THE „PAISII HILENDARSKI“ UNIVERSITY OF PLOVDIV

Annotation of materials and Advanced Habilitation Reference accordance with Article 65 (1) of the Regulations for the development of the academic staff of the "Paisii Hilendarski" UNIVERSITY OF PLOVDIV, including self-evaluation of contributions

on

Chief Assistant Professor Dimitar Georgiev Bozhilov, Ph.D

Department of "Organic Chemistry", Faculty of Chemistry, Plovdiv University "Paisii Hilendarski"

in connection with participation in a competition for the academic position of "**associate professor**" in the field of higher education **4. Natural sciences, mathematics and informatics**,

professional direction **4.2. Chemical sciences**,

scientific specialty **Organic chemistry, Chromatographic analysis,**

announced in the State Gazette, **no. 39 of 02.05.2023**

I. Compliance with the requirements for holding the academic position of "associate professor" as defined in Art. 65. (1) of the regulations for the development of the academic staff of PU "P. Hilendarski"

Since 2010, I have been teaching at Plovdiv University "P. Hilendarski" and I am part of the team of the "Organic Chemistry" department. I defended my **doctoral degree** in the scientific specialty Organic Chemistry in 2017. Since 2018, I have held the academic position of "**chief assistant assistant**".

I present a total of 46 scientific papers that were not used in the procedures for obtaining the educational and scientific degree "Doctor" and for the occupation of the academic position "Chief Assistant Professor" for participation in the competition for the academic position "Associate Professor" in the scientific specialty "Organic Chemistry, Chromatographic Analysis." According to the list of scientific works, they are divided into the following categories:

- ❖ publications in journals referenced in Scopus/Web of Science – 24 issues of articles;
- ❖ publications in specialized scientific journals without an impact factor – 11 issues of articles;
- ❖ publications in conference proceedings – 11 issues of articles;

I. PUBLICATIONS IN SCOPUS/WEB OF SCIENCE REFERRED JOURNALS

1. Georgieva, K., Dagnon, S., Gesheva, E., Bojilov, D., Mihailova, G., & Doncheva, S. (**2017**). Antioxidant defense during desiccation of the resurrection plant Haberlea rhodopensis. *Plant Physiology and Biochemistry*, *114*, 51–59. https://doi.org/10.1016/j.plaphy.2017.02.021

Abstract: Maintaining a strong antioxidant system is essential for preventing drought-induced oxidative stress. Thus, in the present study we investigated the role of some non-enzymic and enzymic antioxidants in desiccation tolerance of Haberlea rhodopensis. The effects of high light upon desiccation on antioxidant capacity was estimated by comparing the response of shade and sun plants. The significant enhancement of the antioxidant capacity at 8% RWC corresponded to an enormous increase in flavonoid content. The important role of ascorbate-glutathione cycle in overcoming oxidative stress during drying of *H. rhodopensis* was established. The antioxidant capacity increased upon dehydration of both shade and sun plants but some differences in non-enzymatic and enzymatic antioxidants were observed. Investigations on the role of polyphenols in desiccation tolerance are scarce. In the present study the polyphenol profiles (fingerprints) of the resurrection plant *Haberlea rhodopensis*, including all compo- nents of the complex are obtained for the first time. It was clarified that the polyphenol complex of H. rhodopensis includes only two types of glycosides - phenylethanoid glucosides and hispidulin 8-*C*-glucosides. Upon desiccation the polyphenol content increase

and the main role of phenylethanoid glucosides in the protection of *H. rhodopensis* was revealed.

2. Dospatliev, L., Lozanov, V., Ivanova, M., Papazov, P., Sugareva, P., Petkova, Z., & Bojilov, D. (**2018**). Comparison of Free Amino Acid Compositions of Stem and Cap in Wild Edible Mushrooms, Bulgaria. *Oxidation Communications*, *41*(4), 542–549. http://www.scibulcom.net/ocr.php?gd=2018&bk=4

Abstract: The aim of this study was to evaluate the differences in amino acid composition between the cap and stem of Morchella esculenta – a wild edible mushroom from the Batak Mountain, Bulgaria. The amino acid composition was determined by Q Exactive mass analyser equipped with TurboFlow LC system and IonMax II electrospray ionisa- tion module (ThermoScientific Co, USA). Data acquisition and processing were carried out with XCalibur 4.2 software package. Twenty free amino acids, histidine, arginine, aspargine, glutamine, serine, aspartic acid, glutamic acid, threonine, glycine, proline, tyrosine, valine, methionine, leucine/isoleucine, phenylalanine, orndihydrochloride, tryptophane, lysine, 4-hydroxyproline and γ-amino butyric acid were determined in mushroom cap and stem. The total free amino acid (TAA) content of the cap was 26.17 mg kg^{-1} and that of the stem – 42.75 mg kg^{-1} . The essential to total amino acids ratios of the cap and the stem were 0.15 and 0.25, respectively. The most substantial difference between Morchella esculenta cap and stem was established in ornithine – 263%, followed by serine – 18.93% and aspargine – 20.28%. The smallest differences in the amino acid composition between cap and stem were demonstrated in proline – 102.77%, followed by glutamine – 104.33% and glutamic acid – 93.12%. Of all 20 amino acids, only 6 were found in larger amounts in the cap than in the stem. The results showed the free amino acid content of the analysed wild edible mushroom was considerable, and that they may be important compounds contributing to the typical mushroom taste, nutritional value, and potent antioxidant properties.

3. Dospatliev, L. K., Petkova, Z. Y., Bojilov, D. G., Ivanova, M. T., Antova, G. A., & Angelova-Romova, M. Y. (**2019**). A comparative study on the methods of antioxidant activity in wild edible mushrooms from the Batak Mountain, Bulgaria. *Bulgarian Chemical Communications*, *51*(Special Issue A), 245–250. http://www.bcc.bas.bg/BCC_Volumes/Volume_51_Special_A_2019/E-pdf-ready/BCC-51-A-2019-245-250-ZhPetkova-E2.pdf

Abstract: A comparative study on the methods of antioxidant activity in methanolic extracts from seven species of wild edible mushrooms (*Boletus pinophilus, Cantharellus aurora, Cantharellus tubaeformis, Cantharellus cibarius, Craterellus cornucopioides, Morchella esculenta* and *Tricholoma equestre*) from the Batak Mountain (Bulgaria) was performed. The total antioxidant capacity was estimated by $ABTS^{\bullet+}$ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)), $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical), FRAP (ferric reducing/antioxidant power) and CUPRAC (cupric ion reducing antioxidant capacity) methods. The total phenolic content (TPC) of the mushrooms was evaluated by Folin-Ciocalteau's phenol reagent and gallic acid was used as standard. Trolox equivalent antioxidant capacity (TEAC) values of the mushrooms ranged from 4.10 to 69.74 mmol TE/g dw, from 1.55 to 20.23 mmol TE/ g dw, from 7.72 to 35.31 mmol TE/g dw, and from 7.97 to 64.25 mmol TE/g dw for $ABTS^{\bullet+}$, $DPPH^{\bullet}$, FRAP and CUPRAC, respectively. TPC ranged from 0.68 to 11.92 mg of gallic acid equivalents (GAE)/g dw. In conclusion, the mushroom with the highest TEAC values was *Boletus pinophilus* and with the lowest was *Cantharellus cibarius*.

4. Dagnon, S., Novkova, Z., Bojilov, D., Nedialkov, P., & Kouassi, C. (**2019**). Development of surrogate standards approach for the determination of polyphenols in *Vernonia amygdalina* Del. *Journal of Food Composition and Analysis*, *82*, 103231. https://doi.org/10.1016/j.jfca.2019.06.003

Abstract: This paper describes an analytical approach for polyphenol components determination in *Vernonia amygdalina* Del. (VA) using surrogate standards, extracts of green coffee and *Achillea asplenifolia* 9602, and rutin as an internal standard. HPLC–PDA fingerprint profiles of the polyphenol complex of VA and surrogate standards were developed. By comparison between the chromatographic and spectral characteristics of VA and the surrogate standards using the relative retention times towards rutin, it was possible to identify the polyphenol components in VA. Additionally, peak identity was confirmed by HRMS spectra, to verify the truthfulness of the procedures. Recoveries of rutin, LOD, LOQ, and RSD% were determined, to validate the method as highly accurate and applicable. The polyphenol complex of VA contained dicaffeoylquinic acids and luteolin glycosides as main components. A method using Nucleosil C_{18} provided the best separation of 1,5- and 3,5-dicaffeoylquinic acids in VA. Their amount reached up to 1.49 ± 0.21 mg g^{-1}. The content of 4,5-dicaffeoylquinic acid was 0.35 ± 0.04 mg g^{-1}. Luteolin glycosides and luteolin were found at 0.40 ± 0.04 mg g^{-1} and 0.14 ± 0.01 mg g^{-1}, respectively. The presence of luteolin 4′-*O*-glucoside, apigenin 7-*O*-rutinoside, apigenin 7-*O*-glucoside and apigenin as minor constituents in VA is reported for the first time. Results suggest the implementation of the surrogate standard approach in food analytical practice as highly advisable.

5. Manolov, S., Ivanov, I., & Bojilov, D. (**2020**). N-(2-(1H-indol-3-yl)ethyl)-2-(6-chloro-9H-carbazol-2-yl)propanamide. *Molbank*, *2020*(4), M1171. https://doi.org/10.3390/M1171

Abstract: The titular compound was prepared by a reaction between tryptamine and carprofen, applying, as a “dehydrating” reagent, N,N'-dicyclohexylcarbodiimide. The newly synthesized compound was fully analyzed and characterized via 1H, 13C-NMR, UV, IR, and mass spectral data.

6. Bojilov, D., Petkova, Z., Manolov, S., Antova, G., & Angelova-Romova, M. (**2020**). Impact of the duration of ultrasound-assisted extraction on total phenolics content and antioxidant activity of lupin seeds. *Bulgarian Chemical Communications*, *52*(Special Issue D), 222–226. http://bcc.bas.bg/BCC_Volumes/Volume_52_Special_D_2020/BCC-52-D-2020-222-226-Petkova-E02.pdf

Abstract: The influence of the duration of ultrasound-assisted extraction on antioxidant activity and total phenolics content of lupin seeds (Lupinus angustifolius L. cultivar ‘Boregine’) was investigated for the first time. Lupin seeds were with German origin but introduced in Bulgaria. They were extracted for 10, 20 and 30 min with absolute methanol and the obtained extracts were evaluated for total phenolic content and antioxidant activity. The polyphenol content of the extracts was found to be in the range of 1.65 – 2.03 mg gallic acid equivalents (GAE)/g dry weight sample, depending on the duration of the extraction process. The antioxidant activity was estimated by $ABTS^{\bullet+}$ (2,2′azinobis-(3- ethylbenzthiazoline-6-sulfonic acid)) (2.28 – 2.89 mmol Trolox Equivalent (TE)/g dw), $DPPH^{\bullet}$ (1,1-diphenyl-2- picrylhydrazyl radical) (2.01 – 2.71 mmol TE/g dw), FRAP (ferric reducing/antioxidant power) (3.76 – 4.36 mmol TE/g dw) and CUPRAC (cupric ion reducing antioxidant capacity) (3.07 – 4.69 mmol TE/g dw) methods. Generally, methanol extracts with 30 min of extraction displayed the highest total phenolic

contents, while 10 min of extraction time was the least efficient ultrasound-assisted method. On the other hand, antioxidant activity of the extracts was highest in 20 min of extraction apart from CUPRAC method where 30 min of extraction depicted more antioxidant capacity of the methanol extract.

7. Bojilov, D., Dagnon, S., Kostadinov, K., & Filipov, S. (**2020**). Polyphenol composition of lettuce cultivars affected by mineral and bio-organic fertilization. *Czech Journal of Food Sciences*, *38*(6), 359–366. https://doi.org/10.17221/97/2020-CJFS

Abstract: Three types of *Lactuca sativa* L. plants (green *lettuces Batavia cv. Maritima* and *cv. Winter Butterhead*, red *lettuce Lolo rosa cv. Tuska*) were investigated for their polyphenol composition. The lettuce plants were grown in po- lyethylene greenhouses and treated with different fertilisers. The qualitative and quantitative polyphenol compositi- on was evaluated according to the use of mineral, organic (Italpollina and Arkobaleno) and bio (Lombricompost and EKOprop NX) fertilisers. The individual polyphenol components (caffeoyl derivatives and quercetin glycosides) were determined by high-performance liquid chromatography and the sample differences were estimated. The differences in the polyphenol content in the green lettuce cultivars in dependence on fertilisation were much higher than those in the red cultivar. In general, the red lettuce Lolo rosa cv. Tuska was characterised by the highest content of polyphenols. The highest content of all components was determined in the samples of red lettuce with the use of organic fertiliser Arkobaleno. In. the red lettuce and the green lettuce cv. Winter Butterhead organic fertilisation resulted in the higher content of polyphenols in comparison with mineral fertilisation and unfertilised samples. An exception was observed in cv. Maritima, where the unfertilised samples showed higher content of polyphenols compared to the fertilised samples.

8. Mollova, S., Fidan, H., Antonova, D., Bozhilov, D., Stanev, S., Kostova, I., & Stoyanova, A. (**2020**). Chemical composition and antimicrobial and antioxidant activity of Helichrysumitalicum (Roth) G.Don subspecies essential oils. *Turkish Journal of Agriculture and Forestry*, *44*(4), 371–378. https://doi.org/10.3906/tar-1909-34

Abstract: The chemical composition and the antimicrobial and antioxidant activity of essential oils from two *Helichrysum italicum* (Roth) G.Don subspecies grown in Bulgaria were investigated. As a result, 95 compounds with concentrations above 0.05% were detected by GC/MS and 46 of them, mainly mono- and sesquiterpenes, representing 79.81% and 85.51% of the total content of the samples, were identified. The main constituents of the essential oil from *Helichrysum italicum subsp. microphyllum* (plant origin from Bosnia) were monoterpene α-pinene (20.84%) and sesquiterpene γ-curcumene (16.53%), followed by β-selinene (5.59%), ar-curcumene (4.39%), trans-caryophyllene (4.35%), β-diketone italidione I (4.32%), α-selinene (4.28%), and neryl acetate (3.81%). The sesquiterpene hydrocarbons were the dominant groups of chemical constituents in the essential oil, followed by the oxygenated aliphatic hydrocarbons. The main constituents in the H. italicum essential oil (plant origin from France) were neryl acetate (33.87%), γ-curcumene (8.84%), rosifoliol (5.46%), geranyl propionate (4.98%), αr-curcumene (4.31%), italidione I (3.56%), α-eudesmol (3.19%), and limonene (3.02%). The main class of chemical compounds was the oxygenated monoterpenes, followed by sesquiterpene hydrocarbons. H. italicum essential oil from France showed more pronounced antimicrobial activity against the Gram-positive bacteria Staphylococcus aureus and Bacillus subtilis, and the fungus Aspergillus brasiliensis, as well as stronger antioxidant potential.

