T.C.

YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
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INVESTIGATION OF CYTOTOXIC AND ANTIOXIDANT ACTIVITIES OF *MICHAUXIA CAMPANULOIDES* L'HÉRIT. EX AITON

M. Sc. THESIS

RENGİN BAYDAR, B Pharm

İstanbul-2024

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APPROVAL

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

21.06.2024

Rengin Baydar

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LIST OF SYMBOLS and ABBREVIATIONS

ABTS: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid

AcOH: Acetic acid

CUPRAC: Cupric reducing antioxidant capacity

DCM: Dichloromethane

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EtOAc: Ethyl acetate

FA: Formic acid

FBS: Fetal bovine serum

FRAP: Ferric reducing antioxidant power assay

GA: Gallic acid

GAE: Gallic acid equivalent

GC-MS: Gas chromatography-mass spectrometry

H_2O: Water

HPLC: High-performance liquid chromatography

HPTLC: High-performance thin-layer chromatography

ICH: International conference on harmonization

LC: Lethal concentration

LOD: Limit of detection

LOQ: Limit of quantification

MCH: *Michauxia campanuloides* aerial parts

MCR: *Michauxia campanuloides* roots

MeOH: Methanol

MIC: Minimum inhibition concentration

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

n-BuOH: *n*-Butanol

NP: Natural product

PCNA: Proliferating cell nuclear antigen

PDA: Photodiode array

PEG: Polyethylene glycol

PMS: Phenazine methosulphate

QE: Quercetin equivalent

R_F : Retardation factor

RPMI: Roswell Park Memorial Institute

SD: Standart deviation

STD MIX: Standart mixture

T1: Treatment 1

T2: Treatment 2

TAC: Total antioxidant capacity

TBA: Thiobarbituric acid

TE: Trolox equivalent

TFC: Total flavonoid content

TLC: Thin-layer chromatography

TPC: Total phenolic content

TPTZ: 2,4,6-tris(2-pyridyl)-s-triazine

t_R : Retention time

UPLC: Ultra-performance liquid chromatography

UV: Ultra violet

ABSTRACT

Baydar, R. (2024). Investigation of Cytotoxic and Antioxidant Activities of *Michauxia campanuloides* L'Hérit. Ex Aiton. Yeditepe University, Institute of Health Sciences, Department of Phytotherapy, MSc Thesis, İstanbul.

Michauxia campanuloides L'Hérit. ex Aiton (Campanulaceae) is one of the traditionally used plants growing in Southern Anatolia. This study aimed to evaluate *in vitro* cytotoxic and antioxidant activities of the extracts derived from *M. campanuloides* aerial parts and roots, as well as to identify their phytochemical composition. The *in vitro* cytotoxic activity of MeOH extract as well as *n*-hexane, EtOAc, *n*-BuOH and H_2O subextracts prepared from roots and aerial parts of *M. campanuloides* were evaluated against colon (HCT116 and SW480), breast (MDA-MB-231 and HCC1937) and liver (HEP3B and HEPG2) cancer cell lines and fibroblastic L929 healthy cell line by MTS assay. EtOAc subextract of roots exhibited the highest cytotoxic activity against all studied cell lines with the IC_{50} values of 10-154.76 µg/mL. ABTS, DPPH, FRAP and CUPRAC methods were used for *in vitro* antioxidant activity studies. Total phenolic and flavonoid contents of the extracts were also determined. EtOAc subextracts of aerial parts (For ABTS test, 353.399±19.206 mg TE/g) and roots (For ABTS test, 367.651±16.382 mg TE/g) showed significant antioxidant activity. Extracts having high TPC and TFC values showed remarkable antioxidant activity. Total flavonoid content of *n*-BuOH subextract of aerial parts was higher than other subextracts. HPTLC was used for comparative identification of compounds in the extracts, and the identified compounds (rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid and quercetin) were also quantified by UPLC. Astragalin (93.37±0.11 mg/g) and isoquercitrin (51.22±0.81 mg/g) were predominant in EtOAc subextract of aerial parts showing the significant antioxidant activity. Although EtOAc root subextract exhibited the best cytotoxicity, most of its metabolites were not characterised. Therefore, the potential major active compounds in EtOAc subextract of roots deserve further isolation and structural elucidation studies.

Keywords: *Michauxia campanuloides*, cytotoxic activity, antioxidant activity, HPTLC, UPLC

ÖZET

Baydar, R. (2024). *Michauxia campanuloides* L'Hérit. Ex Aiton Bitkisinin Sitotoksik ve Antioksidan Aktivitelerinin Araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fitoterapi ABD, Yüksek Lisans Tezi, İstanbul.

Michauxia campanuloides L'Hérit. ex Aiton, Güney Anadolu'da geleneksel olarak kullanılan bir bitkidir. Bu çalışmada, *M. campanuloides*'in toprak üstü kısımları ve köklerine ait ekstrelerin *in vitro* sitotoksik ve antioksidan aktiviteleri ile fitokimyasal içeriklerinin araştırılması amaçlanmıştır. *M. campanuloides*'in kök ve toprak üstü kısımlarından hazırlanan MeOH ekstresi ve *n*-hekzan, EtOAc, *n*-BuOH ve H_2O alt ekstrelerinin *in vitro* sitotoksik aktiviteleri kolon (HCT116 ve SW480), meme (MDA-MB-231 ve HCC1937) ve karaciğer (HEP3B ve HEPG2) kanser hücre hatlarına ve L929 fibroblast sağlıklı hücre hattına karşı MTS testi ile incelenmiştir. Kök kısımlarının EtOAc alt ekstresi, test edilen tüm hücre hatlarına karşı en iyi sitotoksik aktivite göstermiştir (IC_{50}= 10-154.76 µg/mL). *In vitro* antioksidan aktivite deneyleri için ABTS, DPPH, FRAP ve CUPRAC yöntemleri kullanılmıştır. Ekstrelerin toplam fenolik ve flavonoit içerikleri de araştırılmıştır. Toprak üstü kısımların ve köklerin EtOAc alt ekstreleri, diğer ekstrelerden anlamlı oranda yüksek antioksidan aktivite göstermiştir (ABTS testi için sırasıyla 353.399±19.206 mg TE/g ve 367.651±16.382 mg TE/g). Yüksek TPC ve TFC değerlerine sahip ekstreler anlamlı antioksidan aktivite sergilemiştir. Toprak üstü kısımların *n*-BuOH alt ekstresinin toplam flavonoit içeriği diğer ekstrelere kıyasla yüksek olarak belirlenmiştir. Ekstrelerdeki bileşiklerin karşılaştırmalı tanımlaması için HPTLC kullanılmıştır ve rutin, klorojenik asit, neoklorojenik asit, izokersitrin, astragalin, kafeik asit ve kersetinin ekstrelerdeki miktarları UPLC ile saptanmıştır. Yüksek antioksidan özellik gösteren toprak üstü kısımların EtOAc alt ekstresinde, astragalin (93.37±0.11 mg/g) ve izokersitrin (51.22±0.81 mg/g) temel bileşikler olarak belirlenmiştir. Köklerden elde edilen EtOAc alt ekstresi yüksek oranda sitotoksik aktivite göstermesine rağmen birçok metaboliti aydınlatılamamıştır. Bu sebeple, köklerin EtOAc alt ekstresinde bulunan potansiyel majör biyoaktif bileşiklerin izolasyonu ve yapılarının aydınlatılması önem arz etmektedir.

Anahtar kelimeler: *Michauxia campanuloides*, sitotoksik aktivite, antioksidan aktivite, HPTLC, UPLC

1. INTRODUCTION and PURPOSE

Michauxia species (Campanulaceae), known as 'Dart Bellflower', are represented by seven species in the world (*M. campanuloides* L'Hér. ex Aiton., *M. laevigata* Vent., *M. nuda* A.DC., *M. tchihatchewii* Fisch. & C.A.Mey., *M. thyrsoidea* Boiss. & Heldr, *M. koeieana* Rech. and *M. stenophylla* Boiss & Hausskn). They are distributed from Eastern Mediterranean to Caucaus region and Iran. *M. campanuloides*, *M. laevigata*, *M. nuda*, *M. thyrsoidea*, and *M. tchihatchewii* grow wild in the flora of Türkiye (1,2).

M. campanuloides grows in cliffs and stoney sites in usually Southern and rarely Central Anatolia in Türkiye. The plant is 100-150 cm tall; the stem is thick and hairy, leaves are hairy on both sides (3). It is known as 'keçibiciği', 'sütlü', 'keşir' or 'kırtmaç' in Türkiye (4). Traditionally, its stems are consumed as vegetable after cooking in Tarsus, Silifke and Gülnar (5). The leaves of the plant are also used for wound healing by crushing them, externally (6).

Previous studies stated that *Michauxia* species exhibited *in vitro* antioxidant (7), antimicrobial (8), anti-inflammatory (7), cytotoxic (8) and *in vivo* wound healing (7) activities. There is also a study related to the positive effect of *M. campanuloides* on semen quality in rats (9). The biological and phytochemical studies on *M. campanuloides* are limited.

Antioxidants are currently in high demand for various reasons. Particularly, oxidative stress is implicated in the etiology of numerous diseases. Enhancing intracellular oxygenation through the scavenging of reactive oxygen species is a critical mechanism. Consequently, sourcing antioxidant compounds from botanical origins has gained significant importance. To address this need, various methodologies have been developed to quantify the antioxidant potentials in herbal extracts (10). Additionally, it is crucial to screen the anticancer potential of plant extract to discover new drug leads.

M. campanuloides has not been investigated for its cytotoxic activities and phytochemical composition to the best of our knowledge. The purpose of this study is comparatively evaluating the antioxidant and cytotoxic activities of *M. campanuloides* root and aerial parts methanolic (MeOH) extract and their subextracts [*n*-hexane, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH)], along with determining phenolic compounds including rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid and quercetin in the extracts by using chromatographic methods [High-Performance Thin Layer Chromatography (HPTLC) and Ultra-Performance Liquid Chromatography (UPLC)].

2. LITERATURE REVIEW

2.1. General Information on *Michauxia*

2.1.1. Botanical Information

2.1.1.1. Campanulaceae

The genus *Michauxia* L'Hérit belongs to Campanulaceae which is a family in Campanulales order. Campanulaceae family includes 94 accepted genera and more than 2300 species. Systematically 5 subfamilies are known and Campanulaceae is a rich family. These subfamilies are Campanuloideae, Lobelioideae, Cyphioideae, Nemacladoideae and Cyphocarpoideae. Campanulaceae family plants are easy to recognize due to its attractive and glabrous flowers (11, 12).

Campanulaceae family plants are mostly perennial or annual. These species are usually known for their milky secretion (articulated laticifers) and thickened rhizomes. Also, its members are known to store inuline like carbohydrates, polyacetylenes and pyridine alkaloids (especially some Lobelioideae). Stipulates are absent. Leaves are alternate or exstipulate. Flowers are epigynous, hermaphroditic, or actinomorphic, and can be protandrous or cleistogamous. The inflorescence can consist of 1 to many flowers, which may be spicate, racemiform, or paniculate, with flowers often being axillary, solitary, or terminal. The calyx is 5, 8, 9, or 10-lobed, with the calyx-tube adnate to the ovary. The corolla is gamopetalous with 5, 8, 9, or 10 lobes and can be blue, lilac, or whitish, usually campanulate, cylindrical, infundibular, or rotate. The ovary is inferior with numerous ovules. Stamens are 5, with free filaments and anthers, usually broadened and ciliate at the base. The style is elongated and hairy, with a 2-, 3-, or 5-fid stigma. The fruit is a capsule that can be erect or nodding, dehiscing by 2, 3, or 5 basal, lateral, or apical pores or valves. Seeds are numerous, small, and shiny (12, 13).

2.1.1.2. *Michauxia* L'Hérit

Michauxia (in honour of the French botanist, Andre Michaux, 1746–1803) species (also known as 'Dart Bellflower') are distributed from Eastern Mediterranean to Caucaus and Iran. In the flora of Türkiye, five out of seven *Michauxia* species (*M. campanuloides* L'Hér. ex Aiton., *M. laevigata* Vent., *M. nuda* A.DC., *M. thyrsoidea* Boiss. & Heldr. and *M. tchihatchewii* Fisch. & C.A.Mey.) grow naturally (1, 2).

Michauxia species are biennial and they have thick stem, to 150 cm tall. Leaves mostly ovate-oblong. Inflorescence raceme, dense spike or panicle. Corolla white; pollen yellow; stamens free; filaments dilated at base. Style thick and hairy; stigmas 8-10. Fruit a capsule; seeds small (0.8-1x0.3-0.4 mm), ellipsoid, light brown and shiny.

Dichotomous key for *Michauxia* species is below (13);

1. Corolla divided to 4/5; style 30-40 mm (occasionally 20 mm). Pedicels 2-4 cm; corolla lobes 25-45 mm, reflexed

2. Pedicels 2-4 cm; corolla lobes 25-45 mm, reflexed ***campanuloides***

2. Pedicels 1 cm or flowers ± sessile; corolla lobes 15-20 mm, spreading-recurved

laevigata

1. Corolla divided to 1/3 – 1/2; style 15-17 mm

3. Plant 40-120 cm tall, coarsely hairy; Corolla divided to 1/2, lobes stiffly hairy outside on middle nerve and apex ***tchihatchewii***

3. Plant 25-40 cm tall, tomentose; Corolla divided to 1/3, tomentose outside

thyrsoidea

2.1.1.3. *Michauxia campanuloides* L'Hérit. ex Aiton

Plants are 100-150 cm tall; stem thick at base, erect, patent-coarse-hairy; racemose or paniculate inflorescence. Leaves hairy on both sides; flowers subsolitary, 2-4 cm, pedicellate. Calyx lobes 8-10 mm, stiffly hairy on margin and middle nerve outside. Corolla divided to 4/5, lobes lingulate, 25-45 mm, reflexed, stiffly hairy outside on middle nerve. Style 30-40 mm. Filament base broadly ovate, papillose on margins (3). Seeds 1x0.4 mm. *Michauxia campanuloides* L'Hérit. ex Aiton plant is shown in Figure 1.

Altitude: 10-1700 m

Habitat: Rocky outcrops and cliffs, dry open stony sites

Distribution in Türkiye: Mostly Southern and rarely Central Anatolia; hills of Kayseri, Antalya, Mersin, Konya, Mut, Niğde, Adana, Kahramanmaraş, Malatya, Adana, Hatay (Amanos Mountains), and Gaziantep**.**

Distribution in the World: Lebanon-Syria, Palestine, Türkiye.

Figure 1. General appearance of *Michauxia campanuloides*.

2.1.1.4. Traditional Use of *Michauxia* Species

Baytop (5) stated that the roots and stems of *M. campanuloides* and *M. tchihatchewii* was eaten after cooking in Tarsus, Silifke and Gülnar as vegetable.

In areas where *M. campanuloides* plant grows, there are a few ethnobotanical studies reported. A study stated that in Hasanbeyli district, locals have been freshly eaten the aerial parts and roots of *M. campanuloides* are consumed when they are fresh. It is also reported to be used against mycosis (14).

M. campanuloides is known as 'keçibiciği', 'sütlü', 'keşir' or 'kırtmaç' in Türkiye. It is a traditionally used plant according to ethnobotanical studies. A study stated that aerial parts of *M. campanuloides* are consumed in Nigde-Aladaglar, to reduce hunger and thirst (4, 14).

Kayiran and Ozhatay (6) assessed an ethnobotanical study in Andırın, Kahramanmaras. They recorded the plants which have been used for healing by villagers in the area. This study reported that leaves of *M. campanuloides* were crushed and used as wound healing externally.

2.1.2. Bioactivity Studies on *Michauxia* Species

2.1.2.1. Studies on Antioxidant Activity

Hürkul (2) investigated the antioxidant activity of different extracts [MeOH, water (H_2O), dichloromethane (DCM), EtOAc and *n*-BuOH)] prepared from *Michauxia* species using qualitative and quantitative 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Thiobarbituric Acid (TBA) tests for the first time. According to the results of qualitative and quantitative DPPH assays, DCM and EtOAc extracts of the species showed higher antioxidant activity than other extracts. Aerial parts of *M. campanuloides* EtOAc extract showed the highest antioxidant activity (IC_{50}=14.33 ± 0.57 mg/mL). TBA test resulted that EtOAc extract of *M. tchihatchewii* aerial parts (IC_{50}=4.94 ± 6.46 µg/mL) and EtOAc extract of *M. campanuloides* aerial parts (IC_{50}=28.02 ± 1.08 µg/mL) were exhibited high antioxidant activity.

Güvenç et al. (7) examined the antioxidant activity of roots and aerial parts of 5 different *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea, M. nuda*) collected from different provinces of Türkiye, by using qualitative (bioautographical) and quantitative (by measuring absorbance levels) DPPH assays and TBA test. MeOH extracts of the plants were partitioned with DCM, EtOAc and *n*-BuOH. Qualitative DPPH test results showed that MeOH and EtOAc extracts of both root and aerial parts of *M. campanuloides, M. tchihatchewii* and *M. nuda* had significant antioxidant effect by expressing yellow zones on TLC plate. According to quantitative DPPH test, EtOAc extract of the *M. campanuloides* had the highest antioxidant capacity with the IC_{50} value of 14.33 ± 0.57 and 37.34 ± 1.21 mg/mL for aerial parts and roots, respectively. EtOAc extract of aerial parts of *M. tchihatchewii* (IC_{50}=4.94 µg/mL ± 6.46) showed highest activity in TBA assay.

Ebrahimabadi et al. (8) evaluted the antioxidant activity of MeOH extract and essential oils obtained from stems and fruits of *Mindium laevigatum* (Syn.: *Michauxia laevigata*) collected from two different areas (Shahsavaran valley and Rahagh area) in Isfahan province, Iran; by DPPH radical scavenging assay, hydrogen peroxide scavenging assay, reducing power determination test and β-carotene/linoleic acid bleaching assay. Except β-carotene/linoleic acid bleaching assay, the results mostly supported that both extract and volatile compounds of stems of *M. laevigatum* had more antioxidant capacity compared to fruits. Additionally, essential oils of the plant have more antioxidant activity than MeOH extracts, likely because of the deterioration of all volatile materials during

extraction process. According to DPPH assay, IC_{50} value for the fruit of the plant collected from Shahsavaran valley was the lowest value and it was 344.3 ± 2.3 µg/mL.

An *in vivo* study investigated the effect of aqueous extract of *M. campanuloides* aerial parts on granulosa cells about Proliferating Cell Nuclear Antigen (PCNA) expression and ovarian histomorphology in Wistar Albino female rats. Total antioxidant capacity (TAC) was also evaluated. Rats were grouped, (n=6 for each group) as Control, Treatment 1 (T1) and Treatment 2 (T2). 20 mg/kg/day and 40 mg/kg/day dosages were administered to T1 and T2 groups. Based on the results, the extract was positively effective on follicular dynamics and granulosa cell expression level, however it was not effective on production of new follicles. TAC levels of T2 group were significantly higher (0.87 ± 0.14 mmol/L) than T1 group (0.58 ± 0.04 mmol/L). TAC levels of T1 and Control groups (0.28 ± 0.08 mmol/L) were not significantly different from each other. This information supports the idea of the importance of administration dosages on activity (15).

