Macedonian Journal of Chemistry and Chemical Engineering, Vol. **44**, No. 2, pp. xx–xx (2025)

MJCCA9 –

ISSN 1857-5552
e-ISSN 1857-5625

Received: May 19, 2025
Accepted: October 30, 2025

DOI: 10.20450/mjcce.2025.3149
Original scientific paper

GREEN EXTRACTION OF METHYLXANTHINE DERIVATIVES FROM FOOD AND BEVERAGES USING DEEP EUTECTIC SOLVENTS AND IN SILICO STUDIES OF NEUROPROTECTIVE POTENTIAL

Nevena Grujić-Letić,* Branislava Teofilović, Ljiljana Suvajdžić, Emilia Gligorić

University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

nevena.grujic-letic@mf.uns.ac.rs

In the food and pharmaceutical industries, green technologies refer to the use of methods and materials that do not negatively impact the environment and offer safety advantages. The aim of this paper is to extract methylxanthine derivatives from food and beverage samples using ultrasound-assisted deep eutectic solvent extraction (UAE-DES), ultrasound (UAE), and microwave-assisted extraction (MAE), and traditional extraction with chloroform. A chemometric tool was applied for data analysis and molecular docking was used to predict neuroprotective potential. The high-performance liquid chromatography (HPLC) method was employed for methylxanthines determination. The results showed that green approaches effectively extracted theobromine (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) from food and beverages, with UAE-DES emerging as the most effective technique. Caffeine's high binding energy (–6.7 kcal/mol) against the adenosine receptor (A2A) observed by molecular docking suggested that it may have neuroprotective effects and could be used in multiple fields with promising future development possibilities. According to the results, the application of DESs as high dissolving, low cost, and environmental-friendly solvents has significant potential in the pharmaceutical and food industry.

Keywords: methylxanthine; deep eutectic solvent; HPLC; molecular docking; chemometrics

ЗЕЛЕНА ЕКСТРАКЦИЈА НА МЕТИЛКСАНТИНСКИ ДЕРИВАТИ ОД ХРАНА И ПИЈАЛАЦИ СО УПОТРЕБА НА ДЛАБОКО ЕВТЕКТИЧНИ РАСТВОРУВАЧИ И ИСТРАЖУВАЊА IN SILICO НА НЕВРОПРОТЕКТИВНИОТ ПОТЕНЦИЈАЛ

Во прехранбената и фармацевтската индустрија зелените технологии се однесуваат на употреба на методи и материјали кои не влијаат негативно врз животната средина и нудат предности во поглед на безбедноста. Целта на овој труд е екстракција на метилксантински деривати од примероци храна и пијалаци со користење ултразвучно помогната екстракција со длабоко евтектични растворувачи (UAE-DES), ултразвучна екстракција (UAE), микробраново помогната екстракција (MAE), како и традиционална екстракција со хлороформ. За анализа на податоците беа применети хемометриски алатки, а молекуларното докирање беше искористено за предвидување на неврозаштитниот потенцијал. За определување на метилксантините беше применет методот на високоефикасна течна хроматографија (HPLC). Резултатите покажаа дека зелените пристапи ефикасно ги екстрахираат теоброминот (1,3-диметилксантин) и кофеинот (1,3,7-триметилксантин) од храна и пијалаци, при што UAE-DES се покажа како најефикасна техника. Високата енергија на врзување на кофеинот (–6,7 kcal/mol) со аденозинскиот рецептор (A2A), утврдена со молекуларно докирање, укажува дека кофеинот може да има неврозаштитни ефекти и да се применува во повеќе области со ветувачки можности за идниот развој. Според резултатите, примената на длабоко евтектични растворувачи, поради нивната способност за висока растворливост, ниската цена и еколошката прифатливост, има значителен потенцијал во фармацевтската и прехранбената индустрија.

Клучни зборови: метилксантини; длабоко евтектични растворувачи; HPLC; молекуларно докирање; хемометрика

1. INTRODUCTION

Methylxanthine derivatives (caffeine, theobromine, and theophylline), among the most consumed alkaloids in the world, are the subject of many studies due to their stimulating effects on the nervous, cardiovascular, and muscular systems.[1–3] It is believed that blocking adenosine receptors in the brain, inhibiting phosphodiesterase, mobilizing intracellular calcium, and modifying GABA (gamma-aminobutyric acid) receptors are the mechanisms responsible for these stimulating effects in humans. They occur naturally in coffee, tea, and cocoa, or are added to a variety of beverages. Testing the neuroprotective effect of coffee, as well as examining caffeine's contribution to this effect, has recently been the focus of many studies.[4,5]

Traditional methylxanthine extraction from natural products required the use of toxic solvents, which generated large amounts of solvent waste, left unhealthy residues in final products, and resulted in low yields.[6,7] Green technologies are increasingly employed in the pharmaceutical and food industries to promote ecologically acceptable activities that cause little or no harm to humans. Among the 12 principles of green chemistry, one is the use of environmentally friendly solvents and substances.[8]

Ionic liquids (IL) are considered among the most significant green solvents; nonetheless, they have disadvantages, including high cost and manufacturing complexity. Recently, ILs have increasingly been replaced by harmless solvents called deep eutectic solvents (DESs), which are less expensive and more environmentally friendly.[8] DESs consist of combinations of two or three components that form hydrogen bond interactions with each other, creating a eutectic mixture with a lower boiling point than the individual components. At least two components are required to synthesize DESs: a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA).[9]

The most synthesized DESs are miscible with water and hydrophilic organic solvents, which has limited the use of such DESs with lipophilic samples. In recent years, special attention has been paid to the synthesis of hydrophobic DESs, which has expanded their use in the extraction of natural components and pharmaceuticals.[10] In hydrophobic DESs, the HBD is usually a fatty acid due to its hydrophobicity, with moderate ability to participate in hydrogen interactions.[11]

Some studies have investigated using DESs for the extraction of caffeine and theobromine from natural sources, but there are no comprehensive studies that compare using DESs enhanced by ultrasound-assisted extraction (UAE) as extractants with other conventional or green methods.[2,12,13] The aim of this work was to extract methylxanthine derivatives (caffeine and theobromine) from food and beverages using ultrasound-assisted DES extraction (UAE-DES), as well as two other green techniques, ultrasound (UAE) and microwave-assisted extraction (MAE). Traditional extraction with chloroform, the solvent of choice for methylxanthine extraction, was performed to compare the obtained results with green extraction techniques. Chemometric analysis was applied to detect similarities and dissimilarities among the applied extraction methods. Given that there are no previous studies of this kind, this paper also includes in silico molecular docking analysis of caffeine as a blocker of adenosine A2A receptors, and a comparison with istradefylline, a medicine with a similar structure and known neuroprotective effect, indicated in the treatment of Parkinson's disease.

