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ORIGINAL ARTICLE

Isolation of flavonoids from the selected *Trigonella* species

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Abstract: *Trigonella foenum-graecum*, a plant cultivated in Türkiye, is used as fodder, food, spice, perfume, insect repellent, dye, and for medicinal applications. While there are numerous studies on this species in the literature, detailed investigations of some endemic species widely grown in Türkiye are lacking. The phytochemical studies on *T. foenum-graecum* and Türkiye's endemic species, *T. macrorrhyncha* and *T. kotschyi*, were conducted, affording 11 compounds, including three previously undescribed compounds, from aqueous extracts of aerial parts of the endemic species and *T. foenum-graecum*. The isolated new compounds were conclusively identified as quercetin 3-*O*-(2″-*O*-*β*-glucosyl-6″-*O*-acetyl)-*β*-galactoside 7-*O*-*β*-glucoside **(3)**, kaempferol 3-*O*-(2″-*O*-(4‴-*O*-malonyl)-*α*-rhamnosyl)-*β*-glucoside) 7-*O*-*α*-rhamnoside **(10)** and kaempferol 3-*O*-(2″-*O*-(4‴-*O*-methylmalonyl)-*α*-rhamnosyl)-*β*-glucoside) 7-*O*-*α*-rhamnoside **(11)**. Cytotoxic activity of the extracts and the isolated compounds from the active extract was also tested on A549 and PC3 cancer cell lines. Although the *T. kotschyi* extract demonstrated moderate cytotoxicity (IC_{50}: 75.54 and 64.14 µg/mL for A549 and PC-3, respectively), none of the isolated compounds exhibited notable cytotoxicity.

Keywords: Fabaceae, *Trigonella*, fenugreek, quercetin, kaempferol-glycosides

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1 Introduction

Medicinal plants have long served as an important source of bioactive compounds and continue to play a significant role in drug discovery studies (Nahar & Sarker, 2020). The understanding that the biological activities of plants occur through the metabolites they carry has led to the development of the science of phytochemistry (Çalis, 2021). Species of the genus *Trigonella* and especially *T. foenum-graecum* L. (fenugreek) are traditionally used for many purposes (Petropoulos, 2017). The genus *Trigonella* belongs to the family Fabaceae (Huber–Morath, 1970). It comprises approximately 135 species widely distributed in the Eastern Mediterranean, Western Asia, Southern Europe, and Northern and Southern Africa. It is cultivated all over the world, especially in India and Mediterranean countries, and industrial products are prepared from agricultural raw materials. The seeds of the plant are used as feed, food, spice, perfume, insect repellent, dye and for medicinal purposes such as carminative, tonic, aphrodisiac, digestive stimulant agents (Akan et al., 2020; Al-Habori & Raman, 2017; Petropoulos, 2017). According to the monographs of the European Medicines Agency (Committee on Herbal Medicinal Products, 2021), the seeds of *T. foenum-graecum* are also used for the symptomatic treatment of minor skin inflammations due to their anti-inflammatory properties and for the treatment of temporary loss of appetite. Fenugreek is an important plant cultivated in Türkiye for use both as a pharmaceutical raw material and as a spice (Beyzi et al., 2010).

The biological and pharmaceutical effects of fenugreek are due to its steroids, nitrogenous compounds and polyphenolic compounds (Skaltsa, 2017). It has nutraceutical and medicinal uses due to steroidal saponin (diosgenin), amino acids and galactomannan (Zandi et al., 2017). The aboveground parts of *Trigonella* species are rich in flavonoids (Han et al., 2001; Kawashty et al., 1998). Although many studies have been carried out on *T. foenum-graecum*, there are few

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Table 1. The extracts of the aerial parts of three *Trigonella* species

Plant name	Air-dried aerial parts of the plants (g)	Methanol extract (g)	Yield (%)	Aqueous extract (g)
T. kotschyi (TK)	269.4	54.2	20.1	48.1
T. macrorrhyncha (TM)	33.2	8.98	27.1	6.02
T. foenum-graecum (TFG)	419.5	97.7	23.2	58.8

phytochemical studies on other *Trigonella* species. The genus *Trigonella* is presented as 10 sections in Türkiye. The genus *Trigonella* is represented by 32 species and 2 subspecies (34 taxa); 11 of these are endemic, and the rate of endemism in Türkiye is 32.4%. Türkiye is one of the main countries where the genus *Trigonella* and some species of the Fabaceae family grow (Akan et al., 2020).

This study aimed to investigate the phytochemical profiles and to evaluate possible cytotoxic effects of the aerial parts of the aqueous extract of *T. foenum-graecum* and two endemic species, which are evaluated as LC (least concern) among endemic species and on which there are no detailed phytochemical studies. For this purpose, *T. macrorrhyncha* from the Foenum-graecum section and *T. kotschyi* from the Cylindricae section were selected (Akan et al., 2020).

2 Materials and Methods

2.1 Plant Material

The aerial parts of the plants were collected from different regions of Türkiye; *Trigonella kotschyi* Fenzl (Çamlıyayla, Mersin; April 2024), *T. macrorrhyncha* Boiss (Artuklu, Mardin; May 2024), *T. foenum-graecum* L. (Çalış, Avanos, Nevşehir; June 2024). The plant materials were shade-dried in a cool and dry place. Herbarium samples of the plants were prepared and stored at Hacettepe University Faculty of Pharmacy Herbarium (HUEF) with the voucher numbers of HUEF24209, 25002 and 25001, respectively.

