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Scientific paper

Chemical Profile, Antiproliferative and Antioxidant Activities of *Genista januensis* subsp. *lydia* (Boiss.) Kit Tan & Ziel (Fabaceae)

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Abstract

The aim of this study was to investigate the anticancer and antioxidant activities of *Genista januensis* subsp. *lydia* (Fabaceae) extracts and to isolate the phytochemical compounds responsible for these activities. The *G. januensis* plant was extracted by maceration with solvents of increasing polarity. The total phenolic/flavonoid content, antioxidant activities and anticancer activity were investigated by performing cytotoxicity, apoptosis and cell cycle analyses in crude extracts. We found that the EtOAc extract had the highest antioxidant and anticancer activities and this extract was selected for isolation studies. All purified flavonoid compounds from the EtOAc extract were elucidated using 1D and 2D NMR and mass spectroscopic methods and compared with the literature. A new biflavonoid **4** was isolated together with three known isoflavonoids (genistein (**1**), genistin (**2**) and 4'-hydroxyisoflavone (**3**)) from *Genista januensis* subsp. *lydia* (Fabaceae). MTT analysis of compounds **2**, **3**, and **4** was used to evaluate the anticancer activity. Compound **4** was invesigated for its antioxidant activities. These findings suggest that *G. januensis* subsp. *lydia* represents new potential natural sources of effective antiproliferative and antioxidant agents.

Keywords: *Genista januensis* subsp. *lydia*, biflavonoid, antiproliferative effect, isoflavonoids, antioxidant activity.

1. Introduction

Although there are many damage repair mechanisms and checkpoints in the cell, cancer is very common today and is a multifactorial disease with an unknown cause, underlying many molecular mechanisms in the cell.[1] There are more than 200 known types of cancer. Cancer progression is typically attributed to DNA damage or mutations in proto-oncogenes, involved in cell growth mechanisms, and tumor suppressor genes, responsible for stimulating cell growth and apoptosis.[1]

Natural antioxidants found in foods, such as phytochemicals, have been shown to be beneficial and are associated with a reduced risk of developing various health probems.[2] Flavonoids, one of the major subclasses of dietary polyphenols, have potent antioxidant activities and anti-carcinogenic properties.[3]

Leguminosae (Fabaceae) is a family with a wide variety of phytochemicals and is an important source of flavonoid compounds.[4] Plants of the Fabaceae family, especially *Genista* species, are important for human nutrition and health. *Genista* species have been reported to have anti-inflammatory,[5] anti-acetylcholinesterase,[6] antimicrobial,[7] anti-diabetic,[5] estrogenic/antiestrogenic,[8] antioxidant,[9] analgesic,[5] antihyperglycaemic,[5] and hepatoprotective[10]

activities. The plant *Genista januensis* subsp. *lydia* (Boiss.) Kit Tan & Ziel (Fabaceae) is widespread in the Balkans, Syria and Türkiye. It is also prevalent in Trakya and Central Anatolia (Kırklareli, Istanbul, Kutahya, Bolu, Kastamonu, Bursa, Denizli, Izmir, Yozgat, Hatay, and Isparta) in Türkiye. Phytochemical studies on flavonoid compounds and their antiproliferative and antioxidant activities of *G. januensis* subsp. *lydia* have not been reported.

Given the prevalence of research investigating the development of innovative therapeutic targets from natural compounds, this study aims to investigate the antiproliferative effect and antioxidant activity of crude extracts of *G. januensis* subsp. lydia. In addition, this study focused on the isolation and determination of chemical structures of phytochemical compounds of *G. januensis* subsp. *lydia*, and the investigation of bioactivities of isolated compounds.

2. Experimental

2. 1. General Experimental Procedures

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) analysis was recorded using an ELISA reader (Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer). Apoptosis analysis was performed using an ABI Attune Acoustic flow cytometer and associated Roche Iccellinge software. Cell cycle analysis was performed using a Partec brand flow cytometer. Spectrophotometric measurements of antioxidant assays were performed using a Hitachi Model 121-002 spectrophotometer. IR spectra were recorded using a Bruker Vertex 70 ATR spectrophotometer. Optical rotations were measured by using a polarimeter. Thin layer chromatography analysis was carried out using a Schmidt + Haensch Polartronic D. Silica gel $60F_{254}$ plates (Merck) used with chromatograms visualized under UV light using a Vilber Laurmat CN-6 UV lamp. Silica gel 60 (0.063–0.200 mm, Merck) was utilized for column chromatography. Normal phase high-performance liquid chromatography (HPLC) was performed on a Shimadzu Prominence LC-8AD/20A model using an Intersil SIL 100A 5μM column. 1D and 2D NMR spectra were recorded on Bruker Avance III (500 MHz) and Agilent (600 MHz) spectrometers. Mass spectra were recorded using LC-MS QTOF Agilent 6530 + Agilent HPLC and LC-MS/MS (Abi-Sciex 4600 Triple Quadrupole TOF). All chemicals were of reagent grade quality and were purchased from commercial suppliers.

2. 2. Plant Material and Extraction

The collection of *Genista januensis* subsp. *lydia* (Boiss.) Kit Tan & Ziel (Fabaceae) occurred in the Armutveren/Demirkoy/Kırklareli area (41°53'22.0"N 27°33'46.0"E; 41°53'06.3"N 27°31'29.6"E; 41°53'43.4"N 27°33'04.6"E) in the Trakya region in May 2019. Dr. Necmettin Guler of the Biology Department of Trakya University botanically identified the plant material (EDTU 16812) (1828.72 g). The above-ground parts of the plant were dried and divided into small pieces. The plant was macerated in solvents according to the order of increasing polarity: hexane, chloroform, ethyl acetate, and methanol. During the extraction of *G. januensis*, in the methanol extract, some crystals were observed. The crystals that were filtered and labelled as "GJ-crystal" The solvents were evaporated on rotary evaporator. Result of the extraction process: hexane extract (25.982 g), chloroform extract (23.674 g), ethyl acetate extract (11.411 g), methanol extract (193.636 g), and crystal extract (7.748 g) were obtained.

2. 3. Antiproliferative Activity

2. 3. 1. Cell Culture

The human lung carcinoma A549 (ATCC) and human breast cancer cell MCF-7 (ATCC HTB-22) were used to determine the cytotoxicity, apoptosis, and cell cycle analysis of the crude extracts. The MCF-7 cell line was also used for MTT analysis of isolated compounds **2**, **3**, and **4**. The cells were provided by the Faculty of Medicine, Tekirdag Namik Kemal University.

2. 3. 2. Cytotoxicity

The cytotoxic effect of the different extracts of *G. januensis* subsp. *lydia* on MCF-7 and A549 cells was evaluated by clonogenic assay.[11] The anticancer activity of the isolated compounds on MCF-7 cells was determined using the MTT assay according to the Vybrant® MTT Cell Proliferation Assay Kit (V-13154 ThermoInc. USA).