Jafarian et al. (16) evaluated *M. laevigata* for its genetic sources. The study based on genomic DNA extraction and antioxidant capacity of the plant. Proteins, antioxidant enzymes and phylogenetic tree of the plant were identified for the first time.

2.1.2.2. Studies on Antimicrobial Activity

Ebrahimabadi et al. (8) assessed the antimicrobial activity of MeOH extracts of stem and fruit of *M. laevigatum* collected from two different areas (Shahsavaran valley and Rahagh area) in Isfahan province, Iran, against 11 microorganisms. Disc diffusion and micro-well dilution assays were used for the study. Extracts determined to have weak antimicrobial activities except for fruit extract, in both tests. Fruit extract of the plant collected from Rahagh area was effective on *Bacillus subtilis, Staphylococcus epidermidis, Klebsiella pneumonia, Shigella dysenteriae* and *Proteus vulgaris*. For the assays of the fruit extract, maximum inhibition zones and minimum inhibition concentration (MIC) values were in the range of 10–24 mm and >500 µg/mL, respectively. None of the extracts showed any antifungal activity.

2.1.2.3. Studies on Anti-inflammatory Activity

Güvenç et al. (7) used an *in vivo* acetic acid induced increase in capillary permeability test for determining the anti-inflammatory effect of the extracts of roots and aerial parts of 5 *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea, M. nuda*) by using Whittle method. Results showed that *M. nuda* root extract (100 mg/kg dosage) provided 24.39% inhibition of increasing vascular

permeability while indomethacin (positive control) had 48.18% inhibition. *M. nuda* root extract showed the strongest inhibition capacity compared to all extracts of other 4 species.

2.1.2.4. Studies on Wound Healing Activity

Güvenç et al. (7) evaluated the wound healing activity of roots and aerial parts of 5 *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea, M. nuda*) extract by linear incision (measuring tensile strength of wounded and treated skin) and circular excision (monitoring wound contraction and wound closure time) wound models. They applied the test samples in ointment form onto the wounds and compared the results with Madecassol®. At the end of the study, specimen samples of the tissues were isolated for further histopathological examination. According to results, 10 days of application of the ointments prepared from *M. nuda* root and *M. tchihatchewii* aerial parts extracts resulted the best wound tensile strength (27.4% and 25.5%, respectively). The wound tensile strength percentage for Madecassol® was 59.9%. Results of *M. nuda* root and *M. tchihatchewii* aerial parts extracts were similar to the healing activity on circular excision, too.

2.1.2.5. Studies on Cytotoxic Activity

Ebrahimabadi et al. (8) assessed cytotoxicity evaluation of fruit and stem of *M. laevigatum* MeOH extracts collected from different areas (Shahsavaran valley and Rahagh area) by using Brine Shrimp lethality test and cancer cell lines. LC_{50} (Lethal concentration) value for Brine Shrimp lethality bioassay for extracts was >1000 µg/mL. LC_{50} value for colon (HTC116) and prostate (PC-3) cancer cell lines assay was >750 µg/mL. The lowest LC_{50} value for colon (HTC116) cancer cell line belonged to *M. laevigatum* MeOH fruit extract collected from Shahsavaran valley, and the value was 753.8 ± 2.5 µg/mL.

2.1.2.6. Studies on Effecting Semen Parameters

Spermatogenesis process directly gets effected by reactive oxygen species and the deficiency of antioxidant molecules. A study based on this information suggested that administration of lyophilized aqueous extract of *M. campanuloides* would make change on semen quality parameters. For the study, 18 male Wistar albino rats were divided into 3 groups: Control group, T1 and T2. For 21 days, prepared extract was administered orally to T1 group at a dose of 20 mg/kg/day and to T2 group at a dose of 40 mg/kg/day. Results showed that semen density was significantly higher for T2 group compared to control and T1 groups (P=0.002). Also, the weight of seminal gland was affected by the extract

administration; group T1 and group T2 values were higher than control group (P=0.016). Abnormal spermatozoa rates were significantly lower for extract administered groups (T1 and T2) compared to control group (P=0.003). Mentioned parameters proved that lyophilized extract of *M. campanuloides* positively effects semen parameters in rats (9).

2.1.3. Phytochemical Studies on *Michauxia* species

Studies on phytochemical constituents of *Michauxia* species are very limited. In spite of that, Hürkul (2) conducted a study which includes the qualitative reactions of the main phytochemicals which were alkaloids, cardiotonic glycosides, saponosides, flavonoids, anthocyanins, cyanogenic heterosides, tannins, anthracenosides, coumarins, essential oils and mucilages in 5 different *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea, M. nuda*) grows in Türkiye. The study resulted that anthracenosides, essential oil and mucilage were not present in any of the species. However, both root and aerial parts extracts of all species were found to contain flavonoids and cyanogenic glycosides. Additionally, all root extracts were found to contain saponins and all aerial parts extracts were found to contain tannins. Coumarin compounds were detected in both aerial parts and root extracts of *M. campanuloides* (2). However, it is worth to note that, these assays are screening tests, and they are not conclusive.

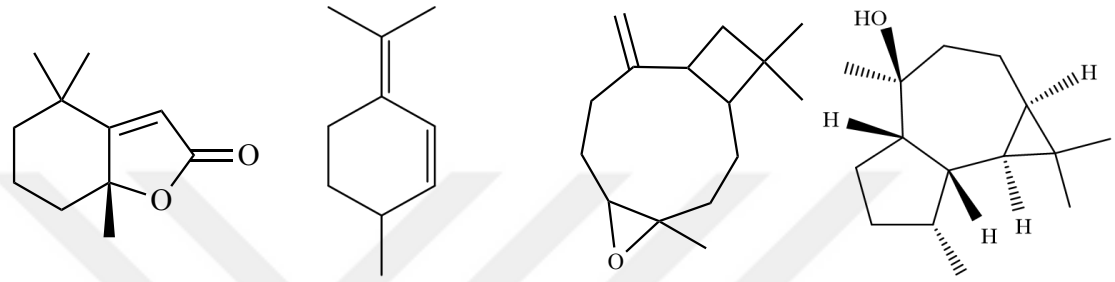
Additionally, total phenolic contents of *Michauxia* species were investigated in the same study. Gallic acid (GA) was employed as the standard reference, and the results were quantified as milligrams of gallic acid equivalents per gram of extract (GAE/g). As a result, EtOAc extract of *M. campanuloides* aerial parts exhibited the highest amount of total phenolic content (439.05±18.70 mg GAE/g extract). Overall, dichloromethane and EtOAc extracts of the species exhibited high amount of total phenolic content (2).

In a study, total phenolic content of 5 different *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea, M. nuda*) aerial parts and roots extracts (H_2O, MeOH, DCM, EtOAc and *n*-BuOH) growing in Türkiye were evaluated. GA was employed as a reference, and the results were quantified as milligrams of gallic acid equivalents per gram of extract. According to results, EtOAc, *n*-BuOH and MeOH extracts were determined to contain more phenolic contents compared to H_2O and DCM extracts. The highest phenolic content was detected in EtOAc extract of *M. campanuloides* aerial parts (439.05 ± 18.70 mg GAE/g) (7).

The content of total phenolic compounds belonging stems and fruits of *Mindium laevigatum* MeOH extract (plants were collected from two different areas: Shahsavaran

valley and Rahagh area) were determined in a study. Results showed that fruit extract of *M. laevigatum* collected from Shahsavaran area had the highest amount of phenolic content (79.6 ±3.6 µg/mg, result showed as GA equivalent) (8).

A study (17) aimed to analyze the volatile principles in *M. laevigatum* by gas chromatography-mass spectrometry (GC-MS). 91 constituents were identified by GC-MS method, characteristic constituents were dihydroactinidolide, alpha-terpinolene, caryophylene oxide and viridiflorol oils (Figure 2).



Dihydroactinidolide α-Terpinolene Caryophylene Oxide Viridiflorol

Figure 2. Characteristic constituents of *M. laevigatum* according to GC-MS results (17).

2.1.4. Methods for Evaluation of Bioactivity

2.1.4.1. Cytotoxicity Studies

There are numerous physical and chemical elements that may harm cell viability and create toxicity via different mechanisms. Disintegration of the cell membrane, irreversible binding to receptors, or enzymatic reactions are some of referred mechanisms. To determine the cell viability levels, cytotoxicity assays are used. These methods help to understand the number of cells that die under different conditions (18).

Cytotoxicity and cell viability methods varies in a large scale. One of the most used methods was tetrazolium salt, which is a colorimetric method, since it was a cheap and easy method. Tetrazolium salts have been used for identifying dead cells from viable cells for long years. Tetrazolium salts mechanism relies on the reduction to formazans by the cytochrome systems of viable cells (19).

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), was the new tetrazolium analogue after 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The difference was the ability of MTS to give a water-soluble formazan product that had maximum absorbance at 490-500 nm in

presence of phenazine methosulphate (PMS) which is an electron coupling reagent, in phosphate buffered saline, which eases the absorbance measuring process compared to MTT. MTT was not water-soluble, that created the need for microscobic observation for determination of the color change (20, 21).

Principle of MTS test is the reduction of tetrazolium salt (MTS) to purple colored formazan crystals by the active metabolism of viable cells. As a result, colored crystals are proportional to viable cells (22).

2.1.4.2. Antioxidant Activity

2.1.4.2.a. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Radical Scavenging Method

ABTS radical scavenging assay is a method used for measurement of antioxidant activity of various substances/compounds. It is referred as a decolorization assay and it is a spectrophotometric method. The forming of $ABTS^+$ radical (blue/green) requires the reaction between potassium persulphate and ABTS. The hydrogen atoms of antioxidants reduce the $ABTS^+$ radical; thus, decolorization occurs. Accordingly, the degree of decolorization is measured based on concentration of antioxidants and reaction time and is calculated according to the reactivity of standard compound under identical conditions (23). The specific maximum UV absorbance (UV_{max}) value for the reaction product is at 734 nm. Earlier, the $ABTS^+$ radical was generated in different way, metmyoglobin and hydrogene peroxide were used (24).

The increased use of $ABTS^+$ radical scavenging method relies on the solubility properties of the compounds used. The ABTS method is useful for both water-soluble and lipophilic compounds because of its solubility in water and organic solvents. The test is also valid across a wide range of pH values (23). One of the drawbacks of this technique is the prerequisite generation of ABTS, which must be produced in advance through a chemical reaction (such as with manganese dioxide or potassium persulfate), an enzymatic process (using peroxidase or myoglobin), or an electrochemical method. Additionally, the reaction kinetics with certain antioxidants can be slow, potentially leading to inaccuracies in the measurement of antioxidant capacity (25). Figure 3 indicates the reaction occurs when ABTS reagent interferes with antioxidant compounds.

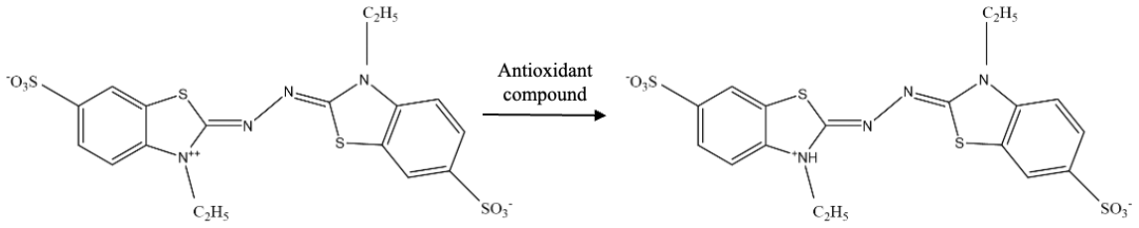


Figure 3. Reaction of $ABTS^{+}$ radical with antioxidant compound (25).

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

DPPH assay was firstly used by Blois (26), the idea behind the experiment was measuring the antioxidant capacity of the sample by observing the decolorization on DPPH (a stable free radical) solution. This method is the simplest antioxidant capacity measuring method. Mechanism of the reaction is based on the reduction of the only electron of nitrogen atom in DPPH by antioxidants to the related compound, hydrazine. UV_{max} value for the reaction product is at 517 nm (27). In Figure 4, it is shown that DPPH free radical is donated a free electron from an antioxidant and become stable by receiving a hydrogen atom. Decolorization occurs when the electron is donated (26). This method is useful for determining total antioxidant content in plant extracts according to its simplicity and speed. On the other hand, DPPH can be decolorized by any reducing agent other than antioxidants. This is a disadvantage of the DPPH assay, as it can lead to unreliable results when assessing antioxidant capacity (28).

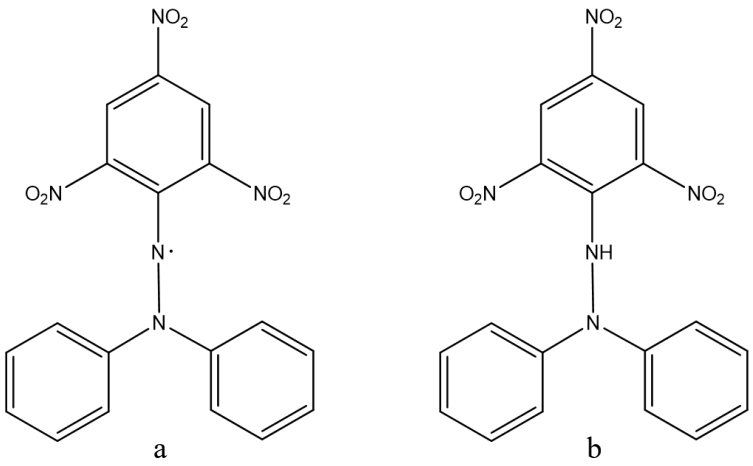
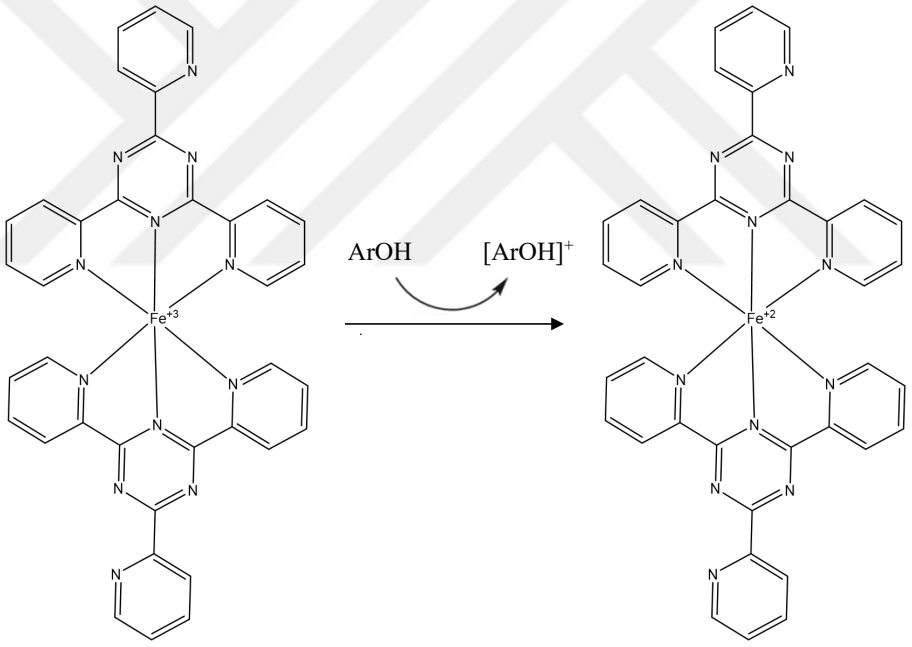


Figure 4. DPPH free radical (a) and its reduced form (b), respectively (25).

2.1.4.2.c. Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP method was referred as direct test of total antioxidant power in 1999 (29). It was used for measuring the antioxidant power of various compounds in natural products. Basically, the mechanism relies on the transformation (reduction) of ferric ion [Fe^{3+} - 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)] to ferrous ion (Fe^{2+} – TPTZ) by antioxidants or other reductants in acidic environment (pH=3.6), intense blue color occurs at 593 nm (30, 31). Reduction of ferric ion is shown in Figure 5.

FRAP assay is simple, fast, stable, and it is also related to the concentration of the antioxidant. Further studies showed that reaction time was also related to the antioxidant capacity results (32). While FRAP assay is widely used for measuring antioxidant capacity of plants, this assay does not interact with thiols because the reduction potential of thiols is generally lower than that of the Fe^{3+}/Fe^{2+} half-reaction (33).



$$[Fe^{III}(TPTZ)_2]^{3+} + ArOH \longrightarrow [Fe^{III}(TPTZ)_2]^{3+} + ARO^{\bullet} + H^{+}$$

Figure 5. Reduction of ferric ion (Fe^{3+} - TPTZ) to ferrous ion (Fe^{2+} – TPTZ) by antioxidants in FRAP assay (10).

2.1.4.2.d. Cupric Reducing Antioxidant Capacity (CUPRAC)

In 2004, Apak et al. (34) named and described CUPRAC assay to directly measure the total antioxidant activity level of compounds in natural products including phenolic

compounds (carotenoids, flavonoids, etc.), together with thiols and synthetic antioxidants. Regarding this method, copper (II)-neocuproine [Cu (II)-Nc] (2,9-dimethyl-1,10-phenanthroline) was used as the chromogenic and oxidizing reagent. As shown in Figure 6, in the presence of antioxidant compound, Cu (II) cation acted as a facilitator of the electron transfer. As a result of the reaction, yellow-orange colored Cu (I) neocuproine chelate occurred. The absorbance of the chelate produced as a result of the reaction was read at 450 nm, at neutral pH. Reagent used in CUPRAC is stable, accessible and responds to lipophilic and hydrophilic antioxidants. However, the oxidation reaction may require a relatively prolonged incubation period (35).

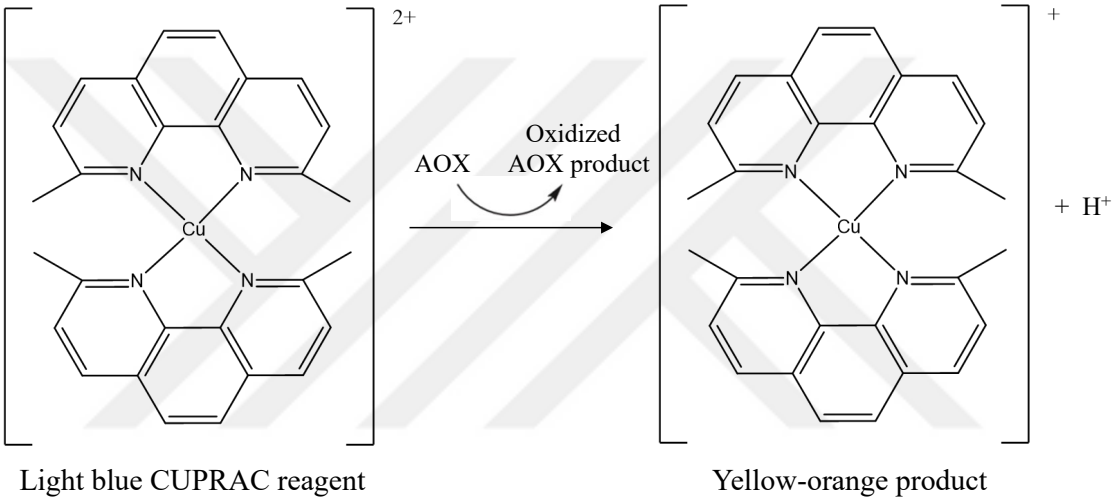


Figure 6. Formation for Cu(I)-Nc cation followed by CUPRAC reaction (35). AOX: Antioxidant.