2.2 Extraction and Isolation

The aerial parts of the plants were ground and extracted with 80% methanol in a water bath at 40°C, with a minimum yield of 20.1% (Table 1). The obtained extracts were combined and evaporated to dryness. To remove lipophilic compounds, the extracts were dissolved in water and partitioned with petroleum ether (Table 1). Thus, aqueous extracts were obtained for phytochemical and biological activity studies.

HPLC studies were conducted on three aqueous extracts to compare phytochemical profiles using the analytical HPLC system (Thermo Scientific Dionex UPLC 3000) with a Phenomenex Luna C_{18} column (5 µM, 150 mm × 4.6 mm) at a flow rate of 1 mL/min at 25°C. The mobile phase consisted of water containing 0.1% trifluoroacetic acid (A) and methanol containing 0.1% trifluoroacetic acid (B). A gradient program was used as follows: 15–50% B in 30 min, brought to 100% B in 5 min, maintained at 100% B for another 10 min, then back to 10% B in 5 min. The UV chromatogram was screened at 280 nM. The injection volume was 20 µL, and the concentration of the samples was 10 mg/mL. The three aqueous extracts were individually subjected to analytical HPLC under the specified conditions to compare their chromatographic profiles and assess the suitability of the method for subsequent preparative HPLC separation.

For isolation of pure compounds, three aqueous extracts were subjected separately to preparative HPLC (Thermo Scientific Dionex UPLC 3000) with a Phenomenex Luna C_{18} column (10 µM, 150 mm × 21.2 mm) at a flow rate of 10 mL/min at 25°C. The concentration of samples was 400 mg/mL, and the injection volume was 400 µL. The gradient system was the same as the method with the analytical HPLC system. The procedure was repeated for each extract until sufficient amounts of the compounds were obtained for structural elucidation. Compounds **1**–**2** and **5** from TFG, **6**–**7** from TK and **8**–**9** from TM were isolated from a reverse-phase preparative HPLC system as pure form. Two fractions from TFG and one from TM were re-purified on a reversed-phase semi-preparative HPLC system (Agilent 1260 Infinity) with an ACE C_{18} column (10 µM, 150 × 10 mm) at a flow rate of 2 mL/min at 25°C. The concentrations of samples were 10–20 mg/mL, and the injection volume was 60 µL. The mobile phase consisted of water containing 0.1% trifluoroacetic acid (A) and acetonitrile containing 0.1% trifluoroacetic acid (B). A gradient program was used individually for the fractions from TFG to afford compounds **3**–**4** as follows: 10–100% B in 30 min, maintained at 100% B for 10 min, then back to 10% B in another 5 min. Another program for the fraction of TM which contains compounds **10**–**11** as a mixture was as follows: 10–70% B in 30 min, brought to 100% B in 5 min, maintained at 100% B for 10 min, then back to 10% B in another 5 min. As a result, the structures of eleven compounds were elucidated from three extracts. For the structure elucidations of the isolated compounds, 1D and 2D NMR spectra were recorded on a Bruker Avance III NMR spectrometer (600 MHz), while high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analyses were performed using an Agilent 6530 LC-HRMS system. The list of elucidated flavonoids is given in Table 2. Compounds **1**–**5** were isolated from *T. foenum-graecum*, **6**–**7** were isolated from *T. kotschyi* and **8**–**11** were isolated from *T. macrorrhynca*.

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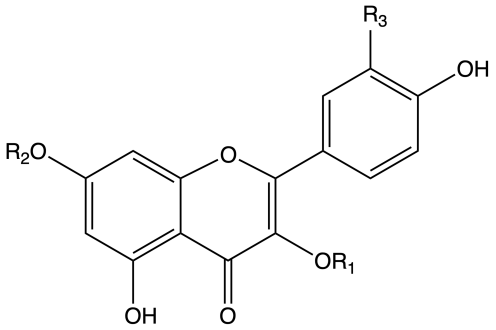
Table 2. Isolated flavonoids from three *Trigonella* species



Comp.s	Code	Name of compound	R_1	R_2	R_3
1	TFG-1	Quercetin 3-*O*-(2″-*O*-*β*-glucosyl)-*β*-galactoside 7-*O*-*β*-glucoside	Glu(1→2)Gal	Glu	OH
2	TFG-2	Kaempferol 3-*O*-(2″-*O*-*β*-glucosyl)-*β*-galactoside 7-*O*-*β*-glucoside	Glu(1→2)Gal	Glu	H
3*	TFG-3*	Quercetin 3-*O*-(2″-*O*-*β*-glucosyl-6″-*O*-acetyl)-*β*-galactoside 7-*O*-*β*-glucoside	Glu(1→2)(6″-Ac)Gal	Glu	OH
4	TFG-4	Kaempferol 3-*O*-(2″-*O*-*β*-glucosyl-6″-*O*-acetyl)-*β*-galactoside 7-*O*-*β*-glucoside	Glu(1→2)(6″-Ac)Gal	Glu	H
6	TK-1	Quercetin 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-galactoside 7-*O*-*β*-glucoside	Rham(1→2)Gal	Glu	OH
7	TK-2	Kaempferol 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-galactoside 7-*O*-*β*-glucoside	Rham(1→2)Gal	Glu	H
8	TM-1	Quercetin 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-glucoside 7-*O*-*β*-rhamnoside	Rham(1→2)Glu	Rham	OH
9	TM-2	Kaempferol 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-glucoside 7-*O*-*β*-rhamnoside	Rham(1→2)Glu	Rham	H
10*	TM-3	Kaempferol 3-*O*-(2″-*O*-(4‴-*O*-malonyl)-*α*-rhamnosyl)-*β*-glucoside) 7-*O*-*α*-rhamnoside	4‴-MalonylRham (1→2)Glu	Rham	H
11*	TM-4	Kaempferol 3-*O*-(2″-*O*-(4‴-*O*-methylmalonyl)-*α*-rhamnosyl)-*β*-glucoside) 7-*O*-*α*-rhamnoside	4‴-(Methylmalonyl) Rham (1→2)Glu	Rham	H

Note: *Previously undescribed compounds.