2. 3. 3. Apoptosis by Annexin V Analysis

Apoptotic cells in MCF-7 and A549 cell lines were determined using the Alexa Fluor 488-Annexin V/PI kit according to the manufacturer's protocol (V13245; Invitrogen). Samples were analyzed by flow cytometry and data analysis was performed using FlowJo software.[12]

2.3.4. Cell Cycle Analysis

This study evaluated and optimized the use of a flow cytometric cell cycle assay as a tool for screening the anticarcinogenic properties of crude extracts, modified from the method used by Otto *et al.*[13]

2. 4. Determination of the Total Phenolic and Flavonoid Contents and Antioxidant Activities

The total flavonoid content (TFC) of *G. januensis* subsp. *lydia* extracts was determined by aluminum chloride colorimetric method.[14] The Folin–Ciocalteu method

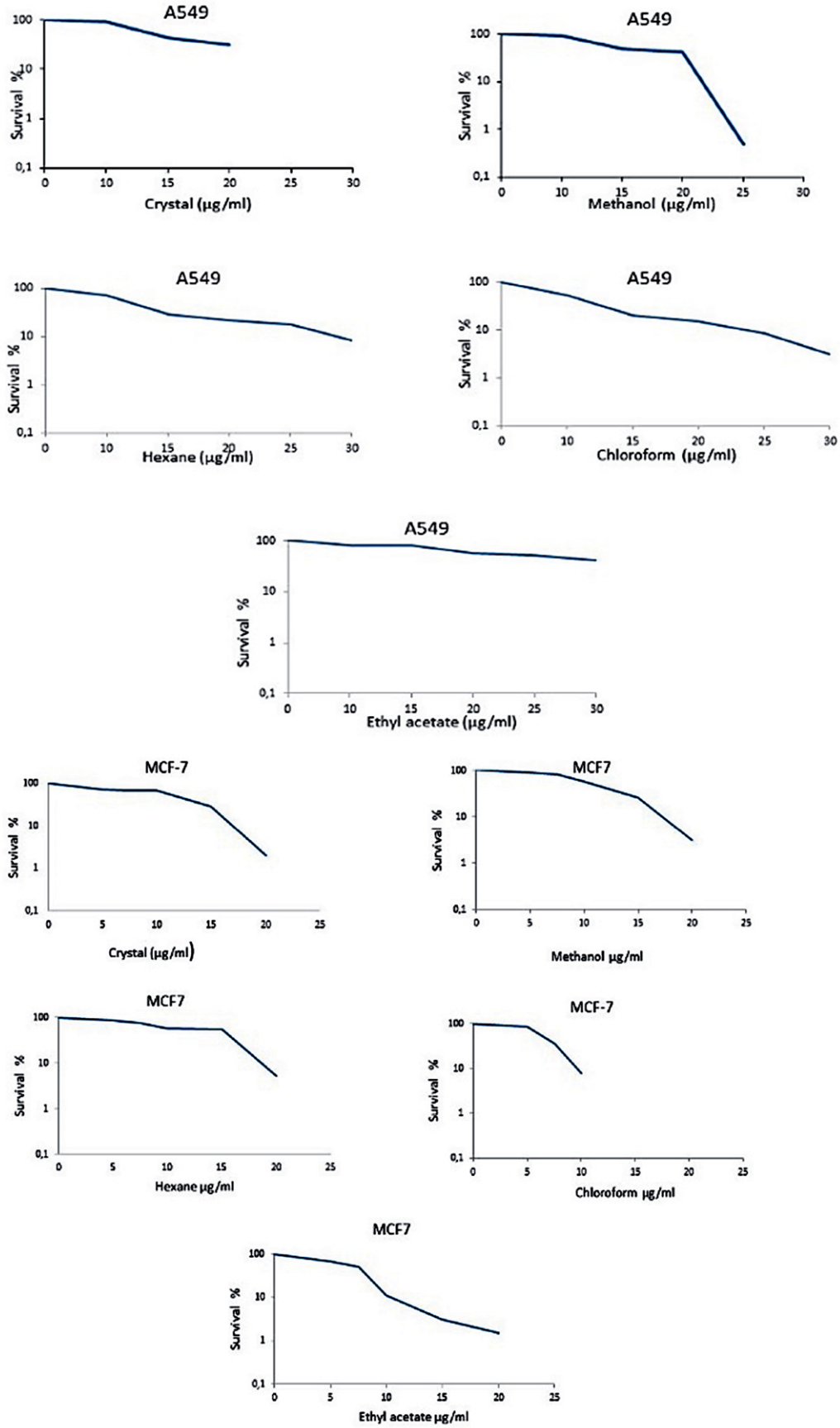

Figure 1. Survival curves of all crude extracts of *G. januensis* subsp. *lydia* applied to human A549 and MCF-7 cells.

was employed to ascertain the total phenolic content (TPC) of the extracts.[15]

The antioxidant activities were evaluated for their reductive abilities using TEAC ($ABTS^{\bullet+}$ cation radical scavenging), ferric-reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The $ABTS^{\bullet+}$ cation radical scavenging (TEAC) was performed according to the method of Re *et al.* (1999).[16] The ferric-reducing antioxidant power (FRAP) was measured according to the method of the study by Benzie and Strain (1996).[17] DPPH experiments were also performed according to Amarowicz *et al.* (2002).[18]

2. 5. Isolation of Compounds

Ethyl acetate crude extract was chosen for isolation studies because it gave the highest anticancer and antioxidant activity results among the crude extracts (Table 2, Figure 6). Different chromatographic methods were used to purify the EtOAc extract of *G. januensis* subsp. *lydia*, including preparative TLC, normal phase HPLC and column chromatography. The ethyl acetate extract (11.411 g) was chromatographed on a silica gel column (column size 60×4 cm) eluted by a gradient system of CH_2Cl_2–EtOAc (1:0–0:1) and a final step of 100% EtOAc to 100% MeOH (by 5% increase in the polarity), as eluent, to give 791 fractions. The 791 fractions were grouped into 32 sub-fractions (JE-1–JE-32) according to the TLC results.

The JE-22 fraction (295 mg) was subjected to a gradient from 90.9% $CHCl_3$ to 100% EtOAc for 45 minutes by HPLC to give compound **1** (genistein) (12 mg) (CH_2Cl_2:EtOAc (91:9)).

Compound **2** (genistin) (10 mg) and **4** (5,4',3'',7'',8'',2''',3''',4'''-octahydroxy-7,5'''-*O*-biflavon) (5 mg) were isolated from fraction JE-23 (155 mg) using column chromatography with the same solvent system (CH_2Cl_2:EtOAc (91:9)).

However, compound **3** (4'-hydroxyisoflavone) (3 mg) was isolated from fraction JE-24 (541 mg) by column chromatography with the solvent system (CH_2Cl_2: EtOAc (91:9 and 85:15)).

All the purified flavonoid compounds were elucidated using methods such as 1D (^1H–^1H and ^{13}C NMR, APT) and 2D (HSQC, HMBC, COSY, and NOESY) NMR and mass (LC-QTOF-MS and LC-ESI-MS) spectroscopy. In addition, spectroscopic data of known compounds (**1**, **2** and **3**) were compared with literature data.

3. Result and Discussions

3. 1. Antiproliferative Activity

Various studies have shown that genistein (**1**) has preclinical activity against many types of human cancer, including bone, bladder, breast, cervical, colon, esophageal, gastric, kidney, lung, liver, neuroblastoma, ovarian, pancreatic, prostate, pituitary, salivary gland, skin, testicular and uterine cancers.[11] In A549 and MCF-7 cells the genistein has been shown in previous studies to be cytotoxic, inducing apoptosis, and cell cycle arrest.[19–22] Therefore, in this study, the antiproliferative effect of compounds **2**, **3**, and **4** on MCF-7 cells was investigated. As the ethyl acetate extract was more effective on MCF-7 cells than on A549 cells and the amount of isolated compounds was not suitable for triplicate study, only MCF-7 cancer cells were tested with isolated compounds.

