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Investigation of phytochemical profile, cytotoxic and antioxidant activities of *Michauxia campanuloides* L'Hérit. Ex Aiton

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ABSTRACT: *Michauxia campanuloides,* a traditionally used plant in Southern Anatolia, was investigated for its bioactivity and phytochemical profiles. Methanolic extracts were first prepared from the roots and aerial parts of *M. campanuloides*, then each extract was suspended in water and partitioned with *n*-hexane, EtOAc and *n*-butanol, respectively. Methanolic extracts, subextracts and the remaining water extracts were evaluated for their *in vitro* cytotoxicity against colon (HCT116, SW480), breast (MDA-MB-231, HCC1937), and liver (HEP3B, HEPG2) cancer cell lines by MTS assay. The EtOAc subextract of roots showed the highest cytotoxic activity against all tested cancer cell lines (IC_{50} = 10–154.76 µg/mL). Antioxidant activities were assessed using ABTS, DPPH, FRAP, and CUPRAC assays. The EtOAc subextracts derived from both aerial parts and roots exhibited the highest antioxidant activity (353.40 ± 19.21 and 367.65 ± 16.38 mg TE/g in ABTS, respectively). HPTLC was used for phytochemical profiling of the extracts, while UPLC was used for the quantification of the investigated phenolic compounds, including rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid, and quercetin. Astragalin (93.37 ± 0.11 mg/g) and isoquercitrin (51.22 ± 0.81 mg/g) were the main compounds of the EtOAc subextract. This is the first report on *M. campanuloides* evaluating its cytotoxic activity and quantification of the marker compounds.

KEYWORDS: *Michauxia campanuloides*; cytotoxicity; antioxidant; HPTLC; UPLC.

1. INTRODUCTION

There are seven species of *Michauxia* genus (Campanulaceae), also known as 'Dart Bellflower' including *M. campanuloides*, *M. laevigata*, *M. nuda*, *M. tchihatchewii*, *M. thyrsoidea*, *M. koeieana*, and *M. stenophylla* distributed across globe, known by now [1]. *M. campanuloides* (Figure 1), a perennial plant, grows naturally in rocky outcrops and dry open sites of Lebanon-Syria, Palestine and Southern Anatolia of Türkiye [2]. The stems and roots of some *Michauxia* species have traditionally been used as a food source [3]. Ethnobotanical studies in Türkiye reported that *M. campanuloides* is an edible and medicinal plant. The crushed leaves are used for wound healing externally [3, 4]. Besides, the plant is also utilized against mycosis [5].

Studies on various *Michauxia* species have revealed diverse bioactivities, including antioxidant, antimicrobial, cytotoxic [6], anti-inflammatory, and wound-healing activities [7]. Specifically, *M. campanuloides* has shown notable antioxidant, anti-inflammatory, and wound-healing activities in previous studies [7-10].

Phytochemical studies on the genus *Michauxia* are limited to only a few investigations. One study identified 91 volatile compounds in *M. laevigata* using GC-MS, including dihydroactinidiolide, α-terpinolene, caryophyllene oxide, and viridiflorol [11]. Another study analyzed five *Michauxia* species (*M. campanuloides*, *M. laevigata*, *M. nuda*, *M. tchihatchewii*, and *M. thyrsoidea*) and determined that *M. campanuloides* had the highest total phenolic content (TPC)[7, 9].

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Figure 1. *Michauxia campanuloides* (Belen, Hatay, Türkiye, taken by Rengin Baydar).

Antioxidants have recently attracted significant attention due to their role in various biological processes. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, is strongly associated with the onset and progression of diseases such as cancer. By neutralizing ROS, antioxidants help mitigate cellular damage, which, if left uncontrolled, can lead to unregulated cell growth and tumour formation [12]. Given this relationship between oxidative stress and cancer, the exploration of plant-derived compounds with both antioxidant and anticancer properties has become increasingly important. Thus, evaluating the anticancer potential of plant extracts has emerged as a crucial approach in the search for new therapeutic agents [13].

To date, there is no available research on the cytotoxic activities and detailed phytochemical composition of *M. campanuloides*. This gap in knowledge highlights the need for further investigation into the potential bioactivities of this plant. This study aims to evaluate the cytotoxic and antioxidant activities along with the phytochemical profile of *M. campanuloides*. The main steps of the study include: 1)Preparing methanolic extracts from the root and aerial parts, 2) partitioning water dispersed methanolic extracts with organic solvents in increasing polarity (*n*-hexane, ethyl acetate, and *n*-butanol); 3) Assessing the cytotoxic effects of the extracts on colon, liver, and breast cancer cell lines; 4) Evaluating antioxidant activity through ABTS, DPPH, CUPRAC, and FRAP assays; 5) Estimating total phenolic (TPC) and flavonoid (TFC) content; 6) Identifying and quantifying phenolic compounds (rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid, and quercetin) using High-Performance Thin-Layer Chromatography (HPTLC) and Ultra-Performance Liquid Chromatography (UPLC).

2. RESULTS

2.1. Extraction and Partition

The dried, powdered roots (50.0 g) and aerial parts (100.0 g) of *M. campanuloides* were extracted with MeOH (0.5 L × 4 h × 2 for roots and 1 L × 4 h × 2 for aerial parts) at 45 °C and then methanol was evaporated using a rotary evaporator (13.2 g, yield: 26.44% for roots; and 22.2 g, yield: 22.2% for aerial parts). The methanolic extracts were dispersed in H_2O (25 mL for roots, and 50 mL for aerial parts) and partitioned successively with equal volumes of *n*-hexane, EtOAc, and *n*-BuOH (25 mL × 3 for roots and 50 mL × 3 for aerial parts). Finally, subextracts of *n*-hexane (0.6 g), EtOAc (0.2 g), and *n*-BuOH (0.8 g) from the roots, and *n*-hexane (5.1 g), EtOAc (0.4 g), and *n*-BuOH (2.4 g) from the aerial parts were obtained. The remaining water extracts (*r*H_2O) were lyophilized and the yields were 10.0 g for roots and 12.9 g for aerial parts.

2.2. Cytotoxic Activity

The cytotoxic potential of *M. campanuloides* methanolic extracts, subextracts, and *r*H_2O extracts from the roots and aerial parts was evaluated using the MTS assay on HEP3B, HEPG2, HCT116, SW480, MDA-MB-231, and HCC1937 cancer cell lines as well as a healthy cell line, L929. Paclitaxel was used as the positive control. The findings are presented in Table 1.

Table 1. Cytotoxic activities of the extract on tested cell lines.