2.2.1 Quercetin 3-O-(2″-O-β-glucosyl-6″-O-acetyl)-β-galactoside 7-O-β-glucoside (3)

Amorphous powder. UV (MeOH) λ_{max}: 354, 256, and 200 nM. ^{1}H and ^{13}C NMR (CD_3OD): Tables 3 and 4; HR-ESI-MS: 829.2081 $[M-H]^-$, $C_{35}H_{41}O_{23}$; calc. 829.2044.

2.2.2 Kaempferol 3-O-(2″-O-(4‴-O-malonyl)-α-rhamnosyl)-β-glucoside) 7-O-α-rhamnoside (10)

Amorphous powder. UV (MeOH) λ_{max}: 347, 266, and 200 nM. ^{1}H and ^{13}C NMR (CD_3OD): Tables 3 and 4; HR-ESI-MS: 825.2125 $[M-H]^-$, $C_{36}H_{41}O_{22}$; calc. 825.2095.

2.2.3 Kaempferol 3-O-(2″-O-(4‴-O-methylmalonyl)-α-rhamnosyl)-β-glucoside) 7-O-α-rhamnoside (11)

Amorphous powder. UV (MeOH) λ_{max}: 349, 266, and 196 nM. ^{1}H and ^{13}C NMR (CD_3OD): Tables 3 and 4; HR-ESI-MS: 839.2285 $[M-H]^-$, $C_{37}H_{43}O_{22}$; calc. 839.2251.

2.3 Acid Hydrolysis of the Compounds for Sugar Identification

The compounds **3**, **10** and **11** were dissolved in 1 mL of methanol and subjected to acid hydrolysis by adding 2 mL of 12% HCl. The reaction mixture was heated at 80°C for one hour and subsequently partitioned with $CHCl_3$ to separate the aglycone portion. The remaining aqueous phase

Table 3. ^{13}C NMR[a] (150 MHz) spectroscopic data for the isolated compounds **3**, **10**, and **11**

	3 δ_C		**10** δ_C	**11** δ_C
Aglycon		**Aglycon**		
2	157.9	**2**	159.3	159.2
3	133.8	**3**	134.8	134.6
4	178.5	**4**	179.5	178.8
5	161.3	**5**	163.0	163.2
6	99.4	**6**	100.5	100.4
7	163.3	**7**	163.4	163.4
8	94.3	**8**	95.5	95.4
9	156.5	**9**	158.0	158.1
10	105.9	**10**	107.6	107.6
1′	121.9	**1′**	122.9	122.8
2′	114.8	**2′**	132.2	132.3
3′	144.5	**3′**	116.2	116.3
4′	148.7	**4′**	161.6	161.6
5′	116.4	**5′**	116.2	116.3
6′	121.2	**6′**	132.2	132.3
3-*O*-Gal		**3-*O*-Glu**		
1″	100.1	**1″**	100.4	100.5
2″	79.4	**2″**	80.4	80.1
3″	73.0	**3″**	78.8	78.9
4″	68.7	**4″**	71.7	71.7
5″	73.3	**5″**	78.5	78.4
6″	62.7	**6″**	62.7	62.7
2″-*O*-Glu		**2″-*O*-Rham**		
1‴	103.9	**1‴**	102.6	102.5
2‴	74.1	**2‴**	72.3	72.3
3‴	76.4	**3‴**	70.4	70.3
4‴	69.8	**4‴**	76.6	76.7
5‴	76.6	**5‴**	67.7	67.7
6‴	60.8	**6‴**	17.3	17.2
7-*O*-Glu		**7-*O*-Rham**		
1⁗	100.2	**1⁗**	99.9	99.8
2⁗	73.3	**2⁗**	71.8	71.9
3⁗	76.5	**3⁗**	72.1	72.1
4⁗	69.5	**4⁗**	73.6	73.6
5⁗	76.9	**5⁗**	71.3	71.2
6⁗	61.0	**6⁗**	18.1	18.1
6″-Ac		**4‴-*O*-Malonyl**		**4‴-*O*-Methyl-malonyl**
1⁗′	170.9	**1⁗′**	169.0	168.1
2⁗′	18.9	**2⁗′**	*	*
		3⁗′	171.1	168.8
		4⁗′		53.2

Note: [a]The residual solvent peak was calibrated as 49.00 ppm.
*Missing because of solvent peak.

was concentrated under reduced pressure and used for sugar analysis (Kutluay et al., 2019b). The sugar residues were compared with authentic standards (D-glucose, D-galactose, and L-rhamnose) by TLC. The analysis was carried out using silica gel plates with n-butanol-acetone-water (4:5:1, v/v/v) as the developing solvent system. The chromatograms were visualized after spraying with anisaldehyde reagent followed by heating at 110°C (EP8, 2013; Pruden et al., 1975).