Cytotoxicity. The outcomes of the colony viability assay for MCF-7 and A549 cells are presented in Figures 1

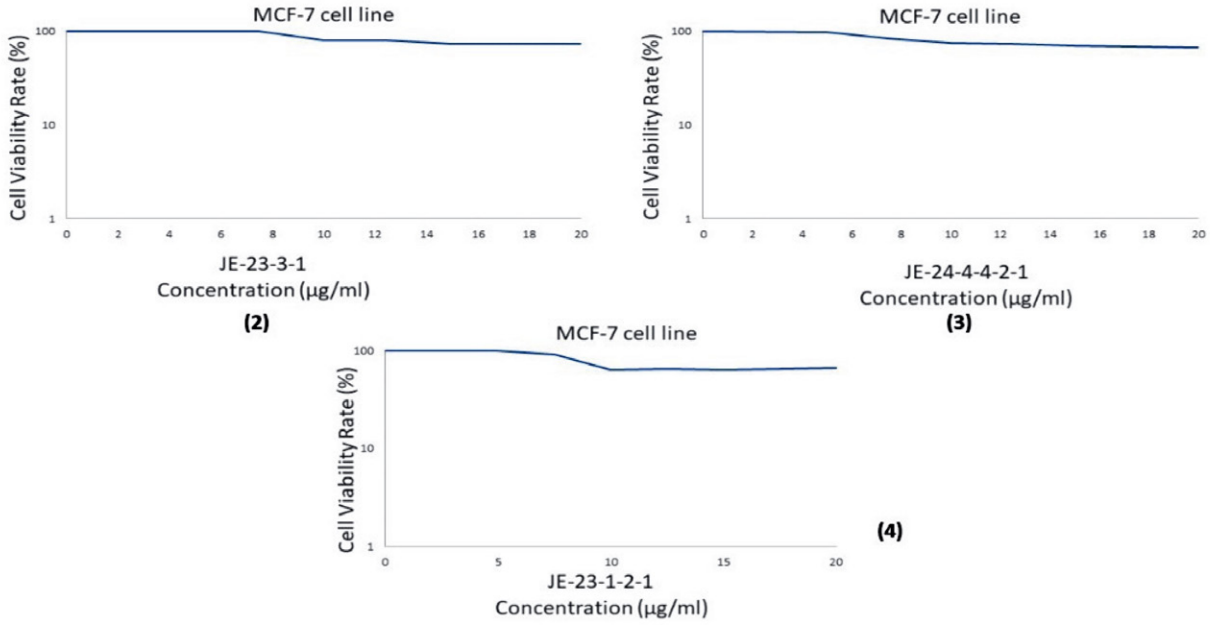

Figure 2. MTT analysis results of isolated compounds in the MCF-7 cell line.

and 2, respectively. A549 cancer cells were exposed to increasing doses (5, 7.5, 10, 15, 20, 25, 30 μg/mL) of crude extracts of the plant *G. januensis*. A colony viability test (clonogenicity) was performed to assess cell survival and proliferation. The results demonstrated that increasing doses of the extract resulted in a reduction in the survival of A549 cells. Approximately 50% cell viability was observed in the A549 cell line, particularly at the doses of 15 μg or 20 μg of all crude extracts. In the MCF-7 cell line, 50% survival was observed at 15 and 20 μg of all extracts except for the chloroform extract. In particular, a gradual decrease in colony formation of MCF-7 cells was observed at concentrations of 5, 7.5, 10, 15, and 20 μg/mL of crude extracts. The MTT analysis showed that compounds **2**, **3**, and **4** did not achieve the 50% mortality rate.

Detection of apoptotic cells. The results of apoptosis of MCF-7 and A549 cells in extracts of *G. januensis* are shown in Figures 3, 4, and S10–S17. A549 cells were treated with increasing doses of crude extracts (10, 15, 20, 25, and 30 μg/mL) for 24 h, but no significant dose-dependent reduction in extract-induced apoptosis of A549 cells was found. MCF-7 cells were treated with increasing doses of crude extracts (5, 7.5, 10, 15, and 20 μg/mL) for 24 h. Especially in GJ-crystal and EtOAc extracts, the rate of cancer cells undergoing apoptosis is quite high. It was found that apoptosis was induced in MCF-7 breast cancer cells at 7.5 and 10 μg/mL concentrations of EtOAc extract.

Cell cycle analysis. The results of the cell cycle analysis for A549 and MCF-7 cells are shown in Figure 5 and S18–S25. A549 and MCF-7 cells were treated with increasing doses of crude extracts (10, 15, 20, 25, and 30 μg/mL for A549 cells; 5, 7.5, 10, 15, and 20 μg/mL for MCF-7 cells) for 24 h.

At a concentration of 10 μg/mL of EtOAc extract from *G. januensis* plant, cell cycle arrest was detected in MCF-7 cells in the G2/M phase compared to the control (29.54% and 25.14%, respectively). In addition, cell cycle arrest was observed in A549 cells in the G2/M phase at a concentration of 7.5 μg/mL of chloroform extract compared to the control.

3. 2. Determination of the TPC, TFC and Antioxidant Activities

In this study, the ethyl acetate extract was found the have the highest total phenolic content (428.56 ± 9.32 μg CAT mg extract^{-1}) and total flavonoid content (216.22 ± 1.68 μg CAT mg extract^{-1}) among the crude extracts. The ethyl acetate extract was found to contain 4.84 times more TPC than the chloroform extract and 2.19 times more

