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Hybrids of 4-aminoquinolines and adamantane as inhibitors of AChE

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Abstract: Alzheimer's disease (AD) is an incurable and progressive neurodegenerative disorder that causes cognitive capabilities and memory loss and damage to brain functionality and structure. From diverse possibilities for drug development, the inhibition of acetylcholinesterase (AChE) remains as dominant treatment of symptoms. In our continued investigation of long-chain derivatives of 4-aminoquinoline containing an adamantyl (Ad) group, six new derivatives that differ in the substitution at the terminal amino group or the Ad moiety were synthesised. Their inhibition of AChE, *in silico* drug-likeness, the potential for passing through the blood-brain barrier (BBB) and possible binding modes with AChE for the most active compounds were investigated. It was shown that introducing OH, Br or acetamide group increases the inhibitory potency compared with less polar compounds containing the benzyl group as the second substituent at the amino group. Analysis of *in silico* obtained parameters defined by Lipinsky's rule showed that neither compound is likely to cross the BBB because of violation of at least one of the rules, in general, log *P* and a number of rotatable bonds (RB). Docking of the most active compounds to AChE suggests that compounds act as dual-binding site inhibitors since they have simultaneous interaction with catalytic and peripheral anionic sites of AChE. The substituents on the Ad could be the ones that determine the mode of binding into the enzyme and provide interactions that stabilise the complex between the compound and AChE.

Keywords: 4-aminoquinolines; adamantane; cholinesterase; AChE.

INTRODUCTION

Alzheimer's disease (AD) is an incurable neurodegenerative disorder which progressively and irreversibly causes damage to the brain functionality and manifes-

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ests with a loss of cognitive capabilities, memory and communication ability of patients. According to the report of the World Health Organization, AD currently affects more than 60 million people worldwide with estimation of 10 million new cases annually. The aetiology of the disease is complex, manifested by the enhanced depletion of the neurotransmitter acetylcholine (ACh), the deposition of amyloid- β peptides ($A\beta$ plaques), the accumulation of hyperphosphorylated τ -protein (neurofibrillary tangles, NFTs), the dyshomeostasis of biometal cations, the oxidative stress and the overstimulation of *N*-methyl-D-aspartate (NMDA) receptors.^{1,2} Although such a complex aetiology usually offers multiple druggable targets, the current treatment of AD is based on partially restoring the patient's cognitive functions by alleviating the symptoms of the disease, which is mainly achieved by the drugs acting as inhibitors of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of ACh.³ Currently, four drugs are approved by the FDA for the treatment of cognitive symptoms: donepezil, rivastigmine, galantamine and benzgalantamine (memogain)⁴ as galantamine prodrug, all of them inhibitors of acetylcholinesterase (AChE).⁵ Although most developed inhibitors decrease the action of AChE by interacting with its catalytic active site (CAS), the inhibition of AChE can also occur through interactions with its peripheral anion site (PAS). Such a mode of inhibition has additional benefits for slowing the progression of AD as AChE PAS is involved in the formation of the stable AChE- $A\beta$ complex that is even more toxic than $A\beta$ peptide aggregates.⁶ Also, the $A\beta$ secretion is under cholinergic control, in the way that increasing the level of ACh causes suppression of amyloid production.⁷ Finally, AChE contributes to the inflammation because of the interference with the cholinergic-based cascade in the response of the immune system.⁸ For these reasons, developing compounds that are capable of interfering both with CAS and PAS and acting as multi-target directed ligands (MTDL) is a very promising strategy for developing new AChE inhibitors.

Recently, our group studied two series of 4-aminoquinoline derivatives as MTDLs whose primary mode of action is the inhibition of human AChE – 4-aminoquinolines (4AQ) structural hybrids with adamantyl (Ad) moiety as the substituent on the terminal amino group⁹ and *N*-benzyl (Bn) substituted 4AQ with 1,8-octanediyl side chain.¹⁰ Both series have low cytotoxicity and exhibited excellent inhibitory potency of AChE in the 75 nM to 9.4 μ M range (4AQ-Ad compounds) and 3.2 nM to 1.2 μ M range (4AQ-Bn) of the enzyme-inhibitor dissociation constant (K_i). According to the calculated drug-likeness properties, it was estimated that compounds have good blood-brain barrier (BBB) penetration potential, *via* passive transport. The obtained kinetic results paired with molecular modelling revealed that the examined compounds are capable of binding to the CAS and the PAS of AChE and as such can also affect the formation of amyloid clusters.

Encouraged by the promising results previously obtained for compounds **1** ($K_i = 75 \text{ nM}$)⁹ and **2** ($K_i = 9.8 \text{ nM}$)¹⁰ our idea is to examine how changes close to the terminal basic amino group or introduction of a substituent on the Ad moiety influence the inhibition of AChE. For that purpose, in this work, a series of six new compounds (Fig. 1) was designed and synthesised to investigate their potency to inhibit AChE. The rationalisation of the observed activity was performed by molecular docking. Besides that, their BBB parameters were calculated. The first set of changes on the encirclement of the terminal group was to extend the distance between the Ad group and the terminal amino group (**3**) and introduce the benzyl group, a simple aryl fragment as an additional substituent (**4** and **5**). The second set (**6–8**) has a hydroxyl group, bromine or an amide group, introduced with the aim to affect the physicochemical properties and to enable additional interactions with the protein chain corresponding to the predecessor **1**.

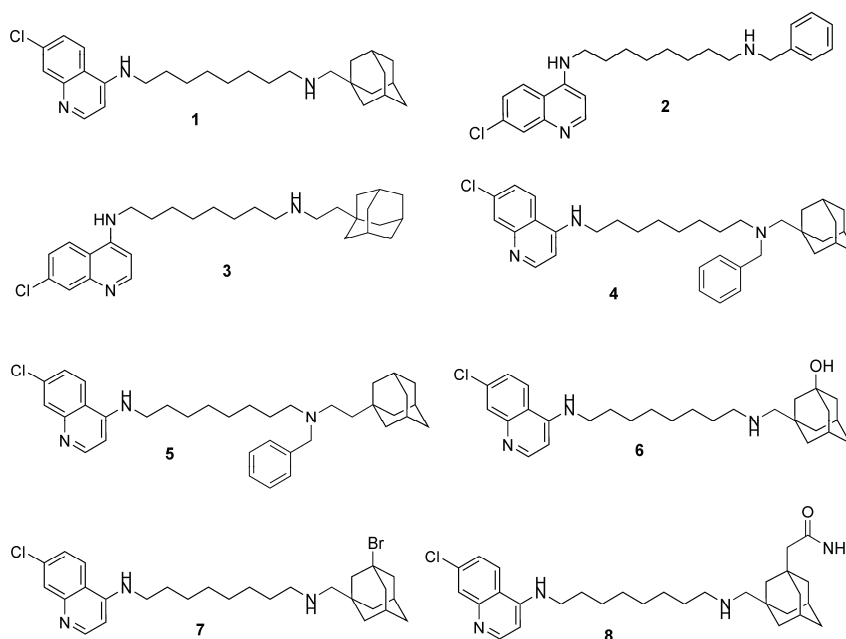


Fig. 1. Structures of **1**, **2** and new compounds examined in this study.

EXPERIMENTAL

Chemistry

All of the chemicals and reagents for the synthesis of 4-aminoquinolines and adamantane hybrid compounds were purchased from commercial sources and used without additional purification. Melting points were recorded on an electrothermal melting point apparatus (1428 6/4 457), England and Boetius PMHK, and were not corrected. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X (Perkin-Elmer, Waltham, MA 02451, USA).

The positions of absorbance band are expressed in cm^{-1} and intensity is labelled as *w* (weak), *m* (medium) or *s* (strong). ^1H - and ^{13}C -NMR spectra were recorded on a Varian/Agilent spectrometer (Agilent Technology, Santa Clara, CA, USA, 95051) at 400 and 100 MHz, for a ^1H - and ^{13}C -NMR spectra, respectively, and on a Bruker Ultrashield Advance III spectrometer (Bruker Scientific Instruments, Billerica, MA, USA, 01821) at 500 and 125 MHz, for a ^1H - and ^{13}C -NMR spectra, respectively, employing indicated solvents, using TMS as the internal standard. The chemical shifts are expressed in ppm (δ) values and coupling constants (*J*) in Hz. The ESI-MS spectra were recorded on an Agilent Technologies 6210 time-of-flight LC-MS instrument with positive ion mode using MeCN/H₂O gradient with 0.2 % HCOOH as the carrying solvent solution. Samples were dissolved in MeOH (HPLC grade purity). GC/MS analysis: Agilent Technologies 7890A gas chromatograph 5975, MSD and FID detector and DB-5 MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μm). LC/MS analysis: Acquity UPLC H-Class LC instrument, Acquity UPLC BEH C18 column (50 mm $\times$ 2.1 mm $\times$ 1.7 μm) and Waters ACQ-TQD MS instrument. Eluent: A = H₂O, B = MeCN. Gradient protocol: 0–1 min (5 % B), 1–10 min (5 $\rightarrow$ 95 % B), 10–11 min (95 % B), 11–12 min (95 $\rightarrow$ 5 % B), 12–13 min (5 % B). Ionisation method: ESI. For the chromatography purification silica gel provided by Merck–Supelco was used, SiO₂ 40–63 μm for the dry-flash chromatography and SiO₂ GF254 or SiO₂ PF254 for the preparative thin-layer chromatography.

HPLC purity

The compounds were analysed for purity using an Agilent 1260 Infinity HPLC system equipped with Quat pump (G1311B), injector (G1329B) 1260 ALS, TCC 1260 (G1316A) and detector 1260 DAD VL+ (G1315C) (Agilent, Santa Clara, CA, USA, 95051). The compounds were dissolved in MeOH, and their final concentrations were $\sim 0.5 \text{ mg mL}^{-1}$. The flow rate was 0.5 mL min^{-1} . Compounds were eluted using gradient protocol: 0–1 min 95 % solvent A, 1–6 min 95 % solvent A $\rightarrow$ 5 % solvent A, 6–11 min 5 % solvent A, 11–14 min 5 % solvent A $\rightarrow$ 95 % solvent A, 14–15 min 95 % solvent A. The analysis was performed at the UV max of the compounds to maximise selectivity. Four different methods were used for HPLC analysis:

Method I – Zorbax Eclipse Plus C18 4.6 mm $\times$ 150 mm, 1.8 μm , S.N. USWKY01594 was used as the stationary phase for the analysis of **3** and **5**. The eluent was made of solvent A = 0.2 % HCOOH in deionised water and solvent B = MeCN;

Method II – Zorbax Eclipse Plus C18 4.6 mm $\times$ 150 mm, 1.8 μm , S.N. USWKY01594 was used as the stationary phase for the analysis of **3** and **5**. The eluent was made of solvent A = 0.2 % HCOOH in deionised water and solvent B = MeOH;

Method III – Zorbax Eclipse Plus C18 4.6 mm $\times$ 100 mm, 1.8 μm was used as the stationary phase for the analysis of **4** and **6–8**. The eluent was made of solvent A = 0.2 % HCOOH in deionised water and solvent B = MeCN;

Method IV – Zorbax Eclipse Plus C18 4.6 mm $\times$ 100 mm, 1.8 μm was used as the stationary phase for the analysis of **4** and **6–8**. The eluent was made of solvent A = 0.2 % HCOOH in deionised water and solvent B = MeOH.

Synthetic procedures, spectral data, and copies of NMR spectra and HPLC chromatograms are given in the Supplementary material to this paper.

Inhibition of AChE

Acetylthiocholine (ATCh) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, MO, USA. ATCh was dissolved in water, DTNB in 0.1 M sodium phosphate buffer (pH 7.4). Stock solutions of 4AQ hybrids with adamantane were dissolved in DMSO, and all further dilutions were made in water. The source of AChE

was human hemolysed erythrocytes. Blood from healthy donors was collected at the Institute for Medical Research and Occupational Health, Zagreb, Croatia, in accordance with the approval from the Ethics Committee of the Institute for Medical Research and Occupational Health. Erythrocytes were separated from the plasma by centrifugation, after which the sedimented erythrocytes were made up to the volume of whole blood by phosphate buffer. The AChE was used without further purification; all enzyme dilutions were done in phosphate buffer. Cholinesterase activity measurements were done following the procedures described previously.^{11,12} Briefly, activities of AChE were measured spectrophotometrically at 0.10 mM ATCh in the absence and presence of a 4AQ hybrid with adamantane (the final concentrations 10 and 1 μ M). At least two experiments for each substrate concentration were conducted.