2.4 Cytotoxic Activity Studies by MTT Method

Cytotoxic activity studies were conducted on two cancer cell lines, A549 (human lung carcinoma) and PC3 (human prostate adenocarcinoma). Considering that lung and prostate cancers are among the most prevalent cancer types worldwide (WHO, 2022), A549 and PC-3 cell lines were selected as suitable *in vitro* models to evaluate the cytotoxic potential of the extracts and isolated compounds. At least 12 wells from three or more different experiments were used to determine the results for each treatment and concentration. Both cell lines were cultured in DMEM with 10% fetal bovine serum and 0.5% penicillin-streptomycin solution. Cell suspension (100 µL; 10^5 cells/mL) was seeded on 96-well plates and incubated at 37°C containing 5% CO_2 for 24 hours. The medium in the wells was aspirated, and 100 µL of sample solutions (5–500 µg/mL for the extract and 2–100 µM for isolated compounds) were added and incubated for an additional 24 hours. At the end of incubation, 10 µL of 5 mg/mL MTT solution in PBS was added. After another 4–6 hours of incubation, the medium was removed, and 100 µL of DMSO was added to resolve formazan crystals. The absorbance was measured at 577/655 nM after shaking for 5 minutes (Al Groshi et al., 2018; Dogan et al., 2023). The IC_{50} value was calculated using the trendline obtained from cell viability and concentration data. Results are expressed as mean cell viability (%) ± SEM (standard error of the mean).

2.5 Statistical Analysis

Each experiment was performed at least three times, with a minimum of 12 data points per group. Statistical analyses were carried out using GraphPad Prism (version 6). Differences between group means were assessed by one-way ANOVA post-hoc Dunnnet's test, and $p < 0.05$ was considered statistically significant.

3 Results and Discussion

3.1 Determination of Isolated Compounds

Previous phytochemical studies on fenugreek have revealed the presence of various bioactive secondary metabolites, particularly polyphenolic constituents and nitrogen-containing compounds (Skaltsa, 2017). Among these metabolites, flavonoids are reported as characteristic constituents of the aerial parts of *Trigonella* species (Ayvazyan et al., 2023; Han et al., 2001; Kawashty et al., 1998). In a study comparing 8 *Trigonella* species grown in Egypt, 70% ethanol extracts of the aerial parts of the plants were found to be rich in kaempferol and quercetin glycosides (Kawashty et al., 1998). In a study on 95% ethanol extract of the aerial parts of *T. foenum-graecum*, 13 pterocarpane-type flavonoids, one of which was previously unreported, were isolated (Wu et al., 2020). In the present study, one previously

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Table 4. [1]H NMR[a] (600 MHz) spectroscopic data for the isolated compounds **3**, **10**, and **11** (δ_H(*J*, Hz)

	3 δ_H (*J*, Hz)		**10** δ_H (*J*, Hz)	**11** δ_H (*J*, Hz)
Aglycon		**Aglycon**		
6	6.52 d (2.1)	**6**	6.48 d (2.2)	6.49 d (2.4)
8	6.80 d (2.1)	**8**	6.77 d (2.2)	6.78 d (2.4)
2′	7.76 d (2.4)	**2′**	8.08 d (9)	8.10 d (9)
3′		**3′**	6.92 d (9)	6.92 d (9)
5′	6.91 d (8.4)	**5′**	6.92 d (9)	6.92 d (9)
6′	7.61 dd (8.4, 1.8)	**6′**	8.08 d (9)	8.1 d (9)
3-*O*-Gal		**3-*O*-Glu**		
1″	5.29 d (7.8)	**1″**	5.7 d (7.2)	5.7 d (7.2)
2″	4.06 t (7.8, 1.8)	**2″**	3.62 m	3.63 m
3″	3.73 m	**3″**	3.57 t (9)	3.57 t (8.4)
4″	3.81 m	**4″**	3.29 t (8.4)	3.29 t (8.4)
5″	3.67 m	**5″**	3.24 m	3.24 m
6″	4.21 dd (12, 7.8)	**6″**	3.75 dd (12, 2.4)	3.75 dd (12, 2.4)
	4.02 dd (12, 4.2)		3.5 dd (12, 6)	3.51 m
2″-*O*-Glu		**2″-*O*-Rham**		
1‴	4.76 d (7.8)	**1‴**	5.27 d (1.8)	5.25 d (1.8)
2‴	3.42 t (7.2)	**2‴**	4.08 dd (3.6, 1.8)	4.06 dd (3, 1.8)
3‴	3.56 m	**3‴**	4.01 dd (10.2, 3.6)	4.00 dd (9.6, 3)
4‴	3.44 m	**4‴**	4.96 t (9.6)	4.94 t (10.2)
5‴	3.36 m	**5‴**	4.24 dd (9.6, 6)	4.25 dd (10.2, 6.6)
6‴	3.83 dd (8.4, 2.4)	**6‴**	0.92 d (6)	0.89 d (6.6)
	3.75 dd (12, 4.8)			
7-*O*-Glu		**7-*O*-Rham**		
1⁗	5.08 d (7.8)	**1⁗**	5.58 d (2.4)	5.59 d (1.8)
2⁗	3.51 t (6)	**2⁗**	4.04 dd (3.6, 1.8)	4.04 dd (3.6, 1.8)
3⁗	3.43 m	**3⁗**	3.85 dd (9.6, 3.6)	3.85 dd (9, 3.6)
4⁗	3.43 m	**4⁗**	3.49 m	3.50 m
5⁗	3.55 m	**5⁗**	3.61 m	3.60 m
6⁗	3.94 dd (12.6, 2.4)	**6⁗**	1.27 d (6)	1.27 d (6)
	3.73 dd (12, 4.2)			
6″-Ac		**4‴-*O*-Malonyl**		**4‴-*O*-Methylmalonyl**
2⁗′	1.77 s	**2⁗′**	3.36 d (15)	3.36*
		4⁗′		3.55 s

Note: [a]The solvent peak was calibrated as 3.32 ppm.
*Not defined because of solvent peak.

undescribed flavonoid from the aqueous extract of *T. foenum-graecum* and two previously undescribed flavonoid glycosides from that of *T. macrorrhynca*, along with eight known compounds, were isolated and characterized.

