

Antiproliferative activity of extracts containing flavonoids from in vitro callus cultures of *Astragalus onobrychis* L.

Preslav Enchev

Medical University-Sofia: Medicinski universitet-Sofia

Yancho Zarev (✉ zarev.yancho@gmail.com)

Medical University-Sofia: Medicinski universitet-Sofia <https://orcid.org/0000-0001-5830-4697>

Lyubomir Marinov

Medical University-Sofia: Medicinski universitet-Sofia

Andrés Rivera-Mondragón

University of Panama: Universidad de Panama

Iliana Ionkova

Medical University-Sofia: Medicinski universitet-Sofia

Research Article

Keywords: *Astragalus onobrychis*, in vitro cultures, flavonoids, LC/MS analysis

Posted Date: June 26th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3005474/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published at Revista Brasileira de Farmacognosia on October 10th, 2023. See the published version at <https://doi.org/10.1007/s43450-023-00466-8>.

Abstract

Astragalus onobrychis (Fabaceae) is a perennial herbaceous plant with a strong taproot and reaches a height of 10–60 cm. The stems have a woody base and grow first spreading and then ascending. The phytochemical composition of the plant has not been fully studied. So far, mainly metabolites from the flavonoid and saponin group have been found. Species of the genus *Astragalus* have been studied and shown a range of pharmacological effects, including antioxidant, anti-inflammatory, immunomodulatory, antitumor, cardioprotective, and antidiabetic. In traditional medicine, *A. onobrychis* is used as a diuretic and diaphoretic agent and as a remedy for rheumatic diseases. The aim of the present study was to obtain valuable information on secondary metabolites related to flavonoids in *in vitro* callus and shoot cultures of *A. onobrychis*. Five fractions were obtained from the methanolic extracts (70% MeOH) of callus and shoot cultures of *A. onobrychis* after column chromatography, each of which was subjected to ultrahigh performance liquid chromatography-high resolution mass spectrometry analysis (UHPLC-HR-MS). The antiproliferative capacity of each fraction was also evaluated. Based on the mass spectral fragmentation data, use of standards, and comparison with literature sources, 15 flavonoid derivatives were characterized.

Introduction

Astragalus Onobrychis L. (Fabaceae) is widespread in Central, Eastern and Southern Europe, Western Siberia, the Caucasus, Central Asia. Its stems are recumbent or ascending, 30–60 cm long, stiff at the base, and above are grassy and strongly branched and fibrous. The leaves are 3–12 cm long, with short stalks or sessile, unpaired. The individual leaflets of the compound leaf are 4–15 cm long, elliptically lanceolate, sharp or obtuse, green on both sides, adjacently fibrous. The fruit (bean) is 7–15 mm long, ovate-lanceolate to oblong-ovate, three-walled, slightly flattened, broadly and deeply grooved on the back, with a long curved beak at the top, two-nested, short white fibrous. Seeds about 1–1.5 cm long, rounded kidney-shaped, olive green. The flavonoids found so far in the species *A. onobrychis* are: rutin, kaempferol-3-*O*-rutinoside, apigenin-7-*O*-rutinoside, kaempferol, quercetin, as well as the phenolic acids caffeic and chlorogenic acid. *A. onobrychis* is used in traditional medicine to treat gynecological diseases. Its use as a diuretic, diaphoretic and rheumatic agent is also known (Benbassat and Nikolov, 1995). Due to its flavonoid composition, it has hypolipidemic and antifungal activity, and triterpene saponins cause immunomodulatory effects (Lysiuk and Darmohray, 2016). The phenolic acids *p*-hydroxybenzoic, chlorogenic, neochlorogenic, ferulic and isoferulic, caffeic, 3-feruloylquinic, 3-*p*-coumaroquinic acids are described in the species *A. subrobustus*, *A. floccosifolius*, *A. quisqualis* and *A. onobrychis* (Pistelli F 2002). *A. onobrychis* leaf extract shows significant antioxidant activity. Moreover, the different activity of antioxidant enzymes depending on the vegetation period during which the plant material was collected has been proven. This shows the influence of external factors on the enzymatic activity of *A. onobrychis*. In spring, peroxidase activity was highest. Catalase and glutathione peroxidase show higher activity in summer. Superoxide dismutase is most active in the autumn (Miladinović et al. 2013). The antifungal activity of an extract of the aerial part of *A. onobrychis* against 12 strains of *Candida* (*C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. inconspicua*, *C. krusei*, *C. lusitaniae*, *C. norvegica*, *C. parapsilosis*, *C. pulcherrima*, *C. zeylanoides*, *C. orthopsilosis*, and *C. metapsilosis*) was also studied. Different solvents for extraction were used, with a difference in the activity of the extracts. The ethanolic extract showed higher antifungal activity (MIC = 2.57 mg/mL) than the corresponding aqueous extract (MIC = 3.32 mg/mL). Significant antifungal activity distinguishes *A. onobrychis* as a promising candidate for the development of antifungal products (Pauliuc and Botau 2013). Methanolic extract of *A. onobrychis* showed inhibitory activity against the enzymes tyrosinase (1.25 ± 0.04) and alpha amylase (4.39 ± 0.15) (Sarikurku et al. 2020). The study showed a correlation between the inhibitory activity of the enzyme tyrosinase and the total phenolic content, as well as the presence of hesperidin in the extract. Alpha-amylase is an enzyme synthesized in the pancreas that is involved in the breakdown of carbohydrates by hydrolysis of alpha bonds in starch. Inhibition of this enzyme indicates anti-diabetic activity, by a mechanism like acarbose (slows the absorption of glucose in the small intestine). No correlation was found between the presence of hesperidin or hyperoside and alpha-amylase inhibitory activity.