9. Manolov, S., Ivanov, I., & Bojilov, D. (**2021**). N-(2,2-Diphenylethyl)-2-(6-methoxynaphthalen-2-yl)propanamide Stanimir. *Molbank*, *2021*(3), M1257. https://doi.org/https://doi.org/10.3390/M1257

Abstract: *N*-(2,2-Diphenylethyl)-2-(6-methoxynaphthalen-2-yl)propanamide was prepared by a reac- tion between 2,2-diphenylethan-1-amine and naproxen in high yield. The newly obtained naproxen derivative was fully analyzed and characterized via ^{1}H, ^{13}C, UV, IR, and mass spectral data.

10. Dragoev, S., Vlahova-Vangelova, D., Balev, D., Bozhilov, D., & Dagnon, S. (**2021**). Valorization of waste by-products of rose oil production as feedstuff phytonutrients. *Bulgarian Journal of Agricultural Science*, *27*(1), 209–219. https://doi.org/10.31220/osf.io/cdf9b

Abstract: The rose (*Rosa damascena* Mill.) flowers characterized with strong antioxidant capacity and antibacterial activity. Because of that it has been suggested the oil-bearing rose by-products and wastes can be discussed as natural antioxidant sources. From this point of view the objectives of this research were to determine the possibilities for dry pressed distilled rose petals valorisation as feed stuff phytonutrients in animal husbandry, to study the chemical composition and radical scavenging activity of polyphenol complex in rose (*Rosa damascena* Mill.) waste products, to identify and quantify the polyphenol composition in dry rose petals, dry pressed distilled rose petals and waste water (liquid aqueous phase after dis- tillation). The polyphenol composition in dry rose petals, dry pressed distilled rose petals and waste water after distillation was identified and quantified. By HPLC-PDA and LC-MS thirteen glycosides of kaempferol, ten glycosides of quercetin, six glycosides of gallic acid and the two flavonol aglycones have been identified in dry rose petals. Those polyphenols pos- sess high antioxidant activity and depending on the dose and length of fattening is expected to have a positively influence on the growth performance of pigs, broilers and lambs.

11. Manolov, S., Ivanov, I., & Bojilov, D. (**2021**). Microwave-assisted synthesis of 1,2,3,4-tetrahydroisoquinoline sulfonamide derivatives and their biological evaluation. *Journal of the Serbian Chemical Society*, *86*(2), 139–151. https://doi.org/10.2298/JSC200802076M

Abstract: Herein we report an alternative eco-friendly method for the synthesis of 1,2,3,4-tetrahydroisoquinoline sulfonamide derivatives. All obtained com- pounds were screened for their in vitro inhibition of albumin denaturation, antioxidant, antitryptic and antibacterial activity, and have shown significant results. The lipophilicity was established using both reversed-phase thin layer chromatography and in silico calculations.

12. Manolov, S., Ivanov, I., Bojilov, D., & Yuliyan Voinikov. (**2021**). Evaluation of antioxidant, anti-inflammatory and anti-arthritic activity of new ibuprofen derivatives. *Bulgarian Chemical Communications*, *53*(1), 66–71. https://doi.org/10.34049/bcc.53.1.5320

Abstract: Herein, we present the synthesis and in-vitro anti-inflammatory, antioxidant, and anti-arthritic activities of new ibuprofen derivatives. All structures were confirmed by spectral analysis (^{1}H NMR, ^{13}C NMR, UV, IR and HRMS). The lipophilicity was established using reversed-phase thin layer chromatography and in silico calculations. The anti- inflammatory and anti-arthritic activities correlated with the lipophilicity of the compounds.

13. Manolov, S., Ivanov, I., & Bojilov, D. (**2021**). *N*-(2-(1H-Indol-3-yl)ethyl)-2-(6-methoxynaphthalen-2-yl)propanamide. *Molbank*, *2021*(1), M1187. https://doi.org/https://doi.org/10.3390/M1187

Abstract: The title compound was obtained in high yield in the reaction between tryptamine and naproxen. The newly synthesized naproxen derivative was fully analyzed and characterized via ^{1}H, ^{13}C-NMR, UV, IR, and mass spectral data.

14. Manolov, S., Ivanov, I., & Bojilov, D. (**2021**). *N*-(2-(1H-Indol-3-yl)ethyl)-2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanamide. *Molbank*, *2021*, M1177. https://doi.org/10.3390/M1177

Abstract: *N*-(2-(1H-Indol-3-yl)ethyl)-2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanamide was prepared by a reaction between tryptamine and flurbiprofen, applying N,N'-dicyclohexylcarbodiimide, as a coupling agent. The obtained new amide has a fragment similar to Brequinar, a compound used in SARS-CoV-2 treatment trials. The newly synthesized compound was fully analyzed and characterized via ^{1}H, ^{13}C-NMR, UV, IR, and mass spectral data.

15. Manolov, S., Ivanov, I., & Bojilov, D. (**2021**). *N*-(Benzo[d]thiazol-2-yl)-2-(2-fluoro-[1,1′-biphenyl]-4-yl)propanamide. *Molbank*. https://doi.org/10.3390/M1260

Abstract: *N*-(Benzo[d]thiazol-2-yl)-2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanamide was prepared by a reaction between benzo[d]thiazol-2-amine and flurbiprofen in high yield. The newly obtained flur- biprofen derivative was fully analyzed and characterized via ^{1}H, ^{13}C, UV, IR, and mass spectral data.

16. Manolov, S., Ivanov, I., & Bojilov, D. (**2022**). (±)-2-(2-Fluoro-[1,1'-biphenyl]-4-yl)-N-(1-phenylpropan-2- yl)propanamide. *Molbank*, *2022*(1), M1319. https://doi.org/10.3390/M1319

Abstract: 2-(2-fluoro-[1,1'-biphenyl]-4-yl)-N-(1-phenylpropan-2-yl)propanamide was synthesized by a reaction between amphetamine and flurbiprofen in high yields. The newly obtained hybrid molecule was fully analyzed and characterized via ^{1}H, ^{13}C, UV, IR, HPLC, and mass spectral data.

17. Manolov, S., Ivanov, I., Bojilov, D., & Nedialkov, P. (**2022**). Synthesis, in silico, and in vitro biological evaluation of new furan hybrid molecules. *Processes*, *10*(10), 1997. https://doi.org/10.3390/pr10101997

Abstract: Herein, we report the synthesis of new hybrid molecules between furan and N-containing heterocyclic compounds such as pyrrolidine, 1,2,3,4-tetrahydroquinoline, 1,2,3,4-tetrahydroisoquinoline, and piperidine. The obtained compounds were fully characterized using ^{1}H- and ^{13}C-NMR, UV-Vis, and HRMS spectra. All compounds were assessed for their anti-inflammatory, anti-arthritic, antioxidant, reducing power ability, and chelating activity. The less lipophilic molecules H2 (60.1 ± 8.16) and H4 (62.23 ± 0.83) had almost 12 times higher ATA compared with the used ketoprofen (720.57 ± 19.78) standard. The inhibition of albumin denaturation results makes the newly obtained hybrids potential anti-inflammatory drugs, as the expressed values are higher than the ketoprofen standard (126.58 ± 5.00), except H3 (150.99 ± 1.16). All four compounds show significant activity regarding the in vitro biological activities, which makes them great candidates for potential future drugs.

18. Manolov, S., Ivanov, I., & Bojilov, D. (**2022**). Synthesis of New 1,2,3,4-Tetrahydroquinoline Hybrid of Ibuprofen and Its Biological Evaluation. *Molbank, 2022*(M1350). https://doi.org/10.3390/M1350

Abstract: Herein we report the obtaining of 1-(3,4-dihydroquinolin-1(2H)-yl)-2-(4-isobutylphenyl) propan-1-one and its characterization. The newly obtained hybrid and its derivatives (hybrids of ibuprofen with 1,2,3,4-tetrahydroisoquinoline, and piperidine) were screened for their in vitro antioxidant, antitryptic, and inhibition of albumin denaturation activity. The lipophilicity was established using both reversed-phase thin layer chromatography and in silico calculations.

19. Manolov, S., Ivanov, I., Bojilov, D., & Nikolova, G. (**2023**). (±)-N-(3-Chlorophenethyl)-2-(6-methoxynaphthalen-2-yl)propanamide. *Molbank*, *2023*, M1625. https://doi.org/10.3390/M1625

Abstract: Herein we report the synthesis of (±)-*N*-(3-chlorophenethyl)-2-(6-methoxynaphthalen-2-yl)propanamide in the reaction between 2-(3-chlorophenyl)ethan-1-amine and (±)-naproxen. The newly obtained bio-functional hybrid molecule was fully characterized via ^{1}H, ^{13}C NMR, UV, IR, and mass spectral data.

20. Manolov, S., Ivanov, I., Bojilov, D., & Kalinova, Y. (**2023**). *N*-(3-Chlorophenethyl)-2-(4-isobutylphenyl)propanamide. *Molbank*, *2023*(1), M1536. https://doi.org/10.3390/M1536

Abstract: *N*-(3-chlorophenethyl)-2-(4-isobutylphenyl)propanamide was synthesized for the first time in the reaction between 2-(3-chlorophenyl)ethan-1-amine and 2-(4-isobutylphenyl)propanoyl chloride with high yield. The newly obtained chlorine-containing ibuprofen derivative was fully analyzed and characterized using ^{1}H-, ^{13}C-NMR, UV, and mass spectral data.

21. Mollova, S., Dzhurmanski, A., Fidan, H., Bojilov, D., Manolov, S., Dincheva, I., Stankov, S., Stoyanova, A., Ercisli, S., Assouguem, A., Marc, R. A., Ullah, R., & Bari, A. (**2023**). Chemical Composition of Essential Oils from *Nepeta transcaucasica* Grossh. and *Nepeta cataria* L. Cultivated in Bulgaria and Their Antimicrobial and Antioxidant Activity. *ACS Omega*, *8*(17), 15441–15449. https://doi.org/10.1021/acsomega.3c00704

Abstract: The genus Nepeta, belonging to the family Lamiaceae, includes about 300 species, most of which are used in folk medicine due to their pronounced biological properties. The aim of the present study was to evaluate the agrobiological characteristics of *Nepeta transcaucasica* (*N. transcaucasica*) Grossh. and *Nepeta cataria* (*N. cataria*) L., cultivated in Bulgaria, and obtain their essential oils and determine their antimicrobial and antioxidant activities. The agrobiological characteristics of the two species growing in Kazanlak were analyzed; therefore, high variability in the population of *N. transcaucasica* and comparative homogeneity in *N. cataria* was shown. The species *N. transcaucasica* contained 0.28% essential oil with main components β-citronellol (52.05%), eucalyptol (7.34%), β-citronellal (6.06%), germacrene D (5.45%), (Z)-β-ocimene (5.14%), and β-caryophyllene (3.06%). The species *N. cataria* consisted of 0.19% essential oil with main components β-citronellol (26.31%), geraniol (15.92%), neral (11.45%), nerol (9.56%), carvacrol (6.04%), and β-citronellal (5.35%). The antibacterial activity against Gram-positive bacteria Listeria monocytogenes and Staphylococcus aureus and Gram-negative bacteria Escherichia coli (E. coli) and Salmonella

enterica subsp. enterica serovar Abony was determined. The essential oils showed antimicrobial activity only against E. coli. The diameters of the inhibition zones were found to be 26 mm for the species *N. transcaucasica* and 10 mm for the species *N. cataria*. The antioxidant activity of the two essential oils was also determined by four different methods, DPPH, ABTS, FRAP, and CUPRAC, with the highest values for the ABTS radical, for the species N. transcaucasica (48.72 μM TE/mL), and the species N. cataria (310 μM TE/mL).

22. Manolov, S., Ivanov, I., Bojilov, D., & Nedialkov, P. (**2023**). Synthesis, In Vitro Anti-Inflammatory Activity, and HRMS Analysis of New Amphetamine Derivatives. *Molecules*, *28*(1), 151. https://doi.org/10.3390/molecules28010151

Abstract: Herein, we report the obtaining of new hybrid molecules of amphetamine with different profens (amfens). The obtained amfens are characterized by their melting points, UV, ^{1}H–, ^{13}C–NMR, and HRMS spectra. A complete and detailed mass spectral analysis of the newly obtained derivatives of amphetamine with ibuprofen, flurbiprofen, ketoprofen, naproxen, and carprofen was performed. In vitro inhibition of albumin denaturation of each new compound was assessed, and they showed significant activity. The IC_{50} values of the obtained amphetamine-profen derivatives ranged from 92.81 to 159.87 μg/mL. This indicates that the new hybrids inherit the anti-inflammatory properties of profens. Using in silico method, the toxicity was also calculated. The obtained results are given in LD_{50} values. Depending on the route of administration, the amfens are less toxic compared to the standard amphetamine.