Docking studies

The ligands for docking were prepared using Maestro from Schrödinger Suite 2021-4.¹³ Ligands were docked as double protonated based on predicted pK_a calculations and the pH of the biological assay (pH 7.4). The enzyme structures were prepared as described previously,¹⁴ starting from the crystal structures of free AChE (PDB ID: 4EY4).¹⁵ Most active ligands were docked into the enzyme active site using the InducedFit docking protocol from Schrödinger Suite 2021-4.¹⁶ The AChE binding site was defined as described in the Supplementary material. Figures were made using DiscoveryStudio Client v18.1. (Dassault Systèmes, Vélizy-Villacoublay, France).

pK_a Calculation

The pK_a value of ionisable sites of the tested compounds was predicted *in silico* using the Chemicalize 2022 platform.¹⁷

In silico prediction of drug-likeness

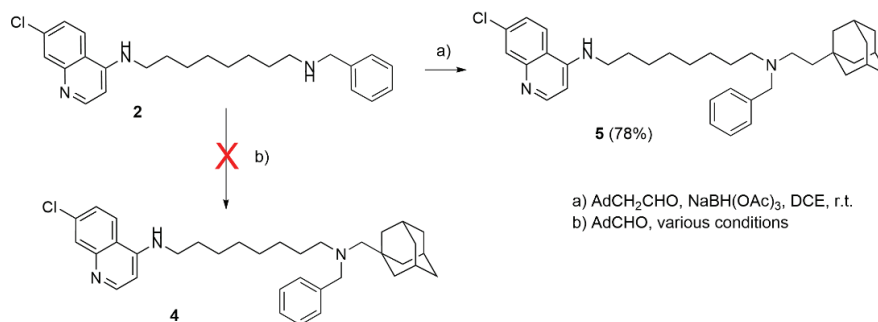
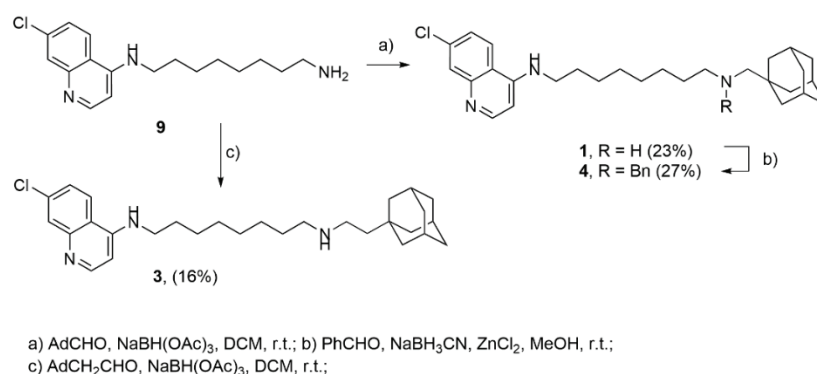
The drug-likeness was estimated for all synthesised compounds, according to the physico-chemical properties important for oral bioavailability: molecular weight (Mw), number of rotatable bonds (RB), number of H-bond acceptors (HBA), number of H-bond donors (HBD), topological polar surface area ($TPSA$) and lipophilicity ($\log P$). For an orally active drug in humans, it is allowed to violate only one of the listed criteria: $180 \leq Mw \leq 500$, $RB \leq 10$, $HBA < 10$, $HBD < 5$, $PSA < 120 \text{ \AA}^2$ and $3 < \log P < 5$.¹⁸ *In silico* calculated data were obtained using Chemicalize 2022 platform.¹⁷

RESULTS AND DISCUSSION

Synthesis of compounds

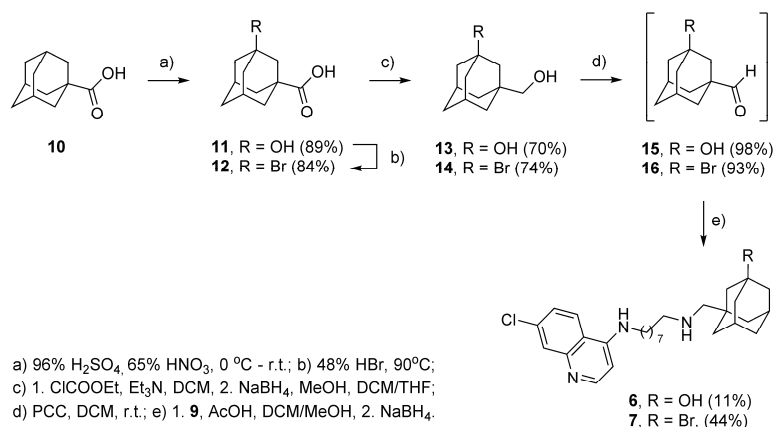
In our previous studies⁹ a low yield of the reaction that attached Ad moiety to the terminal amino group was observed. For that reason, we thought that for the synthesis of the **4** and **5**, the most cost-effective route is to start from **2** and attach the Ad group later (Scheme 1), while **3** will be synthesised directly from **9** (Scheme 2).

The starting compound **2** was prepared following the previously described procedure¹⁹ and derivative **5** was obtained using a corresponding 1-adamantyl-acetaldehyde and $\text{NaBH}(\text{OAc})_3$ in 1,2-dichloroethane (DCE) in a very good yield. Unfortunately, **4** could not be obtained following the planned route. All attempts, which comprise various hydride reagents (NaBH_4 , $\text{NaBH}(\text{OAc})_3$, NaBH_3CN) and solvents (MeOH and DCE), including activating reagents such as ZnCl_2 , were unsuccessful, probably due to high steric demands of Ad fragment.

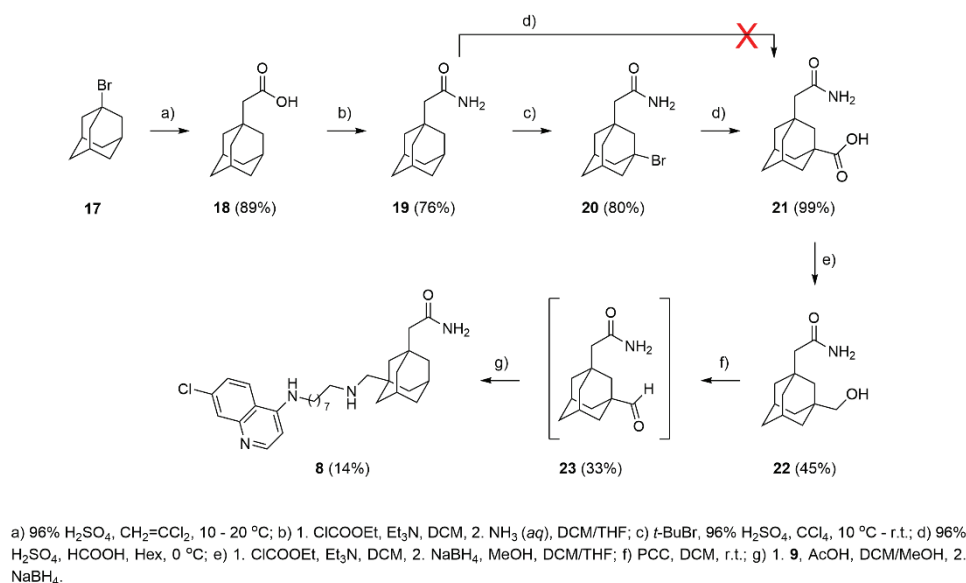
Scheme 1. Synthesis of derivative **5**.Scheme 2. Synthesis of derivatives **3** and **4**.

The derivative **4** was finally obtained by applying the reverse synthetic pathway (Scheme 2). The derivative **1** was obtained from **9** and 1-adamantanecarboxaldehyde using the above-described procedure, and it was transformed into **4** using benzaldehyde and $\text{NaBH}_3\text{CN}/\text{ZnCl}_2$ in MeOH, in a low yield as expected. The derivative **3** was obtained from **9** using 1-adamantylacetaldehyde and $\text{NaBH}(\text{OAc})_3$ in dichloromethane (DCM), also in a low yield.

The synthesis of the derivatives **6** and **7** (Scheme 3) begins with the hydroxylation of 1-adamantanecarboxylic acid **10**, applying the described procedure^{20,21} using the mixture of 96 % sulfuric and 65 % nitric acid. The obtained hydroxyacid **11** was transformed into **12**, using 48 % HBr.²² The carboxylic acids **11** and **12** were transformed into the corresponding mixed anhydrides using ClCOOEt and Et_3N in DCM, which were reduced, without any additional purification, into corresponding alcohols **13** and **14**, using NaBH_4 and MeOH in DCM/THF (THF: tetrahydrofuran). The spectral data for the isolated alcohols **13** and **14** were in accordance with those published.^{23,24} The diols **13** and **14** were oxidised into the corresponding aldehydes **15** and **16**, which were, *via* reductive amination using the amine **9**, transformed into **6** and **7** respectively.

Scheme 1. Synthesis of derivatives **6** and **7**.

Synthesis of the compound **8** (Scheme 4) started from 1-bromoadamantane **17** applying the described procedure,²⁵ using 1,1-dichloroethene in the presence of 96 % sulfuric acid. Obtained 1-adamantaneacetic acid **18** was transformed into **19**,²⁶ applying a modified procedure,²⁷ through intermediary mixed anhydride, using the solution of ammonia. The direct carboxylation of the amide **19** to the acid (**21**) failed. The product **21** was obtained indirectly, after bromination using *t*-BuBr,²⁸ followed by Koch–Haaf carboxylation of **20** in sulfuric acid in the presence of formic acid.²⁹ After the reduction of the carboxyl group in **21** to alcohol **22**, the

Scheme 4. Synthesis of derivative **8**.

obtained product was further oxidised to aldehyde **23**, and by the reductive amination with amine **9**, compound **8** was obtained.

Inhibition of acetylcholinesterase

The ability of compounds to inhibit the action of human AChE is expressed as the percent of the inhibition (%*Inh*) of enzyme activity (Table I). A reversible inhibition was determined for all of the tested derivatives, with the percent of inhibition in the 63–99 % range for 10 μ M and 22–92 % range for 1 μ M concentration of inhibitor.

TABLE I. Inhibition (%*Inh*) of hAChE by 4AQ hybrids with adamantane. All measurements were performed at least in duplicate, and %*Inh* with standard error values were determined from at least two experiments

Compound	Concentration, μ M ^a	
	10	1
1	–	47.0 (0.1 μ M) ^b
2	–	85.0 (0.1 μ M) ^b ; 42.7(0.01 μ M) ^c
3	98.2	82.4
4	84.2	43.8
5	62.7	22.1
6	98.6	87.6
7	99.4	91.9
8	99.0	88.6
Tacrine	–	32 $\pm$ 3 (0.1 μ M) ^b

^aConcentration of the tested compounds **3–8**; ^bconcentration of the tested compound was 0.1 μ M; ^cconcentration of the tested compound was 0.01 μ M

The newly synthesised compounds inhibited AChE activity by more than 80 % when the concentration of inhibitors was 10 μ M, except for the derivative **5**. Reducing inhibitor concentration to 1 μ M, the derivatives with the unsubstituted adamantane moiety exhibited a decrease of inhibition potency, while the derivatives with substituents on adamantane – **6–8** retained a high percentage of inhibition, 99 % at 10 μ M of the inhibitor vs. 88–92 % at 1 μ M. Although the study was carried out at different concentrations for **3** and **1**, it could be seen that the elongation of the linker between adamantane and the terminal amino group led to a lowering of %*Inh*. Derivative **5** proved to be the least active, less than its structural analogue **4**, indicating that replacing the adamantylethyl group in **5** with the adamantylmethyl group in **4** leads to a decrease in enzyme inhibition. Although the presence of one hydrophobic group, like Ad or Bn on the terminal amino group is shown to increase the inhibition potency compared with the corresponding precursor **9** (see Supplementary material) – K_i (**9**) = 0.61 μ M vs. K_i (**1**) = 75 nM vs. K_i (**2**) = 9.8 nM,^{9,10} the observed results suggest that introducing an additional nonpolar moiety decreased inhibitory potency. All three derivatives with a substituent on the Ad group showed high inhibition of AChE activity, with a minor

mutual difference. This indicates that an additional functional group does not disrupt the binding of inhibitors with the enzyme active site and/or can enable new interactions. Although compound **1** is the most active within this set of inhibitors, derivatives **6–8** are also promising candidates for the design of new more potent inhibitors.

Docking results

To rationalise the experimentally determined inhibition potency of the selected compounds and suggest a binding mode, the most active ligands (**6–8**) were docked into the enzyme active site using InducedFit docking procedure. Although the examined compounds showed very similar docking scores (Table S-I, Supplementary material), they have some specific differences in binding.

All docked compounds interacted with the amino acids from CAS and PAS and bound to AChE as dual-binding site inhibitors. The compounds **6** and **8**, are bonded similarly, with the aminoquinoline ring oriented to PAS and forming multiple hydrophobic T-shaped interactions with amino acids Tyr72 and Trp286 [Fig. 2B and C and Figs. S-2 and S-3 of the Supplementary material). The **8** interacted with Asp74, Tyr 124 and Tyr341, further stabilising the AChE-**8** complex. The Ad moiety, in both compounds, was oriented toward inside of the enzyme, forming interaction with some of the amino acids from CAS and choline-binding sites – Ser203, His447, Trp86 and Tyr337. The **7** has a different orientation, in which the protonated aminoquinoline ring was oriented toward CAS, while the adamantane group was directed toward PAS (Fig. 2 and Fig. S-1). The aminoquinoline ring is engaged in multiple π - π stacked and T-shaped interactions (Trp86, Tyr337 and His447), together with electrostatic ones and a conventional H-bond with His447. Bromine atom on Ad moiety directed the orientation of the ligand by forming multiple hydrophobic-stacked interactions with residues of Tyr72, Trp286 and His287 in the PAS. Different orientations between compounds may be accounted to following interactions: a) multiple H-bonds between OH-group with Gly121, Gly122, Ser203 for **6** and amide group with Tyr133 and Glu202 for **8**, b) multiple hydrophobic-stacked interactions of Br-atom with PAS and c) and a set of interaction of unprotonated quinoline moiety of **8** with the PAS. All identified interactions are listed in Tables S-II–S-IV of the Supplementary material.

pK_a and distribution of protonated species

The ionisation constants of the tested aminoquinolines at physiological pH 7.4 are given in Table S-I. Compounds had pK_{a1} (quinoline nitrogen) = 7.7 and pK_{a2} (terminal amino group) in the 9.65–11.46 range. Considering the dynamic equilibrium between monoprotonated and diprotonated forms of the examined compounds at pH 7.4, 67 % is in deprotonated form.