Cell lines (IC_{50}) (µg/mL)	Positive Control and Extracts		
	MCR EtOAc (µg/mL)	MCR *n*-hexane (µg/mL)	Paclitaxel* (nM)
HEP3B	123.32 ± 22.38	ND	6.35 ± 0.48
HEPG2	57.69 ± 7.72	144 ± 12.65	14.67 ± 0.73
HCT116	62.32 ± 2.09	ND	4.67 ± 0.54
SW480	108.57 ± 9.35	ND	2.93 ± 0.33
MDA-MB-231	154.76 ± 12.76	ND	19.31 ± 1.26
HCC1937	10.0 ± 0.53	ND	ND
L929	13.71 ± 1.43	128.92 ± 15.28	ND

The data represents the mean ± SD of three independent experiments. Extracts that did not exert cytotoxic activity within the tested concentration interval are not presented. ND: Not Detected. MCR: *M. campanuloides* root. * Positive Control (nM)

The results revealed that the EtOAc subextract derived from the roots exhibited moderate cytotoxicity, with IC_{50} values ranging between 10.0 - 154.76 µg/mL against studied cancer cell lines. The *n*-hexane subextract of *M. campanuloides* roots showed moderate cytotoxicity against HEPG2 cancer cell line with the IC_{50} value of 144 ± 12.65 µg/mL. On the other hand, the *n*-BuOH subextract from the roots and the *n*-hexane, EtOAc, and *n*-BuOH subextracts from the aerial parts of *M. campanuloides* did not exhibit cytotoxic activity.

2.3. Antioxidant Activity

2.3.1. ABTS Radical Scavenging Assay

The ABTS assay was performed to evaluate the antioxidant capacity of all extracts and subextracts. As shown in Table 2, the EtOAc subextracts of the roots and aerial parts exhibited statistically significant antioxidant activity (367.65 ± 16.78 and 353.40 ± 19.21 mg TE/g, respectively). In contrast, the *r*H_2O extract of the roots showed the lowest activity (16.67 ± 0.46 mg TE/g).

2.3.2. DPPH Radical Scavenging Assay

The DPPH assay was used to assess the antioxidant capacity of *M. campanuloides* extracts and subextracts. According to the results (Table 2), the EtOAc subextract of the aerial parts demonstrated the highest DPPH radical scavenging activity (223.41 ± 4.7 mg TE/g). Conversely, the *r*H_2O extract of the roots exhibited the lowest activity (3.52 ± 0.87 mg TE/g).

2.3.3. Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP assay revealed notable variation among the samples. The EtOAc subextract of the aerial parts exhibited the most significant activity (342.96 ± 22.55 mg TE/g). Similarly, the EtOAc subextract of the roots and the *n*-BuOH subextract of the aerial parts displayed high activity (208.5 ± 4.28 and 105.43 ± 6.94 mg TE/g, respectively). Conversely, the *r*H_2O extract of the roots had the lowest antioxidant activity (1.85 ± 0.27 mg TE/g). Detailed results are presented in Table 2.

2.3.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

The CUPRAC assay results (Table 2) indicated that the EtOAc subextract prepared from aerial parts exhibited remarkable antioxidant activity, with 583.46 ± 7.91 mg TE/g. Similarly, the EtOAc subextract of the roots and the *n*-BuOH subextract of the aerial parts exhibited notable antioxidant potential (341.51 ± 2.11 and 210.16 ± 6.05 mg TE/g, respectively). On the other hand, the *r*H_2O extract of the roots exhibited the lowest activity (5.02 ± 0.54 mg TE/g).

Table 2. Total antioxidant contents of *M. campanuloides* roots and aerial parts.

Extracts	ABTS	DPPH	FRAP	CUPRAC
	mg TE/g			
MeOH	27.531±1.299^{cd}	5.965±0.584^{ef}	6.258±0.482^{ef}	11.567±0.418^{f}
n-Hexane	40.371±0.853^{cd}	21.286±0.459^{def}	13.249±1.742^{ef}	46.685±0.777^{de}
EtOAc	367.651±16.382^{a}	155.827±16.506^{b}	208.501±4.278^{b}	341.506±2.111^{b}
n-BuOH	53.574±1.248^{c}	20.324±0.903^{def}	37.210±0.217^{d}	54.272±0.497^{de}
rH_2O	16.673±0.459^{d}	3.52±0.875^{f}	1.852±0.226^{f}	5.017±0.544^{f}
MeOH	46.631±0.734^{c}	24.031±0.722^{de}	26.673±1.310^{de}	56.214±3.805^{d}
n-Hexane	26.370±0.460^{cd}	16.155±0.804^{def}	11.821±1.181^{ef}	44.349±1.612^{e}
EtOAc	353.399±19.206^{a}	223.413±4.705^{a}	342.957±22.549^{a}	583.461±7.912^{a}
n-BuOH	198.823±16.781^{b}	103.241±13.280^{c}	105.428±6.937^{c}	210.160±6.051^{c}
rH_2O	45.596±0.660^{c}	27.224±1.403^{d}	15.352±1.348^{def}	55.237±1.788^{d}

The data represents the average ± SD of three independent experiments. Different letters within the same column indicate values that are significantly different at a significance level of $p \leq 0.05$.

2.4. Phytochemical Studies

2.4.1. Total Phenolic Content Assay

The Folin-Ciocalteu reagent, which reacts with phenolic compounds to produce a blue chromophore, played a key role in quantifying the TPC of these extracts [14]. Accordingly, the EtOAc subextract of the aerial parts of *M. campanuloides* exhibited the highest TPC with 204.32 ± 27.29 mg GAE/g, followed by EtOAc subextract of the roots (144.94 ± 1.2 mg GAE/g) and *n*-BuOH subextract of the aerial parts (129.73 ± 4.58 mg GAE/g). In contrast, the *r*H_2O extract of the roots exhibited the lowest TPC, with a value of 5.56 ± 0.32 mg GAE/g. These findings indicated the variability in phenolic content among the different extracts and subextracts of *M. campanuloides*. The detailed results of the TPC assay are presented in Table 3.

2.4.2. Total Flavonoid Content Assay

The $FeCl_3$ method enables to quantify flavonoid content by forming a yellow-colored complex between flavonoids and ferric chloride [15]. Based on the results, *n*-BuOH subextract of the aerial parts of *M. campanuloides* exhibited the highest total flavonoid content (TFC) with 29.27 ± 0.9 mg QE/g. Additionally, EtOAc subextract of the aerial parts displayed a statistically comparable TFC, with a value of 28.46 ± 1.18 mg QE/g. In contrast, the *r*H_2O extract of the roots demonstrated the lowest TFC, measured at 0.04 ± 0.009 mg QE/g. The results are given in Table 3.