Material and methods

General Experimental Procedures

Ultrahigh-performance liquid chromatography- high-resolution mass spectrometry (UHPLC-HR-MS) analysis was performed using a Thermo Scientific Dionex Ultimate 3,000 RSLC instrument (ThermoFisher Scientific, Germering, Germany) consisting of a six-channel degasser SRD-3600, a high-pressure gradient pump HPG3400RS, an autosampler WPS-3000TRS and a column compartment TCC-3000RS coupled to a Thermo Scientific Q Exactive Plus mass spectrometer (ThermoFisher Scientific, Bremen, Germany). UHPLC separations were performed on a Kinetex C₁₈ column (Phenomenex Corporation, Torrance, CA, USA) (2.1 mm × 100 mm, 2.6 µm) at 40°C. Each chromatographic run was carried out with a binary mobile phase consisting of water containing 0.1% (v/v) formic acid (A) and acetonitrile also with 0.1% (v/v) formic acid (B). A gradient program was performed as follows: 0–3 min, 10% B; 3–23 min, 10–70% B; 23–25 min, 95% B. The flow rate was 0.3 mL/min, and the sample injection volume was 1 µL. Full scan data were recorded in positive (+) electrospray ionization (ESI) mode from *m/z* 250 to 1000 at a resolution of 70 000 (at *m/z* 200). Full scan dd-MS/MS (top 5) was performed at a resolution of 17500 (at *m/z* 200), AGS target 1e5 with maximum IT 50 ms. Xcalibur software version 4.0 was used for data acquisition and processing.

Plant Material

The plant material in the present study is callus and shoot *in vitro* cultures of *A. onobrychis*, obtained and successfully cultivated in our laboratory. Cultivation of the seed explants on solid MS culture medium (Murashige and Skoog 1962) resulted in shoot cultures, and callus cultures were obtained when shoot explants were cultivated on G₄₈ medium (Ionkova et al. 2010).