23. Bojilov, D., Manolov, S., Nacheva, A., Dagnon, S., & Ivanov, I. (**2023**). Characterization of Polyphenols from *Chenopodium botrys* after Fractionation with Different Solvents and Study of Their In Vitro Biological Activity. *Molecules*, *28*(2), 4816. https://doi.org/10.3390/molecules28124816

Abstract: In the present work, we have investigated the polyphenolic composition of *Chenopodium botrys* from Bulgaria. The polyphenols were fractionated with solvents of varying polarity (n-hexane, chloroform, ethyl acetate, and n-butanol). The fractions were analyzed by HPLC-PDA and UHPLC-MS. The ethyl acetate fraction contained mono- and di-glycosides of quercetin, di-glycosides of kaempferol, and isorhamnetin and monoglycosides of hispidulin and jaceosidine. We found quercetin triglycosides in the butanol fraction. The ethyl acetate and butanol fractions contained 168.82 mg/g Extr and 67.21 mg/g Extr of quercetin glycosides, respectively. The main components of the polyphenolic complex in *C. botrys* were 6-methoxyflavones (355.47 mg/g Extr), which were found in the chloroform fraction. The flavonoids pectolinarigenin, demethylnobiletin, and isosinensetin, and the glycosides of quercetin (triglycosides, acylglycosides), kaempferol, isorhamnetin, hispidiulin, and jaceosidine, were discovered and reported in *Chenopodium botrys* for the first time. We used *in vitro* methods to assess the biological activity against oxidative stress (hydrogen peroxide scavenging activity (HPSA) and hydroxyl radical scavenging activity (HRSA)), nitrosative stress (nitric oxide scavenging activity (NOSA)), anti-inflammatory activity (IAD inhibition), and anti-tryptic activity (ATA). Quercetin mono- and di-glycosides exhibited greater HPSA and HRSA (IC_{50} = 39.18, 105.03 μg/mL), while 6-methoxyflavones had a greater NOSA (IC_{50} = 146.59 μg/mL). The same components showed the highest ATA (IC_{50} ranging from 116.23 to 202.44 μg/mL).

24. Manolov, S., Bojilov, D., Ivanov, I., Marc, G., Bataklieva, N., Oniga, S., Oniga, O., & Nedialkov, P. (**2023**). Synthesis, Molecular Docking, Molecular Dynamics Studies, and In Vitro Biological Evaluation of New Biofunctional Ketoprofen Derivatives with

Different *N*-Containing Heterocycles. *Processes*, *11*(6), 1837. https://doi.org/10.3390/pr11061837

Abstract: Herein, we report the synthesis of four new hybrid molecules between ketoprofen or 2-(3-benzoylphenyl)propanoic acid and *N*-containing heterocyclic compounds, such as piperidine, pyrrolidine, 1,2,3,4-tetrahydroquinoline, and 1,2,3,4-tetrahydroisoquinoline. The obtained hybrid compounds were fully characterized using ^{1}H- and ^{13}C-NMR, UV-Vis, and HRMS spectra. Detailed HRMS analysis is provided for all novel hybrid molecules. The compounds were assessed for their in vitro anti-inflammatory and antioxidant activity. The lipophilicity of the hybrids was determined, both theoretically (cLogP) and experimentally (RM). The affinity of the compounds to the human serum albumin was assessed in silico by molecular docking study using two software, and the stability of the predicted complexes was evaluated by molecular dynamics study. All novel hybrids have shown very good HPSA activity, statistically close when compared to the reference quercetin. The molecular docking confirmed the obtained *in vitro* results. Tetrahydroquinoline derivative **3c** and tetrahydroisoquinoline derivative **3d** have the highest affinity for albumin. They show stronger anti-inflammatory action than their predecessor, ketoprofen and the regularly used ibuprofen.

II. Publications in specialized scientific journals without an impact factor

25. Christoskova, S. G., Stoyanova, M. K., Dimitrov, D. Y., Bojilov, D. G., & Ivanov, K. I. (**2009**). New oxide systems for catalytic abatement of volatile organic compounds and CO in waste gases. *Journal of International Scientific Publication: Ecology and Safety*, *3*(2), 179–187.

Abstract: The promotion effect of Ag on the calalytic behevior of NiOx and CoOx, in reaction of total oxidation of CH_3OH as well as of mixture of CH_3OH, $(CH_3)_2O$ and CO main components of waste gases from production of formaldehyde has been studied. The catalytic samples have been synthesized by thermal decomposition of nitrate salts of the corresponding metals in air. The results obtained reveal that using catalysts investigated a complete catalytic destruction of volatile organic compounds to CO_2 and H_2O qnd oxidqtion of CO to CO_2 is achieved. Moclification of the individual NiOx, and CoOx strongly effects the activity of the catalytic systems causing a decrease of the temperature.for complete conversion qf CH_3OH, $(CH_3)_2O$ and CO to harmless products. In fact, the reaction temperature for different gases studied is as follow: for CH_3OH - 160 - 180 ^oC depending on the catalyst; for CO - 240 ^oC, and 300 ^oC for $(CH_3)_2O$.

26. Topova, S., Bojilov, D., Dagnon, S., & Argirov, O. (**2014**). Hydroxypyridine Formation In Model System Monosodium Glutamate And 2-Furfural. *Applied Science Reports*, *6*(2), 71–77. https://doi.org/10.15192/PSCP.ASR.2014.2.2.7177

Abstract: Monosodum salt of glutamic acid has been approved in Europe as a food additive E 621. It enhances the effect of other taste-active compounds, improving the overall taste of certain foods. There is also an interaction between E 621 and table salt (sodium chloride), and other umami substances such as nucleotides. The aim of the present study was to model a Maillard type reaction of E 621 with carbohydrates during food processing and to analyze the products of this interaction. Formation of the N-containing heterocyclic compounds as hydroxypyridines, imidazole, pyrazine and pyrazinone is a common feature of the Maillard

reaction taking place at temperatures typical for food processing. The formation of 3-hydroxypyridine was observed as reaction product between E 621 and furfural during prolonged but not during short-time heating. The target compound was identified as 3-hydroxypyridine using reverse phase HPLC. The concentration of 3-hydroxypyridine in the reaction mixture was 1 ppm calculated by means of regression analysis. Possible mechanism of this reaction is discussed.

27. Bojilov, D. G., Simeonova, Z. B., Solakov, N. Y., Angelova-Romova, M. Y., Tsvetanova, V. M., & Ivanov, I. I. (**2014**). Chemical composition of *Gleditsia triacanthos* L.-application in phytotherapy of socially significant diseases. *Journal of International Scientific Publications: Materials, Methods and Technologies*, *8*, 382–392. https://www.scientific-publications.net/en/article/1000186/

Abstract: Isolation and identification of biologically active substances from medicinal plant *Gleditsia triacanthos* L. are examined. Development of methods for obtaining medical extracts of *Gleditsia triacanthos* L. is of practical importance in medicine for prevention of cardiovascular system diseases, antispasmodics and biliary clearance acting drugs as well as in medicinal cosmetics.

28. Milina, R., Mustafa, Z., Bojilov, D., Dagnon, S., & Moskovkina, M. (**2016**). Determination and discrimination of biodiesel fuels by gas chromatographic and chemometric methods. *Acta Scientifica Naturalis*, *3*(1), 25–32. https://doi.org/10.1515/asn-2016-0004

Abstract: Pattern recognition method (PRM) was applied to gas chromatographic (GC) data for a fatty acid methyl esters (FAME) composition of commercial and laboratory synthesized biodiesel fuels from vegetable oils including sunflower, rapeseed, corn and palm oils. Two GC quantitative methods to calculate individual fames were compared: Area % and internal standard. The both methods were applied for analysis of two certified reference materials. The statistical processing of the obtained results demonstrates the accuracy and precision of the two methods and allows them to be compared. For further chemometric investigations of biodiesel fuels by their FAME-profiles any of those methods can be used. PRM results of FAME profiles of samples from different vegetable oils show a successful recognition of biodiesels according to the feedstock. The information obtained can be used for selection of feedstock to produce biodiesels with certain properties, for assessing their interchangeability, for fuel spillage and remedial actions in the environment.

29. Edreva, A., Vitkova, A., Dagnon, S., Konakchiev, A., Gesheva, E., & Bojilov, D. (**2017**). Field-cultivated medicinal plants of Achillea millefolium group: a source of bioactive compounds. *Genetics and Plant Physiology*, *7*(1–2), 22–33. https://pdfs.semanticscholar.org/d807/9dc49e9ae3b557fd9706c84d1984eca58372.pdf

Abstract: Medicinal plants of Achillea millefolium group in Bulgarian wild flora are widely used in both traditional medicine and pharmaceutical, cosmetic and nutritive industries. They are intensively collected from the natural habitats, thus leading to a number of negative effects, such as ecological risk of extinction of native sources, and high heterogeneity of the collected market samples. We explored the possibilities for field cultivation of wild *A. millefolium* group species as a promising approach to produce homogenous samples of valuable plants without damage to the natural environment. Our preliminary studies have identified as promising two species of *A. millefolium* group in Bulgaria - *A. collina* and *A. asplenifolia*. The species *A. collina* and *A. asplenifolia*, each with two populations, were studied, while cv. "Proa", bred for

field cultivation, was used as a standard. Seeds were collected from the natural habitats, and those of cv. Proa were purchased from the firm "Pharmasaat GmbH", Germany. Seedlings were produced in a greenhouse and then transferred to the experimental field of the Institute of Plant Physiology and Genetics near Sofia. Plants were harvested at full blossoming stage. Essential oil yield and chamazulene content in the oil, contents of total flavonoids and chlorogenic (3-O-caffeoyl quinic) acid as well as antiradical and antioxidant activities in flower heads were determined. Principal Component Analysis (PCA) of data was performed. The results showed that field-grown plants of the studied *A. millefolium* group species produced significant amounts of bioactive compounds, particularly flavonoids and chlorogenic acid which highly correlated with the antiradical activity. PCA pointed to the relatedness of populations within each species, and the distant positions of the species. The populations of *A. asplenifolia* were distinguished by higher contents of total flavonoids, essential oil, chamazulene and chlorogenic acid as well as by higher antiradical and antioxidant activities as compared to A. collina, while being closer by all parameters to cv. "Proa". The data point to *A. asplenifolia* as a promising species for field cultivation.

30. Dagnon, S., Bojilov, D., Docheva, M., & Edreva, A. (**2018**). The Relationship between Main Polyphenol Components and Free Radical Scavenging Activity of Selected Medicinal Plants. *International Journal of Pharmaceutical Sciences and Drug Research*, *10*(03), 131–138. https://doi.org/10.25004/IJPSDR.2018.100305

Abstract: This study investigates the relationships between the main polyphenols and DPPH radical scavenging activity (RSA) in extracts from some of the most common wild and cultivated species from the *Achillea millefolium* group, Mentha, *Chenopodium botrys*, *Clinopodium vulgare* and *Artemisia vulgaris*. Another aspect of the study is the effects of choosing an extraction solvent, highlighting the importance of selecting an appropriate one for obtaining extracts with maximum manifested bioactivity. Chemical characterization of compounds is performed. It is based on HPLC-PDA fingerprint profiles, obtained from 70% aqueous methanol extracts. Hydroxycinnamic acids, glycosides of luteolin (LG), apigenin (AG), their aglycones and methoxyflavones are found to be the most abundant components, which have the highest impact on radical scavenging activity (RSA) of the extracts. The data allow supposing high correlation relationships between caffeoylquinic acids and RSA of extracts from four *A. millefolium* group species and cv. Proa. Cl. vulgare methanol extract, rich in rosmarinic acid, LG and AG, showed the highest RSA with IC_{50} 10.37μg.mL^{-1}. In contrast, extracts of *Ch. botrys* which lack phenolic acids, contain low amount of glycosides and are rich in methoxyflavone aglycones, possess very weak RSA. For multicomponent analysis, our data suggest 70% methanol as the most appropriate solvent for extraction of compounds belonging to the polyphenol complex. Based on the high quantities of rosmarinic acid, luteolin and apigenin glycosides, 66% ethanol solvent is found to be the best choice for medicinal consumption extracts.

31. Bojilov, D. G., Manolov, S. P., Bazadzhiev, B. V., Stremski, J. I., & Ivanov, I. I. (**2018**). Antioxidant activity in two species common beans (*Phaseolus vulgaris* L.) from village of Smilyan, Bulgaria. *Journal of International Scientific Publication: Agriculture & Food*, *6*, 314–324. https://www.scientific-publications.net/en/article/1001751/

Abstract: Smilyan beans are called bean seeds, one of the few Bulgarian foods protected by a trademark patent for its cultivation in the area of the upper valley of Arda River. Smilyan beans have a unique taste and are famous both in Bulgaria and abroad. Common beans (*Phaseolus vulgaris* L.) from two different cultivars from village of Smilyan were sieve analyzed and assessed for antioxidant activities.

32. Manolov, S. P., Ivanov, I. I., Mollov, V. A., & Bojilov, D. G. (**2019**). Synthesis and *in silico* study of new ketoprofen derivatives. *Materials, Methods & Technologies*, *13*, 250–061. https://www.scientific-publications.net/get/1000036/1568823180682021.pdf

Abstract: Ketoprofen is a widespread non-steroidal anti-inflammatory agent used in treatment of acute pain and chronic arthritis. On the other hand, isoquinoline alkaloids are very well known with their wide range of biological activities. From this point of view it is of interest to us to obtain new isoquinolines containing a ketoprofen residue in their structure as new substances with potential biological activity. To estimate general efficacy and safety of the newly obtained compounds, their biological potential has been evaluated

33. Bojilov, D. G., Manolov, S. P., Ivanov, I. I., & Mollova, S. L. (**2019**). Investigation of antioxidant activity of different extracts of *Helichrysum italicum* from Bulgaria. *Journal of International Scientific Publications: Materials, Methods & Technologies*, *13*, 241–249. https://www.scientific-publications.net/en/article/1001898/

Abstract: *Helichrysum italicum* from the daisy family Asteraceae have been used in the folk medicine for many years for preventive purposes and for restoring the body after a heart attack. The plant material from H. italicum was provided by the Institute of Roses and Essential Oils, Kazanlak, Bulgaria. The antioxidant activity of different H. italicum extracts was evaluated by various methods, such as ABTS, CUPRAC, FRAP and DPPH.