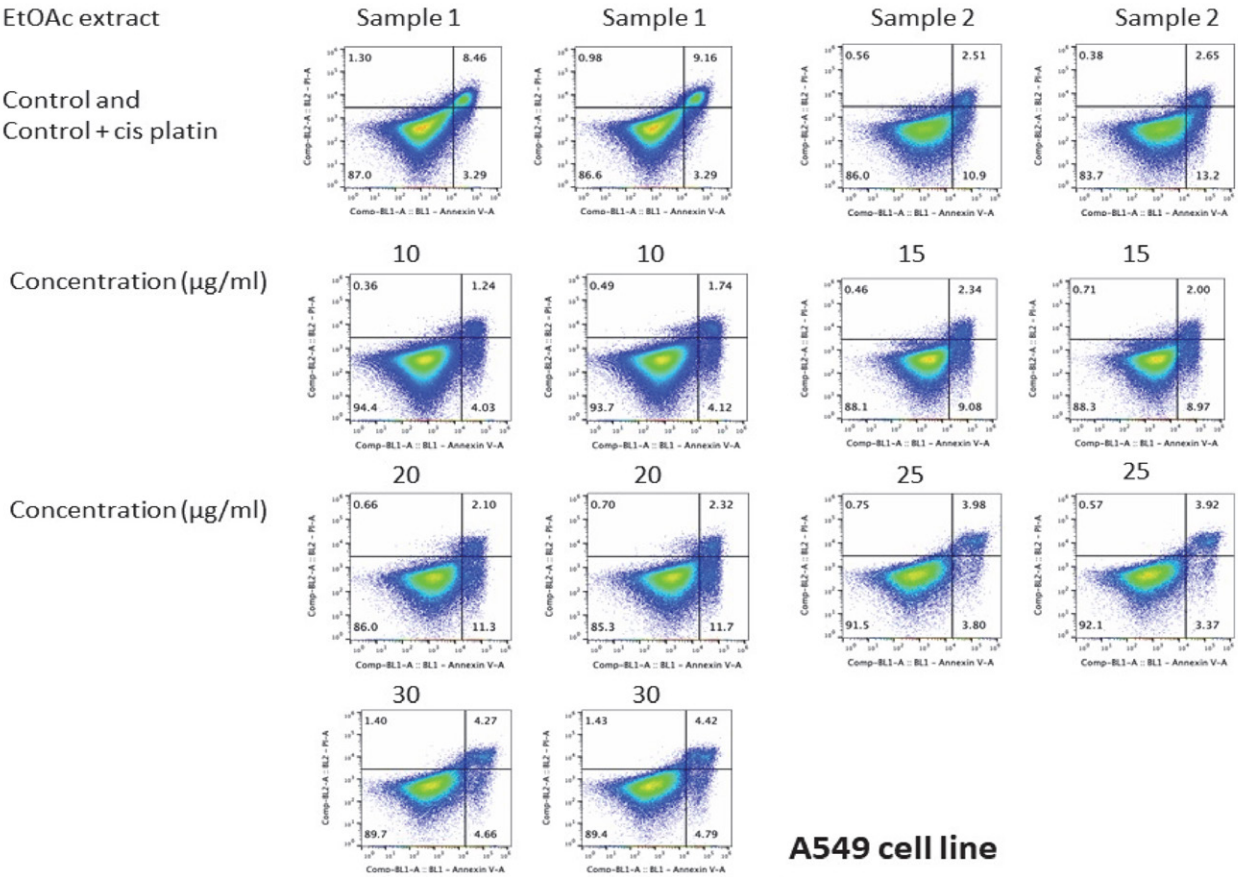

Figure 3. Dose-dependent apoptosis graphs in human A549 cancer cells in EtOAc extract of *G. januensis* extract applications.

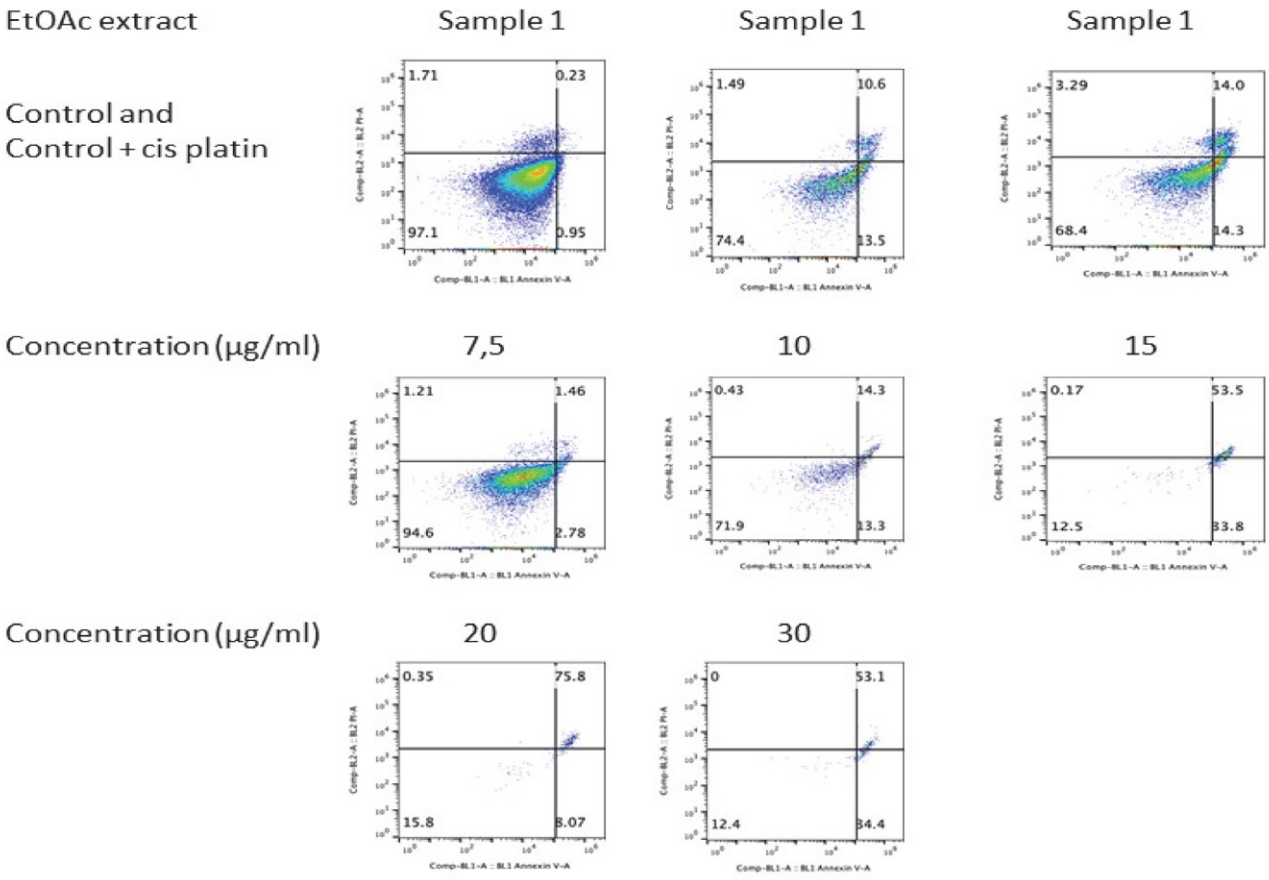

Figure 4. Dose-dependent apoptosis graphs in human MCF-7 cancer cells in EtOAc extract of *G. januensis* extract applications.

TPC than the MeOH extract. Similarly, in our previous research, TPC, TFC, and antioxidant activity were found to be lower in the hexane extract of *G. januensis* subsp. *lydia*.[23] When examining the antioxidant results, it was found that ethyl acetate extract had the highest antioxidant activity followed by chloroform and MeOH extracts. The $ABTS^{\bullet+}$ scavenging activity was determined to be 2.133 mmol Trolox g^{-1} as shown in Table 1. The FRAP capacity of the plant in ethyl acetate extract was found to be 2683.02 µmol Fe^{2+} g^{-1}. According to the results of EC_{50} value of DPPH, which is defined as the concentration required for *50*% scavenging of DPPH radicals of different extracts, it is possible to conclude that the ethyl acetate extract showed the highest radical scavenging activity (0.015 mg/mL) among the examined extracts (Table 1). When all the results were evaluated, the ethyl acetate extract showed the highest total phenolic and flavonoid content and higher antioxidant activities than the other extracts.

Antioxidant activity assays of compound **4** in comparison to synthetic antioxidant components are shown in Table 1. The $ABTS^{\bullet+}$ scavenging activity was found to be 0.345 mmol Trolox g^{-1}, the FRAP capacity was found to be 229.34 µmol Fe^{2+} g^{-1}, and the EC_{50} value of DPPH was found to be detected.

3. 3. Compound Characterization

This is the first report on the bioactivity of the chemical compositions of *G. januensis* subsp. *lydia*. Compounds **1**, **2**, and **3** isolated from *G. januensis* subsp. *lydia* are known compounds, but this is the first time these compounds have been isolated from *G. januensis* subsp. *lydia*. In accordance with spectral data and literature, these compounds were identified as genistein (**1**),[24] genistin (**2**),[25] and 4'-hydroxyisoflavone (**3**).[26,27] These compounds have been previously isolated from various natural sources and other *Genista* species.[9,28–30]

Compound 4. Amorphous powder: $[\alpha]^{D}_{26.5}$ –0.027 (*c* 0.3333, MeOH); UV λ_{max} (MeOH) 208 (Ar-), 259 (Ar-O-), 306, 281 nm; IR (nujol) ν_{max} 1220, 1458, 1670, 3361 cm^{-1}, ^{1}H and ^{13}C NMR spectral data: see Table 2; LC-ESI-MS and LC-QTOF-MS *m/z* (rel. int. %): 587 $[M+H]^+$ (55.84%), 585 $[M–H]^-$ (3.41%), 393 $[M–C_9H_5O_5]^-$ (100%), 478 $[M–C_6H_4O_2]^-$ (5.12%), 252 $[(M–H)–C_{15}H_9O_9]^-$ (3.01%), 269 $[M–C_{15}H_9O_8]^-$ (17.18%), 151 $[(M–H)–C_{23}H_{14}O_9]^-$ (6.83%), 431 $[(M–3H)–C_7H_4O_4]^-$ (5.71%).