In line with the ideas of sustainable development, the use of effective and environmentally friendly extraction techniques for methylxanthines may help reduce energy use and pollution emissions during production processes. Apart from obtaining caffeine-containing green, non-toxic extracts, it is important to provide answers regarding their potential medical uses beyond those that are already well-established. Caffeine is currently only known to be used in supplements to improve alertness, athletic performance, and fatigue levels.[14,15] It can also be incorporated into analgesics (pain medications) to increase their efficacy as an analgesic adjuvant.[16] Due to the lack of clinical studies and the limited number of animal experiments on caffeine's neuroprotective effect, there are currently no approved supplements that could be used to prevent neurological diseases or alleviate the symptoms of those that already exist.[17–21] In light of these findings, this work focused on in silico tests that aimed to contribute to the current scientific base on the role of caffeine and its similarities with medicines already used in the prevention and treatment of conditions linked to adenosine A2A

receptors, with the goal of developing innovative supplements to improve public health.

2. EXPERIMENTAL

2.1. *Samples*

Commercial samples of food and beverages used in this work were available for sale at markets in the Republic of Serbia: green tea (Macval), black tea (Teekanne), instant coffee (Jacobs), black coffee (Grand), Red Bull® (Red Bull GmbH), Guarana® (Knjaz Miloš a.d.), ice tea (Sola), Coca-Cola® (Coca-Cola Company), and organic cocoa (Sanaterra).

2.2. *Standard substances and reagents*

Standards of caffeine and theobromine (> 99 %), menthol, methyl salicylate, and dodecanoic acid were obtained from Sigma Aldrich (Deisenhofen, Germany). HPLC-grade acetonitrile, chloroform, tetrahydrofuran, and sodium hydroxide were purchased from Fluka (Bluchs, Switzerland). Distilled deionized water (dd H_2O) was used throughout the experiments. DESs, menthol:dodecanoic acid in a molar ratio of 2:1, and menthol:methyl salicylate in a ratio of 1:1, were prepared from an appropriate amount of each component, using an analytical scale (Sartorius, Germany) with an accuracy of 0.1 mg. It was determined that the relative standard uncertainty of the mole fraction of the measured components was less than ±0.03. The water content of the pure components was determined by Karl-Fischer titration using an 831 Karl-Fischer coulometer (Metrohm, Switzerland), and the results were 42 ppm, 41 ppm, and 12 ppm for menthol, methyl salicylate, and dodecanoic acid, respectively. Purity for all components was 99 %. Although pure menthol was a solid component at room temperature, DESs containing dodecanoic acid and methyl salicylate were liquid at room temperature.

2.3. *Ultrasound-assisted DES extraction*

For the extraction of solid food and beverage samples, 0.3 g of solid sample, 5 ml of distilled water, and 2 ml of DES were mixed in a test tube. The mixture was vortexed (Ibx Instruments, Switzerland) for 30 s. The tubes were then placed in an ultrasonic bath (Clifton, UK), and the extraction was performed for 20 minutes (input power of 250 W and a frequency of 40 kHz). The contents were then centrifuged for 15 minutes (Sigma 2-7, SIGMA Laborzentrifugen GmbH, Germany). The supernatant was drawn with a syringe and filtered through a nylon membrane filter (RC-45/25, 0.45 μm) directly into the vial for high-performance liquid chromatography (HPLC) analysis.

The procedure for the extraction of liquid food and beverage samples involved the evaporation of 10 ml of the liquid sample in a beaker. Five milliliters (5 ml) of water (pH = 8) was added to the dry residue, and the sample was transferred to a test tube. Two milliliters (2 ml) of DES was added, and the mixture was vortexed for 30 s. The tubes were then placed in an ultrasonic bath, and the extraction was carried out for 20 minutes (180 W). After this procedure, the contents were centrifuged for 15 minutes. The supernatant was drawn with a syringe and filtered through a nylon membrane filter (0.45 μm) directly into the vial prior to HPLC analysis. Liquid samples, when necessary, were degassed in an ultrasonic bath for 10 minutes. The methods for sample preparation and extraction of methylxanthines are described in the literature.[2,22]

2.4. *Ultrasound-assisted extraction*

UAE involved weighing 0.3 g of solid or measuring 10 ml of the liquid sample that had been previously evaporated to dryness, adding 7 ml of water (pH = 8), and mixing on a vortex mixer for 30 s. The tubes were then placed in an ultrasonic bath (Clifton, UK), and the sample was extracted for 20 minutes. Samples were filtered through a nylon membrane filter (RC-45/25, 0.45 μm) directly into the vial and analyzed by HPLC. Liquid samples containing dissolved gases were pretreated in an ultrasonic bath for 10 minutes.

2.5. *Microwave-assisted extraction*

For the MAE of solid samples, 7 ml of water (pH = 8) was added to a beaker containing 0.3 g of solid sample and mixed thoroughly using a vortex mixer for 30 s. Extraction was carried out in a microwave oven (Midea, China), with temperature and pressure control, in sealed vessels at a power of 400 W for 30 s. Samples were filtered through a nylon membrane filter (0.45 μm) into the vial prior to HPLC analysis.

The liquid samples were first pretreated, if necessary, in an ultrasonic bath for 10 minutes. Ten milliliters (10 ml) of the liquid sample was evaporated to dryness under reduced pressure, then prepared and treated using the same procedure as for the solid samples.

2.6. *Extraction with chloroform*

The samples (5 g or 10 ml of the liquid sample, evaporated to dryness) were transferred to a laboratory beaker, 200 ml of warm water was added, and maceration was carried out for 30 minutes at room temperature with occasional stirring. The extract was separated by filtration, 10 ml was collected for analysis, and the extract was purified on an SPE (solid-phase extraction) column (Supelco, Supelclean™ LC-18 SPE Tubes, 6 ml (0.5 g)).

For purification on SPE, methanol (2 × 10 ml) was passed through the SPE column, followed by HPLC-grade distilled water (2 × 10 ml). The sample (10 ml) was then passed through the column. Methylxanthines were extracted from the column with chloroform (10 ml), and the solvent was removed by evaporation under reduced pressure. The dry residue was dissolved in 2 ml of water (pH = 8.0), and methylxanthines were analyzed using HPLC by injecting 2 µl of the solution into the system.[23]

2.7. *HPLC analysis*

Methylxanthine analysis was performed using an HPLC method described in the literature, employing an HPLC-DAD model Agilent HP 1100 system with an autosampler injector (Waldbron, Germany).[23] HPLC analysis was conducted using a Zorbax C-8 column (4.6 mm × 150 mm, 5 µm particle size), with a mobile phase consisting of 0.5 % THF (pH = 8) and acetonitrile in a 95:5 ratio, a flow rate of 0.8 ml/min, UV detection at 273.4 nm, a column temperature of 25 °C, and a total analysis time of 10 minutes.