2.5. Chromatography

2.5.1. High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC enables simultaneous analysis of multiple samples by comparing band colors and retention factors (R_F). In this study, compounds were identified based on their characteristic band colors and R_F values. Orange-colored bands at $R_F \approx 0.37$, ≈ 0.6, and ≈ 0.95 corresponded to rutin, isoquercitrin, and quercetin, respectively. Similarly, blue-colored bands at $R_F \approx 0.48$, ≈ 0.53, and ≈ 0.91 were identified as ChA, NeoChA, and caffeic acid. Additionally, a green-colored band at $R_F \approx 0.67$ was determined as astragalin (Figure 2).

The methanolic extract of *M. campanuloides* aerial parts was found to contain rutin, ChA, NeoChA, isoquercitrin, and astragalin. The *n*-hexane subextract exhibited a similar profile, with the addition of a red band at $R_F \approx 0.98$. In the EtOAc subextract, all compounds were detected along with a blue-colored band between $R_F \approx 0.82$–0.90 and a green-colored band at $R_F \approx 0.98$. The *n*-BuOH subextract contained ChA, NeoChA, isoquercitrin, and astragalin, as well as an unknown compound at $R_F \approx 0.82$. In contrast, only ChA and NeoChA were detected in the *r*H_2O extract.

Table 3. The results of total phenolic content and total flavonoid content assays.

	Extracts	Total Phenolic Content	Total Flavonoid Content
		mg GAE/g	mg QE/g
Roots	MeOH	10.658±1.236^{d}	0.548±0.0242^{de}
	n-Hexane	19.078±0.3^{cd}	1.349±0.098^{de}
	EtOAc	141.942±1.202^{b}	9.768±0.140^{c}
	n-BuOH	36.570±1.557^{c}	1.750±0.029^{d}
	H_2O	5.563±0.321^{d}	0.040±0.009^{e}
Aerial parts	MeOH	30.085±2.445^{cd}	8.383±0.427^{c}
	n-Hexane	22.002±0.728^{cd}	14.413±0.533^{b}
	EtOAc	204.320±27.290^{a}	28.462±1.183^{a}
	n-BuOH	129.733±4.581^{b}	29.276±0.905^{a}
	H_2O	25.104±3.509^{cd}	1.429±0.111^{de}

The results were given as average ± SD. mg GAE/g: mg gallic acid equivalent per g extract. mg QE/g: mg quercetin equivalent per g extract. Different letters within the same column indicate values that are significantly different at a significance level of $p \leq 0.05$.

The methanolic extract of *M. campanuloides* roots contained ChA and a green-colored band at $R_F \approx 0.98$. A similar green-colored band was also observed in the *n*-hexane subextract. The EtOAc subextract contained ChA, caffeic acid, and quercetin, along with two blue-colored bands at $R_F \approx 0.71$ and ≈ 0.85, and a green-colored band at $R_F \approx 0.98$. The *n*-BuOH subextract exhibited ChA and a blue-colored band at $R_F \approx 0.85$, while no investigated compounds were detected in the *r*H_2O extract.

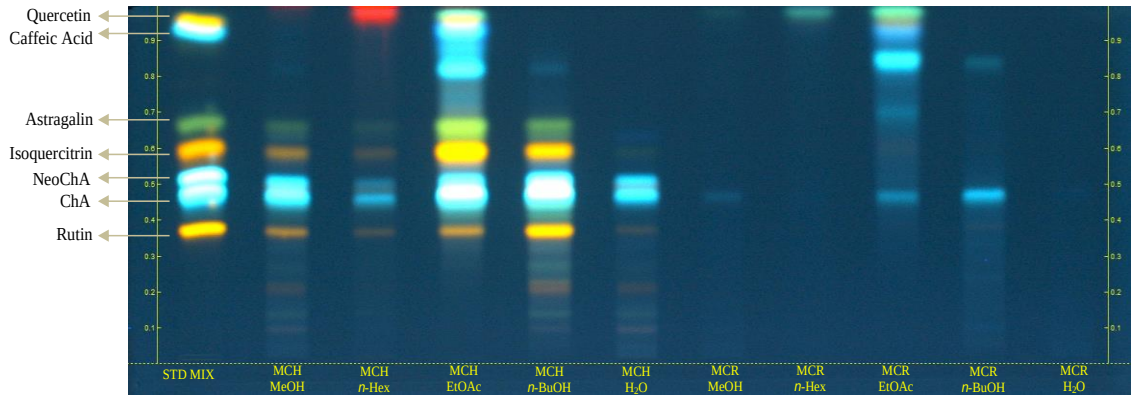

Figure 2. HPTLC chromatogram of root and aerial parts of *M. campanuloides* at 366 nm after derivatization with NP/PEG reagents. Developing solvent system: EtOAc-AcOH-FA-H_2O (100:11:11:26, *v*/*v*/*v*/*v*), ChA: Chlorogenic acid, MCH: *M. campanuloides* aerial parts, MCR: *M. campanuloides* roots, NeoChA: Neochlorogenic acid, STD MIX: Standard mixture.

The cytotoxicity results of the EtOAc and *n*-hexane subextracts from *M. campanuloides* roots were not related with the investigated compounds. This result showed that other bioactive compounds could be responsible for the cytotoxic activity. To screen for these unidentified compounds, the plate was derivatized with anisaldehyde and visualized under white light. As shown in Figure 3, the results revealed the presence of other compounds, highlighting the need for further research to identify and characterize these bioactive constituents.

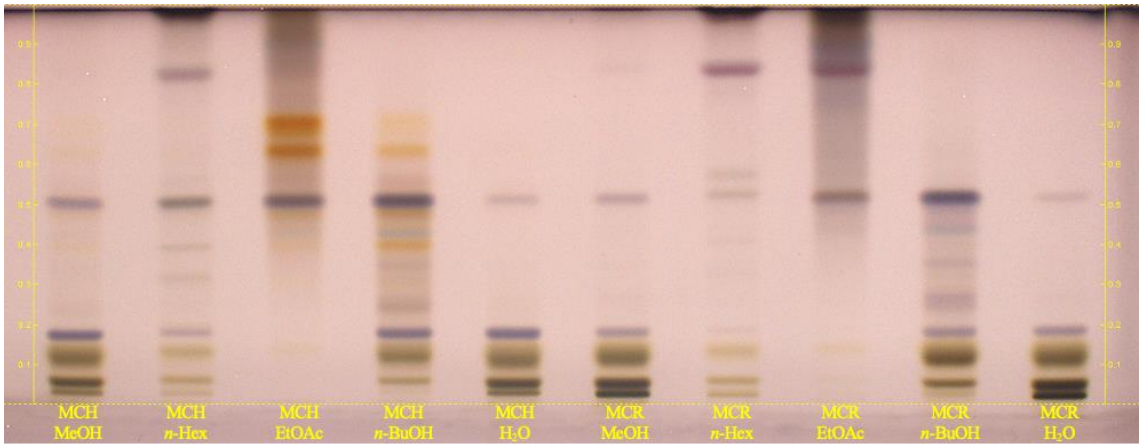

Figure 3. HPTLC chromatogram of root and aerial parts of *M. campanuloides* at white light after derivatization with anisaldehyde reagent. Developing solvent system: EtOAc-AcOH-FA-H_2O (100:11:11:26, $v/v/v/v$), MCH: *M. campanuloides* aerial parts, MCR: *M. campanuloides* roots.

