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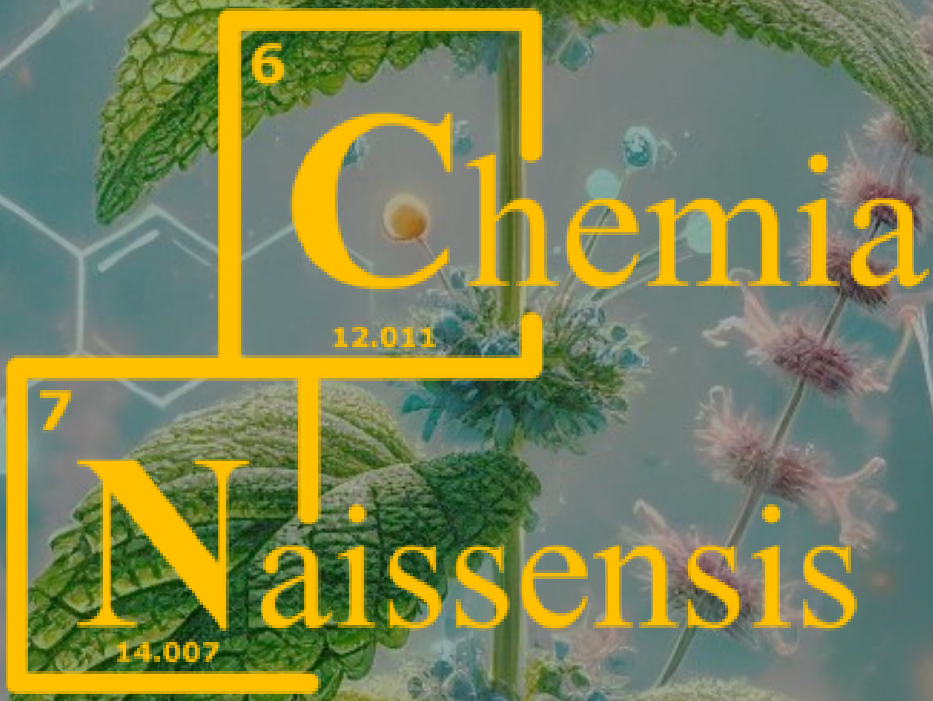


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# Selected nutrient elements in commercial samples of salads

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## ABSTRACT

This study investigates the element composition, including potassium, sodium, calcium, magnesium, and selenium, in six different leafy vegetable species commonly consumed in salads. The vegetables analyzed were *Lactuca sativa* (lettuce), *Spinacia oleracea* (spinach), *Cichorium intybus* (radicchio), *Valerianella locusta* (lamb's lettuce), *Cichorium endivia* (endive), and *Eruca sativa* (eruca). The contribution of the mentioned elements in the diet by consuming a portion of 100 g fresh salad was evaluated by the recommended daily intake. The results revealed significant variations in element content among the species. Given the recommended daily intake of selenium, these vegetables can contribute significantly to meeting daily selenium requirements (35.9% – 60.5%). The elevated selenium concentrations in *L. sativa* (3.327 mg/kg) and *V. locusta* (2.467 mg/kg) indicate that regularly consuming these foods may be an effective means of boosting dietary selenium intake, thereby supporting its vital role in human health. Other results demonstrate the diverse element content across different leafy vegetables, which can provide varying health benefits depending on the mineral requirements. Based on the elemental content across the samples, *L. sativa* has the greatest content of calcium (12302 mg/kg), while *S. oleracea* has the highest magnesium and potassium content (6131 mg/kg and 22854 mg/kg), *C. endivia* stands out for its high sodium content (20840 mg/kg).

*Keywords: nutrient elements, commercial salads, ICP OES*

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## Introduction

Plants have always been used for food, as spices, teas, and in traditional medicine; therefore, it is important to examine their chemical content. Diet rich in fruits and vegetables is widely recommended for its health-promoting properties (Barmmer, 2022). Fruits and vegetables have historically been in dietary guidance because of their concentrations of vitamins (i.e. vitamins C and A), minerals (electrolytes), and more recently phytochemicals, especially antioxidants (Revoredo-Giha, 2021). Additionally, fruits and vegetables are recommended as a source of dietary fiber (Slavin et al., 2012). In addition to organic, plants also contain various inorganic components. Inorganic components are less abundant in plants, but they are equally important. The trace elements found in living organisms may be essential, i.e., indispensable for growth and health, or they may be nonessential, fortuitous reminders of our geochemical origins or indicators of environmental exposure (Özcan, 2004). Minor elements have very important functions, and they are proven as a key component of proteins such as hemoprotein and hemoglobin which play a role in biochemical functions and essential enzyme systems even in low doses (Imelouane et al., 2011). Although the required levels of micronutrients are usually permanently fixed, they can vary depending on many factors, such as plant species, genotype and growth conditions, different organs and tissues of the same plant (Ražić et al., 2005).

Salads can be a vital part of a healthy diet, offering numerous nutritional benefits that support overall well-being, such as a wide array of vitamins, minerals, and antioxidants. These nutrients help protect the body from oxidative stress and support immune function. The variety of salads provides numerous flavors, textures, and nutritional benefits. The diversity of ingredients in salads makes them suitable for different dietary preferences and can help achieve various health goals, from weight management to boosting immunity.

Six plant samples purchased in the same megamarket were used in this research: *Lactuca sativa* (fam. Asteraceae) (lettuce), *Spinacia oleracea* (fam. Amaranthaceae) (spinach), *Cichorium intybus* var. foliosum (fam. Asteraceae) (radicchio), *Valerianella locusta* (fam. Valerianaceae) (lamb's lettuce), *Cichorium endivia* var. Crispum (fam. Asteraceae) (endive)and *Eruca sativa* (fam. Brassicaceae) (eruca). The aim of this study was to determine the content of potassium, sodium, calcium, magnesium, and selenium, in these samples, and the contribution of the mentioned elements in the diet by consuming a portion of salad.

## Experimental

### Chemicals and instruments

All reagents used for digestion were analytical grade, purchased from Sigma-Aldrich Chemical Company (Germany).
The measurements of elements were carried out in an Inductively Coupled Plasma Optical Emission Spectroscopy, ICP-OES (Thermo Scientific,UK), model 6500 Duo, equipped with a CID86 chip detector. Ultra-scientific ICP multi-element standard solutions were used as a stock solution for calibration.

### Sample collection

All samples *Lactuca sativa* L. (fam. Asteraceae), *Spinacia oleracea* L. (fam. Amaranthaceae), *Cichorium intybus* L. var. foliosum (fam. Asteraceae), *Valerianella locusta* L. (fam. Valerianaceae), *Cichorium endivia* L. var. Crispum (fam. Asteraceae) and *Eruca sativa* L. (fam. Brassicaceae)) purchased in the local market.

### Sample preparation

Prior to analysis, plants were air-dried at room temperature. Once dried, the samples were ground into a fine powder using a stainless-steel grinder and stored in polypropylene bags until further analysis. Samples were prepared through wet digestion, following a modified version of the procedure outlined by Tüzen, 2003. One gram of each salad species was combined with 15 mL oxi-acidic mixture composed of $HNO_3$, $H_2SO_4$, and $H_2O_2$ in a 4:1:1 ratio. The mixture was then heated to 150 °C for 4 hours before being diluted to a final volume of 25 mL with deionized water. A blank sample was processed in the same manner for comparison.

## Results and Discussion

The obtained results of each element (mg/kg dry weight) represent a mean value of three measurements ± SD (Table 1). The results indicated differences in element concentrations based on the species.

**Table 1.** Element content (mg/kg dry weight) of analyzed salads as mean ± standard deviation

| Samples | K | Na | Ca | Mg | Se |
|---|---|---|---|---|---|
| *L. sativa* | 19832±438 | 16523±324 | 12302±153 | 3981±59 | 3.3±0.12 |
| *S. oleracea* | 22854±115 | 3668±44 | 6745±122 | 6131±126 | 2.04±0.03 |
| *C. intybus* | 12468±327 | 14480±196 | 8334±65 | 1942±100 | 1.97±0.01 |
| *V. locusta* | 20607±4883 | 8605±122 | 8269±76 | 2775±78 | 2.47±0.02 |
| *C. endivia* | 14806±130 | 20840±35 | 4628±86 | 1915±102 | 2.328±0.009 |
| *E. sativa* | 15866±287 | 2086±44 | 14243±116 | 2240±102 | 1.89±0.07 |

Also, the content of elements in the selected types of salad was calculated per 100 g of fresh salad (USDA, Food Data Centrtal, 2020) and expressed as a percentage of the daily intake. When recalculating the nutritional content of a fresh salad, it was taken that approximately 90% of its weight is water. It was obtained using the mean value of three measurements for each element, and the results are shown in Table 2. A portion of salads contribution is considered to be significant if it provides 15% of the recommended daily intake (RDI) of nutritionally valuable elements (Stefanović, 2016).

**Table 2.** The element content in the analyzed samples shown per 100 g of fresh salad and % of daily intake

| Sample | K | | Na | | Ca | | Mg | | Se | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg K/100g fs | % | mg Na/100 g fs | % | mg Ca/100g fs | % | mg Mg/100 g fs | % | mg Se/100g fs | % |
| *L. sativa* | 198.3 | 9.9 | 165.2 | 11.0 | 123.0 | 15.4 | 39.8 | 10.0 | 0.033 | 60.5 |
| *S. oleracea* | 228.5 | 11.4 | 36.7 | 2.4 | 67.5 | 8.4 | 61.3 | 15.3 | 0.020 | 37.1 |
| *C. intybus* | 124.7 | 6.2 | 144.8 | 9.7 | 83.3 | 10.4 | 19.4 | 4.9 | 0.020 | 35.9 |
| *V. locusta* | 206.1 | 10.3 | 86.1 | 5.7 | 82.7 | 10.3 | 27.8 | 6.9 | 0.025 | 44.9 |
| *C. endivia* | 148.1 | 7.4 | 208.4 | 13.9 | 46.3 | 5.8 | 19.2 | 4.8 | 0.023 | 42.3 |
| *E. sativa* | 158.7 | 7.9 | 20.9 | 1.4 | 142.4 | 17.8 | 22.4 | 5.6 | 0.019 | 34.4 |

fs-fresh salad

The analysis of element content in various leafy vegetables reveals notable differences across the samples. While potassium does not become a part of the chemical structure of plants, it plays many important regulatory roles in development. It increases crop yield and improves quality. It is required for numerous plant growth processes (Prajapati et al., 2012). Potassium is the third most abundant mineral in the human body and plays an important role in many metabolic processes, including the proper functioning of the brain, heart and muscles. It is the most abundant cation in the human body, yet only 2% of total body potassium is contained in the extracellular fluid, a compartment accessible to clinical assessment. Its concentration in extracellular fluid is tightly regulated between 3.5 and 5.0 mmol/L. Most of the potassium (98%) is located intracellularly (mainly in muscle) at concentrations between 100 and 150 mmol/L, depending on cell type (Besouw et al., 2019).

Among the analyzed species, *S. oleracea* had the highest concentration of potassium (22854 mg/kg dw), and *C. intybus* had the lowest (12468 mg/kg dw). The RDI for potassium for adults is 2000 mg (EEC, 2008). Table 2 shows that all analyzed samples do not have a significant contribution of potassium by consuming a portion of 100 g, as it does not exceed 15% of the daily intake. Consuming a portion of 100 g of fresh species provides from 6.2% (*C. intybus*) to 11.4% (*S. oleracea*) of the daily K requirement.

A proper balance of sodium levels in every part of our body is vital; the osmotic pressure of the extracellular fluids of our body is dictated for 90% by sodium ions and their counter-ions, largely chloride. Tight regulation of the sodium levels in our blood plasma and interstitial fluids is crucial for the essential physiological functions of virtually all cells in our body, as many transport processes depend on it. Slight deviations affect the electrical activity of muscle and nerve cells, renal function, capillary exchange, and cardiac output, impacting blood pressure in multiple ways. (Dötsch et al., 2009)

All analyzed samples showed different sodium levels, ranging from 2086-20840 mg/kg dw. *C. endivia* is had the highest sodium content (20840 mg/kg dw), whereas the lowest was in *E. sativa* (2086 mg/kg dw). The RDI for sodium is 1500 mg. (EFSA, 2019) Consuming a portion of 100 g of fresh salad provides from 1,39 % to 13,9% sodium per day.

Calcium is an essential plant nutrient. $Ca^{2+}$ it is required for structural roles in the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol. (Marschner, 1995) It is an essential nutrient necessary for many human health functions. Calcium is the most abundant mineral in the body with 99% found in teeth and bone. Only 1% is found in serum. The serum calcium level is tightly monitored to remain within normal range by a complex metabolic process. Calcium metabolism involves other nutrients, including protein, vitamin D, and phosphorus. (Beto, 2015)

Calcium occurs in very similar concentrations in almost all samples, in the 4628 - 8334 mg/kg dw range. The *L. sativa* and *E. sativa* were separated with a higher calcium content, 12302 mg/kg dw and 14243 mg/kg dw, respectively. The RDI of calcium for adults is 800 mg. (EC, 2008) From Table 2 it can be seen some analyzed samples significantly contribute calcium from a portion of 100 g of fresh salad because it exceeds 15 %. Consuming a portion of 100 g of fresh *E. sativa* and *L. sativa* can provide a maximum of 17.8% and 15.4% of calcium daily intake.

Magnesium is most important to plants; about 75% of the leaf magnesium is involved in protein synthesis, and an amount between 15 % and 20 % of total Mg is associated with chlorophyll pigments, mainly acting as a cofactor of a series of enzymes involved in photosynthetic carbon fixation and metabolisms. (Guo et al., 2016) There are two major roles for magnesium in biological systems. It can form chelates with important intracellular anionic ligands, notably ATP, and it can compete with calcium for binding sites on proteins and membranes. There are about 300 magnesium activated enzymes. Among the enzyme-catalysed reactions in which magnesium acts as an essential cofactor are those concerned with glycolysis, cell respiration and transmembrane transport of other cations such as sodium and calcium. In particular, the activity of membrane bound Na-K-ATPase depends on magnesium. (Ryan, 1991)

The concentration of Mg in the analyzed samples ranged from 1915 mg/kg dw (*C. endivia*) to 6131 mg/kg dw (*S. oleracea*). Among the tested samples, *S. oleracea* had the highest content of

magnesium, 6131 mg/kg, and consuming a portion of 100 g of fresh *S. oleracea*, can provide a significant of 15.3% of magnesium daily intake.

Selenium cycles through food systems, being removed from soils by plants and micro-organisms which can take up the element into their tissue proteins and convert some of it to volatile metabolites (e.g. dimethylselenide) that enter the atmosphere ultimately to be brought down with precipitation and airborne particulates (Shrift, 1964; Allaway et al. 1967; Stork et al. 1999). As selenocysteine, the 21$^{st}$ amino acid, selenium is a component of selenoproteins, some of which have important enzymic functions.

Among the tested samples, the highest content of selenium was shown by the species *L. sativa* (3.327 mg/kg dw). In contrast, the minimum content was observed in the *E. sativa* (1.89 mg/kg dw). The Institute of Medicine of the American National Academies (U.S. National Academies, Institute of Medicine, 2001) recommends a daily Se intake of 0.055 mg. Based on the results, it can be noticed that all analyzed samples have a significant contribution to the daily intake of selenium (> 15 %), and the species *L. sativa* has the highest value, 60.5 %.

## Conclusion

These results demonstrate the diverse nutrient compositions across different leafy vegetables, which can provide varying health benefits depending on the mineral requirements. Among the vegetables tested, *L. sativa* exhibited the highest concentrations of selenium and calcium, making it a rich source of these essential minerals. A portion of this salad provides a significant contribution of these elements, especially selenium (60.5%). *S. oleracea*, on the other hand, had a relatively higher magnesium and potassium content, along with a balanced distribution of selenium, which constitutes a significant daily intake. *C. intybus* and *V. locusta* presented moderate levels of minerals. *C. endivia* had a unique profile, with higher sodium content compared to other samples, while *E. sativa* had a low selenium and sodium, but higher calcium content. Overall, these findings highlight the diverse mineral compositions in leafy vegetables, with each offering specific nutritional benefits depending on the mineral of interest.

## Acknowledgement

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## Conflict-of-Interest Statement

The authors declare no conflict of interest.

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# Odabrani hranljivi elementi u komercijalnim uzorcima salata

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## SAŽETAK

Cilj ovog istraživanja bio je određivanje sadržaja kalijuma, natrijuma, kalcijuma, magnezijuma i selena, u šest različitih vrsta lisnatog povrća koje se obično koristi za pravljenje salata. Analizirano povrće je *L. sativa, S. oleracea*, *C. intibus*, *V. locusta, C. endivia* i *E. sativa*. Doprinos navedenih elemenata u ishrani konzumacijom porcije od 100 g sveže salate određen je na osnovu preporučenih dnevnih potreba. Rezultati su otkrili značajne varijacije u sadržaju elemenata među vrstama. S obzirom na preporučeni dnevni unos selena, ovo povrće može značajno doprineti zadovoljavanju dnevnih potreba za selenom (35,9% – 60,5%). Konkretno, veće koncentracije selena u *L. sativa* (3,327 mg/kg) i *V. locusta* (2,467 mg/kg) sugerišu da bi njihova redovna konzumacija mogla biti efikasan način da se poveća unos selena ishranom, podržavajući njegovu suštinsku ulogu u ljudskom zdravlju. Drugi rezultati pokazuju različit sadržaj elemenata u različitom lisnatom povrću. Na osnovu sadržaja elemenata u uzorcima, *L. sativa* ima najveći sadržaj kalcijuma (12302 mg/kg), dok *S. oleracea* ima najveći sadržaj magnezijuma i kalijuma (6131 mg/kg i 22854 mg/kg), *C. endivija* se izdvaja po visokom sadržaju natrijuma (20840 mg/kg).

*Ključne reči: nutritivni elementi, komercijalni uzorci salata, ICP OES*

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# Éléments nutritifs sélectionnés dans des échantillons commerciaux de salades

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## RÉSUMÉ

Cette étude examine la composition en éléments, notamment le potassium, le sodium, le calcium, le magnésium et le sélénium, de six espèces différentes de légumes à feuilles couramment consommées en salade. Les légumes analysés étaient *Lactuca sativa*, *Spinacia oleracea*, *Cichorium intybus*, *Valerianella locusta*, *Cichorium endivia* et *Eruca sativa*. La contribution des éléments mentionnés dans l'alimentation en consommant une portion de 100 g de salade fraîche a été évaluée par l'apport quotidien recommandé. Les résultats ont révélé des variations significatives de la teneur en éléments entre les espèces. Compte tenu de l'apport quotidien recommandé en sélénium, ces légumes peuvent contribuer de manière significative à répondre aux besoins quotidiens en sélénium (35,9 % – 60,5 %). Plus précisément, les concentrations plus élevées de sélénium dans *L. sativa* (3,327 mg/kg) et *V. locusta* (2,467 mg/kg) suggèrent que leur consommation régulière pourrait être un moyen efficace d'améliorer l'apport alimentaire en sélénium, soutenant ainsi son rôle essentiel dans la santé humaine. D'autres résultats démontrent la diversité des teneurs en éléments des différents légumes à feuilles, qui peuvent apporter des avantages variables pour la santé en fonction des besoins en minéraux. Sur la base de la teneur en éléments des échantillons, *L. sativa* a la plus grande teneur en calcium (12302 mg/kg), tandis que *S. oleracea* a la plus forte teneur en magnésium et en potassium (6131 mg/kg et 22854 mg/kg), *C. endivia* se distingue par sa teneur élevée en sodium (20840 mg/kg).