34. Miladinova, R. R., Manolov, S. P., Ivanov, I. I., & Bojilov, D. G. (**2021**). Green synthesis of new hybrid molecules of ibuprofen and 1,2,3,4-tetrahydroisoquinoline. *Journal of International Scientific Publications Materials, Methods and Technologies*, *15*, 195–201. https://www.scientific-publications.net/en/article/1002211/

Abstract: Herein we report an eco-friendly method for synthesis of 1,2,3,4-tetrahydroisoquinoline derivatives of ibuprofen. All five newly obtained compounds are fully characterized with their ^{1}H,^{13}C-NMR, IR, HRMS, and are interesting for the purpose of studying their biological activities.

35. Manolov, S. P., Ivanov, I. I., & Bojilov, D. G. (**2021**). *In vitro* and *in silico* biological activity of new ibuprofen hybrids. *Journal of International Scientific Publications Materials, Methods and Technologies*, *15*, 202–211. https://www.scientific-publications.net/en/article/1002212/

Abstract: Herein we report the biological evaluation of new hybrids of 1,2,3,4-tetrahydroisoquinoline with ibuprofen. The hybrids are screened for their in vitro inhibition of albumin denaturation, antioxidant, and antitryptic activity. The tested compounds have shown significant results. The lipophilicity was established using both reversed-phase thin layer chromatography and in silico calculations.

A group of metrics	Content	Доцент	Score
B	Georgieva, K., Dagnon, S., Gesheva, E., Bojilov, D., Mihailova, G., & Doncheva, S. (**2017**). Antioxidant defense during desiccation of the resurrection plant *Haberlea rhodopensis*. ***Plant Physiology and Biochemistry***, 114, 51–59.	Q1	25
	Dagnon, S., Novkova, Z., Bojilov, D., Nedialkov, P., & Kouassi, C. (**2019**). Development of surrogate standards approach for the determination of polyphenols in *Vernonia amygdalina* Del. ***Journal of Food Composition and Analysis***, 82, 103231.	Q1	25
	Bojilov, D., Dagnon, S., Kostadinov, K., & Filipov, S. (2020). Polyphenol composition of lettuce cultivars affected by mineral and bio-organic fertilization. ***Czech Journal of Food Sciences***, 38(6), 359–366.	Q3	15
	Mollova, S., Fidan, H., Antonova, D., Bozhilov, D., Stanev, S., Kostova, I., & Stoyanova, A. (**2020**). Chemical composition and antimicrobial and antioxidant activity of *Helichrysum italicum* (Roth) G.Don subspecies essential oils. ***Turkish Journal of Agriculture and Forestry***, 44(4), 371–378.	Q1	25
	Dragoev, S., Vlahova-Vangelova, D., Balev, D., Bozhilov, D., & Dagnon, S. (**2021**). Valorization of waste by-products of rose oil production as feedstuff phytonutrients. ***Bulgarian Journal of Agricultural Science***, 27(1), 209–219.	Q3	15
	Mollova, S., Dzhurmanski, A., Fidan, H., Bojilov, D., Manolov, S., Dincheva, I., … Bari, A. (**2023**). Chemical Composition of Essential Oils from Nepeta transcaucasica Grossh. and Nepeta cataria L. Cultivated in Bulgaria and Their Antimicrobial and Antioxidant Activity. ***ACS Omega***, 8(17), 15441-15449.	Q1	25
	Bojilov, D., Manolov, S., Nacheva, A., Dagnon, S., & Ivanov, I. (**2023**). Characterization of Polyphenols from Chenopodium botrys after Fractionation with Different Solvents and Study of Their In Vitro Biological Activity. ***Molecules,*** 28(2), 4816.	Q1	25
			155

Advanced Habilitation Reference

Contents

1. Introduction

My research activity is related to the development and optimization of chromatographic conditions in order to obtain a fingerprint of the polyphenolic complex of medicinal plants and foods of important biological importance. This also includes the identification of secondary metabolites (polyphenols and components of essential oils) using mass spectral methods – GC-MS and UHPLC-MS/MS.

The polyphenol complex of *Habarlea rhodopensis*, *Chenopodium botrys*, *Vernonia amygdalina*, *Rosa damascena* Lactuca spp – *Lactuca sativa Batavia cv. Maritima*, *Lolo rosa cv. Tuska* and *cv. Winter Butterhead* was investigated. This includes analysis, identification, quantification of major polyphenolic components and *in vitro* biological activity. In addition, the essential oil composition of *Nepeta transcaucasica* and *Nepeta cataria* was investigated. The antioxidant activity of the essential oils of *Helichrysum italicum* and both Nepeta spp.

Polyphenols play an extremely important role in the defense mechanisms of plants against various stress factors. It is polyphenols that are strong antioxidants and protect resurgent plants from oxidative stress and death under extreme conditions such as drought. Agroclimatic and soil conditions are important factors influencing the content of polyphenols in plants [Bojilov et al. 2020].

Oxidative and nitrosative stress are the most harmful consequences that are responsible for a number of pathological conditions. They are responsible for the generation of ROS and RNS [Chalana et al. 2022; Galano et al. 2010; Jagetia, 2004; Lala, 1998]. The strength of the antioxidant capacity determines the fate of the cells of living organisms.

2. HPLC-PDA analysis of polyphenols

2.1. Chromatographic fingerprint profiles of non-hydrolyzed extracts.

In the study of the polyphenolic composition of *Haberlea rhodopensis*, *Vernonia amygdalina*, three types of lettuce, *Rosa damascena* and *Chenopodium botrys*, I used experimental methods that included different extraction procedures and different detectors. I used solvents of different polarity for fractionation of the polyphenol complex and HPLC-PDA and UHPLC-MS/MS chromatographic equipment for analysis and identification of the components of the polyphenol composition. Polyphenols are polar compounds containing more than one hydroxyl group, which is why 70% methanol is recommended for their extraction as

the most selective and suitable solvent for high-precision HPLC analyzes (Dagnon et al., 2018). Proper identification and quantification of compounds is required to establish the polyphenolic composition needed to develop HPLC-PDA chromatographic fingerprint profiles. For this reason, through the analysis of a specific extract, the analysis time, the type of acid in the mobile phase and its concentration, as well as the slope of the gradient require optimization. The fingerprint profile is unique and contains a maximum number of well-separated peaks (Dagnon et al., 2018). It serves to identify the polyphenols and trace their amount in different stages of plant development and different extracts.

For the separation and quantification of the polyphenolic components in the investigated plant species, we optimized the chromatographic conditions in order to obtain a "fingerprint". We used Purospher, Kromasil, Sinergi and Nucleosil RP C18 chromatographic columns and mobile phases supplemented with formic and trifluoroacetic acid (TFA). Using these acids, we developed methods to separate the components of the polyphenol complex. [Bojilov et al. 2020, 2023; Dagnon et al. 2019; Dragoev et al. 2021; Georgieva et al. 2017].

2.2. Chromatographic profile of *Habarlea rhodopensis* and quantification of major components. Effect of drying conditions on polyphenolic composition.

Habarlea rhodopensis belongs to the group of resurrection plants. It is endemic to Bulgaria because it grows only in the Rhodopes. It is known that it does not die under extreme natural conditions, after which it recovers under favorable conditions. For example, when drying due to dehydration, it loses its green color and acquires a brown color. It appears that it has dried up, but under conditions of hydration it resurrects ie. restores its vital functions. In this work, we investigated the polyphenolic composition and the influence of extreme conditions on the amount of polyphenolic composition.

Chromatographic separation conditions and detection with a PDA detector established a dominating peak with absorption 280-282 nm. The main component in the polyphenol composition is the phenylethanoid myconoside. In addition, we detected the presence of paucifloside, whose spectrum is characteristic of caffeic acid (λ_{max} 288 nm and 329 nm). Other important compounds in the polyphenol profile show λ_{max} at 274-275 nm and 336-339 nm. They belong to the group of hispidulin 8-*C*-glucosides: namely hispidulin 8-*C*-(2-*O*-syringoyl-*β*-glucopyranoside) (HSG), hispidulin 8-*C*-(6-*O*-acetyl-*β*-glucopyranoside) (HAG) and hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*β*-glucopyranoside) (HASG) [Georgieva et al. 2017].

Quantitative changes in the polyphenols

Extreme conditions affect the polyphenol composition. The research is based on the fact that the plant is subjected to the influence of various stress factors, especially drought. That is why the composition and quantitative changes resulting from the change in conditions were investigated. The total amount of polyphenols was higher in well-hydrated sun plants compared to shade plants, and this was mainly due to twice the amount of myconoside and paucifloside. The amount of hispidulin glucosides was also higher in the well-hydrated sun plants, but the differences were not significant. Furthermore, their amount does not change significantly as a result of drought stress [Georgieva et al. 2017]. Under drought, plants growing under low radiation responded with a greater increase in myconoside and paucifloside than plants growing under high light intensity. Despite some reduction in their amount in moderately dehydrated sun plants (50% RWC), further water loss up to 8% RWC resulted in an increased amount of myconoside and paucifloside compared to their initial values. In contrast, shaded plants responded to drought with a continuous increase in both myconoside and paucifloside, and their amount increased significantly in dry shaded plants (8% RWC). After rehydration of both shaded and sunny plants, the amount of miconoside and paucifloside significantly decreased, reaching the values of well-hydrated plants [Georgieva et al. 2017].

The antioxidant capacity of medicinal plants is known to be due to their polyphenol content and may play an important role in the regenerative properties of plants.[Bojilov et al. 2023; Dagnon et al. 2019; Georgieva et al. 2017].

Our results confirm the increasing polyphenol content upon desiccation and reveal the main role of phenylethanoid glucosides in the protective mechanisms of *H. rhodopensis*. It has been demonstrated that polyphenols protect membranes against desiccation-induced damage as well as against free radical-induced oxidation by intercalating into the lipid bilayer. Thus, it could be concluded that myconoside and paucifluside protect membranes during desiccation of *H. rhodopensis* against oxidative stress [Georgieva et al. 2017].

2.3. Chromatographic profile of *Vernonia amygdalina* Del and quantification of major components.

To obtain representative chromatographic profiles, we used 70% methanol at a ratio of 20:1 for *Vernonia amygdalina* and 50:1 for the surrogate standards (green coffee and *A. asplenifolia* 9602).

Green coffee as a surrogate standard was chosen because of its caffeoylquinic acid profile, which is extremely well characterized (Clifford, 2017). The polyphenolic complex of species of the *Achillea millefolium* group, in particular *A. asplenifolia* 9602, was investigated.

We found many similarities between its chromatographic profiles and UV spectra with those of *Vernonia amygdalina*, especially regarding 3,5- and 4,5-dicaffeoylquinic acids and luteolin-7-*O*-glucoside (Dagnon et al., 2018).

Previously, using two columns – Symmetry and Symmetry Shield C18 – 14 peaks of caffeoylquinic acids were documented in the polyphenolic profile of *Vernonia amygdalina*, although good resolution was not achieved. Data are lacking for luteolin and luteolin glycosides (Johnson et al., 2011). Seventeen peaks with better resolution were achieved on a Synergi max C12 column, (150 × 4 mm (4 μm)) (Phenomenex, Torrance, CA), with 13 peaks identified, including mono- and dicaffeoylquinic acid, rutin, luteolin, and apigenin glycosides, and aglycone luteolin (Ola et al., 2009).

Three conventional C18 columns (Purospher, Kromasil and Nucleosil) were tested in this chromatographic approach [Dagnon et al. 2019]. Different mobile phases and multistep gradients were applied to develop a profile of *Vernonia amygdalina* polyphenols and surrogate standards. Rutin was chosen as the internal standard due to its structural similarity to the analytes and its absence in the polyphenolic profile of *Vernonia amygdalina*. The application of the identification technique using surrogate standards qualitatively established the polyphenol composition in *Vernonia amygdalina* [Dagnon et al. 2019].

Polyphenolic composition of Vernonia Amygdalina

Usually in chromatography a combination of different techniques is required for adequate identification, summarizing retention time data, UV and MS spectra and previously published information. In our experiments, we used two surrogate standards of known composition: green coffee extracts and *A. asplenifolia* 9602 [Dagnon et al. 2019].

In the chromatographic profile of green coffee, the main compounds refer to 3-, 4- and 5-caffeoylquinic acids, as well as 3,4-, 3,5- and 4,5-dicaffeoylquinic acids [Dagnon et al. 2019]. The polyphenolic complex of green coffee was studied in detail by Clifford et al. (1987, 2005) and many other authors [Craig et al., 2016]. Mono- and dicaffeoylquinic acids are the main polyphenolic components in green coffee. In this study, through carefully controlled method conditions, supported by UV spectra from the PDA detector and relative retention times to the

internal standard, by comparing them with the data of the surrogate standard, the phenolic components of *Vernonia amygdalina* were identified. The use of relative retention time in HPLC is preferable because of the variability of chromatographic conditions. In this work, the peak of the internal standard rutin was used to calculate the relative retention time of the compounds [Dagnon et al. 2019].

Undoubtedly, it can be seen that the retention of peaks of *Vernonia amygdalina* coincides with those of the green coffee surrogate standard and the standard mixture of 5-caffeoylquinic acid and luteolin. The UV absorption spectra of the *Vernonia amygdalina* peaks were identical to those of the green coffee profile and the standards 5-caffeoylquinic acid and luteolin. Luteolin-7-*O*-glucoside is a characteristic polyphenolic component of species belonging to the *Achillea millefolium* group [Innocenti et al., 2007]. This fact and the careful study of *A. asplenifolia* 9602 was the main reason for recommending it as a surrogate standard. Regarding peak identity conformation, ethyl acetate and chloroform fractions of *Vernonia amygdalina* and surrogate standards were investigated by mass spectrometry [Dagnon et al. 2019].

The other major components in the *Vernonia amygdalina* profile are 1,5-, 3,5- and 4,5-dicaffeoylquinic acids [Dagnon et al. 2019]. Regarding caffeoylquinic acids, our data confirm, extend and refine previously published data (Ola et al., 2009; Johnson et al., 2011). Furthermore, our data more precisely establish the main polyphenolic components of *Vernonia amygdalina* based on better chromatographic resolution and MS/MS structure elucidation of luteolin glycosides and 1,5-, 3,5-, and 4,5-dicaffeoylquinic acids in the profile of *Vernonia amygdalina* [Dagnon et al. 2019]. Additionally, the presence of luteolin 4′-*O*-glucoside, apigenin 7-*O*-rutinoside, apigenin 7-*O*-glucoside and apigenin as minor constituents in *V. amygdalina* has been described [Dagnon et al. 2019].