2.8. *Chemometric analysis*

Principal component analysis (PCA) was performed using Statistica v.12 software (Stat Soft Inc., Tulsa, USA). The extraction techniques (UAE-DES 1, UAE-DES 2, UAE, MAE, and chloroform) were treated as cases, while the detected amounts of caffeine and theobromine in the analyzed samples were treated as variables. All data were standardized prior to calculation.

2.9. *Molecular docking analysis*

The chemical structures of ligand molecules in this study were obtained from the PubChem database.[24] Three-dimensional crystallographic structures were retrieved from the Protein Data Bank (PDB).[25] Ligands and water molecules were removed, polar hydrogen atoms were added, and partial atomic charges were calculated using the Gasteiger method [26] with AutoDock Tools (AutoDock v. 4.2.3, United States). The grid box dimensions were set to 60 × 60 × 60 with a spacing of 0.375 Å between points. Molecular docking was conducted using the AutoDock 4.2.3. program package, employing the Lamarckian genetic algorithm and the standard docking procedure for a rigid receptor and flexible ligand, with 25 independent runs per ligand. Other parameters were set to their default values. Conformations of docked structures with the lowest binding energy were considered the most favorable docking pose. Discovery Studio Visualizer (v. 4.5, United States) was used to visualize the results and generate the figures.

2.10. *Statistical analysis*

Statistical analyses were performed using IBM SPSS (v. 22, United States). The data were presented as mean values ± standard deviation (SD). Mean values of the measured parameters were subjected to a one-way analysis of variance (ANOVA) using Duncan's multiple range test to determine significant differences among samples, with level of significance $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. *HPLC-DAD determination of methylxanthines*

Caffeine and theobromine were determined by comparing the retention times and UV spectrum with standards of caffeine and theobromine. As shown in the Figure 1, it can be seen that there are no interferences or overlapping peaks from other active substances present in the samples, demonstrating that the applied extraction and purification methods, as well as the analytical method, were suitable for the determination of caffeine and theobromine in food and beverages.

The results of caffeine content in food and beverage samples, obtained using different extraction techniques, are summarized in Table 1. The chemical structures of DESs (HBA and HBD) and a representative HPLC chromatogram are presented in Figures 1A and 1B, respectively.

T a b l e 1

Caffeine (1,3,7-trimethylxanthine) content in food and beverages

Sample number	Sample name	Caffeine (mg/100 ml)					Declaration
		Microwave	Ultrasound	DES 1	DES 2	Chloroform	
1	Red Bull	28.31 ± 0.11[a]	20.22 ± 0.07[b]	29.25 ± 0.08[a]	31.83 ± 0.08[c]	30.75 ± 0.08[a]	≥32
2	Guarana	21.75 ± 0.07[a]	19.88 ± 0.08[b]	23.81 ± 0.09[c]	24.75 ± 0.06[d]	23.45 ± 0.06[e]	≥25
3	Coca Cola	8.28 ± 0.03[a]	6.32 ± 0.02[b]	9.74 ± 0.03[c]	11.12 ± 0.04[d]	10.52 ± 0.09[e]	≥15
4	Ice tea	8.89 ± 0.03[a]	5.48 ± 0.01[b]	9.21 ± 0.03[a]	10.44 ± 0.04[c]	9.33 ± 0.06[a]	–
		Caffeine (mg/100 g)					
Sample number	Sample name	Microwave	Ultrasound	DES 1	DES 2	Chloroform	
5	Green tea	210.00 ± 0.55[a]	174.57 ± 0.66[b]	547.35 ± 0.33[c]	596.64 ± 0.45[d]	550.22 ± 0.25[e]	–
6	Black tea	554.6 ± 0.25[a]	454.76 ± 0.75[b]	559.15 ± 0.33[a]	682.14 ± 0.92[c]	557.45 ± 0.92[a]	–
7	Black coffee	2683.64 ± 3.12[a]	2669.84 ± 5.11[a]	2855.10 ± 4.22[b]	5196.38 ± 2.25[c]	4027.33 ± 4.75[d]	–
8	Ness coffee	1809.99 ± 4.15[a]	1755.28 ± 4.33[a]	2075.96 ± 3.25[b]	4056.41 ± 5.33[c]	3985.25 ± 3.28[c]	–
9	Cocoa	79.95 ± 0.12[a]	73.18 ± 0.15[b]	92.57 ± 0.14[c]	98.52 ± 0.25[d]	78.22 ± 0.25[e]	–

Data are presented as mean of triplicate measurements ± SD. Different superscript letters within the same rows indicate significant differences of means at the 0.05 level.
DES 1: menthol : dodecanoic acid 2 : 1; DES 2: menthol : methyl salicylate 1 : 1.

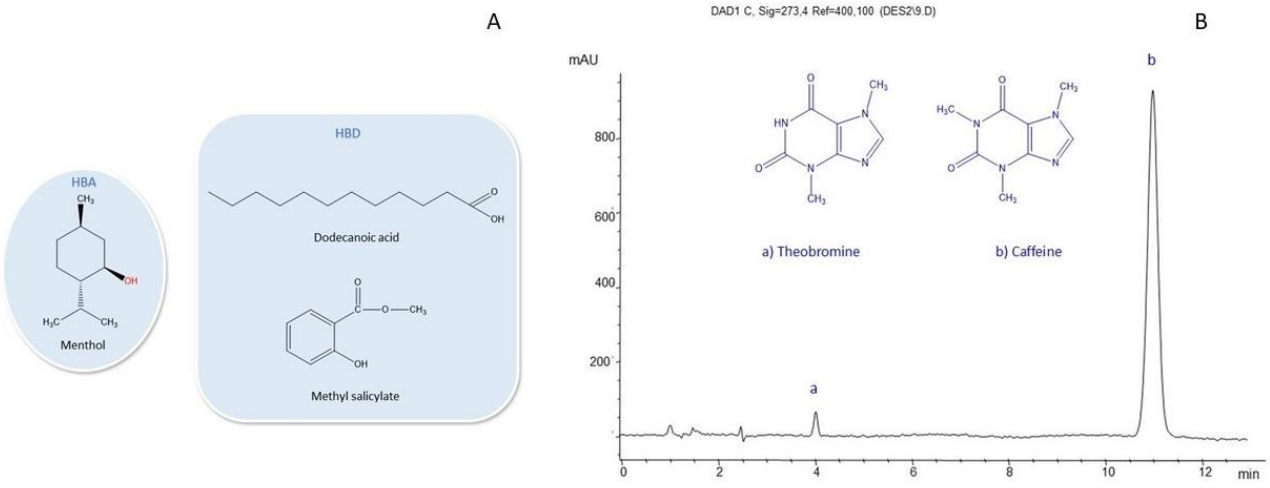

Fig. 1. A) Chemical structures of DESs (HBA and HBD); B) Representative HPLC chromatogram of sample 1 UAE-DES 2 extraction: a) theobromine, b) caffeine

Caffeine concentrations in food and beverage samples varied significantly depending on the extraction method used. The caffeine content in sample 1 ranged from 20.22 to 31.83 mg/100 ml, with UAE-DES 2 yielding a 1.57-fold higher amount than UAE. Additionally, the amount of caffeine matching the declared value was obtained using UAE-DES 2. Similar findings were observed for samples 2 and 3, where the caffeine concentration ranged from 19.88 to 24.75 mg/100 ml and from 6.32 to 11.12 mg/100 ml, respectively. UAE-DES 2, as the most effective technique, yielded caffeine levels in these samples that matched the declared values.

