained catechin (3.65 µg/mg), rutin (1.09 µg/mg) and quercetin (23.97 µg/mg). Betulinic acid was not detected in the fruits of *R. pisiformis* subsp. *pisiformis*. Cytotoxic activity was observed only in the leaf, root and stem of our endemic species *R. pisiformis* subsp. *pisiformis*.

Rose species, including rose hips, are wild plants primarily originating in Asia with less occurrences in Europe, North America, and Northwest Africa. These plants contain medicinal compounds traditionally used to treat various diseases. While it is widely distributed in Türkiye with 27 *Rosa* species, there are two endemic species. Of these, *Rosa pisiformis* subsp. *pisiformis* (Christ.) D. Sosn. is endemic to Northeast and East Anatolia, and in the provinces of Kars, Ağrı, Erzincan, Erzurum, Gümüşhane, Bayburt. *Rosa dumalis* BECHST subsp. *boissieri* (CREPIN) Ö. NILSSON. var. *antalyensis* (MANDEN.) Ö. NILSSON, on the other hand, is naturally distributed in Southwest Anatolia.

Rosa pisiformis (Christ.) D. Sosn, a medicinal perennial shrub in the Rosaceae family, a medicinal perennial shrub in the Rosaceae family, is endemic to various regions, including the evil eye rose around Istanbul and Van, rosehip in the town of Güzelsu, Gürpınar district of Van, and Şilan in the Malazgirt district of Muş[56,57]. This species is originated from different parts of Europe, Asia and the America's and Northwest Iran. The leaves and fruits of the plant are rich in vitamins (A, E, C), trace elements (Fe, Cu, Zn, Mn, Cr, Se) and minerals (Ca, K, Mg, Na). It is stated that it has protective effects on heart tissue antioxidant enzymes (GSH-Px, SOD, CAT) during stress[58]. Leaves and fruits of *Rosa pisiformis* subsp. *pisiformis* (Christ.) D. Sosn, found in Van province, are rich in vitamins (A, E, C), trace elements (Fe, Cu, Zn, Mn, Cr, Se), and minerals (Ca, K, Mg, Na). It has also been reported to have protective effects on cardiac tissue by enhancing antioxidant enzymes (GSH-Px, SOD, CAT) during oxidative stress induced by isoproterenol[58]. It was desmostrated that treatment with *Rosa pisiformis* (Christ.) D. Sosn. (R.PS) fruits reduced oxidative damage and improved the levels of Zn, Mn, Co, Mg, and Na in rat heart tissue. Moreover, the pharmacological effects of *R. pisiformis* fruit are attributed to its phytochemical composition, including caffeic and p-coumaric acids[59].

Betulinic acid, an immune system modulator, has been shown to have anticancer effects in *Rosa canina* L.[37]. It was demonstrated that betulinic acid mitigated toxin-induced lung damage by reducing oxidative stress, inhibiting inflammation, and decreasing apoptosis through down-regulation of the mitochondrial apoptotic pathway[60]. The fact that the seeds of *Rosa canina* and *R. pisiformis* contain higher amounts of fatty acids than their fruits[22,61] is important because fatty acids have the property of controlling the growth of pathogens and cancer cells[13,62]. In addition, another important feature of *Rosa pisiformis* seeds and fruits is that their vitamin E content is higher than that of *Rosa canina*. Specifically, the amounts of δ- and α-tocopherol were (17.60 µg/g) in *R. pisiformis*, whereas in *R. canina*, they are only 7.15 µg/g, indicating a significantly lower level[22].

The investigation of *Rosa* species for their vitamin C, polyphenols, and antioxidant activity[63–65] led us to explore betulinic acid—a triterpenic acid with significant pharmacological effects, such as anticarcinogenic properties— both qualitatively and quantitatively in different organs of the endemic *Rosa pisiformis* subsp. *pisiformis* (Christ.) D. Sosn, including the root, stem, leaf, and fruit. According to the Box-Benchen method, the optimisation data indicated a solvent ratio of 65% and five maceration cycles with 75 ml of solvent. The highest amount of catechin was found in the leaf (DCM) at 15.61 µg/mg. The stem was found to be rich in rutin (28.96 µg/mg) and quercetin (39.90 µg/mg). Betulinic acid was detected for the first time in the stem (11.84 µg/mg) and root (9.72 µg/mg) in hexane extracts.