According to 1D, 2D NMR spectra and pseudomolecular ion peaks, known compounds determined as quercetin 3-*O*-(2″-*O*-*β*-glucosyl)-*β*-galactoside 7-*O*-*β*-glucoside (**1**), kaempferol 3-*O*-(2″-*O*-*β*-glucosyl)-*β*-galactoside 7-*O*-*β*-glucoside (**2**), kaempferol 3-*O*-(2″-*O*-*β*-glucosyl-6″-*O*-acetyl)-*β*-galactoside 7-*O*-*β*-glucoside (**4**) (Han et al., 2001), phenylalanine (**5**) (Leyva et al., 2021), quercetin 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-galactoside 7-*O*-*β*-glucoside (**6**), kaempferol 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-galactoside 7-*O*-*β*-glucoside (**7**) (Kaouadji et al., 1990), quercetin 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-glucoside 7-*O*-*β*-rhamnoside (**8**), kaempferol 3-*O*-(2″-*O*-*α*-rhamnosyl)-*β*-glucoside 7-*O*-*β*-rhamnoside (**9**) (Ayvazyan et al., 2023; Fiorentino et al., 2009; Yang et al., 2015). Compounds **1**–**5** were isolated from *T. foenum-graecum*, **6**–**7** were isolated from *T. kotschyi*, **8**–**11** were isolated from *T. macrorrhynca*. The list of elucidated flavonoids is given in Table 2. Quercetin 3-*O*-(2″-*O*-*β*-glucosyl-6″-*O*-acetyl)-*β*-galactoside 7-*O*-*β*-glucoside (**3**) from *T. foenum-graecum*, kaempferol 3-*O*-(2″-*O*-(4‴-*O*-malonyl)-*α*-rhamnosyl)-*β*-glucoside) 7-*O*-*α*-rhamnoside (**10**) and kaempferol 3-*O*-(2″-*O*-(4‴-*O*-methylmalonyl)-*α*-rhamnosyl)-*β*-glucoside) 7-*O*-*α*-rhamnoside (**11**) from *T. macrorrhynca* were identified as previously unreported compounds.

To the best of our knowledge, compound **3** has not been previously isolated from any natural source. Its occurrence has only been suggested based on HPLC-Q-TOF-MS/MS analysis in a patent application (CN113912654A) in fenugreek leaf alcoholic extract, without isolation or structural confirmation (Yanfeng et al., 2022). The fragmentation pattern of acylated flavonoid glycosides does not yield conclusive information on the location of the acyl group on the sugar moiety (Beszterda & Franski, 2015). Accordingly, this study provides the first report of its isolation and complete structural characterization, including comprehensive NMR data. Furthermore, the compounds **10** and **11** were determined to be an unrecorded molecules after searching the SciFinder database.

The molecular formula of compound **3** was determined to be $C_{35}H_{42}O_{23}$ based on the ^{13}C NMR data and the major pseudomolecular ion peak (*m/z* 829.2081 $[M-H]^-$) in the (−)-HR-ESI-MS spectrum (Table 3). In the ^1H NMR spectrum, three aromatic proton signals observed at δ_H 7.76 (1H, d, J = 2.4 Hz, H-2′), 6.91 (1H, d, J = 8.4 Hz, H-5′), and 7.61 ppm (1H, dd, J = 8.4, 1.8 Hz, H-6′) indicated an ABX system, showing that the B-ring is 3′,4′-disubstituted. In addition, the signals observed at δ_H 6.52 (1H, d, J = 1.8 Hz, H-6) and 6.80 ppm (1H, d, J = 2.4 Hz, H-8), displaying meta coupling, are characteristic of a 5,7-disubstituted A-ring. The absence of the C-ring H-3 signal in the aromatic region confirmed that the aglycone of the structure is quercetin.

Three anomeric proton signals observed at δ_H 5.29 (1H, d, J = 7.8 Hz, H-1″), 4.76 (1H, d, J = 7.8 Hz, H-1‴), and 5.08 ppm (1H, d, J = 7.8 Hz, H-1⁗) indicated that the compound is a quercetin triglycosides. Among the 35 resonances observed in the ^{13}C NMR spectrum, 15 belong to quercetin and 18 signals originate from the three hexose moieties. Following acid hydrolysis and TLC analysis, compound **3** was confirmed to contain glucose and galactose units. The ^1H NMR (δ_H 1.77, 3H, s) and ^{13}C NMR (δ_C 18.9 and 170.9 ppm) spectral data indicated the presence of an additional acetyl group.

All sugar signals were assigned starting from the anomeric proton signals with the aid of the COSY spectrum. The anomeric coupling constant values (J = 7.8 Hz) indicated the β-bond configuration. After comparison with the literature, together with HMQC and ^{13}C NMR data, the sugars were identified as two β-D-glucose (Glc) and one β-D-galactose (Gal) residues (Han et al., 2001). Evaluation of the unsubstituted galactose data revealed that the downfield shift of approximately 6 ppm at the carbon resonance of galactose C-2″ (δ_C 79.4), the upfield shift of approximately 5 ppm at C-1″ (δ_C 100.1), and the HMBC correlation between δ_H 4.76 (H-1‴) and δ_C 79.4 (C-2″) clearly demonstrated that glucose is linked to the C-2 position of galactose (Cho et al., 2008). Furthermore, the downfield shift of approximately 2.5–3 ppm at the carbon resonance of galactose C-6″ (δ_C 62.7), the upfield shift of approximately 2–2.5 ppm at C-5″ (δ_C 73.3), and the HMBC correlation between δ_H 1.77 (Ac-$COCH_3$) and δ_C 62.7 (C-6″) indicated that acylation occurs at the C-6 position of galactose (Han et al., 2001; Yasukawa & Takido, 1988). Correlations between the galactose proton H-1″ (δ_H 5.29) and the C-3 carbon of the aglycone C-ring, as well as between glucose proton H-1⁗ (δ_H 5.08) and C-7 of the A-ring in the HMBC spectrum, confirmed the substitution. As a result, based on COSY, HSQC, and HMBC spectral analyses, the structure of the compound **3** was elucidated as quercetin 3-*O*-β-D-glucosyl(1→2)-(6″-*O*-acetyl)-β-D-galactoside 7-*O*-β-D-glucoside (Figure 1).