Caffeine levels in sample 4 ranged from 8.89 to 10.44 mg/100 ml, with UAE-DES 2 providing 1.17 times more caffeine than UAE. In the case of solid samples, the caffeine content was as follows: in sample 5, 174.57 – 596.64 mg/100 g; in sample 6, 454.76 – 682.14 mg/100 g; in sample 7, 2669.84 – 5196.38 mg/100 g; in sample 8, 1755.28 – 4056.41 mg/100 g; and in sample 9, 73.18 – 98.52 mg/100 g. In these solid samples, the UAE-DES 2 technique yielded 3.42, 1.5, 1.95, and 1.35 times more caffeine than UAE, respectively.

After UAE-DES 2, the conventional method of extracting caffeine using chloroform was the next most effective technique in the majority of the samples studied, as presented in Table 1. Since the results demonstrated a strong influence of extraction technique on caffeine content within the same samples, there was a clear need for careful selection of methods that employ non-toxic solvents and yield the highest concentration of the target compound.

When green extraction methods were considered, it was evident that UAE-DES extraction produced caffeine levels consistent with the declared values of the products, while other green extraction methods yielded significantly lower values. This difference was likely due to the hydrophobic DES used in this study, which served as an effective extractive medium for non-polar organic compounds.[27,28]

When comparing the two different DESs used, both of which show promise as green solvents in the food industry, higher caffeine yields were achieved with DES 2. This increase was attributed to stronger hydrogen bonding interaction between caffeine and DES 2.[2] As shown in Figure 1A, the structure of methyl salicylate enabled more hydrogen bonding interactions with caffeine than dodecanoic acid. Recent research has also confirmed that methyl salicylate is an excellent solvent for caffeine, showing that caffeine solubility in methyl salicylate is nearly 70 % higher than in water. Computational simulation demonstrated that the clathrate-like organization of methyl salicylate molecules around caffeine effectively prevented its self-aggregation in solution.[22]

The results of our study were slightly higher than those reported in a similar study on caffeine extraction from coffee beans using UAE-DES (betaine and sorbitol), which yielded 229 – 514 mg/100 g.[12] This difference may be attributed to the higher efficiency of the DESs used in our study, as well as variations in coffee samples and general extraction conditions. Additionally, our results were comparable to those of a study employing automated homogeneous liquid–liquid microextraction based on DES (choline chloride with phenol at 1:3 molar ratio) for HPLC-UV determination of caffeine in beverages, where caffeine concentrations in energy drinks ranged from 27 to 32.5 mg/100 ml.[29]

The results of theobromine content in the analyzed samples are presented in Table 2 and Figure 1B.

T a b l e 2

Theobromine (3,7-dimethylxanthine) content in food and beverages

Sample number	Sample name	Theobromine (mg/100 ml)				
		Microwave	Ultrasound	DES 1	DES 2	Chloroform
1	Red Bull	< LOD	< LOD	< LOD	< LOD	< LOD
2	Guarana	< LOD	< LOD	< LOD	< LOD	< LOD
3	Coca Cola	< LOD	< LOD	< LOD	< LOD	< LOD
4	Ice tea	< LOD	< LOD	< LOD	< LOD	< LOD
Sample number	Sample name	Theobromine (mg/100 g)				
		Microwave	Ultrasound	DES 1	DES 2	Chloroform
5	Green tea	25.66 ± 0.05^{b}	23.75 ± 0.09^{b}	32.98 ± 0.17^{a}	33.45 ± 0.09^{a}	31.00 ± 0.15^{a}
6	Black tea	26.66 ± 0.05^{b}	21.45 ± 0.08^{b}	34.75 ± 0.09^{a}	36.50 ± 0.19^{a}	33.52 ± 0.10^{a}
7	Black coffee	< LOD	< LOD	< LOD	< LOD	< LOD
8	Ness coffee	< LOD	< LOD	< LOD	< LOD	< LOD
9	Cocoa	411.28 ± 0.35^{b}	392.50 ± 0.50^{b}	450.66 ± 0.62^{a}	468.92 ± 0.50^{a}	455.75 ± 0.45^{a}

Data are presented as mean of triplicate measurements ± SD. Different superscript letters within the same rows indicate significant differences of means at the 0.05 level.
DES 1: menthol : dodecanoic acid 2 : 1; DES 2: menthol : methyl salicylate 1 : 1.
LOD – limit of detection

Theobromine was only present in solid samples of green tea, black tea, and cocoa (samples 5, 6, and 9), while samples of coffee and energy drinks (samples 1, 2, 3, 4, 7, and 8) contained theobromine below the detection limit. In sample 5, theobromine ranged from 23.75 to 33.45 mg/100 g; in sample 6, from 21.45 to 36.5 mg/100 g; and in sample 9, from 392.5 to 469.92 mg/100 g. In sample 5, UAE-DES 2 yielded 1.41 times more theobromine than UAE. In sample 6, UAE-DES 2 provided 1.7 times more, and in sample 9, 1.19 times higher theobromine content compared to UAE.

UAE-DES 1, followed by traditional extraction with chloroform, were the next most effective techniques after UAE-DES 2. Among green extraction techniques, DES-based extraction proved to be the most effective. Similar to the case of caffeine, DES 2 exhibited a greater affinity for theobromine extraction than DES 1, which may be attributed to the formation of more intense hydrogen bonding interactions between DES 2 and theobromine.

Extraction of theobromine using DESs has been rarely reported in the literature. Although alternative DESs have been used, the theobromine content obtained in our study matched the results of Pavlović et al. DES-MAE-assisted theobromine extraction from cocoa beans (24.65 – 49.12 mg/100 g).[13] In contrast to choline chloride : butane-1,4 diol (1:2), DES 2 (menthol : methyl salicylate, 1:1) selected in our research demonstrated comparable efficacy as an extractant.