*Mots-clés : éléments nutritifs, salades commerciales, ICP OES*

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# Избранные питательные элементы в коммерческих образцах салатов

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## Резюме

Данное исследование посвящено анализу элементного состава, включая калий, натрий, кальций, магний и селен, в шести различных видах листовых овощей, которые часто употребляются в салатах. В анализе рассматривались следующие овощи: *Lactuca sativa* (салат латук), *Spinacia oleracea* (шпинат), *Cichorium intybus* (цикорий), *Valerianella locusta* (полевой салат), *Cichorium endivia* (эндивий) и *Eruca sativa* (руккола). Вклад указанных элементов в рацион оценивался на основе их содержания в порции свежего салата массой 100 г с учетом рекомендованной суточной нормы (РСН). Результаты показали значительные различия в содержании элементов между видами. Учитывая рекомендованную суточную норму селена, эти овощи могут существенно способствовать покрытию потребности в этом элементе (35,9% – 60,5%). В частности, более высокое содержание селена обнаружено в *L. sativa* (3,327 мг/кг) и *V. locusta* (2,467 мг/кг), что свидетельствует о том, что их регулярное употребление может быть эффективным способом повышения уровня селена в рационе, поддерживая его важную роль в здоровье человека. Другие результаты продемонстрировали разнообразие содержания элементов в различных видах листовых овощей, что может обеспечивать различные преимущества для здоровья в зависимости от минеральных потребностей. Например, на основе элементного состава: *L. sativa* имеет наибольшее содержание кальция (12 302 мг/кг); *S. oleracea* содержит больше всего магния и калия (6 131 мг/кг и 22 854 мг/кг соответственно); *C. endivia* выделяется высоким содержанием натрия (20 840 мг/кг).

*Ключевые слова: питательные элементы, коммерческие салаты, ICP OES*

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# Ausgewählte Nährstoffelemente in kommerziellen Salatproben

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## ABSTRAKT

In dieser Studie wird die Zusammensetzung der Elemente Kalium, Natrium, Kalzium, Magnesium und Selen, in sechs verschiedenen Blattgemüsesorten untersucht, die üblicherweise in Salaten verzehrt werden. Die analysierten Gemüsesorten waren *L. sativa, S. oleracea, C. intybus, V. locusta, C. endivia* und *E. sativa.* Der Beitrag der genannten Elemente in der Ernährung durch den Verzehr einer Portion von 100 g frischem Salat wurde anhand der empfohlenen Tagesdosis bewertet. Die Ergebnisse zeigten signifikante Unterschiede im Elementgehalt zwischen den einzelnen Arten. Angesichts der empfohlenen Tagesdosis an Selen können diese Gemüsearten erheblich zur Deckung des täglichen Selenbedarfs beitragen (35,9 % – 60,5 %). Insbesondere die höheren Selenkonzentrationen in *L. sativa* (3,327 mg/kg) und *V. locusta* (2,467 mg/kg) legen nahe, dass ihr regelmäßiger Verzehr eine effektive Möglichkeit sein könnte, die Aufnahme von Selen in der Ernährung zu erhöhen und damit dessen wichtige Rolle für die menschliche Gesundheit zu unterstützen. Weitere Ergebnisse zeigen die unterschiedliche Elementgehalte der verschiedenen Blattgemüsesorten, die je nach Mineralstoffbedarf unterschiedliche gesundheitliche Vorteile bieten können. Basierend auf dem Elementgehalt der Proben weist *L. sativa* den höchsten Kalziumgehalt (12302 mg/kg) auf, während *S. oleracea* den höchsten Magnesium- und Kaliumgehalt (6131 mg/kg bzw. 22854 mg/kg) hat. *C. endivia* zeichnet sich durch einen hohen Natriumgehalt (20840 mg/kg) aus.

*Schlüsselwörter: Nährstoffelemente, kommerzielle Salate, ICP OES*

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# Elemental composition of acacia honey samples from Serbia

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## ABSTRACT

This study examines the concentration of essential minerals and toxic heavy metals in acacia honey samples collected in Serbia, highlighting their health implications and environmental influences. Among the 23 elements analyzed, calcium levels ranged from 38.35 to 1148.5 mg/kg, averaging 411.5 mg/kg, higher than previous findings for acacia honey, underscoring its nutritional value. Potassium averaged 208 mg/kg, consistent with the literature, whereas Na content was notably higher than the values reported in the literature. Zinc levels were higher than typical (average 11.57 mg/kg), likely due to pollution of acacia plant. Lead was the only detected toxic metal, with concentrations below the European safety threshold of 1 mg/kg. The findings highlight honey's nutritional benefits and emphasize the need for monitoring environmental contaminants to ensure honey's safety and quality.

*Keywords: acacia honey, elemental composition, ICP OES*

## Introduction

Honey is a natural sweetener produced by *Apis mellifera* (honey bees) from the nectar of flowers. It has been valued not only for its flavor but also for its nutritional and medicinal properties. Honey is a source of simple carbohydrates. Its composition on average is 17.1% water, 82.4% total carbohydrate and 0.5% proteins, amino acids, vitamins and minerals. (Khan et al., 2007) Researches indicates that honey have several health-beneficial effects including antioxidant, anti-

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inflammatory, antibacterial, antidiabetic, respiratory, gastrointestinal, cardiovascular, and nervous system protective effects. (Samarghandian et al., 2017) Understanding the elemental composition of honey is essential for assessing its quality, geographical origin, and potential health benefits.

Inductively Coupled Plasma (ICP) techniques, such as ICP-OES (Optical Emission Spectroscopy) and ICP-MS (Mass Spectrometry), have become indispensable techniques in the precise determination of trace elements in honey samples (Solayman et al., 2015). Given the growing concern over food safety and authenticity, applying ICP techniques to investigate the elemental profile of honey can provide critical insights into its traceability and quality.

The elemental composition of honey can be influenced by various factors, including the botanical and geographical origin of the nectar, environmental conditions, and anthropogenic activities. Relatively small mineral content (0.1–1.0% w/w) in honey can be a reliable indicator for tracing its geographical and botanical origins. Trace elements such as Na, K, Ca, Mg, Fe, Zn and Cu are naturally present in honey and contribute to its nutritional value. However, the presence of toxic elements like lead, cadmium, and arsenic can indicate environmental contamination and pose health risks. Various studies have been conducted to evaluate the elemental composition of honey samples worldwide (Pisani et al., 2008; Chudzinska & Baralkiewicz, 2010, 2011; Marghitas et al., 2010; Golob et al., 2005; Fernandez-Torres et al., 2005; Bilandzic et al., 2011; Spiric et al., 2019).

This study aims to analyze the elemental composition of five acacia honey samples using the ICP-OES technique to identify their mineral content and detect potential contaminants. By comparing the elemental profiles of honey from different regions, we seek to establish patterns that may aid in the authentication and quality control of honey products. Additionally, this research contributes to a broader understanding of how environmental and geographical factors impact the elemental composition of honey.

## Experimental

### Honey samples

Honey samples were purchased from local productors in vicinity of Niš, Serbia. The areas where samples were collected are relatively unpolluted, because of the distance between large industrial plants and major railways and highways. Samples were collected in a steel container and kept a few days until maturation is achieved. After that, samples were bottled in decontaminated glass vessels and stored at room temperature until analysis.

### Chemicals and instruments

Hydrochloric acid, nitric acid (65%), and hydrogen peroxide (30%), were purchased from Merck (Darmstadt, Germany). Multi - element standard solutions (20.00 ± 0.10 mg L-1) used for ICP

analysis was purchased from Ultra Scientific (North Kingstown, RI, U.S.A.). Samples were prepared using Milestone ETHOS EASY advanced microwave digestion system.
The measurements were carried out with an ICP-OES iCAP 6000, Thermo Scientific.

## Samples preparation

Digestion was done using a Milestone ETHOS EASY advanced microwave digestion system. Samples (0.3 g) were mixed with 9 mL of $HNO_3$ and 1 mL of $H_2O_2$ and subjected to digestion. The instrumental parameters used are shown in Table 1.

**Table 1.** Instrumental parameters for the microwave oven digestion.

| Step | Power (W) | Temperature (°C) | Time (min) |
|---|---|---|---|
| 1 | 1800 | 210 | 20 |
| 2 | 1800 | 210 | 15 |

After digestion, the PTFE vessels were left to cool to room temperature. The samples were quantitatively transferred into 25 mL volumetric flasks and filled up to the mark with ultrapure water. The honey samples were digested in triplicate. Blank samples were prepared with the same procedure.

## Parameters of ICP-OES instrument and characteristics of the calibration curve

The contents of the tested elements in soil and plant material samples were determined by the ICP-OES technique (iCAP 6000 series, ThermoScientific, Cambridge, United Kingdom) at the following optimal instrument parameters: flush pump rate-100 rpm, analysis pump rate-50 rpm, RF power-1150 W, nebulizer gas flow-0.7 L/min, coolant gas flow-12 L/min, auxiliary gas flow 0.5 L/min, plasma view-axial, washing time-30 s. Multielement certified standard solution IV (Al, As, Ba, Be, B, Cd, Cr, Co, Cu, Fe, Pb, Mn, Ni, Se, Tl, V, and Zn; TraceCERT, Fluka Analytical, Switzerland) was used. The correlation coefficient (r), the limit of detection (LOD), and the limit of quantification (LOQ) of the working calibration curve for each tested element are shown in Table 2.

**Table 2.** ICP-OES validation parameters

| Element | λ (nm) | r | LOD (ppm) | LOQ (ppm) |
|---|---|---|---|---|
| Al | 308.215 | 0.991447 | 0.001955 | 0.006516 |
| As | 189.042 | 0.999202 | 0.002142 | 0.007139 |
| B | 249.678 | 0.998918 | 0.000871 | 0.002902 |
| Ba | 455.403 | 0.999804 | 0.000027 | 0.000090 |
| Be | 234.861 | 0.999777 | 0.000060 | 0.000199 |
| Ca | 393.366 | 0.997494 | 0.000147 | 0.000490 |
| Cd | 226.502 | 0.999744 | 0.000085 | 0.000282 |
| Co | 228.616 | 0.999677 | 0.000216 | 0.000721 |

| | | | | |
|---|---|---|---|---|
| Cr | 283.563 | 0.99971 | 0.000429 | 0.00149 |
| Cu | 324.754 | 0.997565 | 0.000399 | 0.001329 |
| Fe | 259.940 | 0.999494 | 0.000370 | 0.001234 |
| Hg | 184.950 | 0.995259 | 0.000341 | 0.001136 |
| K | 766.490 | 0.997001 | 0.050090 | 0.166965 |
| Mg | 279.553 | 0.998979 | 0.000160 | 0.000532 |
| Mn | 257.610 | 0.998056 | 0.000068 | 0.000228 |
| Na | 589.592 | 0.998974 | 0.016701 | 0.055670 |
| Ni | 221.647 | 0.996075 | 0.000216 | 0.000720 |
| P | 177.495 | 0.999885 | 0.003288 | 0.010959 |
| Pb | 220.353 | 0.999355 | 0.001051 | 0.003504 |
| Se | 196.090 | 0.999167 | 0.003321 | 0.011068 |
| Si | 251.611 | 0.996822 | 0.001034 | 0.003448 |
| Tl | 190.856 | 0.999842 | 0.001340 | 0.004468 |
| V | 309.311 | 0.99982 | 0.000285 | 0.000950 |
| Zn | 213.856 | 0.998308 | 0.000054 | 0.000181 |

## Results and Discussion

The analysis of the elemental composition of acacia honey samples revealed the presence of various essential and trace elements. Table 1 shows the mean content of detected elements of three consecutive measurements ± standard deviation SD (mg/kg of honey) for each of the tested samples.

Out of 23 elements analyzed, four of them (Cd, Co, Hg and Tl) were below the limit of detection.

The calcium content ranged from 1148 – 38.3 mg/kg, with an average concentration of 411.5 mg/kg. Calcium is essential for bone health and metabolic functions. Alvarez-Suarez et al. (2014) showed calcium levels between 10 to 45 mg/kg for Cuban honey samples, indicating variability based on geographical and botanical origin. Concentrations of Ca in acacia honey samples found by Alvarez-Suarez et al. (2014) were lower, compared to our results. Two samples from our study showed extremely high content of Ca (773 and 1148 mg/kg), whereas the other three were in the range found in the literature (Elbagerma et al., 2019). Various factors affect Ca content in honey samples. Different plants have varying capacities for absorbing and accumulating calcium, which is reflected in the honey produced from their nectar. Soil composition and environmental factors in different regions affect the mineral content of plants, and consequently, the honey (Schmidlová et al., 2024). The calcium levels found in honey reinforce its role as a nutritious food product with additional health benefits. The recommended daily intake (RDI) of calcium for adults ranges from 1000 to 1300 mg, depending on age and gender (National Institutes for Health). While acacia honey is not a primary source of calcium, its regular consumption can contribute to the overall dietary intake, particularly when included as part of a balanced diet.

**Table 3.** The mean content of detected elements ± standard deviation SD (mg/kg) in acacia honey samples

| Sample (mg/kg) | Acacia honey 1 | Acacia honey 2 | Acacia honey 3 | Acacia honey 4 | Acacia honey 5 |
|---|---|---|---|---|---|
| Al | 17.8±0.9 | 4.59±0.06 | 3.07±0.04 | 9.4±0.1 | 0.801±0.005 |
| As | 0.013±0.001 | 0.020±0.002 | 0.132±0.005 | 0.187±0.004 | 0.127±0.005 |
| B | 5.23±0.06 | 11.9±0.6 | 2.73±0.09 | 31.8±0.8 | 2.18±0.06 |
| Ba | 1.25±0.05 | 1.34±0.03 | 0.191±0.005 | 0.227±0.002 | 5.02±0.06 |
| Ca | 773±4 | 1148±23 | 46±1 | 51±2 | 38.3±0.9 |
| Cd | n.d. | n.d. | n.d. | n.d. | n.d. |
| Co | n.d. | n.d. | n.d. | n.d. | n.d. |
| Cr | 0.099±0.001 | 0.19±0.08 | 0.081±0.003 | 0.033±0.002 | 0.028±0.003 |
| Cu | 3.99±0.04 | 5.13±0.05 | 0.213±0.004 | 0.374±0.002 | 0.309±0.003 |
| Fe | 49±1 | 46.1±0.8 | 13.5±0.6 | 8.01±0.06 | 11.20±0.06 |
| Hg | n.d. | n.d. | n.d. | n.d. | n.d. |
| K | 197±6 | 231±7 | 183±6 | 217±8 | 209±8 |
| Mg | 27±0.9 | 44.2±0.8 | 12.00±0.09 | 9.89±0.06 | 6.36±0.04 |
| Mn | 0.877±0.009 | 0.998±0.007 | 0.279±0.003 | 0.294±0.002 | 0.246±0.001 |
| Na | 222±4 | 301 ±6 | 218±6 | 235±5 | 235±3 |
| Ni | 1.68±0.05 | 0.654±0.005 | 0.242±0.002 | 0.661±0.006 | 0.450±0.005 |
| P | 25.5±0.4 | 32.3±0.3 | 32.7±0.6 | 57.7±0.7 | 28.1±0.8 |
| Pb | 0.178±0.003 | 0.398±0.005 | 0.110±0.001 | 0.140±0.002 | 0.021±0.004 |
| Se | 0.237±0.005 | 0.472±0.004 | 0.191±0.002 | 0.153±0.004 | 0.211±0.003 |
| Si | 29±1 | 353±7 | 29±2 | 253±8 | 68±4 |
| Tl | n.d. | n.d. | n.d. | n.d. | n.d. |
| V | 0.158±0.004 | 0.155±0.002 | 0.037±0.001 | 0.027±0.002 | 0.028±0.001 |
| Zn | 3.52±0.06 | 9.057±0.07 | 5.45±0.06 | 37.95±0.03 | 1.877±0.01 |

n.d. – below LOD

Potassium is the most abundant element in the honey samples, found in literature, with an average concentration of 350 mg/kg. Our study reported an average value of 208 mg/kg, which is in agreement with the values reported in the literature. Alqarni et al. (2014) found 450 mg/kg of K in Saudi Arabia acacia honey samples, which is almost twice as higher than values reported in our study, indicating that K level can reflect geographic origin of honey samples. A high level of potassium is typical in honey and is beneficial for human health, playing a crucial role in maintaining fluid balance and normal cell function. Studies have shown that acacia honey generally contains lower levels of potassium compared to darker honey varieties such as buckwheat or heather honey (Bogdanov et al., 2004). This is attributed to the floral source, as darker honey often come from plants that grow in mineral-rich soils.

Interestingly, sodium level was higher in honey samples analyzed in our study, compared to literature data. Pseudo Acacia honey exhibited the lowest Na content (15.69 mg/kg), whereas Somrah honey had the highest (26.93 mg/kg). (Alqarni et al., 2014). Those values were ten times lower compared to values found in our study (average value 243 mg/kg). Na content in a study by Pavlin et al. (2023) was in broad concentration range from 2.4 ± 10% mg/kg to 142 ± 3% mg/kg.

Sodium level might be more subjected to geographical origin than botanical, so it could not be used to distinguish honey samples based on Na content data.

Magnesium is one of the essential minerals found in honey, although its concentration can vary depending on the floral source, geographical origin, and environmental factors. (Schmidlová et al., 2024) Magnesium content in honey is an indicator of its nutritional quality and can provide health benefits when consumed as part of a balanced diet. Magnesium plays several critical roles in maintaining health, including muscle and nerve function, bone health, cardiovascular health, energy production and blood sugar control. (Volpe, 2013) Mg content in honey samples analyzed in our study varied between 6.36-44.22 mg/kg, with an average value of 20.11 mg/kg. Content of Mg varied between199.30 mg/kg and 80.70 mg/kg in a study by Alqarni et al. (2014), whereas Pavlin et al. (2023) reported Mg values 6.8-110 mg/kg. Such differences in Mg concentrations might be a consequence of Mg concentrations in soil, as one of main soil element, where plants for honey production are grown.

Trace elements in honey, though present in small amounts, are crucial for human health. They contribute to various physiological functions, offer antioxidant protection, and support the immune system. (Barreiros et al., 2024) Understanding the factors influencing the concentration of these trace elements can help in selecting high-quality honey and maximizing its health benefits. Trace elements in honey include minerals such as iron, zinc, copper, manganese, selenium, and chromium.

Iron content in analyzed acacia honey samples varied between 8.01 to 49 mg/kg, with an average value of 20.11. mg/kg. Atanassova et al. (2012) reported Fe values in Bulgarian honey acacia (0.8 mg/kg), linden (1.6 mg/kg), and coriandrum (1.3 mg/kg), which is lower than our findings. Iron helps prevent anemia and boosts energy levels and immune function. The recommended daily intake (RDI) of iron varies by age, sex, and physiological status (e.g., pregnancy). According to the National Institutes of Health (NIH) RDI for iron varies between 7 and 27 mg per day. While honey does contain iron, its contribution to the daily iron intake is relatively modest due to its low concentration. Consuming honey alone would not be sufficient to meet the RDI for iron, especially for groups with higher iron requirements such as pregnant women and adolescent girls. Therefore, it is essential to include a variety of iron-rich foods in the diet, to meet the recommended daily intake of iron.

On average, the zinc content in honey typically ranges from 0.1 to 3.5 mg/kg (Terab et al., 2004; Tuzen and Soylak, 2005), which is lower than average value for Zn content in our study (11.57 mg/kg). Industrial pollution, agricultural practices, urbanization, and waste disposal can lead to increased levels of zinc in honey. Understanding these influences is essential for maintaining the quality and safety of honey as a dietary source of zinc. Tuzen et al. (2007) concluded that trace element concentrations in honey are generally correlated with the degree of trace element contamination of the environment. Cu content in analyzed honey samples varied between 0.213 to 5.13 mg/kg, which is in accordance for Cu values found in literature. (Sitarz-Palczak et al., 2015)

The presence of toxic heavy metals in honey is influenced by environmental pollution, from anthropogenic activities. Regular monitoring and assessment of honey for heavy metals are essential to ensure its safety and quality. While honey can serve as an environmental bioindicator, the potential health risks from heavy metal contamination highlight the need for stricter regulations and pollution control measures. Heavy metals that have no proven biological function and are considered to be toxic are Hg, Cd and Pb. Out of the mentioned elements, only Pb was found in analyzed acacia honey samples, in concentrations between 0.021 and 0.398 mg/kg. The European Commission suggests an acceptable maximum level of 1 mg/kg for Pb (Bogdanov et al., 2007), which is higher than values for Pb concentration in our study, confirming acacia honey analyzed in this study is safe for usage regarding toxic heavy metals.