The ^1H NMR spectra of compounds **10** and **11** revealed four types of aromatic proton signals, including H-6 and H-8, as well as an AB system consisting of four protons, which is characteristic of a monosubstituted B-ring, thereby indicating a kaempferol aglycone. The ^1H NMR spectrum further suggested the presence of a flavonoid glycoside with three sugar moieties. Compounds **10** and **11** were confirmed to contain glucose and rhamnose units based on comparison of their Rf values and colour reactions with authentic sugar standards. The positions of the sugar proton signals were assigned based on COSY correlations, starting from the anomeric protons. Based on the spectral data and comparison with the literature, the three sugar moieties were identified as one glucose and two rhamnose units (Ayvazyan et al., 2023; Norbæk & Kondo, 1999; Yahara et al., 2000; Yang et al., 2015).

In the HMBC spectrum, compound **10**, the anomeric proton of glucose (δ_H 5.70, H-1″) showed a correlation with the C-3 (δ_C 134.8). HMBC correlation between the anomeric proton of rhamnose (δ_H 5.27, H-1‴) and the C-2″ of glucose (δ_C 80.4) indicated the glycosidic linkage between these two sugar units. The correlation of another rhamnose anomeric proton (δ_H 5.58, H-1⁗) with the aglycone C-7 (δ_C 163.4) established the molecular structure. In contrast to compounds **8** and **9**, a downfield shift of approximately 2.5 ppm at C-4‴ and the HMBC correlation between H-4‴ (δ_H 4.96, t, J = 9.6) and the carbonyl carbon of the malonyl group (δ_C 169.0) indicated substitution by the malonyl unit (Figure 1) (Atay et al., 2015; Fiorentino et al., 2009; Kutluay et al., 2019a; Wang et al., 2016). Additionally, the fragmentation pattern of the compound was consistent with the literature. HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 825.2125 $[M-H]^-$ corresponding to the molecular formula $C_{36}H_{42}O_{22}$. In particular, the pseudomolecular ion peak together with the fragment ions at m/z 781 $[M-CO_2-H]^-$ and *m/z* 739 [M-malonyl-H]$^-$ confirmed the presence of a malonyl substituent in the structure (Kazuma et al., 2003; Stein & Zinsmeister, 1990). The methylene proton signals were observed at δ_H 3.36 ppm (J = 15 Hz) in methanol-d_4. The absence of the methylene carbon signal in methanol-d_4 was independently confirmed by ^{13}C NMR spectroscopy recorded in pyridine-d_6, where the corresponding signal appeared at δ_C 43.1 ppm (Figure S13 in supporting information) (Kazuma et al., 2003; Saito et al., 2012; Takeda et al., 1993). Thus, compound **10** was identified as kaempferol 3-*O*-(2″-*O*-(4‴-*O*-malonyl)-α-rhamnosyl)-β-glucoside) 7-*O*-α-rhamnoside (Figure 1).

The mass spectral data of compound **11** showed a mass increase of 14 Da compared to the related compound **10**, suggesting the presence of a methyl group. The pseudomolecular ion peak at *m/z* 839.2285 $[M-H]^-$ corresponding to the molecular formula $C_{37}H_{44}O_{22}$ together with the fragment ions at *m/z* 825 $[M-CH_3]^-$ and 739 [M-CH_3-malonyl-H]$^-$

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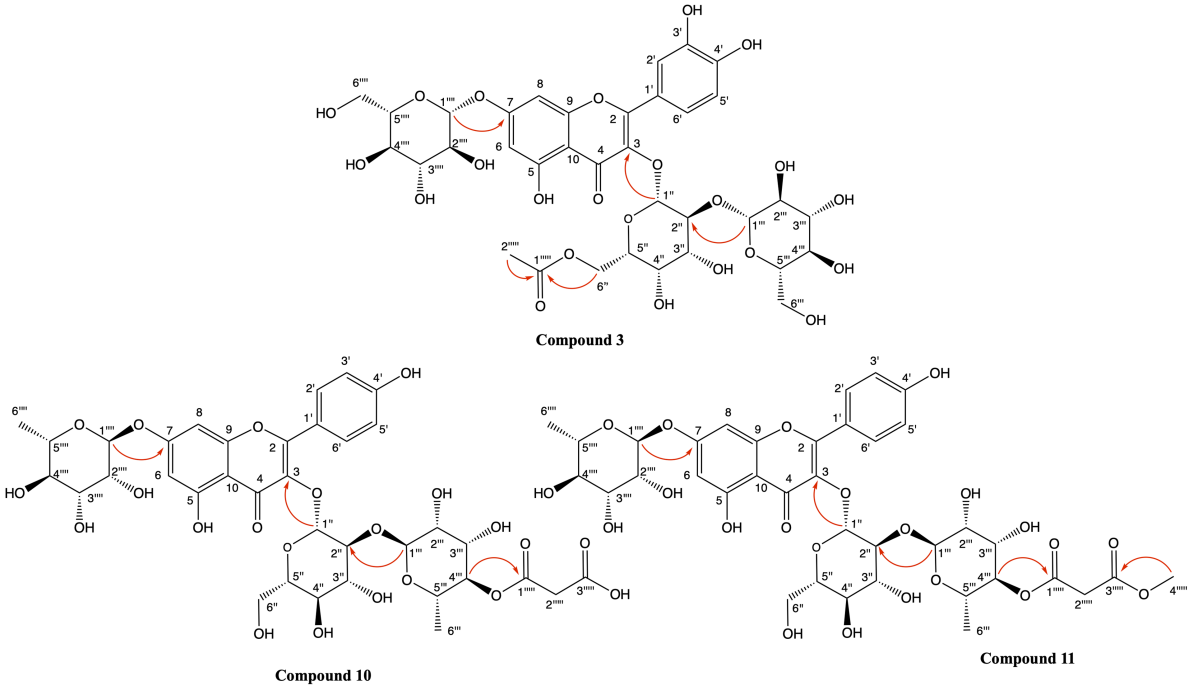