3.2. *Chemometric analysis*

The use of chemometrics has given significant results in many fields, such as biological, food, and pharmaceutical research, by predicting and identifying similarities and differences among the examined samples.[30] Principal component analysis (PCA) was applied in this study to identify the most suitable method for caffeine and theobromine extraction from food and beverage samples. Within the initial data matrix, different extraction techniques represented the objects of PCA, while the amounts of caffeine and theobromine in the analyzed samples represented the variables. New variables, or principal components (PC), were obtained through PCA by decomposing the original data into loading and score vectors. The first two PCs (PC1 and PC2), which accounted for most of the variation in the dataset, explained 92.06% of the total variance in the initial data matrix (Fig. 2A).

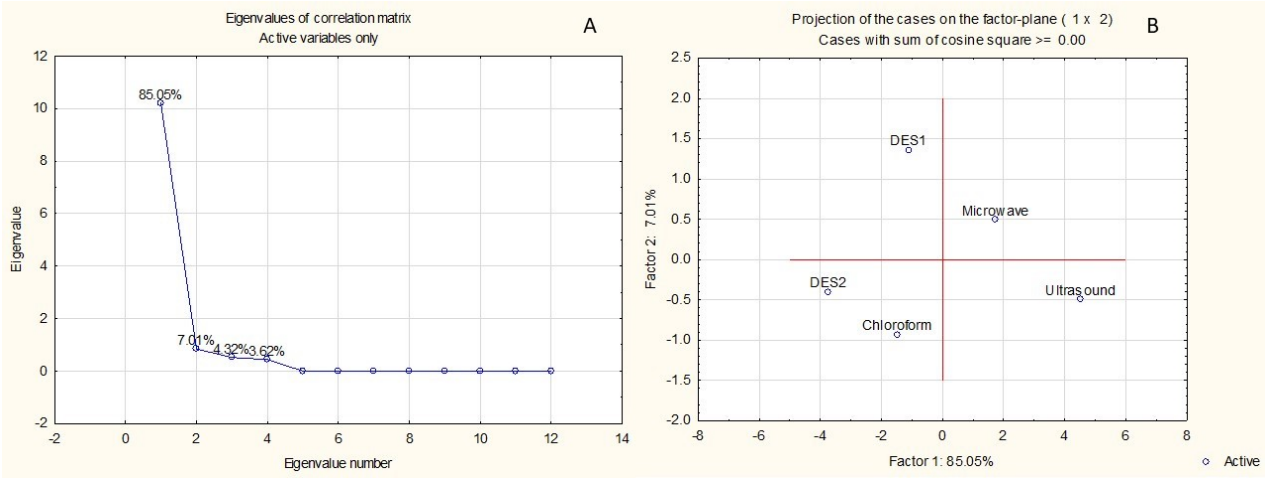

Fig. 2. A) Eigenvalues of correlation matrix; B) Principal component analysis.
Projection of cases – extraction techniques in the space defined by the first two principal components (PC1 and PC2)

PC1 allowed the distinction between two groups of extraction techniques based on the caffeine and theobromine content of the analyzed samples (Fig. 2B). The first group consisted of microwave- and ultrasound-assisted extraction methods, which proved to be less efficient for caffeine and theobromine extraction from the analyzed samples compared to the other techniques. Extraction using DES 1, DES 2, and chloroform comprised the second group, suggesting similar efficiency of these solvents for methylxanthine extraction. PC2 enabled further separation of DES 1 from the other two solvent-based extractions.

When selecting an appropriate extraction method, it was important to consider the characteristics of the chosen solvent. Given the disadvantages of using organic solvents such as chloro-

form, including toxicity, lack of recyclability, and cost, the preferred method should be UAE-DES 2.

3.3. *Molecular docking*

Molecular docking was applied to investigate and compare the interactions between caffeine and istradefylline with the A2A receptor, and the results are presented in Table 3 and Figures 3 and 4.

Molecular docking was used as an effective tool to illustrate the interactions between ligands and proteins, enabling the analysis of small molecule behavior at the protein binding site, and it played a major role in the discovery of new drugs.[31,32] The obtained results were compared with those of istradefylline, a medicine with neuroprotective effects and clinical applications, which, like caffeine, is a xanthine derivative that enhances dopaminergic activity by inhibiting the A2A receptor.[33] As shown in Table 3, it was observed that both caffeine and istradefylline exhibited high inhibitory potential against A2A, with binding energies of –6.3 and –8.9 kcal/mol, respectively.

T a b l e 3

Results of molecular docking analysis of caffeine and istradefylline against A2A receptor

Compound	Binding energy (kcal/mol)	Interaction site		
		Hydrogen bond	Distance (Å)	Other interactions (Pi-cation/Pi-alkyl/Pi-sigma, Pi-Pi)
Caffeine	–6.3	AsnA 253	3.33	GluA 169[a], PheA 168[a], LeuA 249[b], IleA 66[b], AlaA 63[b], IleA 274[c]
Istradefylline	–8.7	AsnA 253 SerA 67 TyrA 271 GluA 169	3.40 3.59 3.29 3.78	LeuA 249[b], IleA 274[b], MetA 270[b], LeuA 267[b], PheA 168[d]

[a]π-cation, [b]π-alkyl, [c]π-σ, [d]π-π

These results indicated that caffeine possessed significant binding energy, consistent with literature reports in which binding energies less than –6 kcal/mol demonstrated good inhibitory potential against the A2A receptor.[34,35] Hydrogen bonds and hydrophobic interactions contributed most significantly to the binding energies.[36] By applying molecular docking, it was observed that both caffeine and istradefylline participated in the formation of hydrogen bonds as well as numerous hydrophobic interactions with the binding site on the receptor A2A.

In Figure 3, it was evident that the carbonyl group of caffeine formed a hydrogen bond with the amino acid residue asparagine (AsnA 253), as a part of the interaction site on the A2A receptor. In addition, π-cation, π-alkyl, and π-σ interactions were observed with the amino acids glutamine (GluA 169), phenylalanine (PheA 168), leucine (LeuA 249), isoleucine (IleA 66 and IleA 274), and alanine (AlaA 63). The hydrophobic cleft formed by methionine (MetA 270 and MetA 177), tryptophan (TrpA 246), and valine (ValA 84) provided additional stability of the ligand within the active site of A2A.

Istradefylline, as presented in Figure 4, formed four hydrogen bonds via its carbonyl and hydroxyl groups with amino acids residues of asparagine (AsnA 253), serine (SerA 67), tyrosine (TyrA 271), and glutamine (GluA 169). π-alkyl, π-σ, and π-π interactions were formed with leucine (LeuA 249 and LeuA 267), isoleucine (IleA 274), methionine (MetA 270), and phenylalanine (PheA 168). Additional stabilization of the ligand was provided by hydrophobic clefts formed by leucine (LeuA 167 and LeuA 85), alanine (AlaA 63), valine (ValA 84), tryptophan (TrpA 246), methionine (MetA 174), and histidine (HisA 264).