Compound **4** was obtained as yellow crystals. Q-TOF mass spectroscopy revealed an ion at *m/z* 587.0741 $[M+H]^+$ (calcd 587.0747) and 585.0743 $[M–H]^-$ (calcd

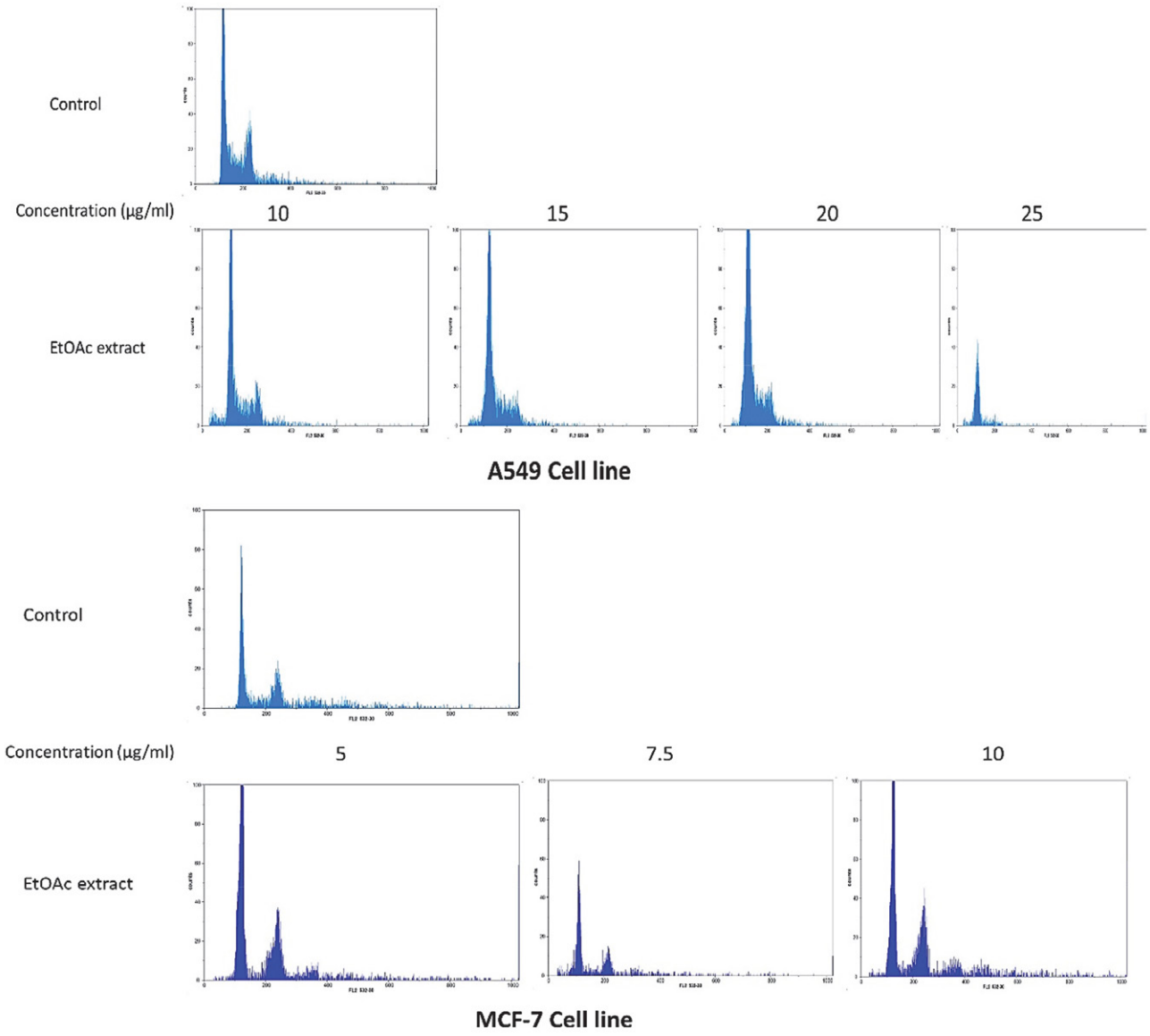

Figure 5. Dose-dependent cell cycle analysis graphs in human A549 and MCF-7 cell lines, respectively, for applications of EtOAc extract of *G. januensis*.

Table 1. Total flavonoid content and antioxidant capacity (ABTS$^{\bullet+}$, FRAP, DPPH) of the crude extract, and comparison of compound **4** isolated from *G. januensis* subsp. *lydia* with reference compounds.

Extracts	TPC (µg CAT* mg extract^{-1})	TFC (µg CAT mg extract^{-1})*	ABTS$^{\bullet+}$ (mmol Trolox g^{-1})	FRAP (µmol Fe^{2+} g^{-1})	DPPH EC_{50} (mg/mL)
Chloroform	88.53 ± 1.44**	Nd	0.555 ± 0.04	900.31 ± 5.70	0.099 ± 0.03
Ethyl acetate	**428.56 ± 9.32**	**216.22 ± 1.68**	**2.133 ± 0.16**	**2683.02 ± 5.99**	**0.015 ± 0.01**
MeOH	195.23 ± 4.42	114.03 ± 0.98	1.281 ± 0.22	2481.54 ± 8.35	0.027 ± 0.01
GJ-crystal	189.70 ± 5.08	80.77 ± 1.76	0.949 ± 0.17	1272.87 ± 4.32	0.048 ± 0.02
Compound					
Compound (4)			0.345	229.34	0.461 ± 0.04
BHA			2.161	4276.18	0.0087 ± 0.01
BHT			1.328	2270.76	0.048 ± 0.01
Ascorbic Acid			2.041	4535.92	0.018 ± 0.01
Quercetin			2.150	4185.22	0.0091 ± 0.01

*Total Phenolic/Flavonoid Content as Catechin Equivalent. **Data are expressed as the mean ± standard deviation ($n \geq 3$). Values in the same column having different letters differ significantly ($P < 0.05$). Nd: Not determined. BHA: Butylated hydroxyanisole; BHT: Butylated hydroxytolune.

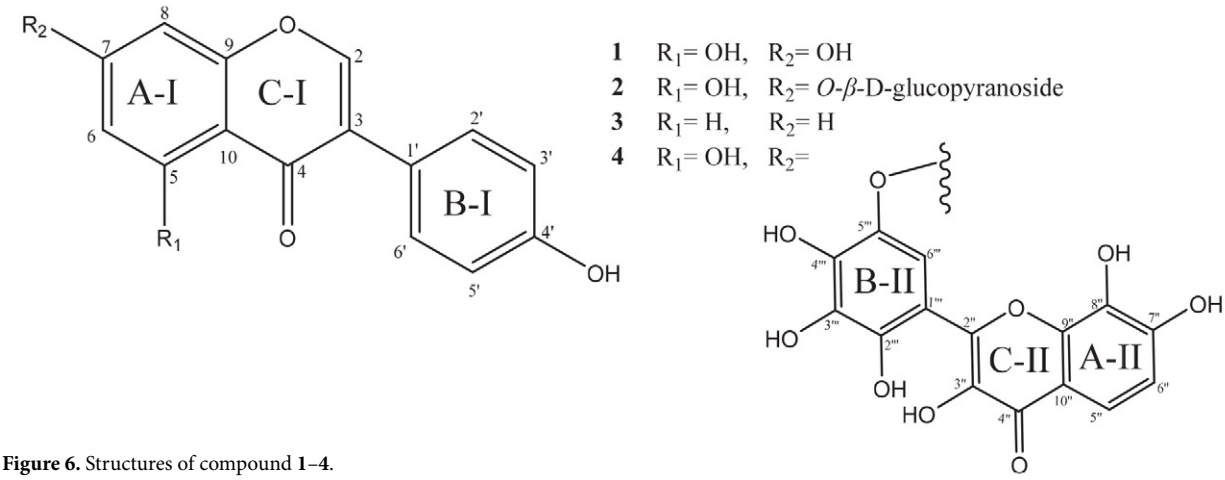

Figure 6. Structures of compound **1**–**4**.