## Conclusion

This study provides a comprehensive analysis of the elemental composition of acacia honey samples, emphasizing both essential minerals and toxic heavy metals. The findings highlight the nutritional benefits of honey, particularly its contributions of calcium, potassium, magnesium, and zinc, which play vital roles in various physiological functions and overall health. The significant variability in mineral content among samples underscores the influence of geographical origin, botanical source, and environmental factors. Notably, the calcium content in our samples was considerably higher than previously reported values, suggesting a robust potential for honey to contribute to dietary calcium intake. Magnesium and zinc concentrations further demonstrated the impact of environmental conditions and pollution on mineral content. The detection of lead as the only toxic heavy metal, with concentrations below the European Commission's safety threshold, indicates that the analyzed acacia honey samples are safe for consumption regarding heavy metal contamination. This reinforces the importance of regular monitoring and stringent quality control measures to ensure honey safety. Overall, this study confirms honey's role as a nutritious food product, offering essential minerals while highlighting the need for continuous assessment of environmental pollutants to safeguard its quality.

## Acknowledgement

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## Conflict-of-Interest Statement

The authors declare no conflict of interest.

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# Elementni sastav uzoraka bagremovog meda iz Srbije

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## SAŽETAK

Cilj ovog rada je analiza esencijalnih elemenata i teških metala u uzorcima bagremovog meda prikupljenim u Srbiji. Analizirana su 23 elementa, i zaključeno je da su koncentracije kalcijuma bile u granici od 38,35 do 1148,5 mg/kg, u proseku 411,5 mg/kg, što je više od prethodnih rezultata za bagremov med. Srednja koncentracija za kalijum iznosila je 208 mg/kg, i pronađene vrednosti bile su u saglasnosti sa rezultatima iz prethodnih istraživanja, dok je sadržaj Na bio znatno viši od vrednosti navedenih u literaturi. Koncentracije cinka su takođe bile više od uobičajenih (prosečno 11,57 mg/kg), verovatno zbog zagađenja biljke bagrema. Olovo je bio jedini detektovani toksični metal, sa koncentracijama ispod propisane maksimalno dozvoljene koncentracije od 1 mg/kg. Dobijeni rezultati ističu nutritivne prednosti meda i naglašavaju potrebu za praćenjem toksičnih elemenata kako bi se osigurala bezbednost i kvalitet meda.

*Ključne reči: bagremov med, elementni sastav, ICP OES*

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# Composition élémentaire d'échantillons de miel d'acacia de Serbie

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## RÉSUMÉ

Cette étude examine la concentration de minéraux essentiels et de métaux lourds toxiques dans des échantillons de miel d'acacia collectés en Serbie, soulignant leurs implications pour la santé et leurs influences environnementales. Parmi les 23 éléments analysés, les teneurs en calcium variaient de 38,35 à 1148,5 mg/kg, avec une moyenne de 411,5 mg/kg, supérieure aux résultats précédents pour le miel d'acacia, soulignant sa valeur nutritionnelle. Le potassium était en moyenne de 208 mg/kg, ce qui est conforme à la littérature, tandis que la teneur en Na était nettement supérieure aux valeurs rapportées dans la littérature. Les niveaux de zinc étaient supérieurs à la normale (en moyenne 11,57 mg/kg), probablement en raison de la pollution de la plante d'acacia. Le plomb était le seul métal toxique détecté, avec des concentrations inférieures au seuil de sécurité européen de 1 mg/kg. Les résultats soulignent les avantages nutritionnels du miel et soulignent la nécessité de surveiller les contaminants environnementaux pour garantir la sécurité et la qualité du miel.

*Mots-clés : miel d'acacia, composition élémentaire, ICP OES*

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# Элементный состав образцов акациевого меда из Сербии

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## Резюме

Целью данной работы являлся анализ содержания основных элементов и тяжелых металлов в образцах акациевого меда, собранных в Сербии. Было проанализировано 23 элемента. Установлено, что концентрации кальция варьировались от 38,35 до 1148,5 мг/кг, в среднем составляя 411,5 мг/кг, что превышает данные предыдущих исследований для акациевого меда. Средняя концентрация калия составила 208 мг/кг, что согласуется с результатами предыдущих исследований, тогда как уровень натрия оказался значительно выше значений, упомянутых в литературе. Концентрации цинка также были выше обычных (в среднем 11,57 мг/кг), вероятно, из-за загрязнения акациевого растения. Свинец был единственным обнаруженным токсичным металлом, однако его концентрация оставалась ниже установленного максимального допустимого уровня в 1 мг/кг. Полученные результаты подчеркивают питательные преимущества акациевого меда и акцентируют необходимость контроля токсичных элементов для обеспечения его безопасности и качества.

*Ключевые слова: акациевый мед, элементный состав, ICP OES*

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# Elementare Zusammensetzung von Akazienhonigproben aus Serbien

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## ABSTRAKT

In diesem Beitrag wird der Gehalt an essentiellen Mineralien und toxischen Schwermetallen in Akazienhonigproben aus Serbien untersucht, um deren gesundheitliche Auswirkungen und Umwelteinflüsse aufzuzeigen. Unter den 23 analysierten Elementen lagen die Kalziumwerte zwischen 38,35 und 1148,5 mg/kg, wobei der Durchschnitt bei 411,5 mg/kg lag und damit höher ist als in frühere Ergebnisse für Akazienhonig, was seinen Nährwert unterstreicht. Der Kaliumgehalt betrug im Durchschnitt 208 mg/kg und entsprach den Literaturangaben, während der Natriumgehalt deutlich über den in der Literatur berichteten Werten lag. Die Zinkkonzentrationen waren höher als üblich (Durchschnitt 11,57 mg/kg), was vermutlich auf die Verschmutzung der Akazienpflanze zurückzuführen ist. Blei war das einzige nachgewiesene toxische Metall, wobei die Konzentrationen unterhalb der europäischen Sicherheitsgrenze von 1 mg/kg lagen. Die Ergebnisse heben die ernährungsphysiologischen Vorteile von Honig hervor und betonen die Notwendigkeit, Umweltkontaminanten zu überwachen, um die Sicherheit und Qualität von Honig zu gewährleisten.

*Schlüsselwörter: Akazienhonig, Elementarzusammensetzung, ICP OES*

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# Traditional Ethnobotanical Applications of *Melissa officinalis* for Niš district

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## ABSTRACT

*Melissa officinalis* (lemon balm) has been utilized for centuries in traditional medicine for its diverse therapeutic properties. This study explores its traditional applications and evaluates the pharmacological evidence supporting its use in treating various ailments. The bioactive compounds present in *M. officinalis*, such as rosmarinic acid, flavonoids, and essential oils, are responsible for its notable effects, including anxiolytic, antimicrobial, antioxidant, and digestive benefits. Traditional uses of *M. officinalis* in Niš District, southeastern Serbia, spanning from alleviating stress, anxiety and sleep problems to treating gastrointestinal discomfort, align with findings from contemporary scientific research. Despite its extensive history of use and integration in modern medicinal therapeutic practices, further clinical investigations are needed to substantiate its safety, efficacy, and mechanisms of action. This paper highlights the significance of *M. officinalis* as a bridge between traditional knowledge and modern medicinal applications, as well as the importance of preserving traditional knowledge.

*Keywords: Melissa officinalis, ethnobotany, medicinal plants, therapeutic effects, traditional medicine, traditional knowledge*

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## Introduction

Ethnobotany has an important role in understanding traditional medicinal knowledge and its applications in healthcare systems. Traditional medicinal herbs have long been an essential part of indigenous cultures all over the world, frequently serving as the main source of healthcare in specific areas (Mekonnen et al., 2022). These plants not only treat a variety of illnesses, but they also provide information about sustainable drug research and discovery (Fabricant and Fansworth, 2001). Ethnobotanical studies aim to document and analyze traditional knowledge, emphasizing the cultural significance, preparation methods, and therapeutic uses of plants. Furthermore, it has been shown that integrating ethnobotanical knowledge with modern pharmacology can help to combine traditional and scientific medical practices (Cunnigham, 2014).

*Melissa officinalis* L. is perennial herb from the family Lamiaceae, where many plants from this family are used as medicinal plants in traditional medicine due to their contributions to human health. For decades, *M. officinalis*, has been used in folk medicine to cure a variety of illnesses (Table 1), especially those related to anxiety, digestive issues, and sleep disorders. In recent decades, scientific study on its pharmacological qualities has developed despite its widespread usage in traditional herbal medicine, revealing a vast range of bioactive compounds that contribute to its health benefits (Chung-Hsiung et al., 2024; Shakeri et al., 2016). Among the most significant constituents are terpenes, flavonoids and phenolic acids. The primary active ingredients include citronellal, geranial, neral, and rosmarinic and caffeic acid (Miraj et al., 2017). The pharmacologicaly important components contribute to the herb's antioxidant, anti-inflammatory, antimicrobial, and anxiolytic properties.

The essential oil (EO) of *M. officinalis* is rich in monoterpenes such as citronellal and geranial, which are responsible for antimicrobial (Dukić et al., 2004), and anti-inflammatory activity (Bounihi et al., 2013). Additionally, the anti-inflammatory properties of *M. officinalis*'s EO rich in citral and rosmarinic acid have also been demonstrated, (Miraj et al., 2017). The lemon balm essential oil’s relaxing effects make it effective in reducing anxiety and promoting sleep (Sharifi-Rad et al., 2021).

Polar extracts of *M. officinalis* contain rosmarinic and caffeic acids in significant amounts, which are responsible for antioxidant and antimicrobial activities. Previous studies have identified several flavonoids, including luteolin and apigenin, as well as phenolic acids (Miraj et al., 2017). These mentioned compounds are well-known as antioxidants, which importance is the protection of cells from oxidative stress (https://www.reviewsll.com/gluco-shield-pro-review-a-comprehensive-analysis-of-the-pros-and-cons/) and contribute to the herb's anti-inflammatory and antidiabetic activities (Draganić, 2022).

**Table 1.** Biological properties and pharmacological uses of *M. officinalis.*

| Properties/Use | Description | References |
|---|---|---|
| Antioxidant | Scavenge free radicals and prevent oxidative damage. | Miraj et al., 2016 |
| Antimicrobial | Inhibits the growth of bacteria and fungi. | Carvalho et al., 2021 |
| Anti-inflammatory | Reduces inflammation. | Bounihi et al., 2013 |
| Antiviral | Effective against virus and viral infections. | Allahverdyev et al., 2004 |
| Anxiolytic | Reduces anxiety and promotes relaxation. | Stojanović et al., 2023 |
| Sedative | Enhances sleep quality. | Demirci et al., 2015 |
| Digestive aid | Reduces gastrointestinal discomfort. | Kapalka, G. M., 2010 |
| Cognitive enhancer | Improves concentration and memory | Naseri et al, 2021 |
| Gastroprotective | Alleviate gastric disorders | Juee et al., 2023 |
| Wound healing | Applied as a poultice to treat wounds and prevent infections. | Arbastan et al., 2014 |
| Menstrual pain relief | Used to alleviate menstrual cramps and hormonal imbalances. | Bounihi et al., 2013 |
| Culinary use | Leaves and essential oils added for flavoring food and beverages. | Carvalho et al., 2023 |

The subject of this study was to determine whether and to what extent is *M. officinalis* used in traditional medicine in Niš district. The aim was to gather knowledge about application of *M. officinalis* and in which forms it is prepared. The results were collected through surveys and consisted of general data about respondents (gender, age, place of living) and questions about application of plants, preparation methods, and treated illnesses.

## Experimental

The study was conducted in the Niš district, a region located in southeastern Serbia. The specific areas surveyed include rural villages and urban settings where traditional knowledge about medicinal plants is still actively practiced. A field survey was conducted for 2023 and 2024 year. In total, 100 participants were tested through an online survey. Before collecting data, informed consent was secured from all participants in compliance with ethical guidelines for ethnobotanical investigation (Jones, 2017). The study protocol was approved by the scientific project Ethno-

pharmacological study of the region of southeastern Serbia, O-02-17, supported by the Serbian Academy of Sciences and Arts.

Among 100 participants, 14 reported using *M. officinalis* leaves for tea with the intention of curing the nervous system and gastrointestinal tract.

## Results and Discussion

The present study included 100 participants aged 18 to 70, with an average age of 50. Ladies represented 60% and men 40%. Most participants had been exposed to traditional herbal practices, indicating a deep cultural inheritance of ethnobotanical knowledge in Nis District. Among 100 participants, 14 reported the use of lemon balm, 6 of them bought the tea in the market, and 8 collect a fresh plant. Amidst these participants that mentioned the use of lemon balm, many of them (9 participants) heard about the medicinal uses of this plant from other people which contributes to the preservation of traditional application knowledge of this medicinal plant. All participants consume *M. officinalis* in the form of tea using leaves of dried plant, where 4 participants reported using local wild plants and 4 of the participants mentioned they cultivate *M. officinalis* plant while the rest of them reported that they buy lemon balm tea (Table 2).

**Table 2.** Traditional use of *M. officinalis* in Niš District

| City/Village | Urban or Rural | Gender | Bought or harvested | Part of the plant | Consumed form | Use |
|---|---|---|---|---|---|---|
| Niš | Urban | Female | Bought | Leaves | Tea | Calming effect |
| Niš | Urban | Female | Harvested | Leaves | Tea | Insomnia |
| Svrljiško selo | Rural | Female | Harvested | Leaves | Tea, Tincture | Insomnia |
| Draguša | Rural | Female | Harvested | Leaves | Tea | Gastritis |
| Bujanovac | Rural | Female | Bought | Leaves | Tea | Stomach pains |
| Niš | Urban | Female | Harvested | Leaves | Tea | Stomach pains, calming the nervous system |
| Niš | Urban | Female | Harvested | Leaves | Tea | Depression, insomnia, mental health conditions |
| Niš | Urban | Male | Bought | Leaves | Tea | Stomach pains, sore throat, insomnia, calming effect |

| | | | | | | |
|---|---|---|---|---|---|---|
| Zaplanje | Rural | Female | Harvested | Leaves | Tea | Cold |
| Niš | Urban | Female | Harvested | Leaves | Tea | Calming effect, migraine |
| Niš | Urban | Female | Bought | Leaves | Tea | Stomach pains, analgetic effect, overall calming effect |
| Niš | Urban | Female | Harvested | Leaves | Tea | Stomach pains, overall calming effect |
| Niš | Urban | Female | Bought | Leaves | Tea, Oil | Stomach pains, calming effect, insomnia |
| Zaplanje | Rural | Female | Bought | Leaves | Tea, Tincture | Restlessness, insomnia |

A majority of 14 respondents consider tea, EO and tinctures respectively of *M. officinalis* as a remedy for anxiety, stress and sleep-related problems (Figure 1). Some of the participants reported using *M. officinalis* in digestive disorders-related problems (6 participants), while 1 participant mentioned the use of *M. officinalis* tea for treating gastritis, and 1 participant mentioned the use of *M. officinalis* tea for menstrual pain relief.

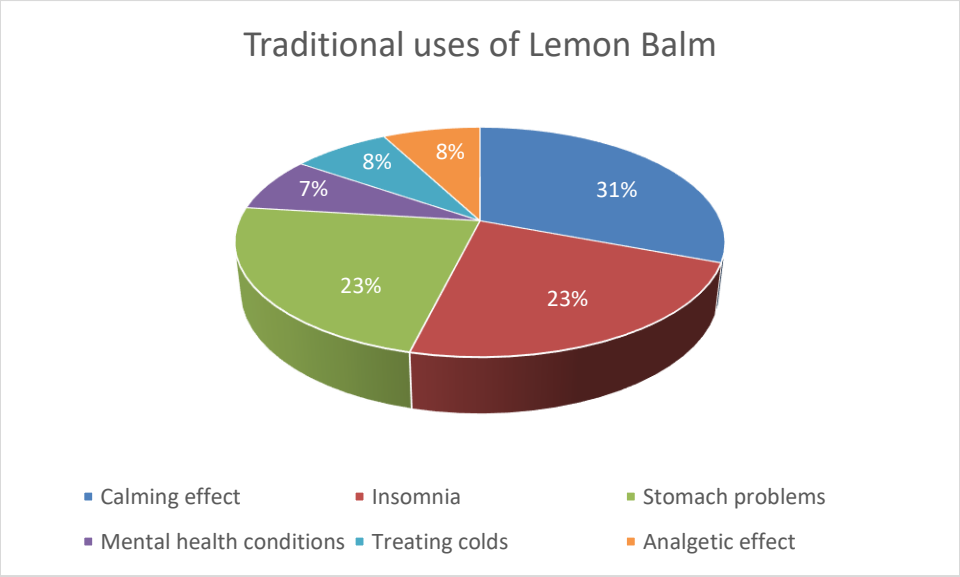


Figure 1. Traditional application of *M. officinalis*

The lemon balm's prominence in traditional practices highlights its deep cultural and ethnobotanical significance. The documented applications align with pharmacological studies

mentioned in Table 1 demonstrating the potential integration of this plant into modern medicine. It is a plant utilized either in its dried form or through oil extraction, and it serves as an antibacterial, antiviral, and antioxidant agent (https://foodly.tn/tips/4-790/). It is also used as a treatment for nervousness and insomnia, and it is commonly used in traditional medicine to treat anxiety, and gastric disorders and is sometimes used for menstrual irregularities (Kapalka, 2011). The traditional use of lemon balm for managing anxiety and stress is supported by numerous studies, for example, Gihazizadeh et al. (2020) reported a positive effect in treating nervous system condition testing on a laboratory animal, while a clinical study approved the integration of *M. officinalis* remedies into modern medicine (Cases et al., 2010).

## Conclusion

The traditional medicinal applications of *M. officinalis* (lemon balm) underscore its enduring significance in herbal medicine and its integration into the therapeutic practices of modern medicine. Historically used for its calming, antiviral, and digestive properties, *M. officinalis* has demonstrated pharmacological activity supported by emerging scientific evidence. The bioactive compounds, including rosmarinic acid, flavonoids, and essential oil's components like terpenes and other volatile compounds, contribute to its anxiolytic, antimicrobial, and antioxidant effects. While traditional knowledge and scientific studies offer valuable insights into potential applications of *M. officinalis*, further research is essential to validate these insights through more clinical studies, optimize treatment methods, and explore its full pharmacological potential. Integrating traditional wisdom with modern science not only strengthens the reliability of herbal medicine but also offers a sustainable avenue for developing accessible and effective treatments, contributing to both public health and the conservation of ethnomedicinal practices. Besides proven bioactivity, in our study, *M. officinalis* was not often mentioned in our survey. The awareness among the people about the importance of *M. officinalis* can be raised by popular lectures, workshops, and promotional activities.

## Acknowledgement

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## Conflict-of-Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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# Tradicionalne etnobotaničke primene *Melissa officinalis* za niški okrug

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## SAŽETAK

*Melissa officinalis (M. officinalis)*, opšte poznata kao matičnjak, vekovima se koristila u tradicionalnoj medicini zbog svojih raznovrsnih terapeutskih svojstava. Ovaj rad prikazuje rezultate istraživanja tradicionalne primene matičnjaka koji dokazuju njegovu upotrebu u lečenju različitih bolesti. Bioaktivna jedinjenja u ovoj biljci, kao što su ruzmarinska kiselina, flavonoidi i etarska ulja, odgovorna su za njegove primetne efekte, uključujući anksiolitičke, antimikrobne, antioksidativne efekte, kao i pozitivne efekte u lečenju digestivnog trakta. Tradicionalna upotreba *M. officinalis* u Niškom okrugu (jugoistočna Srbija) od ublažavanja stresa, anksioznosti i problema sa spavanjem do lečenja gastrointestinalnih tegoba, usklađena je sa nalazima savremenih naučnih istraživanja. Uprkos njegovoj opsežnoj istoriji upotrebe i integraciji u savremene medicinske terapijske prakse, potrebna su dalja klinička ispitivanja da bi se potvrdila njegova bezbednost, efikasnost i mehanizmi delovanja. Ovaj rad naglašava značaj *M. officinalis* kao mosta između tradicionalne i savremene medicinske primene, naglašavajući potrebu očuvanja tradicionalnog znanja.