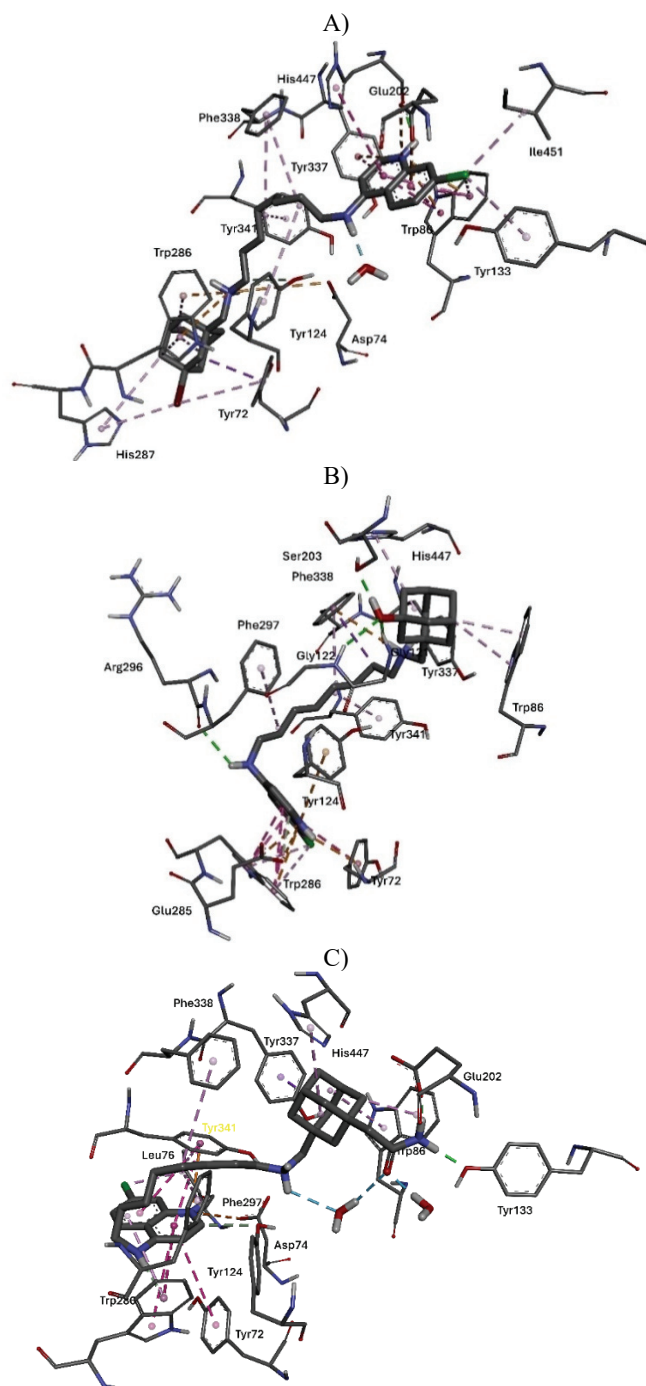


Fig. 2. Binding modes for complexes between compound 7 (A), 6 (B) and 8 (C) and AChE.

In silico prediction of drug-likeness

The important physicochemical properties that influence the passive transport of compounds into the central nervous system (CNS) after oral administration are: M_w , 1-octanol/water $\log P$, HBD , HBA , RB and $TPSA$. Although the key parameters are not experimentally determined, we applied two *in silico* platforms, Chemicalize 2022 and CNS multiparameter optimised algorithm (CNS MPO),^{18b,30} to calculate key parameters and to estimate drug-likeness properties of the examined compounds. The corresponding *in silico* data are given in Table S-I. From the calculated values of physicochemical properties, all tested compounds violate two of the six recommended values (Fig. 3). The first is $\log P$ and the second is RB . The largest deviation from the recommended $\log P$ values has been shown for **4** and **5**, which at the same time have the lowest activity. Further, the same compounds also have the highest values of M_w , and in combination with other parameters, they could be excluded as potential CNS-active compounds. The deviation in RB from the recommended value cannot be discussed without considering the necessity for conformational flexibility of compounds to attain the optimum occupation of CAS and interaction with PAS so that the most effective inhibition of AChE for the hydrolysis of ACh and interference with A β peptide aggregates could be achieved. All the tested compounds met the HBA , HBD , and $TPSA$ requirements.

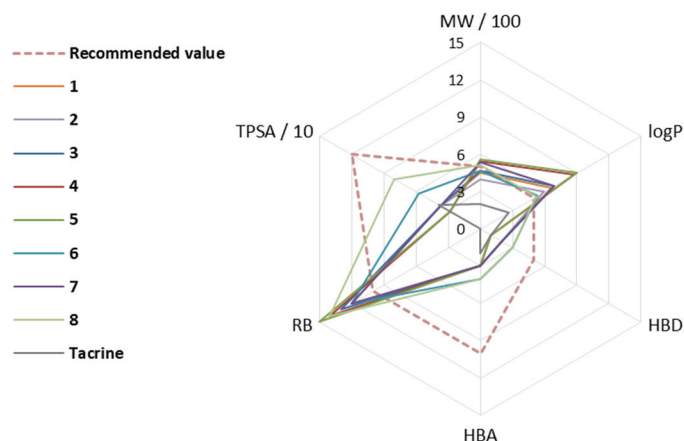


Fig. 3. Radar plot with calculated values of physicochemical properties of the tested compounds. The dashed red line represents the recommended values of M_w , $\log P$, HBD , HBA , RB and $TPSA$.

Since compounds have two basic groups, which will be ionised in the blood (pH 7.4), the contribution of the pH-dependent partition coefficient ($\log D$) and pK_a the values of ionisable groups should not be excluded from the evaluation of the drug-likeness properties. In order to additionally estimate the potential of the

compounds to be CNS-active, we analysed them using central nervous system multiparameter optimization (CNS MPO) algorithm, which is based on a set of six interrelated fundamental physicochemical parameters (*clog P*, *clog D*, *Mw*, *TPSA*, *HBD* and *pK_a* (the most basic group in the molecule)) and a variation of Harrington's optimisation method. All physicochemical properties were weighted equally, with a desirability score ranging from 0.0 to 1.0 for each property and a total CNS MPO desirability score ranging from 0.0 to 6.0. A higher score denoted more desirable overall parameters and for the majority of CNS-drugs or candidates, this score is in the $3 < \text{CNS MPO} \leq 5$ range. We used the parameters calculated on the Chemicalize 2022 platform for the prediction of *CNS MPO* score (Table II). The compounds have low scores which are in the 1.2–2.4 range, which are lower than for the starting compound **2**. Compounds **6** and **8** have the highest scores among synthesised compounds, 2.4 and 2.2, respectively. Tacrine, the drug used in AD therapy expectedly has the highest score, 5.1.

TABLE II. CNS MPO score of the evaluated compounds

Compound	1	2	3	4	5	6	7	8	Tacrine
CNS MPO	2.2	2.6	1.9	1.2	1.4	2.4	1.7	2.2	5.1

CONCLUSION

In this study, the influence of changes close to the terminal amino group or the introduction of an additional substituent on the AD moiety on the inhibitory potential of AChE, the drug-likeness properties and the capability of compounds to penetrate BBB was investigated. The introduction of an additional hydrophobic benzyl group, or methylene group in the side chain leads to a decrease in the inhibitory activity of the compounds. On the other hand, the introduction of hydroxyl, bromine or amide group enhanced the potency for the inhibition of AChE, to more than 99 % inhibition at 10 μM or 92–88 % range at 1 μM concentration of the compound. *In silico* obtained indices that determine the drug-likeness of compounds revealed that all synthesised compounds have higher values of *log P* and an acceptable number of RB. Considering a new parameter, *CNS MPO*, that estimates the similarity of compounds with drugs or drug candidates, it was shown that new compounds have low scores which are in the 1.2–2.4 range, compared with tacrine with *CNS MPO* = 5.1. Docking of the most active compounds to AChE revealed their capacity to act as dual-binding site inhibitors due to simultaneous interaction with the amino acids from CAS and PAS. Further, our expectations regarding additional substituents on the adamantane moiety were justified, since it was observed that new compounds **6–8** have additional interactions with amino acid residues in comparison to **1**. These interactions determine the binding mode of compounds into the enzyme and provide additional stabilisation of the complex with AChE. Although the reported results of *in silico* parameters and drug-likeness

properties of the tested compounds suggest the low potential to be CNS candidates and to penetrate BBB, at the same time these results give the guidelines for the improvements of the structures of the compounds. They should be improved in terms of increasing compound polarity, which would result in increased TPSA and lowering log *P* and log *D*. At the same time, having in mind that the criteria for CNS drugs are more restrictive than for other orally bioavailable drugs, careful selection of potential structural changes, such as the introduction of polar functional groups (ethers, amides, esters, CF₃, *etc.*) should be made to avoid violation of additional parameters such as p*K*_a or HBD.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13230>, or from the corresponding author on request.

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ИЗВОД

ХИБРИДИ 4-АМИНОХИНОЛИНА И АДАМАНТАНА КАО ИНХИБИТОРИ АСhЕ

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Алцхајмерова болест (AD) је неизлечиви и прогресивни неуродегенеративни поремећај који узрокује губитак когнитивних способности и памћења и оштећење функционалности и структуре мозга. Од различитих могућности за развој лекова, инхибиција ацетилхолин-естеразе (AChE) остаје доминантан третман симптома. У наставку наших истраживања дуголанчаних деривата 4-аминохинолина који садрже адамантил (Ad) групу као инхибитор AChE, истражили смо шест нових деривата који се разликују по супституцији терминалне аминок групе или по Ад остатку. Истражена је њихова способност инхибиције AChE, *in silico* сличност лековима, потенцијал за пролазак кроз БББ и могући начини везивања са AChE код најактивнијих једињења. Показало се да увођење хидроксилне групе, брома или ацетамидне групе повећава инхибиторну моћ у поређењу са мање поларним једињењима која садрже бензил групу као други супституент на аминок групи. Анализа *in silico* добијених параметара дефинисаних правилом Липинског показала је да сва једињења нарушавају log *P* и број веза око којих је могућа ротација (RB). Докинг најактивнијих једињења на AChE сугерише да једињења делују као двоструки

инхибитори због истовремених интеракција са каталитичким и периферним местом везивања. Супституенти на Ad одређују начин везивања за ензиме и обезбеђују интеракције које стабилизују комплекс између једињења и AChE.

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SUPPLEMENTARY MATERIAL TO
Hybrids of 4-aminoquinolines and adamantane as inhibitors of AChE

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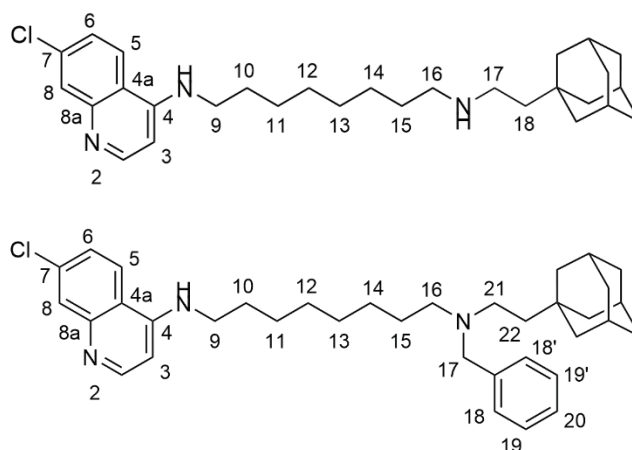
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1. SYNTHESIS

1.1. Synthetic procedures and spectral data

Numeration method:



*N*¹-Benzyl-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (**2**).

Compound **2** was synthesized according to the published procedure.¹ Yield 84,1 mg (74%), pale yellow solid, Mp = 61-65 °C. IR (ATR): 3315m, 2927s, 2852s, 1580s, 1452m, 1367m, 1241m, 1212m, 1158m, 1134m, 1081m, 1026w, 899w, 875w, 850w, 804m, 754m, 697m, 598w, 525w cm⁻¹. ¹H NMR (400 MHz, CD₃OD, δ): 8.32 (d, *J* = 5.7 Hz, 1H, H-2), 8.11 (d, *J* = 9.0 Hz, 1H, H-5), 7.76 (d, *J* = 2.1 Hz, 1H, H-8), 7.44 – 7.38 (m, 6H, H-6 and H-18-20), 6.53 (d, *J* = 5.8 Hz, 1H, H-3), 4.08 (s, 2H, H-17), 3.37 (t, *J* = 7.2 Hz, 2H, H-9), 2.94 – 2.87 (m, 2H, H-16), 1.74 (quint, *J* = 7.2 Hz, 2H, H-10), 1.69 – 1.58 (m, 2H, H-15), 1.49 – 1.34 (m, 8H, 4× -CH₂, H-(11-14)).

*N*¹-(2-(Adamantan-1-yl)ethyl)-*N*¹-benzyl-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (**5**).