Extraction and sample preparation

The dried plant material of callus (0.5234 g) and shoot cultures (0.5291 g) of *A. onobrychis* was ground and sieved through a sieve with a pore size of 0.80 mm and transferred to centrifuge tubes. After addition of 15 mL of 70% methanol, the vials were placed in an ultrasonic bath at 45 degrees for 30 minutes. Total extracts were obtained after repeating the procedure three times. Total extracts were purified by open column chromatography (15 × 350 mm) using

Diaion® HP -20 75–150 µm particle size, Mitsubishi Chemical Co., Tokyo, Japan (30 g). Total extracts were dissolved in acidified water (formic acid 0.1%) and the column was eluted with water and increasing concentrations of methanol (10%, 30%, 50%, 70% and 90%). Each fraction of 30 mL was eluted into 50 mL volumetric flasks and 50% methanol was added up to the mark. The fractions obtained were transparent solutions, of which a 1 mL aliquot was used for LC-MS analysis.

LC-HR-MS data processing

In order to describe the possible methoxylation of flavonoids a KMD plot was created [Kendrick Mass Defect (KMD) against the Kendrick nominal mass (KNM)] in which the ions that differ only by a CH₂ group (e.g., methylation) can have the same KMD value, i.e., they form a homologous series (Zarev et al. 2021).

Biological evaluation

The cytotoxic activity of the different fractions of the extracts was investigated on a human tumor HUT-78 cell line (cutaneous T-lymphocytes) acquired from CLS Cell Line Service GmbH, Dr.-Eckener-Straße 8, 69214, SKW-3 (T-leukemia cell line- BCR-ABL-negative) T-24 (HTB-4 -human bladder cancer cell line) and BV-173 (human B-cell leukemia) acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). Cells were grown in a controlled environment – cell culture flasks at 37°C in an incubator with humidified atmosphere and 5% CO₂. The growth medium was 90% RPMI-1640 (Invitrogen, USA) + 10% inactivated FBS (fetal bovine serum). Cells were passaged two to three times per week to keep them in log phase before treating. Cellular viability after exposure to the fractions was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay according to Mosmann 1983. The assay is based on the ability of the mitochondrial succinate dehydrogenase of viable cells to metabolize the water-soluble yellow tetrazolium salt MTT to violet MTT-formazan crystals. Cells were seeded in 96- well plates at a density of 1 × 10⁵ cells and incubated for 24 h incubation at 37°C. Then, the cells were treated with different fractions 10%, 30%, 50%, 70% and 90% (0.1–400 µg/ml) for 72 h. For each concentration a set of at least 8 wells was used. After 72 h MTT-solution (10 mg/ml in PBS) was added and the plates were incubated for 3 h at 37°C. The MTT-formazan crystals formed were dissolved through the addition of 100 µl/well of 2-propanol acidified with 5% formic acid. The absorbance was measured in a multiplate reader Synergy 2 (BioTek Instruments) at 570 nm (690 nm for background absorbance). Cells survival fractions were calculated as a percentage of the untreated control (set as 100% viability) and the IC₅₀ values were calculated.

Statistical Analysis

The IC₅₀ values and their respective confidence intervals were calculated by means of the GraphPad Prism software (Version 6.00, for Windows, GraphPad Software, La Jolla California, San Diego, CA, USA).

Result and discussion

LC/MS analysis of flavonoids from extracts of callus and shoot in vitro cultures of *A. onobrychis*

In the LC-ESI-MS analysis three of 22 standards (flavonoids and phenolic acid) were found in extracts from *in vitro* cultures of *A. onobrychis*. By comparing the data obtained with the standards, as well as with data from the scientific literature, we make assumptions about the structure of 15 phenolic metabolites (Table 1). Compound 1 was observed as deprotonated molecular ion [M-H][−] with *m/z* 147.0439, molecular formula C₉H₇O₂ and retention time *Rt*= 10.70 min, with MS² spectra showing a fragment ion with *m/z* 119.05 corresponding to the loss of the group CO. Comparing the retention time and the observed spectrum with that of the cinnamic acid standard, it can be concluded that the observed metabolite is identical to the standard.

Table 1
Secondary metabolite identified in *in vitro* shoot and callus cultures from *A. onobrychis*.