2.4. Chromatographic profile of *Rosa damascena* Mill and quantification of major components.

Chromatographic profiles of the polyphenolic complex of dry rose petals, dry-pressed distilled rose petals and wastewater after the hydrodistillation of the essential oil showed absorbance at 280 nm and 352 nm [Dragoev et al. 2021]. In the three fingerprint profiles, the UV spectra from the PDA detector showed mainly peaks of gallic acid, quercetin derivatives and kaempferol. Chromatographic profiles of dry rose petals and distilled waste by-products – dry pressed distilled rose petals and waste water contain components with identical spectra,

therefore no qualitative differences in polyphenolic composition are observed. Careful chromatographic analysis revealed the abundance of polyphenolic compounds in dry rose petals and dry pressed distilled rose petals. Different proportions of three main groups of polyphenolic compounds were found in dry rose petals, in dry pressed distilled rose petals and in wastewater after the isolation of essential oil from *Rosa damascena* [Dragoev et al. 2021]. The lowest content of all components was found in the wastewater. Dry-pressed distilled rose petals contain a significant amount of glycosides as well as kaempferol [Dragoev et al. 2021]. Approximately 50% of all polyphenolic compounds identified in rose petals were found in dry pressed distilled rose petals. Dry pressed distilled rose petals are characterized by the highest kaempferol content. On the contrary, the profile of polyphenols in the wastewater after hydrodistillation of the essential oil showed a low content of kaempferol.

Similar to our results, the presence of flavonol glycosides in distilled rose petals was reported by Schieber et al. (2005). They identified 22 kaempferol and quercetin glycosides, in contrast to our data, which identified 30 polyphenolic compounds: 13 kaempferol glycosides, 10 quercetin glycosides, 6 gallic acid glycosides, and 2 flavonol aglycones. Our data show the presence of the same compounds in dry rose petals with the exception of 6 gallic acid glycosides (1-6) and additional isomers of quercetin galloyl hexoside and kaempferol disaccharide, which are identified for the first time. The data in our study lead to the suggestion that most of the bioactive flavonoid glycosides are preserved unchanged in DDRP after distillation, mainly involving kaempferol derivatives. However, the amounts of quercetin and kaempferol glycosides are quite similar. Like us, Abdel-Hameed et al. (2012) reported that Taif rose residues possess antioxidant activity. Abdel-Hameed et al. (2012) confirmed that phenolic compounds, especially flavonols, were the main antioxidant active components in Taif rose residues.

2.5. Chromatographic profile of Lactuca spp. and quantification of major components.

Three lettuce cultivars (*Batavia cv. Maritima*, *Lolo rosa cv. Tuska* and *cv. Winter Butterhead*) were investigated for distribution and quantification of major polyphenolic compounds. Plants are subjected to various fertilization practices including mineral, organic and bio-fertilizers. The granular organic fertilizers (Italpollina and Arkobaleno) and the two biofertilizers (Lombricompost – granular and EKOprop NX – liquid) were applied for the first time in an experiment aimed at studying the polyphenolic complex in lettuce varieties. In the last decade, polyphenolic compounds of lettuce have been intensively investigated using

HPLC-PDA-MS methods targeting chromatographic profiles and major components [Llorach et al. 2008]. Our research shows that the chromatographic profiles of the three lettuce cultivars (control samples, unfertilized) are the same as those obtained previously [Romani et al. 2002]. Regarding the other chromatographic methods, it should be mentioned the reverse order of elution of 2,3-dicaffeoyltartaric acid (2,3-diCVQ) and quercetin glycosides, which depends on the type of column [Llorach et al. 2008, Materska et al. 2019].

Major polyphenolic compounds in lettuce cultivars (unfertilized samples)

Chromatographic fingerprint profiles of polyphenols in three lettuce cultivars (*Batavia cv. Maritima, Lolo rosa cv. Tuska* and *cv. Winter Butterhead*) (unfertilized control) showed identical peaks belonging mainly to caffeic acid derivatives [Bojilov et al. 2020]. The main components of the polyphenols are 2,3-dicaffeoyltartaric acid (chicory acid) (2,3diQVK), 5-caffeoylquinic acid (5-KHK) and caffeoyltartaric acid (KVK) and two quercetin glycosides [Bojilov et al . 2020]. Three derivatives of caffeic acid in smaller amounts have also been identified by their UV spectra [Romani et al. 2002, Llorach et al. 2008, Materska et al. 2019].

Only quantitative differences in the polyphenols of the three cultivars were recorded from the analysis. The main component is 2,3-diKVK, and its content varies from 1.28 to 5.6 mg/g DW [Bojilov et al. 2020]. The 5-CHC content also varied over a very wide range from 0.79 to 4.4 mg/g DW. The content of quercetin-3-*O*-(6"-*O*-malonyl)-glucoside ranged from 0.09 to 5.2 mg/g DW, which was highest in *Lolo rosa cv. Tuska* [Bojilov et al. 2020]. Recently, the same claim, regarding the high content of quercetin-3-*O*-(6"-*O*-malonyl)-glucoside and its bioactivity, was made in a study on the modification of the phenolic composition in red lettuce by reducing nitrogen supply, which enhances its antiproliferative effects on colorectal cancer cells [Zhou et al. 2019]. Quercetin-3'-*O*-glucuronide (1.71 mg/g DW) and the sum of caffeic acid (CA) derivatives (0.79 mg/g DW) also showed the highest content in red lettuce, while the content of KVK was almost the same in the three cultivars [Bojilov et al. 2020].

Effect of mineral and bioorganic fertilization.

The effect of mineral, organic and bio-fertilizers on the content of polyphenols was evaluated by applying Duncan's test. It allows to compare the data of the type of fertilizers used with those of the control. Duncan's test allows to evaluate the differences between the components in lettuce varieties, on the one hand, and on the other hand, to evaluate how the content of polyphenols changes depending on the type of fertilization. The data show that fertilization of the green cultivars (*Batavia cv. Maritima* and *cv. Winter Butterhead*) changed

the polyphenol content dramatically compared to the control, while smaller differences were observed in red lettuce (*Lolo rosa cv. Tuska*) [Bojilov et al. 2020]. The two green cultivars responded differently to the fertilizer treatment, respectively in cv. Maritima with reduction of polyphenols and in cv. Winter Butterhead as their content increases. Quantitative differences in quercetin glycosides led to qualitative differences, as quercetin-3-*O*-(6''-*O*-malonyl)-glucoside was absent in two samples (cv. Maritima-3 and cv. Winter Butterhead-5). Most affected due to fertilization in cv. Maritima were 2,3-diKVK (0.37 ÷ 2.22 mg/g DW) and quercetin-3'-*O*-glucuronide (0.23 ÷ 0.97 mg/g DW), their content drastically decreasing after use of Italpollina. Previously, a reduction in polyphenols due to different fertilization practices was also observed in Maravilla de Verano lettuce in outdoor experiments [Sofo et al. 2016].

No significant changes in the polyphenolic complex were registered when applying the biofertilizer EKOprop NX [Bojilov et al. 2020]. Therefore, it can be assumed that it is appropriate to use fields without fertilization or fields fertilized with EKOprop NX to grow lettuce cv. Maritima with preserved bioactivity. The data show that the red lettuce *Lolo Rosa cv. Tuska* is characterized by the highest content of polyphenols, which is less affected by the type of fertilization. Therefore, the use of mineral, organic and bio-fertilizers has a rather weak influence on the content of the main polyphenolic components 5-CAC, 2,3-diCVAC and quercetin-3-*O*-(6''-*O*-malonyl)-glucoside [Bojilov et al. 2020]. In the pursuit of lettuce production with high bioavailability and without chemical fertilizers, the use of organic and biofertilizers is recommended. In cv. Winter Butterhead, the response of polyphenolic compounds due to organic and biofertilization was aimed to increase the content depending on the type of fertilizer. The use of organic fertilizer Arcobaleno and biofertilizer EKOprop NX significantly increases the content of the main polyphenols. The high content of polyphenols is directly related to antioxidant and other biological activities. Proven biological properties of 2,3-diKVK, which include antiviral, anti-inflammatory, glucose and lipid homeostasis, neuroprotection and antioxidant effects [Peng et al. 2019].

The data showed that the polyphenolic complex in the three lettuce varieties was quantitatively affected by fertilization depending on the type of lettuce and fertilizers. The content of the main polyphenolic compounds in red lettuce type *Lolo Rosa cv. Tuska* was less affected, while polyphenols in the two green cultivars (*Batavia cv. Maritima* and *cv. Winter Butterhead*) changed dramatically differently depending on the type of fertilization. Therefore, the choice of fertilizer is a very important task for the production of high-quality lettuce with high biological activity without chemical treatment, which is important for human health.

2.6. Chromatographic profile of the fractionated polyphenolic composition of *Chenopodium botrys* and quantification of major components.

Fractionation is a key step in both polyphenol analysis, particularly with UHPLC-MS/MS, and biological activity assessment. The components of the polyphenolic complex are separated by polarity using fractionation. Solvents with varying polarities were used, as follows: hexane, chloroform, ethyl acetate, and butanol.

This stage allows for the completion of the two major tasks. The first objective is connected with removing the matrix's impact and facilitating the identification of components. The second goal is to determine which component of the polyphenolic complex is high in vitro biological activity, and to establish a link between structure and activity. Therefore, in the fractionation of the polyphenolic complex, we used solvents of different polarity - hexane, chloroform, ethyl acetate and butanol.

Content of Total Phenols (TPC) and Tannins (TTC) in the Fractions

Polyphenols, as secondary metabolites, are abundant in the plant world, and contribute considerably to antioxidant and other properties that may benefit human health. As a result, when researching a specific herb, it is critical to assess the total phenolic content and total tannin content of the various extracts, fractions, and tinctures.

In the three fractions, the TPC values were between 18.41 and 159.84 mg GAE/g Extr, while TTC values ranged between 10.40 and 133.08 mgTAE/g Extr. Nevertheless, the ethyl acetate fraction was the smallest, and the TPC and TTC data showed the highest yield of phenolic compounds. Thus, the ethyl acetate fraction was found to be the richest in total phenolics (159.84 mg GAE/g Extr) and total tannins (133.08 mgTAE/g Extr). The biological activity is directly proportional to the quantity of polyphenols in the extracts [Bojilov et al. 2023].

Content of 6-methoxy flavones and quercetin glycosides in the fractions

An important goal of the study was estimating the total amount of flavonoids and glycosides in the fractions that contained the polyphenolic complex from *C. botrys*. TPC is a generic indication and does not provide detailed information about the polyphenolic composition. Chromatographic analysis offers detailed information on the concentration and distribution of the herb's different polyphenol classes. Previously, we examined the 70% methanol extract of *C. botrys* and established the fingerprint profile of the polyphe-nolic

complex, which consists mainly of 6-MFs and a small amount of quercetin glycosides [Bojilov et al. 2017].

In the present research, the polyphenolic complex was fractionated by polarity using several organic solvents, including hexane, chloroform, ethyl acetate, and butanol. The last three fractions were analyzed by HPLC-PDA [Bojilov et al. 2023]. From the chromatographic profiles, we have found that quercetin glycosides were most effectively extracted with polar solvents, such as ethyl acetate and butanol, while 6-MFs were most effectively extracted with the less polar chloroform [Bojilov et al. 2017].

Using a combination of HPLC-PDA and UHPLC-MS/MS analysis, we have discovered that ethyl acetate was able to extract mono- and di-glycosides of quercetin, kaempferol, isorhamnetin, hispidulin, and jaceosidine, while butanol was able to extract the more polar quercetin triglycosides. Quercetin glycosides are shown to be the main con-stituents in both fractions.

The content of quercetin glycosides and 6-methoxy flavones was determined relative to quercetin and hispidulin, respectively. The study results reveal that the ethyl acetate fraction has the highest level of quercetin glycosides (168.82 mg/g Extr), whereas the butanol fraction has a 2.5 fold lower content. The glycosides of kaempferol, isorhamnetin, hispidulin, and jaceosidine are present in low concentrations in the same fraction. The amount of 6-MFs in the chloroform fraction reached 355.47 mg/g Extr.

3. UHPLC-MS Identification of polyphenols by UHPLC-MS

3.1. Identification and structural characterization of polyphenols from *Habarlea rhodopensis*

The identification of the components of the polyphenol composition was confirmed by the mass spectral data obtained for the chloroform and ethyl acetate fractions and the fragmentation of protonated and deprotonated ($[M+H]^+$, $[M-H]^-$) molecular ions [Georgieva et al. 2017].

In the present work, we investigated the structure of polyphenols in negative mode [Georgieva et al. 2017]. The phenylethanoid glucosides miconoside and paucifloside have molecular ions at *m/z* 743 and *m/z* 741, respectively [Georgieva et al. 2017]. Fragmentation of the molecular ion of myconoside revealed fragment ions *m/z* 181 and *m/z* 163, characteristic of dihydrocaffeic

acid. The fragment ions observed for paucifloside m/z 179 and m/z 161 are characteristic of caffeic acid [Georgieva et al. 2017].

In flavonoid-*C*-glycosides, the carbohydrate moiety is directly linked to the flavone core *via* a strong C-C bond. Until now, of the *C*-glycosides, only those at the C-6 and C-8 positions of the flavonoid nucleus are known, so the main task is reduced to the differentiation between 6-*C*- and 8-*C*-glycosides. MS/MS analysis in combination with CID allows the characterization of *C*-glycosides with both negative and positive mode ionization. Analyzes performed by UHPLC-MS/MS revealed the presence of 8-*C*-glycosides. Mass spectral analysis proved the presence of hispidulin 8-*C*-glucosides: namely hispidulin 8-*C*-(2-*O*-syringoyl-*β*-glucopyranoside) (HSG), hispidulin 8-*C*-(6-*O*-acetyl-*β*-glucopyranoside) (HAG) and hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*β*-glucopyranoside) (HASG) [Georgieva et al. 2017].