While the neuroprotective effect of caffeine was investigated in a limited number of epidemiological and animal studies, no clinical studies have addressed this topic to date. Prior epidemiological research examined the potential neuroprotective effects of caffeine, as well as the potential health benefits of consuming foods and beverages high in caffeine, in relation to cognitive function and symptoms of Alzheimer's disease. These studies demonstrated, through in vivo and in vitro tests, positive neuroprotective effects of caffeine, but more detailed investigations into the mechanism of action were required.[4,37]

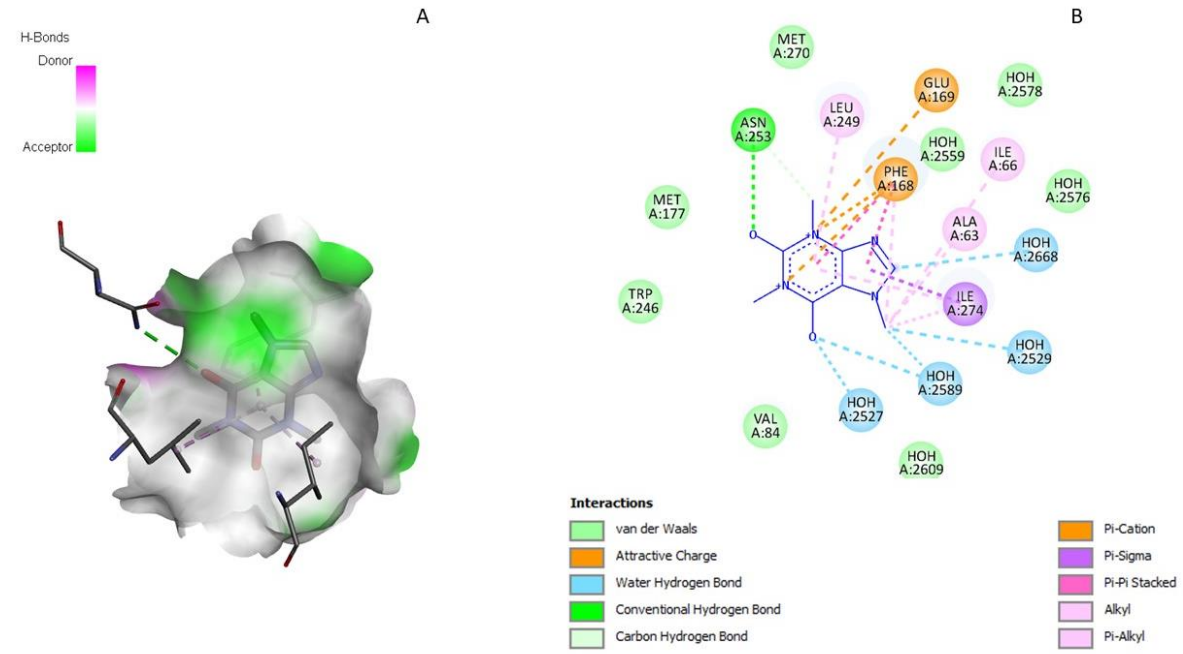

Fig. 3. Caffeine interactions at the active site of A2A: A) Three-dimensional display; B) Two-dimensional display

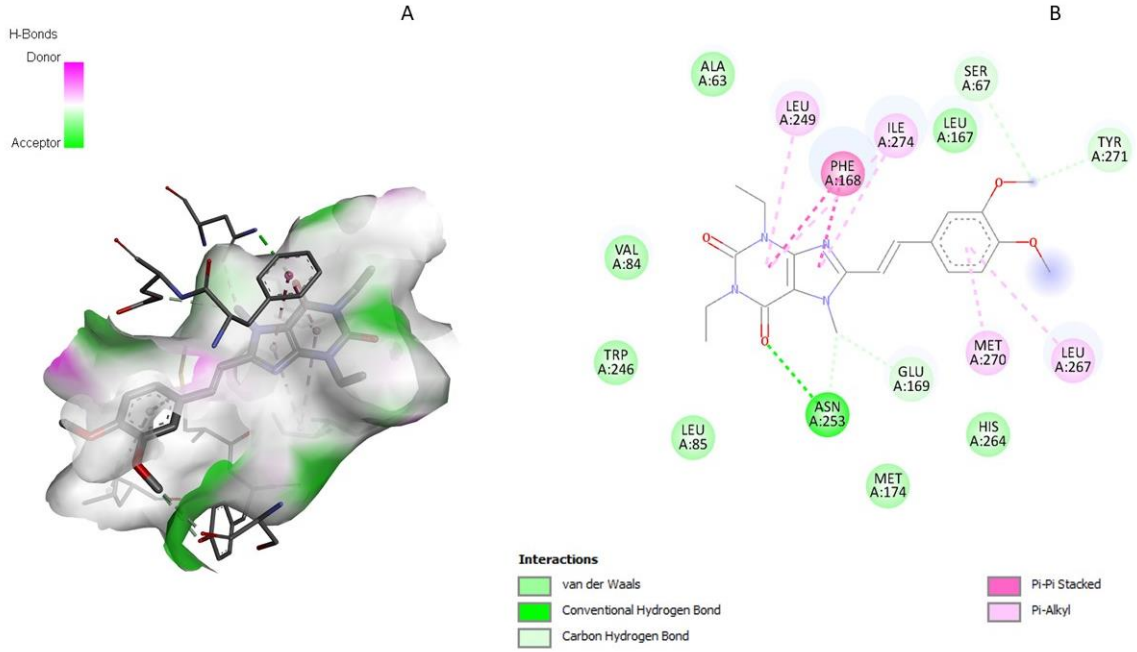

Fig. 4. Istradefylline interactions at the active site of A2A: A) Three-dimensional display; B) Two-dimensional display

According to an epidemiological study that included 13,137 cognitively healthy subjects over the age of 65, it was concluded that consuming 1 – 2 cups of coffee per day had a moderate neuroprotective effect, especially among women and non-smokers.[38] Another study included 2,513 participants over 60 years of age, in which caffeine intake was monitored using two 24-hour dietary recall questionnaires, and cognition was assessed using the CERAD (Consortium to Establish a Registry for Alzheimer's Disease) test, animal fluency test, and DSST(DANTES Subject Standardized Tests). It was reported that participants with a caffeine intake of 226.4 – 495 mg/day performed significantly better on the DSST compared to those who did not consume coffee and caffeine.[39]

A previous animal study demonstrated that in an 18 – 19 month mouse model of Alzheimer's disease, memory significantly improved after 4 – 5 weeks of caffeine administration at a dose of 1.5 mg/day (the human equivalent of 500 mg/day) compared to the control group (4 weeks: 217 %, 5

weeks: 198 %).[40] Additionally, other studies showed that caffeine consumption dramatically enhanced the performance of the Alzheimer's disease mice model, highlighting its protective effects against cognitive decline and its role in improving memory retention.[19,20]

There were no in silico studies of the neuroprotective effect of caffeine or comparisons with medicines used in the treatment of neurological disorders against which the data from our research could be evaluated. The obtained results only served as a supplement to existing knowledge, indicating that caffeine may possess neuroprotective properties and could be applied across various disciplines, with promising prospects for further research.