2.1.5. Methods for Evaluation of Total Amounts of Phenolic Compounds

2.1.5.1. Total Phenolic Content Assay

Phenolics are present in many plants as their largest group of secondary metabolites. They are expected to neutralize free radicals, based on their structural properties. Determination of total phenolic content of different kinds of plants is commonly use as a preliminary analysis before phytochemical studies. Folin-Ciocalteu method was found by Folin and Vintila Ciocalteu and has been used for quantitative analysis of phenolic compounds for years.

The mechanism of Folin-Ciocalteu method refers to the reduction of phosphomolybdic/phosphotungustic acid complexes which were the ingredients of Folin

reagent, and phenolic/non-phenolic reducing compounds in the basic condition (pH>7) (36). As a result of electron interferences between the complexes and reducing agents, dark blue color occurs. Darkness of the blue color is correlated to the reducing capacity of antioxidant compound and measured the absorbance at 765 nm. It is a cheap and simple method to determine total phenolic content in extracts and different kinds of samples. It is preferably used for aqueous phase, therefore it is not a suitable procedure for lipophilic compounds. Folin-Ciocalteu reagent is not specific, accordingly it does not detect all phenolic compounds (36, 37). The reaction between gallic acid used as a reference compound in the assay and molybdenum is indicated in Figure 7.

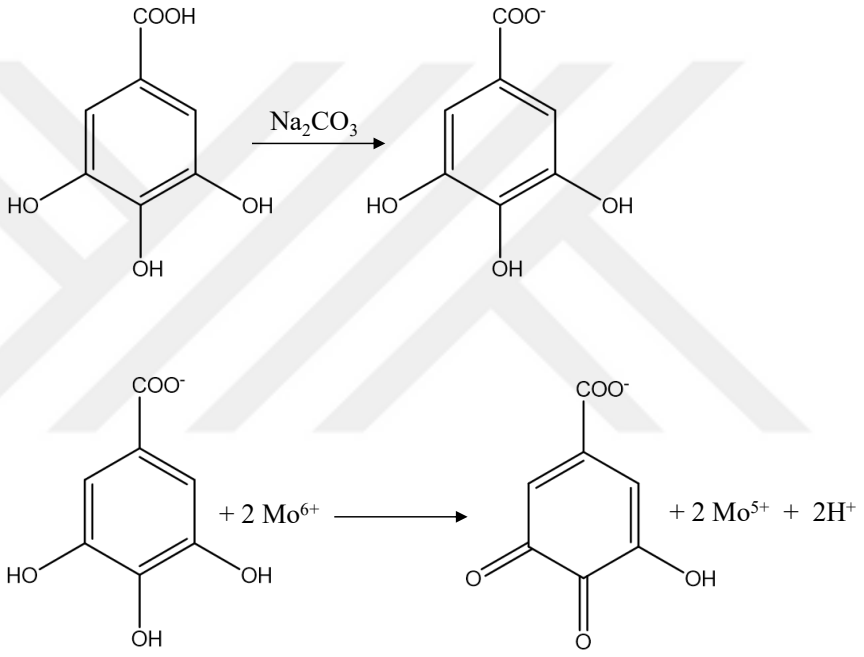


Figure 7. The reaction mechanism of Folin-Ciocalteu (38).

2.1.5.2. Total Flavonoid Content Assay

Flavonoids are crucial for the pharmacological activites of plants as a secondary metabolite. They have a high bioactivity potential such as antioxidant, anti-cancer and anti-inflammatory activity (39). Earlier, Barnum (40) stated that this assay was used for only ortho-diphenols. Then, the method was adapted, and a colorimetric assay was developed based on chelate formation (complexation). The mechanism of total flavonoid content assay basically related with the acid-stable complex formation between aluminium chloride and keto and hydroxyl groups of flavones or flavonols. The

absorption of yellow-colored product was measured at the range of 300-600 nm. Different flavonols (quercetin, rutin, galangin, etc.) were used as standard compounds. Sodium acetate was used for balancing pH level (41). The reaction between quercetin and $AlCl_3$ is shown in Figure 8.

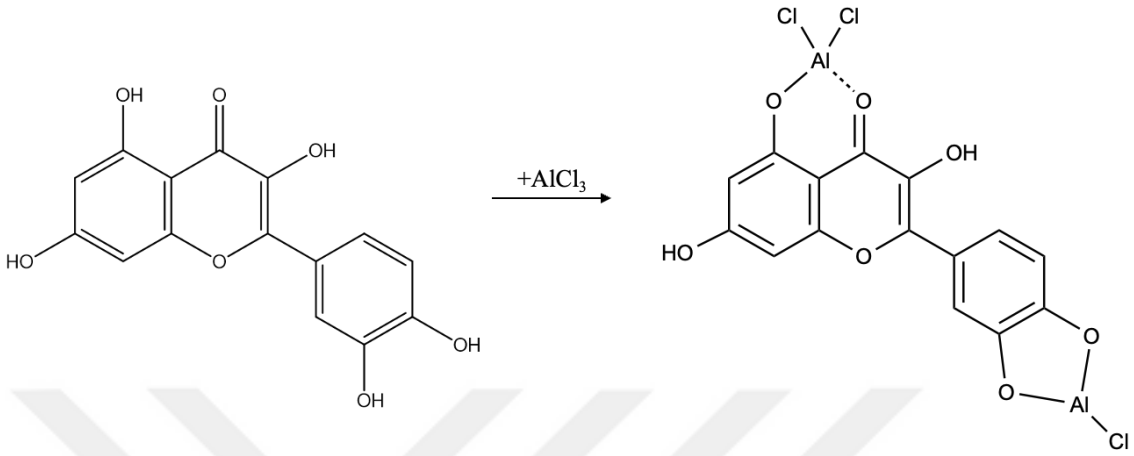


Figure 8. The mechanism of Total Flavonoid Content Assay (42).

2.1.6. Chromatography

For the first time, chromatography was used by M. S. Tswett who is a well-known Russian Botanist in 1903 to separate the green leaf pigments. He used the name 'chromatography' as its meaning: 'color writing'. After years, column liquid chromatography was redefined by Lederer and after that, chromatography was improved for years (43).

Chromatography referred as a whole system that used for separation of the compounds in a complex mixture from each other and for qualitative and quantitative analysis of these compounds. (44).

2.1.6.1. Thin Layer Chromatography (TLC) and High-Performance Thin-Layer Chromatography (HPTLC)

TLC is a chromatographic method for the separation of components within a mixture, and it requires several distinct steps. It is a simple method compared to other chromatographic methods. Initially, sample solution is applied to a plate (stationary phase) coated with an adsorbent material (silica gel, alumina, etc.). Subsequently, the plate is placed in a sealed glass chamber containing a mobile phase move through the stationary phase. By the help of capillary action, compounds having different polarities separate on the stationary phase. After development, the plate is replaced and dried, and the sample components are visualized by using ultra-violet (UV) cabinets. To visualize

the components, derivatization reagents may be used. Derivatization reagents help the samples to be seen under different wavelengths of light (254 and 366 nm) (45).

HPTLC is fully equipped version of thin layer chromatography. It provides automation and singularity for application on the plate and full optimization for every single step of the method. HPTLC is a valuable analyzing tool because it results a chromatographic fingerprint of the analyte. It is suitable for qualitative and quantitative analysis. After obtaining fingerprint chromatogram, appropriate usage of derivatization techniques on HPTLC plate and measuring retardation factor (R_F) values gives a considerable result (fingerprint) about mixture (46).

2.1.6.2. High-Performance Liquid Chromatography (HPLC) and Ultra-Performance Liquid Chromatography (UPLC)

In the 1970s, packed columns were used, and pumps were only able to create 35 bar pressure through the column. Then, technological advancements accelerated, and the pressure limit was raised to 400 bar for newly developed devices. Following this development, advancements in the entire device followed one another, and the acronym HPLC came to stand for High-Performance Liquid Chromatography (47).

HPLC system is capable to separation, identification, and quantification the compounds in any mixture or solutions. HPLC method is suitable for a wide range of working field. Chromatograms are outputs of HPLC system, they represent a series of peaks that each of them belongs to a distinct compound in the sample. This graphical representation is generated and plotted by the computer data station (47).

Normal-phase HPLC separation technique involves using a polar stationary phase in combination with a mobile phase that is significantly less polar or non-polar. In contrast to normal-phase chromatography, reversed-phase HPLC employs a polar mobile phase and a non-polar stationary phase. This approach is prevalent in HPLC methods, making up about 75% of all applications due to its reproducibility and wide-ranging applicability.

UPLC is more advantageous form of HPLC in many aspects. Particle size of the column, speed and pressure are main differences in the name of UPLC. For UPLC systems, smaller particle sized columns are suitable. This creates significantly better resolution of the compounds in a relatively short period of time and increases the sensitivity of the system compared to HPLC. The decrease in particle size in the column created significantly high system back-pressure. Because of the highly increased pressure, separation occurs under up to 1000 bar. Efficiency has significantly increased

because all components of the UPLC system (pump, column, detector, injector systems) have been reinforced accordingly (48).

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Chemicals and Solvents

Detailed information about the chemicals and solvents used in this study is shown in Table 1.

Table 1. Chemicals and solvents.

Chemicals and Solvents	Trademark
2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent	Sigma Aldrich
ABTS reagent	Roche Diagnostics
Acetic acid	Isolab
Acetonitrile	Chemlab
Aluminum chloride hexahydrate	Sigma Aldrich
Ammonium acetate	Fluka
Anisaldehyde	Sigma Aldrich
Astragalin	Sigma Aldrich
Caffeic acid	Sigma Aldrich
Chlorogenic acid	Sigma Aldrich
Copper (II) chloride dihydrate	Sigma Aldrich
Dichloromethane	Carlo Erba
Ethanol	Haker, Carlo Erba
Ethyl acetate	Sigma Aldrich
Ferric chloride	Fluka
Folin Ciocalteu's phenol reagent	Merck
Gallic acid	Sigma Aldrich
Hydrochloric acid	Isolab
Isoquercitrin	HWI
Methanol	Carlo Erba
n-Butanol	Carlo Erba
n-Hexane	Isolab
2-aminoethyl diphenyl borinate	Sigma Aldrich
Neochlorogenic acid	Sigma Aldrich

Table 1. Continued.

Chemicals and Solvents	Trademark
Neocuproine	Sigma Aldrich
o-phosphoric acid	Isolab
Polyethylene glycol (PEG) 400	Merck
Potassium persulfate	Sigma Aldrich
Quercetin	Sigma Aldrich
Rutin	Sigma Aldrich
Sodium acetate trihydrate	Riedel-de-Haën
Sodium carbonate	Tekkim
Sulphuric acid	Isolab
TPTZ	Sigma Aldrich
Trolox	Sigma Aldrich

3.1.2. Equipments and Instruments

Detailed information about the equipments and instruments that areused for the study is shown in Table 2.

Table 2. Equipments and instruments.

Equipments and instruments	Trademark
96-well plate	TPP; Germany 92096
Agilent Poroshell 120 EC-C18 column	Agilent Chemical
Balance	Ohaus Pioneer
Deep freezer (-20°C)	Arcelik
Eppendorf tubes (1, 5, 15 mL)	Eppendorf
Grinder	Arcelik
HPTLC Plate	Merck
HPTLC System	CAMAG
Immersion device	CAMAG Immersion Device III
Laboratory glassware	Isolab
Lyophilizator	Christ Alpha 2-4 LD
Micropipette	Eppendorf (20-200, 100-1000)
Microplate Incubator	Miulab
Microplate Reader	Thermo Scientific, Massachusetts, ABD
pH meter	Mettler-Toledo
Refrigerator	Arcelik
Rotary-evaporator	Heidolph, Laborota 4001
Syringe filter (0.22 μm)	Chrom Fil PTFE-L
TLC Developing Tank	CAMAG
TLC Plate Heater	CAMAG
Ultrapure Water Device	Millipore
Ultrasonic bath	Sonorex; Germany RK156BH
Ultra-Performance Liquid Chromatography	Schimadzu
UV cabinet	CAMAG

3.1.3. Plant Materials

The aerial parts and roots of *M. campanuloides* 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). Documented specimen 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.

3.2. Methods

3.2.1. Extraction and Partition

The aerial parts and roots were separated and dried at room temperature. Then, these parts were powdered. 50 g roots and 100 g aerial parts were macerated with 500 mL and 1000 mL MeOH, respectively for 3 days in an erlenmeyer flask, seperately. Then, the plant materials were extracted at 45°C for 4 hours by using rotary evaporator without vacuuming. After filtration, the solvent was evaporated under reduced pressure to yield crude MeOH extract. The same procedure was repeated, and the pooled extracts were combined. Then, obtained extracts belong to aerial parts and roots were dispersed in H_2O and freeze-dried, separetely.

For partition process; *n*-hexane, EtOAc and *n*-BuOH were the solvents of choice, respectively. Lyophilized root extract was dispersed in H_2O (root: 25 mL, aerial parts: 50 mL), transferred to a separatory funnel and aqueous phase was partitioned with 25 mL of *n*-hexane, EtOAc and *n*-BuOH three times, respectively. The same partition procedure using *n*-hexane, EtOAc and *n*-BuOH was repeated for the extract belong to lyophilized aerials parts after dispersing it with 50 mL of the H_2O. Partition process and yields are given as schematic expression in Figure 9. After partition process, *n*-BuOH and H_2O layers were lyophilized, while *n*-hexane and EtOAc subextracts were dried under reduced pressure.

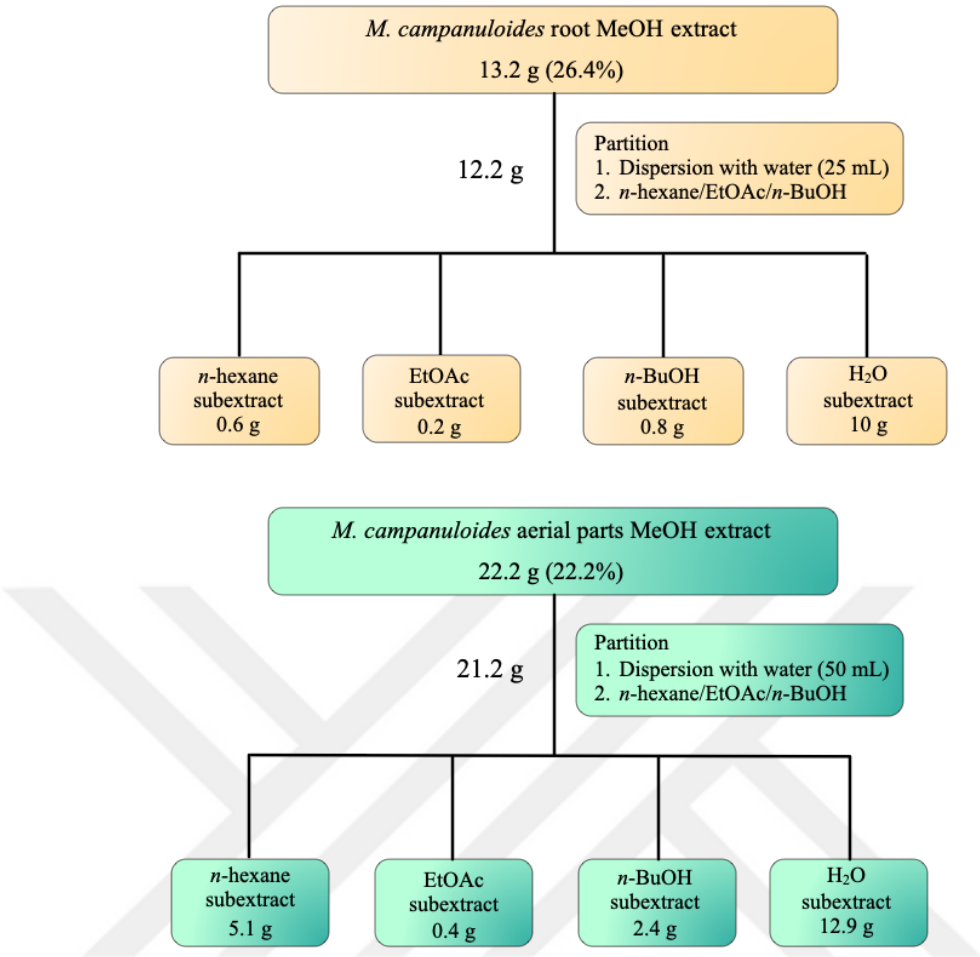


Figure 9. Schematic expression of partition processes.

3.2.2. Preparation of Sample Test Solutions

For cytotoxicity investigations, each extract was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution at a concentration of 100 mg/mL. Subsequently, this stock solution was further diluted.

The crude MeOH extract and subextracts (*n*-hexane, EtOAc, *n*-BuOH, H_2O) were dissolved in MeOH to prepare sample test solutions (stock solutions). Further, stock sample test solutions (5 mg/mL) were diluted with MeOH to perform antioxidant activities and phytochemical studies.

For ABTS and DPPH assays, prepared stock solutions were further diluted to concentrations ranging from 200 µg/mL to 2 mg/mL using MeOH. For FRAP and CUPRAC assays, prepared stock solutions were further diluted to concentrations ranging from 200 µg/mL to 5 mg/mL using MeOH.

Further dilutions for Total Phenolic Content assay were performed at the concentrations from 200 µg/mL to 2 mg/mL by using MeOH. For Total Flavonoid Content assay, extracts were further diluted by using methanol at the concentration range of 5 mg/mL and 50 mg/mL.

For HPTLC studies, 20 mg/mL of sample test solutions were prepared by using MeOH.

For UPLC method, root extracts were diluted to the concentration of 1 mg/mL with MeOH. Aerial parts extracts were diluted in the range of 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL with the same solvent.

3.2.3. Preparation of Standard Solutions

The standard stock solutions of gallic acid and quercetin used for total phenolic content assay and total flavonoid assay, respectively were prepared at the concentration of 1 mg/mL by using H_2O and ethanol. Additionally, trolox used in antioxidant assays were at prepared at the concentration of 1 mg/mL by using ethanol. These solutions were further diluted to prepare working standard solutions (8-125 µg/mL) to be used in the assays.

For HPTLC studies; rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid and quercetin were dissolved in MeOH and used as reference compounds. These compounds were mixed in equal volumes to obtain standard mixture solution at the concentration of 50 µg/mL.

For UPLC studies, rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid and quercetin stock solutions were prepared at the concentration of 350 µg/mL by using MeOH.

3.2.4. Bioactivity Studies

3.2.4.1. Cell Lines and Cell Cultures

For cell culture studies, totally 6 different cancer cell lines including colon (HCT116 and SW480), breast (MDA-MB-231 and HCC1937) and liver (HEP3B and HEPG2) were used. Fibroblastic L929 cell line was used as healthy cell line. Medium contents of choice were included Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific #11995065) (L929, SW480, MDA-MB-231), Roswell Park Memorial Institute (RPMI) (Thermo Fisher Scientific #11875085) (HCC1937) or DMEM/F12 (Thermo Fisher Scientific #11320033) (HCT116), 10% fetal bovine serum (FBS, Sigma-Aldrich #11320033) (HCT 116) and as antibiotic; 1% penicillin/streptomycin solution

(Capricorn Scientific, #PS-B). Trypsin (Thermo Fisher Scientific #25200056) was used for passaging process, when the cells reached 80% confluency.