2.5.2. Ultra-Performance Liquid Chromatography (UPLC)

The UPLC method was utilized for the qualitative and quantitative analysis of the samples and standard compounds, including NeoChA, ChA, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin. The method employed multiple wavelengths selected based on the absorption spectra of the standards. NeoChA and ChA were detected at 325 nm, caffeic acid at 323 nm, rutin and isoquercitrin at 256 nm, astragalin at 264 nm, and quercetin at 371 nm.

Method validation was performed following ICH guidelines, focusing on specificity, linearity, precision, and recovery parameters. The specificity of the developed method was demonstrated by the absence of standard compounds (NeoChA, ChA, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin) in the blank chromatogram. The retention times (t_R) for the standards were determined as follows: NeoChA ($t_R \approx 2.9$), ChA ($t_R \approx 3.7$), caffeic acid ($t_R \approx 5.0$), rutin ($t_R \approx 8.5$), isoquercitrin ($t_R \approx 10.3$), astragalin ($t_R \approx 15.1$), and quercetin ($t_R \approx 20.6$), shown in Figure 4.

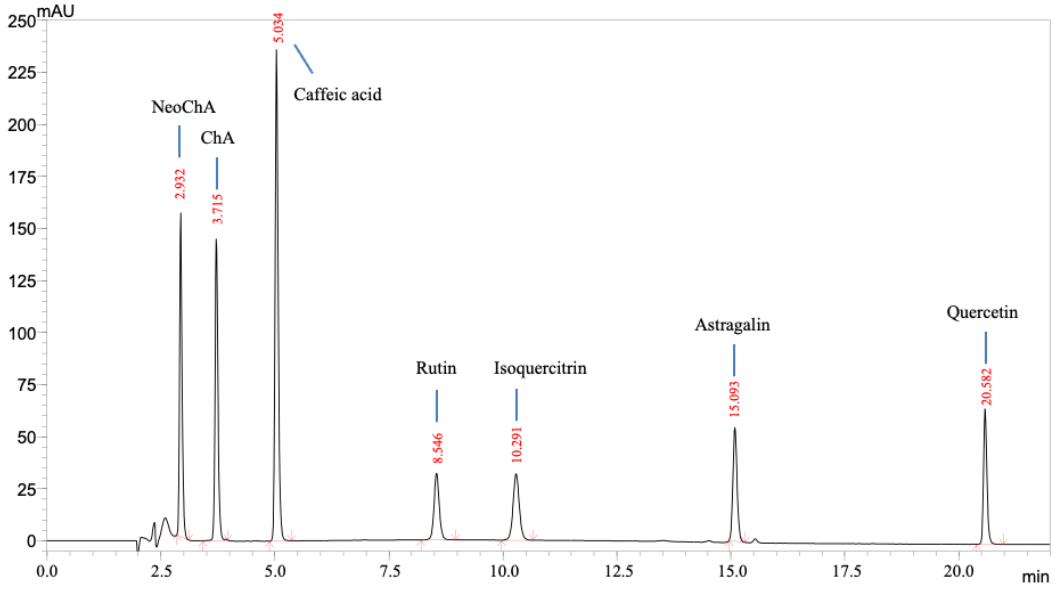

Figure 4. UPLC chromatogram of standards at 256 nm.

NeoChA: neochlorogenic acid, ChA: chlorogenic acid.

The limits of quantification (LOQ) and detection (LOD) were calculated based on the linearity data, using the formulas LOQ = 10 × (SD/S) and LOD = 3 × (SD/S), where SD is the standard deviation and S is the slope of the calibration curve. The calculated LOQ and LOD values are presented in Table 4.

Table 4. Linearity data of the calibration curve, LOD and LOQ values for the investigated standards.

Standards	Parameters					
	Linearity range µg/mL	r^2	b	a	LOD µg/mL	LOQ µg/mL
NeoChA	0.5-50	1	11924	10.868	0.025	0.084
ChA	0.5-50	1	12845	-1306.7	0.071	0.235
Caffeic acid	0.5-50	0.9998	22350	2732.9	0.178	0.592
Rutin	0.5-50	0.9997	8299	292.67	0.058	0.194
Isoquercitrin	0.5-50	0.9998	10176	615.88	0.028	0.094
Astragalin	0.5-50	1	9622.9	261.36	0.062	0.195
Quercetin	0.5-50	1	15061	-2902.7	0.089	0.295

LOQ: Limit of quantification, LOD: Limit of detection, NeoChA: neochlorogenic acid, ChA: chlorogenic acid, the calibration equation was 'y=a+bx'.

Precision was assessed through intraday (within-day) and interday (between-day) analyses. Standard solutions containing 5 µg/mL of NeoChA, ChA, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin were analyzed three times within a single day to evaluate intraday precision. Interday precision was determined by repeating the analysis different days. The results of the precision studies are summarized in Table 5.

Table 5. Repeatability and precision data for the standards.

Standards (5 µg/mL)	Intraday Precision		Interday Precision		Interday Precision	
	Average (µg/mL ± SD) *n*=3	RSD	Average (µg/mL ± SD) *n*=3	RSD	Average (µg/mL ± SD) *n*=3	RSD
NeoChA	4.969 ± 0.01	0.21				
	4.944 ± 0.02	0.42	4.96 ± 0.04	0.84	4.93 ± 0.01	0.27
	4.921 ± 0.01	0.24				
ChA	4.970 ± 0.02	0.35				
	4.940 ± 0.01	0.21	4.942 ± 0.04	0.88	4.930 ± 0.00	0.07
	4.930 ± 0.00	0.08				
Caffeic acid	5.060 ± 0.00	0.07				
	5.020 ± 0.01	0.17	5.030 ± 0.01	0.28	5.01 ± 0.00	0.05
	5.020 ± 0.02	0.36				
Rutin	5.090 ± 0.03	0.57				
	5.030 ± 0.05	0.98	5.080 ± 0.05	0.90	5.090 ± 0.01	0.23
	5.060 ± 0.04	0.71				
Isoquercitrin	4.890 ± 0.07	1.52				
	4.830 ± 0.00	0.08	4.870 ± 0.08	1.73	4.880 ± 0.08	1.62
	4.850 ± 0.04	0.86				
Astragalin	5.130 ± 0.03	0.61				
	5.110 ± 0.03	0.51	5.120 ± 0.01	0.28	5.130 ± 0.05	0.97
	5.090 ± 0.04	0.84				
Quercetin	5.000 ± 0.03	0.60				
	4.960 ± 0.04	0.77	4.980 ± 0.07	1.33	5.00 ± 0.03	0.51
	4.940 ± 0.03	0.65				

The results were presented as average ± SD.