*Ključne reči: Melissa officinalis, ethnobotanika, medicinske biljke, terapeutski efekti, tradicionalna medicina, tradicionalno znanje*

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# Applications ethnobotaniques traditionnelles de *Melissa officinalis* dans le district de Niš

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## RÉSUMÉ

*Melissa officinalis* (*M. officinalis*), communément appelée mélisse officinale, est utilisée depuis des siècles en médecine traditionnelle pour ses diverses propriétés thérapeutiques. Cette étude explore ses applications traditionnelles et évalue les preuves pharmacologiques soutenant son utilisation dans le traitement de diverses affections. Les composés bioactifs de *M. officinalis*, tels que l'acide rosmarinique, les flavonoïdes et les huiles essentielles, sont responsables de ses effets notables, notamment ses bienfaits anxiolytiques, antimicrobiens, antioxydants et digestifs. Les utilisations traditionnelles de *M. officinalis* dans le district de Niš, dans le sud-est de la Serbie, vont du soulagement du stress, de l'anxiété et des problèmes de sommeil au traitement de l'inconfort gastro-intestinal, ce qui concorde avec les résultats de la recherche scientifique contemporaine. Malgré sa longue histoire d'utilisation et son intégration dans les pratiques thérapeutiques médicinales modernes, des recherches cliniques supplémentaires sont nécessaires pour étayer sa sécurité, son efficacité et ses mécanismes d'action. Cet article souligne l'importance de *M. officinalis* en tant que pont entre les connaissances traditionnelles et les applications médicinales modernes, en insistant sur la nécessité de préserver les connaissances traditionnelles.

*Mots-clés : Melissa officinalis, ethnobotanique, plantes médicinales, effets thérapeutiques, médecine traditionnelle, connaissances traditionnelles*

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# Традиционное этноботаническое использование *Melissa officinalis* в Нишском округе

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## Резюме

*Melissa officinalis* (*M. officinalis*), широко известная как мелисса, на протяжении веков использовалась в традиционной медицине благодаря своим разнообразным терапевтическим свойствам. В данной работе представлены результаты исследования традиционного использования мелиссы, подтверждающие ее применение в лечении различных заболеваний. Биоактивные соединения, содержащиеся в этом растении, такие как розмариновая кислота, флавоноиды и эфирные масла, обусловливают его заметные эффекты, включая анксиолитическое, антимикробное, антиоксидантное действие, а также положительное влияние на лечение заболеваний желудочно-кишечного тракта. Традиционное применение *M. officinalis* в Нишском округе (юго-восточная Сербия), от снятия стресса, тревожности и проблем со сном до лечения желудочно-кишечных расстройств, согласуется с выводами современных научных исследований. Несмотря на долгую историю использования и интеграцию в современные медицинские терапевтические практики, необходимы дополнительные клинические исследования для подтверждения ее безопасности, эффективности и механизмов действия. Данная работа подчеркивает значение *M. officinalis* как моста между традиционным и современным медицинским применением, акцентируя необходимость сохранения традиционных знаний.
*Ключевые слова: Melissa officinalis, этноботаника, лекарственные растения, терапевтические эффекты, традиционная медицина, традиционные знания*

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# Traditionelle ethnobotanische Anwendungen von *Melissa officinalis* im Bezirk Niš

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## ABSTRAKT

*Melissa officinalis* (*M. officinalis*), allgemein bekannt als Zitronenmelisse, wird seit Jahrhunderten in der traditionellen Medizin aufgrund ihrer vielfältigen therapeutischen Eigenschaften verwendet. Diese Arbeit untersucht ihre traditionellen Anwendungen und bewertet die pharmakologischen Belege, die ihre Wirksamkeit bei der Behandlung verschiedener Beschwerden unterstützen. Die bioaktiven Verbindungen in *M. officinalis*, wie Rosmarinsäure, Flavonoide und ätherische Öle, sind für ihre bemerkenswerten Wirkungen verantwortlich, darunter anxiolytische, antimikrobielle, antioxidative und verdauungsfördernde Eigenschaften. Traditionelle Anwendungen im Bezirk Niš im Südosten Serbiens reicht von der Linderung von Stress, Angstzuständen und Schlafstörungen bis hin zur Behandlung von Magen-Darm-Beschwerden und stimmen mit Erkenntnissen der modernen wissenschaftlichen Forschung überein. Trotz der langen Nutzungsgeschichte und der Integration in moderne medizinische therapeutische Praktiken sind weitere klinische Untersuchungen erforderlich, um Wirkungsmechanismen umfassend zu belegen. Diese Arbeit betont die Bedeutung von *M. officinalis* als Brücke zwischen traditionellem Wissen und modernen medizinischen Anwendungen und unterstreicht die Notwendigkeit, traditionelles Wissen zu bewahren.

*Schlüsselwörter: Melissa officinalis, Ethnobotanik, Heilpflanzen, therapeutische Wirkungen, traditionelle Medizin, traditionelles Wissen*

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# Do machine learning and molecular dynamics reveal key insights into GABA-sulfonamide conjugates as carbonic anhydrase inhibitors?

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## ABSTRACT

Carbonic anhydrase (CA) enzymes are critical to numerous physiological processes, making them valuable therapeutic targets. Aromatic and heterocyclic sulfonamides have demonstrated exceptional inhibitory activity, with significant applications in managing glaucoma, a complex and progressive neurodegenerative condition. This study employs an integrative approach combining machine learning, specifically Multiple Linear Regression (MLR) modeling, with molecular dynamics simulations to investigate a series of γ-aminobutyric acid (GABA)-conjugated sulfonamides. The MLR model effectively identified key structural and physicochemical features governing inhibitory activity against carbonic anhydrase isoforms II and IV, enabling precise predictions of biological efficacy. Molecular dynamics simulations were conducted exclusively on the most active GABA conjugate identified, in complex with CA II and CA IV enzymes. These simulations revealed atomistic details of enzyme-ligand interactions, highlighting critical binding interactions, dynamic stability, and conformational behavior driving potent inhibitory effects. By integrating machine learning techniques and targeted molecular dynamics simulations, this study not only deepens our understanding of sulfonamide activity but also provides a robust foundation for the rational design of next-generation inhibitors with enhanced therapeutic potential against glaucoma.

*Keywords: Machine learning, Molecular dynamics, GABA, Sulfonamides, Carbonic anhydrase*

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## Introduction

Carbonic anhydrases (CAs) are a family of metalloenzymes that catalyse the reversible hydration of carbon dioxide to bicarbonate and protons, a reaction fundamental to various physiological processes, including respiration, acid-base balance, and electrolyte secretion (Supuran & Scozzafava, 2007). In humans, 15 CA isoforms have been identified, each exhibiting distinct tissue distributions and physiological roles (Supuran & Scozzafava, 2007; Jaitak et al., 2024). Notably, aberrant CA activity is implicated in several pathological conditions, such as glaucoma, epilepsy, and certain cancers, rendering CAs significant therapeutic targets (Supuran, 2021; Naeem et al., 2024). Glaucoma, a neurodegenerative disorder and leading cause of irreversible blindness, is managed by lowering intraocular pressure, with CA inhibitors playing a key role in reducing aqueous humor production (Nocentini & Supuran, 2019). The inhibition of CA II and CA IV isoforms is critical for glaucoma treatment, as supported by the development of novel dual-tail sulfonamide inhibitors that exhibit effective and sustained intraocular pressure reduction (Angeli et al., 2024).

Sulfonamides, particularly aromatic and heterocyclic variants, have been extensively studied as CA inhibitors (Angeli et al., 2023). Inhibition of CAs by sulfonamides occurs through multiple mechanisms, with primary action involving direct binding to the active site zinc ion (Supuran, 2016a; Supuran, 2016b). Furthermore, modifications to their scaffolds with functional groups enable interactions with residues near the active site entrance, facilitating enhanced isoform selectivity and diverse inhibition profiles (Nocentini & Supuran, 2019). The incorporation of γ-aminobutyric acid (GABA) moieties into sulfonamide structures represents a significant advancement in CA inhibitor design (Mincione et al., 1999). This study has shown that GABA-sulfonamide conjugates exhibit different inhibition constants (Ki) for CA II and CA IV, underscoring their potential for isoform-specific inhibition.

Advancements in computational methodologies have revolutionised drug discovery processes (Xu, 2024). Machine learning (ML) techniques, including Multiple Linear Regression (MLR) modeling, are extensively utilised to predict biological activity from chemical structures, thereby facilitating the identification of potential drug candidates (Rodríguez-Pérez & Bajorath, 2021). For example, ML models have demonstrated considerable efficacy in accurately predicting the activity and selectivity profiles of human CA inhibitors, significantly enhancing the efficiency and precision of the drug development pipeline (Tinivella et al., 2021). Complementary to ML approaches, molecular dynamics (MD) simulations provide atomistic insights into the dynamic behaviour of enzyme-inhibitor complexes (Wei & McCammon, 2024). These simulations elucidate the conformational flexibility and stability of such complexes, offering a deeper understanding of binding interactions and inhibition mechanisms (Ilić, 2020; Ilić et al., 2021). Furthermore, a recent study elegantly highlights the pivotal role of molecular dynamics in providing deeper insights into the interactions between CA and sulfonamide inhibitor (Pagnozzi et al., 2022). Integrating ML and MD techniques enables a comprehensive analysis of potential

inhibitors by combining predictive modeling with detailed mechanistic insights (Frasnetti et al., 2024).

This study aims to investigate the inhibitory activity of a series of GABA-sulfonamide conjugates against CA II and CA IV isoforms, leveraging an integrative approach that combines ML-based MLR modeling and MD simulations. By identifying key structural and physicochemical parameters governing inhibitory activity, the research seeks to elucidate the molecular determinants underlying isoform selectivity and binding affinity. These findings are expected to provide a robust foundation for the rational design of next-generation CA inhibitors with enhanced therapeutic efficacy, particularly for the treatment of glaucoma.

## Experimental

### Machine learning

A dataset comprising 52 sulfonamides featuring the GABA moiety (Figure 1), along with their inhibitory activity values against the CA II and CA IV isoforms, was obtained from the study by Mincione et al. (1999). The dataset was processed using the DTC Lab Tools software, developed by the Drug Theoretics and Cheminformatics Laboratory at Jadavpur University, Kolkata, following the methodology described by Banerjee & Roy (2023). For modeling purposes, the compounds were divided into a training set (41 compounds) and a test set (11 compounds), ensuring a robust dataset partitioning for ML-based MLR modeling. The inhibitory activity values for the CA II and CA IV isoforms, originally reported as Ki values (nM), were converted to pKi values to ensure methodological compatibility with the MLR modeling process. The partitioning of the dataset, along with the pKi values for the training and test sets, is summarized in Table 1.

To develop the MLR model, 484 two-dimensional (2D) molecular descriptors were computed using E-Dragon, the remote version of Dragon, developed by the Milano Chemometrics and QSAR Research Group (Tetko et al., 2005). These descriptors encompassed a wide range of chemical and structural properties, including topological indices, walk and path counts, connectivity indices, functional group counts, and atom-type E-state indices (Todeschini & Consonni, 2009). Subsequently, a rigorous data pretreatment procedure was applied to refine the descriptor dataset. Specifically, descriptors with a standard deviation below 0.0001 were excluded as they lack significant variation across the dataset, rendering them uninformative and unnecessarily increasing computational complexity. Additionally, descriptors with pairwise correlation coefficients exceeding 0.95 were removed to eliminate multicollinearity, as such redundancy can lead to overfitting and compromise the interpretability and reliability of the MLR model. This preprocessing step reduced the initial descriptor pool to 87 variables deemed suitable for inclusion in the MLR modeling process.

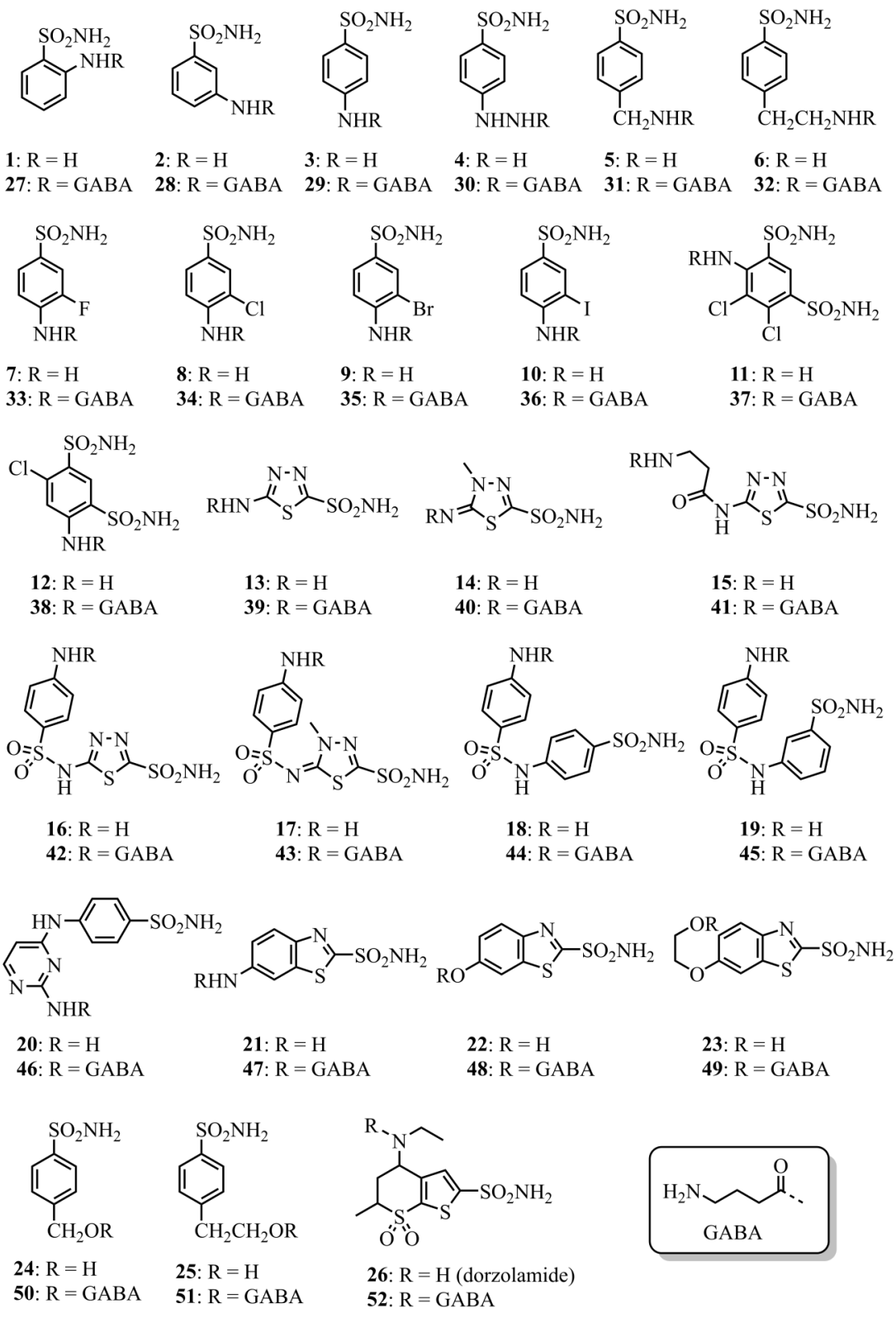


**Figure 1.** Aromatic and heterocyclic sulfonamides and their GABA conjugates as CA inhibitors

**Table 1.** Experimental and predicted inhibition constants for CA inhibitors

| Inhibitor | CA II isoform inhibition | | | CA IV isoform inhibition | | |
|---|---|---|---|---|---|---|
| | **Ki (nM)** | **pKi (nM)** | **pKi* (nM)** | **Ki (nM)** | **pKi (nM)** | **pKi* (nM)** |
| **1**[#] | 295 | 6.53 | 6.24 | 1310 | 5.88 | 5.68 |
| **2** | 240 | 6.62 | 6.48 | 2200 | 5.66 | 5.74 |
| **3** | 300 | 6.52 | 6.65 | 3000 | 5.52 | 5.77 |
| **4** | 320 | 6.49 | 6.66 | 3215 | 5.49 | 5.94 |
| **5** | 170 | 6.77 | 6.66 | 2800 | 5.55 | 5.74 |
| **6** | 160 | 6.80 | 6.67 | 2450 | 5.61 | 5.75 |
| **7**[#] | 60 | 7.22 | 7.25 | 180 | 6.74 | 6.43 |
| **8** | 110 | 6.96 | 7.25 | 320 | 6.49 | 6.31 |
| **9**[#] | 40 | 7.40 | 7.25 | 66 | 7.18 | 6.75 |
| **10**[#] | 70 | 7.15 | 7.25 | 125 | 6.90 | 6.78 |
| **11** | 28 | 7.55 | 7.72 | 175 | 6.76 | 6.97 |
| **12** | 75 | 7.12 | 7.18 | 160 | 6.80 | 6.60 |
| **13** | 60 | 7.22 | 7.46 | 540 | 6.27 | 6.34 |
| **14** | 19 | 7.72 | 7.62 | 355 | 6.45 | 6.48 |
| **15** | 3 | 8.52 | 7.99 | 125 | 6.90 | 7.21 |
| **16** | 2 | 8.70 | 8.60 | 5 | 8.30 | 8.06 |
| **17**[#] | 6 | 8.22 | 8.58 | 8 | 8.10 | 8.28 |
| **18**[#] | 6 | 8.22 | 8.01 | 50 | 7.30 | 7.61 |
| **19** | 9 | 8.05 | 7.99 | 53 | 7.28 | 7.52 |
| **20**[#] | 12 | 7.92 | 8.06 | 154 | 6.81 | 7.68 |
| **21** | 9 | 8.05 | 8.15 | 19 | 7.72 | 7.57 |
| **22** | 8 | 8.10 | 8.15 | 17 | 7.77 | 7.71 |
| **23**[#] | 7 | 8.15 | 8.29 | 15 | 7.82 | 7.92 |
| **24**[#] | 125 | 6.90 | 6.66 | 560 | 6.25 | 5.99 |
| **25** | 110 | 6.96 | 6.67 | 450 | 6.35 | 6.03 |
| **26** | 9 | 8.05 | 8.28 | 45 | 7.35 | 7.34 |
| **27** | 197 | 6.71 | 6.90 | 243 | 6.61 | 6.47 |
| **28** | 182 | 6.74 | 7.04 | 215 | 6.67 | 6.54 |
| **29**[#] | 112 | 6.95 | 7.09 | 164 | 6.79 | 6.58 |
| **30** | 212 | 6.67 | 7.21 | 305 | 6.52 | 6.70 |
| **31** | 32 | 7.49 | 7.21 | 69 | 7.16 | 6.58 |
| **32** | 30 | 7.52 | 7.22 | 62 | 7.21 | 6.56 |
| **33** | 10 | 8.00 | 7.60 | 38 | 7.42 | 7.20 |
| **34** | 31 | 7.51 | 7.60 | 63 | 7.20 | 7.06 |
| **35** | 30 | 7.52 | 7.60 | 60 | 7.22 | 7.52 |
| **36** | 27 | 7.57 | 7.60 | 56 | 7.25 | 7.56 |
| **37** | 10 | 8.00 | 8.01 | 50 | 7.30 | 7.44 |
| **38** | 9 | 8.05 | 7.54 | 50 | 7.30 | 7.12 |
| **39** | 8 | 8.10 | 8.10 | 43 | 7.37 | 7.20 |
| **40** | 10 | 8.00 | 8.00 | 42 | 7.38 | 7.52 |
| **41** | 5 | 8.30 | 8.36 | 15 | 7.82 | 7.96 |
| **42** | 3 | 8.52 | 8.54 | 9 | 8.05 | 7.83 |
| **43** | 4 | 8.40 | 8.46 | 14 | 7.85 | 7.94 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **44** | 14 | 7.85 | 7.86 | 75 | 7.12 | 7.23 |
| **45** | 13 | 7.89 | 7.85 | 67 | 7.17 | 7.13 |
| **46** | 11 | 7.96 | 8.07 | 50 | 7.30 | 7.55 |
| **47** | 5 | 8.30 | 8.40 | 12 | 7.92 | 7.79 |
| **48**[#] | 54 | 7.27 | 8.40 | 12 | 7.92 | 8.02 |
| **49** | 4 | 8.40 | 8.31 | 11 | 7.96 | 7.88 |
| **50** | 73 | 7.14 | 7.21 | 180 | 6.74 | 6.83 |
| **51** | 66 | 7.18 | 7.22 | 155 | 6.81 | 6.81 |
| **52** | 6 | 8.22 | 8.17 | 32 | 7.49 | 7.63 |

pKi*****- pKi value predicted by the MLR model
[#]- inhibitors of the test set analyzed using the MLR model

Using the DTC Lab Tools software and following the methodology described by Banerjee & Roy (2023), an extensive modeling process was conducted, resulting in the development of over 225 million MLR models for the CA II isoform and more than 274 million MLR models for the CA IV isoform. The selection of optimal models for both isoforms was based on rigorous validation using multiple statistical metrics. The validation process involved assessments performed on the training set, test set, and through the Y-randomization test. Key statistical metrics used for model evaluation included the coefficient of determination ($R^2$), adjusted coefficient of determination ($R_a^2$), cross-validated coefficient of determination ($Q^2$), scaled $r_{m(training)}^2$ and $\Delta r_{m(training)}^2$ for training set validation. For test set validation, predictive $R^2$ ($R_{pred}^2$), $Q_{F1}^2$, $Q_{F2}^2$, concordance correlation coefficient (CCC), $r_{m(test)}^2$, and $\Delta r_{m(test)}^2$ were calculated. Furthermore, average $R^2$, average $Q^2$, and ${}^{C}Rp^2$ were derived from the results of the Y-randomization test. The developed MLR models were assessed for acceptability based on the criteria recommended by Golbraikh & Tropsha (2002), ensuring their robustness and reliability. Additionally, the predictive quality of the models was evaluated using mean absolute error (MAE)-based criteria and categorized as 'Good', 'Moderate', or 'Bad', as recommended by Roy et al. (2016).