3.2.4.2. Evaluation of Cytotocixity Studies

To evaluate the cytotoxicity of the extracts on cell lines, colorimetric MTS method was used. Trypan blue was used for determination of cell count per mililiter. Cells and trypan blue were mixed (1:1 *v/v*) and JuLi imaging station was used for monitoring the results. 5×10^3 cells per well were seeded in 96-well plates in triplicates and incubated overnight at 37°C in humid cell culture incubator in order to provide optimal condition for attaching the cells.

Treatment concentrations prepared in complete media by serial dilution method to obtain test solutions at the concentrations of 200, 100, 50, 25 and 12.5 µg/mL. Cell culture medium was used as blank, and untreated cells were employed as positive control. As cytotoxic control, paclitaxel was used at the concentrations of 1, 2.5, 5, 10 and 20 nM. Cells were incubated with the test solutions for 48 hours at indicated concentrations. After the incubation, 10% MTS reagent (Abcam #ab197010) was added to each well and plates were further incubated for two hours. Absorbance of the culture media was read at 490 nm with a spectrophotometer (Epoch, BioTek, USA) and viability was evaluated using the formula given below. Dose-response curves were drawn for the extracts, the IC_{50} values of the extracts were calculated for each cell line.

$$Viability\ (\%) = \frac{Test\ OD - Blank\ OD}{Positive\ Control\ OD - Blank\ OD} \times 100$$

3.2.4.3. Evaluation of Antioxidant Activity

3.2.4.3.A. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Radical Scavenging Method

ABTS assay was carried out according to Re et al.'s study to determine the antioxidant capacity of *M. campanuloides* root and aerial parts extracts with minor changes (23).

The ABTS reagent was prepared by dissolving 9 mg ABTS and 1.6 mg potassium persulphate in 5 mL of H_2O. The prepared ABTS reagent was kept in a dark place at room temperature for 12 to 16 hours. Color of the reagent changed from turquoise to dark blue. After awaiting process, ABTS reagent was checked at 734 nm and its absorbance was found 0.7. Then, this reagent was used for further studies. 5 mL of prepared reagent was

dissolved in 45 mL of ethanol. To each well, 20 µL sample or blank (MeOH) and 280 µL ABTS reagent were added. At room temperature, the plate was incubated for 6 minutes. Absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific, Massachusetts, ABD) at 734 nm, after the incubation. The results of ABTS were expressed as mg of trolox equivalents per g of extract (mg TE/g extract).

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

Blois et al.'s study was used for analyzing antioxidant capacity of the extracts by DPPH method with minor changes (26).

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 the mixture was kept in a dark environment for 30 minutes for incubation. Followed by the incubation period, absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 520 nm. The results of DPPH assay were documented as mg of trolox equivalents per g of extract (mg TE/g extract).

3.2.4.3.C. Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP assay was performed according to study performed by Benzie and Strain (29) with minor changes.

FRAP solution was prepared from three different solutions in the ratio of 1:1:10 as follows; 2×10^{-2} M $FeCl_3$, 1×10^{-2} M TPTZ and pH 3.6 sodium acetate buffer solution, respectively. In order to prepare $FeCl_3$ solution, 1 mL of 37% HCl solution was added to required amount of $FeCl_3$ to increase the solubility of $FeCl_3$ in distilled water.

To each well, 20 µL sample test solution or blank (distilled water) and 280 µL freshly prepared FRAP reagent were added and incubated for 6 minutes at room temperature. At the end of the incubation period, absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 595 nm. FRAP assay results were given as mg of trolox equivalents per g of extract (mg TE/g extract).

3.2.4.3.D. Cupric Reducing Antioxidant Capacity (CUPRAC)

Apak et al.'s (34) study named and explained the CUPRAC assay in 2004 and this method was used in this study with minor changes.

For the preparation of required solutions, $1x10^{-2}$ M copper (II) chloride dihydrate and 1 M ammonium acetate was prepared with MeOH (pH: 7), $7.5x10^{-3}$ M neocuproine was prepared with ethanol. pH level of ammonium acetate solution was adjusted by using 37% HCl solution.

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 of each well 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).

3.2.5. Phytochemical Evaluation

3.2.5.1. Total Phenolic Content Assay

Folin-Ciocalteu method (49) was used for the determination of the total phenolic content in *M. campanuloides* root and aerial parts extracts.

Folin reagent was prepared by dissolving the reagent 1:10 (50 mL) with distilled water. The reagent was kept away from the light. Sodium carbonate (Na_2CO_3) solution (7.5%) was prepared with distilled water.

To each well, 25 µL sample or blank (distilled water), 25 µL distilled water, 125 µL Folin-Ciocalteu reagent and 100 µL 7.5% Na_2CO_3 solution was added. The plate was placed in an incubator set at 37°C for 30 minutes. After the incubation period, absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 760 nm. Total phenolic content assay results were expressed as mg of gallic acid equivalents per g of the extract (mg GA/g extract).

3.2.5.2. Total Flavonoid Content Assay

Total flavonoid content was detected by using the Woisky et al.'s (50) study with minor changes.

To each well, 30 µL sample test solution or blank (ethanol), 30 µL 10% aluminium chloride (prepared with 96% ethanol to avoid precipitation at the bottom of the plate), 30 µL sodium acetate trihydrate (dissolved in 96% ethanol) and 210 µL MeOH was added. Increased volume of solvent (MeOH) was used to avoid the precipitation and error on absorption values. Plate was incubated at 37°C for 15 minutes. At the end of the incubation period, absorbances of each well were measured by Varioskan Lux Microplate Reader (Thermo Scientific) at 415 nm. Results of total flavonoid content assay were presented as mg of quercetin equivalents per g of extract (mg QE/g).

A picture of laboratory working area and Varioskan Lux Microplate Reader (Thermo Scientific) is shown in Figure 10.

Figure 10. Preparation for total flavonoid content assay.

3.2.6. Chromatography

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

Semi-automatic Linomat 5 (CAMAG, Muttenz, Switzerland) equipped with 100 µL Hamilton syringe was used for the applications of sample and standard solutions on 20 x 10 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with silica gel 60 F_{254}. Accordingly, 20 mg/mL of each sample test solutions was applied as 5 µL whereas 50 µg/mL of standart mixture solution containing rutin, chlorogenic acid, neochlorogenic acid, isoquercitrin, astragalin, caffeic acid and quercetin was applied as 2 µL with 8 mm band lenght. Developing process was performed in a glass twin-trough chamber. EtOAc – acetic acid (AcOH) – formic acid (FA) – H_2O (100:11:11:26 *v/v/v/v*) was used as a developing solvent system. Firstly, 10 mL of developing solvent system was placed in one trough having filter paper for the saturation of 20 minutes. Then, the plate was developed up to 7 cm. After the development, the plate was dried with a cold air for 2 minutes.

For the visualization, two different derivatization reagents were used:

a. Natural Product [2-aminoethyl diphenylborinate (1 g) was dissolved in EtOAc (200 mL)] - Polyethylene Glycol 400 [(Polyethylene glycol 400 (10 g) was dissolved in DCM (200 mL) (51)] solutions named as NP/PEG reagents. The developed plate was first heated with TLC Plate Heater III at 105°C for 3 min. Then, the plate was dipped into NP and PEG reagents, respectively using CAMAG Chromatogram Immersion Device III.

b. 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 (51)] was used as a derivatization reagent. The developed plate was dipped into anisaldehyde reagent by using CAMAG Chromatogram Immersion Device III. After derivatization, the plate was heated at 105°C for 3 min by using TLC Plate Heater III.

The plates were documented at 254 nm, 366 nm, and white light using TLC Visualizer. HPTLC system was controlled by WinCATS software.

For identifying and evaluating the investigated standards in samples, retardation factors (R_F) and the band colors were used.

3.2.6.2. Ultra-Performance Liquid Chromatography (UPLC)

For the identification and quantitative analysis of neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin and quercetin in roots and aerial parts of *M. campanuloides* extracts, Schimadzu UPLC-PDA (Photodiode Array) system (Kyoto, Japan) was utilized. Agilent Poroshell 120 EC-C18 column (3.0 x 150 mm) with 2.7 µm particle size was used for the chromatographic separation, with a temperature of 20°C. The filtered (0.22 µm size) and degassed mobile phase A [*o*-phosphoric acid-water (0.1:99.9, *v*/*v*)] and mobile phase B (acetonitrile) were used for gradient elution as shown in Table 3.

Table 3. Detailed information on gradient elution of mobile phases in UPLC method.

Min	Flow Rate (mL/min)	Mobile Phase A	Mobile Phase B
0	0.3	85	15
1	0.3	83	17
3	0.5	82.8	17.2
11	0.5	82.7	17.3
12	0.5	80	20
22	0.5	60	40
23	0.5	30	70
25	0.3	85	15
30	0.3	85	15

The newly developed UPLC method was additionally validated in accordance with the guidelines of the International Conference on Harmonization (ICH) (52).

Specificity, linearity, LOD, LOQ, precision, and accuracy parameters were used for the validation of the UPLC method. Table 4 presents the detection wavelength selected according to their UV_{max} values for each analyte.

Table 4. Detection wavelenght of the analytes for UPLC analysis.

Analyte	Wavelenght
neochlorogenic acid, chlorogenic acid	325 nm
caffeic acid	323 nm
rutin, isoquercitrin	256 nm
astragalin	264 nm
quercetin	371 nm

Standard solutions were prepared from stock solutions of neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin and quercetin at the concentrations of 5 to 50 µg/mL with MeOH.

3.2.7. Statistical Analysis

Each test was conducted three times, and the average value and its corresponding 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 $p \leq 0.05$.

4. RESULTS

The aim of this study was the determination of cytotoxic and antioxidant activity of *M. campanuloides* extract as well as characterize some of the compounds of the extracts. Crude MeOH extracts prepared from roots and aerial parts were then partitioned with *n*-hexane, EtOAc and *n*-BuOH. For cell culture studies; colon (HCT116 and SW480), breast (MDA-MB-231 and HCC1937) and liver (HEP3B and HEPG2) cancer cell lines were used, cytotoxicity studies was held by using colorimetric MTS method. ABTS, DPPH, FRAP and CUPRAC methods were used for the evaluation of antioxidant activity of the extracts. Total phenolic and flavonoid contents of the extracts were also studied for supporting the data from antioxidant activity assays. Additionally, chromatographic methods were used in order to determine the major components in the extracts. HPTLC and UPLC studies were carried out using neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin as standard compounds. Detailed results are given in following sections.

4.1. Extraction and Partition

Mainly two methanolic extracts were prepared from 50 g roots and 100 g aerial parts of *M. campanuloides* (26.4% and 22.2% yield, respectively). Both extracts were lyophilized before partition. After lyophilization, 1 g of each extract were kept for further experiments. Partition was applied on crude MeOH extracts of root and aerial parts of *M. campanuloides* plant by using *n*-hexane, EtOAc and *n*-BuOH, respectively. Lyophilized root and aerial parts extracts were dispersed with 25 mL and 50 mL of water, respectively. For root extract, 25 mL of organic solvents and for aerial parts extract, 50 mL of organic solvents were used for each extraction for three times. Information for the extraction process is summerized and given in Table 5.

Table 5. Extraction.

Part of the plant	Amount of the plant (g)	Solvent	Amount of the solvent (mL)	Amount of the extract after lyophilization (g)	Yield
Aerial parts	100	MeOH	1000	22.2	22.2%
Root	50	MeOH	500	13.2	26.44%

4.2. Bioactivity studies

4.2.1. Cytotoxicity Activity Studies

The *in vitro* cytotoxic effects of *M. campanuloides* root and aerial parts of MeOH extracts and their subextracts (*n*-hexane, EtOAc, *n*-BuOH and H_2O subextracts) were tested on colon (HCT116, SW480), breast (MDA-MB-231, HCC1937) and liver (HEP3B, HEPG2) cancer cell lines and L929 healthy cell line by MTS method. As a positive control, an anticancer drug paclitaxel was used.

The MTS study results revealed that EtOAc subextract of *M. campanuloides* roots had moderate cytotoxic effect with the IC_{50} values ranging from 57.69-154.76 µg/mL on HEP3B, HEPG2, HCT116, SW480, MDA-MB-231, HCC1937 cancer cell lines. However, this extract was also toxic to normal cell lines, L929. Another extract which had significant cytotoxic effect was *n*-hexane subextract of *M. campanuloides* roots. It exhibited cytotoxicity against HEPG2 cancer cell line with the IC_{50} value 144 ± 12.65 µg/mL. Despite that, other extracts (*n*-hexane, EtOAc and *n*-BuOH subextracts of aerial parts and *n*-BuOH subextract of *M. campanuloides* root part) did not show significant cytotoxic activity. Table 6 is given for detailed information about *in vitro* MTS assay.

Table 6. Cytotoxic activities of EtOAc and *n*-hexane subextract of *M. campanuloides* roots against HEP3B, HEPG2, HCT116, SW480, MDA-MB-231, HCC1937 and L929 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 ± 0.53	ND	ND
L929	13.71 ± 1.43	128.92 ± 15.28	ND

The data represent the mean ± SD of three independent experiments, each conducted in triplicate. ND: Not Detected. MCR: *M. campanuloides* root.

* Positive Control (nM)

MeOH extracts, *n*-BuOH subextracts, EtOAc and *n*-hexane subextracts of aerial parts did not show cytotoxicity in the tested concentrations (IC_{50} > 200 µg/mL).

4.2.2. Antioxidant Activity Studies

4.2.2.1. ABTS Radical Scavenging Assay

Calibration curve of trolox which used as a standard for ABTS radical scavenging method is given in Figure 11. The obtained absorbances of the sample test solutions were subjected to calibration curve equation below to calculate the results as mg of trolox equivalents per g of extract (mg TE/g extract). According to the results of ABTS radical scavenging method, EtOAc subextract of *M. campanuloides* roots and aerial parts showed statistically similar and significant antioxidant activity (367.65 ± 16.78 and 353.40 ± 19.21 mg TE/g, respectively). On the other hand, H_2O subextract of *M. campanuloides* roots showed the lowest antioxidant activity (16.67 ± 0.46 mg TE/g). Figure 12 demonstrates the decolorization of ABTS reagent. The results of antioxidant activity performed by ABTS method were given in Table 7.

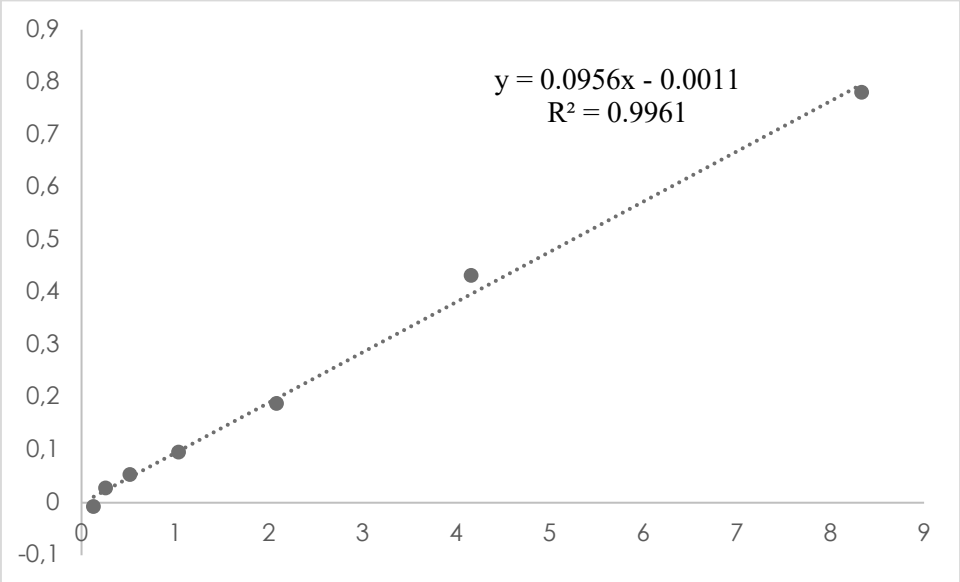


Figure 11. Trolox calibration curve (8-125 µg/mL) used in ABTS assay.

Figure 12. The color change occurred by ABTS reagent. A → B: Antioxidant activity from lowest to highest.

4.2.2.2. DPPH Radical Scavenging Assay

Calibration curve of trolox which was used as a standard for DPPH radical scavenging method is given in Figure 13. Absorbance results of the extracts were calculated by using the calibration curve equation below. According to the results of DPPH radical scavenging method, EtOAc subextract of *M. campanuloides* aerial parts showed statistically significant antioxidant activity (223.41 ± 4.7 mg TE/g). Although, H_2O subextract of *M. campanuloides* roots showed the lowest antioxidant activity (3.52 ± 0.87 mg TE/g). The color change of DPPH reagent was shown in Figure 14. Detailed results of DPPH method were given in Table 7.

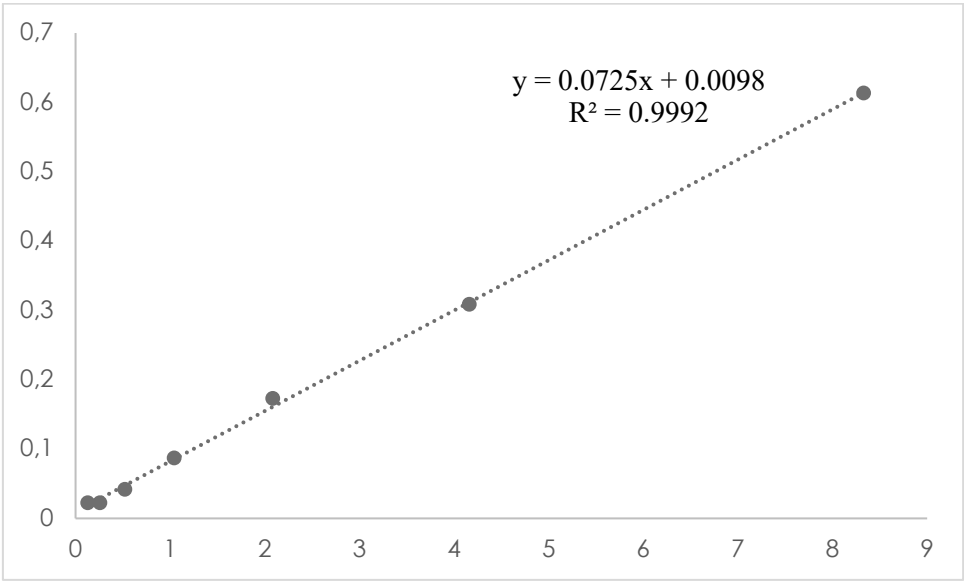


Figure 13. Trolox calibration curve (8-125 µg/mL) used in DPPH assay.

Figure 14. Discoloration of the DPPH reagent. A → B: Antioxidant activity from lowest to highest.

4.2.2.3. Ferric Reducing Antioxidant Power Assay (FRAP)

Calibration curve of Trolox which used as a standard for FRAP assay is given in Figure 15. According to the results of FRAP assay, EtOAc subextract of *M. campanuloides* aerial parts showed statistically significant antioxidant activity (342.96 ± 22.55 mg TE/g). Respectively, EtOAc subextract of *M. campanuloides* roots and *n*-BuOH subextract of aerial parts were statistically different but still showed high antioxidant activity compared to other extracts (208.5 ± 4.28 and 105.43 ± 6.94 mg TE/g, respectively). Although, H_2O subextract of *M. campanuloides* roots showed the lowest antioxidant activity (1.85 ± 0.27 mg TE/g). Coloring reaction occured by FRAP assay was shown in Figure 16. Detailed FRAP assay results were given in Table 7.