No.	Compound	Rt (min)	Molecular formula	[M-H] ⁻ m/z	RDB	δ (ppm)	ESI MS/MS	callus						Shoot Total
								Total	10%	30%	50%	70%	90%	
1	Cinnamic acid	10.70	C ₉ H ₇ O ₂	147.04396	6.5	-0.653	119.05 [M-H-28 (-CO)] ⁻	+				+	+	+
2	Apigenin-7- <i>O</i> -glucoside	11.65	C ₂₁ H ₁₉ O ₁₀	431.09811	12.5	1.941	268.038[M-H-162(hex)] ⁻	+			+	+	+	+
3	Naringenin-7- <i>O</i> -hexoside	12.43	C ₂₁ H ₂₁ O ₁₀	433.11429	11.5	7.312	271.06[M-H-162(hex)] ⁻	+				+	+	+
4	5,7-dihydroxy-4'-methoxyisoflavone- <i>O</i> -hexoside	13.85	C ₂₂ H ₂₁ O ₁₀	445.11389	12.5	0.940	283.06[M-H-162(hex)] ⁻ , 268.04, 267.03, 239.04, 224.05, 211.19, 132.02	+				+	+	+
5	Kaempferol-3- <i>O</i> -hexoside	9.32	C ₂₁ H ₁₉ O ₁₁	447.09421	12.5	4.523	285.03[M-H-162(hex)] ⁻						+	
6	Luteolin-7- <i>O</i> -hexoside	12.28	C ₂₁ H ₁₉ O ₁₁	447.09332	12.5	2.532	285.03[M-H-162(hex)] ⁻			+				
7	5-hydroxy-7-methoxy-2',5'-dihydroxyisoflavone- <i>O</i> -hexoside	17.35	C ₂₂ H ₂₁ O ₁₁	461.10983	12.5	3.131	299.05[M-H-162(hex)] ⁻ , 298.05, 283.02, 255.03, 227.03					+	+	
8	Quercetin-3- <i>O</i> -hexoside	11.21	C ₂₁ H ₁₉ O ₁₂	463.08817	11.5	2.305	301.04[M-H-162(hex)] ⁻			+				
9	Hesperetin hexoside	12.99	C ₂₂ H ₂₃ O ₁₁	463.12711	11.5	5.631	301.07 [M-H-162(hex)] ⁻					+	+	
10	Unknown flavonoid glycoside	12.43	C ₂₈ H ₃₅ O ₁₃	579.20947	12.5	2.942	417.16[M-H-162(hex)] ⁻				+	+	+	
11	Luteolin-3- <i>O</i> -rutinoside	12.30	C ₂₇ H ₂₉ O ₁₅	593.14960	13.5	-0.837	285.03[M-H-308(rut)] ⁻							+
12	Quercetin-3- <i>O</i> -rutinoside	11.02	C ₂₇ H ₂₉ O ₁₆	609.14551	13.5	0.819	301.02[M-H-308(rut)] ⁻							
13	Isoramnetin-3- <i>O</i> -rutinoside	12.66	C ₂₈ H ₃₁ O ₁₆	623.16278	13.5	-3.163	315.05[M-H-308(rut)] ⁻							+
14	Luteolin derivative	9.56	C ₃₉ H ₄₉ O ₂₄	901.26392	15.5	2.822	285.03[M-H-616] ⁻							+
15	Quercetin derivative	9.28	C ₃₈ H ₄₇ O ₂₅	903.24243	15.5	2.337	301.03[M-H-603] ⁻							+

Similarly, compound **2** was observed as deprotonated molecular ion [M-H]⁻ with *m/z* 431.0981, molecular formula C₂₁H₂₀O₁₀ and retention time *Rt* = 11.65 min, with MS² spectra showing a fragment ion with *m/z* 268.04, corresponding to the loss of a hexose residue (162 Da). Comparing the retention time and observed spectrum with that of the apigenin-7-*O*-glucoside standard, we can conclude that the observed metabolite is identical to the standard. LC/MS data for compound **12**, the deprotonated molecular ion [M-H]⁻ with *m/z* 609.1455, molecular formula C₂₇H₂₉O₁₆ and retention time *Rt* = 11.02 min, were identical to those of the apigenin-7-*O*-glucoside standard.