## Molecular dynamics simulation

The molecular dynamics simulation of the most potent GABA-sulfonamide conjugate, compound **42**, bound to the CA II (PDB: 4M2U) and CA IV (PDB: 3FW3) isoforms was performed using Desmond molecular dynamics software (version 2018.4). Developed by D. E. Shaw Research in New York, Desmond relied on PDB files obtained from the Protein Data Bank. The simulation protocol, with minor modifications, followed the methodology described by Ilić (2020) and Ilić et al. (2021). The water molecules in the system were modeled using the simple point charge (SPC) solvent model. Chloride ions ($Cl^-$) were introduced to neutralize the system, ensuring a net zero charge in the simulation box. The final system consisted of approximately 30,000 atoms. Before initiating the MD simulation, the system underwent a six-step relaxation protocol to ensure stability. The relaxed system was then subjected to a 100 ns simulation using a normal pressure-temperature (NPT) ensemble. A Nosé–Hoover thermostat maintained the temperature at 300 K, while a Martyna–Tobias–Klein barostat controlled the pressure at 1.01325 bar. Atomic coordinate data and system energies were recorded at intervals of 1 ps. To evaluate the dynamic behavior and

stability of the inhibitor-isoform complexes, root mean square deviation (RMSD) and root mean square fluctuation (RMSF) analyses were conducted over the entire simulation period. These metrics provided insights into the structural fluctuations and conformational stability of the complexes during the simulation.

## Results and Discussion

### Machine learning

The most effective MLR model for GABA-sulfonamide conjugates targeting CA II isoform is illustrated in Figure 2. The model demonstrates high predictive accuracy, as evidenced by the corresponding correlation parameters in Figure 2, and predicted pKi* values in Table 1. The statistical robustness and reliability of the model are substantiated by its compliance with the criteria recommended by Golbraikh & Tropsha (2002).

pKi*= 3.00709(+/-0.79181) - 0.00018(+/-0.00005) × Wap - 12.81387(+/-4.49435) × PW4 + 0.30171(+/-0.0486) × SRW05 + 0.95161(+/-0.14585) × piID - 0.31387(+/-0.07068) × nCb- + 0.81704(+/-0.11941) × nArX

Training set validation parameters:

$R^2$ = 0.89052, $R_a^2$ = 0.8712, $Q^2$ = 0.85603, $r_{m(training)}^2$ = 0.79956, $\Delta r_{m(training)}^2$ = 0.08739

Test set validation parameters:

$R_{pred}^2$ = 0.73695, $Q_{F1}^2$ = 0.55258, $Q_{F2}^2$ = 0.51286, CCC = 0.82018, $r_{m(test)}^2$ = 0.64512, $\Delta r_{m(test)}^2$ = 0.18128

Y-randomization test results:

average $R^2$ = 0.233384, average $Q^2$ = -0.14606, ${}^{C}Rp^2$ = 0.804388

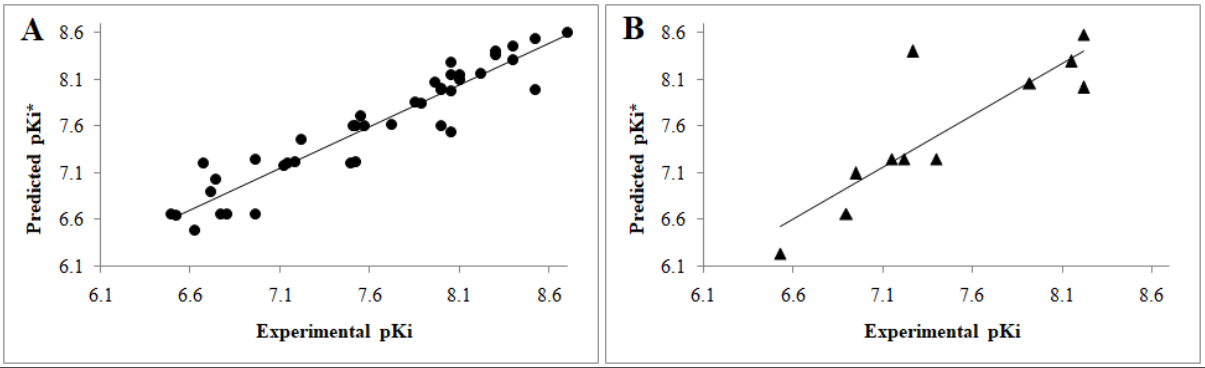


**Figure 2.** The MLR model, correlation parameters, and plots of experimental versus predicted pKi values for (**A**) the training set and (**B**) the test set of CA II inhibitors

**Table 2.** Calculated molecular descriptors of CA inhibitors

| Inhibitor | Wap | PW4 | SRW05 | piID | nCb- | nArX | ZM1V | Qindex | SaasC | SddssS | SsCl |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1**[#] | 383 | 0.15 | 0 | 6.18 | 2 | 0 | 194 | 8 | 0.15 | -3.66 | 0 |
| **2** | 383 | 0.13 | 0 | 6.16 | 2 | 0 | 194 | 8 | 0.43 | -3.61 | 0 |
| **3** | 383 | 0.12 | 0 | 6.15 | 2 | 0 | 194 | 8 | 0.59 | -3.58 | 0 |
| **4** | 490 | 0.13 | 0 | 6.31 | 2 | 0 | 210 | 8 | 0.70 | -3.60 | 0 |
| **5** | 490 | 0.13 | 0 | 6.31 | 2 | 0 | 190 | 8 | 1.13 | -3.55 | 0 |
| **6** | 618 | 0.14 | 0 | 6.45 | 2 | 0 | 194 | 8 | 1.22 | -3.55 | 0 |
| **7**[#] | 480 | 0.12 | 0 | 6.33 | 3 | 1 | 250 | 9 | -1.18 | -3.84 | 0 |
| **8** | 480 | 0.12 | 0 | 6.33 | 3 | 1 | 250 | 9 | 0.47 | -3.68 | 5.57 |
| **9**[#] | 480 | 0.12 | 0 | 6.33 | 3 | 1 | 250 | 9 | 1.04 | -3.63 | 0 |
| **10**[#] | 480 | 0.12 | 0 | 6.33 | 3 | 1 | 250 | 9 | 1.31 | -3.60 | 0 |
| **11** | 1139 | 0.16 | 0 | 7.20 | 5 | 2 | 430 | 14 | -2.66 | -8.49 | 11.20 |
| **12** | 986 | 0.15 | 0 | 7.08 | 4 | 1 | 374 | 13 | -1.61 | -8.28 | 5.56 |
| **13** | 273 | 0.13 | 2.40 | 5.69 | 0 | 0 | 244 | 8 | -0.17 | -3.72 | 0 |
| **14** | 353 | 0.13 | 2.40 | 5.89 | 0 | 0 | 236 | 9 | 0.13 | -3.70 | 0 |
| **15** | 864 | 0.14 | 2.40 | 6.52 | 0 | 0 | 312 | 9 | -0.22 | -3.85 | 0 |
| **16** | 2965 | 0.14 | 2.40 | 8.27 | 2 | 0 | 436 | 16 | 0.33 | -7.99 | 0 |
| **17**[#] | 3327 | 0.15 | 2.40 | 8.36 | 2 | 0 | 446 | 17 | 0.34 | -8.02 | 0 |
| **18**[#] | 3469 | 0.14 | 0 | 9.13 | 4 | 0 | 408 | 16 | 0.41 | -7.71 | 0 |
| **19** | 3469 | 0.15 | 0 | 9.17 | 4 | 0 | 408 | 16 | 0.14 | -7.88 | 0 |
| **20**[#] | 2665 | 0.15 | 0 | 8.43 | 2 | 0 | 310 | 13 | 1.39 | -3.67 | 0 |
| **21** | 1550 | 0.15 | 2.40 | 8.00 | 3 | 0 | 278 | 13 | 0.49 | -3.71 | 0 |
| **22** | 1550 | 0.15 | 2.40 | 8.00 | 3 | 0 | 294 | 13 | -0.07 | -3.75 | 0 |
| **23**[#] | 2658 | 0.15 | 2.40 | 8.36 | 3 | 0 | 338 | 13 | 0.43 | -3.77 | 0 |
| **24**[#] | 490 | 0.13 | 0 | 6.31 | 2 | 0 | 214 | 8 | 0.71 | -3.61 | 0 |
| **25** | 618 | 0.14 | 0 | 6.45 | 2 | 0 | 218 | 8 | 0.98 | -3.60 | 0 |
| **26** | 3300 | 0.17 | 2.40 | 7.67 | 0 | 0 | 350 | 17 | 0.66 | -7.30 | 0 |
| **27** | 1280 | 0.15 | 0 | 7.07 | 2 | 0 | 266 | 9 | 0.13 | -3.83 | 0 |
| **28** | 1280 | 0.14 | 0 | 7.00 | 2 | 0 | 266 | 9 | 0.40 | -3.74 | 0 |
| **29**[#] | 1280 | 0.13 | 0 | 6.97 | 2 | 0 | 266 | 9 | 0.57 | -3.68 | 0 |
| **30** | 1524 | 0.13 | 0 | 7.05 | 2 | 0 | 282 | 9 | 0.60 | -3.68 | 0 |
| **31** | 1524 | 0.13 | 0 | 7.05 | 2 | 0 | 270 | 9 | 0.89 | -3.66 | 0 |
| **32** | 1795 | 0.13 | 0 | 7.13 | 2 | 0 | 274 | 9 | 1.04 | -3.64 | 0 |
| **33** | 1471 | 0.14 | 0 | 7.11 | 3 | 1 | 322 | 10 | -1.25 | -3.95 | 0 |
| **34** | 1471 | 0.14 | 0 | 7.11 | 3 | 1 | 322 | 10 | 0.39 | -3.79 | 5.87 |
| **35** | 1471 | 0.14 | 0 | 7.11 | 3 | 1 | 322 | 10 | 0.97 | -3.74 | 0 |
| **36** | 1471 | 0.14 | 0 | 7.11 | 3 | 1 | 322 | 10 | 1.24 | -3.71 | 0 |
| **37** | 2636 | 0.16 | 0 | 7.80 | 5 | 2 | 502 | 15 | -2.84 | -8.77 | 11.70 |
| **38** | 2389 | 0.15 | 0 | 7.71 | 4 | 1 | 446 | 14 | -1.74 | -8.55 | 5.77 |
| **39** | 1040 | 0.14 | 2.40 | 6.61 | 0 | 0 | 316 | 9 | -0.20 | -3.85 | 0 |
| **40** | 1208 | 0.14 | 2.40 | 6.61 | 0 | 0 | 326 | 10 | 0 | -3.85 | 0 |
| **41** | 2208 | 0.13 | 2.40 | 7.06 | 0 | 0 | 392 | 10 | -0.34 | -3.92 | 0 |
| **42** | 6516 | 0.15 | 2.40 | 8.91 | 2 | 0 | 508 | 17 | 0.31 | -8.15 | 0 |
| **43** | 7198 | 0.15 | 2.40 | 9.00 | 2 | 0 | 518 | 18 | 0.33 | -8.17 | 0 |
| **44** | 7424 | 0.14 | 0 | 9.77 | 4 | 0 | 480 | 17 | 0.33 | -7.86 | 0 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **45** | 7424 | 0.15 | 0 | 9.81 | 4 | 0 | 480 | 17 | 0.06 | -8.04 | 0 |
| **46** | 6074 | 0.15 | 0 | 9.11 | 2 | 0 | 382 | 14 | 1.29 | -3.73 | 0 |
| **47** | 4018 | 0.15 | 2.40 | 8.70 | 3 | 0 | 350 | 14 | 0.48 | -3.80 | 0 |
| **48**[#] | 4018 | 0.15 | 2.40 | 8.70 | 3 | 0 | 370 | 14 | 0.20 | -3.82 | 0 |
| **49** | 6005 | 0.15 | 2.40 | 8.90 | 3 | 0 | 414 | 14 | 0.40 | -3.81 | 0 |
| **50** | 1524 | 0.13 | 0 | 7.05 | 2 | 0 | 290 | 9 | 0.75 | -3.68 | 0 |
| **51** | 1795 | 0.13 | 0 | 7.13 | 2 | 0 | 294 | 9 | 0.96 | -3.66 | 0 |
| **52** | 6291 | 0.17 | 2.40 | 8.15 | 0 | 0 | 436 | 19 | 0.17 | -7.64 | 0 |

[#]- inhibitors of the test set analyzed using the MLR model

Furthermore, the quality of the model was evaluated using the MAE-based criteria proposed by Roy et al. (2016), and an MAE value of 0.18025 confirms that the model satisfies the requirements for classification as a 'Good' model. This categorization underscores its applicability in reliably predicting the pKi values of novel GABA-sulfonamide conjugates, paving the way for rational design of potent CA II inhibitors. To provide deeper insights into the molecular determinants of the observed inhibitory activity, the calculated values of the descriptors used in the MLR model (Wap, PW4, SRW05, piID, nCb-, nArX) are presented in Table 2 for each GABA-sulfonamide conjugate. The descriptors in the MLR model and their directional contributions reveal key design principles for optimizing inhibitory potency against the CA II isoform (Figure 2).

The negative contribution of Wap (All-Path Wiener Index) to the inhibitory activity suggests that highly connected or compact molecular structures diminish the effectiveness of GABA-sulfonamide conjugates as CA II inhibitors. This likely occurs because overly condensed or less branched molecules may hinder proper alignment within the enzyme's active site or reduce binding flexibility. To enhance spatial compatibility and binding efficiency, introducing moderate branching or elongation into the molecular structure is recommended, allowing for improved interaction with the enzyme's active site.

The negative contribution of PW4 (Path/Walk 4 - Randić Shape Index) indicates that more complex molecular shapes over short paths of four bonds adversely affect inhibitory activity. This suggests that excessive local branching or structural irregularities may disrupt proper docking or cause steric clashes within the enzyme's active site. To address this, simplifying local molecular shapes by minimizing unnecessary branching or bulky substituents near the core structure is recommended to enhance binding efficiency and inhibition.

The positive contribution of SRW05 (Self-Returning Walk Count of Order 5) underscores the significance of local symmetry and structural stability in enhancing inhibitory activity. Molecules capable of maintaining symmetry over 5-bond paths are likely to stabilize interactions with the zinc ion and surrounding residues within the enzyme's catalytic center. To optimize this effect, it is advisable to preserve or enhance molecular symmetry, particularly in regions directly involved in enzyme binding.

The positive contribution of piID (Conventional Bond Order ID Number) indicates that stronger or more polarizable bonds, such as sulfonamide bonds, play a crucial role in enhancing inhibitory activity. This enhancement likely arises from improved coordination with the zinc ion or other critical interactions within the enzyme's active site. To optimize binding affinity, the design should prioritize molecules featuring strong, polar bonds, particularly high-quality sulfonamide groups.

The negative contribution of nCb- (Number of Substituted Benzene $C(sp^2)$) suggests that an increased number of substituents on the benzene ring can introduce steric hindrance or disrupt the electronic balance required for effective binding. To mitigate these issues and improve docking efficiency, it is advisable to limit the number of substituents on the benzene ring, ensuring the molecule remains compact and well-suited for interaction with the active site.

The positive contribution of nArX (Number of X on Aromatic Ring) highlights the beneficial impact of functionalizing the aromatic ring with suitable groups, such as -OH, -Cl, or $-CH_3$, on inhibitory activity. These modifications likely enhance interactions with the enzyme through improved hydrogen bonding, dipole interactions, or hydrophobic effects. To optimize these benefits, it is recommended to introduce functional groups that strengthen specific interactions while avoiding excessive bulk or disruptions to the molecule's electronic balance.

The MLR model developed for GABA-sulfonamide conjugates targeting the CA IV isoform is both robust and highly predictive. Its accuracy is demonstrated through the correlation parameters shown in Figure 3 and the predicted pKi* values detailed in Table 1. Statistical validation confirms the model’s reliability, meeting the criteria established by Golbraikh & Tropsha (2002), while an MAE value of 0.22307, categorizes it as a 'Good' model. The descriptor values (ZM1V, Qindex, Wap, SaasC, SddssS, SsCl), which serve as the foundation of the MLR model, are presented in Table 2 to provide a detailed analysis of molecular factors influencing inhibitory activity against the CA IV isoform.

The positive contribution of ZM1V (Zero-Order Molecular Connectivity Index) indicates that greater connectivity in the molecular graph plays a critical role in enhancing binding affinity. Molecules with well-connected atomic structures are likely to align their functional groups more effectively, facilitating optimal interactions with the CA IV active site. To maximize inhibitory activity, the design should focus on creating conjugates with dense and well-connected molecular scaffolds.

The positive contribution of the Q-index (Quadratic Index) highlights the role of molecular complexity and quadratic contributions in enhancing inhibitory activity. Higher Q-index values suggest that conjugates with carefully distributed atomic connections are structurally well-suited to fit the CA IV binding pocket. To optimize activity, it is important to maintain molecular complexity while avoiding excessive branching, ensuring an effective balance between structure and functionality.

The negative contribution of Wap suggests that fewer or less accessible molecular paths enhance inhibitory activity, indicating that excessive molecular flexibility or extended networks can reduce binding efficiency by introducing conformational instability. To optimize binding to CA IV, designing compact and rigid molecules is recommended, as these structures promote stability and improve interaction with the active site.

pKi*= 2.83126(+/-0.34758) + 0.01346(+/-0.00133) × ZM1V + 0.23813(+/-0.03506) × Qindex - 0.00038(+/-0.00005) × Wap + 0.10417(+/-0.08338) × SaasC + 0.41842(+/-0.04781) × SddssS - 0.06358(+/-0.02217) × SsCl

Training set validation parameters:

$R^2$ = 0.89695, $R_a^2$ = 0.87876, $Q^2$ = 0.86014, $r_{m(training)}^2$ = 0.80295, $\Delta r_{m(training)}^2$ = 0.08654

Test set validation parameters:

$R_{pred}^2$ = 0.83926, $Q_{F1}^2$ = 0.71703, $Q_{F2}^2$ = 0.71468, CCC = 0.8907, $r_{m(test)}^2$ = 0.67378, $\Delta r_{m(test)}^2$ = 0.14716

Y-randomization test results:

average $R^2$ = 0.224263, average $Q^2$ = -0.13319, $^{C}Rp^2$ = 0.822077

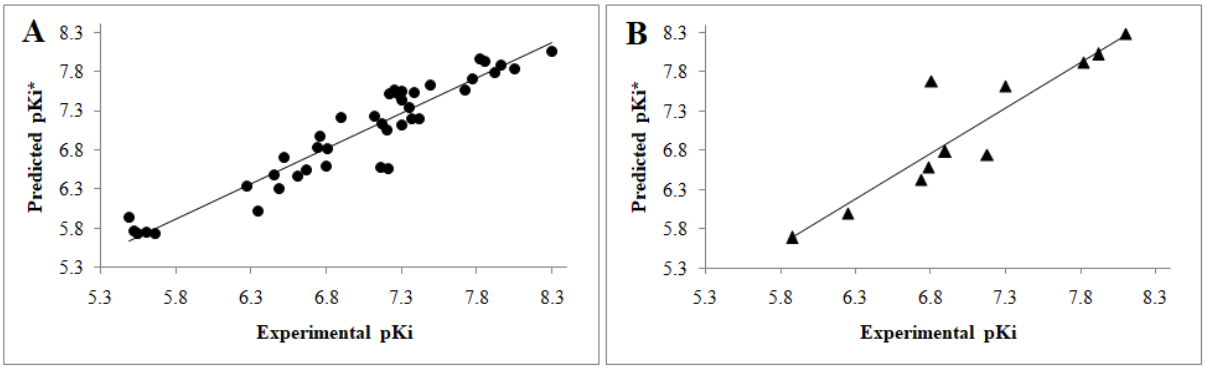


**Figure 3.** The MLR model, correlation parameters, and plots of experimental versus predicted pKi values for (**A**) the training set and (**B**) the test set of CA IV inhibitors

The positive contribution of SaasC (Sum of Atomic Electronegativity for Carbon Atoms) underscores the importance of the carbon framework, particularly in the GABA backbone and sulfonamide group, in stabilizing interactions with CA IV through hydrogen bonding or hydrophobic effects. A strong carbon-based backbone is essential for enhancing inhibitory activity.