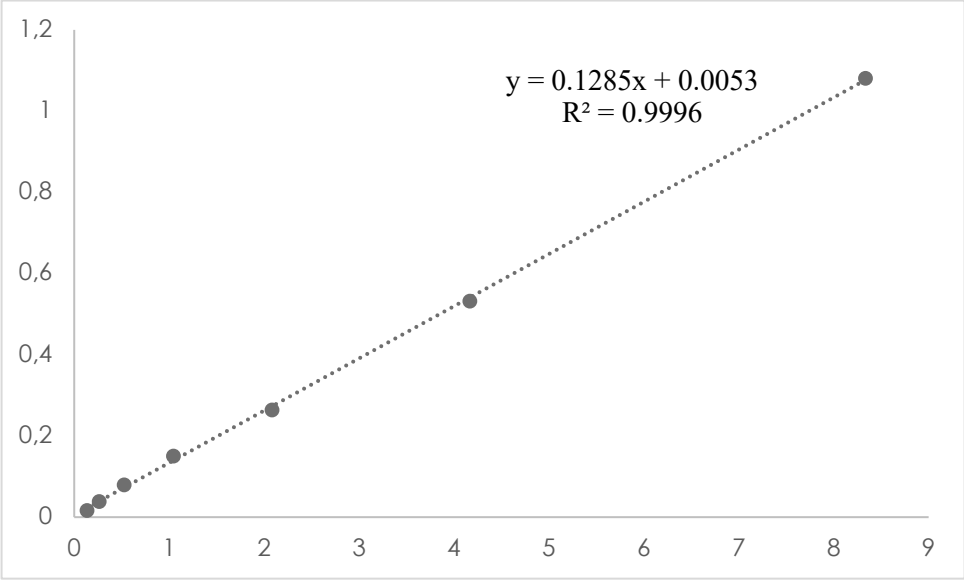


Figure 15. Trolox calibration curve (8-125 µg/mL) used in FRAP assay.

Figure 16. The color change occurred as a result of FRAP assay. A → B: Antioxidant activity from lowest to highest.

4.2.2.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

Calibration curve of trolox which used as a standard for CUPRAC assay is given in Figure 17. According to the results of CUPRAC assay, EtOAc subextract of *M. campanuloides* aerial parts showed statistically significant antioxidant activity (583.46 ± 7.91 mg TE/g). Respectively, EtOAc subextract of *M. campanuloides* roots and *n*-BuOH subextract of aerial parts were statistically different but still showed high antioxidant activity compared to other extracts (341.51 ± 2.11 and 210.16 ± 6.05 mg TE/g, respectively). Although, H_2O subextract of *M. campanuloides* roots showed the lowest antioxidant activity (5.02 ± 0.54 mg TE/g). Yellow colored reaction occured by CUPRAC assay was shown in Figure 18. All details for CUPRAC assay shown in Table 7.

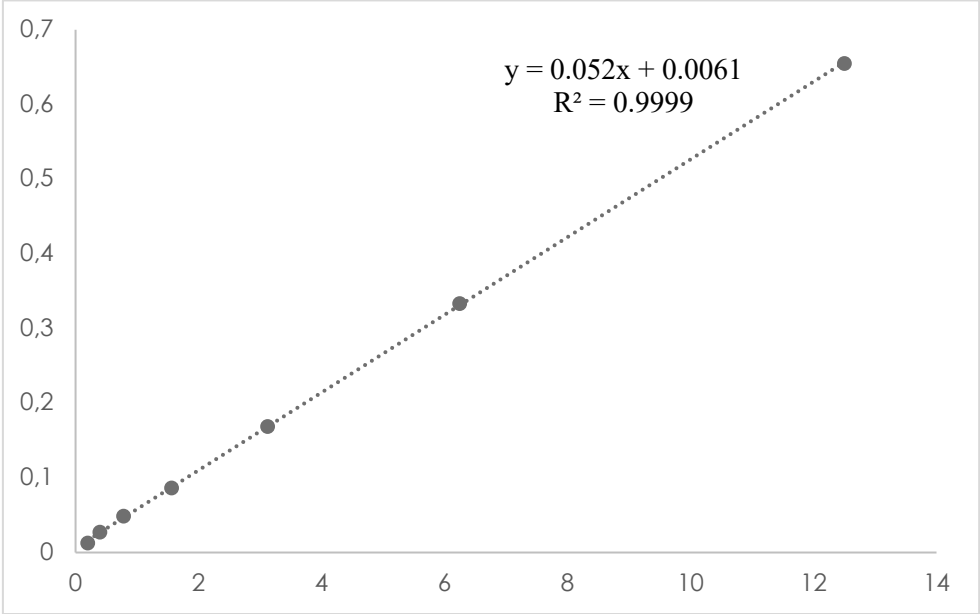


Figure 17. Trolox calibration curve (8-125 µg/mL) used in CUPRAC assay.

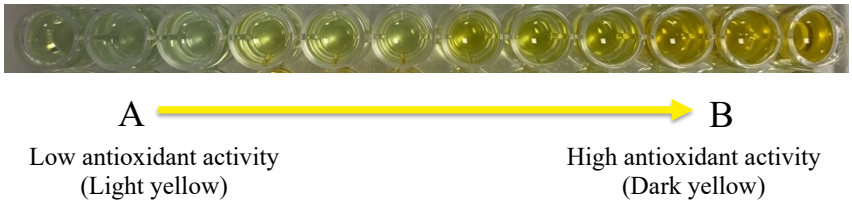


Figure 18. The color change occurred as a result of CUPRAC assay. A → B: Antioxidant activity from lowest to highest.

Detailed results regarding antioxidant assays are given in Table 7.

Table 7. Total antioxidant contents of *M. campanuloides* roots and aerial parts.

	Extracts	ABTS	DPPH	FRAP	CUPRAC
		mg TE/g			
Roots	**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}
	H_2O	16.673±0.459^{d}	3.52±0.875^{f}	1.852±0.226^{f}	5.017±0.544^{f}
Aerial parts	**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}
	H_2O	45.596±0.660^{c}	27.224±1.403^{d}	15.352±1.348^{def}	55.237±1.788^{d}

mg TE/g: mg trolox equivalent per g extract. Different letters in the same column indicate significantly different values at $p \leq 0.05$

4.3. Phytochemical Studies

4.3.1. Total Phenolic Content Assay

Calibration curve of gallic acid which used as a standard for total phenolic content assay is given in Figure 19. Total phenolic content of MeOH extracts and subextracts of roots and aerial parts were stated in Table 8. Statistically the highest total phenolic content belonged to EtOAc subextract of *M. campanuloides* aerial parts (204.32 ± 27.29 mg

GAE/g) followed by EtOAc subextract of roots (144.94 ± 1.2 mg GAE/g) and *n*-BuOH subextract of aerial parts (129.73 ± 4.58 mg GAE/g). The lowest total phenolic content was belonged to H_2O subextract of *M. campanuloides* roots (5.56 ± 0.32 mg GAE/g). The color change occured in 96-well plate after the reaction shown in Figure 20. All detailed results for total phenolic content assay shown in Table 8.

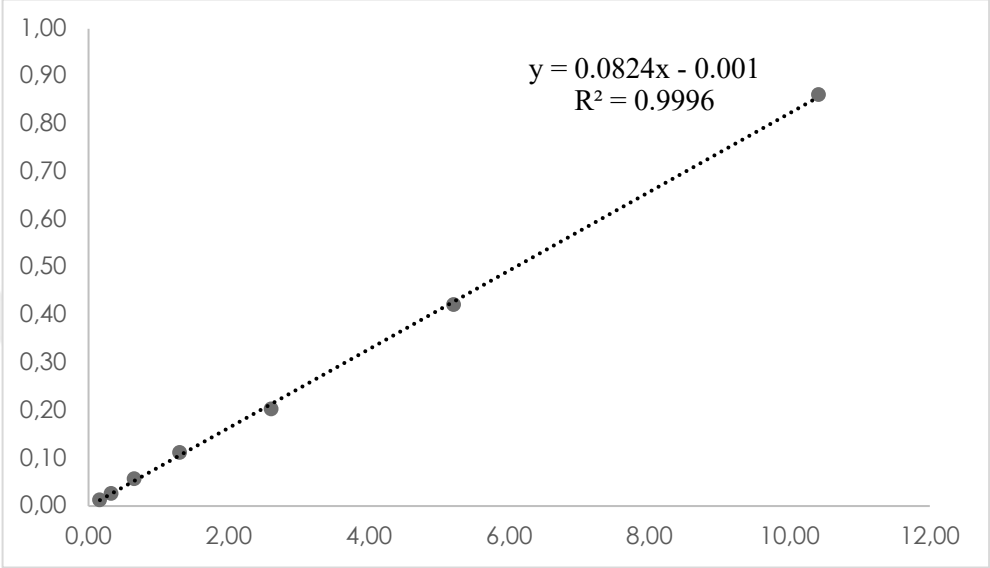


Figure 19. Calibration curve of gallic acid.

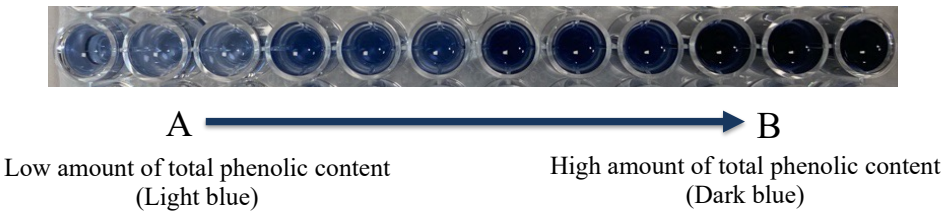


Figure 20. The color change occurred by total phenolic content assay. A → B: TPC content from lowest to highest.

4.3.2. Total Flavonoid Content Assay

Calibration curve of quercetin which used as a standard for total flavonoid content assay is given in Figure 21. Total flavonoid content of MeOH extracts and subextracts of roots and aerial parts were stated in Table 8. *n*-BuOH subextract of *M. campanuloides* aerial parts had the highest quantity of total flavonoid content (29.27 ± 0.9 mg QE/g) and

statistically, it had similar total flavonoid content with EtOAc subextract of *M. campanuloides* aerial parts (28.46 ± 1.18 mg QE/g). Similar to other results, lowest total flavonoid content was belonged to H_2O subextract of *M. campanuloides* roots (0.04 ± 0.009 mg GAE/g) among all extracts. Yellow-colored test results have shown in Figure 22. All detailed results for total flavonoid content assay shown in Table 8.

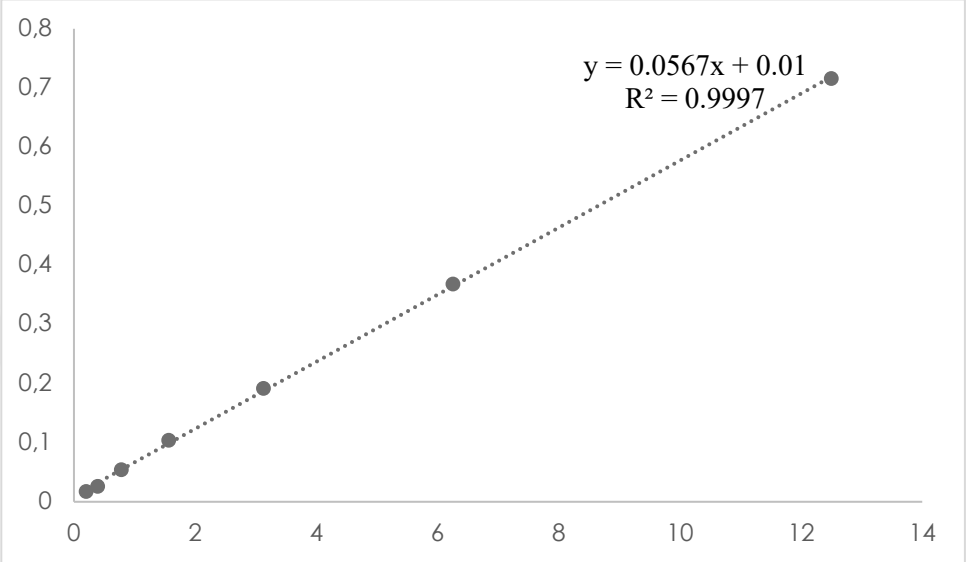


Figure 21. Calibration curve of quercetin.

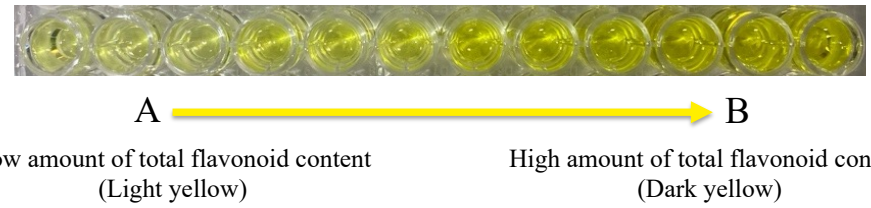


Figure 22. The yellow color resulting from the reaction of flavonoids present in the medium with aluminum chloride. A → B: TFC content from lowest to highest.

Table 8. Total phenolic and flavonoid contents of *M. campanuloides* roots and aerial parts.

	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}

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 $p \leq 0.05$.

4.4. Chromatographic Analysis

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

MeOH extracts and subextracts of roots and aerial parts of *M. campanuloides* were analyzed by HPTLC for chemical fingerprinting of the extracts. The results were shown in Figure 23, 24 and 25 as HPTLC chromatograms.

R_F values for standards determined on the HPTLC chromatogram was: rutin ($R_F \approx$ 0.37, orange band color), chlorogenic acid ($R_F \approx 0.48$, blue band color), neochlorogenic acid ($R_F \approx 0.53$, blue band color), isoquercitrin ($R_F \approx 0.6$, orange band color), astragalin ($R_F \approx 0.67$, green band color), caffeic acid ($R_F \approx 0.91$, blue band color) and quercetin (R_F ≈ 0.95, orange band color).

a. HPTLC plate was derivatized using NP/PEG and followed by measurement of the R_F values and band colors of the standards at 366 nm. According to HPTLC plate derivatized with NP/PEG (Figure 23), all subextracts of aerial parts of *M. campanuloides* were determined to contain rutin ($R_F \approx 0.37$), chlorogenic acid (R_F

≈ 0.48), neochlorogenic acid ($R_F \approx 0.52$, above chlorogenic acid) and isoquercitrin ($R_F \approx 0.6$,). EtOAc and *n*-BuOH subextracts of aerial parts of *M. campanuloides* seemed to have a rich phytochemical profile compared to other subextracts. Among subextracts of the roots; MeOH, EtOAc and *n*-BuOH subextracts were determined to have chlorogenic acid ($R_F \approx 0.47$,). Additionally, EtOAc and *n*-BuOH subextracts of roots had unidentified compound ($R_F \approx 0.85$, blue band color). EtOAc subextracts of both root and aerial parts of the plant determined to have unknown main compound ($R_F \approx 8.3$, blue band color). Among all subextracts, *n*-Hexane and H_2O subextracts of roots were found to have non of the standard compounds.

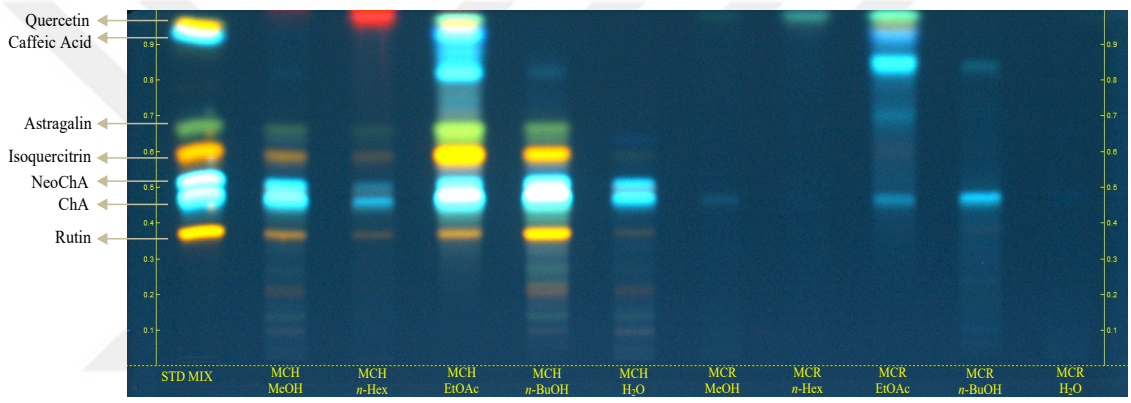


Figure 23. HPTLC chromatogram of root and aerial parts of *M. campanuloides* at 366 nm. Developing solvent system: EtOAc-AcOH-FA-H_2O (100:11:11:26, *v/v/v/v*), ChA: Chlorogenic acid, MCH: *M. campanuloides* aerial parts, MCR: *M. campanuloides* roots, NeoChA: Neochlorogenic acid, STD MIX: Standart mixture.

b. Figure 24 stated the HPTLC chromatogram of the samples. For this method, derivatization was applied using anisaldehyde and the R_F values and band color of the standards were measured at white light. The HPTLC chromatogram also showed that *n*-hexane and EtOAc subextracts of roots were determined to have unidentified compounds ($R_F \approx 0.84$, purple band color). Additionally, all subextracts had a common compound ($R_F \approx 0.52$, navy blue band color). HPTLC chromatogram of the samples derivatized by anisaldehyde and visualized at 366 nm as shown in Figure 25.

Due to the absence of the investigated compounds in the active extract (EtOAc and n-hexane subextracts of the roots) in cytotoxicity study, it was hypothesized

that different compounds were present, leading to the need to investigate various reagents. Anisaldehyde reagent is generally investigated for monoterpenes, triterpenes and steroids. Based on this result, it was demonstrated that different compounds were present, indicating the necessity for further research.

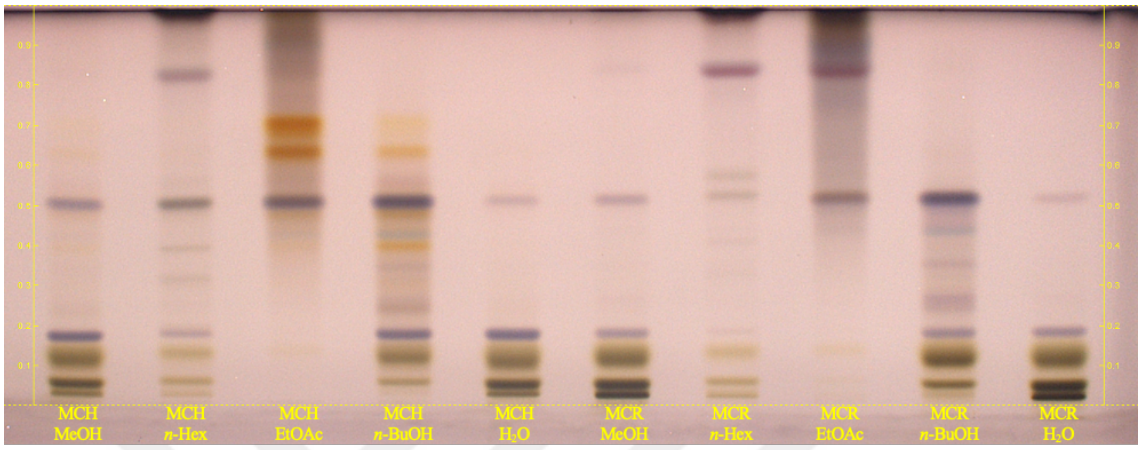


Figure 24. HPTLC chromatogram of root and aerial parts of *M. campanuloides* at white light. Developing solvent system: EtOAc-AcOH-FA-H_2O (100:11:11:26, *v/v/v/v*), MCH: *M. campanuloides* aerial parts, MCR: *M. campanuloides* roots.