A fragment ion with m/z 271.06 was observed in the MS² spectrum of deprotonated molecular ion [M-H]⁻ with m/z 433.1143, molecular formula C₂₁H₂₁O₁₀ and retention time R_t = 12.43 min. which is corresponding to the loss of one hexose residue (162 Da). Comparing the observed spectrum with the literature data, it can be seen that the main fragment ions of the aglicon: m/z 151, 119, 107 correspond to naringenin (Bystrom et al. 2008). Based on these considerations, tentatively the structure of compound **3** was described as naringenin-7-*O*-hexoside.

Compound **4** was observed as deprotonated molecular ion [M-H]⁻ with m/z 445.1139, molecular formula C₂₂H₂₁O₁₀ and retention time R_t = 13.85 min. In the MS² spectrum, a fragment ion with m/z 283.06 was observed, corresponding to the loss of one hexose residue (162 Da), which suggests the presence of one hexose unit in the molecule of compound **4**. The same pattern of fragmentation was observed also for compound **7**, deprotonated molecular ion [M-H]⁻ with m/z 461.1098, molecular formula C₂₂H₂₁O₁₁ and retention time R_t = 17.35 min, with main fragment ion at MS² spectra m/z 299.06. Figure 1 and Table S1 clearly shows the homologous series between protonated molecular ions of flavonoid aglicons with m/z 283.06 and 299.06 which differ each other only by one CH₂, which is also visible in the corresponding molecular formulas of the observed ions C₁₅H₉O₆ and C₁₆H₁₁O₆, respectively. Comparing the MS² spectra of compound **7** with the results of our previous study of flavonoids from *in vitro* cultures of *A. vesicarius* described its aglicon as 5-hydroxy-7-methoxy-2', 5'-dihydroxyisoflavone (Popova et al. 2021). Respectively, the aglicon of compound **4** is expected to be just demethoxylated. Thus, compounds **4** and **7** are described as 5,7-dihydroxy-4'-methoxyisoflavone-*O*-hexoside and 5-hydroxy-7-methoxy-2', 5'-dihydroxyisoflavone-*O*-hexoside, respectively.

Compound **5** was observed as deprotonated molecular ion [M-H]⁻ with m/z 447.0942, molecular formula C₂₁H₁₉O₁₁ and retention time R_t = 9.32. In the MS² spectrum, a fragment ion with m/z 285.03 was observed, corresponding to the loss of one hexose residue (162 Da), which suggests the presence of one hexose unit in the molecule of the substance. Comparing the observed spectrum with the literature data, the main fragment ions of the aglicon: m/z 151, 107 correspond to kaempferol. Because of these considerations, the presumed structure of compound **5** is kaempferol-3-*O*-hexoside. Similarly, compound **6** with main fragment ion in MS² spectra of m/z 285, 255 and 277 was defined as Luteolin-7-*O*-hexoside (Wang et al. 2017). Within the MS² spectra of compounds **11**, **12** and **13** losses of 308 Da was observed which may be related to glycosidic residue of rutinose. Comparing the fragmentation patterns of aglycones with literature data and analyzed standards defined those metabolites as: luteolin-3-*O*-rutinoside, quercetin-3-*O*-rutinoside and isorametnetin-3-*O*-rutinoside, respectively.

Screening of antiproliferative activity

The antioxidants quercetin, kaempferol, apigenin, luteolin and their metabolites are known to exhibit anticancer effects which are associated with modulation of redox state in cancer cells. This can be explained by their antioxidative effects and the capacity to scavenge ROS and their prooxidative activity which generates ROS (Lee et. al 2021). Furthermore, recent studies have shown that natural triterpenoidal saponins display anticancer activities by targeting different transcriptional factors, growth factors, proteins and signaling pathways (Arslan and Cenzano, 2020). Thus, we studied the effects of the fractions (0.1–400 µg/ml) in four tumor cell lines. Table 2 summarizes the results obtained after 72h exposure by MTT assay. The 10%, 30% and 50% fractions have shown low cytotoxic effects (IC₅₀ > 151 µg/ml). Only fraction 50% has demonstrated higher cytotoxicity in cell line BV-173 (IC₅₀ = 56.3 µg/ml). In contrast, the other two fractions 70% and 90% showed more pronounced cytotoxic effects in all four tumor cell lines with IC₅₀ values between 24.39–340.30 µg/ml. The BV-173 cells were most sensitive as evidenced by IC₅₀ values ranging from 24.39 to 309.8 µg/ml followed by T-24, SKW-3 and HUT-28 cell line (most resistant).