The accuracy of the developed UPLC method was assessed using standard solutions at concentrations of 4, 8, and 12 µg/mL, containing NeoChA, ChA, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin. To evaluate accuracy, the measured concentrations obtained from the calibration curve were compared to the theoretical values. The results, which demonstrate the accuracy of the method, are summarized in Table 6.

Table 6. Comparative results of theoretical values and obtained results (%) by UPLC method.

Standards	Theoretical value (µg/mL)	Amount found (µg/mL ± SD, *n*=3)	Recovery (%)
NeoChA	4	4.19 ± 0.02	104.73
	8	8.38 ± 0.11	104.78
	16	16.95 ± 0.17	105.95
ChA	4	3.88 ± 0.01	96.89
	8	7.91 ± 0.03	98.84
	16	16.06 ± 0.14	100.36
Caffeic acid	4	3.92 ± 0.01	98.07
	8	8 ± 0.03	100.06
	16	16.21 ± 0.12	101.33
Rutin	4	4.01 ± 0.02	100.29
	8	8.02 ± 0.02	100.30
	16	16.41 ± 0.08	102.55
Isoquercitrin	4	3.82	95.57
	8	7.51 ± 0.04	93.88
	16	15.25 ± 0.11	95.31
Astragalin	4	4.04 ± 0.05	100.99
	8	8.21 ± 0.04	102.68
	16	15.95 ± 0.1	100.156
Quercetin	4	3.95 ± 0.02	98.80
	8	8.03 ± 0.03	100.42
	16	15.54 ± 0.09	97.14

The results were presented as average ± SD.

The UPLC analysis of *M. campanuloides* root and aerial part methanolic extracts, subextracts and *r*H_2O extracts revealed that the aerial parts contained higher amounts of investigated compounds compared to the roots (Table 7).

Table 7. Quantitative UPLC results of the standards and *M. campanuloides* roots and aerial parts extracts.

	Standards						
Extracts	**NeoChA**	**ChA**	**Caffeic acid**	**Rutin**	**Isoquercitrin**	**Astragalin**	**Quercetin**
Roots							
MeOH	ND	0.65±0.01^{g}	ND	ND	ND	ND	ND
***n*-hexane**	ND	ND	ND	ND	ND	ND	ND
EtOAc	ND	0.99±0.01^{f}	1.09±0.01^{b}	ND	ND	ND	ND
***n*-BuOH**	ND	2.64±0.02^{e}	ND	ND	ND	ND	ND
***r*H_2O**	ND	0.49±0.01^{g}	ND	ND	ND	ND	ND
Aerial parts							
MeOH	3.18±0.01^{b}	11.45±0.11^{c}	0.22±0.02^{c}	2.51±0.02^{c}	3.28±0.03^{c}	3.55±0.11^{c}	ND
***n*-hexane**	0.73±0.0^{e}	2.76±0.01^{e}	ND	0.56±0.02^{d}	0.87±0.03^{d}	1.11±0.02^{d}	ND
EtOAc	2.68±0.04^{d}	38.21±0.03^{a}	6.67±0.09^{a}	5.51±0.06^{b}	51.22±0.81^{a}	93.37±0.11^{a}	5.79±0.04^{a}
***n*-BuOH**	6.67±0.16^{a}	37.23±0.21^{b}	ND	15.28±0.04^{a}	16.32±0.09^{b}	12.28±0.13^{b}	ND
***r*H_2O**	2.85±0.03^{c}	7.32±0.03^{d}	ND	0.39±0.02^{e}	0.89±0.02^{d}	ND	ND

Results were given as mg/g extract, ChA: chlorogenic acid, ND: not detected, NeoChA: neochlorogenic acid, MCR: *M. campanuloides* roots, MCH: *M. campanuloides* aerial parts. Different letters within the same column indicate values that are significantly different at a significance level of $p \leq 0.05$.

Among the subextracts, the EtOAc subextract of the aerial parts was particularly notable, containing all the standard compounds (Figure 5), many of which were present in significantly higher amounts than other extracts (Table 7). Astragalin (93.37 ± 0.11 mg/g), isoquercitrin (51.22 ± 0.81 mg/g), and chlorogenic acid (38.21 ± 0.03 mg/g) were identified as the major constituents. Furthermore, quercetin was only quantified as 5.79 ± 0.04 mg/g in the EtOAc subextract of the aerial parts.

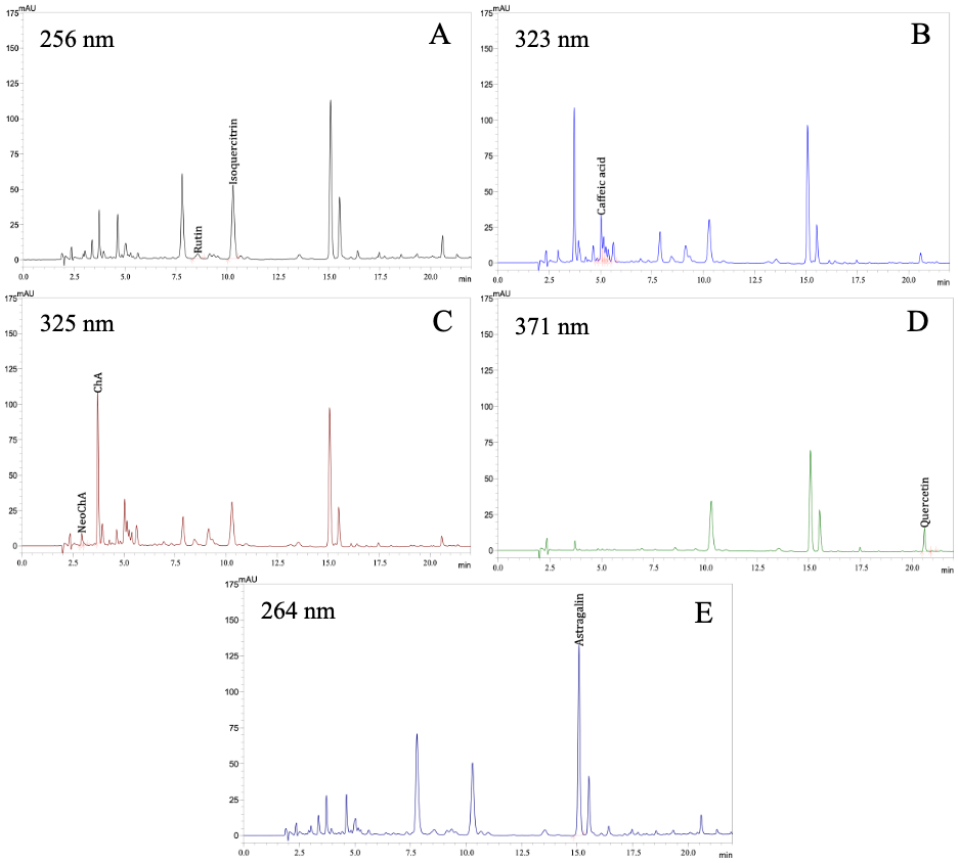

Figure 5. UPLC chromatograms of *M. campanuloides* aerial parts EtOAc subextract at different wavelenghts.