Table 2. 1D and 2D NMR spectral data of compound **4**[a]

Position	δ ^{1}H	δ ^{13}C	δ HMBC	δ ^{1}H–^{1}H COSY	δ NOESY
2	8.03 (1H, s)	153.3	C-3, C-4, C-9, C-10, C-1', C-2'/6'	–	H-3'/5'
3	–	123.3	–	–	–
4	–	180.8	–	–	–
5	–	158.3	–	–	–
6	6.32 (1H, d, *J*= 2.14 Hz)	93.3	C-4, C-5, C-7, C-8, C-9, C-10, C-5'''	–	–
7	–	164.5	–	–	–
8	6.20 (1H, d, *J*= 2.14 Hz)	98.7	C-6, C-7, C-10, C-5'''	–	–
9	–	157.4	–	–	–
10	–	104.8	–	–	–
1'	–	121.9	–	–	–
2'/6'	7.35 (2H, d, *J*= 8.55 Hz)	129.9	C-3, C-4, C-1', C-3'/5', C-4'	H-3'/5'	H-3'/5'
3'/5'	6.83 (2H, d, *J*= 8.70 Hz)	114.8	C-3, C-1', C-4'	H-2'/6'	H-2, H-2'/6'
4'	–	157.4	–	–	–
2"	–	144.5	–	–	–
3"	–	134.1	–	–	–
4"	–	176.4	–	–	–
5"	7.35 (1H, d, *J*= 8.55 Hz)	129.9	C-3", C-4", C-6", C-7"/8", C-9"	H-6"	H-6"
6"	6.83 (1H, d, *J*= 8.70 Hz)	114.8	C-7"/8", C-9", C-10"	H-5"	H-5"
7"/8"	–	157.4	–	–	–
9"	–	158.3	–	–	–
10"	–	104.8	–	–	–
1'''	–	123.3	–	–	–
2'''/3'''/4'''	–	134.1	–	–	–
5'''	–	162.4	–	–	–
6'''	8.08 (1H, s)	129.1	C-7, C-2", C-1''', C-2'''/3'''/4'''	–	–

[a] at 500 MHz in CD_3OD.

585.0747) consistent with the molecular formula of $C_{30}H_{18}O_{13}$. Table 2 shows the complete 1D and 2D NMR assignments of compound **4**. When we examined ^{1}H NMR spectrum in detail, the *meta*-coupled protons resonated at δ 6.32 (1H, d, J = 2.14 Hz, H-6) and δ 6.20 (1H, d, J = 2.14 Hz, H-8) for ring A-I comprising the AX system (Figure S1). The singlet at δ 8.03 (1H, s, H-2) in C-I ring indicated the presence of an isoflavonoid. Furthermore, four *ortho*-coupled protons forming the AA'XX' system at δ 7.35 (2H, d, J = 8.55 Hz, H-2'/6') and δ 6.83 (2H, d, J = 8.70 Hz, H-3'/5') were assigned to the 1,4-disubstituted aromatic ring B-I, while the peaks at 7.35 (1H, d, J = 8.55 Hz, H-5'') and 6.83 (1H, d, J = 8.70 Hz, H-6'') forming the AX system suggested the presence of the tetrasubstituted aromatic ring A-II. Also COSY interactions of the H-2'/6', H-3'/5' and H-5'' and H-6'' protons supported this. The numerous proton interactions detected in the aromatic area and mass spectra suggest the presence of a biflavonoids structure. However, the presence of a singlet at 8.08 (1H, s, H-6''') supported the B-II aromatic ring. The HMBC spectrum showed cross-peaks of H-2 (δ 8.03) with C-3 (δ 123.32), C-4 (δ 180.84), C-9 (δ 157.42), and C-1' (δ 121.90) of ring

C-I; while H-6 was correlated with C-4 (δ 180.84), C-5 (δ 158.31), C-7 (δ 164.55), C-8 (δ 98.72), C-9 (δ 157.42), and C-10 (δ 104.89). Other important HMBC cross peaks that confirmed the molecular framework included those that supported the assignment of the A-II ring, namely, H-5'' (δ 7.35) to C-3'' (δ 134.15), C-4'' (δ 176.43), and C-6'' (δ 114.86); as well as H-6'' (δ 6.83) to C-7''/8'' (δ 157.42), C-9'' (δ 158.31), and C-10'' (δ 104.89). The HMBC spectrum of compound **4** confirmed the formation of an inter-flavonoid ether linkage between C-7 and C-5''' with correlations from H-8 to C-5''' and from H-6''' to C-7 (Table 2, Figures 6 and S5). In addition, the *m/z* 269 $[M–(C_{15}H_9O_8)]^+$ peak of compound **4** observed in (–) LC-ESIMS and (+/–) LC-QTOF-MS supported that flavonoid-I and flavonoid-II rings are linked at C-7 and C-5'' positions. Moreover, a cross peak was detected in both COSY and NOESY spectrum between H-3'/5' and H-2'/6'; as well as H-5'' and H-6'' resonances (Table 2, Figures 6, S3, and S6). In addition, peaks at *m/z* 151 $[(M–H)–C_{23}H_{14}O_9]^-$ and 431 $[(M–3H)–C_7H_4O_4]^-$ supported the Diels-Alder degradation of in the flavonoid-II structure (Table 2, Figure 6). The compound **4** was thus identified as 5,4',3'',7'',8'',2''',3''',4'''-octahydroxy-7,5'''-*O*-biflavon (Figure 6).

3. 4. Discussion

The polarity-dependent increase in antioxidant activity, reducing properties and free radical scavenging activity of the plant may be attributed to the high affinity of antioxidant compounds in plant extracts for more polar solvents as compared to nonpolar ones. The hexane extract of *G. januensis* subsp. *lydia* may have shown lower activity than other extracts due to its lower polarity. The results of this study demonstrated that the ethyl acetate extract of *G. januensis* subsp. *lydia* may contain several components with reducing activity that act as free radical scavengers compared to the other extracts. This is consistent with the encouraging results obtained in various experiments on antiproliferative assays. Previous research has shown that the antioxidant properties of phenolic and flavonoid constituents of medicinal plants play an important role in the fight against cancer. Many studies have shown that plants

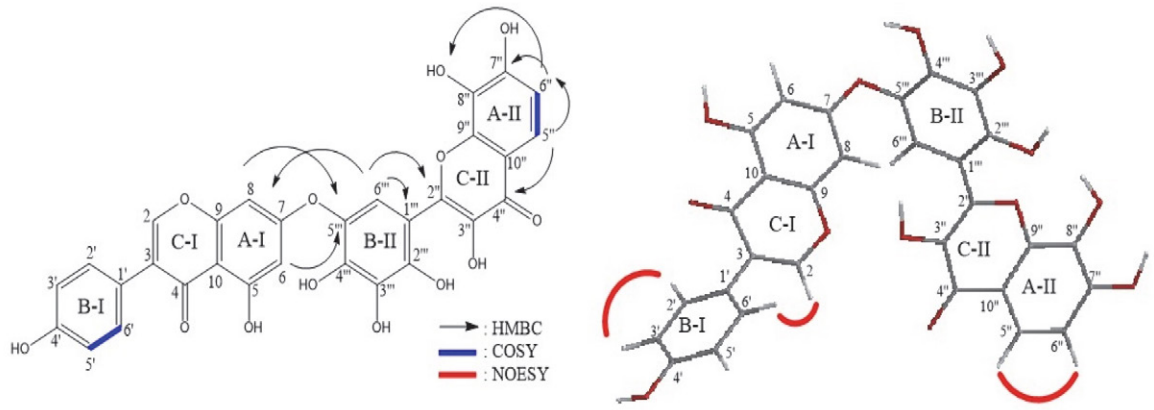

Figure 7. Key HMBC, $^1H–^1H$ COSY and NOESY correlations of compound **4**.