Table 2
IC₅₀ values (µg/ml) of the five fractions following 72 h exposure in the panel of tumor cell lines.

Fractions	HUT-28	SKW-3	T-24	BV-173
10%	> 400	334.8	329.0	309.8
30%	> 400	337.9	> 400	285.3
50%	> 400	286.8	151.3	56.3
70%	286.0	340.3	159.9	46.9
90%	140.4	87.83	116.5	24.4

Discussion

After obtaining and fractionating total extracts from *in vitro* callus and aboveground cultures of *A. onobrychis*, an LC-HR-ESI-MS analysis was carried out which showed that both types of cultures produced glycosylated flavonoids, derivatives of quercetin, kaempferol, apigenin, luteolin and others. Differences in flavonoid production were found within the two types of *in vitro* cultures. Luteolin-7-*O*-hexoside and quercetin-7-*O*-hexoside were detected only in the *in vitro* callus cultures, while luteolin-3-*O*-rutinoside, quercetin-3-*O*-rutinoside, isoramnetin-3-*O*-rutinoside and luteolin and quercetin derivatives with 4 sugar residues were found in the *in vitro* shoot cultures. The result of our study shows that the different degree of differentiation of the *in vitro* cultures significantly influences the processes of secondary metabolism. Callus cultures of *A. onobrychis* can serve as a source of monoglycosylated flavonoid derivatives, while the higher degree of organogenesis in above-ground *in vitro* cultures of *A. onobrychis* yields more complex metabolites, rutinose derivatives and polyglycosylated flavonoids.

The study also highlights the potential anticancer effects of certain antioxidants, specifically quercetin, kaempferol, apigenin, and luteolin, and especially their glycosylated metabolites. These compounds are known to modulate the redox state in cancer cells, which contributes to their anticancer properties. This

modulation is achieved through their antioxidative effects, where they scavenge reactive oxygen species (ROS), as well as their prooxidative activity, which generates ROS. Additionally, the study explores the anticancer activities of natural triterpenoid saponins, which have been found to target various transcriptional factors, growth factors, proteins, and signaling pathways associated with cancer. This study investigated the effects of different fractions (ranging from 0.1–400 µg/ml) on four tumor cell lines. Based on the results obtained after 72 hours of exposure using the MTT assay, it was found that the 10%, 30%, and 50% fractions exhibited low cytotoxic effects, with IC₅₀ values greater than 151 µg/ml. However, the 50% fraction demonstrated higher cytotoxicity specifically in the BV-173 cell line, with an IC₅₀ value of 56.3 µg/ml.

On the other hand, the 70% and 90% fractions showed more pronounced cytotoxic effects across all four tumor cell lines, with IC₅₀ values ranging from 24.39 to 340.30 µg/ml. Among the tested cell lines, BV-173 cells displayed the highest sensitivity, with IC₅₀ values ranging from 24.39 to 309.8 µg/ml. The T-24, SKW-3, and HUT-28 cell lines showed varying degrees of resistance to the fractions. These findings suggest that the 70% and 90% fractions possess stronger cytotoxic effects on the tested tumor cell lines, with BV-173 cells being the most sensitive. This information could be valuable in further research and development of potential anticancer therapies utilizing these natural compounds.

Declarations

Acknowledgements

This study is financed by the European Union-NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project № BG-RRP-2.004-0004-C01. Thanks, are also due to the incentive program of the National Investigation System (SNI) of the Republic of Panama for sup-porting Dr. Rivera-Mondragón.