Into the solution of **2** (18.3 mg, 0.0462 mmol) and 1-adamantaneacetaldehyde (16.5 mg, 0.0926 mmol) in DCE (1,5 mL), NaBH(OAc)₃ (29.4 mg, 0.1387 mmol) was added. The mixture was stirred at room temperature for 2 h, in an Ar atmosphere. The reaction was quenched with sat. aqueous solution of NaHCO₃, transferred into a separatory funnel and the aqueous layer was extracted three times with DCM. The organic layers were combined, washed with brine and dried over anhyd. Na₂SO₄. The solvent was evaporated and the product was isolated after chromatographic purification (preparative thin-layer chromatography, silica gel, DCM/MeOH). Yield 20.2 mg (78%), dark yellow oil. IR (ATR): 3263w, 3062w, 3026w, 2904s, 2847s, 1611m, 1580s, 1540m, 1492w, 1451m, 1368m, 1332m, 1281w, 1249w, 1203w, 1136w, 1101w, 1080w, 1028w, 968w, 902w, 877w, 852w, 806w, 768w, 737m, 699w, 645w, 623w, 430w cm⁻¹. ¹H NMR (400 MHz, CDCl₃, δ) 8.51 (d, *J* = 5.4 Hz, 1H, H-2), 7.95 (s, 1H, H-8), 7.68 (d, *J* = 9.0 Hz, 1H, H-5), 7.38 – 7.18 (m, 6H, H-6, H-18, H-18', H-19, H-19' and H-20), 6.39 (d, *J* = 5.4 Hz, 1H, H-3), 5.14 (s, 1H, -NH), 3.57 (s, 2H, H-17), 3.29 (q, *J* = 6.5 Hz, 2H, H-9), 2.51 – 2.37 (m, 4H, H-16 and H-21), 1.90 (s, 3H, 3× -CH(Ad)), 1.78 – 1.56 (m, 8H, 3× -CH₂(Ad) and -CH₂ H-10), 1.50 – 1.25 (m, 18H, 3× -CH₂(Ad) and 6× -CH₂ H-(11-15) and H-22). ¹³C NMR (100 MHz, CDCl₃, δ) 151.91, 149.97, 148.97, 139.54, 135.00, 129.09, 128.68, 128.22, 126.90, 125.36, 121.10, 117.18, 99.12, 58.43, 53.69, 47.57, 43.39, 42.64, 40.43, 37.28, 31.99, 29.51,

29.41, 28.95, 28.78, 27.42, 27.19, 26.92. ESI-HRMS (m/z): calc. for $[C_{36}H_{48}ClN_3+H]^+$ 558.3610, found 558.3611.

*N*¹-(7-Chloroquinolin-4-yl)octane-1,8-diamine (9).

Compound **9** was synthesized according to the published procedure.² Yield 2,6513 g (78%), pale yellow solid, Mp = 104–108 °C. IR (ATR): 3324m, 3260m, 3175m, 2928s, 2851s, 1612m, 1576s, 1536m, 1476m, 1463m, 1450m, 1428m, 1391m, 1369m, 1328m, 1281w, 1251w, 1218w, 1199w, 1166w, 1133m, 1079w, 1026w, 981w, 954w, 925w, 903w, 875w, 853m, 812m, 796w, 770w, 727w, 648w, 622w, 597w, 527w, 495w, 441w cm⁻¹. ¹H NMR (400 MHz, CD₃OD, δ): 8.33 (d, *J* = 5.6 Hz, 1H, H-2), 8.08 (d, *J* = 9.0 Hz, 1H, H-5), 7.76 (s, 1H, H-8), 7.40 – 7.34 (m, 1H, H-6), 6.47 (d, *J* = 5.7 Hz, 1H, H-3), 3.35 – 3.28 (m, 2H, H-9, overlapped with CD₃OD), 2.61 (t, *J* = 7.2 Hz, 2H, H-16), 1.73 (quint, *J* = 7.3 Hz, 2H, H-10), 1.51 – 1.28 (m, 10H, 5 × -CH₂).

*N*¹-((Adamantan-1-yl)methyl)-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (1).

Compound **1** was synthesized according to the published procedure.³ Yield 395.3 mg (26 %), yellow oil. IR (ATR): 3277m, 3064w, 2905s, 2849s, 1610m, 1581s, 1541w, 1453m, 1368w, 1332m, 1282w, 1250w, 1137w, 878w, 851w, 806w. ¹H NMR (400 MHz, CD₃OD, δ) 8.33 (d, *J* = 5.6 Hz, 1H, H-2), 8.10 (d, *J* = 9.0 Hz, 1H, H-5), 7.76 (d, *J* = 1.4 Hz, 1H, H-8), 7.38 (dd, *J*₁ = 9.0 Hz, *J*₂ = 1.8 Hz, 1H, H-6), 6.49 (d, *J* = 5.6 Hz, 1H, H-3), 3.35–3.34 (m, 2H, H-9 overlapped with MeOH solvent), 2.68 – 2.60 (m, 2H, H-16), 2.35 (s, 2H, H-17), 2.00 – 1.93 (m, 3H, 3 × -CH(Ad)), 1.81 – 1.63 (m, 8H, 3 × -CH₂(Ad) and -CH₂ H-10), 1.61 – 1.30 (m, 16H, 3 × -CH₂(Ad) and 5 × -CH₂).

*N*¹-((Adamantan-1-yl)methyl)-*N*¹-benzyl-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (4).

Into the solution of **1** (64 mg, 0.1409 mmol) and benzaldehyde (30 μL, 0.2954 mmol) in dry MeOH (1.7 mL), the mixture of NaBH₃CN (9.7 mg, 0.1544 mmol) and ZnCl₂ (9.6 mg, 0.0704 mmol) in dry MeOH (1.7 mL) was added. After stirring the reaction mixture at room temperature for 24 h, the solvent was evaporated under reduced pressure. Sat. aqueous solution of NaHCO₃ and DCM were added, the mixture was transferred into a separatory funnel and layers were separated. The water layer was extracted with DCM and combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. The solvent was evaporated and the product was isolated after chromatographic purification (dry-flash chromatography, silica gel, gradient Hex/EA, then preparative thin-layer chromatography, silica gel, EA/MeOH, then dry-flash chromatography, silica gel, gradient DCM/MeOH). Yield 20.7 mg (27%), yellow oil. IR (ATR): 3261w, 3062w, 3026w, 2904s, 2848s, 1611m, 1581s, 1540m, 1492w, 1452m, 1368m, 1332m, 1282w, 1247w, 1205w, 1137w, 1100w, 1081w, 1028w, 983w, 902w, 877w, 852w, 806w, 768w, 736w, 698w, 645w cm⁻¹. ¹H NMR (500 MHz, CDCl₃, δ) 8.51 – 8.46 (m, 1H, H-2), 7.95 (d, *J* = 1.9 Hz, 1H, H-8), 7.68 (dd, *J*₁ = 9.0 Hz, *J*₂ = 3.3 Hz, 1H, H-5), 7.38 (d, *J* = 7.3 Hz, 2H, H-18 and H-18'), 7.36 – 7.32 (m, 1H, H-6), 7.29 (t, *J* = 7.5 Hz, 2H, H-19 and H-19'), 7.21 (t, *J* = 7.3 Hz, 1H, H-20), 6.38 (dd, *J*₁ = 5.4 Hz, *J*₂ = 1.9 Hz, 1H, H-3), 5.33 – 5.17 (m, 1H, Ar-NH), 3.58 (s, 2H, H-17), 3.28 (q, *J* = 7.1 Hz, 2H, H-9), 2.34 – 2.26 (m, 2H, H-16), 2.13 (s, 2H, H-21), 1.97 – 1.90 (m, 3H, 3 × -CH(Ad)), 1.76 – 1.58 (m, 8H, H-10 and 3 × -CH₂(Ad)), 1.51 (d, *J* = 2.2 Hz, 6H, 3 × -CH₂(Ad)), 1.45 – 1.37 (m, 4H, H-11 and H-15), 1.35 – 1.28 (m, 2H, H-14), 1.27 – 1.18 (m, 4H, H-12 and H-13). ¹³C NMR (125 MHz, CDCl₃, δ) 151.42, 150.25, 148.49, 141.14, 135.27, 128.68, 128.30, 128.14, 126.64, 125.51, 121.22, 117.09, 99.06, 68.21, 62.10, 56.45, 43.47, 41.60, 37.44, 35.17, 29.59, 29.45, 28.96, 28.74, 27.70, 27.37, 27.22. ESI-HRMS (m/z): calc. for $[C_{35}H_{46}ClN_3+H]^+$ 544.3453, found 544.3456.

*N*¹-(2-(Adamantan-1-yl)ethyl)-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (3).

Into the solution of **9** (153 mg, 0.5003 mmol) and 1-adamantaneacetaldehyde (89.2 mg, 0.5004 mmol) in DCM (11.5 mL), NaBH(OAc)₃ (212.1 mg, 1.0008 mmol) was added. After stirring the mixture for 22 h, at room temperature, the reaction was quenched with aqueous NaOH (1 M). The organic layer was separated and the aqueous layer was extracted with DCM. The combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. The solvent was evaporated and the product was isolated after chromatographic purification (dry-flash chromatography, silica gel, gradient prepared from DCM and mixture [MeOH/NH₃(aq)=9/1], then multiple preparative thin-layer chromatography, silica gel, EA/MeOH/NH₃(aq)). Yield 36.4 mg (16%), pale yellow oil. IR (ATR): 3278m, 2904s, 2848s, 1611m, 1581s, 1541m, 1451m, 1428w, 1368m, 1332w, 1282w, 1203w, 1137w, 1080w, 901w, 878w, 852w, 806w, 768w, 738w, 646w cm⁻¹. ¹H NMR (400 MHz, CDCl₃, δ) 8.52 (d, *J* = 5.3 Hz, 1H, H-2), 7.95 (s, 1H, H-8), 7.67 (d, *J* = 8.9 Hz, 1H, H-5), 7.35 (d, *J* = 8.9 Hz, 1H, H-6), 6.40 (d, *J* = 5.4 Hz, 1H, H-3), 5.07 (s, 1H, Ar-NH), 3.29 (q, *J* = 6.7 Hz, 2H, H-9), 2.67 – 2.53 (m, 4H, H-16 and H-17), 1.93 (s, 3H, 3× -CH(Ad)), 1.79 – 1.58 (m, 8H, 3× -CH₂(Ad) and -CH₂ H-10), 1.54 – 1.40 (m, 10H, 3× -CH₂(Ad) and 2× -CH₂ H-15 and H-11), 1.40 – 1.24 (m, 8H, H-18 and 3× -CH₂ H-(12-14)). ¹³C NMR (100 MHz, CDCl₃, δ) 152.17, 149.83, 149.22, 134.90, 128.90, 125.32, 121.02, 117.22, 99.17, 50.33, 44.71, 44.48, 43.37, 42.75, 37.26, 31.96, 30.15, 29.55, 29.38, 28.97, 28.78, 27.44, 27.19. ESI-HRMS (*m/z*): calc. for [C₂₉H₄₂ClN₃+H]⁺ 468.3140, found 468.3141.

3-Hydroxyadamantane-1-carboxylic acid (11).⁴

1-Adamantanecarboxylic acid **10** (1 g, 5.5478 mmol) was introduced portionwise into the ice-cold mixture of 96% H₂SO₄ (4 mL) and 65% HNO₃ (0.5 mL). Then, the reaction mixture was spontaneously warmed to room temperature and stirred overnight. Water was added in drops at 10 °C and the formation of precipitate was completed after additional stirring and cooling. The product was isolated after filtration, rinsed with water and dried. Yield 970.1 mg (89%), white solid, Mp = 195–200 °C. IR (ATR): 3440s, 2949s, 2909s, 2862m, 2809m, 2640m, 1708s, 1454m, 1392w, 1341m, 1273s, 1248s, 1227m, 1193w, 1147w, 1120m, 1046w, 1018m, 978w, 941m, 915m, 883w, 724m, 670w, 564w, 524w, 439w cm⁻¹. ¹H NMR (500 MHz, CD₃OD, δ) 2.25 – 2.19 (m, 2H, 2× -CH), 1.84 – 1.74 (m, 6H, 3× -CH₂), 1.73 – 1.65 (m, 4H, 2× -CH₂), 1.64 – 1.59 (m, 2H, -CH₂). ¹³C NMR (125 MHz, CD₃OD, δ) 180.42, 68.72, 47.23, 45.05, 44.89, 39.01, 36.22, 31.71.

3-Bromoadamantane-1-carboxylic acid (12).⁵

The mixture of **11** (1.8264 g, 9.3068 mmol) and 48% HBr (18.42 mL, 162.8227 mmol) was stirred in an Ace pressure tube at 90 °C for 6 h and then cooled at 4 °C. The product was isolated after filtration, rinsed with cold water and dried. Yield 2.0389 g (84%), white solid, Mp = 140–143 °C. IR (ATR): 2918s, 2862m, 2654w, 2327w, 1690s, 1478w, 1453w, 1416w, 1329w, 1315m, 1288m, 1171w, 1148w, 1107w, 1083w, 956w, 895w, 831w, 763w, 746w, 691w, 671w, 535w cm⁻¹. ¹H NMR (500 MHz, CDCl₃, δ) 2.49 (s, 2H, -CH₂), 2.37 – 2.27 (m, 4H, 2× -CH₂), 2.25 – 2.20 (m, 2H, 2× -CH), 1.91 (d, *J* = 2.9 Hz, 4H, 2× -CH₂), 1.75 – 1.67 (m, 2H, -CH₂). ¹³C NMR (125 MHz, CDCl₃, δ) 181.88, 63.29, 49.44, 48.15, 44.80, 37.01, 34.53, 31.73.