Our results clearly shows that the extraction efficiencies of phenolic compounds (catechin, rutin, quercetin) and triterpenic acids (betulinic and ursolic acids) varied significantly among the solvents of ethanol, DCM and hexane. DCM exhibited superior efficiency for the extraction of polyphenolic compounds, especially rutin and quercetin. This is likely due to the moderate polarity of DCM, which is well-suited to dissolve a wide range of moderately polar to nonpolar compounds, making it effective for flavonoid extraction. Ethanol, despite being a commonly used solvent for phenolic extraction due to its polarity and ability to dissolve hydrophilic compounds, showed lower efficiency compared to DCM. The reduced efficiency in ethanol extractions might be attributed to the higher polarity of ethanol, which does not solubilize moderately polar compounds as effectively as DCM. Hexane, being a nonpolar solvent, was inefficient at extracting hydrophilic phenolic compounds such as catechin and rutin. The extraction of these polyphenols was low across all plant organs, e.g., catechin in hexane root extract was 9.43 µg/mg, and quercetin was only 2.32 µg/mg. However, hexane excelled in extracting nonpolar compounds like ursolic acid (e.g., 206.40 µg/mg in stem extract), reflecting its strong affinity for lipophilic substances such as triterpenic acids.

The lower overall extraction efficiencies, particularly in the ethanol extractions, can be explained by solvent polarity and solvent-specific affinity. Ethanol is less effective at dissolving moderate hydrophobic phenolic compounds like quercetin compared to the less polar DCM. Its high polarity also limits its ability to extract nonpolar compounds like betulinic and ursolic acids, which are better extracted by hexane. Each solvent's chemical affinities impact extraction efficiency. DCM's intermediate polarity allows it to extract both polar and nonpolar compounds more effectively than ethanol, which primarily extracts hydrophilic substances. Hexane is highly effective in extracting nonpolar compounds like ursolic acid but is less effective for polar phenolics. Different solvents target specific chemical classes, so a single solvent may not achieve high extraction efficiencies for all compounds.

The observed low extraction efficiencies, especially with ethanol, are due to the polarity features between the solvent and the target compounds. These findings align with known solvent affinities in the literature and suggest that solvent polarity plays a critical role in extraction efficiency[66–68].

Cytotoxic activities were most pronounced in the DCM leaf, which is rich in phenolics such as catechin (15.61 µg/mg), rutin (12.80 µg/mg), and quercetin (20.70 µg/mg). Additionally, cytotoxic effects were observed in the root (hexane) and stem (hexane), primarily against prostate and lung carcinoma cells, as determined by the ABTS assay.

Conclusion

The presented study demonstrates the potential of *Rosa pisiformis* subsp. *pisiformis* as an alternative source of pharmacologically significant compounds, particularly betulinic acid. The optimized extraction conditions revealed substantial variation in the yield of phenolic and triterpenic compounds, depending on the solvent and plant organ used. Dichloromethane proved to be the most effective solvent for extracting polyphenolic compounds, while hexane demonstrated better performance in isolating betulinic and ursolic acids from stems and roots. Notably, betulinic acid was detected for the first time in the stems and roots of *R. pisiformis*, providing a new path for the bioactive utilization of this endemic species.

Based on our study, to obtain betulinic acid efficiently from *Rosa pisiformis* plant material, future applications should utilize the Box-Behnken optimized extraction parameters: a 65% solvent ratio and a five-times maceration process with 75 ml of ethanol, dichloromethane, or hexane, depending on the targeted compound. Following these guidelines allows for high-yield extraction, especially from stems and roots, which contain notable concentrations of betulinic acid. These methods provide a reliable approach for isolating betulinic acid and other bioactive compounds from *Rosa pisiformis* with maximized efficiency. For future works, specific *Rosa* plant parts identified as high-yield sources may serve as alternative betulinic acid sources. Additionally, propagation of these specific plant parts through tissue culture could be employed to increase betulinic acid yield, providing an efficient and sustainable approach for betulinic acid production.

The cytotoxic activity observed in various extracts, especially those from stems and leaves, further emphasizes the plant's therapeutic potential. These findings offer a promising foundation for further exploration into the bioactivity and pharmaceutical applications of *R. pisiformis*, positioning it as a valuable source of natural triterpenic acids with anti-cancer properties.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Declarations

Competing interests

The authors declare no competing interests.

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