4. CONCLUSION

In this study, active components with a wide range of pharmacological activity and significant medicinal value were extracted using deep eutectic solvents, which were environmentally friendly and energy-efficient. The DES composed of menthol:methyl salicylate in a 1:1 molar ratio was identified as the optimal solvent for methylxanthine extraction, and the UAE-DES 2 method yielded up to 3.4 times higher amounts of methylxanthine compared to UAE. These findings demonstrated that this novel, non-toxic solvent was a potent extractant capable of efficiently extracting non-polar organic compounds, and could be widely applied for effective methylxanthine extraction from food and beverages.

Additionally, molecular docking was employed to explore whether the obtained green extracts could be utilized in applications beyond those already recognized. The study examined their structural characteristics, neuroprotective properties, and potential mechanisms of action, providing data that contributed to a rational and scientific grounded basis for future development and application of methylxanthine derivatives.

Acknowledgments: The authors are grateful for the financial support of Provincial Secretariat for Higher Education and Scientific Research, grant number: 003077388 2024 09418 003 000 000 001/1.

REFERENCES

(1) Grujić-Letić, N.; Gligorić, E.; Teofilović, B.; Vraneš, M.; Gadžurić, S., Ibuprofen as an organic pollutant in the danube and effects on aquatic organisms. *Acta Chim. Slov.* **2023**, *70*, 59–64. https://doi.org/10.17344/acsi.2022.7831.

(2) Cai, C.; Li, F.; Liu, L.; Tan, Z., Deep eutectic solvents used as the green media for the efficient extraction of caffeine from Chinese dark tea. *Sep.Puri. Technol.* **2019**, *227*, 115723. https://doi.org/10.1016/j.seppur.2019.115723

(3) Gudić, S.; Oguzie, E. E.; Radonić, A.; Vrsalović, L.; Smoljko, I.; Kliškić, M., Inhibition of copper corrosion in chloride solution by caffeine isolated from black tea. *Macedonian J.Chem.Chem. Eng.* **2014**, *33*, 13–25. https://doi.org/10.20450/mjcce.2014.441

(4) M Yelanchezian, Y. M.; Waldvogel, H. J.; Faull, R. L.; Kwakowsky, A., Neuroprotective effect of caffeine in Alzheimer's disease. *Molecules* **2022**, *27*, 3737. 10.3390/molecules27123737

(5) Panza, F.; Solfrizzi, V.; Barulli, M. R.; Bonfiglio, C.; Guerra, V.; Osella, A.; Seripa, D.; Sabba, C.; Pilotto, A.; Logroscino, G., Coffee, tea, and caffeine consumption and prevention of late-life cognitive decline and dementia. A systematic review. *J. Nutr. Health Aging* **2015**, *19*, 313–328. https://doi.org/10.1007/s12603-014-0563-8

(6) Loukri, A.; Sarafera, C.; Goula, A. M.; Gardikis, K.; Mourtzinos, I., Green extraction of caffeine from coffee pulp using a deep eutectic solvent (DES). *Appl. Food Res.* **2022**, *2*, 100176. https://doi.org/10.1016/j.afres.2022.100176

(7) Memon, M. M.; Idress, M., Optimization of caffeine extraction from various tea types using dichloromethane as an organic solvent. *Eng. Proc.* **2024**, *67*, 81. https://doi.org/10.3390/engproc2024067081

(8) Halder, A. K.; Cordeiro, M. N. D., Probing the environmental toxicity of deep eutectic solvents and their components: An in silico modeling approach. *ACS Sustain. Chem. Eng.* **2019**, *7*, 10649–10660. https://doi.org/10.1021/acssuschemeng.9b01306

(9) Wypych, G., *Handbook of Solvents*, Volume 2: *Use, Health, and Environment*; Elsevier, 2019.

(10) Cao, J.; Su, E., Hydrophobic deep eutectic solvents: The new generation of green solvents for diversified and colorful applications in green chemistry. *J. Clean. Prod.* **2021**, *314*, 127965. https://doi.org/10.1016/j.jclepro.2021.127965

(11) Van Osch, D. J.; Zubeir, L. F.; Van den Bruinhorst, A.; Rocha, M. A.; Kroon, M. C., Hydrophobic deep eutectic solvents as water-immiscible extractants. *Green Chem.* **2015**, *17*, 4518–4521. https://doi.org/10.1039/C5GC01451D

(12) Syakfanaya, A. M.; Saputri, F. C.; Mun'im, A., Simultaneously extraction of caffeine and chlorogenic acid from Coffea canephora bean using natural deep eutectic solvent-based ultrasonic assisted extraction. *Phcog.y J.* **2019**, *11*. https://doi.org/10.5530/pj.2019.11.41

(13) Pavlović, N.; Jokić, S.; Jakovljević, M.; Blažić, M.; Molnar, M., Green extraction methods for active compounds from food waste — Cocoa bean shell. *Foods* **2020**, *9*, 140. https://doi.org/10.3390/foods9020140

(14) Guest, N. S.; VanDusseldorp, T. A.; Nelson, M. T.; Grgic, J.; Schoenfeld, B. J.; Jenkins, N. D.; Arent, S. M.; Antonio, J.; Stout, J. R.; Trexler, E. T., International

society of sports nutrition position stand: caffeine and exercise performance. *J. Int. Soc. Sports Nutr.* **2021**, *18*, 1. https://doi.org/10.1186/s12970-020-00383-4.

(15) Kalisz, O.; Studzińska, S.; Bocian, S., A determination of the caffeine content in dietary supplements according to green chemistry principles. *Foods* **2023**, *12*, 2474. https://doi.org/10.3390/foods12132474.

(16) Derry, C. J.; Derry, S.; Moore, R. A., Caffeine as an analgesic adjuvant for acute pain in adults. *Cochrane Database Syst. Rev*. **2014**. https://doi.org/10.1002/14651858.CD009281.pub3.