t_R values; Rutin: 5.563, Isoquercitrin: 10.283, Caffeic acid: 5.034, Neochlorogenic acid: 2.935, Chlorogenic acid: 3.720, Quercetin: 20.585, Astragalin: 15.084.

In the *n*-BuOH subextract of the aerial parts, chlorogenic acid (ChA) was determined as the predominant compound, with 37.23 ± 0.21 mg/g. However, caffeic acid and quercetin were not detected in this subextract. These results highlighted that the aerial parts of *M. campanuloides* had a remarkable phenolic composition, particularly in the EtOAc and *n*-BuOH subextracts. Additionally, ChA was found to be the main constituent in the methanolic extract, *n*-hexane subextracts and *r*H_2O extract, with respective amounts of 11.45 ± 0.11 mg/g, 2.76 ± 0.01 mg/g, and 7.32 ± 0.03 mg/g (Table 7).

Among the extracts and subextracts of *M. campanuloides* roots, all of them except *n*-hexane subextract contained ChA. The highest ChA was found in *n*-BuOH subextract as 2.64 ± 0.02 mg/g. The EtOAc subextract was the only one containing caffeic acid (1.09 ± 0.01 mg/g). Other investigated compounds were not determined in the extracts and subextracts of of *M. campanuloides* roots.

3. DISCUSSION

There are limited bioactivity and phytochemical studies on *M. campanuloides*. A study investigated the wound-healing activity of *M. campanuloides* [7], linking this activity to the traditional use of its leaves for wound treatment [4]. Previous studies have provided limited evaluations, including TPC determination [7, 9], as well as anti-inflammatory [7] and antioxidant activities [7, 8, 10]. However, *M. campanuloides* has not been investigated in detail in terms of anticancer and antioxidant activities and its chemical composition has not been revealed quantitatively in previous studies.

In this study, roots and aerial parts of *M. campanuloides* were subjected to MTS cytotoxicity assay. As a result, the EtOAc extract of the roots demonstrated the highest cytotoxic activity against the breast cancer cell line (HCC1937), with an IC_{50} of 10 µg/mL. This study represents the first investigation on the anticancer potential of *M. campanuloides*. Among the tested extracts, the EtOAc subextract of the roots need further bioactivity-guided fractionation studies to isolate potent cytotoxic compounds.

The DPPH assay results of the present study align with previous findings, which demonstrated that the EtOAc subextract of *M. campanuloides* aerial parts exhibited higher antioxidant potential compared to

other subextracts (H_2O, MeOH, DCM, EtOAc, and *n*-BuOH) [7]. In contrast, the H_2O subextract of the roots showed the lowest antioxidant activity, with an IC_{50} value of 159.84 mg/mL, consistent with our results. ABTS, DPPH, CUPRAC and FRAP assays were used together to determine the antioxidant capacity of *M. campanuloides* roots and aerial parts extracts and subextracts, for the first time. Similarly, the highest TPC was reported in the EtOAc subextract of the aerial parts in the same study (439.05 ± 18.70 mg/g) [7]. Based on the results of our study, EtOAc subextract of the aerial parts had significantly high amount of TPC and TFC (204.32±27.29 mg GA/g and 28.46±1.18 mg QE/g, respectively). TPC and TFC in the extracts were found to be proportional to their antioxidant capacities which was in line with the literature [16]. This study represents the total flavonoid content of *M. campanuloides* for the first time.

HPTLC and UPLC methods were used to identify marker compounds in the extracts. All tested extracts contained chlorogenic acid, but the root extracts lacked neochlorogenic acid, rutin, isoquercitrin, astragalin, and quercetin. Only the EtOAc subextract of *M. campanuloides* aerial parts contained all the investigated compounds used, namely NeoChA (2.68±0.04 mg/g), ChA (38.21±0.03 mg/g), caffeic acid (6.67±0.09 mg/g), rutin (5.51±0.06 mg/g), isoquercitrin (51.22±0.81 mg/g), astragalin (93.37±0.11 mg/g), and quercetin (5.79±0.04 mg/g). Qualitative screening tests previously revealed the presence of saponins in the roots of *M. campanuloides*, which may contribute to the cytotoxic activity observed in the EtOAc subextract of the roots [9]. Additionally, flavonoids, cyanogenic glycosides, and coumarins were identified by screening tests (*in vitro*) in both the roots and aerial parts of the plant in a previous study [9]. To the best of our knowledge, astragalin, caffeic acid, ChA, gallic acid, isoquercitrin, NeoChA, quercetin, and rutin were detected and quantified in *M. campanuloides* by using HPTLC and UPLC methods, respectively for the first time.

4. CONCLUSION

In conclusion, this is the first comprehensive study of evaluating cytotoxic activity and antioxidant activity using different methods of *M. campanuloides* root and aerial part extracts and subextracts. In addition, main phenolic compounds of *M. campanuloides* root and aerial part extracts and subextracts were quantified for the first time to the best of our knowledge.

The EtOAc subextract of the roots showed moderate cytotoxic potential at the tested concentrations. As the extracts are mixtures of many secondary and primary metabolites, the potential cytotoxic compounds needs to be isolated and purified by sequential chromatographic methods. Additionally, the EtOAc subextract of the aerial parts having the highest astragalin content among the investigated compounds exhibited the strongest antioxidant activity.