Figure 1. HMBC correlations (red arrows) for compounds **3**, **10** and **11**

supported this assumption. This assignment was further supported by the ^{1}H and ^{13}C NMR spectrum, which exhibited a characteristic methoxy signal at δ_H 3.55 and δ_C 53.2. Moreover, the HMBC correlation between the methoxy protons and malonyl carboxyl carbon at 168.8 ppm confirmed the position of methylation (Lv et al., 2023). Accordingly, with the help of HMBC spectrum, compound **11** was identified as kaempferol 3-*O*-(2″-*O*-(4‴-*O*-methylmalonyl)-*α*-rhamnosyl)-*β*-glucoside 7-*O*-*α*-rhamnoside (Figure 1).

To the best of our knowledge, the isolation of secondary metabolites from the selected endemic species has never been reported before. However, all the isolated flavonoids were found to be triglycosides of quercetin or kaempferol, which are found in several Fabaceae family members (Han et al., 2001; Spanou et al., 2008; Spanou et al., 2012). Previous studies have indicated that *Trigonella* species are particularly rich in kaempferol and quercetin glycosides (Ayvazyan et al., 2023; Han et al., 2001; Noviany et al., 2023; Skaltsa, 2017). In addition, chemotaxonomic investigations within the genus have demonstrated that flavonoid glycoside distribution may vary among different sections. Especially, the occurrence of 3- and 7-glycosylated quercetin derivatives has been associated with specific sections such as Cylindraceae and Foenum-graecum. The isolation of quercetin and kaempferol triglycosides in the present study is therefore consistent with the previously reported chemotaxonomic characteristics of the genus and further supports the significance of flavonoid glycosides as useful chemotaxonomic markers within *Trigonella* species (Kawashty et al., 1998; Saleh et al., 1982). Additionally, previous studies on Fabaceae species have demonstrated the occurrence of flavonoid derivatives substituted with organic acids such as malonic and acetic acids. However, these compounds have generally been reported based on tentative LC-MS identifications (Khalil et al., 2023).

3.2 Cytotoxic Activities of the Extracts and Isolated Compounds

The cytotoxic effects of the extracts were evaluated against A549 and PC-3 cell lines. The cytotoxicity assay was performed following a 24 h incubation period, which was considered suitable for preliminary screening of the extracts and isolated compounds, although longer incubation times may reveal additional late-stage apoptotic effects. The aqueous extract of *T. kotschyi* (TK) exhibited an IC_{50} value of 75.54 µg/mL and 64.14 µg/mL for A549 and PC-3 cell lines, respectively. The extract significantly reduced cell viability within the concentration range of 50–500 µg/mL ($p < 0.0001$) (Figure 2). Based on these findings, two flavonoids (compounds **6** and **7**) isolated from the active extract, together with trigonelline, an alkaloid commonly reported in the *Trigonella* genus and frequently associated with the plant's biological activities, were further evaluated for their cytotoxic potential in comparison with the positive control, doxorubicin (Figure 3). Nevertheless, no significant cytotoxic activity was shown by any of the investigated substances, indicating that the aqueous extract's reported effect might be due to the synergistic interactions among multiple constituents rather than a single dominant compound. These results are consistent with earlier observations highlighting the importance of complex phytochemical compounds in biological activity, suggesting that minor components or combination actions of the extract may contribute to the observed cytotoxicity (Dogan et al., 2022).