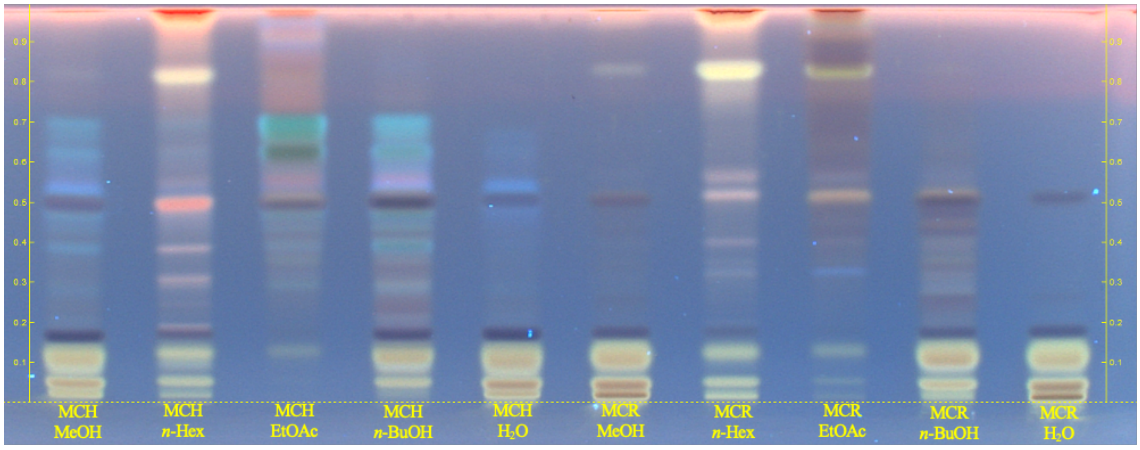


Figure 25. HPTLC chromatogram of root and aerial parts of *M. campanuloides* at 366 nm. Developing solvent system: EtOAc-AcOH-FA-H_2O (100:11:11:26, *v/v/v/v*), MCH: *M. campanuloides* aerial parts, MCR: *M. campanuloides* roots.

4.4.2. Ultra-Performance Liquid Chromatography (UPLC)

To examine the quantity of certain standard compounds in root and aerial parts of *M. campanuloides* plant, UPLC method was used. Neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin and quercetin were used as standards. Neochlorogenic acid and chlorogenic acid were detected in 325 nm, caffeic acid was detected in 323 nm, rutin and isoquercitrin was detected in 256 nm, astragalin was detected in 264 nm and quercetin was in 371 nm to reach appropriate results based on absorption graphs of the standards (Figure 26).

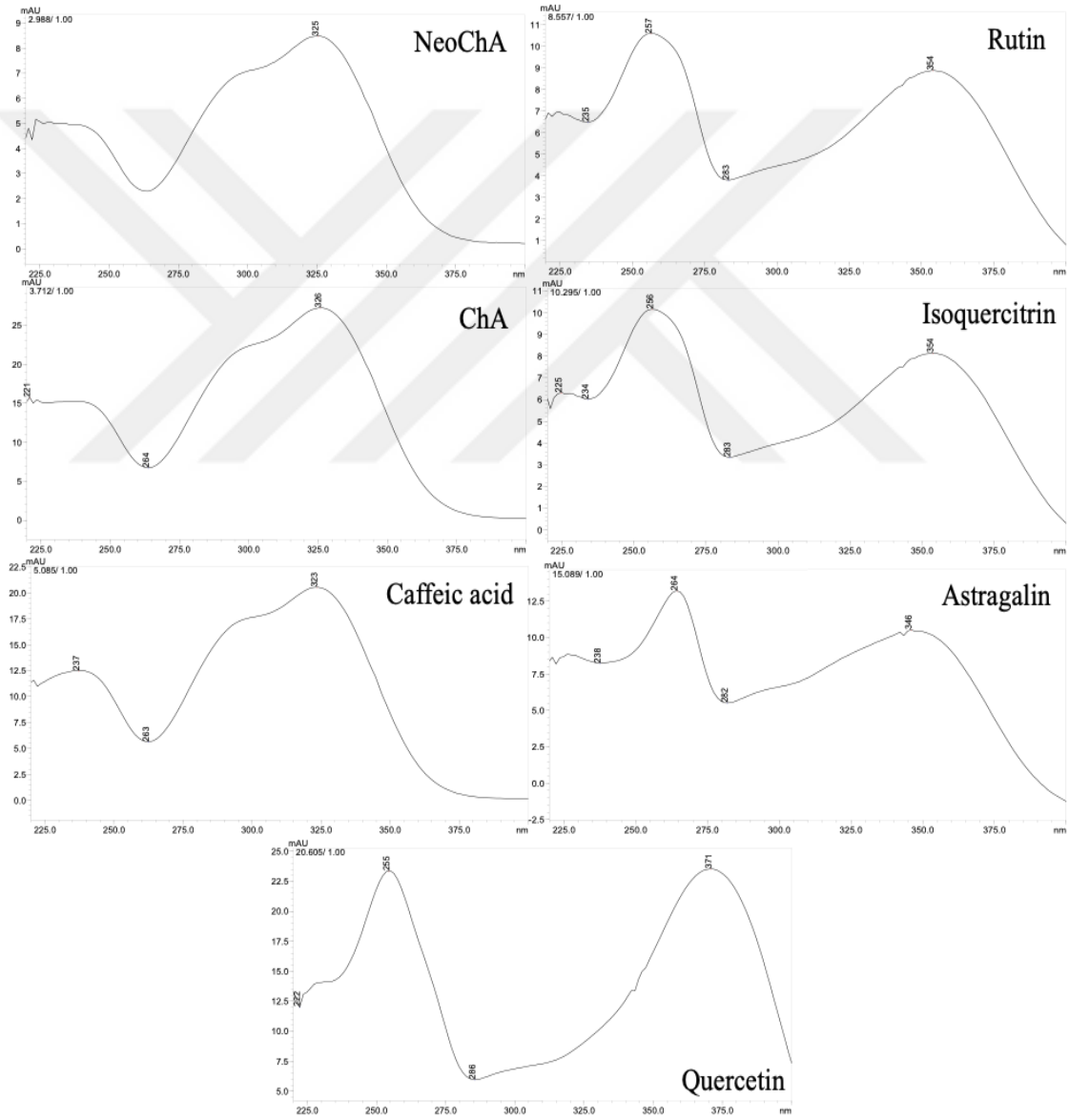


Figure 26. UV spectra of the reference compounds (200-400 nm).

The standard compounds; neochlorogenic acid (t_R: 2.935), chlorogenic acid (t_R: 3.719), caffeic acid (t_R: 5.037), rutin (t_R: 8.546), isoquercitrin (t_R: 10.292), astragalin (t_R: 15.089), and quercetin (t_R: 20.582) were not detected in the blank chromatogram, demonstrating the specificity of the developed method.

The linearity data, the limit of quantification (LOQ) calculated as 10 x (SD/S) and limit of detection (LOD) values calculated by 3 x (SD/S), are given in Table 9.

Table 9. 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**
Neochlorogenic acid	0.5-50	1	11924	10.868	0.025	0.084
Chlorogenic acid	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

The calibration equation was ‘y=a+bx’

The precision of the developed UPLC method was assessed to determine the repeatability and reliability of the analytical results. Precision was evaluated through intraday (within-day) and interday (between-day) measurements of the standard solution (neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin) at the concentration of 5 µg/mL. The within-day precision was determined by conducting the analysis three times in a single day, while the between-day precision was measured by performing the analysis on three consecutive days. The results have given in Table 10.

Table 10. Repeatability and precision data for the standard compounds.

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
Neochlorogenic acid	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				
Chlorogenic acid	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 accuracy (recovery) of the developed method has been done with the concentration of 4, 8, 12 µg/mL of standard solutions (neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin, and quercetin). The found values of these concentrations using the obtained calibration curve were compared with the theoretical values for the evaluation of the method accuracy. The results have shown in Table 10.

Table 11. Comparative results of theoretical values and obtained results (%) by UPLC method.

Standards	Theoretical value (µg/mL)	Amount found (µg/mL ± SD, *n*=3)	Recovery (%)
Neochlorogenic acid	4	4.19 ± 0.02	104.73
	8	8.38 ± 0.11	104.78
	16	16.95 ± 0.17	105.95
Chlorogenic acid	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

According to UPLC results shown in Table 12, all samples belonging *M. campanuloides* aerial parts contained neochlorogenic acid. *n*-BuOH subextract had the highest quantity of neochlorogenic acid (6.67 ± 0.16 mg/g), followed by MeOH extract (3.18 ± 0.01 mg/g), H_2O (2.85 ± 0.03 mg/g) and EtOAc subextracts (2.68 ± 0.04 mg/g), respectively. The lowest amount of neochlorogenic acid was found in *n*-Hexane subextract (0.73 ± 0.0 mg/g). Despite of that, nechlorogenic acid was not present in any samples for roots of the plant. Chlorogenic acid was found in all samples except *n*-Hexane subextract of the roots. Chlorogenic acid was detected predominantly in EtOAc subextract (38.21 ± 0.03 mg/g) and followed by *n*-BuOH subextract (37.23 ± 0.21 mg/g) of aerial parts. MeOH extract (11.45 ± 0.11 mg/g) and H_2O subextracts (7.32 ± 0.03 mg/g) of aerial parts found to had relatively higher amounts of chlorogenic acid compared to other samples. The lowest amount of chlorogenic acid was detected in H_2O subextract of roots (0.49 ± 0.01 mg/g). Caffeic acid was found only in MeOH and EtOAc subextracts of aerial parts and EtOAc extract of roots. The highest amount of caffeic acid was detected in EtOAc subextract of aerial part (6.67 ± 0.09 mg/g), the lowest amount was found in

MeOH extract of aerial parts (0.22 ± 0.02 mg/g). Rutin was found only in samples of aerial parts of the plant. Predominantly, *n*-BuOH subextract (15.28 ± 0.04 mg/g) found to contain rutin followed by EtOAc subextract (5.51 ± 0.06 mg/g). Among samples of aerial parts, rutin was detected at the lowest concentration in *n*-Hexane subextract (0.56 ± 0.02 mg/g). Isoquercitrin was also detected only in samples of aerial parts. Predominantly, isoquercitrin was detected in EtOAc subextract (51.22 ± 0.81 mg/g). *n*-BuOH subextract (16.32 ± 0.09 mg/g) and MeOH extract (3.28 ± 0.03 mg/g) had lower amounts of isoquercitrin, respectively. *n*-Hexane subextract were found to had lowest amount of isoquercitrin (0.87 ± 0.03 mg/g), H_2O subextract had also low amount of isoquercitrin (0.89 ± 0.02 mg/g). Astragalin was present in all extracts of aerial parts of the plant. The amount of astragalin present in EtOAc subextract was significantly higher compared to other subextracts (93.37 ± 0.11 mg/g). The lowest amount of astragalin was found in *n*-hexane subextract (1.11 ± 0.02 mg/g). Quercetin was only found in EtOAc subextract of *M. campanuloides* aerial parts (5.79 ± 0.04 mg/g). None of the other subextracts did not find to have quercetin.

It was suprising to detect some phenolic acids and flavonoids (neochlorogenic acid, chlorogenic acid, rutin, isoquercitrin, astragalin) as trace amounts in the *n*-hexane subextract of the aerial parts, which are not expected to pass *n*-hexane subextract. It could be due to the phase separation problem during partition process, between H_2O and *n*-hexane.

Table 12. Quantitative UPLC results of the standards and *M. campanuloides* roots and aerial parts extracts.

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

Results were given as mg/g extract, ND: not detected, 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 $p \leq 0.05$.

Figures 27-37 show the UPLC-PDA chromatograms of both the standard mixture and the samples, which include the retention time (t_R) values of the standards.

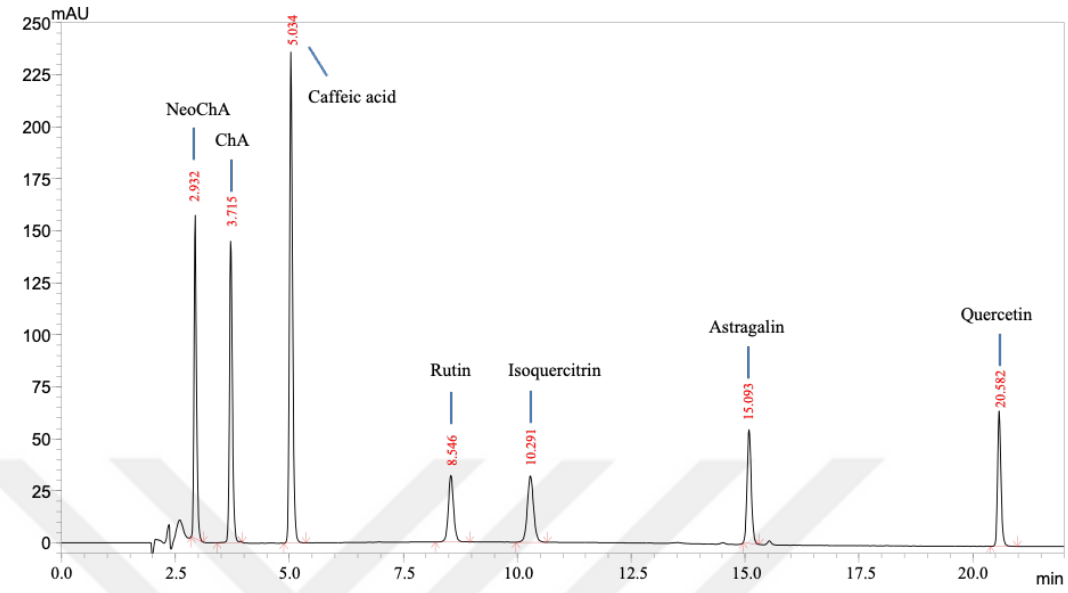


Figure 27. UPLC-PDA chromatogram of standards at 256 nm.

NeoChA: neochlorogenic acid, ChA: chlorogenic acid. t_R values; NeoChA: 2.935, ChA: 3.719, caffeic acid: 5.037, rutin: 8.546, isoquercitrin: 10.292, astragalin: 15.089, quercetin: 20.582

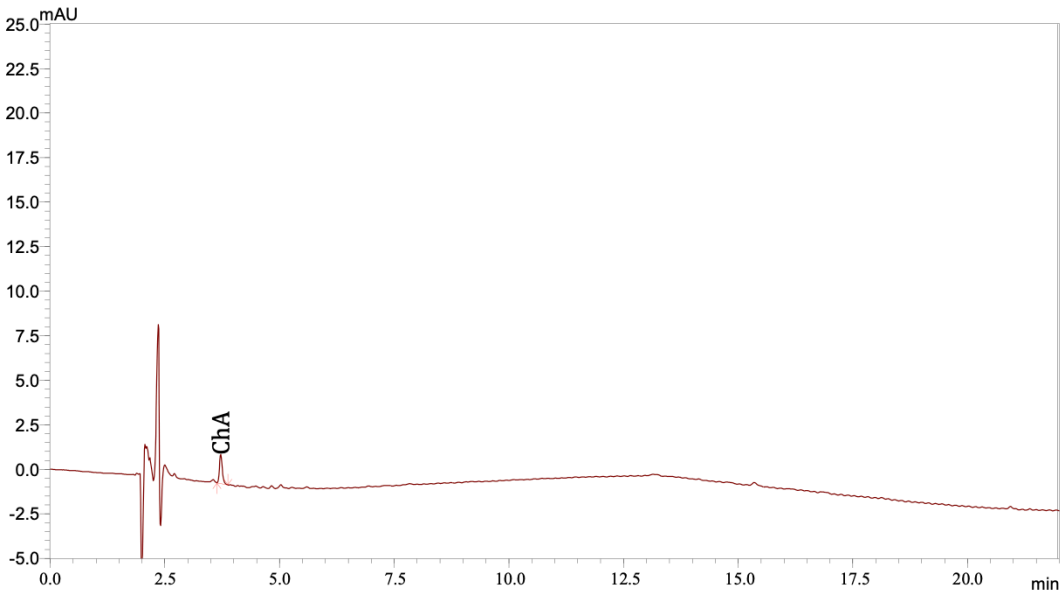


Figure 28. UPLC-PDA chromatogram of *M. campanuloides* root MeOH extract at 325 nm.

ChA: chlorogenic acid, t_R: 3.720

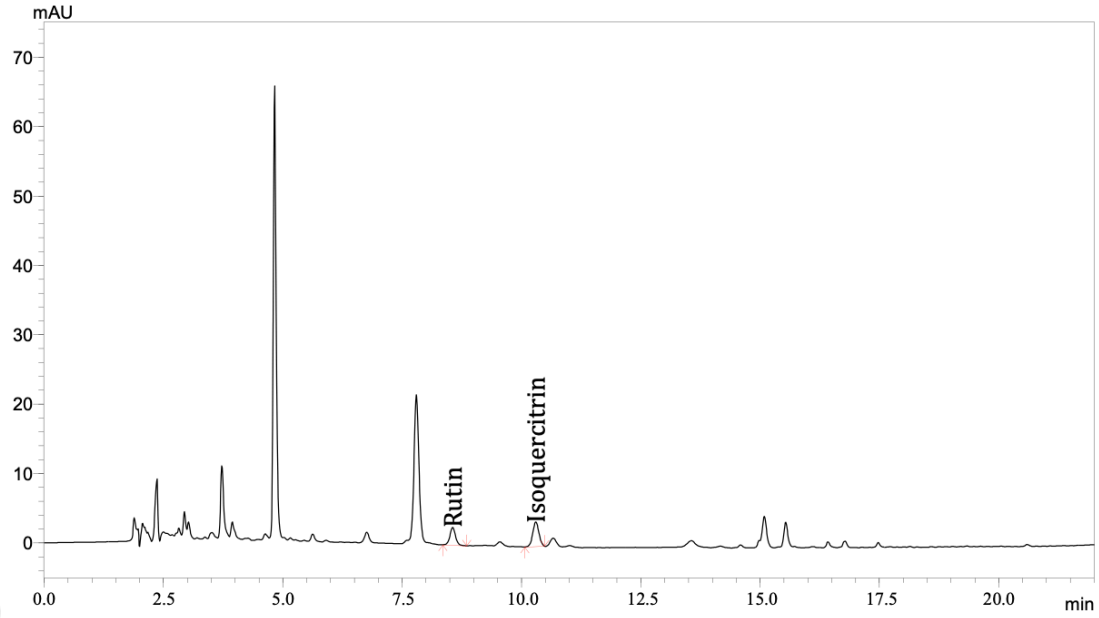


Figure 29. UPLC-PDA chromatogram of *M. campanuloides* aerial parts MeOH extract at 256 nm.