5. MATERIALS AND METHODS

5.1. Chemicals and solvents

HPLC grade methanol (MeOH), and analytical grade of ethanol (EtOH), *n*-hexane, ethyl acetate (EtOAc), dichloromethane, acetonitrile, *n*-BuOH, and HCl were purchased from Sigma-Aldrich (Steinheim, Germany); glacial acetic acid, sulphuric acid and *o*-phosphoric acid was from Isolab (Eschau, Germany). Dulbecco's Modified Eagle's Medium (DMEM) (#11995065), Roswell Park Memorial Institute (RPMI) 1640 (#11875093), and DMEM/F12 (#11320033) were purchased from Gibco (Massachusetts, ABD). Fetal Bovine Serum (FBS) (#F7524) and dimethyl sulfoxide (DMSO, #D2650) were obtained from Sigma Aldrich (Steinheim, Germany). Penicillin streptomycin antibiotic solution (#PS-B) was purchased from Capricorn Scientific (Hessen, Germany), and trypsin-ethylenediaminetetraacetic acid (EDTA) (#25200056) was from Thermo Fisher Scientific (Massachusetts, ABD). 10% (*v/v*) 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent (MTS, # ab197010) was obtained from Abcam (Cambridge, UK). The standards as astragalin, caffeic acid, chlorogenic acid (ChA), gallic acid, isoquercitrin, neochlorogenic acid (NeoChA), quercetin, rutin and trolox were obtained from Sigma-Aldrich (Steinheim, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and Folin's reagents were purchased from Sigma-Aldrich (Steinheim, Germany), Roche (Basel, Switzerland), and Merck (Hohenbrunn, Germany), respectively. Aluminum chloride hexahydrate ($AlCl_3.6H_2O$), anisaldehyde copper (II) chloride dihydrate ($CuCl_2.2H_2O$), 2-aminoethyl diphenyl borinate, neocuproine, potassium persulfate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate ($C_2H_7NO_2$) and ferric chloride ($FeCl_3$) were obtained from Fluka (Steinheim, Germany). Polyethylene glycol (PEG) 400

was obtained from Merck (Hohenbrunn, Germany). Sodium acetate trihydrate ($C_2H_9NaO_5$) was purchased from Riedel-de-Haën (Seelze, Germany).

5.2. Plant materials

M. campanuloides roots and aerial parts were collected from Belen, Hatay in May 2023, at 200 m height (30.50068°N, 36.16592°E). Plant materials were identified by Prof. Dr. Hayri Duman (Gazi University, Faculty of Sciences). A voucher sample of *M. campanuloides* (YEF 23002) has been kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, Istanbul, Türkiye. All parts of the plant were dried in a dark and cool place.

5.3. Extraction and partition

The roots (50 g) and aerial parts (100 g) of *M. campanuloides* were separated and dried at room temperature, followed by powdering process. Plant materials were macerated with 500 and 1000 mL of MeOH separately for three days. After evaporating the solvent, the root and aerial parts extracts (yields: 26.4% and 22.2%, respectively) were freeze-dried (Christ Alpha 2-4 LD Lyophilizator). The freeze-dried extracts were dispersed in H_2O and partitioned with *n*-hexane, EtOAc and *n*-butanol (*n*-BuOH), respectively. Following the partition process, *n*-BuOH subextract and *r*H_2O extract were lyophilized; the solvents belonging to *n*-hexane and EtOAc were evaporated using a rotary evaporator [17].

5.4. Preparation of the Sample Test Solutions

5.4.1. Bioactivity studies

Extracts and subextracts were dissolved in dimethyl sulfoxide (DMSO) (100mg/mL) and further diluted to perform cytotoxicity studies. Stock test solutions for antioxidant activity tests were prepared by using MeOH at the concentration of 5 mg/mL, then further diluted to 200 µg/mL - 2 mg/mL range of concentration for ABTS and DPPH assays, diluted to 200 µg/mL - 5 mg/mL range of concentration for FRAP and CUPRAC assays.

5.4.2. Phytochemical studies

The crude methanolic extract and its subextracts (*n*-hexane, EtOAc, *n*-BuOH, and H_2O) were dissolved in methanol to prepare stock solutions at a concentration of 5 mg/mL. These solutions were subsequently diluted for antioxidant and phytochemical assays. For the TPC assay, dilutions were prepared in the range of 200 µg/mL to 2 mg/mL, whereas for the TFC assay, concentrations ranged from 5 mg/mL to 50 mg/mL. For HPTLC and UPLC studies, 20 mg/mL and 0.25 mg/mL - 0.5 mg/mL - 1 mg/mL sample test solutions were prepared with MeOH, respectively.

5.5. Preparation of the Standard Solutions

5.5.1. Bioactivity studies

Trolox stock solution (1 mg/mL) used for all antioxidant assays was prepared with EtOH and further diluted to 8-125 µg/mL range of concentration.

5.5.2. Phytochemical studies

Gallic acid and quercetin stock solutions (1 mg/mL) were used as standards for TPC and TFC assays which were prepared by using water and ethanol, respectively. Stock solutions were further diluted (8-125 µg/mL). Rutin, ChA, NeoChA, isoquercitrin, astragalin, caffeic acid and quercetin solutions were prepared with MeOH and mixed in equal volumes (50 µg/mL) in order to use as standard mixture for HPTLC studies. Same standards and solvent (350 µg/mL) were used for UPLC studies, further dilution was applied at the range of 5 to 50 µg/mL concentrations.

5.6. Cytotoxicity Studies

For cytotoxicity screening, colon (HCT116 and SW480), breast (MDA-MB-231 and HCC1937) and liver (HEP3B and HEPG2) cancer cell lines and a fibroblastic healthy cell line (L929) were used. SW480, MDA-MB-231, HEP3B, HEPG2 and L929 were cultured in DMEM**;** HCT116 was cultured in DMEM/F12 and HCC1937 was cultured in RPMI 1640 media, all supplemented with 10% FBS and 1% penicillin streptomycin antibiotic solution. Cells were detached with trypsin-EDTA and seeded for viability screening at the concentration of 5×10^3 cells per well in triplicate. Extracts were dissolved in DMSO and test solutions were prepared at the

concentrations of 200, 100, 50, 25, and 12.5 µg/mL in respective complete culture medium. Cells seeded in culture plate wells were treated with extracts, or paclitaxel (1-20 nM) for 48 hours. At the end of incubation period, 10% (*v/v*) MTS was added to wells [18]. Untreated cells were included as negative control whereas plain culture medium was included as blank. At the end of the incubation period, absorbance at 490 nm was read with spectrophotometer (Epoch, BioTek). Viability was calculated using the formula (Eq. 1) below, and IC_{50} values were determined with GraphPad Prism software (version: 8).

$$\text{(Eq. 1).} \qquad Viability\ (\%) = \frac{Test\ OD - Blank\ OD}{Positive\ Control\ OD - Blank\ OD}\ x\ 100$$

5.7. Antioxidant Activity Studies

5.7.1. 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Radical Scavenging Method

The ABTS reagent was prepared by using ABTS, potassium persulphate and water, and kept in a dark place for 12 hours. The color change was observed from turquoise to dark blue. The absorbance level of the reagent was checked at 734 nm (≈ 0.7), the used for further studies. 5 mL of prepared reagent was dissolved in ethanol (%10). In the beginning, 20 µL sample or blank (MeOH) and 280 µL ABTS reagent were added to each well. The plate was incubated for 6 minutes at room temperature, then absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific, Massachusetts, ABD) at 734 nm. The results were expressed as mg of trolox equivalents per g of extract (mg TE/g extract) [19].