The positive contribution of SddssS (Sum of Electronic Contributions for Sulfur Atoms with Specific Bonding Patterns) highlights the crucial role of sulfur atoms in the sulfonamide group. This descriptor emphasizes that these atoms contribute significantly to coordinating the zinc ion in the CA IV active site. To optimize inhibitory activity, the design should prioritize the inclusion

of sulfur atoms within the sulfonamide moiety, as they are essential structural components for effective binding.

The negative contribution of SsCl (Sum of Electronic Contributions for Chlorine Atoms) indicates that minimal electronic influence from chlorine atoms enhances inhibitory activity. This suggests that excessive electron-withdrawing effects or steric hindrance caused by chlorine substitution can disrupt optimal interactions with the CA IV active site. To maximize activity, chlorine should be used sparingly to modulate properties without introducing excessive halogen substitution.

## Molecular dynamics simulation

Understanding 2D and 3D molecular properties is critical in drug development to achieve a detailed characterization of molecular behavior and interactions. While 2D descriptors capture essential topological and electronic features, 3D properties such as spatial orientation and conformational flexibility are indispensable for accurate modeling of binding affinities, steric effects, and molecular dynamics (Cho & Choi, 2019). By leveraging 3D molecular characteristics, molecular dynamics reveals dynamic conformational changes, binding mechanisms, and interaction networks, offering a powerful framework for optimizing drug candidates (De Vivo et al., 2016; Do et al., 2018).

The molecular dynamics simulation of the most potent GABA-sulfonamide conjugate, compound **42**, in complex with the CA II isoform over a 100 ns trajectory unveiled significant details regarding the stability and interaction dynamics of the complex (Figure 4). The RMSD and RMSF values for Cα atoms, side chains, and heavy atoms consistently remained below the 2 Å threshold, in agreement with Liu & Kokubo (2017). These findings highlight minimal structural rearrangements and conformational changes, confirming the stability of the compound **42**/CA II complex. The interactions observed throughout the 100 ns molecular dynamics simulation underscored the critical roles of Glu69, His94, His119, Phe131, Thr199, Thr200, and Pro201 in the formation and stabilization of the compound **42**/CA II complex (Figure 4). The involvement of these residues in CA enzyme inhibition is thoroughly documented (Supuran & Scozzafava, 2007; Supuran, 2016a; Jaitak et al., 2024; Naeem et al., 2024). Hydrogen bonding is a central feature of ligand stabilization, as demonstrated in Figure 4A. Thr199 and His119 are critical contributors to these interactions, underscoring their importance in maintaining stability. Glu69 further supports the ligand through a combination of hydrogen bonding and ionic interactions, highlighting the role of charge complementarity. Additional stabilization arises from hydrophobic contacts with His94 and Phe131, as well as water-mediated bridges involving Thr200 and Pro201. As observed in Figure 4C, compound **42** stabilizes the complex through strong hydrogen bonds formed by the sulfonamide group via its oxygen atom with Thr199 and its nitrogen atom with His119. The amino group of the GABA tail interacts with Glu69 through hydrogen bonding and ionic interactions, playing a critical role in electrostatic stabilization. Hydrophobic interactions occur between the benzene ring and Phe131, as well as the thiadiazole ring and His94, strengthening the molecular interface through van der Waals forces. Additionally, the nitrogen

atoms of the thiadiazole ring form water-mediated bridges with Thr200 and Pro201, providing further stability to the complex.

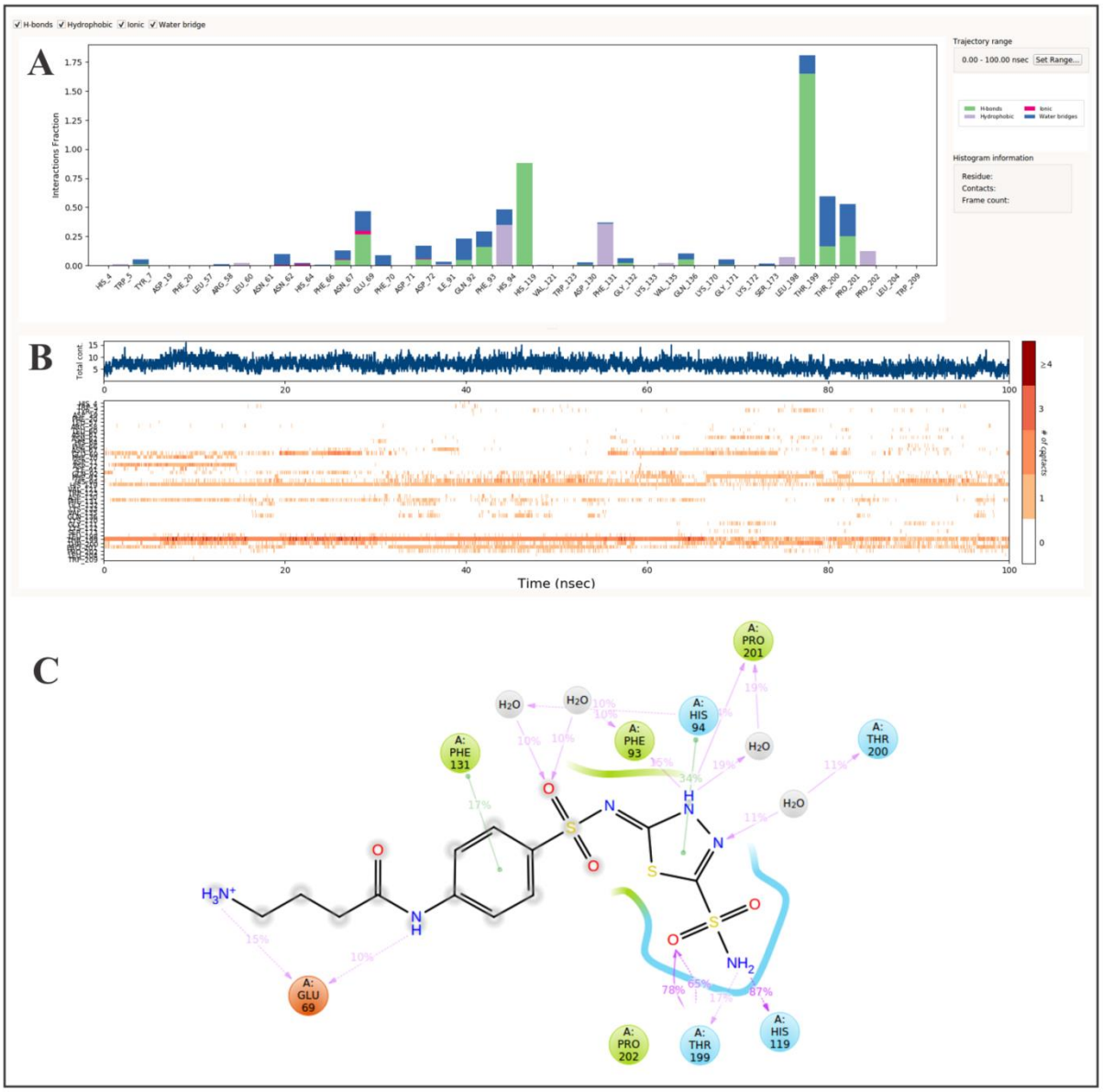


**Figure 4.** Interaction analysis of the GABA-sulfonamide conjugate **42** with CA II isoform: frequency distribution (**A**), interaction timeline (**B**), and spatial contact map (**C**) from a 100 ns molecular dynamics simulation

Based on the results, the design of novel GABA-sulfonamide conjugates targeting the CA II isoform can focus on replacing the thiadiazole ring with a heterocyclic scaffold capable of forming direct hydrogen bonds with Thr200 and Pro201. This substitution could eliminate reliance on water-mediated interactions, thereby enhancing binding affinity and the activity of the compounds (Fischer & Riedl, 2013). Additionally, the incorporation of hydrophobic substituents, such as

methyl or ethyl groups, on the benzene ring could strengthen van der Waals interactions with Phe131, further contributing to increased activity.

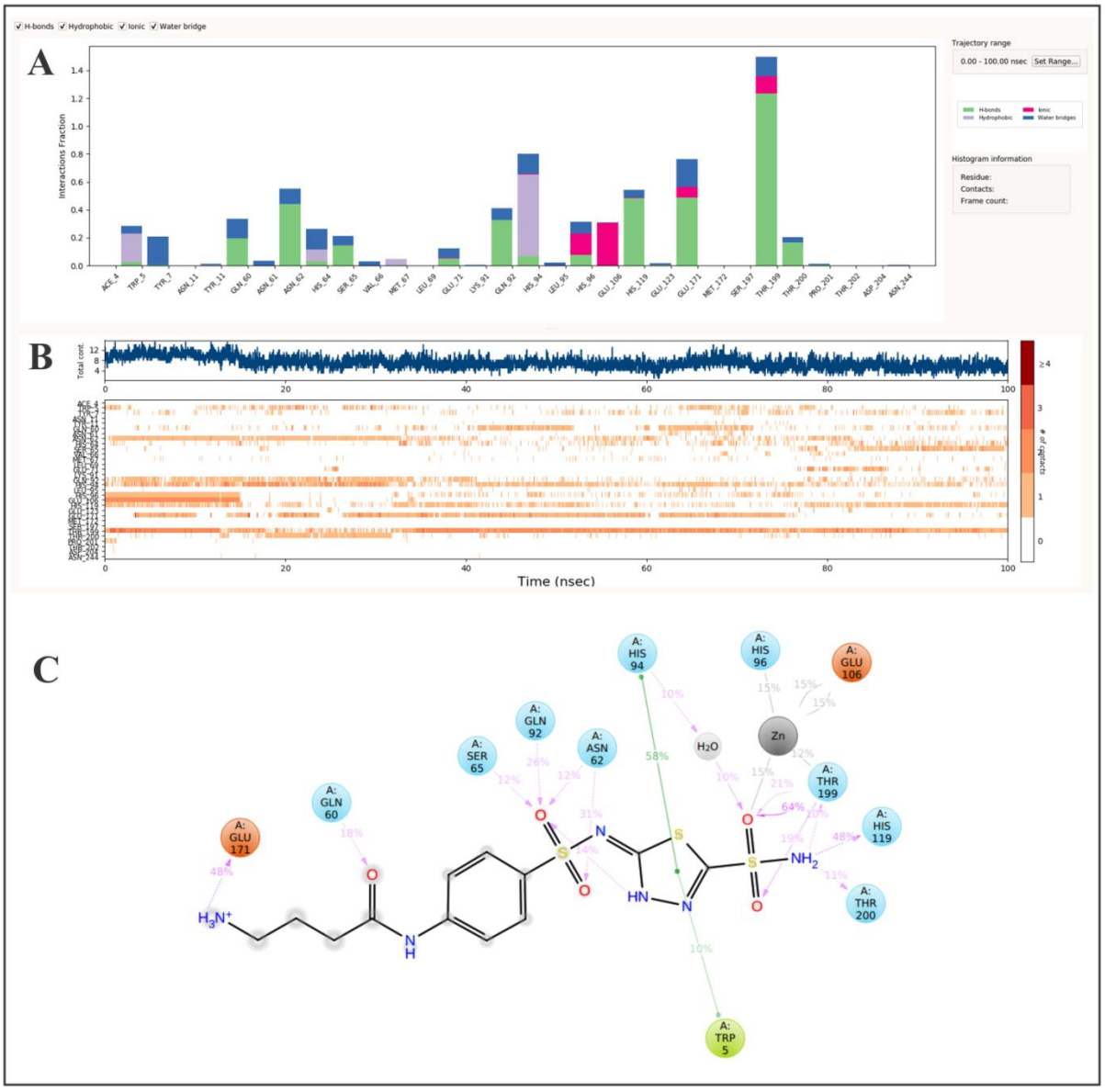


**Figure 5.** Interaction analysis of the GABA-sulfonamide conjugate **42** with CA IV isoform: frequency distribution (**A**), interaction timeline (**B**), and spatial contact map (**C**) from a 100 ns molecular dynamics simulation

The molecular dynamics simulation of compound **42** with the CA IV isoform revealed stability, with RMSD and RMSF values consistently below 2 Å (Figure 5). Key interactions were observed with residues Trp5, Gln60, Asn62, His64, Ser65, Gln92, His94, His96, Glu106, His119, Glu171, Thr199, and Thr200, emphasizing their roles in CA IV enzyme inhibition, as previously reported (Supuran & Scozzafava, 2007; Supuran, 2016a; Jaitak et al., 2024; Naeem et al., 2024).

The contributions of distinct interaction types are quantified in Figure 5, highlighting their roles in stabilizing compound **42**. The sulfonamide group in compound **42** forms strong hydrogen bonds with Thr199 via its oxygen atoms and with His119 and Thr200 through its nitrogen atom, while also coordinating to the zinc ion via an oxygen atom. A water-mediated bridge connects the sulfonamide group oxygen to His94, contributing to the stabilization of the complex. In contrast, the sulfonamide moiety, located between the benzene and thiadiazole rings, exhibits significant hydrogen bonding interactions with Asn62, Ser65, and Gln92 through its sulfonyl group, highlighting its distinct role in binding. The amino group of the GABA tail engages in strong hydrogen bonds with Glu171 and also demonstrates a degree of ionic interactions. Furthermore, the carbamoyl group of the GABA fragment interacts through hydrogen bonds with Gln60. The thiadiazole ring, as part of the structure, participates in persistent hydrophobic interactions with His94, while additional hydrophobic interactions are observed with Trp5, emphasizing the overall stabilizing contributions of compound **42** within the binding site. From the perspective of rational design, one potential approach for developing more potent inhibitors containing the GABA fragment toward the CA IV isoform is the replacement of the sulfonamide group with a hydroxamate, which would enhance coordination with the zinc ion and optimize hydrogen bonding (Di Fiore et al., 2012; Hou et al., 2021).

## Conclusion

This study provides a comprehensive analysis of GABA-sulfonamide conjugates as effective CA inhibitors, specifically targeting the CA II and CA IV isoforms. By integrating ML-based MLR modeling with MD simulations, key structural and physicochemical determinants of inhibitory activity were identified. The developed MLR models demonstrated exceptional predictive accuracy and statistical robustness, enabling the identification of critical molecular descriptors that influence CA inhibition. Simultaneously, MD simulations offered atomistic insights into the dynamic stability and specific binding interactions of the most potent GABA-sulfonamide conjugate within enzyme-ligand complexes. The findings highlight the importance of molecular connectivity, symmetry, and functional group modifications in optimizing inhibitor design. Structural modifications, such as substituting the thiadiazole ring or sulfonamide group, could further enhance binding affinity and isoform selectivity. This integrative approach enhances our mechanistic understanding of sulfonamide-based CA inhibitors while providing a robust framework for the rational design of next-generation inhibitors with improved therapeutic efficacy, particularly for the treatment of glaucoma, a progressive neurodegenerative disorder. Future research focusing on scaffold optimization and novel functional groups will likely yield inhibitors with enhanced isoform selectivity and potency.

## Acknowledgement

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## Conflict-of-Interest Statement

The auth

## Informed Consent Statement

Informed consent was obtained from all individual participants included in the study.

## Human and Animal Rights Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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# Da li mašinsko učenje i molekularna dinamika otkrivaju ključne uvide u GABA-sulfonamid konjugate kao inhibitore karboanhidraze?

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## SAŽETAK

Enzimi karboanhidraze (CA) ključni su za brojne fiziološke procese, što ih čini vrednim terapijskim ciljevima. Aromatični i heterociklični sulfonamidi pokazali su izuzetnu inhibicijsku aktivnost, sa značajnim primenama u lečenju glaukoma, složenog i progresivnog neurodegenerativnog oboljenja. Ova studija koristi integrativni pristup koji kombinuje mašinsko učenje, posebno modelovanje višestruke linearne regresije (MLR) sa simulacijama molekularne dinamike, radi istraživanja serije sulfonamida konjugovanih sa γ-aminobuteranom kiselinom (GABA). MLR model je efikasno identifikovao ključne strukturne i fizičkohemijske karakteristike koje upravljaju inhibicijskom aktivnošću protiv izoformi karboanhidraze II i IV, omogućavajući precizna predviđanja biološke efikasnosti. Simulacije molekularne dinamike sprovedene su isključivo na najaktivnijem GABA konjugatu identifikovanom u kompleksu sa enzimima CA II i CA IV. Ove simulacije otkrile su atomističke detalje interakcija između enzima i liganda, ističući ključne vezujuće interakcije, dinamičku stabilnost i konformacijsko ponašanje koji omogućavaju snažnu inhibicijsku aktivnost. Integracijom tehnika mašinskog učenja i ciljano usmerenih simulacija molekularne dinamike, ova studija ne samo da produbljuje razumevanje aktivnosti sulfonamida već pruža čvrstu osnovu za racionalni dizajn sledeće generacije inhibitora sa unapređenim terapijskim potencijalom u lečenju glaukoma.

*Ključne reči: Mašinsko učenje, Molekularna dinamika, GABA, Sulfonamidi, Karboanhidraza*

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# L'apprentissage automatique et la dynamique moléculaire révèlent-ils des informations clés sur les conjugués GABA-sulfonamide en tant qu'inhibiteurs de l'anhydrase carbonique?

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## RÉSUMÉ

Les enzymes d'anhydrase carbonique (CA) sont essentielles à de nombreux processus physiologiques, ce qui en fait des cibles thérapeutiques précieuses. Les sulfamides aromatiques et hétérocycliques ont démontré une activité inhibitrice exceptionnelle, avec des applications importantes dans la gestion du glaucome, une maladie neurodégénérative complexe et progressive. Cette étude utilise une approche intégrative combinant l'apprentissage automatique, en particulier la modélisation par régression linéaire multiple (MLR), avec des simulations de dynamique moléculaire pour étudier une série de sulfamides conjugués à l'acide γ-aminobutyrique (GABA). Le modèle MLR a identifié efficacement les principales caractéristiques structurelles et physicochimiques régissant l'activité inhibitrice contre les isoformes II et IV de l'anhydrase carbonique, permettant des prédictions précises de l'efficacité biologique. Des simulations de dynamique moléculaire ont été réalisées exclusivement sur le conjugué GABA le plus actif identifié, en complexe avec les enzymes CA II et CA IV. Ces simulations ont révélé des détails atomistiques des interactions enzyme-ligand, mettant en évidence les interactions de liaison critiques, la stabilité dynamique et le comportement conformationnel à l'origine de puissants effets inhibiteurs. En intégrant des techniques d'apprentissage automatique et des simulations de dynamique moléculaire ciblées, cette étude approfondit non seulement notre compréhension de l'activité des sulfamides, mais fournit également une base solide pour la conception rationnelle d'inhibiteurs de nouvelle génération avec un potentiel thérapeutique amélioré contre le glaucome.

*Mots-clés : Apprentissage automatique, Dynamique moléculaire, GABA, Sulfonamides, Anhydrase carbonique*

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# Раскрывают ли машинное обучение и молекулярная динамика ключевые аспекты о конъюгатах ГАМК-сульфонамида как ингибиторов карбоангидразы?