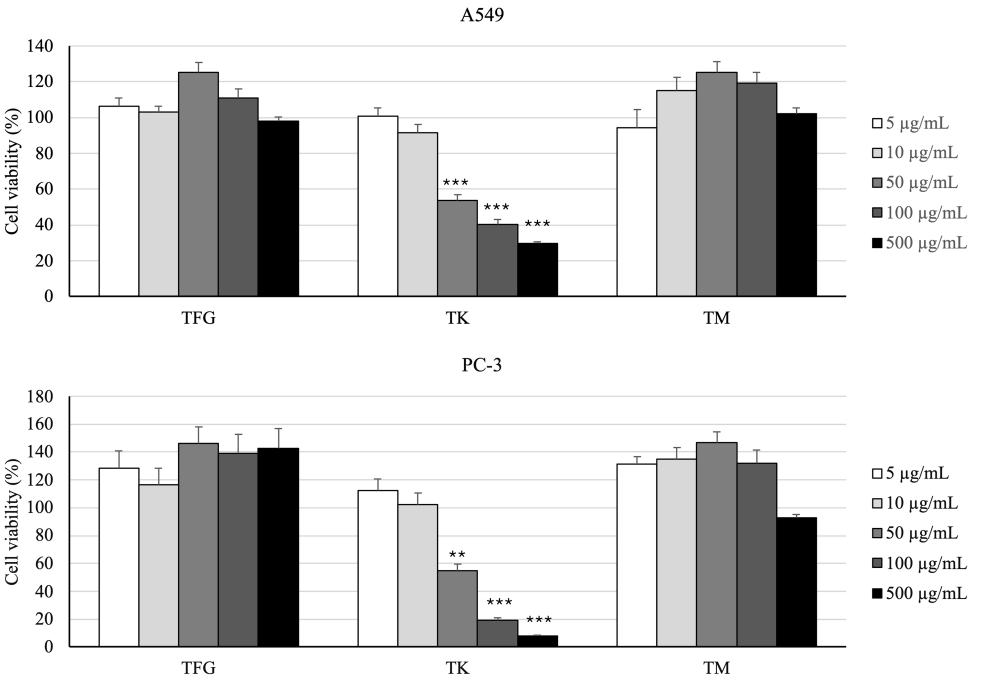

Figure 2. Cytotoxic activity of the selected *Trigonella* extracts on A549 and PC-3 cell lines. (**$p < 0.001$, ***$p < 0.0001$)

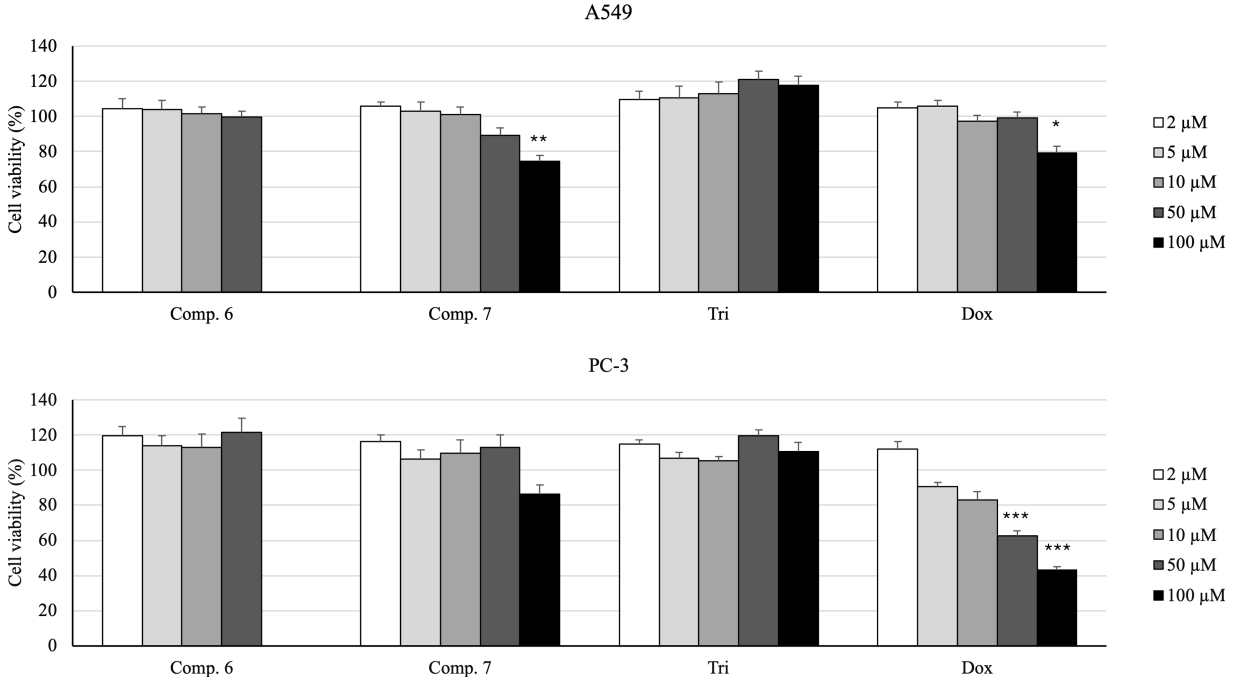

Figure 3. Cytotoxic activity of compounds **6**–**7**, trigonelline (Tri) and doxorubicin (Dox) on A549 and PC-3 cell lines. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$)

Previous studies on *Trigonella* species have demonstrated notable cytotoxic activities, particularly for seed extracts. Ahmad et al. (2025) investigated the cytotoxic effects of *T. foenum-graecum* seed extracts collected from Egypt, India, Saudi Arabia, Yemen, and Iraq against HT29, MCF7, MRC5, and HepG2 cell lines. Among the tested extracts, methanolic extracts showed relatively stronger activities compared to hexane and chloroform extracts, and the

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methanolic extract of the Iraqi sample exhibited a potent cytotoxic effect against HepG2 cells with an IC_{50} value of 8.2 µg/mL. The authors suggested that flavonoids and alkaloids may contribute significantly to the biological activities of fenugreek seeds. In another study, the hexane, ethanol, and aqueous extracts of the whole plants of *T. filipes* Boiss, *T. strangulata* Boiss., and *T. uninata* Banks and Sol. were evaluated against HeLa and MCF7 cell lines, showing IC_{50} values ranging from 39.6 to 542.0 µg/mL (Mojarradgandoukmolla et al., 2024). When considered together with the findings of the present study, these results suggest that the seeds of *Trigonella* species may exhibit stronger cytotoxic effects than the aerial parts.

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Author Contributions

Zeynep Dogan: Writing-original draft, Investigation, Data curation. Ozan Kaplan: Data curation, Writing-review & editing. Murat Kilic: Data curation, Writing-review & editing. Lutfun Nahar: Writing-review & editing, Supervision, Project administration. Satyajit D. Sarker: Writing-review & editing, Supervision, Project administration.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journals/records-of-natural-products.

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