Ethical Approval Not applicable since commercial cell lines were used.

Conflict of interest The authors declare no conflict of interests.

References

1. Arslan I, Cenzano AM (2020) *N*-triterpene saponins in cancer therapy: A review of mode of action. *Revista Brasileira de Farmacognosia*, 30(1), 1-6.
2. Benbassat N, Nikolov S (1995) Flavonoids from *Astragalus onobrychis*. *Planta Med.* Feb;61(1):100. doi: 10.1055/s-2006-958024. PMID: 17238067
3. Bystrom LM, Lewis BA, Brown DL, Rodriguez E, Obendorf RL (2008) Characterization of phenolics by LC-UV/vis, LC-MS/MS and sugars by GC in *Melicoccus bijugatus* Jacq. 'Montgomery' fruits. *Food Chem*; 111(4):1017-1024. doi:10.1016/j.foodchem.2008.04.058
4. Ionkova I, Antonova I, Momekov G, Fuss E (2010) Production of podophyllotoxin in *Linum linearifolium* *in vitro* cultures. *Pharmacognosy Magazine* 6(23): 180–185. <https://doi.org/10.4103/0973-1296.66932>
5. Lee Y, Lee J, Lim C (2021) Anticancer activity of flavonoids accompanied by redox state modulation and the potential for a chemotherapeutic strategy. *Food Science and Biotechnology*, 30(3), 321-340.
6. Lysiuk R, Darmohray R (2016). Pharmacology and Ethnomedicine of the Genus *Astragalus*. *International journal of pharmacology, phytochemistry and ethnomedicine*. 3. 46-53. 10.18052/www.scipress.com/IJPPE.3.46
7. Miladinović DL, Ilić BS, Najman SJ, Cvetković OG, Šajnović AM, Miladinović MD, Nikolić ND (2013). Antioxidative responses to seasonal changes and chemiluminescence assay of *Astragalus onobrychis* leaves extract. *Central European Journal of Chemistry*, 11(2), 123-132
8. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65(1-2), 55-63.
9. Murashige T, Skoog FA (1962) Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Plant Physiology*: 473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052>
10. Pauliuc I, Botau D. (2013), Antifungal activity of *Astragalus onobrychis* L. extracts. *Scientific Bulletin. Series F. Biotechnologies*, Vol. XVII, ISSN 2285-1364, 53-56
11. Pistelli F (2002) "Secondary metabolites of genus *Astragalus*: Structure and biological activity." *Studies in natural products chemistry*. Vol. 27. Elsevier, 443-545.
12. Popova P, Zarev Y, Mihaylova R, Momekov G, Ionkova I (2021) Antiproliferative activity of extract from *in vitro* callus cultures of *Astragalus vesicarius* ssp. *carniolicus* (A. Kern.) Chater. *Pharmacia* 68(1): 217-221. <https://doi.org/10.3897/pharmacia.68.e63421>
13. Sarikurkcü C, Şahinler S, Tepe B (2020). *Astragalus gymenolobus*, *A. leporinus* var. *hirsutus*, and *A. onobrychis*: Phytochemical analysis and biological activity. *Industrial Crops and Products*. 150. 112366. 10.1016/j.indcrop.2020.112366.
14. Wang J, Jia Z, Zhang Z, Wang Y, Liu X, Wang L, Lin R (2017). Analysis of Chemical Constituents of *Melastoma dodecandrum* Lour. by UPLC-ESI-Q-Exactive Focus-MS/MS. *Molecules*, 22(3), 476. doi:10.3390/molecules22030476
15. Zarev Y, Popova P, Foubert K, Ionkova I, Pieters L (2021) Comparative LC-MS analysis of tropolone alkaloids from *in vitro* cultures and native sources of *Gloriosa superba* by Kendrick mass defect plots. *Phytochem Anal.* Jul; 32(4):446-456. doi: 10.1002/pca.2992. Epub 2020 Sep 4. PMID: 32888246.