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## Резюме

Ферменты карбоангидразы (CA) играют ключевую роль во множестве физиологических процессов, что делает их важными терапевтическими мишенями. Ароматические и гетероциклические сульфонамиды демонстрируют высокую ингибирующую активность и находят значительное применение в лечении глаукомы, сложного и прогрессирующего нейродегенеративного заболевания. В этом исследовании используется интегративный подход, сочетающий машинное обучение, в частности моделирование множественной линейной регрессии (MLR), с симуляциями молекулярной динамики для изучения серии сульфонамидов, конъюгированных с γ-аминомасляной кислотой (GABA). MLR-модель эффективно выявила ключевые структурные и физико-химические характеристики, определяющие ингибирующую активность против изоформ карбоангидразы II и IV, обеспечивая точные прогнозы биологической эффективности. Симуляции молекулярной динамики проводились исключительно для наиболее активного GABA-конъюгата, идентифицированного в комплексе с ферментами CA II и CA IV. Эти симуляции выявили атомистические детали взаимодействий между ферментами и лигандами, подчеркивая ключевые связи, динамическую стабильность и конформационное поведение, которые способствуют высокой ингибирующей активности. Интеграция методов машинного обучения и целевых симуляций молекулярной динамики не только углубляет понимание активности сульфонамидов, но и предоставляет надежную основу для рационального дизайна следующего поколения ингибиторов с улучшенным терапевтическим потенциалом для лечения глаукомы.

*Ключевые слова : машинное обучение, молекулярная динамика, GABA, сульфонамиды, карбоангидраза*

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# Enthüllen maschinelles Lernen und Molekulardynamik wichtige Informationen über GABA-Sulfonamid-Konjugate als Carboanhydrase-Inhibitoren?

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## ABSTRAKT

Die Enzyme der Kohlensäureanhydrase (CA) sind für zahlreiche physiologische Prozesse von entscheidender Bedeutung, was sie zu wertvollen therapeutischen Ansatzpunkten macht. Aromatische und heterozyklische Sulfonamide haben außergewöhnliche hemmende Wirkungen gezeigt und finden breite Anwendung bei der Behandlung von Glaukomen, einer komplexen und progressiven neurodegenerativen Erkrankung. Diese Studie verfolgt einen integrativen Ansatz, das maschinelles Lernen, insbesondere die Methode der multiplen linearen Regression (MLR), mit Molekulardynamiksimulationen kombiniert, um eine Reihe von γ-Aminobuttersäure (GABA)-konjugierten Sulfonamiden zu untersuchen. Das MLR-Modell identifizierte erfolgreich die wesentlichen strukturellen und physikochemischen Merkmale, die die hemmende Wirkung gegen die Isoformen II und IV der Kohlensäureanhydrase steuern, und ermöglichte präzise Vorhersagen der biologischen Wirksamkeit. Molekulardynamik-simulationen wurden ausschließlich für das aktivste identifizierte GABA-Konjugat durchgeführt, das mit den Enzymen CA II und CA IV interagiert. Die Simulationen lieferten atomistische Details zu Enzym-Ligand-Interaktionen und zeigten kritische Bindungswechsel-wirkungen, dynamische Stabilität und Konformationsverhalten auf, die die starken hemmenden Effekte begünstigen. Durch die Integration von maschinellen Lerntechniken und gezielten Molekulardynamiksimulationen vertieft diese Studie nicht nur unser Verständnis der Sulfonamid-Aktivität, sondern bietet auch eine solide Grundlage für das rationale Design von Inhibitoren der nächsten Generation mit verbessertem therapeutischem Potenzial gegen Glaukome.

*Schlüsselwörter: Maschinelles Lernen, Molekulardynamik, GABA, Sulfonamide, Kohlensäureanhydrase*

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# The content of heavy metal(oid)s, total phenols, total flavonoids, rosmarinic acid, and antioxidant activity of lemon balm leaves (*Melissa officinalis* L.)

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## ABSTRACT

Lemon balm is a plant widely used across various industries worldwide, and it contains a significant number of phenolic compounds that can positively affect human health. Given its extensive use today, this study determined the total polyphenols and flavonoid contents, antioxidant activities, rosmarinic acid content, and heavy metal(oid)s levels in the leaves of the pot-grown lemon balm. The heavy metal(oid)s content was compared with the maximum permissible concentrations for medicinal plants, as the World Health Organization (WHO) recommended. The results showed that all analyzed elements (As, Cd, Co, Cu, Zn, Mn, Ni and Pb) concentrations were within the recommended limits. The phenolic compound content of lemon balm was compared with literature data for wild-grown lemon balm samples.

*Keywords: lemon balm, phenolic compounds, flavonoids, rosmarinic acid, antioxidant activity, heavy metal(oid)s*

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## Introduction

A medicinal plant, lemon balm (*Melissa officinalis* L.) is cultivated worldwide today due to its various positive effects on human health and its applications in the food and cosmetic industries. It is known that lemon balm has antioxidant, anti-inflammatory, antimicrobial, antiviral, and sedative properties. Additionally, it has a pleasant taste and good aroma, which makes it a popular additive in various products to improve their quality (Turhan, 2006).

Considering the properties of the lemon balm and its application in various industrial products, there is a significant need for its analysis and quality control. This includes the assessment of heavy metal(oid)s content, which could pose health risks to consumers of lemon balm and its derivatives, as well as the determination of total phenols, total flavonoids, antioxidant activity, and rosmarinic acid content. It is known that rosmarinic acid has a positive effect on the *Herpes simplex* virus, contributing to the medicinal effects of lemon balm (Petersen and Simmonds, 2003).

As the products of secondary metabolism in plants, phenolic compounds play a crucial role in their defence from various stress factors such as low temperatures, pathogen infections, nutrient deficiencies, heavy metal-induced stress, and others. These stressors trigger the production of free radicals and other molecules that cause oxidative stress (Lattanzio, 2013). Phenolic compounds contents in plants are crucial for the efficacy of medicinal plant-based products because they are natural antioxidants.

Given that different factors can influence the phenolic content, such as the presence of heavy metal(oid)s (Márquez-García et al., 2012) and cultivation conditions (Nurzyńska-Wierdak, 2023), there is need to explore these effects. Since cultivation conditions can significantly impact phenolic content, one of the goals of this study is to examine the influence of growing lemon balm in pots on phenolic content and compare it with literature data on wild-grown lemon balm samples. This comparison will be used as a basis for further research, such as the impact of elevated concentrations of heavy metal(oid)s on the antioxidant activity and phenolic compound content of lemon balm grown in pots, as well as the uptake of other heavy metal(oid)s present, both essential and potentially toxic.

Rosmarinic acid, an ester of caffeic acid, was first isolated from rosemary (*Rosmarinus officinalis*) in 1958. It is found in numerous plants, such as *Ocimum tenuiflorum* L., *Thymus mastichina* L., and others from the *Lamiaceae* family. Rosmarinic acid is believed to exhibit antioxidant, anti-aging, anti-inflammatory, antibacterial, antiviral, anticancer, antidiabetic, cardioprotective, hepatoprotective, nephroprotective, antidepressant, antiallergic properties (Nadeem et al., 2019). Since rosmarinic acid has antioxidant and antimicrobial activities and positively affects human health, it is used in the cosmetic and food industry to extend the expiry date of products.

Heavy metal(oid)s are naturally present in plants and can be categorized into essential and non-essential elements. Essential elements are necessary for plant growth and development. However, excessive levels of some essential elements can also negatively influence plant growth. In contrast, non-essential elements are not required for plant growth, and their elevated presence leads to harmful effects, such as leaf chlorosis, reduced plant growth, and other symptoms (Asati et al.,

2016). Determining the content of heavy metal(oid)s in plants is of great importance, as excessive levels can negatively affect the health of people consuming these plants.

## Experimental

Lemon balm leaf samples were obtained by growing the plant in pots under natural conditions from June until August 2023. The soil used for cultivation was collected near Niš, from an area where no agro-technical measures had been applied for many years, to minimize the influence of these factors on the parameters being investigated. The collected soil was first air-dried for two weeks, sieved, and then mixed with commercially available Hawita Professional substrate in a 1:1 volumetric ratio to improve soil fertility. One-month-old lemon balm seedlings, grown from seeds purchased from the Dr. Josif Pančić Institute, were planted in this prepared soil. The collected lemon balm samples were air-dried before further analysis.

The extraction of phenolic compounds from the lemon balm samples was performed as described in Adamczyk-Szabela et al. (2023). Briefly, approximately 0.5 g of dried plant material was accurately weighed and transferred to an Erlenmeyer flask. In the flask, 50 mL of 70% methanol was added, and the extraction was carried out by placing the flask on a shaker for 2 h at room temperature. After extraction, the liquid was separated from the solid by centrifugation at 3000 rpm for 10 min in plastic tubes. The resulting extract was then filtered using microfilters (0.45 µm) and stored in a refrigerator until further analysis.

### Determination of heavy metal(oid)s content using ICP OES

To determine the heavy metal(oid)s content, dried lemon balm leaf samples were prepared using the microwave digestion (ETHOS EASYmicrowave digestion system, Milestone, Bergamo, Italy) method as follows: approximately 0.35 g of the sample was weighed, transferred to digestion vessels, and 3 mL of concentrated $H_2O_2$ and 6 mL of concentrated $HNO_3$ were added. The digestion program was as follows: a temperature of 180 °C was reached within 20 min and then maintained for 10 min. After the digestion process was completed, the samples were allowed to cool. The entire content from the vessels was then quantitatively transferred to volumetric flasks (25 mL) and filled to the mark with deionized water.

For the ICP OES analysis, an external calibration curve method was used, with the wavelength selection for reading the results based on the relative intensity of the emission lines, the correlation coefficient value, spectral interferences, and the matrix effect on emission. The following heavy metal(oid)s were determined in the lemon balm leaves: As, Cd, Co, Cu, Zn, Mn, Ni, and Pb. ICP analysis was performed using ICP OES, series iCAP 6000 (ThermoScientific, Cambridge, United Kingdom).

## Determination of total polyphenolic compounds content

The total polyphenol content in the lemon balm samples was determined using the Folin-Ciocalteu method. 0.1 mL of the extract, obtained by the previously described method (Adamczyk-Szabela et al., 2023), was measured in a 10 mL volumetric flask. Then, 0.5 mL of Folin-Ciocalteu reagent was added, followed by 2 mL of a saturated $Na_2CO_3$ solution after 5 minutes. The volumetric flask was then filled with deionized water to a final volume of 10 mL, and left in the dark for 30 min, and the absorbance of the sample was measured at 760 nm, with deionized water as a blank using UV VIS Perkin Elmer Lambda 15 spectrometer. The calibration curve was constructed by measuring the absorbance of standard gallic acid solutions to which 0.5 mL of Folin-Ciocalteu reagent and 2 mL of saturated $Na_2CO_3$ solution were added, and the volume was adjusted with deionized water, resulting in final gallic acid concentrations ranging from 1 to 9 µg/mL. After 30 minutes in the dark, the absorbance of the standard solutions was measured, and the calibration curve was obtained: $A = 0.04385 + 0.10517c_{\text{gallic acid}}$, $r^2 = 0.99986$ (Singleton et al., 1999; Stratil et al., 2006; Huang et al., 2005). The results of the extract analysis were expressed as mg of gallic acid equivalents per g of dry lemon balm sample.

## Determination of total flavonoid content

0.1 mL of the lemon balm extract was measured into a volumetric flask (10 mL), and 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 1.5 mL of $AlCl_3$ solution was added, and after another 5 min, 2 mL of 1M NaOH was added. The volumetric flask was then filled with deionized water to 10 mL. The absorbance was measured at 510 nm, with deionized water as a blank. A series of working solutions of catechin was prepared from a stock solution of catechin at a concentration of 0.5 mg/mL and used to create a calibration curve, which showed linearity in the concentration range from 1 to 10 mg/L. The calibration equation was $A = 0.03612 + 0.00491c_{\text{catechin}}$. Based on this equation, the total flavonoid content was calculated and expressed as mg of catechin equivalents per g of dry sample (mg CE/g).

## Determination of antioxidant activity using the DPPH method

0.5 mL of the obtained extract was diluted to 50 mL with 70% (v/v) methanol. The DPPH method was used, as described by Brand-Williams et al. (1995), with minor modifications. A solution of 2,2-diphenyl-2-picrylhydrazyl (DPPH) with a concentration of $1 \cdot 10^{-4}$ mol/L in methanol was prepared. A 5.0 mL aliquot of this solution was placed in a volumetric flask (10 mL), along with 0.5 mL of the diluted sample. The flask was then filled to 10 mL with methanol. After 30 min, the colour change of the DPPH radical was determined spectrophotometrically at 520 nm. A calibration curve was created using Trolox solutions based on the decrease in absorbance ($\Delta A = A_{\text{blank}} - A$), which corresponded to the DPPH radical scavenging activity. The results were reported as µg of Trolox equivalents (TE) per g of dry lemon balm (µg TE/g).

## Determination of rosmarinic acid

An Agilent 1200 model (Agilent Technologies, Santa Clara, California, USA) was employed for HPLC analysis. The analytical column used was a C18 Zorbax Eclipse XDBC18, 5 µm, 4.6×150 mm (Agilent Technologies, Santa Clara, California, USA). The mobile phase flow rate was set to 0.8 mL/min, and the analysis time was 40 min. Solvent A was 5% HCOOH in deionized water, and Solvent B was 5% HCOOH in 80% acetonitrile, with the following gradient program: 0% B during the first 10 min, 0–25% B from 10 to 20 min, 25–40% B from 20 to 30 min, 40–70% B from 30 to 35 min, and 70–80% B from 35 to 40 min.

## Results and Discussion

The content of heavy metal(oid)s in the lemon balm leaves is provided in Table 1.

**Table 1.** Content of heavy metal(oid)s in lemon balm leaves and selected wavelengths for determination

| Element/λ[nm] | Concentration [mg/kg] | Element/λ[nm] | Concentration [mg/kg] |
|---|---|---|---|
| As/189.0 | 0.5 ± 0.2 | Zn/202.5 | 71.7 ± 0.2 |
| Cd/214.4 | 0.08 ± 0.00 | Mn/257.6 | 30.0 ± 0.2 |
| Co/228.6 | 0.48 ± 0.02 | Ni/221.6 | 3.24 ± 0.03 |
| Cu/324.7 | 10.45 ± 0.06 | Pb/220.3 | 0.84 ± 0.02 |

Among the analyzed elements, Zn, Mn, Cu, and Ni are the most prevalent, which can be explained by the fact that these are essential elements necessary for plant growth (Sarwar et al., 2017). In addition, Co is an essential metal, but its concentration is significantly lower than the previously mentioned elements. Pb, As, and Cd are less prevalent as they are non-essential elements that plants do not require. The content of the analyzed elements is shown in Figure 1.

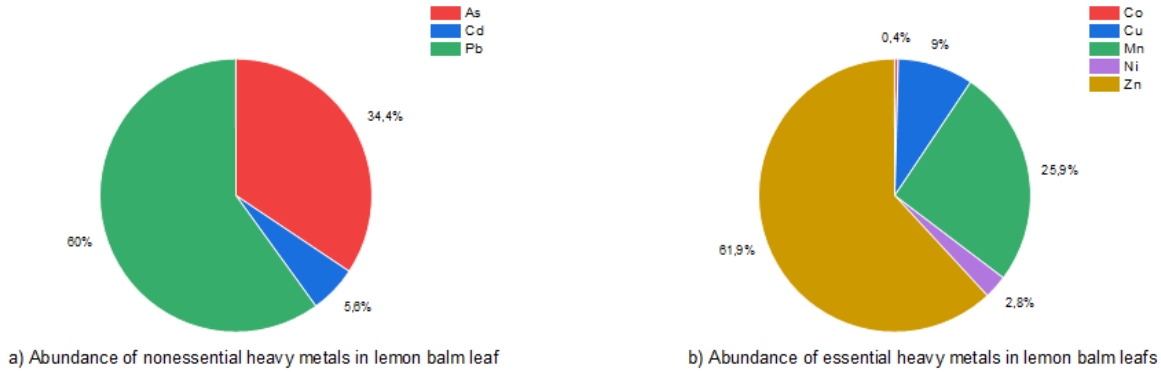


Figure 1. Distribution of heavy metal(oid)s in lemon balm leaves

As seen in Figure 1, the order of prevalence for essential elements is as follows: Zn > Mn > Cu > Ni > Co, and for non-essential elements, Pb > As > Cd. The concentrations of Cd and Pb are within the permissible limits for medicinal plants, based on the recommendations of the World Health Organization, as well as the values for Canada and China (WHO, 2007). The obtained concentrations of the analyzed elements in lemon balm are similar to the results obtained in the study by Sussa et al. (2022), where lemon balm was also grown in pots with the slightly lower concentration of Pb and Ni and somewhat higher the concentration of Zn.

The concentration of total polyphenols was 69 ± 3 mg/g gallic acid equivalent, expressed on a dry mass basis. In the study by Spiridon et al. (2011), the concentration of total phenols was 54.9 ± 2.14 mg/g, expressed on the dry extract mass of lemon balm. According to Boneza and Niemeyer (2018), the total phenol content ranged from 5.50 to 26.87 mg/g gallic acid equivalents, depending on the cultivar. In the works of Spiridon et al. (2011) and Boneza and Niemeyer (2018), samples from nature were used.

The concentration of total flavonoids, expressed on a dry mass basis of the plant, was 78 ± 2 mg/g catechin equivalents. According to Spiridon et al. (2011), the total flavonoid content in lemon balm was 25.8 ± 6.26 mg/g rutin equivalents, expressed on the dry extract mass, while for oregano (*Origanum vulgare*), the value for total flavonoids was 31.6 ± 4.25 mg/g rutin equivalents. Lin et al. (2012) determined total flavonoids in lemon balm leaves dried in two ways: warm air and freeze-drying. These authors extracted lemon balm leaves using ethanol and obtained results for the freeze-dried sample of 54.32 ± 4.13 mg/g catechin equivalents, and for the warm air-dried sample, 48.45 ± 3.24 mg/g catechin equivalents. Hassan et al. (2019) compared the impact of different solvents for extraction on the total flavonoid content in lemon balm extracts, obtaining the following results for the methanol extract: 72.38 mg/g dry extract of quercetin equivalents (QE), for methylene chloride extract: 59.76 mg/g QE eq., for ethyl acetate extract: 124.96 mg/g QE eq., and for butanol extract: 84.96 mg/g QE eq. The results of Hassan et al. (2019) clearly indicate that depending on the solvent used for extraction, the total flavonoid content varies, but the results obtained in this study, where a 70% (v/v) methanol extract was used, are similar to those obtained by Hassan et al. (2019) when methanol was used for extraction. All samples in the literature data include wild lemon balm.

The antioxidant activity of lemon balm leaves is one of its crucial properties, contributing to a wide range of applications. Thus, determining and controlling the antioxidant activity of lemon balm is very important. The antioxidant activity of lemon balm, determined by the DPPH method for the potted lemon balm leaves, is 379 ± 4 μmol/g Trolox equivalents, expressed on the dry mass of the lemon balm sample.

Rosmarinic acid is one of the most significant phenolic compounds in lemon balm. Based on the HPLC analysis, the content is 26.9 ± 0.1 mg/g of dried sample for potted lemon balm. Dastmalchi et al. (2008) reported a 96.45 ± 0.13 mg/g value for rosmarinic acid in their lemon balm samples. Arceusz and Wesolowski (2013), analyzing 19 lemon balm samples from Poland, found the concentration of rosmarinic acid to range from a minimum of 0.158 mg/g to a maximum of 48.608 mg/g, with an average of 27.05 mg/g, which is approximately consistent with the results obtained

in this study. This suggests no significant difference in rosmarinic acid content between potted lemon balm and wild lemon balm. Wang et al. (2004) found a value of 27.4 mg/g of rosmarinic acid in their lemon balm analysis, further supporting the earlier conclusion that there is no substantial difference between potted lemon balm and field-grown lemon balm regarding rosmarinic acid content.

Future studies should include research on the impact of elevated concentrations of some heavy metal(oid)s in soil on phenolic compounds content, antioxidant activity, and the content of heavy metal(oid)s (essential and potentially toxic) of lemon balm. Another focus of further research should be on improving the uptake of heavy metal(oid)s by lemon balm using different complexing agents, the prevention of heavy metal(oid)s uptake using zeolites, and the effect of different factors such as pH of soil, content of organic matter in soil, and the presence of some pesticides on heavy metal(oid)s uptake by lemon balm. This research will be conducted as pot experiments since concentrations of heavy metal(oid)s, phenolic compounds, flavonoids, and rosmarinic acid are similar in lemon balm grown in pots and naturally grown lemon balm.