3-(Hydroxymethyl)adamantan-1-ol (13).⁶

A solution of **11** (380 mg, 1.9364 mmol), Et₃N (0.32 mL, 2.3085 mmol) and ClCOOEt (0.22 mL, 2.3007 mmol) in DCM (10 mL) was stirred at room temperature for 2 h. The reaction mixture was evaporated to dryness and the residue was dissolved in 20 mL DCM/THF = 3/1, followed by the addition of NaBH₄ (293 mg, 7.7452 mmol) and MeOH (6 mL) in small portions. After 21 h, the reaction was quenched by pouring into ice/dilute HCl mixture and layers were

separated. The organic layer was washed with sat. NaHCO_3 and brine. The aqueous layer was neutralized to pH 7, extracted twice with EA and the combined organic layers were washed with brine. Finally, both organic layers were combined and dried over anhydrous Na_2SO_4 . The solvent was evaporated and the product was isolated after chromatographic purification (dry-flash chromatography, silica gel, gradient Hex/EA). Yield 245.8 mg (70%), white crystals, $\text{Mp} = 108\text{--}112\text{ }^\circ\text{C}$. IR (ATR): 3367s, 3302s, 2912s, 2850s, 2668w, 1512w, 1454m, 1430m, 1342m, 1315m, 1249w, 1209w, 1168m, 1149m, 1112m, 1056s, 1026m, 980w, 959w, 941m, 920w, 898w, 833w, 797w, 774w, 735w, 708w, 648m, 618w, 596w, 549w, 462w, 430w cm^{-1} . ^1H NMR (500 MHz, CD_3OD , δ) 3.17 (s, 2H, $-\text{CH}_2\text{OH}$), 2.21 – 2.15 (m, 2H, 2 $-\text{CH}$), 1.72 – 1.54 (m, 6H, 3 $\times$ $-\text{CH}_2$), 1.47 – 1.39 (m, 6H, 3 $\times$ $-\text{CH}_2$). ^{13}C NMR (125 MHz, CD_3OD , δ) 72.96, 69.30, 47.50, 45.61, 39.40, 39.07, 36.89, 31.87. EI-GC/MS m/z (%): 182.1 (13) $[\text{M}]^+$, 151.1 (100) $[\text{M} - \text{CH}_2\text{OH}]^+$, 107.0 (16), 95.0 (36).

(3-Bromoadamantan-1-yl)methanol (14).⁷

A solution of **12** (2.0389 g, 7.8679 mmol), Et_3N (1.3 mL, 9.3784 mmol) and ClCOOEt (0.9 mL, 9.4069 mmol) in DCM (41.5 mL) was stirred at room temperature for 150 min. The reaction mixture was evaporated to dryness and the residue was dissolved in DCM/THF = 3/1 (81.3 mL), followed by the addition of NaBH_4 (1.1906 g, 31.4724 mmol) and MeOH (25 mL) in small portions. After 24 h, the reaction was quenched by pouring into ice/dilute HCl mixture and layers were separated. The aqueous layer was extracted twice with DCM and the combined organic layers were washed with sat. NaHCO_3 , then with brine and dried over anhydrous Na_2SO_4 . The solvent was evaporated and the product was isolated after chromatographic purification (dry-flash chromatography, silica gel, gradient Hex/EA). Yield 1.4274 g (74%), pale yellow crystals, $\text{Mp} = 73\text{--}77\text{ }^\circ\text{C}$. IR (ATR): 3426m, 3320s, 2906s, 2854s, 1449m, 1397w, 1371w, 1347w, 1333w, 1302m, 1280w, 1233w, 1196w, 1158w, 1139m, 1105w, 1038s, 1016m, 972m, 945w, 911w, 813m, 790w, 735w, 675m, 657w, 602w, 482w cm^{-1} . ^1H NMR (500 MHz, CDCl_3 , δ) 3.26 (s, 2H, $-\text{CH}_2\text{OH}$), 2.37 – 2.25 (m, 4H, 2 $\times$ $-\text{CH}_2$), 2.22 – 2.17 (m, 2H, 2 $\times$ $-\text{CH}$), 2.15 (s, 2H, $-\text{CH}_2$), 1.75 – 1.61 (m, 2H, $-\text{CH}_2$), 1.58 – 1.49 (m, 4H, 2 $\times$ $-\text{CH}_2$). ^{13}C NMR (125 MHz, CDCl_3 , δ) 72.45, 65.92, 50.78, 48.90, 40.10, 37.30, 35.23, 32.22. EI-GC/MS m/z (%): 213.0 (2) $[\text{M} - \text{CH}_2\text{OH}]^+$, 165.1 (100) $[\text{M} - \text{Br}]^+$, 147.1 (29), 105.1 (36), 91.1 (26).

3-(((8-((7-Chloroquinolin-4-yl)amino)octyl)amino)methyl)adamantan-1-ol (6).

General procedure: A solution of **13** (200 mg, 1.0973 mmol) in dry DCM (15 mL), with added PCC (354.8 mg, 1.6459 mmol) was stirred at room temperature for 150 min. The crude product was purified by column chromatography (dry-flash, silica gel, gradient DCM/MeOH) affording 193.8 mg of **15** (98%), which was used in the next step without prior characterization.

Into the solution of **15** (197.8 mg, 1.0973 mmol) and **9** (335.6 mg, 1.0973 mmol) in DCM/MeOH = 1/2 (21 mL), AcOH (0.094 mL, 1.6436 mmol) was added in drops and the reaction mixture stirred overnight. Afterwards, NaBH_4 (249 mg, 6.5821 mmol) was carefully added and stirring continued for another 72 h. The solvent was removed under reduced pressure and the residue partitioned between DCM and NH_3 (aq, 2 M). The organic layer was washed with water, then brine, dried over anhydrous Na_2SO_4 and the solvent was evaporated. The crude product was purified by column chromatography (dry-flash, silica gel, gradient DCM/MeOH). Yield 57.1 mg (11%), pale yellow oil. IR (ATR): 3313m, 2922s, 2850s, 1611m, 1581s, 1540m, 1453m, 1428m, 1368m, 1333m, 1312w, 1282w, 1204w, 1161w, 1137m, 1114w, 1080w, 1049w, 946w, 901w, 878w, 852w, 806w, 768w, 737w, 704w, 646w, 553w cm^{-1} . ^1H NMR (400 MHz, CDCl_3 , δ) 8.52 (d, $J = 5.3$ Hz, 1H, H-2), 7.95 (d, $J = 1.7$ Hz, 1H, H-8), 7.67 (d, $J = 8.9$ Hz, 1H, H-5), 7.34 (dd, $J_1 = 8.9$, $J_2 = 1.7$ Hz, 1H, H-6), 6.40 (d, $J = 5.4$ Hz, 1H, H-3), 5.06 (s,

1H, Ar-NH), 3.29 (q, $J = 6.8$ Hz, 2H, H-9), 2.57 (t, $J = 7.2$ Hz, 2H, H-16), 2.32 (s, 2H, H-17), 2.20 (s, 2H, $2 \times -CH(Ad)$), 1.80 – 1.62 (m, 6H, $2 \times -CH_2(Ad)$ and $-CH_2$ H-10), 1.58 – 1.52 (m, 2H, $-CH_2(Ad)$), 1.50 – 1.30 (m, 16H, $3 \times -CH_2(Ad)$ and $5 \times -CH_2$, H-11-15). ^{13}C NMR (100 MHz, $CDCl_3$, δ) 152.15, 149.84, 149.25, 134.90, 128.94, 125.32, 121.01, 117.24, 99.18, 69.00, 61.97, 51.15, 48.63, 45.06, 43.39, 39.73, 37.41, 35.82, 30.72, 30.07, 29.55, 29.41, 29.00, 27.33, 27.20. ESI-HRMS (m/z): calc. for $[C_{28}H_{40}ClN_3O+H]^+$ 470.2933, found 470.2939.

*N*¹-((3-Bromoadamantan-1-yl)methyl)-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (7).

Following the above-described general procedure, **14** (150 mg, 0.6118 mmol) reacted with PCC (197.8 mg, 0.9176 mmol) in dry DCM (8.2 mL). The crude product was purified by column chromatography (dry-flash, silica gel, DCM) affording 139 mg of **16** (93%), which was used in the next step. Aldehyde **16** (139 mg, 0.5717 mmol) and amine **9** (209.8 mg, 0.6860 mmol) in DCM/MeOH = 1/2 (12 mL), were transformed into **7**, using AcOH (0.05 mL, 0.8743 mmol) and $NaBH_4$ (129.8 mg, 3.4311 mmol). The crude product was purified by column chromatography (dry-flash, silica gel, gradient prepared from DCM and mixture [MeOH/ $NH_3(aq)$ =9/1]). Yield 133.8 mg (44%), yellow oil. IR (ATR): 3267w, 3064w, 2928s, 2854m, 1611m, 1580s, 1540m, 1453m, 1368m, 1332m, 1304w, 1282w, 1250w, 1203w, 1138w, 1080w, 902w, 878w, 852w, 813w, 768w, 738w, 680w cm^{-1} . 1H NMR (400 MHz, $CDCl_3$, δ) 8.53 (d, $J = 5.4$ Hz, 1H, H-2), 7.95 (d, $J = 2.1$ Hz, 1H, H-8), 7.67 (d, $J = 9.0$ Hz, 1H, H-5), 7.35 (dd, $J_1 = 8.9$ Hz, $J_2 = 2.1$ Hz, 1H, H-6), 6.41 (d, $J = 5.4$ Hz, 1H, H-3), 5.04 (s, 1H, Ar-NH), 3.30 (q, $J = 7.1$ Hz, 2H, H-9), 2.57 (t, $J = 7.2$ Hz, 2H, H-16), 2.37 – 2.22 (m, 6H, H-17 and $2 \times -CH_2(Ad)$), 2.21 – 2.10 (m, 4H, $2 \times -CH(Ad)$ and $-CH_2(Ad)$), 1.80 – 1.30 (m, 18H, $3 \times -CH_2(Ad)$ and $6 \times -CH_2$, H-10-15). ^{13}C NMR (100 MHz, $CDCl_3$, δ) 152.10, 149.88, 149.18, 134.97, 128.91, 125.38, 120.99, 117.23, 99.20, 66.62, 61.66, 52.48, 51.11, 48.96, 43.42, 39.13, 39.08, 35.26, 32.50, 30.00, 29.56, 29.43, 29.02, 27.34, 27.22. ESI-HRMS (m/z): calc. for $[C_{28}H_{39}BrClN_3+H]^+$ 532.2089, found 532.2094.

1-Adamantaneacetic acid (18).⁸

1-Bromoadamantane **17** (5 g, 23.2407 mmol) was slowly added to 96% H_2SO_4 (20 mL) at 10 °C. Afterwards, 1,1-dichloroethene (17.6 mL, 221.1347 mmol) was added dropwise through the septum, over 1 h. Stirring was continued for another 3 h at the same temperature and another 3 h at 20 °C. The reaction mixture was poured into the ice/water mixture, transferred into a separatory funnel and extracted twice with diethyl ether. The combined organic layers were washed with aqueous NaOH (pH 9). Then, the separated aqueous layer was acidified to pH 3, during which a precipitate appeared, that was extracted twice with diethyl ether. Combined organic layers were washed with water, then brine and dried over anhydrous Na_2SO_4 . The product was isolated after the removal of the solvent under reduced pressure. Yield 4.0170 g (89%), white solid, $Mp = 123-130$ °C. IR (ATR): 2905s, 2848s, 2661m, 2191w, 2167w, 2106w, 1707s, 1645m, 1448m, 1404m, 1364w, 1346w, 1313m, 1269s, 1203w, 1145m, 1097w, 990w, 907m, 650w, 630w cm^{-1} . 1H NMR (500 MHz, $CDCl_3$, δ) 2.10 (s, 2H, $-CH_2COOH$), 2.02 – 1.95 (m, 3H, $3 \times -CH$), 1.74 – 1.61 (m, 12H, $6 \times -CH_2$). ^{13}C NMR (125 MHz, $CDCl_3$, δ) 178.57, 48.89, 42.43, 36.83, 32.84, 28.74.

1-Adamantaneacetamide (19).⁹

Into the ice-cold solution of **18** (1.5 g, 7.7212 mmol) in DCM (40 mL), in the presence of Et_3N (1.28 mL, 9.2341 mmol), $ClCOOEt$ (0.89 mL, 9.3076 mmol) was added slowly. After stirring at room temperature for 1 h 45 min, the mixture was evaporated to dryness and the residue was dissolved in DCM/THF = 3/1 (70.5 mL). Then, NH_3 (aq, 30%) (7.25 mL) was added dropwise and stirring of the mixture was continued for another 1 h. The solvents were

evaporated under reduced pressure, followed by the addition of water and EA. The aqueous layer was extracted once again with EA, and combined organic layers were washed with sat. NaHCO_3 , then with brine and dried over anh. Na_2SO_4 . The solvent was evaporated and the product was isolated after chromatographic purification (dry-flash, silica gel, gradient Hex /EA). Yield 1.13 g (76%), white solid, $\text{Mp} = 168\text{--}170\text{ }^\circ\text{C}$. IR (ATR): 3372s, 3190m, 2902s, 2849s, 2678w, 1665s, 1626s, 1447m, 1402m, 1363m, 1344w, 1313w, 1246w, 1207w, 1167w, 1130w, 1101w, 987w, 947w, 887w, 805w, 749w, 674w, 601w, 461w, 418w cm^{-1} . ^1H NMR (500 MHz, CDCl_3 , δ) 5.72 – 5.27 (m, 2H, $-\text{NH}_2$), 2.02 – 1.95 (m, 5H, $2\times -\text{CH}$ and $-\text{CH}_2\text{CONH}_2$), 1.75 – 1.62 (m, 12H, 6 $-\text{CH}_2$). ^{13}C NMR (125 MHz, CDCl_3 , δ) 173.64, 51.24, 42.70, 36.86, 32.69, 28.76. EI-GC/MS m/z (%): 193.1 (30) $[\text{M}]^+$, 135.1 (100) $[\text{M} - \text{CH}_2\text{CONH}_2]^+$, 93.1 (17), 79.1 (16).

3-Bromo-(1-adamantaneacetamide) (20).