(17) Wasim, S.; Kukkar, V.; Awad, V. M.; Sakhamuru, S.; Malik, B. H., Neuroprotective and neurodegenerative aspects of coffee and its active ingredients in view of scientific literature. *Cureus* **2020**, *12*. https://doi.org/10.7759/cureus.9578

(18) Kolahdouzan, M.; Hamadeh, M. J., The neuroprotective effects of caffeine in neurodegenerative diseases. *CNS Neurosci. Ther*. **2017**, *23*, 272–290. https://doi.org/10.1111/cns.12684

(19) Chu, Y.-F.; Chang, W.-H.; Black, R. M.; Liu, J.-R.; Sompol, P.; Chen, Y.; Wei, H.; Zhao, Q.; Cheng, I. H., Crude caffeine reduces memory impairment and amyloid β_{1-42} levels in an Alzheimer's mouse model. *Food Chem*. **2012**, *135*, 2095–2102. https://doi.org/10.1016/j.foodchem.2012.04.148

(20) Pedata, F.; Pugliese, A. M.; Coppi, E.; Dettori, I.; Maraula, G.; Cellai, L.; Melani, A., Adenosine A2A receptors modulate acute injury and neuroinflammation in brain ischemia. *Med. Inflamm.* **2014**, 805198. https://doi.org/10.1155/2014/805198

(21) Waheeb, T. S.; Abdulkader, M. A.; Ghareeb, D. A.; Moustafa, M. E., Neuroprotective efficacy of berberine and caffeine against rotenone-induced neuroinflammatory and oxidative disturbances associated with Parkinson's disease via inhibiting α-synuclein aggregation and boosting dopamine release. *Inflammopharmacology* **2025**, 1–22. https://doi.org/10.1007/s10787-025-01661-w

(22) Vraneš, M.; Borović, T. T.; Panić, J.; Bešter-Rogač, M.; Janković, N.; Papović, S., Is methyl salicylate the perfect organic solvent for caffeine? *Sustain. Chem. Pharm.* **2024**, *37*, 101361. https://doi.org/10.1016/j.scp.2023.101361

(23) Srdjenovic, B.; Djordjevic-Milic, V.; Grujic, N.; Injac, R.; Lepojevic, Z., Simultaneous HPLC determination of caffeine, theobromine, and theophylline in food, drinks, and herbal products. *J. Chrom. Sci.* **2008**, *46*, 144–149. https://doi.org/10.1093/chromsci/46.2.144.

(24) PubChem database http://pub-chem.ncbi.nlm.nih.gov (accessed 2025 -02- 11).

(25) Protein DataBank (PDB) http://www.rcsb.org (accessed 2025 -02- 11).

(26) Gasteiger, J.; Marsili, M., A new model for calculating atomic charges in molecules. *Tetrahedron Lett.* **1978**, *19*, 3181–3184. https://doi.org/10.1016/S0040-4039(01)94977-9

(27) El Achkar, T.; Greige-Gerges, H.; Fourmentin, S., Basics and properties of deep eutectic solvents: a review. *Environ. Chem. Lett.* **2021**, *19*, 3397–3408. https://doi.org/10.1007/s10311-021-01225-8

(28) Dwamena, A. K., Recent advances in hydrophobic deep eutectic solvents for extraction. *Separations* **2019**, *6*, 9. https://doi.org/10.3390/separations6010009

(29) Shishov, A.; Volodina, N.; Nechaeva, D.; Gagarinova, S.; Bulatov, A., An automated homogeneous liquid-liquid microextraction based on deep eutectic solvent for the HPLC-UV determination of caffeine in beverages. *Microchem. J.* **2019**, *144*, 469–473. https://doi.org/10.1016/j.microc.2018.10.014

(30) Bystrzanowska, M.; Tobiszewski, M., Chemometrics for selection, prediction, and classification of sustainable solutions for green chemistry — A review. *Symmetry* **2020**, *12*, 2055. https://doi.org/10.3390/sym12122055

(31) Gligorić, E.; Igić, R.; Suvajdžić, L.; Teofilović, B.; Turk-Sekulić, M.; Grujić-Letić, N., Methodological aspects of extraction, phytochemical characterization and molecular docking studies of Salix caprea L. bark and leaves. *Acta Chim. Slov.* **2019**, *66*, 821–830. https://doi.org/10.17344/acsi.2018.4829

(32) Meng, X.-Y.; Zhang, H.-X.; Mezei, M.; Cui, M., Molecular docking: a powerful approach for structure-based drug discovery. *Curr. Comput.-Aided Drug Des.* **2011**, *7*, 146–157. https://doi.org/10.2174/157340911795677602

(33) Berger, A. A.; Winnick, A.; Welschmeyer, A.; Kaneb, A.; Berardino, K.; Cornett, E. M.; Kaye, A. D.; Viswanath, O.; Urits, I., Istradefylline to treat patients with Parkinson's disease experiencing "off" episodes: A comprehensive review. *Neurol. Int.* **2020**, *12*, 109–129. https://doi.org/10.3390/neurolint12030017

(34) Ivanova, L.; Karelson, M., The impact of software used and the type of target protein on molecular docking accuracy. *Molecules* **2022**, *27*, 9041. https://doi.org/10.3390/molecules27249041

(35) Gligorić, E.; Igić, R.; Teofilović, B.; Grujić-Letić, N., Phytochemical screening of ultrasonic extracts of Salix species and molecular docking study of Salix-derived bioactive compounds targeting pro-inflammatory cytokines. *Int. J. Mol. Sci.* **2023**, *24*, 11848. https://doi.org/10.3390/ijms241411848

(36) Wei, R.; Qin, X. M.; Li, Z., Comparison of the inedible parts of white and green asparagus based on metabolomics and network pharmacology. *Food Funct.* **2023**. https://doi.org/10.1039/d3fo01797d

(37) Zhai, Y.; Yelanchezian, Y. M. M.; Kwakowsky, A., Is caffeine a potential therapeutic intervention for Alzheimer's disease? *Brain Net. Modul.* **2023**, *2*, 36. https://doi.org/10.4103/2773-2398.379339

(38) Sugiyama, K.; Tomata, Y.; Kaiho, Y.; Honkura, K.; Sugawara, Y.; Tsuji, I., Association between coffee consumption and incident risk of disabling dementia in elderly Japanese: The Ohsaki Cohort 2006 Study. *J.Alzheimer's Dis.* **2016**, *50*, 491–500. https://doi.org/10.3233/JAD-150693

(39) Dong, X.; Li, S.; Sun, J.; Li, Y.; Zhang, D., Association of Coffee, decaffeinated coffee and caffeine intake from coffee with cognitive performance in older adults: national health and nutrition examination survey (NHANES) 2011–2014. *Nutrients* **2020**, *12*, 840. https://doi.org/10.3390/nu12030840

(40) Arendash, G. W.; Mori, T.; Cao, C.; Mamcarz, M.; Runfeldt, M.; Dickson, A.; Rezai-Zadeh, K.; Tan, J.; Citron, B. A.; Lin, X., Caffeine reverses cognitive impairment and decreases brain amyloid-β levels in aged Alzheimer's disease mice. *J. Alzheimer's Dis*. **2009**, *17*, 661–680. https://doi.org/10.3233/JAD-2009-1087