5.7.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Method

To each well, 20 µL sample test solution or blank (ethanol), and 280 µL 0.1 mM ethanolic DPPH solution (1.9 mg DPPH was dissolved in 50 mL ethanol) were added and incubated in a dark environment for 30 minutes. Absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 520 nm. The results were documented as mg of trolox equivalents per g of extract (mg TE/g extract) [12,20].

5.7.3. Ferric Reducing Antioxidant Power Assay (FRAP)

2×10^{-2} M $FeCl_3$, 1×10^{-2} M TPTZ and pH 3.6 sodium acetate buffer solutions were used in 1:1:10 ratio to prepare FRAP reagent. First, 20 µL sample test solution or blank (distilled water) and 280 µL freshly prepared FRAP reagent were added to each well, and incubated for six minutes at room temperature. At the end of the incubation period, the absorbances were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 595 nm. The results were presented as mg of trolox equivalents per g of extract (mg TE/g extract) [21, 22].

5.7.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

$1x10^{-2}$ M copper (II) chloride dihydrate and 1 M ammonium acetate solutions were prepared with MeOH (pH: 7, adjusted by using 37% HCl solution), $7.5x10^{-3}$ M neocuproine solution was prepared with ethanol. To each well, 30 µL sample test solution or blank (distilled water), 80 µL from each prepared solution (copper (II) chloride dihydrate, ammonium acetate and neocuproine), and 30 µL MeOH were added, respectively. Absorbances were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 450 nm. The results were stated as mg of trolox equivalents per g of extract (mg TE/g extract) [23].

5.8. Phytochemical Analysis

5.8.1. Total Phenolic Content Assay (TPC)

Folin-Ciocalteu method was used for determination of the quantity of total phenolic content in the extracts. Folin reagent was prepared by dissolving the reagent with distilled water (1:10), and stored in a dark place. To each well, 25 µL sample or blank (distilled water), 25 µL distilled water, 125 µL Folin reagent and 100 µL 7.5% Na_2CO_3 solution were added, the plate was incubated for 30 minutes at 37°C. Absorbances were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 760 nm. The results were expressed as mg of gallic acid equivalents per g of the extract (mg GA/g extract) [14].

5.8.2. Total Flavonoid Content Assay (TFC)

To each well, 30 µL sample test solution or blank (EtOH), 30 µL 10% aluminium chloride solution, 30 µL sodium acetate trihydrate solution, and 210 µL MeOH were added, and the plate was incubated at 37°C

for 15 minutes. Absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 415 nm. The results were presented as mg of quercetin equivalents per g of extract (mg QE/g) [15].

5.9. Chromatographic Analysis

5.9.1. High-Performance Thin-Layer Chromatography (HPTLC)

Sample test solutions (20 mg/mL) were applied on 20 x 10 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with silica gel 60 F_{254}, as 5 µL whereas standard mixture solution (50 µg/mL) containing rutin, ChA, NeoChA, isoquercitrin, astragalin, caffeic acid and quercetin was applied as 2 µL with 8 mm band length using Hamilton syringe (100 µL) by Semi-automatic Linomat 5 (CAMAG, Muttenz, Switzerland). EtOAc – acetic acid (AcOH) – formic acid (FA) – H_2O (100:11:11:26 *v/v/v/v*) [24] was used as a developing solvent system. For the saturation of twin-trough chamber, one trough was filled with 10 mL and in another trough, in which filter paper was placed, was filled with 15 mL of developing solvent system and waited for 20 minutes. After, the plate was developed up to 7 cm, then dried with cold air for 2 minutes.

For the visualization, Natural Product/Polyethylene Glycol 400 (NP/PEG) and anisaldehyde reagents were used separately as follows:

1) The developed plate was first heated with TLC Plate Heater III at 105°C for 3 min, then dipped into NP (2-aminoethyl diphenylborinate (1 g) was dissolved in EtOAc) and PEG [(Polyethylene glycol 400 (10 g) was dissolved in DCM (200 mL)] reagents, respectively using CAMAG Chromatogram Immersion Device [24].
2) Another developed plate was first dipped into anisaldehyde reagent (Ice cooled mixture of 170 mL MeOH and 20 mL of acetic acid was prepared, then, 10 mL of sulphuric acid was added. Consequently, 1 mL anisaldehyde was mixed with this solution.) and heated at 105°C for 3 min [25].

TLC Visualizer was used and the plates were visualized at 254 nm (before development), 366 nm (before and after development) and white light (after development). WinCATS software (Camag, Version 128 1.4.8.2031) was used to operate the HPTLC equipments. Retardation factors (R_F) and band colors were used for identification of the compounds in samples.

5.9.2. Ultra-Performance Liquid Chromatography (UPLC)

Schimadzu UHPLC-PDA (Photodiode Array) system (Kyoto, Japan) was utilized for the identification and quantitative analysis of NeoChA, ChA, caffeic acid, rutin, isoquercitrin, astragalin and quercetin in roots and aerial parts extracts of *M. campanuloides*. Agilent Poroshell 120 EC-C_{18} column (3.0 x 150 mm) with 2.7 µm particle size was used for the chromatographic separation, with a temperature of 20°C. Mobile phase A [*o*-phosphoric acid-water (0.1:99.9, *v/v*)] and mobile phase B (acetonitrile) were filtered (0.22 µm size) and degassed. The gradient pattern was used as follows: 15-17% B (0-1 min), 17-17.2% B (1-3 min), 17.2-17.3% B (3-11 min), 17.3-20% B (11-12 min), 20-40% B (12-22 min), 40-70% B (22-23 min), 70-15% B (23-25 min), 15% B (25-30 min). UPLC method was validated by using specificity, linearity, LOD, LOQ, precision, and accuracy parameters according to International Conference on Harmonization (ICH) [26]. Different detection wavelenghts were used according to the UV_{max} values of analytes (NeoChA, ChA, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin) as shown in Table 8.

Table 8. Detection wavelenght of the analytes.

Analyte	Wavelenght
NeoChA, ChA	325 nm
caffeic acid	323 nm
rutin, isoquercitrin	256 nm
astragalin	264 nm
quercetin	371 nm

5.10. Statistical Analysis

Each test was conducted three times, and the average value and standard deviation (SD) were calculated. The results were expressed as the average ± SD. Statistical analyses were performed using Microsoft Excel and Minitab 17. Statistical significance was determined using one-way analysis of variance (one-way ANOVA) with a significance threshold of $p \leq 0.05$.

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