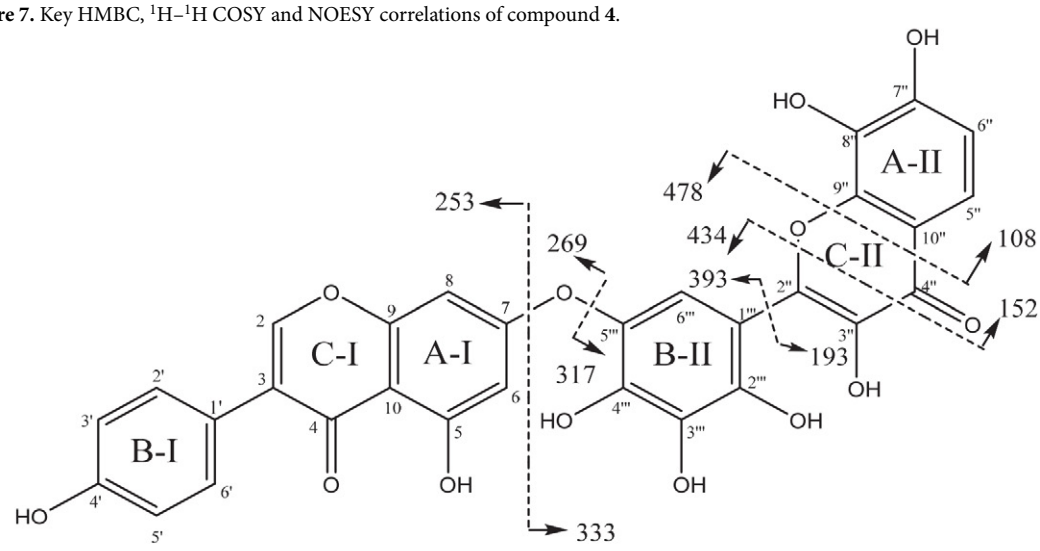

Figure 8. Mass spectral fragmentations of compound **4**.

are a rich source of antioxidant compounds that induce cytotoxic or cytostatic effects in cancer cell lines, making them good candidates for the development of new anticancer drugs.[31–36] Our study, as well as previous studies on the antioxidant and anticancer effects of the different plants, is important in this context.

4. Conclusions

For the first time in crude extracts (hexane, chloroform, ethyl acetate, methanol, GJ-crystal) obtained from *G. januensis* subsp. *lydia* cytotoxicity, apoptosis, and cell cycle analyses were performed on A549 lung cancer and MCF-7 breast cell lines. It was found that the anticancer effect of the EtOAc extract is more effective than other extracts. According to the data obtained the crude extracts of the *G. januensis* subsp. *lydia* plant are believed to have anticancer effects. Although crude extracts are thought to have a cytotoxic effect, their anticancer activity needs to be supported by further studies. Genistein, genistin, and 4'-hydroxyisoflavone compounds did not show effective cytotoxicity.

In antioxidant activity studies, we also found that this ethyl acetate extract had the highest antioxidant activity among the crude extracts. These findings suggest that *G. januensis* subsp. *lydia* has the potential for further investigations as a natural source of antiproliferative and antioxidant agents. Natural flavonoids are considered an essential function of food in the prevention and treatment of chronic diseases. Thanks to *G. januensis* subsp. *lydia* and its antioxidant activity and antiproliferative effect represent a benefit and provide further impetus for multi-disciplinary investigations.

Supplementary Material

Supplementary material for this article is available online, along with Figures S1–S25.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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5. References

1. C. B. Blackadar, *World J. Clin. Oncol.* **2016**, *7(1)*, 54–86. **DOI:**10.5306/wjco.v7.i1.54
2. J. da Silva Mendonça, R. de Cássia Avellaneda Guimarães, V. A. Zorgetto-Pinheiro, C. Di Pietro Fernandes, G. Marcelino, D. Bogo, K. de Cássia Freitas, P. A. Hiane, E. S. de Pádua Melo, M. L. B. Vilela, V. A. do Nascimento, *Molecules* **2022**, *27*, 3563. **DOI:**10.3390/molecules27113563
3. C. Rodríguez-García, C. Sánchez-Quesada, J. J. Gaforio, *Antioxidants* **2019**, *8(5)*, 137. **DOI:**10.3390/antiox8050137
4. M. Usman, W. R. Khan, N. Yousaf, S. Akram, G. Murtaza, K. A. Kudus, A. Ditta, Z. Rosli, M. N. Rajpar, M. Nazre, *Molecules* **2022**, *27(12)*, 3863. **DOI:**10.3390/molecules27123863
5. D. Batista, P. L. Falé, M. L. Serralheiro, M. E. Araújo, P. J. A. Madeira, C. Borges, I. Torgal, M. Goulart, J. Justino, A. Martins, A. P. Rauter, *Nat. Prod. Bioprospect.* **2015**, *5*, 277–285. **DOI:**10.1007/s13659-015-0077-z
6. A. P. Rauter, A. Martins, C. Borges, J. Ferreira, J. Jorge, M. R. Bronze, A. V. Coelho, Y. H. Choi, R. Verpoorte, *J. Chromatogr. A* **2005**, *1089*, 59–64. **DOI:**10.1016/j.chroma.2005.06.046
7. C. Caleja, T. Finimundy, C. Pereira, L. Barros, R. C. Calhelha, M. Sokovic, M. Ivanov, A. M. Carvalho, E. Rosa, I. C. F. R. Ferreira, *Food Funct*. **2019**, *10*, 5939–5951. **DOI:**10.1039/C9FO01473J
8. N. Fokialakis, X. Alexi, N. Aligiannis, A. Boulaka, A. K. Meligova, G. Lambrinidis, E. Kalpoutzakis, H. Pratsinis, A. Cheilari, D. J. Mitsiou, S. Mitakou, M. N. Alexis, *PloS One 14.* **2019**, e0210247. **DOI:**10.1371/journal.pone.0210247
9. M. A. M. Simoes, D. C. G. A. Pinto, B. M. R. Neves, A. M. S. Silva, *Molecules* **2020**, *25*, 81. **DOI:**10.3390/molecules25040812
10. A. Latreche, K. Chebbah, N. Boubekri, R. Bendjedou, D. Zama, R. Mekkiou, F. Benayache, S. Benayache, *Int. J. Pharm. Sci. Rev. Res.* **2016**, *38(17)*, 102–106.
11. N. A. Franken, H. M. Rodermond, J. Stap, J. Haveman, C. van Bree, *Nat. Prot*. **2006**, *1(5)*, 2315–2319. **DOI:**10.1038/nprot.2006.339
12. H. Ho, J. Aruri, R. Kapadia, H. Mehr, M. A. White, A. K. Ganesan, *Cancer Res*. **2012**, *72(21)*, 5516–5528. **DOI:**10.1158/0008-5472.CAN-12-0775
13. C. Otto, T. Hahlbrock, K. Eich, F. Karaaslan, C. Jürgens, C. T. Germer, A. Wiegering, U. Kämmerer, *B. M. C. Complement. Alter. Med*. **2016**, *16*, 160. **DOI:**10.1186/s12906-016-1138-5
14. J. Valcarcel, K. Reilly, M. Gaffney, N. M. O'Brien, *Potato Res.* **2015**, *58*, 221–244. **DOI:**10.1007/s11540-015-9299-z
15. R. Amarowicz, M. Karamać, S. Weidner, S. Abe, F. Shahidi, *J. Food Lipids*. **2002**, *9(3)*, 201–210. **DOI:**10.1111/j.1745-4522.2002.tb00219.x
16. R. Re, N. Pellegrini, A. Proteggente, *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. **DOI:**10.1016/S0891-5849(98)00315-3
17. I. F. F. Benzie, J. J. Strain, *Anal. Biochem*. **1996**, *239*, 70. **DOI:**10.1006/abio.1996.0292
18. M. Wróbel, M. Karamać, R. Amarowicz, E. Frączek, S. Weidner, *Acta Physiol. Plant*. **2005**, *27(3A)*, 313–320. **DOI:**10.1007/s11738-005-0008-4
19. J. Sharifi-Rad, C. Quispe, M. Imran, A. Rauf, M. Nadeem, T. A. Gondal, B. Ahmad, M. Atif, M. S. Mubarak, O. Sytar, O. M. Zhilina, E. R. Garsiya, A. Smeriglio, D. Trombetta, D. G.