A suspension of *t*-BuOH (7.5 mL, 78.9261 mmol) and LiBr (10.28 g, 118.3650 mmol) was cooled in an ice bath and 48% HBr (17.8 mL) was added in drops during 30 min. After stirring the mixture at room temperature for 3 h, the top organic layer was separated, washed with water and dried over anh. Na_2SO_4 . The product was isolated after solvent removal under reduced pressure to give 7.3142 g of *t*-BuBr (68%), which was used in the next step without prior characterization.

Into the mixture of CCl_4 (14.8 mL) and 96% H_2SO_4 (19.7 mL) at $10\text{ }^\circ\text{C}$, **19** (1.11 g, 5.7427 mmol) and *t*-BuBr (3.93 g, 28.6819 mmol) were added. After stirring at room temperature for 2 h, the reaction mixture was poured onto ice and diluted with DCM. The separated aqueous layer was extracted once again with DCM, combined organic layers were washed with water, then brine and dried over anh. Na_2SO_4 . The solvent was evaporated and the product was isolated after chromatographic purification (dry-flash, silica gel, gradient Hex /EA). Yield 1.2414 g (80%), brownish crystals, $\text{Mp} = 104\text{--}110\text{ }^\circ\text{C}$. IR (ATR): 3486m, 3369m, 3167s, 2909s, 2853m, 1675s, 1448m, 1395m, 1336m, 1298m, 1278m, 1186m, 1102m, 1010w, 976m, 933m, 894w, 813m, 711m, 674m, 604m, 524w, 482m cm^{-1} . ^1H NMR (500 MHz, CDCl_3 , δ) 5.58 (d, $J = 153.4\text{ Hz}$, 2H, $-\text{NH}_2$), 2.36 – 2.23 (m, 6H, $3\times -\text{CH}_2$), 2.21 – 2.15 (m, 2H, $2\times -\text{CH}$), 2.03 (s, 2H, $-\text{CH}_2\text{CONH}_2$), 1.72 – 1.61 (m, 6H, $3\times -\text{CH}_2$). ^{13}C NMR (125 MHz, CDCl_3 , δ) 172.73, 65.38, 53.86, 49.68, 48.47, 40.55, 37.42, 34.76, 32.47. EI-GC/MS m/z (%): 192.1 (100) $[\text{M} - \text{Br}]^+$, 133.1 (23), 91.0 (23).

3-(2-amino-2-oxoethyl)adamantane-1-carboxylic acid (21).

Into the 96% H_2SO_4 (22.2 mL), cooled to $0\text{ }^\circ\text{C}$, **20** (1.2 g, 4.4088 mmol) was added, followed by Hex (3.12 mL) and HCOOH (2.7 mL). The mixture was spontaneously warmed to room temperature and stirred for 22 h. The reaction was quenched by carefully pouring onto ice and the pH was adjusted to 3 using aqueous NaOH, during which a precipitate appeared. The product was isolated after filtration and dried under reduced pressure. Yield 1.04 g (99%), white solid, $\text{Mp} = 224\text{--}228\text{ }^\circ\text{C}$. IR (ATR): 3423s, 3340m, 3203m, 2926s, 2851m, 2513w, 2168w, 1940w, 1682s, 1645s, 1577m, 1450m, 1410m, 1361w, 1327m, 1276s, 1164m, 1026w, 999w, 744w, 703w, 672w, 633w, 610w, 516w, 469w, 420w cm^{-1} . ^1H NMR (500 MHz, CD_3OD , δ) 2.14 – 2.09 (m, 2H, $2\times -\text{CH}$), 2.04 (s, 2H, $-\text{CH}_2\text{CONH}_2$), 1.92 – 1.79 (m, 6H, $3\times -\text{CH}_2$), 1.74 – 1.62 (m, 6H, $3\times -\text{CH}_2$). ^{13}C NMR (125 MHz, CD_3OD , δ) 181.26, 176.51, 50.70, 44.86, 42.62, 42.47, 39.40, 36.77, 33.79, 29.93. UPLC-MS m/z (%): 473.71 (100) $[2\text{M} - \text{H}]^-$, 236.41 (50) $[\text{M} - \text{H}]^-$.

2-(3-(Hydroxymethyl)adamantan-1-yl)acetamide (22).

A solution of **21** (1.025 g, 4.3194 mmol) in DCM (23.5 mL), with Et₃N (0.72 mL, 5.1942 mmol) was cooled in an ice bath and ClCOOEt (0.5 mL, 5.2290 mmol) was added in drops. After stirring at room temperature for 90 min, the mixture was evaporated to dryness and the residue was dissolved in DCM/THF = 3/1 (42.1 mL), followed by the addition of NaBH₄ (653.6 mg, 17.2773 mmol) and MeOH (12.7 mL) in small portions. After 20 h, the reaction was quenched by pouring into ice/dilute HCl mixture and layers were separated. The aqueous layer was neutralized to pH 7, using aqueous NaHCO₃, extracted three times with EA and the combined organic layers were washed with brine. The product was isolated after the solvent was evaporated under reduced pressure. Yield 434.1 mg (45%), white crystals, Mp = 161-163 °C. IR (ATR): 3749w, 3305s, 3181s, 2904s, 2847s, 1666s, 1626s, 1541w, 1513w, 1453m, 1404m, 1363m, 1339m, 1315w, 1199w, 1165m, 1063m, 1036m, 608m cm⁻¹. ¹H NMR (400 MHz, CD₃OD, δ) 3.13 (s, 2H, -CH₂OH), 2.09 – 2.01 (m, 2H, 2× -CH), 1.98 (s, 2H, -CH₂CONH₂), 1.70 – 1.33 (m, 12H, 6× -CH₂). ¹³C NMR (125 MHz, CD₃OD, δ) 176.82, 73.60, 51.02, 45.30, 43.29, 39.53, 37.45, 36.54, 33.95, 30.18. EI-GC/MS *m/z* (%): 223.1 (10) [M]⁺, 192.1 (100) [M - CH₂OH]⁺, 133.1 (27), 91.1 (28).

2-(3-(((8-((7-Chloroquinolin-4-yl)amino)octyl)amino)methyl)adamantan-1-yl)acetamide (8).

Following the above-described general procedure, **22** (434.1 mg, 1.9438 mmol) reacted with PCC (628.6 mg, 2.9161 mmol) in dry DCM (27.7 mL). The crude product was purified by column chromatography (dry-flash, silica gel, gradient DCM/MeOH) affording 140.6 mg of **23** (33%), which was used in the next step. Aldehyde **23** (140.6 mg, 0.6353 mmol) and amine **9** (233 mg, 0.7624 mmol) in DCM/MeOH = 1/2 (15 mL), were transformed into **8**, using AcOH (0.073 mL, 1.2764 mmol) and NaBH₄ (144.2 mg, 3.8118 mmol). The crude product was purified by column chromatography (dry-flash chromatography, silica gel, gradient DCM/MeOH, then multiple preparative thin-layer chromatography, silica gel, developed 2 times with MeOH). Yield 46 mg (14%), yellowish oil. IR (ATR): 3337m, 2927s, 2852m, 1667m, 1612m, 1580s, 1541w, 1452m, 1369w, 1333w, 1282w, 1138w, 1081w, 904w, 879w, 853w, 808w, 738w, 647w cm⁻¹. ¹H NMR (500 MHz, CDCl₃, δ) 8.53 (d, *J* = 5.4 Hz, 1H, H-2), 7.95 (d, *J* = 2.2 Hz, 1H, H-8), 7.70 (d, *J* = 8.9 Hz, 1H, H-5), 7.36 (dd, *J*₁ = 8.8 Hz, *J*₂ = 2.2 Hz, 1H, H-6), 6.41 (d, *J* = 5.4 Hz, 1H, H-3), 5.62 (bs, 2H, -NH₂), 5.15 – 5.06 (m, 1H, Ar-NH), 3.30 (q, *J* = 7.1 Hz, 2H, H-9), 2.58 (t, *J* = 7.4 Hz, 2H, H-16), 2.30 (s, 2H, H-17), 2.09 – 2.03 (m, 2H, 2× -CH(Ad)), 2.00 (s, 2H, -CH₂CONH₂), 1.75 (quint, *J* = 7.3 Hz, 2H, H-10), 1.63 – 1.41 (m, 16H, 6× -CH₂(Ad) and 2× -CH₂, H-11 and H-15), 1.41 – 1.29 (m, 6H, 3× -CH₂, H-(12-14)). ¹³C NMR (125 MHz, CDCl₃, δ) 173.48, 152.20, 149.89, 149.28, 134.93, 128.94, 125.35, 121.10, 117.27, 99.19, 62.39, 51.06, 50.82, 45.69, 43.38, 42.23, 40.20, 36.36, 34.26, 33.26, 29.52, 29.47, 29.34, 28.95, 28.85, 27.28, 27.16. ESI-HRMS (*m/z*): calc. for [C₃₀H₄₃ClN₄O+H]⁺ 511.3198, found 511.3197.

2. LIST OF INTERACTIONS BETWEEN THE TESTED COMPOUNDS AND AChE

2.1. *Binding site of AChE*

The first is the catalytic anionic site (CAS) of the AChE, which is at the bottom of the gorge and composed of a catalytic triad, oxyanion hole, and a choline-binding site. A peripheral anionic site (PAS) is located at the entrance and consists of Tyr72, Tyr124, Asp74, Tyr341, and Trp286.¹⁰

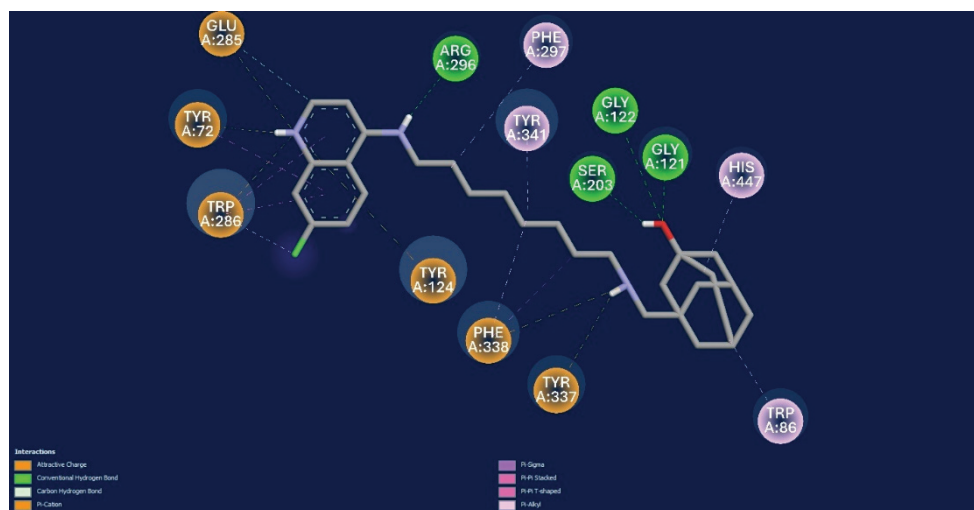
The binding site for docking was determined as the centre of the following residues: Tyr72, Trp86, Tyr124, Tyr133, Ser203, Trp236, Trp286, Phe297, Phe297, Glu334, Tyr337, Phe338, Tyr341, His447, and Tyr449.

Abbreviations for types of interaction

ES – electrostatic; H-Bond – hydrogen bond; Hpho – hydrophobic; Attractive Ch – attractive charge; H₂O H-Bond – water hydrogen bond; Conv. H-Bond – conventional hydrogen bond; π – π -orbitals; HD – H-Donor; HA – H-Acceptor; + – positive; – – negative

TABLE S-I. Scores for docked ligands.

Comp.	6	7	8
IFDScore (kJ mol ⁻¹)	-923.44	-919.54	-919.05

Fig. S1. Map of interactions of compound **6** with AChE.TABLE S-II. Ligand interactions of **6** with AChE.