Figures

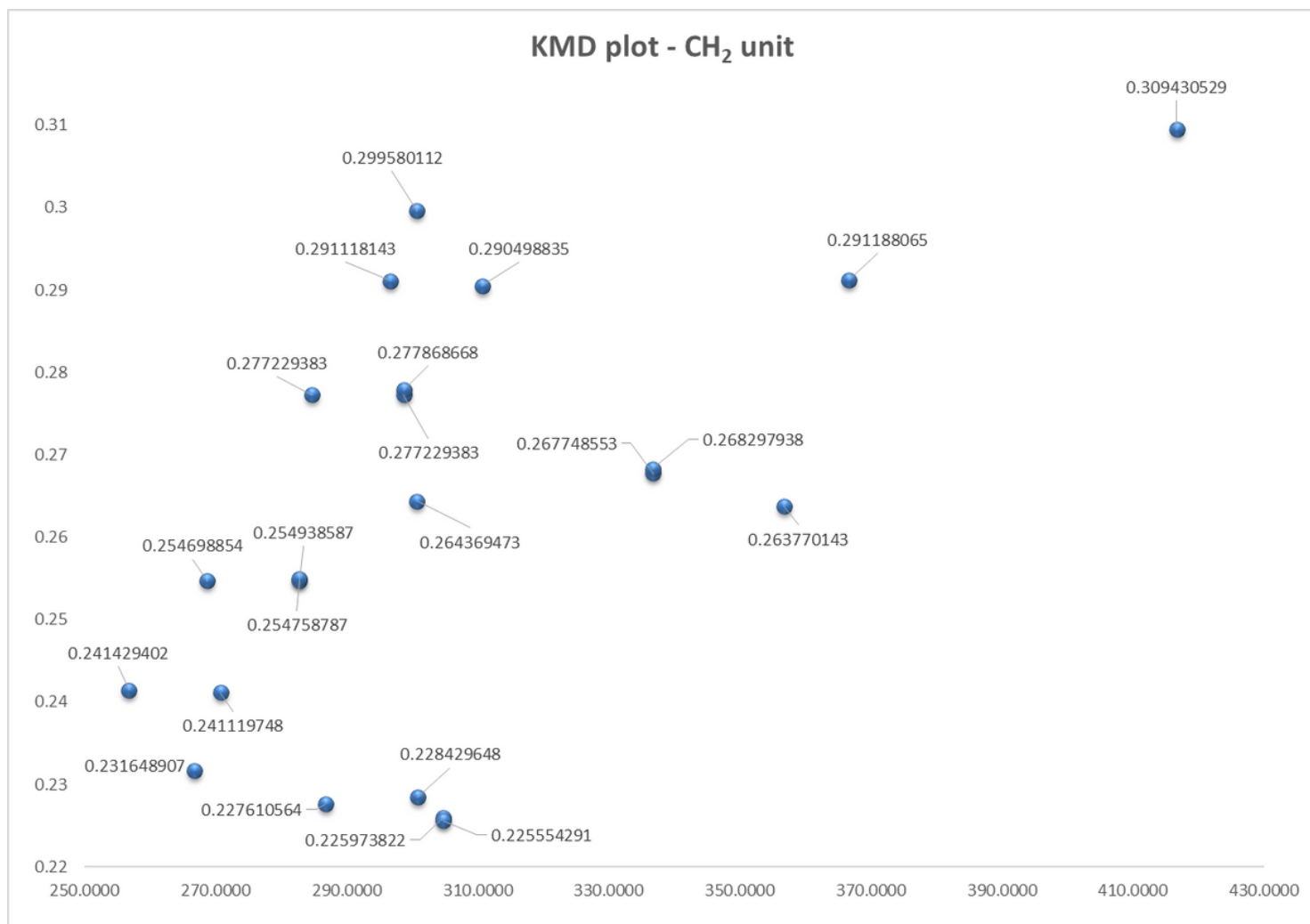


Figure 1

Kendrick plot presenting the formation of a homologous series of the observed metabolites which are structural analogues differing from each other just by one CH₂ group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphicalabstract.tiff](#)
- [TableS1.docx](#)