3.2. Identification and structural characterization of polyphenols from *Vernonia amygdalina* Del.

A total of 17 compounds were identified in the polyphenolic composition of *Vernonia amygdalina* and 9 of them were present in *A. asplenifolia* 9602. The results of the identification of mono- and dicaffeoylquinic acids using the surrogate standard green coffee were confirmed by mass spectral analysis. Furthermore, MS spectra confirmed the presence of luteolin-7-*O*-glucuronide and luteolin-7-*O*-glucoside [Dagnon et al. 2019]. Characteristic ions m/z 285, m/z 151, m/z 133 and m/z 107 prove the presence of luteolin glycosides. The m/z 269, m/z 151, m/z 149 and m/z 117 ions are characteristic of both apigenin and apigenin glycosides [Dagnon et al. 2019]. Ambiguities in the data published so far on the polyphenolic composition of *Vernonia amygdalina* relate to the presence or absence of luteolin and luteolin glycosides. Our results are supported by MS data confirming the presence of the two glycosides luteolin-7-*O*-glucuronide and luteolin-7-*O*-glucoside as major flavonoid components in the polyphenolic complex of *Vernonia amygdalina* [Dagnon et al. 2019].

3.3. Identification and structural characterization of polyphenols from *Rosa damascena* Mill.

Fractionation of the polyphenolic complex of dry rose petals resulted in a low yield of the chloroform fraction (3.79%), in which the aglycones quercetin and kaempferol were identified. In the ethyl acetate fraction (yield 23.67%) only glycosides were found [Dragoev et al. 2021]. In summary, thirty polyphenolic components were identified in dry rose petals,

[Dragoev et al. 2021]. Mainly three groups of polyphenolic compounds in the following order comprise the polyphenolic complex of *Rosa damascena* dry leaves: kaempferol glycosides > quercetin glycosides > gallic acid glycosides [Dragoev et al. 2021]. Thirteen kaempferol glycosides, ten quercetin glycosides, six gallic acid glycosides and two flavonols were identified. MS/MS data revealed the predominant number of kaempferol glycosides in the polyphenolic complex of *Rosa damascena* petals. Fragmentation of the glycosides with the application of low collision energy leads to the cleavage of the glucosyl bond and the loss of 162 Da, 146 Da, 152 Da, 132 Da and 42 Da, which are hexose, (glucose, galactose, rhamnose), galloyl group, pentose and acetyl group. ESI-MS/MS spectra showed the ions of quercetin (*m/z* 301), kaempferol (*m/z* 285) and gallic acid (*m/z* 169). Our data show the presence of flavonol glycosides, mainly kaempferol and quercetin in dry rose petals, except for six gallic acid glycosides and additional isomers of quercetin galloyl hexoside and kaempferol disaccharide, which were identified for the first time. Galloyl glycosides undergo fragmentation with the elimination of the galloyl group (152 Da) and the hexose (162 Da). Characteristic ions for gallic acid are *m/z* 125 and *m/z* 107 [Dragoev et al. 2021].

3.4. Identification and structural characterization of polyphenols from *Chenopodium botrys*.

Flavonoids Identification

In our previous studies, we used UHPLC-MS in the positive mode and reported for the first time 6-MFs as major components in *C. botrys* [Bojilov et al. 2017]. Until now, this group of polyphenols has gained little attention. Therefore, in the present work, we have investigated their structure under the conditions of ESI-MS/MS in the negative mode. Under these conditions, lower noise was observed. Both ionization modes are useful and have aided in the elucidation of the compounds' complex structures. In addition, different fragmentation pathways were observed. Upon further investigation, in addition to the known compounds, we completed the group of methoxyflavones with pectolinarigenin, demethylnobiletin, and the sinensetin isomer isosinensetin, which have not yet been reported in the polyphenolic composition of *C. botrys* [Bojilov et al. 2023]. The fragmentation of 6-MFs showed a well-defined relationship, but the fragmentation mechanism of polymethoxylated flavones was reliant on the quantity of methoxy groups.

On the labels ^{i,j}A and ^{i,j}B refer to the fragments containing A^{-} and B^{-} rings respectively, and the superscripts i and j indicate the C-ring bonds that have been broken. The $^{i,j}A^{-}$ and $^{i,j}B^{-}$ ions undergo further fragmentation by losing a methyl group (–CH_3), –HCO group or small neutral molecules, such as CO and CO_2 [Bojilov et al. 2023].

Initially the 6-MFs molecule ion loses a methyl radical to give the ion $[M–H–CH_3]^{-}$. This ion loses a radical –CHO by producing $[M–H–CH_3–CHO]^{-}$ or $[M\text{-}2H\text{-}CH_3\text{-}CO]^{-}$ ions ([Bojilov et al. 2023]. The resulting ion $[M–H–CH_3–CHO]^{-}$proves the presence of methoxy group at the sixth position in ring A. Furthermore, the same ion systematically undergoes loss of small neutral molecules, such as CO and CO_2 leading successively to formation of ion *m/z* 243, ion *m/z* 215 and ion *m/z* 227 [Bojilov et al. 2023].

Loss of neutral molecules CO and CO_2 may be attributed to the ring C. Similar undergoes the fragmentation of hispidulin, jaceosidin, eupatilin and pectolinarigenin [Bojilov et al. 2023].

The fragmentation of nepetin involves cleavage of C–C bonds at position *1/3* and *0/2* of the C ring and gives structural informative $^{1,3}A^{-}$ and $^{1,3}B^{-}$ and $^{0,2}A^{-}$ ions [Bojilov et al. 2023]. The intensity of the fragment ions depends on the collisional energy (CE). RDA fragmentation may occur in nepetin producing *m/z* 164 and *m/z* 133 ions [Bojilov et al. 2023]. In the high CE spectrum of deprotonated nepetin the ion *m/z* 164 corresponds to the fragment [–H–CH_3-$^{1,3}A^{-}$/–CH_4-$^{1,3}A^{-}$], which is characterizing by a direct loss of methyl group and it is an not intensive peak. Ion *m/z* 133 shows low intensity corresponding to the fragment $^{1,3}B^{-}$. Furthermore, ion [–H–CH_3-$^{1,3}A^{-}$/–CH_4-$^{1,3}A^{-}$] systematically undergoes loss of CO leading to the more intensive ion with *m/z* 136 [–CH_4 -$^{1,3}A^{-}$-CO] [Bojilov et al. 2023]. Ion *m/z* 136 additionally loses CO and CO_2, resulting in the formation of the more intense ion with *m/z* 65 [–CH_4 -$^{1,3}A^{-}$-2CO-CO_2].

The intensity of ion *m/z* 136 depends on the presence of polar groups in ring B. The presence of OH groups leads to an increase in its intensity. The *m/z* 163 ion was obtained by two pathways. One is the retrocyclization of ring C (*0/2*), and the resulting ion is –CH_3 $^{0.2}A^{-}$, and the second pathway involves the cleavage of the C2–C1′ bond between rings C and B, yielding an ion $[M–H–CH_3–HCO–\text{ring B}]^{-}$. Which one is the most likely to be seen is difficult to determine, since the elemental composition of the two ions is the same, i.e., isomeric ions [Bojilov et al. 2023].

The intensity of ion *m/z* 163 depends on the functional groups (OH or OCH_3) in ring B. Therefore, in nepetin and hispidulin, ion *m/z* 163 is of low intensity due to the presence of OH groups. The presence of OCH_3 groups increases the intensity of ion *m/z* 163. The same ion in jaceosidine, eupatilin and pectolinarigenin loses a neutral molecule CO, and an ion *m/z* 135 [–

CH_3 $^{0.2}A^-$–CO] or [M–H–CH_3–HCO–ring B–CO]$^-$ is obtained. The ions *m/z* 164, *m/z* 163, *m/z* 136 and *m/z* 65 are specific to the fragmentation of nepetin in negative mode. They contribute to the identification of 6-MFs being characteristic for the substitution in the A ring. For 6-MFs, these ions characterize the substituents in the A ring. Significant differences in the fragmentation of 6-MFs appear only in the substitution in the B ring [Bojilov et al. 2023].

In addition to 6-MFs, with the capabilities of UHPLC-MS/MS, we have identified the polymethoxylated flavones (PMFs) demethylnobiletin and an isomer of sinensetin [Bojilov et al. 2023]. Previously, we reported the presence of sinensetin and nobiletin [Bojilov et al. 2017]. The loss of CH_3 groups in PMFs under ESI-MS/MS negative mode results in stable quinoid forms [Bojilov et al. 2023]. The same forms undergo rearrangement with the release of neutral molecules CO and CO_2. The resulting fragment ions correspond to [M–H–*i*CH_3–*i*CO]$^-$ and [M–H–*i*CH_3–*i*CO–CO_2]$^-$[Bojilov et al. 2023].

Sinensetin and its isomer have similar spectra, which can be seen in both ionization modes [Bojilov et al. 2023]. The mass spectra of both isomers show that the fragmentation mechanism approaches that of the 6-MFs. The reason for this is that ring A contains three substituents, and under the conditions of ESI-MS/MS (negative and positive mode), after the cleavage of CH_3 groups, ring A regroups in a stable quinoid form [Bojilov et al. 2023]. In order to more thoroughly examination of the structure of the two isomers, we have used two ionization regimes. The cleavage of CH_3 groups and the subsequent RDA cyclization (*1/3*) of ring C leads to the formation of ion *m/z* 168 ([–3CH_3 $^{1.3}A^+$]), which systematically loses neutral molecules CO and leads to the receiving of the ions *m/z* 140 ([-3CH_3 $^{1.3}A^+$–CO]) and *m/z* 112 ([–3CH_3 $^{1.3}A^+$–2CO]). After ion 168 underwent retrocyclization, the product ion 99 was produced [Bojilov et al. 2023]. The second product ion from RDA cyclization has *m/z* 147 ([–CH_3 $^{1.3}B^+$]), which also loses CO, and the product fragment (*m/z* 119) corresponds to ([–CH_3 $^{1.3}B^+$–CO]) [Bojilov et al. 2023]. In the negative mode, the same RDA cyclization is also seen [Bojilov et al. 2023]. In this instance, the resultant fragments differ structurally. Important details about the two isomers' structures are revealed by both modes. We suggest that the isomer is most likely isosinensetin.

The fragmentation of PMFs in negative mode is triggered by an increase in methoxy groups, and is carried out by the mechanisms of nobiletin and demethylnobiletin. In this instance, characteristically, only methyl groups are cleaved in order to produce a stable quinoid form. The majority of the molecules eliminated are neutral molecules, such as CO and CO_2. RDA cyclization yields the ions *m/z* 132 and *m/z* 133 [Bojilov et al. 2023].

As a consequence of this research, we can infer that in *C. botrys*, three novel compounds (pectolinarigenin, sinensetin isomer, and demethylnobiletin) were identified that have not been reported before now.

Identification of Glycosides

C. botrys metabolism is quite diversified, since the main components are 6-MFs, and the glycosides are mostly quercetin, followed by kaempferol, isorhamnetin, and in tiny amounts jaceosidine and hispidulin [Bojilov et al. 2023]. The glycosides were investigated using ESI-MS/MS in negative mode. From the mass spectra, we have found that the fragmentation of the molecular ion $[M–H]^-$ produces an ion (Y_o) due to the loss of carbohydrate units. The fragmentation data were compared to the fragmentation of the standards rutin, quercetin 3-*O*-galactopyranoside, and quercetin 3-*O*-glucopyranoside [Bojilov et al. 2023]. In the ESI-MS spectrum, the molecular ion of rutin $[M–H]^-$ with *m*/*z* 609 was observed [Bojilov et al. 2023]. Its fragmentation produces an ion with *m*/*z* 301 ($[M–H–146–162]^-$) that is related with the loss of the disaccharide rutinose (308 Da). In addition, we detected an ion with *m*/*z* 463, which, following fragmentation, produces an ion with *m*/*z* 301, but one carbohydrate unit of 162 Da (glucose, galactose) is lost. The ESI-MS/MS experiment and fragmentation of ion 301 showed the formation of ions with *m*/*z* 179 ($[^{1,2}A^-]$), *m*/*z* 151 ($[^{1,2}A^–CO]$) and *m*/*z* 107 ($[^{1,2}A^–CO–CO_2]$), which are characteristic of the aglycone quercetin [Bojilov et al. 2023]. This method simplifies the identification and fragmentation of the glycosides found in the two polar fractions. In the butanol fraction and ethyl acetate fractions, 16 new glycosides were identified, which are reported for the first time in the polyphenolic composition of *C. botrys*.

Peak **1** with t_R = 12.11 min [Bojilov et al. 2023] was identified as quercetin 3-*O*-(2″-*O*-apiofuranosyl-6″-*O*-glucopyranosyl)-*β*-D-glucopyranoside. In the MS/MS experiment, the molecular ion *m*/*z* 757 lost a pentose unit of 132 Da, and an ion *m*/*z* 625 ($[M–H–132]^-$) was obtained. The same ion lost 162 Da, and the resulting ion corresponds to $[M-H-132-162]^-$ with *m*/*z* 463, which lost another 162 Da, and the resulting ion 301 fragmented into the characteristic quercetin ions *m*/*z* 179 ($[^{1,2}A^-]$), *m*/*z* 151 ($[^{1,2}A^–CO]$) and *m*/*z* 107 ($[^{1,2}A^–CO–CO_2]$). The derivatives of the aglycone quercetin were discovered to be peaks **2**–**4** [Bojilov et al. 2023]. All three glycosides are isomers with the molecular ion *m*/*z* 841. According to mass spectrum analyses, the molecular ion's structure comprises two units of rhamnose and one hexose (glucose and galactose). Furthermore, the glycoside is acylated with malonic acid. During the MS/MS experiment, the molecular ion sequentially lost two units of rhamnose (2 × 146 Da) and malonic acid, yielding a *m*/*z* 463 ion. The resulting ion from the MS/MS experiment was

quercetin, and its specific fragment ions were *m/z* 179 [$^{1,2}A^-$], *m/z* 151 [$^{1,2}A^-$–CO] and *m/z* 107 [$^{1,2}A^-$–CO–CO_2]. Peak **5** with t_R 13.77 min is molecular ion *m/z* 883, and was identified as Quercetin 3-*O*-*α*-L-rhamnopyranosyl-*α*-L-rhamnopyranosyl-*β*-D-malonylglucopyranoside (acyl). The molecular ion lost an acyl group, yielding the ion *m/z* 841 ([M–H–acyl]$^-$). This indicates that the molecular ion was acylated with acetic and malonic acids. The same ion was structurally identical to the one before it, and fragments in the same way.