TYPE	Category	Type	FROM	TO
6:N3 - GLU285:OE1	ES	Attractive Charge	6:N3, +	GLU285:OE1, -
GLY121:HN - 6:O1	H-Bond	Conv. H-Bond	GLY121:HN, HD	6:O1, HA
GLY122:HN - 6:O1	H-Bond	Conv. H-Bond	GLY122:HN, HD	6:O1, HA
6:H3 - ARG296:O	H-Bond	Conv. H-Bond	6:H3, HD	ARG296:O, HA
6:H35 - SER203:OG	H-Bond	Conv. H-Bond	6:H35, HD	SER203:OG, HA
6:C26 - GLU285:OE1	H-Bond	Carbon H-Bond	6:C26, HD	GLU285:OE1, HA
6:N2 - PHE338	ES	π -Cation	6:N2, +	PHE338, π
6:N3 - TYR124	ES	π -Cation	6:N3, +	TYR124, π
6:N3 - TRP286	ES	π -Cation	6:N3, +	TRP286, π
6:N3 - TRP286	ES	π -Cation	6:N3, +	TRP286, π
6:H20 - TYR337	H-Bond; ES	π -Cation; π -Donor H-Bond	6:H20, +;HD	TYR337, π ; π
6:H42 - TYR72	H-Bond; ES	π -Cation; π -Donor H-Bond	6:H42, +;HD	TYR72, π ; π
6:C12 - PHE338	Hpho.	π -Sigma	6:C12, C-H	PHE338, π
TRP286 - 6	Hpho.	π - π Stacked	TRP286, π	6, π
TRP286 - 6	Hpho.	π - π Stacked	TRP286, π	6, π
TRP286 - 6	Hpho.	π - π Stacked	TRP286, π	6, π
TRP286 - 6	Hpho.	π - π Stacked	TRP286, π	6, π
TYR72 - 6	Hpho.	π - π T-shaped	TYR72, π	6, π
TRP86 - 6	Hpho.	π -Alkyl	TRP86, π	6, Alkyl
TRP86 - 6	Hpho.	π -Alkyl	TRP86, π	6, Alkyl
TRP286 - 6:CL1	Hpho.	π -Alkyl	TRP286, π	6:CL1, Alkyl
TRP286 - 6:CL1	Hpho.	π -Alkyl	TRP286, π	6:CL1, Alkyl
PHE297 - 6	Hpho.	π -Alkyl	PHE297, π	6, Alkyl
PHE338 - 6	Hpho.	π -Alkyl	PHE338, π	6, Alkyl
TYR341 - 6	Hpho.	π -Alkyl	TYR341, π	6, Alkyl
HIS447 - 6	Hpho.	π -Alkyl	HIS447, π	6, Alkyl

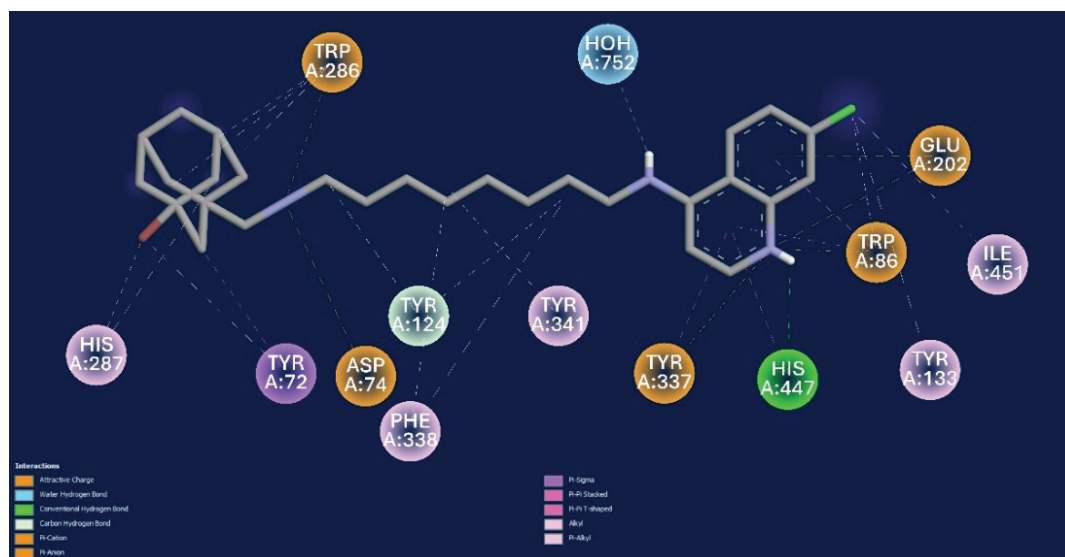
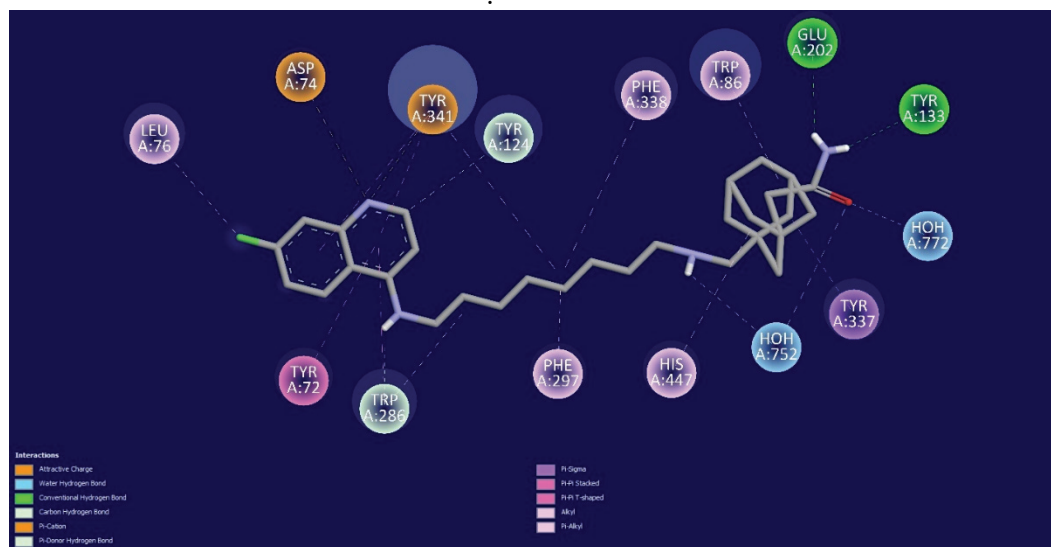
Fig. S2. Map of interactions of compound **7** with AChE.

TABLE S-III. Interactions of compound **7** with AChE.

TYPE	Category	Type	FROM	TO
7:N2 - ASP74:OD2	ES	Attractive Ch.	7:N2, +	ASP74:OD2, –
7:N3 - GLU202:OE2	ES	Attractive Ch.	7:N3, +	GLU202:OE2, –
7:H3 - HOH752:OH2	H-Bond	H ₂ O H-Bond; Conv. H-Bond	7:H3, HD	HOH752:OH2, HA
7:H41 - HIS447:O	H-Bond	Conv. H-Bond	7:H41, HD	HIS447:O, HA
7:C13 - TYR124:OH	H-Bond	Carbon H-Bond	7:C13, HD	TYR124:OH, HA
7:N2 - TRP286	ES	π -Cation	7:N2, +	TRP286, π
7:N2 - TRP286	ES	π -Cation	7:N2, +	TRP286, π
7:N3 - TRP86	ES	π -Cation	7:N3, +	TRP86, π
7:N3 - TRP86	ES	π -Cation	7:N3, +	TRP86, π
7:N3 - TYR337	ES	π -Cation	7:N3, +	TYR337, π
GLU202:OE1 - 7	ES	π -Anion	GLU202:OE1, -	7, π
7:C24 - TYR72	Hpho	π -Sigma	7:C24, C-H	TYR72, π
TRP86 - 7	Hpho	π - π Stacked	TRP86, π	7, π
TRP86 - 7	Hpho	π - π Stacked	TRP86, π	7, π
TRP86 - 7	Hpho	π - π Stacked	TRP86, π	7, π
TRP86 - 7	Hpho	π - π Stacked	TRP86, π	7, π
TYR337 - 7	Hpho	π - π T-shaped	TYR337, π	7, π
HIS447 - 7	Hpho	π - π T-shaped	HIS447, π	7, π
7:CL1 - ILE451	Hpho	Alkyl	7:CL1, Alkyl	ILE451, Alkyl
TYR72 - 7:BR1	Hpho	π -Alkyl	TYR72, π	7:BR1, Alkyl
TRP86 - 7:CL1	Hpho	π -Alkyl	TRP86, π	7:CL1, Alkyl
TYR124 - 7	Hpho	π -Alkyl	TYR124, π	7, Alkyl
TYR133 - 7:CL1	Hpho	π -Alkyl	TYR133, π	7:CL1, Alkyl
TRP286 - 7	Hpho	π -Alkyl	TRP286, π	7, Alkyl
TRP286 - 7	Hpho	π -Alkyl	TRP286, π	7, Alkyl
TRP286 - 7:C23	Hpho	π -Alkyl	TRP286, π	7:C23, Alkyl
TRP286 - 7	Hpho	π -Alkyl	TRP286, π	7, Alkyl
HIS287 - 7:C23	Hpho	π -Alkyl	HIS287, π	7:C23, Alkyl
HIS287 - 7:BR1	Hpho	π -Alkyl	HIS287, π	7:BR1, Alkyl
PHE338 - 7	Hpho	π -Alkyl	PHE338, π	7, Alkyl
PHE338 - 7	Hpho	π -Alkyl	PHE338, π	7, Alkyl
TYR341 - 7	Hpho	π -Alkyl	TYR341, π	7, Alkyl

Fig. S3. Map of interactions of compound **8** with AChE.TABLE S-IV. Ligand interactions of **8** with AChE.

TYPE	Category	Type	FROM	TO
8:N4 - ASP74:OD1	ES	Attractive Charge	8:N4, +	ASP74:OD1, -
HOH752:H1 - 8:O1	H-Bond	H ₂ O H-Bond; Conv. H-Bond	HOH752:H1, HD	8:O1, HA
HOH772:H1 - 8:O1	H-Bond	H ₂ O H-Bond; Conv. H-Bond	HOH772:H1, HD	8:O1, HA
8:H20 - HOH752:OH2	H-Bond	H ₂ O H-Bond; Conv. H-Bond	8:H20, HD	HOH752:OH2, HA
8:H37 - GLU202:OE1	H-Bond	Conv. H-Bond	8:H37, HD	GLU202:OE1, HA
8:H38 - TYR133:OH	H-Bond	Conv. H-Bond	8:H38, HD	TYR133:OH, HA
8:C28 - TYR124:OH	H-Bond	Carbon H-Bond	8:C28, HD	TYR124:OH, HA
8:N4 - TYR341	ES	π -Cation	8:N4, +	TYR341, π -Orbitals
8:H3 - TRP286	H-Bond	π -Donor H-Bond	8:H3, HD	TRP286, π -Orbitals
8:C20 - TYR337	Hpho.	π -Sigma	8:C20, C-H	TYR337, π -Orbitals
TYR341 - 8	Hpho.	π - π Stacked	TYR341, π -Orbitals	8, π -Orbitals
TYR341 - 8	Hpho.	π - π Stacked	TYR341, π -Orbitals	8, π -Orbitals
TYR72 - 8	Hpho.	π - π T-shaped	TYR72, π -Orbitals	8, π -Orbitals
TRP286 - 8	Hpho.	π - π T-shaped	TRP286, π -Orbitals	8, π -Orbitals
TRP286 - 8	Hpho.	π - π T-shaped	TRP286, π -Orbitals	8, π -Orbitals
8:CL1 - LEU76	Hpho.	Alkyl	8:CL1, Alkyl	LEU76, Alkyl
TRP86 - 8	Hpho.	π -Alkyl	TRP86, π -Orbitals	8, Alkyl
TRP86 - 8	Hpho.	π -Alkyl	TRP86, π -Orbitals	8, Alkyl
TRP286 - 8	Hpho.	π -Alkyl	TRP286, π -Orbitals	8, Alkyl
PHE297 - 8	Hpho.	π -Alkyl	PHE297, π -Orbitals	8, Alkyl
PHE338 - 8	Hpho.	π -Alkyl	PHE338, π -Orbitals	8, Alkyl
TYR341 - 8	Hpho.	π -Alkyl	TYR341, π -Orbitals	8, Alkyl
HIS447 - 8	Hpho.	π -Alkyl	HIS447, π -Orbitals	8, Alkyl

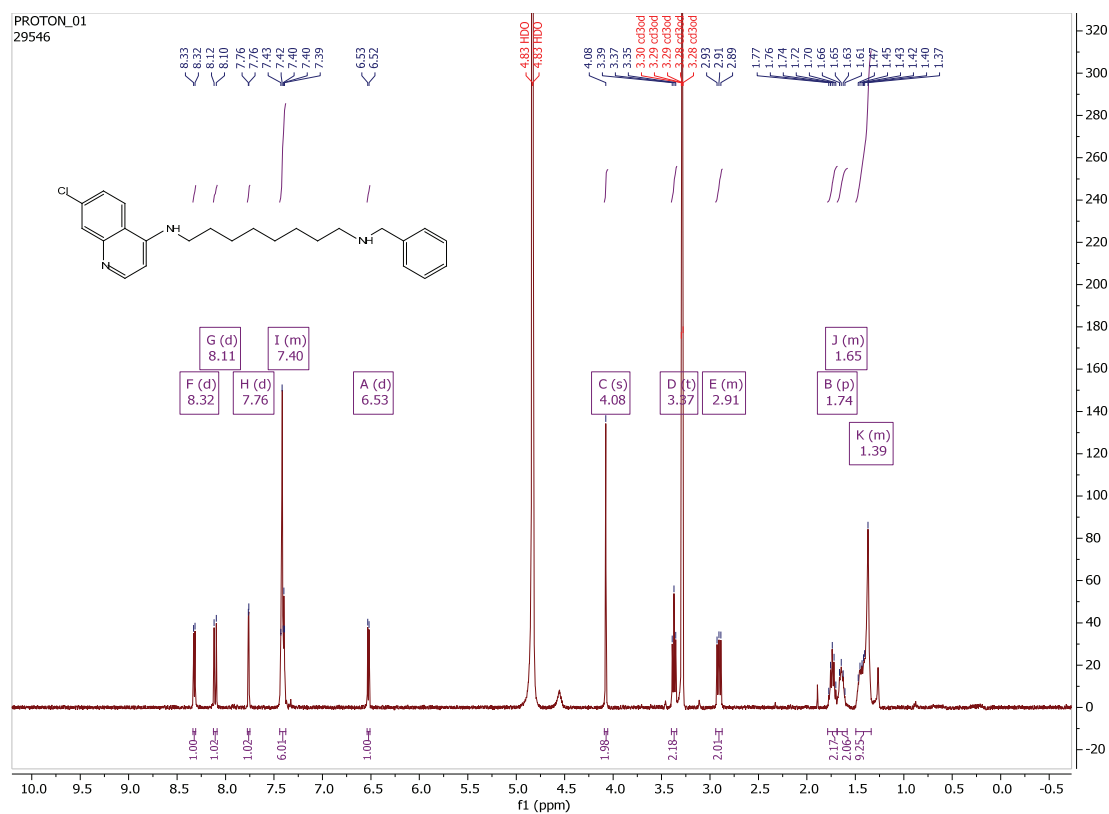
4. *IN SILICO* CALCULATIONS

TABLE S-V. In silico physicochemical properties of 4-AQ hybrids with adamantane.