Pons, M. Martorell, S. M. Cardoso, A. F. A. Razis, U. Sunusi, R. M. Kamal, L. S. Rotariu, M. Butnariu, A. O. Docea, D. Calina, *Oxid. Med. Cell. Longev*. **2021**, *2021*, 36. **DOI:**10.1155/2021/3268136
20. L. Fioravanti, V. Cappelletti, P. Miodini, E. Ronchi, M. Brivio, G. Di Fronz, *Cancer Lett*. **1998**, *130*, 143–152. **DOI:**10.1016/S0304-3835(98)00130-X
21. J. Zhang, H. Su, Q. Li, J. Li, Q Zhao, *Mol. Med. Rep.* **2017**, *15*, 2296–2302. **DOI:**10.3892/mmr.2017.6260
22. H. S. Tuli, M. J. Tuorkey, F. Thakral, K. Sak, M. Kumar, A. K. Sharma, U. Sharma, A. Jain, V. Aggarwal, A. Bishayee, *Front. Pharmacol.* **2019**, *6(10)*, 1336.
23. C. Cakır, H. H. Orak, M. Argon, H. Caliskan, M. Ozturk, *Bulg. Chem. Commun*. **2022**, *54(3)*, 268–270.
24. J. M. Kim, R. K. Ko, D. S. Jung, S. S. Kim, N. H. Lee, *Phytother. Res*. **2010**, *24*, 70–75. **DOI:**10.1002/ptr.2870
25. S. Z. Nishanbaev, K. M. Bobakulov, D. A. Narbutaeva, S. F. Aripova, Z. A. Khushbaktova, N. D. Abdullaev, *Chem. Natural. Compd.* **2020**, *56*, 729–731. **DOI:**10.1007/s10600-020-03132-w
26. S. H. Jung, S. H. Cho, T. H. Dang, J. H. Lee, J. H. Ju, M. K. Kim, S. H. Lee, J. C. Ryu, Y. Kim, *Eur. J. Med. Chem.* **2003**, *38(5)*, 537–545. **DOI:**10.1016/S0223-5234(03)00064-3
27. A. R. Ibrahim, Y. J. Abul-Hajj, *Xenobiotica*. **1990**, *20(4)*, 363–373. **DOI:**10.3109/00498259009046853
28. A. Venditti, C. Frezza, S. Foddai, M. Serafini, A. Bianco, *Arab. J. Chem.* **2019**, *12(8)*, 3921–3926. **DOI:**10.1016/j.arabjc.2016.02.012
29. P. Bontempo, D. Rigano, A. Doto, C. Formisano, M. Conte, A. Nebbioso, V. Carafa, G. Caserta, V. Sica, A. M. Molinari, L. Altucci, *Cell Prolif.* **2013**, *46*, 183–192. **DOI:**10.1111/cpr.12022
30. M. Skalicky, J. Kubes, V. Hejnak, L. Tumova, J. Martinkova, J. Martin, H. Hnilickova, *Molecules* **2018**, *23*, 1619. **DOI:**10.3390/molecules23071619
31. J. Alzeer, B. R. Vummidi, R. Arafeh, W. Rimawi, H., Saleem, N. W. Luedtke, *J. Med. Plant Res*. **2014**, *8*, 408–415.
32. E. T. Arung, B. D. Wicaksono, Y. A. Handoko, I. W. Kusuma, D. Yulia, F. Sandra, T. *J. P. R.* **2009**, *8*, 317–324.
33. K. Brusselemens, R. Vrolix, G. Verhoeven, J. V. Swinnen, *J. B. C*. **2005**, 280, 5636–5646. **DOI:**10.1074/jbc.M408177200
34. J. W. Chai, U. R Kuppusamy, M. S. Kanthimathi, *M. J. M. B. R.* **2008**, *16*, 28–30.
35. D. Chodon, N. RamamurtyD. Sakthisekaran, *Toxicol. In Vitro* **2007**, *21*, 887–891. **DOI:**10.1016/j.tiv.2007.01.023
36. G. R. Pettit, M. S. Hoard, D. L. Doubek, J. M. Schmidt, R. K. Pettit, L. P. Tackett, J. C. Chapuis, *J. Ethnopharmacol.* **1996**, *53*, 57–63. **DOI:**10.1016/S0378-8741(96)01421-3

Povzetek

Cilj študije je bil raziskati protirakavo in antioksidativno delovanje ekstraktov *Genista januensis* subsp. *lydia* (Fabaceae) ter izolirati fitokemijske spojine, odgovorne za te aktivnosti. Ekstrakcije iz rastline *G. januensis* smo izvedli z maceriranjem v topilih z naraščajočo polarnostjo. Določili smo celokupno vsebnost fenolov in flavonoidov ter s pomočjo analize citotoksičnosti, apoptoze in analize celičnega cikla za surove ekstrakte ugotovili antioksidativne in protirakave aktivnosti. Ugotovili smo, da ima EtOAc ekstrakt največjo antioksidativno in protirakavo aktivnost, zato smo ta ekstrakt izbrali za nadaljnjo izvedbo izolacije. Strukture vseh očiščenih flavonoidnih spojin iz EtOAc ekstrakta smo raziskali s pomočjo 1D in 2D NMR ter z masno spektroskopijo in rezultate primerjali s podatki v literaturi. Skupaj s tremi že znanimi spojinami (genistein (**1**), genistin (**2**) in 4'-hidroksizoflavon (**3**)) smo iz *Genista januensis* subsp. *lydia* (Fabaceae) izoliali še nov biflavonoid **4**. Da smo določili protirakavo delovanje smo za spojine **2**, **3** in **4** izvedli MTT analizo, za spojino **4** pa smo določili še antioksidativne lastnosti. Rezultati kažejo, da rastlina *G. januensis* subsp. *lydia* predstavlja nov naravni vir učinkovitih antiproliferativnih in antioksidantivnih spojin z obetajočim potencialom.