Based on the examination of quercetin glycosides in the ethyl acetate fraction and MS/MS data, we discovered that the fragmentation of the di-glycosides was identical to that of rutin [Bojilov et al. 2023]. We discovered mono- and di-acyl glycosides of quercetin (peaks **8**, **9**, **15**) in the polyphenol composition. Peak **8** (t_R 12.31 min) [Bojilov et al. 2023] was identified as Quercetin-*O*-(acyl)-hexoside-hexoside with molecular ion *m/z* 667. It loses an acyl group and produced the ion *m/z* 625 ([M–H–acyl]$^-$), which is an ion with two hexoses in its structure. The resulting ion gradually lost 162 Da, generating the ions *m/z* 463 ([M–H–acyl–162]$^-$) and *m/z* 301 ([M–H–acyl–162–162]$^-$). Fragment ions *m/z* 179 [$^{1,2}A^-$], *m/z* 151 [$^{1,2}A^-$–CO] and *m/z* 107 [$^{1,2}A^-$–CO–CO_2] prove that the molecular ion *m/z* 667 is a quercetin glycoside. Peaks **9** and **15** are distinguished by the same molecular ion, *m/z* 709. We identified them as quercetin diacyl glycosides using MS/MS data. The molecular ion lost an acyl group, yielding an ion *m/z* 667 ([M–H–acyl]$^-$), which then lost a neutral molecule CO, yielding an ion *m/z* 639 ([M–H–acyl–CO]$^-$). Furthermore, the molecular ion lost 204 Da (acyl+hexose) and produced ion *m/z* 505 ([M–H–acyl–hexose]$^-$). The same ion lost another acyl group, and the resulting ion was *m/z* 463 ([M–H–2acyl–hexose]$^-$). The resulting ion from the MS/MS experiment was quercetin, and its characteristic ions were *m/z* 179 [$^{1,2}A^-$], *m/z* 151 [$^{1,2}A^-$–CO] and *m/z* 107 [$^{1,2}A^-$–CO–CO_2].

Peak **18** (t_R 14.85 min) [Bojilov et al. 2023] was identified as Kaempferol-*O*-(acyl)-hexoside-*O*-(acyl)-hexoside with molecular ion *m/z* 693. It lost 204 Da immediately, yielding the *m/z* 489 ion, and then lost an acyl group, yielding the *m/z* 447 ion. The same ion lost 162 Da and produced an ion with *m/z* 285. Under ESI-MS/MS conditions, it fragmented to the ions *m/z* 151 [$^{1,3}A^-$], *m/z* 125 [$^{1,4}A^-$] and *m/z* 107 [$^{1,3}A^-$-CO_2].

Peaks **10**, **13** and **16** [Bojilov et al. 2023] were identified as isorhamnetin glycosides. Peaks **10** and **13** have the molecular ion *m/z* 623, indicating a di-glycoside comprising glucose and rhamnose. They were identified as Isorhamnetin 3-*O*-glucopyranoside-7-*O*-rhamnopyranoside and Isorhamnetin 3-*O*-glucopyranoside-rhamnopyranoside. The glycoside **10** (Peak **10**) lost 162 Da and produced ion *m/z* 461 [M–H–162]$^-$. The same ion lost rhamnose moiety, and the ion 315 [M–H–162–146]$^-$ corresponds to isorhamnetin. Glycoside **13** (Peak **13**) successively lost 146 Da; the product ion 477 corresponded to [M–H–146]$^-$, which then lost

another 146 Da. The resulting ion [M–H–146–162]$^-$ was isorhamnetin. The characteristic ions for isorhamnetin are *m/z* 151[$^{1.3}$A$^-$], *m/z* 137 [–CH$_3$ $^{0.2}$B]$^-$, *m/z* 125 [$^{1.4}$A$^-$] and *m/z* 107 [$^{1.3}$A$^-$–CO$_2$]. Peak **16** was identified as Isorhamnetin 3-*O*-glucopyranoside with molecular ion *m/z* 477. The fragmented ion was 315. The characteristic ions for the same flavonol are *m/z* 151[$^{1.3}$A$^-$], *m/z* 137 [–CH$_3$ $^{0.2}$B]$^-$, *m/z* 125 [$^{1.4}$A$^-$] and *m/z* 107 [$^{1.3}$A$^-$–CO$_2$].

Peaks **14** and **19** [Bojilov et al. 2023] were identified as Jaceosidin 7-*O*-glucopyranosid and Jaceosidin 4′-*O*-glucopyranosid. These are isomers with molecular ion *m/z* 491. During the fragmentation, it produces stable quinoid ion 328. The specific ions that characterize the aglycon jaceosidin are *m/z* 164 [CH$_3$ $^{1.3}$A$^-$–H], *m/z* 163 [CH$_3$ $^{0.2}$A$^-$], *m/z* 147 [$^{1.3}$B$^-$], *m/z* 136 [CH$_3$ $^{1.3}$A$^-$–CO] and *m/z* 133 [–CH$_3$ $^{1.3}$B$^-$].

Peak **17** [Bojilov et al. 2023] was identified as Hispidulin 7-*O*-glucopyranoside with molecular ion *m/z* 461. An ion (*m/z* 297) with a stable quinoid form was produced during ESI-MS/MS. Hispidulin as 6-MF generated the characteristic ions *m/z* 164 [CH$_3$ $^{1.3}$A$^-$–H], *m/z* 163 [CH$_3$ $^{0.2}$A$^-$], *m/z* 136 [CH$_3$ $^{1.3}$A$^-$–CO] and *m/z* 117 [$^{1.3}$B$^-$].

Fractionation of polyphenols is an effective approach. In the polar fractions (ethyl acetate and butanol), we identified glycosides (mono-, di-, tri-) of quercetin, diglycosides of kaempferol, isorhamnetin, and monoglycosides of isorhamnetin, hispidulin, and yaceosidine, reported for the first time. While in the chloroform fraction we identified pectolinarigenin, demethylnobiletin and isosinsetin. With HPLC analysis, we found that from the group of glycosides, quercetin glycosides are the main components.

4. GC-MS analysis of essential oil

4.1. Composition of the essential oil of Nepeta spp.

From the analysis, 51 components were identified, constituting 99.72% of the total content of *N. ranscaucasica* oil. Thirteen of them were in concentrations over 1%, and the rest of the 38 constituents were in concentrations under 1%. The major constituents of the oil are as follows: *β*-citronellol (52.05%), eucalyptol (7.34%), *β*-citronellal (6.06%), germacrene D (5.45%), (*E*)-*β*-ocimene (5.14%), and *β*-caryophyllene (3.06%) [Mollova et al. 2023].

Forty one compounds were identified in the composition of *N. cataria*, representing 99.72% of the total content identified in the oil. Ten of them were in a concentration over 1%, and the rest of the 30 constituents were in concentrations under 1%. The major constituents (about 3%) of the oil are as follows: *β*-citronellol (26.31%), geraniol (15.92%), neral (11.45%), nerol (9.56%), carvacrol (6.04%), and *β*-citronellal (5.35%) [Mollova et al. 2023].

The chemical composition of the two investigated oils is similar, with the identified components differing only in quantity. The *N. transcaucasica* essential oil has a higher content of oxygenated monoterpenes eucalyptol and *β*-citronellol, of the monoterpene hydrocarbon *β*-ocimene, of the sesquiterpene hydrocarbon germacrene D, and nepetalactones. A higher amount of monoterpene alcohols nerol and geraniol, aldehydes neral and geranial, and phenol carvacrol was found in the oil of the *N. cataria* species [Mollova et al. 2023].

The analysis data show that oxygenated monoterpenes dominate (78.38 and 84.93%) in the composition of the studied essential oils, followed by sesquiterpene hydrocarbons (10.02% and 3.53%).

The comparative analysis of the chemical composition of *N. transcaucasica* essential oil shows that the amounts of the main components differ from the data in the literature. According to Baranauskienė the main compounds were oxygenated monoterpenes such as citronellol (17.69 %), geranial (9.05 %), and geranyl acetate (8.20 %) [Baranauskienė et al. 2019]. Mishurova determined that the main compounds were monoterpene hydrocarbons such as *α*-pinene (2.2 %), *β*-pinene (11.8 %), myrcene (0.5 %), limonene (7.4 %), and *γ*-terpinene (7.81 %) [Mishurova & Shikhiev 1977]. According to Pelyakh the main compounds were oxygenated monoterpenes such as citral (8.1−24.6%) and citronellol (21.6−54.8%) [Pelyakh et al. 2016]. According to Kilic the main compound was oxygenated monoterpene 1,8-cineole (14.4%) [Kilic, 2018].

In the essential oil studied by us, the amounts of nepetalactones were deficient, in contrast to the data of Baranauskienė (14.34%), Kilic (18.5%), Karakuş (93.75%), and İşcan (over 65%) [Baranauskienė et al. 2019; Kilic, 2018; Karakuş et al. 2019; Işçan et al. 2011].

In the case of *N. cataria* essential oil, differences were also found with the data from the literature regarding the amounts of oxygenated monoterpenes such as *β*-citronellol, geraniol, neral, nerol, carvacrol, and *β*-citronellal. In *Nepeta cataria* essential oil, the content of nepetalactones is low compared to the data reported in the literature, reaching 70-81% [Baranauskienė et al. 2019; Tiwari et al. 2023; Azizian et al. 2021]. These differences can be explained mainly by how essential oils are obtained. In the steam distillation used in this study, only the primary oil is separated in the receiver of the apparatus, and the resulting distillates are discarded. In laboratory glassware, where the raw material is processed by water distillation, primary and secondary oils are separated in the receiver. The raw material boils with the distillation water, which can change the oil's chemical composition. The influence of various technological factors, such as temperature, pressure, and duration, on the chemical composition,

including the content of nepetalactone, was investigated in the preparation of extracts with CO_2 and essential oil of the species Nepeta persica [Khajeh et al. 2010].

5. Investigation *in vitro* biological activity

5.1. Investigation of antioxidant activity

The extremely important Cu^{2+} and Fe^{2+} ions take part as cofactors in a variety of enzymes and physiological processes in the human body. However, when they are present in their free state, they have a harmful impact, because Cu^{2+} ions accelerate the oxidation of ascorbic acid, which produces ROS such superoxide radicals ($O_2^{\bullet-}$) and H_2O_2. Additionally, the Fenton reaction causes the Cu^{2+} and Fe^{2+} ions to interact with H_2O_2 and produce hydroxyl radicals ($^{\bullet}OH$) [Chalana et al. 2022]. Proteins, DNA, and essential biological components like phospholipids are all damaged by ROS. This chemical damage is known as oxidative stress, and it is thought to be the cause of many health problems, including cancer, cardiovascular disease, atherosclerosis, and Alzheimer's disease [Galano et al. 2010].

In addition to the oxidative stress mechanisms, the inflammatory process promotes and increases the generation of ROS. The most common cause of inflammation is the generation of superoxide anion radicals, which is linked to the creation of other ROS species such as H_2O_2. It also participates in reduction decomposition processes (known as the Haber–Weiss reaction) and organic hydroperoxides' ROOH [Halliwell, 1985]. The most potent oxidant is the hydroxyl radical ($^{\bullet}OH$), which is produced by ROS. It is produced by one-electron reductions in molecular oxygen (O_2) in cellular metabolism, and is the primary cause of cytotoxicity in aerobic species, including humans [Bektaşoğlu et al. 2006].

5.1.1. Investigation of the antioxidant activity of essential oils from *Helichrisum italicum* and Nepeta spp.

In the present work, we investigated the antioxidant activity of the essential oil of two species of Nepeta and *Helichrysum italicum* from Bosnia and France. We used different methods to evaluate the ability to deactivate free radicals and the ability to reduce copper (Cu^{2+}) and iron (Fe^{3+}) ions.

Depending on the mechanism of the antioxidant reaction, the methods were divided into hydrogen atom transfer (HAT) and single electron transfer (SET) techniques. The HAT method is based on the antioxidant's ability to quench free radicals via a hydrogen donor. SET methods are characterized by the reductive ability of one electron transfer of the tested antioxidant to a

radical species. FRAP and CUPRAC are single electron transfer methods. ABTS and DPPH methods are considered as methods using both hydrogen and single electron transfer [Jimenez et al, 2004; Prior et al, 2005].

We used the ABTS, DPPH, CUPRAC, and FRAP methods to evaluate the antioxidant activity of the essential oil of *Helichrysum italicum* and of Nepeta spp. The antioxidant action of the oils is explained by the high content of oxygen containing monoterpenes [Mollova et al. 2020, 2023].

5.1.2. Investigation of the antioxidant activity of the fractionated polyphenolic composition from *Chenopodium botrys.*

In the present work, we investigated the possibility that polyphenols from *Chenopodium botrys* could prevent the harmful effects of hydrogen peroxide, hydroxyl radicals, and nitroxide radicals. It should be kept in mind that ROS and RNS are products of oxidative and nitrosative stress, respectively, which occur in aerobic organisms, including humans. To evaluate the antioxidant effect, the polyphenolic complex was fractionated using solvents of different polarities. In sections 2.6 and 3.4 the polyphenol composition is discussed in detail. Polyphenolic components in all fractions were analyzed and identified by HPLC-PDA and UHPLC-MS. In this section, we explore the potential biological activity as well as the structure-activity relationship.

Hydrogen Peroxide Scavenging Activity (HPSA)

We compared the results obtained for the antioxidant activity of the individual fractions with natural compounds with proven antioxidant properties such as ascorbic acid and quercetin. The antioxidant activity values of the obtained fractions ranged from 39.18 *μg/mL* to 978.07 *μg/mL* [Bojilov et al. 2023].