Rutin t_R: 8.560, Isoquercitrin t_R: 10.302

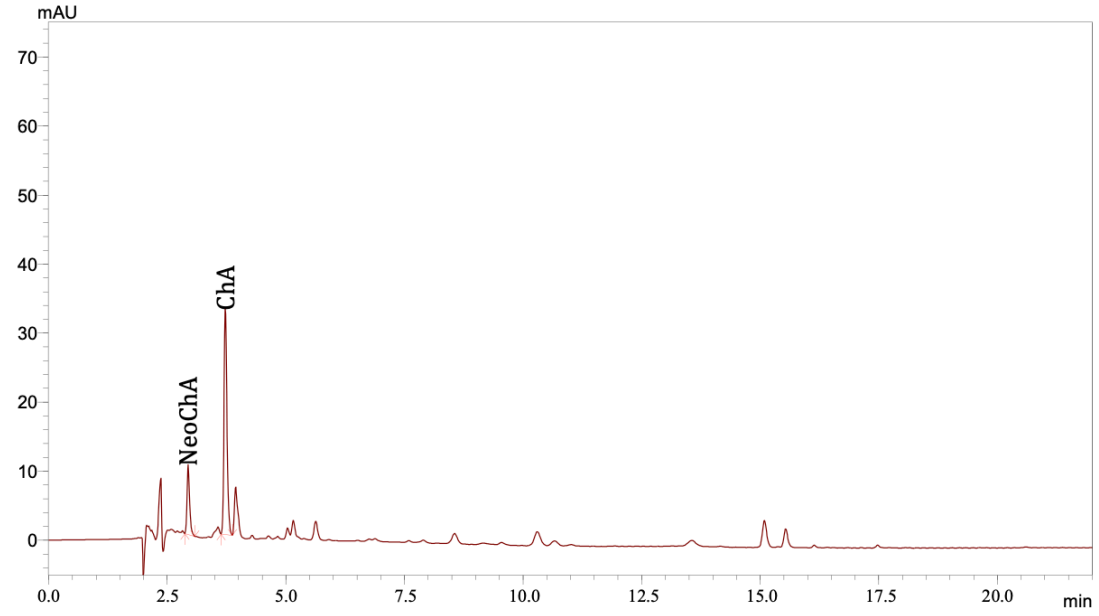


Figure 30. UPLC-PDA chromatogram of *M. campanuloides* aerial parts MeOH extract at 325 nm.

Neochlorogenic acid t_R: 2.942, Chlorogenic acid t_R: 3.727

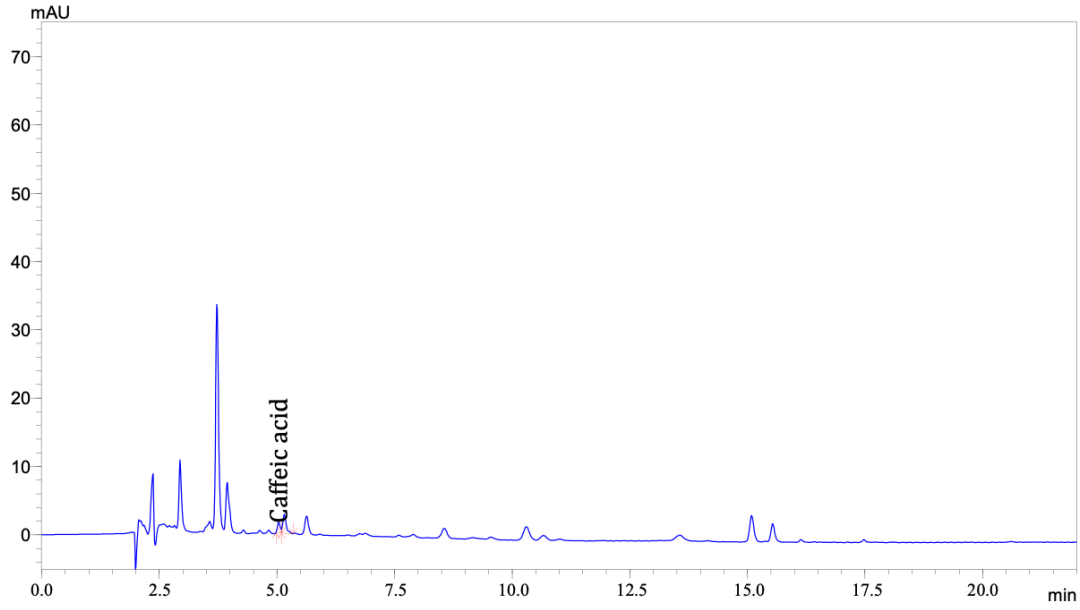


Figure 31. UPLC-PDA chromatogram of *M. campanuloides* aerial parts MeOH extract at 323 nm.

Caffeic acid t_R: 5.038

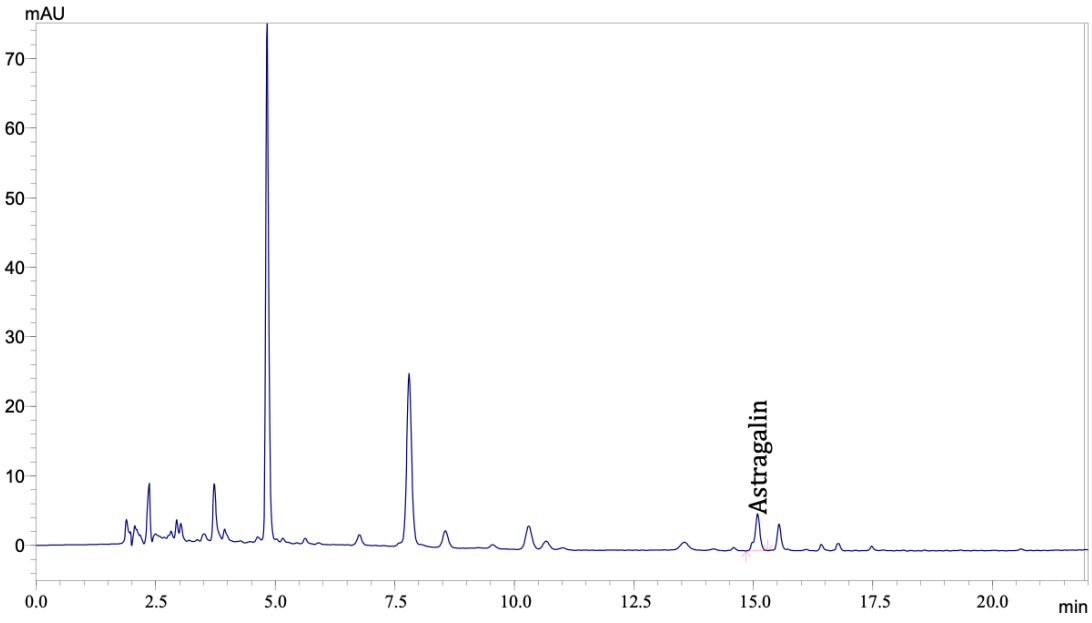


Figure 32. UPLC-PDA chromatogram of *M. campanuloides* aerial parts MeOH extract at 264 nm.

Astragalin t_R: 15.088

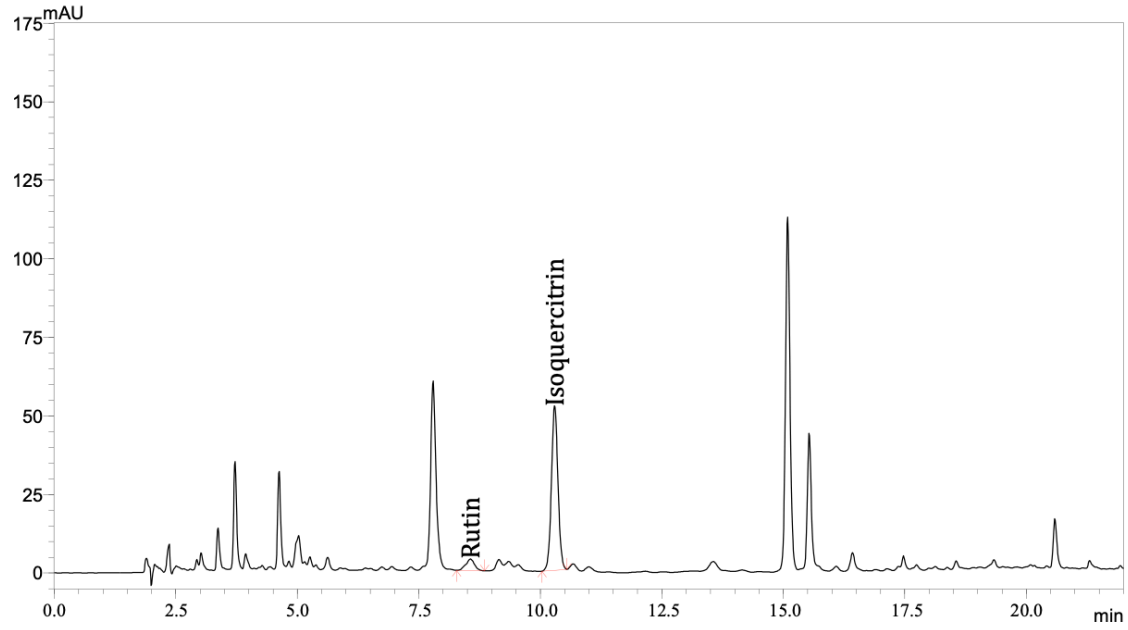


Figure 33. UPLC-PDA chromatogram of *M. campanuloides* aerial parts EtOAc subextract at 256 nm.

Rutin t_R: 5.563, Isoquercitrin t_R: 10.283

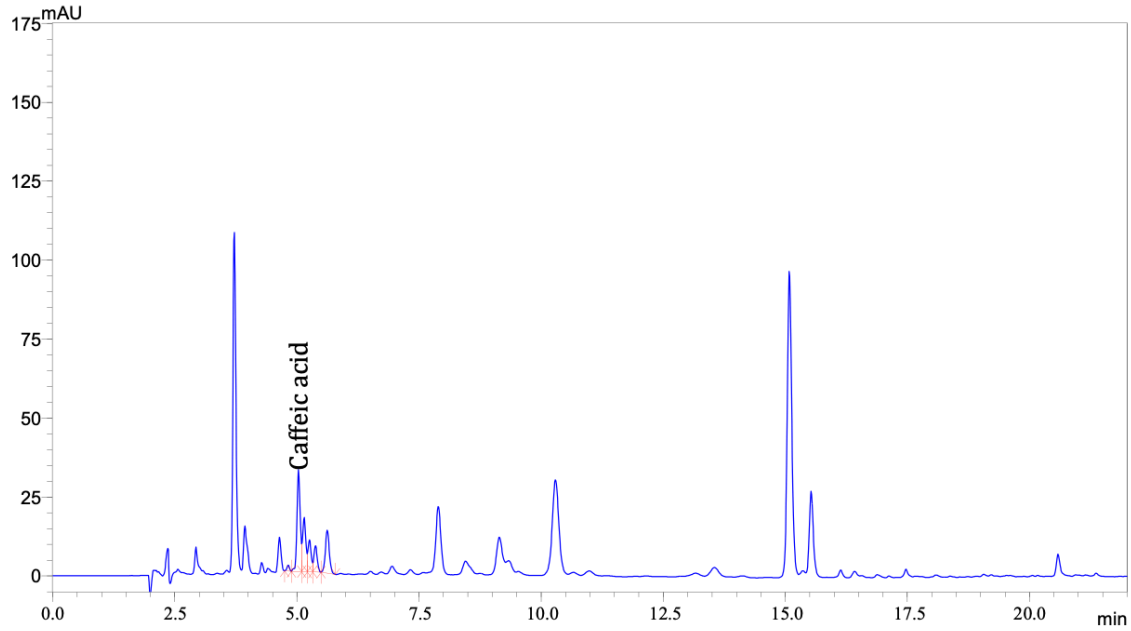


Figure 34. UPLC-PDA chromatogram of *M. campanuloides* aerial parts EtOAc subextract at 323 nm.

Caffeic acid t_R: 5.034

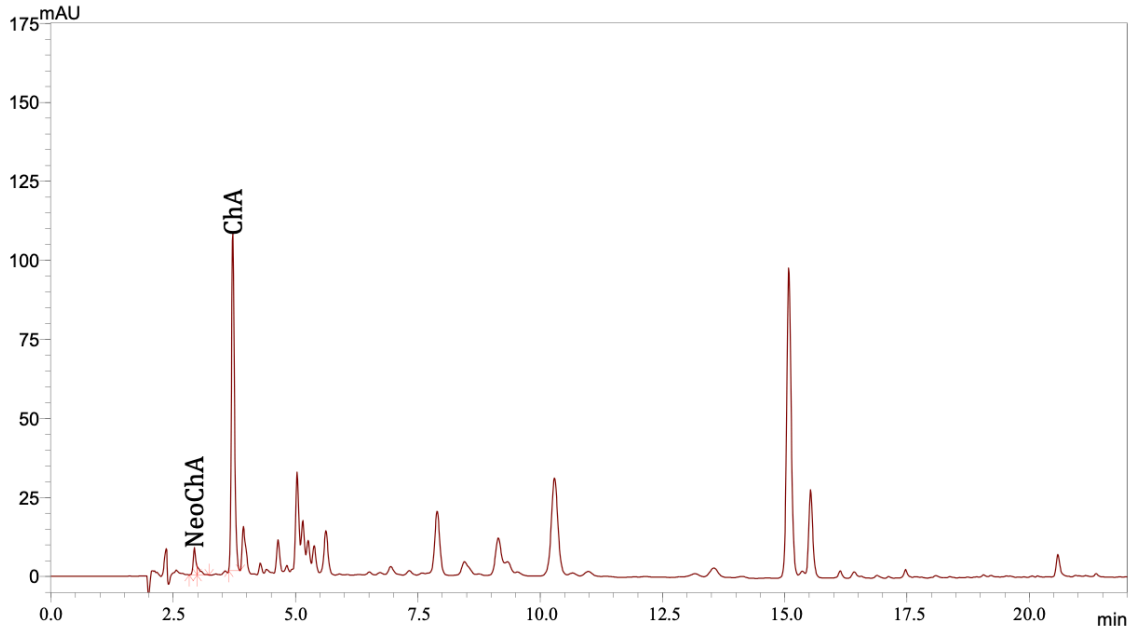


Figure 35. UPLC-PDA chromatogram of *M. campanuloides* aerial parts EtOAc subextract at 325 nm.

Neochlorogenic acid t_R: 2.935, Chlorogenic acid t_R: 3.720

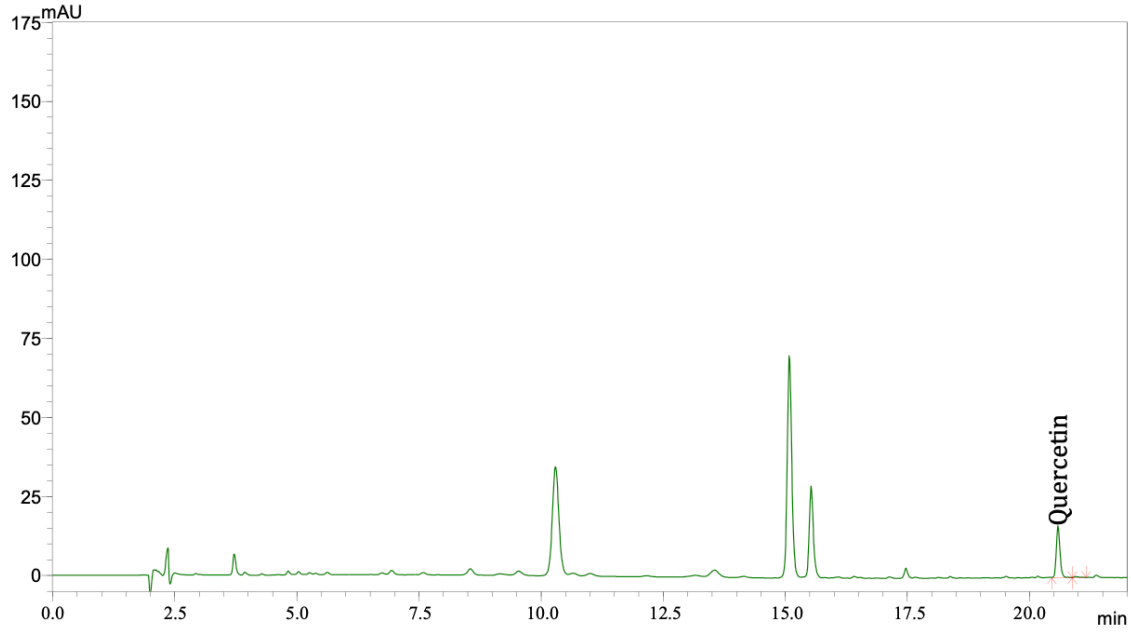


Figure 36. UPLC-PDA chromatogram of *M. campanuloides* aerial parts EtOAc subextract at 371 nm.

Quercetin t_R: 20.585

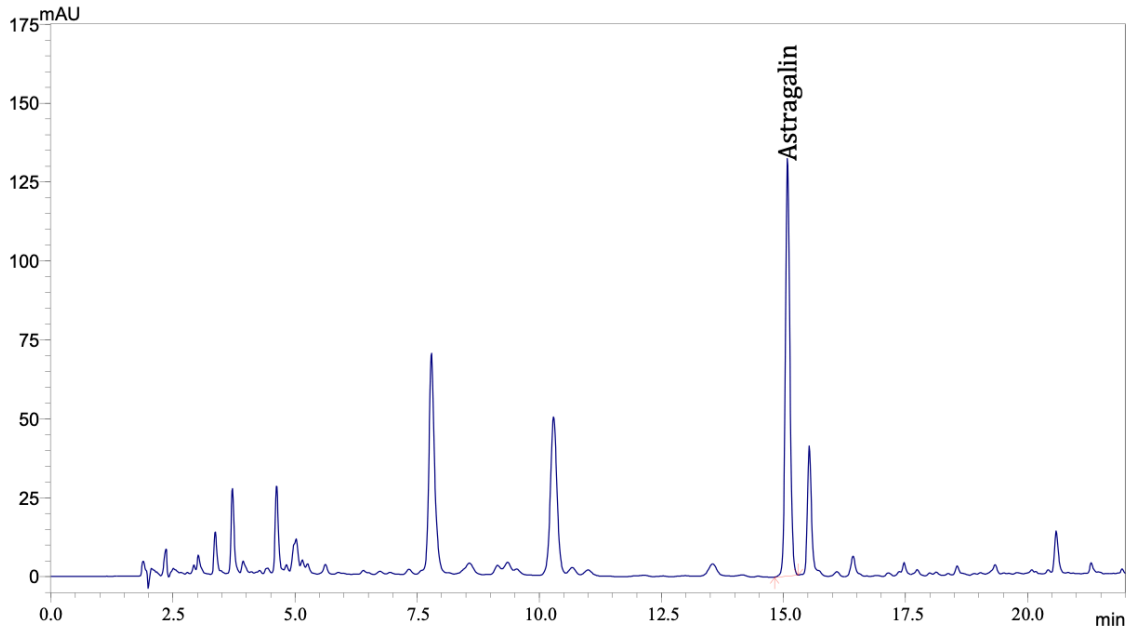


Figure 37. UPLC-PDA chromatogram of *M. campanuloides* aerial parts EtOAc subextract at 325 nm.