## Conclusion

In this study, the content of total polyphenols, total flavonoids, antioxidant activity, rosmarinic acid content, and the presence of eight heavy metal(oid)s (As, Cd, Co, Cu, Zn, Mn, Ni, and Pb) in lemon balm leaves was determined. The content of all heavy metal(oid)s was below the maximum allowed concentrations according to the World Health Organization (WHO) guidelines. Essential elements were present in higher quantities than non-essential. The total polyphenol content was 69 ± 3 mg/g gallic acid equivalents, total flavonoid content was 78 ± 2 mg/g catechin equivalents, antioxidant activity measured by the DPPH method was 379 ± 4 μmol/g Trolox equivalents, and the rosmarinic acid content was 26.9 ± 0.1 mg/g. All these values, as well as the values for heavy metal(oid)s content in lemon balm grown in pots in this study, are comparable to those found in the literature for wild lemon balm samples.

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## Conflict-of-Interest Statement

The authors declare no conflict of interest.

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# Sadržaj teških metal(oid)a, ukupnih fenola, ukupnih flavonoida, rozmarinske kiseline i antioksidativne vrednosti listova matičnjaka (*Melissa officinalis* L.)

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## SAŽETAK

Matičnjak je biljka koja se koristi širom sveta u različitim granama industrije i koja sadrži veliki broj fenolnih jedinjenja koja mogu pozitivno uticati na ljudsko zdravlje. U ovom radu određen je sadržaj ukupnih polifenola, flavonoida, antioksidativna aktivnost, sadržaj ruzmarinske kiseline i teških metal(oid)a u listu matičnjaka. Sadržaj teških metal(oid)a upoređivan je sa maksimalno dozvoljenim koncentracijama u lekovitim biljkama a na osnovu preporuka Svetske zdravstvene organizacije. Dobijeni rezultati su pokazali da su koncentracije analiziranih elemenata u skladu sa preporukama. Sadržaj fenolnih jedinjenja matičnjaka upoređivan je sa literaturnim podacima za uzorke matičnjaka iz prirode.

*Ključne reči: matičnjak, fenolna jedinjenja, flavonoidi, rozmarinska kiselina, DPPH, teški metal(oid)i*

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# Teneur en métaux lourds, phénols totaux, flavonoïdes totaux, acide rosmarinique et activité antioxydante des feuilles de mélisse officinale (*Melissa officinalis* L.)

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## RÉSUMÉ

La mélisse officinale est une plante largement utilisée dans diverses industries à travers le monde et contient un nombre important de composés phénoliques qui peuvent avoir un effet positif sur la santé humaine. Compte tenu de son utilisation intensive aujourd'hui, cette étude a déterminé la teneur totale en polyphénols et flavonoïdes, l'activité antioxydante, la teneur en acide rosmarinique et les niveaux de métaux lourds dans les feuilles de mélisse officinale cultivée en pot. La teneur en métaux lourds a été comparée aux concentrations maximales autorisées pour les plantes médicinales, comme le recommande l'Organisation mondiale de la santé. Les résultats ont montré que toutes les concentrations d'éléments analysés se situaient dans les limites recommandées. La teneur en composés phénoliques de la mélisse officinale a été comparée aux données de la littérature pour des échantillons de mélisse officinale cultivés à l'état sauvage.

*Mots-clés : mélisse officinale, composés phénoliques, flavonoïdes, acide rosmarinique, DPPH, métaux lourds*

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# Содержание тяжелых металлов(оидов), общих фенолов, флавоноидов, розмариновой кислоты и антиоксидантной активности листьев мелиссы (*Melissa officinalis* L.)

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## Резюме

Мелисса — это растение, широко используемое во многих отраслях промышленности, содержащее множество фенольных соединений, которые могут положительно влиять на здоровье человека. В данной работе были определены содержание общих полифенолов, флавоноидов, антиоксидантная активность, содержание розмариновой кислоты и тяжелых металлов(оидов) в листьях мелиссы. Содержание тяжелых металлов(оидов) сравнивалось с максимально допустимыми концентрациями в лекарственных растениях в соответствии с рекомендациями Всемирной организации здравоохранения. Полученные результаты показали, что концентрации анализируемых элементов соответствуют установленным рекомендациям. Содержание фенольных соединений мелиссы сравнивалось с литературными данными для образцов мелиссы, собранных в природных условиях.

*Ключевые слова: мелисса, фенольные соединения, флавоноиды, розмариновая кислота, DPPH, тяжелые металлы(оиды)*

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# Der Gehalt an Schwermetall(oiden), Gesamtphenolen, Gesamtflavonoiden, Rosmarinsäure und antioxidativer Aktivität in Blättern der Zitronenmelisse (*Melissa officinalis* L.)

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## ABSTRAKT

Die Zitronenmelisse ist eine Pflanze, die weltweit in verschiedenen Industriezweigen verwendet wird und eine bedeutende Anzahl phenolischer Verbindungen enthält, die sich positiv auf die menschliche Gesundheit auswirken können. Angesichts ihrer weit verbreiteten Nutzung untersucht diese Studie den Gesamtgehalt an Polyphenolen und Flavonoiden, die antioxidative Aktivität, den Rosmarinsäuregehalt sowie den Schwermetallgehalt in den Blättern von im Topf gezogener Zitronenmelisse. Der Gehalt an Schwermetallen wurde mit den von der Weltgesundheitsorganisation empfohlenen Höchstkonzentrationen für Heilpflanzen verglichen. Die Ergebnisse zeigten, dass alle analysierten Elementkonzentrationen innerhalb der empfohlenen Grenzwerte lagen. Der Gehalt an phenolischen Verbindungen in der Zitronenmelisse wurde mit Literaturdaten für wildwachsender Zitronenmelisse-Proben verglichen.

*Schlüsselwörter: Zitronenmelisse, phenolische Verbindungen, Flavonoide, Rosmarinsäure, DPPH, Schwermetalle*

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# Presentation of activities of European Cooperation in Science and Technology (COST) action CA22105-BeSafeBeeHoney

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## ABSTRACT

Honey has been popular for its nutritional and health properties since ancient times due to the presence of bioactive compounds with antioxidant, antimicrobial, anti-inflammatory and anti-cancer properties. The European bee (*Apis mellifera*) is the most important pollinator and extremely important in agriculture. The number of bees is decreasing worldwide due to anthropogenic activities, climate change and the application of pesticides. This led to a decrease in honey production. Europe imports 40% of the honey it consumes annually. The EU tries to prevent and help to achieve international cooperation and exchange of innovative scientific and professional knowledge and achievements in the following topics: honey and bee products' nutritional and medicinal properties, abiotic stressors and anthropogenic contaminants in the environment using hive products as indicators, prevalent diseases and biotic stressors threatening honeybee colonies, honeybees as pollinators in agriculture and consequences of lost colonies in agrarian ecosystems and policy research and market analysis related with beekeeping activities. The COST action CA22105 brings together beekeeping, veterinary, agriculture engineering, chemistry, biology, nutrition, economy and policy to deliver scientific developments. Cost action takes care of achieving gender equality, young researchers. The implementation of the tasks of this COST action is carried out within several working groups.

*Keywords: bee, honey, bee products, dissemination of knowledge*

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## Overview of working groups (WG) inside CA22105 (COST Association, 2024; Nebot et al., 2024)

### WG1: Nutritional and medicinal properties of honey and by-products: quality criteria in motion

This working group focuses on bee products, their nutritional and medicinal properties and aspects related to their authenticity. The objective is to have an overview of current knowledge, emerging data, as well as the analytical methods available and applicable in different laboratories. Information regarding honey, royal jelly, wax, propolis and pollen, among others, must be reviewed and compiled to explain and promote the benefits of these products on human health.

### WG2: Biomonitoring of abiotic stressors or anthropogenic contaminants in the environment

This working group's goals are to monitor the environment, collect specific data on the use of contaminants, and contribute to the global improvement of environmental pollution monitoring.

As a prerequisite, inside this working group, it is necessary to review anthropogenic contaminants in honeybees and their products, select contaminants more prevalent and problematic in different regions, assess the modern methodologies for the identification of contaminants in beehive products, provide food safety challenges related to honey and honeybee products and their importance depending on their origin and implement Geographic Information System (GIS) by organizing collected data in a specific database.

### WG3: Evaluation of prevalent diseases and biotic stressors that threaten honeybee colonies - endemic and possible imported threats

Veterinary medicine protects the health of bees. Regular meetings and training for veterinarians and beekeepers have strengthened ties between the veterinary profession and the beekeeping sector, promoting the responsible distribution and use of veterinary medicines. Given the dynamic nature of beekeeping in agriculture, beekeepers must adhere to good practices. BeSafeBeeHoney supports beekeeping associations and individual beekeepers.

The tasks within this working group are mapping diseases and biotic stressors in European honeybee colonies, assessing crossover threats, then identifying implemented prevention national and international control strategies, including novel control agents for treatment in beekeeping. Evaluation of novel and emerging real-time sensors that can be used for field pathogen detection by beekeepers in their honeybee colonies is also of interest here, and the assessment of challenges in parasite/virus control in laboratory/research and regulatory/market, with the aim of the data integration into the Geographic Information System (GIS) database.

**WG4: Honeybees as pollinators in agriculture: consequences of lost colonies in agrarian ecosystems**

Bees are crop pollinators, and farmers use pesticides that harm bees and potentially contaminate bee products. One of WG4's goals is to encourage those interested in overcoming these problems and finding sustainable solutions with the help of scientists and experts.

WG4's tasks also include summarizing current knowledge of interactions between agriculture and bees/bee products, encouraging stakeholders involved in the interactions to engage in discussion, gathering stakeholders' opinions on the interactions between bees and agriculture and potential conflicts, and working on solutions with stakeholders, including estimating the impact of agricultural pollutants on bee products.

**WG5: Biomonitoring of abiotic stressors or anthropogenic contaminants in the environment**

This group aims to design and formulate European policies to promote bee production, including preventing, reducing and controlling biotic and abiotic stressors for bees. It will be realised through mapping existing national and EU policies and regulations regarding honey and related products as developed, an online survey to analyse the applicability and implementation of the beekeeping policies at international and national levels, and an assessment of the effect, use and effectiveness of current policies to support apiculture. It also implies the analysis of the current market for honey in European regions, including the degree of self-sufficiency, import and export, the market operators and other relevant market indicators, including the loss of colonies. The final goal is the development of recommendations of best practices related to production, market, research and policies for sustaining further social and economic development and expansion of the European apiculture sector and the design of a BeeSafeQATool to comfort the social and economic development and expansion of the European apiculture sector.

**WG6: Coordination, dissemination, and stakeholder involvement**

The activity and achievements of BeSafeBeeHoney should be widely disseminated to the general public and specific target groups that could use the Action results for their future work. The goal of this WG is to maximise the visibility and attractiveness of the Action, create and continuously update the website and social media profiles, publish the newsletter of the CA22105 to be delivered twice a year, organise international conferences to promote and spread the latest achievements and, define relevant changes and additions to create the BeSafeBeeHoney protocols.

## Achieved results up to now in CA22105

1. The 1st International Conference BeSafeBeeHoney was held on May 28 and 29, 2024, in Larissa, Greece. The event featured esteemed keynote speakers, fruitful oral communicators, and poster presenters, bringing together approximately 100 bee experts, researchers, and specialists.

2. Dissemination of activities of BeSafeBeeHoney through social media:

LinkedIn: https://www.linkedin.com/company/besafebeehoney/posts/?feedView=all

Facebook: https://www.facebook.com/BeSafeBeeHoney.CA22105

Instagram: https://www.instagram.com/besafebeehoney/

3. TheBeeLetter contains the latest news of the Cost Action "Beekeeping products valorization and biomonitoring for the safety of bees and honey" (BeSafeBeeHoney, CA22105).

4. Dissemination through other media, like blogs:
https://www.pmf.ni.ac.rs/chemianaissensis/new-actions-to-preserve-honeybees-and-honest-beekeeping/

## Our experience as a recommendation to participate in CA22105

Although launched in September 2023, CA22105 has achieved significant results up to now because it is a very dynamic group of people dedicated to collaborating and even generation, during the duration of the project, visible results not only for scientists but also for the general public. Prof. dr Danijela Kostić and Dr Biljana Arsić are involved in the activities of several working groups. Prof. dr Danijela Kostić takes part in the tasks related to WG1 and WG5, and dr Biljana Arsić in the activities of WG1, WG5 and WG6. There are so many online meetings with useful frequent trainings related to achieving results related to *e.g.*, the generation of systematic reviews and using software tools. They both participate now in two systematic reviews, and dr Biljana Arsic is also now a coordinator for writing the systematic review related to propolis.

## Conclusion

Honey production has declined drastically in recent years, both in quantity and quality. This action aims to protect bees, increase the number of hives, produce honey and honey products, and improve their quality, with the significant involvement of science and the professional community at the European level. All interested parties are welcome to take part in this COST action to implement its tasks.

## Acknowledgement

CA22105

## Conflict-of-Interest Statement

The authors declare no conflict of interest.

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# Prezentacija aktivnosti akcije evropske saradnje u nauci i tehnologiji (engl. COST) CA22105-BeSafeBeeHoney

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## SAŽETAK

Med je usled prisustva bioaktivnih jedinjenja sa antioksidativnim, antimikrobnim, antiinflamatornim i antikancerogenim svojstvima od davnina popularan zbog svojih nutritivnih i zdravstvenih vredosti. Evropska pčela (*Apis mellifera*) najvažniji je oprašivač i izuzetno važna u poljoprivredi. Broj pčela u svetu se smanjuje zbog antropogenih aktivnosti, klimatskih promena i primene pesticida. To je dovelo do smanjenja proizvodnje meda. Evropa uvozi 40% meda koji godišnje potroši. EU nastoji da spreči i pomogne u ostvarivanju međunarodne saradnje i razmene inovativnih naučnih i stručnih znanja i dostignuća u sledećim temama: nutritivna i lekovita svojstva meda i pčelinjih proizvoda, abiotički stresori i antropogeni zagađivači u životnoj sredini koji koriste proizvode iz košnica kao indikatore, rasprostranjene bolesti i biotički stresori koji ugrožavaju pčelinja društva, pčele kao oprašivači u poljoprivredi i posledice izgubljenih zajednica u agrarnim ekosistemima i istraživanje politike i analiza tržišta u vezi sa pčelarskim aktivnostima. COST akcija CA22105 objedinjuje pčelarstvo, veterinu, poljoprivrednu tehniku, hemiju, biologiju, ishranu, ekonomiju i politiku radi ostvarivanja naučnog razvoja. COST akcija brine o postizanju rodne ravnopravnosti, kao i o mladim istraživačima. Realizacija zadataka ove COST akcije sprovodi se u okviru nekoliko radnih grupa.

*Ključne reči: pčela, med, pčelinji proizvodi, širenje znanja*

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# Présentation des activités de l'action CA22105-BeSafeBeeHoney de la Coopération européenne en science et technologie (COST)

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## RÉSUMÉ

Le miel est apprécié pour ses propriétés nutritionnelles et sanitaires depuis l'Antiquité en raison de la présence de composés bioactifs aux propriétés antioxydantes, antimicrobiennes, anti-inflammatoires et anticancéreuses. L'abeille européenne (Apis mellifera) est le pollinisateur le plus important et extrêmement important dans l'agriculture. Le nombre d'abeilles diminue dans le monde entier en raison des activités anthropiques, du changement climatique et de l'application de pesticides. Cela a entraîné une diminution de la production de miel. L'Europe importe 40 % du miel qu'elle consomme chaque année. L'UE s'efforce de promouvoir et de favoriser la coopération internationale et l'échange de connaissances et de réalisations scientifiques et professionnelles innovantes dans les domaines suivants : propriétés nutritionnelles et médicinales du miel et des produits de la ruche, facteurs de stress abiotiques et contaminants anthropiques dans l'environnement en utilisant les produits de la ruche comme indicateurs, maladies courantes et facteurs de stress biotiques menaçant les colonies d'abeilles, abeilles en tant que pollinisatrices dans l'agriculture et conséquences de la disparition des colonies dans les écosystèmes agraires, recherche politique et analyse de marché liées aux activités apicoles. L'action COST CA22105 rassemble l'apiculture, la médecine vétérinaire, l'ingénierie agricole, la chimie, la biologie, la nutrition, l'économie et la politique pour réaliser des développements scientifiques. L'action COST s'occupe de l'égalité des sexes et des jeunes chercheurs. La mise en œuvre des tâches de cette action COST est réalisée au sein de plusieurs groupes de travail.

*Mots-clés : abeille, miel, produits de la ruche, diffusion des connaissances*

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# Представление деятельности акции европейского сотрудничества в науке и технике (на англ. COST) CA22105-BeSafeBeeHoney

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## Резюме

Мёд популярен благодаря своим питательным и оздоровительным свойствам с давних времен благодаря содержанию биоактивных веществ с антиоксидантными, антимикробными, противовоспалительными и антираковыми свойствами. Европейская пчела (Apis mellifera) является важным опылителем и чрезвычайно важна в сельском хозяйстве. Численность пчел в мире сокращается из-за антропогенных воздействий, климатических изменений и применения пестицидов. Это привело к снижению производства мёда. Европа импортирует 40% мёда, который ежегодно потребляет. ЕС стремится предотвратить и содействовать международному сотрудничеству и обмену инновационными научными и профессиональными знаниями и достижениями в следующих областях: питательные и лечебные свойства мёда и продуктов пчеловодства, абиотические стрессоры и антропогенные загрязнители в окружающей среде, использующие продукты из ульев в качестве индикаторов, распространенные болезни и биотические стрессоры, угрожающие пчелиным сообществам, пчелы как опылители в сельском хозяйстве и последствия утраты сообществ в аграрных экосистемах, а также исследования политики и анализ рынка в области пчеловодства. Акция COST CA22105 объединяет пчеловодство, ветеринарию, сельскохозяйственные технологии, химию, биологию, питание, экономику и политику для достижения научного прогресса. Акция COST уделяет внимание достижению гендерного равенства, поддержке молодых исследователей. Реализация задач этой акции COST осуществляется в рамках нескольких рабочих групп.

*Ключевые слова: пчела, мёд, пчелиные продукты, распространение знаний*

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# Präsentation der Aktivitäten der Europäischen Zusammenarbeit in Wissenschaft und Technologie (COST) Aktion CA22105-BeSafeBeeHoney

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## ABSTRAKT

Honig wird aufgrund seiner ernährungsphysiologischen und gesundheitlichen Eigenschaften seit der Antike geschätzt, da er bioaktive Verbindungen mit antioxidativen, antimikrobiellen, entzündungshemmenden und krebshemmenden Wirkungen enthält. Die Europäische Honigbiene (*Apis mellifera*) ist der wichtigste Bestäuber und spielt eine zentrale Rolle in der Landwirtschaft. Die Zahl der Honigbienen nimmt weltweit aufgrund anthropogener Einflüsse, des Klimawandels und des Einsatzes von Pestiziden ab, was zu einem Rückgang der Honigproduktion geführt hat. Europa importiert 40 % des jährlich konsumierten Honigs. Die EU versucht, die internationale Zusammenarbeit und den Austausch innovativer wissenschaftlicher und fachlicher Erkenntnisse und Errungenschaften in folgenden Bereichen zu unterstützen: Ernährungs- und medizinische Eigenschaften von Honig und Bienenprodukten, abiotische Stressfaktoren und anthropogene Verunreinigungen in der Umwelt unter Verwendung von Bienenstockprodukten als Indikatoren, weit verbreitete Krankheiten und biotische Stressfaktoren, die Honigbienenvölker bedrohen, Honigbienen als Bestäuber in der Landwirtschaft und die Folgen des Verlusts von Bienenvölkern in Agrarökosystemen sowie politische Forschung und Marktanalysen im Zusammenhang mit der Bienenzucht. Die COST-Aktion CA22105 vereint Fachwissen aus den Bereichen Imkerei, Veterinärmedizin, Agrarwissenschaft, Chemie, Biologie, Ernährung, Wirtschaft und Politik, um wissenschaftliche Fortschritte zu erzielen. Sie legt besonderen Wert auf Geschlechtergleichheit und die Förderung junger Forschender. Die Aufgaben dieser COST-Aktion werden in mehreren Arbeitsgruppen umgesetzt.

*Schlüsselwörter: Biene, Honig, Bienenprodukte, Wissensverbreitung*

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