Compared to ascorbic acid (24.84 *μg/mL*) and quercetin (69.25 *μg/mL*), the obtained fractions demonstrated low HPSA except for the ethyl acetate fraction. It shows a significantly higher antioxidant activity compared to the other fractions and about two times higher than that of the standard quercetin [Bojilov et al. 2023]. Antioxidant activity depends on the structure of flavonoids, the presence of glycosyl residues and methoxy groups [Russo, 2018]. Therefore, quercetin mono- and di-glycosides are characterized by a higher HPSA, compared to methoxy flavones and quercetin triglycosides. Methoxy flavones show low activity. The presence and increase in the number of methoxy groups in ring B decreases the degree of inhibition of

hydrogen peroxide. But they have higher activity than quercetin triglycerides. As the mass of the glycosyl residue in the C-3 position increases, the antioxidant activity decreases [Xiao, 2017]. This is the reason why the butanol fraction shows a low HPSA. From the analysis, we found that antioxidant activity is very well associated with the content of total phenols, tannins and quercetin glycosides. The HPSA of the fractions decreased in the following order: EtOAc > CHCl3 > n-Hexane > BuOH [Bojilov et al. 2023].

Hydroxyl Radical Scavenging Activity (HRSA)

The structure of polyphenols influences *HRSA* levels. Our standards are structurally distinct. The polyphenolic content of the obtained fractions has a flavonoid core structure. For this reason, as a reference, we utilized quercetin. The concentration gradient of quercetin glycosides is directly proportional to the antioxidant activity. Quercetin mono- and di-glycosides present in the ethyl acetate fraction showed significantly higher *HRSA* (IC_{50} = 105.03 µg/mL) [Bojilov et al. 2023], while the other three fractions showed *HRSA* that was statistically close to quercetin. From the analysis, we have established that the polar and non-polar polyphenols of *C. botrys* have a higher propensity to scavenge the highly active OH radicals [Bojilov et al. 2023]. This is explained by the presence of free OH groups (C-3′ and C-4′) in ring B.

6-Methoxy flavones differ in the functional groups in ring B. They contain OH groups (hispidulin and nepetin) and OCH_3 groups (C-3′ for jaceosidine and C-3′, C-4′ for eupatilin). The *O*-methylation of OH groups in ring B leads to a decrease in HRSA [Russo, 2018; Treml, 2016]. Quercetin triglycosides showed low HRSA compared to quercetin mono- and di-glycosides. This is due to the fact that the *O*-glycolation of the C-3 OH group and the increase in carbohydrate units leads to a decrease in antioxidant capacity [Russo, 2018, Xiao, 2017]. The mass spectrum analysis confirms this result, demonstrating that the C-3 OH group is conjugated with carbohydrate units. This fact is most likely due to the low HPSA and HRSA of the butanol fraction.

Nitric Oxide Scavenging Activity (NOSA)

NO is produced from the terminal guanido nitrogen atom of L-arginine by NO synthases (NOS), which are NADPH-dependent enzymes. NO is a bioregulatory molecule that is necessary for a variety of physiological activities, such as brain signal transmission, immunological response, vasodilation, blood pressure control, etc. By interacting with the NO

molecule, the superoxide ion (O^{2-}) generates intermediate products such as NO_2, N_2O_4 and N_3O_4, and stable compounds such as nitrate and nitrite. It is the formation of reactive nitrogen species (RNS) known as nitrosative stress that is responsible for several pathological conditions, including cancer [Jagetia, 2004; Lala, 1998]. In comparison to quercetin (IC_{50} = 64.82 µg/mL), the fractions had a lower NOSA. This demonstrates that the flavonoid core is more effective in quenching NO radicals. The IC_{50} values for the different fractions varied from 146.59 to 227.12 µg/mL [Bojilov et al. 2023].

Similar reliance is proven for both HPSA and HRSA, i.e., quercetin mono- and di-glycosides are more active. We can deduce from this that they have the power to counteract ROS. An inverse association was seen in the deactivation of NO radicals, showing that 6-methoxyflavones displayed better NOSA than quercetin glycosides. Aglycones were discovered to have a higher affinity for NO radicals, and the presence of OCH3 groups in ring B led to the increased antioxidant activity [Matsuda et al. 2003]. This might explain why the chloroform fraction has a high NOSA.

We may derive valuable knowledge on the characteristics of the physiologically active components present in *C. botrys* from the experimental results. Aerobic organisms can be shielded from the negative effects of ROS by quercetin mono- and di-glycosides, which are potent oxidants. 6-мethoxy flavones, on the other hand, are more effective in scavenging NO radicals.

5.2. In vitro study of anti-inflammatory and anti-arthritic activities of the fractionated polyphenolic composition from *Chenopodium botrys*.

Inhibition of Albumin Denaturation (IAD)

Inflammation is the process by which living tissues respond to stimuli caused by inflammatory factors such as physical damage, heat, microbial infections, and harsh chemical irritants. The response of the cells to inflammation will lead to certain pathological manifestations characterized by redness, heat, swelling, and pain, even causing impairments in physiological functions. Numerous disorders, including arthritis, stroke, and cancer, include inflammation as a pathogenic factor. Protein denaturation is closely linked to the initiation of the inflammatory response, which results in a variety of inflammatory disorders, including arthritis [Osman et al.2016]. According to Opie [Opie, 1962], tissue injury during life might be related to the denaturation of the protein constituents of cells or of intercellular substances. Hence, the ability of a substance to inhibit the denaturation of protein represents its potential for anti-inflammatory activity.

The resultant *C. botrys* fractions were evaluated for albumin denaturation inhibition. This approach determines the extent to which albumin may be preserved against denaturation by heating. Human albumin was employed for this purpose. The study's results are shown as IC_{50} values. We opted to utilize ibuprofen and ketoprofen as standards to examine the performance of different *C. botrys* fractions, since they have documented anti-inflammatory capabilities. The IC_{50} values of ibuprofen and ketoprofen estimated as IAD were 81.50 µg/mL and 126.58 µg/mL, respectively [Bojilov et al. 2023]. The obtained data reveal that the IC_{50} values of the various fractions ranged from 461.41 to 945.06 µg/mL [Bojilov et al. 2023].

The fractions show a lower degree of protection of albumin from denaturation compared to the standards. When evaluating the degree of protection of albumin in conformity with the polarity of the fractions, the ethyl acetate fraction showed the highest activity. This suggests that the configuration of polar quercetin mono- and di-glycosides has a higher propensity for allosteric binding to the albumin molecule. In this way, it is stabilized and resistant, and prevents the denaturation of albumin. As a result, the ethyl acetate fraction is ideal for extracting physiologically active quercetin mono- and di-glycosides. The activity of quercetin triglycosides did not differ significantly from that of 6-methoxy flavones.

Antitryptic Activity (ATA)

Proteinases have been identified as the root cause of arthritic disorders. Neutrophils are known to be a significant source of proteinases, with a substantial number of serine proteinases in their lysosomal granules. Proteinase, which is found in leukocytes, is known to play a major role in tissue damage during inflammatory responses, and proteinase inhibitors give considerable protection [Oyedapo & Famurewa, 1995].

In vitro anti-arthritic activity was assessed as anti-tryptic activity [Oyedapo & Famurewa, 1995]. The purpose of this study is related to the inhibition of the enzyme trypsin, which is also from the group of serine proteinases.

The IC_{50} values for *ATA* varied from 116.23 to 561 µg/mL. The results of the experiment reveal that the polar fractions had a stronger inhibitory efficacy than ibuprofen and ketoprofen. Higher *ATA* (116.23–202.44 µg/mL) was observed in ethyl acetate, butanol, and chloroform fractions [Bojilov et al. 2023].. As a result of the investigation, we discovered that quercetin glycosides and 6-methoxy flavones have more activity than ordinary profens. The catalytic core of the enzyme is made up of three polar amino acids: serine, aspartate, and histidine.

According to scientific evidence, quercetin creates H-bonds with the polar amino acids involved in the active site of trypsin [Xue et al. 2017]. As a result, we assume that the quercetin

core of the glycosides, as well as the methoxyflavones, create H-bonds, inhibiting the action of the enzyme. The high activity of quercetin glycosides is due to the fact that the quercetin core is compatible with the active center of trypsin. The following lists the anti-tryptic activities of *C. botrys* fractions in decreasing order: EtOAc > BuOH > $CHCl_3$ > *n*-Hexane. This indicates that quercetin mono- and di-glycosides are more active in inhibiting trypsin, followed by quercetin triglycosides and 6-methoxy flavones.

Quercetin glycosides and 6-methoxy flavones are characterized by high in vitro anti-arthritic activity assessed by ATA, but only mono- and di-glycosides demonstrate high *in vitro* anti-inflammatory activity. As a result, fractionation is an important stage of the analysis, in which the components are separated based on polarity, and each class of molecules may more accurately reveal its medicinal and pharmacological purpose.

6. Self-assessment of contributions

1. Development of a chromatographic fingerprint profile of the polyphenolic composition of the investigated medicinal plants and three types of lettuce. Chromatographic profiles are used to establish a characteristic chemical pattern for the plant, fraction or extract. The chromatographic profile is of utmost importance in the development of methods for the analysis of foods, medicinal plants and herbal medicines. The key points in generating chromatographic profiles of polyphenol fingerprints are the correct extraction procedure and chromatographic conditions leading to the maximum number of well-separated peaks. Typically, 60–70% aqueous methanol is used in HPLC analysis for the extraction of phenolic acids and flavonoid glycosides [Georgieva et al. 2017; Dagnon et al. 2019; Bojilov et al. 2020, 2023; Dragoev et al. 2021].
2. Use of surrogate standards to identify polyphenols in unexplored herbs. With their help, caffeoylquinic acids (mono and di), apigenin and luteolin glycosides were identified [Dagnon et al. 2019].
3. Fractionation of polyphenols is a useful step in their analysis by HPLC-PDA and UHPLC-MS-MS [Georgieva et al. 2017; Dagnon et al. 2019; Bojilov et al. 2023; Dragoev et al. 2021].
4. The findings have scientific and applied contributions in HPLC-MS/MS for identification and structure elucidation of biologically active compounds. The structure of 6-methoxy flavones and glycosides was investigated in negative ionization mode. The polyphenol composition in medicinal plants has been

identified [Georgieva et al. 2017; Dagnon et al. 2019; Bojilov et al. 2023; Dragoev et al. 2021]. Glycosides and flavones, which have not been reported so far in the composition of *Chenopodium botrys*, have been identified [Bojilov et al. 2023]. In the polyphenolic composition of *Rosa damascena*, 6 gallic acid glycosides, quercetin galloyl hexoside and kaempferol disaccharide were identified, which were reported for the first time [Dragoev et al. 2021]. Caffeoylquinic acids, apigenin and luteolin glycosides have been identified in *Vernonia amygdalina*.

5. The composition of the essential oil of two species of Nepeta - *Nepeta transcaucasica* and *Nepeta cataria* - was studied. In both oils, the oxygen content of monoterpenes is the highest. The main components in *N. transcaucasica* are *β*-citronellol, eucalyptol, *β*-citronellal, germacrene D, (*E*)-*β*-ocimene and *β*-caryophyllene, while in *N. cataria* are *β*-citronellol, geraniol, neral, nerol, carvacrol and *β*-citronellal [Mollova et al. 2023].
6. The antioxidant activity of the essential oils of *Helichrysum iatalicum* and Nepeta spp. The essential oils of *H. italicum* (French cultures) and *N. cataria* are characterized by high antioxidant activity due to the high content of oxygen-containing monoterpenes.
7. The fractionated polyphenolic composition of *C. botrys* was tested for *in vitro* biological activity. The fractions were found to exhibit antioxidant activity assessed by HPSA, HRSA and NOSA. Quercetin diglycosides exhibit an affinity for scavenging ROS, while 6-methoxy flavones are more active for scavenging nitroxide radicals. Quercetin glycosides and 6-methoxy flavones are characterized by high *in vitro* anti-arthritic activity assessed by ATA, but only mono- and diglycosides demonstrate high *in vitro* anti-inflammatory activity.
8. The content of three types of lettuce - *Lactuca sativa Batavia cv. Maritima*, *Lolo rosa cv. Tuska* and *cv. Winter Butterhead*. The relationship between different fertilization practices and polyphenol composition was investigated. It was found that the application of different fertilization practices influenced the content of polyphenols. The use of organic fertilizers leads to an increase in their content in *Lolo rosa cv. Tuska* and *cv. Winter Butterhead*. *Batavia cv. Maritima* is the most sensitive to the changes, with their content in the unfertilized samples being higher.
9. The antioxidant activity of 7 types of wild growing edible mushrooms (*Boletus pinophilus*, *Cantharellus aurora*, *Cantharellus tubaeformis*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Morchella esculenta,* and *Tricholoma equestre*)

collected from the Rhodope Mountains in the area of the city of Batak was determined. DPPH, ABTS, FRAP and CUPRAC were used as methods to evaluate their antioxidant activity. Values are presented as TEAC. Trolox was used as a standard. *Boletus pinophilus* is characterized by the highest activity.

10. A mass spectral analysis of variously substituted 2-phenylethylamines and their cyclic analogues was performed. Also of tryptamine hybrid molecules with a different prophene residue.
11. *In vitro* biological activity of newly synthesized biofunctional hybrid molecules was investigated. Antioxidant activity assessed by hydrogen peroxide deactivation (HPSA) was performed. These compounds contain a profen fragment in their structure, and therefore *in vitro* anti-inflammatory activity (assessed by inhibition of albumin denaturation - IAD) and in vitro anti-arthritic activity (assessed by anti-tryptic activity - ATA) were performed. The analysis found that all the hybrid molecules inherited the anti-inflammatory and anti-arthritic properties of prophenes.
12. *In vitro* biological activity of 1,2,3,4-Tetrahydroisoquinoline sulfonamide derivatives was investigated. Newly synthesized sulfone amides are characterized by significant antioxidant (HPSA), in vitro anti-inflammatory activity (IAD) and anti-arthritic activity (ATA). This makes them reliable and effective drugs.

7. References

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