Astragalin t_R: 15.084

5. DISCUSSION and CONCLUSION

Plants are currently a prominent source for the development of many drugs used to treat various diseases. Cancer, the primary cause of death in developed nations, and reactive oxygen species, implicated in a wide range of diseases, are among the most extensively researched subjects in medicine today (10, 53). As an complementary treatment for these indications, plants are one of the most crucial resources in the developing world. Besides the plants already under investigation, examining previously unstudied plants and demonstrating their applicability in necessary fields is of great importance.

M. campanuloides is a traditionally used plant as vegetable and for wound healing externally (6). Regarding this knowledge, there is only one study about wound healing activity of the plant (7). Except for determining total phenolic content (2, 7) and evaluating antioxidant activities (2, 7, 9) with limited methods, *M. campanuloides* has not been investigated in detail before.

In the current study, plant materials (roots and aerial parts) were macerated with MeOH, dispersed in water and partitioned against *n*-hexane, EtOAc, and *n*-BuOH, respectively. The crude MeOH extract and *n*-hexane, EtOAc, *n*-BuOH and H_2O subextracts were seperately investigated to determine their cytotoxicity on six different cancer cell lines (colon (HCT116, SW480), breast (MDA-MB-231, HCC1937) and liver (HEP3B, HEPG2)) as well as L929 healthy cell line by MTS method. Results indicated that only EtOAc subextract prepared from the roots displayed cytotoxicity against all tested cancer cell lines. The highest cytotoxic activity was determined towards breast cancer cell line (HCC1937) with an IC_{50} of 10 µg/mL, followed by fibroblast healthy cell line (L929) with an IC_{50} of 13.71 µg/mL. *n*-hexane subextract of the roots also showed weak cytotoxicity on liver (HEPG2) cell line with an IC_{50} of 144 µg/mL and healthy (L929) cell line with an IC_{50} of 128.92 µg/mL. This is the first study on the cytotoxic potential of *M. campanuloides*. Among the tested extracts, EtOAc subextract of the roots deserve further bioactivity-guided fractionation studies in order to isolate potential cytotoxic compounds.

In the present study, *in vitro* antioxidant activities of MeOH extract, *n*-hexane, EtOAc, *n*-BuOH and H_2O subextracts of *M. campanuloides* roots and aerial parts were investigated by using ABTS, DPPH, FRAP, and CUPRAC methods. Based on the results, EtOAc subextracts of both roots and aerial parts exhibited significantly strong antioxidant

activity compared to other subextracts. Except ABTS assay, EtOAc subextracts of roots resulted to have stronger activity than aerial parts. The results of the ABTS assay for the EtOAc subextracts were as follows: aerial parts, 367.651 mg TE/g; roots, 353.399 mg TE/g. For aerial parts, *n*-hexane subextract (DPPH assay, 16.155 mg TE/g) and for roots, H_2O subextract (DPPH assay, 3.52 mg TE/g) were expressed least antioxidant activity among all extracts. There is no available study that used ABTS, FRAP or CUPRAC assays for evaluating the antioxidant activity of *M. campanuloides* plant.

Güvenç et al. (7) conducted a study on the antioxidant activity of root and aerial part extracts (H_2O, MeOH, DCM, EtOAc, and *n*-BuOH) from five different *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea,* and *M. nuda*) using qualitative and quantitative DPPH tests and TBA tests. According to the quantitative DPPH test results, *M. campanuloides* showed the highest antioxidant activity for roots and aerial parts (IC_{50} value for DCM extract of *M. campanuloides* aerial parts = 14.33 mg/mL). It was also determined through DPPH and TBA tests that the EtOAc extracts of all species were more active. H_2O extract of *M. campanuloides* roots showed the least antioxidant activity with an IC_{50} of 159.84 mg/mL in DPPH assay. Our results were found to be consistent with the previous DPPH assays.

Ebrahimabadi et al. (8) evaluated the antioxidant activity of MeOH extract and essential oil obtained from stems and roots of *Mindium laevigatum* (Syn.: *Michauxia laevigata*) collected from two different places (Shahsavaran valley and Rahagh area). The strongest antioxidant capacity belonged to the *M. laevigatum* collected from Shahsavaran valley with an IC_{50} of 344.3 µg/mL. The difference in IC_{50} values may be caused by climate changes or soil types in different areas that plants were collected.

Additionally, total phenolic content and total flavonoid content of the extracts were investigated in the current study. The highest phenolic content was observed in EtOAc extract of *M. campanuloides* aerial parts (204.320±27.290 mg GAE/g), followed by EtOAc extract of *M. campanuloides* roots (141.94±1.202 mg GAE/g). H_2O subextract of *M. campanuloides* roots found to contain the least amount of phenolic content (5.563±0.321 mg GAE/g).

Güvenç et al. (7) evaluated the total phenolic content of root and aerial part extracts (H_2O, MeOH, DCM, EtOAc, and *n*-BuOH) from five different *Michauxia* species (*M. campanuloides, M. laevigata, M. tchihatchewii, M. thyrsoidea*, and *M. nuda*) using the Folin-Ciocalteu method, as the current study. The results of referred study stated that the EtOAc extract of *M. campanuloides* aerial parts had the highest amount of

phenolic content (439.05 ± 18.70 mg/g), compared to other extracts. According to the results of *M. campanuloides* roots and aerial parts, *n*-BuOH extract of aerial parts was found to contain 210.8 ± 9.42 mg/g total phenolic content.

Total flavonoid contents of the extracts were also determined in this study. The results showed that *n*-BuOH and EtOAc subextract of aerial parts had significantly similar amount of flavonoid content (29.276±0.905 mg QE/g and 28.462±1.183 mg QE/g, respectively). Based on total phenolic and flavonoid content assay results EtOAc subextracts had significantly high amount of total phenol and total flavonoid content (values for EtOAc subextract of aerial parts were 204.32±27.29 mg GA/g and 28.46±1.18 mg QE/g, respectively). EtOAc subextracts were also found to have strongest antioxidant capacity according to ABTS, DPPH, FRAP and CUPRAC assays. In our study, total phenol and flavonoid contents in the extracts were found to be proportional to their antioxidant capacities which was in line with the literature (54). This study represents the total flavonoid content of *M. campanuloides* for the first time.

To identify the major compounds in the prepared extracts, HPTLC and UPLC methods were used. Due to the absence of prior studies on this topic, a comparative analysis of the results was not possible. In this current study, neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, astragalin and quercetin were used as standards for both chromatographic methods. All extracts were determined to have chlorogenic acid in different concentrations. As an overall result, extracts of roots determined not to have any neochlorogenic acid, rutin, isoquercitrin, astragalin and quercetin. The EtOAc extract of *M. campanuloides* aerial parts was the only extract detected to contain all used standards (neochlorogenic acid, 2.68±0.04 mg/g; chlorogenic acid, 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; quercetin, 5.79±0.04 mg/g extract).

In conclusion, cytotoxic activities of *M. campanuloides* roots and aerial parts extracts were evaluated. It is known that *M. campanuloides* is effective in wound healing (7); however, the cytotoxicity potential of the extract was not studied before. Antioxidant activities of the extracts were evaluated by using four different methods. Additionally, total phenolic content and total flavonoid content of the extracts were investigated to make a correlation between phenolic contents and antioxidant activities of the extracts. HPTLC and UPLC methods were used to identify the major compounds present in the extracts. All results were found to be statistically significant. Although some major

compounds and antioxidant activities were detected in EtOAc subextract of aerial parts of the plant; the effective extract against all studied cancer cell lines was determined as EtOAc subextract of roots. In order to discover the active compounds in cytotoxic subextract, further chromatographic and spectroscopic methods are needed. There is no available study regarding detailed phytochemistry of *M. campanuloides* plant. The current study analyzed the total flavonoid content *of M. campanuloides*, evaluated its cytotoxic activity against six different cancer cell lines, and used HPTLC and UPLC methods for the first time.

6. REFERENCES

1. Al-Zein MS, Musselman LJ. *Michauxia* (Campanulaceae): A Western Asian genus honoring a North American pioneer botanist. Castanea. 2004; 69(sp2): 200-205.
2. Hürkul MM. Türkiye'de Yetişen *Michauxia* L'Herit. (Campanulaceae) Türleri Üzerinde Farmasötik Botanik Yönünden Araştırmalar. Ankara, Ankara University, 2011.
3. Ertekin AS, Aksoy A, Duran A, Yaprak A, Karavelioğulları AF, Kahraman A, ed. *A Checklist of the Flora of Turkey (Vascular Plants)*. 1st ed. İstanbul; 2012.
4. Özdemir E, Alpinar K. The Wild Edible Plants of Western Nigde Aladaglar Mountains (Central Turkey). J Fac Pharm Istanbul. 2010; 41: 66-74.
5. Baytop T. *Türkçe Bitki Adları Sözlüğü*. Ankara: Türk Dil Kurumu Yayınları; 1994.
6. Demirci Kayıran S, Ozhatay N. Wild Plants Used For Medicinal Purpose in Andırın. *Turk J Pharm Sci*. 2012; 9(1): 75-92.
7. Güvenç A, Küpeli Akkol E, Hürkul MM, Süntar I, Keleş H. Wound healing and anti-inflammatory activities of the *Michauxia* L'Hérit (Campanulaceae) species native to Turkey. *J Ethnopharmacol*. 2012; 139(2): 401-408.
8. Ebrahimabadi AH, Movahedpour MM, Batooli H, Ebrahimabadi EH, Mazoochi A, Mobarak Qamsari M. Volatile compounds analysis and antioxidant, antimicrobial and cytotoxic activities of *Mindium laevigatum*. *Iran J Basic Med Sci*. 2016; 19(12): 1337-1344.
9. Koca RH, Hurkul MM, Kurt S, Koroglu A. The effect of *Michauxia campanuloides* L'Her. on Some Semen Parameters in Wistar Albino Rats. *Ataturk Univ Vet Bilim Derg.* 2020; 15(3): 138-144.
10. Gulcin İ. Antioxidants and antioxidant methods: an updated overview. *Arch Toxicol*, 2006. 94(3): p. 651-715.
11. Crowl AA, Miles NW, Visger CJ, et al. A global perspective on Campanulaceae: Biogeographic, genomic, and floral evolution. Am J Bot. 2016; 103(2): 233-245.
12. Pires de Godoy SA. Neotropical Campanulaceae. Neotropikey — Interactive Key and Information Resources for Flowering Plants of the Neotropics. Plants of the World Online. 2009; Accessed date (23.09.2023), www.kew.org/science/tropamerica/neotropikey/families/Campanulaceae.htm.
13. Davis PH. *Flora of Turkey and East Agean Islands*. Edinburg: Edinburg University Press; 1975.

14. Mart S. Bahçe ve Hasanbeyli (Osmaniye) Halkının Kullandığı Doğal Bitkilerin Etnobotanik Yönden Araştırılması. Adana, Çukurova University, 2006.
15. Kurt S, Koca RH, Hürkul MM, Seker U, Köroğlu A. The antioxidant effect of *Michauxia campanuloides* on rat ovaries. J Hellenic Vet Med Soc. 2021; 72(3): 3163-3170.
16. Jafarian V, Taalloli A, Vafadar M. New Molecular and Biochemical Records for Mindium Laevigata at Its Various Developmental Stages. Journal of Plant Process and Function. 2017; 5(18).
17. Masoum S, Ghasemi-Estarki H, Seifi H, Ebrahimabadi EH, Parastar H. Analysis of the volatile chemical constituents in *Mindium laevigatum* by gas chromatography - Mass spectrometry and correlative chemometric resolution methods. Microchemical Journal. 2013; 106: 276-281.
18. Aslantürk ÖS. *In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages and Disadvantages,* in Genotoxicity - A Predictible Risk to Our Actual World, Larramendy ML, Soloneski S, eds. London: British Library Cataloguing-in-Publication Data. 2018. p. 1-3.
19. Barltrop JA, Owen TC, Cory AH, Cory JG. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. Bioorg Med Chem Lett. 1991; 1(11): 611-614.
20. Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an Aqueous Soluble Tetrazolium/Formazan Assay for Cell Growth Assays in Culture. Cancer Commun. 1991; 3(7): 207-212.
21. Longosorbello G, Saydam G, Banerjee D, Bertino J. *Cytotoxicity and Cell Growth Assays*, in Cell Biology, Celis JE, ed. Elsevier. 2006. p. 315-324.
22. Kamiloglu S, Sari G, Ozdal T, Capanoglu E. Guidelines for cell viability assays. Food Front. 2020;1(3): 332-349.
23. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radic Biol Med. 1999; 26: 1231-1237.
24. Pietta P, Simonetti P, Mauri P. Antioxidant Activity of Selected Medicinal Plants. *J* Agric Food Chem. 1998; 46(11): 4487-4490.

25. Hernandez-Rodriguez, P.,Baquero, L. P., Larrota, H. R. *Flavonoids: Potential Therapeutic Agents by Their Antioxidant Capacity*, in: Bioactive Compounds, Woodhead Publishing. 2019. p. 265-288
26. Blois MS. Antioxidant Determinations by the Use of a Stable Free Radical. Nature. 1958; 181(4617): 1199-1200.
27. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol. 2011; 48(4): 412-422.
28. Prior RL, Wu X, Schaich K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. J Agric Food Chem. 2005; 53(10): 4290-4302.
29. Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 1999; 299: 15-27.
30. Benzie IFF, Strain JJ. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal Biochem. 1996; 239(1): 70-76.
31. Then M, Szentmihályi K, Sárközi Á, Varga IS. Examination on Antioxidant Activity in the Greater Celandine (*Chelidonium majus* L.) Extracts by FRAP Method. Acta Biol Szeged. 2003; 47(1-4): 115-117.
32. Soural I, Švestková P, Híc P, Balík J. Different values obtained by the FRAP method for the determination of slowly and rapidly reacting phenols. Acta Aliment. 2022; 51(1): 84-92.
33. Halvorsen BL, Holte K, Myhrstad MCW, et al. A Systematic Screening of Total Antioxidants in Dietary Plants. J Nutr. 2002; 132(3): 461-471.
34. Apak R, Güçlü K, Özyürek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. J Agric Food Chem. 2004; 52(26): 7970-7981.
35. Özyürek M, Güçlü K, Apak R. The main and modified CUPRAC methods of antioxidant measurement. TrAC. 2011; 30(4): 652-664.
36. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc. 2007; 2(4): 875-877.

37. Attard E. A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. Open Life Sci. 2013; 8(1): 48-53.
38. Pereira X, Souza F, da S. Almeida JRG, et al. *Biological Oxidations and Antioxidant Activity of Natural Products* in Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health. InTech; 2012.
39. Shraim AM, Ahmed TA, Rahman MM, Hijji YM. Determination of total flavonoid content by aluminum chloride assay: A critical evaluation. LWT. 2021; 150: 111932.
40. Barnum DW. Spectrophotometric determination of catechol, epinephrine, dopa, dopamine and other aromatic vic-diols. Anal Chim Acta. 1977; 89: 157–166.
41. Pękal A, Pyrzynska K. Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. Food Anal Methods. 2014; 7(9): 1776-1782.
42. Azizah DN, Kumolowati E, Faramayuda F. Penetapan Kadar Flavonoid Metode $AlCl_3$ Pada Ekstrak Metanol Kulit Buah Kakao (*Theobroma cacao* L.). Kartika: Jurnal Ilmiah Farmasi. 2014; 2(2): 45-49.
43. Poole CF. *The Essence of Chromatography*. Amsterdam, Elsevier; 2003.
44. Özcimder M, Demirci A. *Gaz ve Sıvı Kromatografileri*. Ankara; 2004.
45. Striegel Mary F, Hill J. *Thin-Layer Chromatography for Binding Media Analysis*. Los Angeles: The Getty Conservation Institue; 1996.
46. Srivastava M, ed. *High-Performance Thin-Layer Chromatography (HPTLC)*. Springer: Berlin Heidelberg; 2011.
47. Chandra S, Saklani S. *Tools and Techniques of Chromatography*. Saarbrücken: Lambert Academic Publishing; 2012.
48. Eswarudu MM, Eswaraiah MC, Kumar KP, Sudhakar K. Ultra Performance Liquid Chromatography (UPLC): A preeminent technique in pharmaceutical analysis. RJPT. 2012; 5(12).
49. Folin O, Ciocalteu V. On Tyrosine and Tryptophane Determinations in Proteins. J Biol Chem. 1927; 73(2): 627-648.
50. Wolsky RG, Salatino A. Analysis of propolis: some parameters and procedures for chemical quality control. J Apic Res. 1998; 37(2): 99-105.
51. Reich E, Schibli A, ed. *High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants*. New York: Thieme Medical Publishers; 2007.
52. ICH. Guideline. Validation of analytical procedures: text and methodology Q2 (R1) International Conference on Harmonization 2005 Geneva, Switzerland.

53. Khazir J, Mir BA, Pilcher L, Riley DL. Role of plants in anticancer drug discovery. Phytochem Lett. 2014; 7: 173-181.
54. Guzelmeric E, Reis R, Sen NB, Celik C, Özhan Y, Petrikaite V, Sipahi H, Aydın A, Yesilada E. Insights into the Anti-inflammatory, Analgesic, and Anticancer Potentials of the Standardized Extracts From Three *Cistus* L. Species. J Herb Med. 2023; 41: 100724.

7. CURRICULUM VITAE

Personal Information

Name	Rengin	**Surname**	Baydar
Place of Birth	-	**Date of Birth**	-
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Education

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M.Sc.	Phytotherapy	Yeditepe University	2024
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High School	-	İstiklal Makzume Anatolian High School	2016

Language Skill

Language	Exam Degree
English	YÖKDİL (09.09.2022): 83.75

Work Experience

Position	Institution	Dates
Pharmacist	Aqua Pharmacy	2021-2022
Research Assistant	İstanbul Medipol University, Department of Pharmacognosy	2022-

Computer Skills

Program	Level
Microsoft Office	Very good
Mendeley	Very good

Scientific works
The articles published in the journals indexed by SCI, SSCI, AHCI

-

The articles published in other journals

Baydar R., Karadağ A.E., Biltekin S.N., Güzelmeriç E., Demirci F. (2024). *In vitro* Anti-Inflammatory Activities of *Tanacetum parthenium* L. Extract and Its Major Metabolite Parthenolide. *Acta Pharmaceutica Sciencia*, 62(1), 183-192.

International Conference Proceedings

Baydar R., Biltekin S.N., Karadağ A.E., Demirci F. *In vitro* Anti-Inflammatory and Anticancer Activities of *Tanacetum parthenium* L. Extract and Its Major Metabolite Parthenolide. International Multidisciplinary Symposium on Drug Research and Development (DRD). 2023, May 4-6, İzmir, Türkiye. (Oral Presentation)
Baydar R., Biltekin S.N., Karadağ A.E. Comparison of *In vitro* Anti-inflammatory, COX-1/COX-2 and LOX Inhibitory Activities of *Malva sylvestris* L. Leaves and Flowers Extract. 71st International Congress and Annual Meeting of The Society for Medicinal Plant and Natural Product Research (GA). 2023, July 2-5. Dublin, Ireland. (Poster Presentation)

National Conference Proceedings

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Projects

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