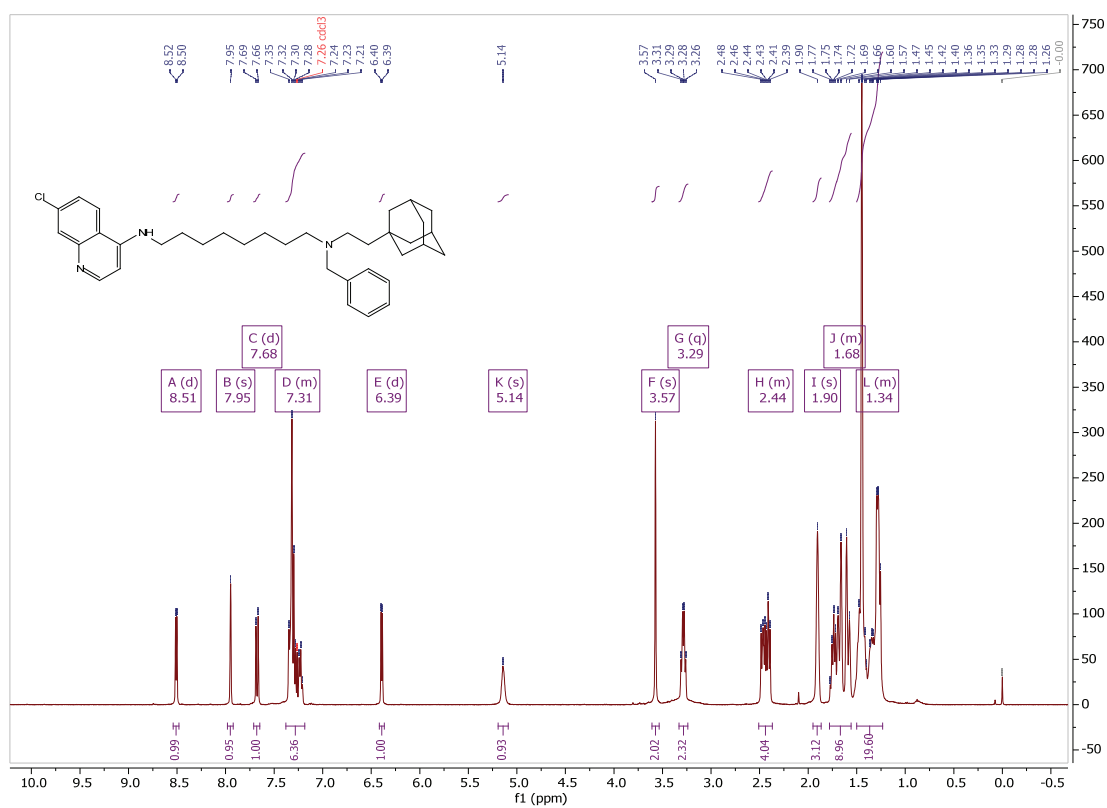
Comp.	MW (g/mol)	RB	HBA	HBD	TPSA (Å ²)	log <i>P</i>	p <i>K</i> _{a1} ^a	p <i>K</i> _{a2} ^a	log <i>D</i> ^b	CNS MPO ^c
1	454.09	12	3	2	36.95	6.625	7.71	11.46	3.00	2.2
2	395.98	12	3	2	36.95	5.923	7.70	9.65	3.28	2.6
3	468.12	13	3	2	36.95	6.913	7.71	11.38	3.30	1.9
4	544.21	14	3	1	28.16	8.732	7.71	10.24	5.54	1.2
5	558.24	15	3	1	28.16	9.021	7.70	9.65	6.36	1.4
6	470.09	12	4	3	57.18	5.31	7.71	11.15	1.74	2.4
7	532.99	12	3	2	36.95	6.85	7.71	11.45	3.23	1.7
8	511.14	14	4	3	80.04	5.242	7.71	11.45	1.62	2.2
Tacrine	198.26	0	2	1	38.91	2.628	9.35	-	1.05	5.1

^a p*K*_{a1} belongs to the quinoline ring, p*K*_{a2} belongs to the terminal basic amino group; ^b log *D* value at pH = 7.4; ^c CNS

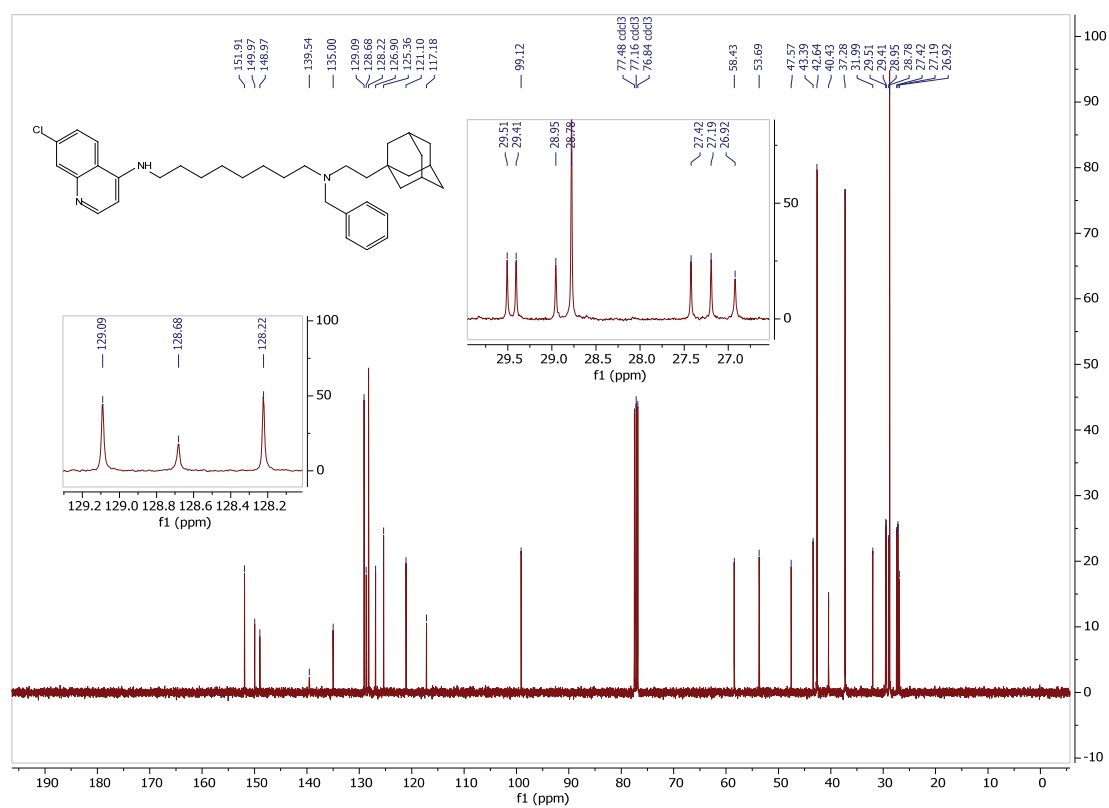
MPO data were generated following instruction published in reference T. T. Wager, X. Hou, P. R. Verhoest, A. Villalobos, *ACS Chem. Neurosci.*, **1** (2010) 435. <https://doi.org/10.1021/cn100008c>;

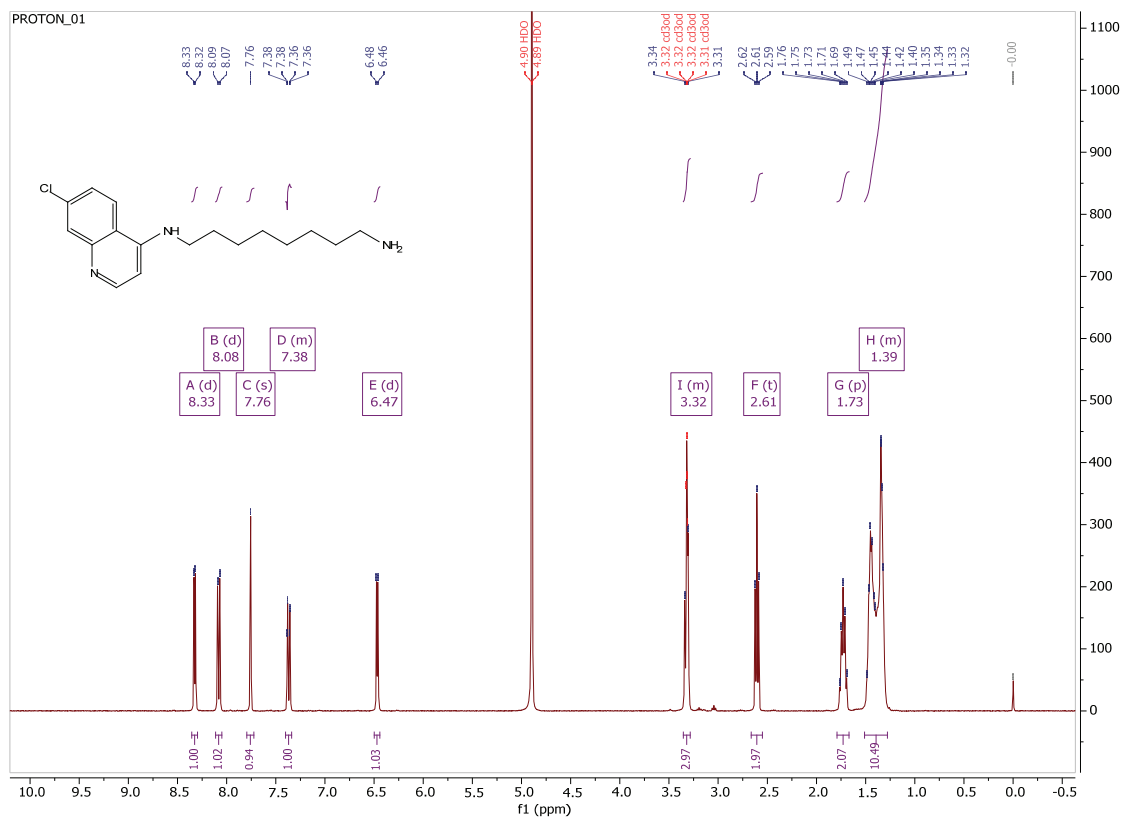
4. ^1H AND ^{13}C NMR SPECTRA ^1H NMR spectrum of N^1 -benzyl- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (2)

¹H NMR spectrum of *N*¹-(2-adamantan-1-yl)ethyl)-*N*¹-benzyl-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (5)

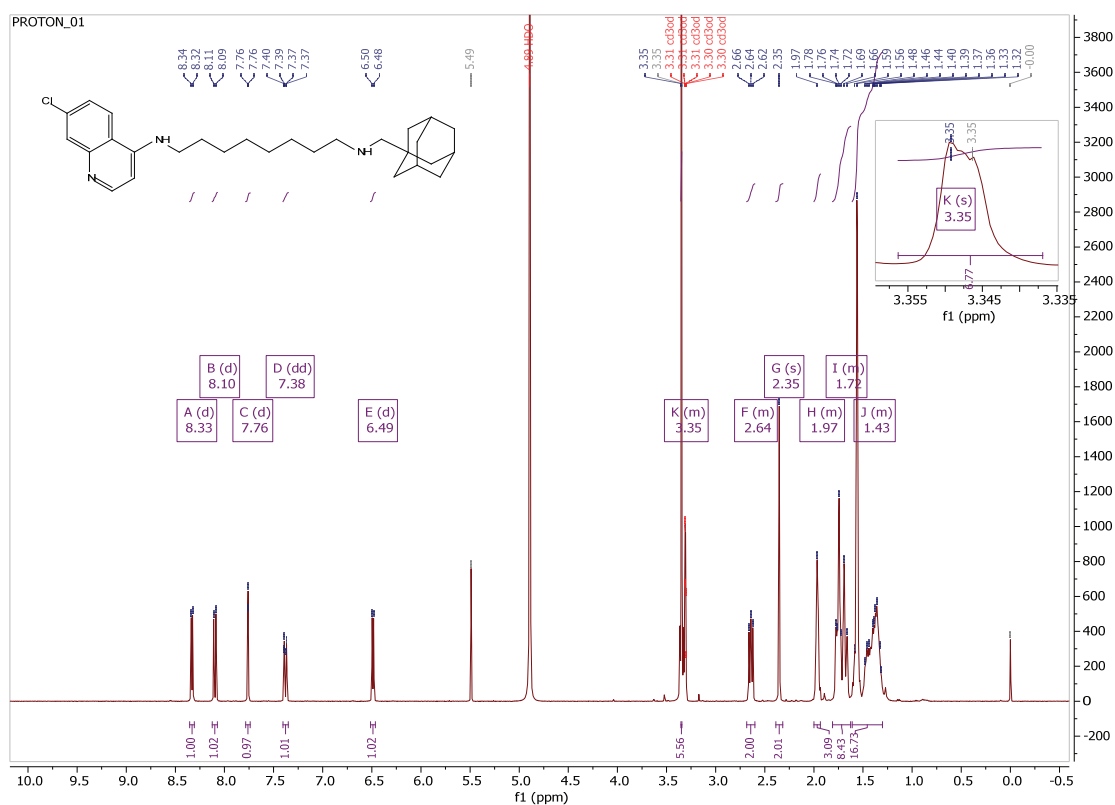


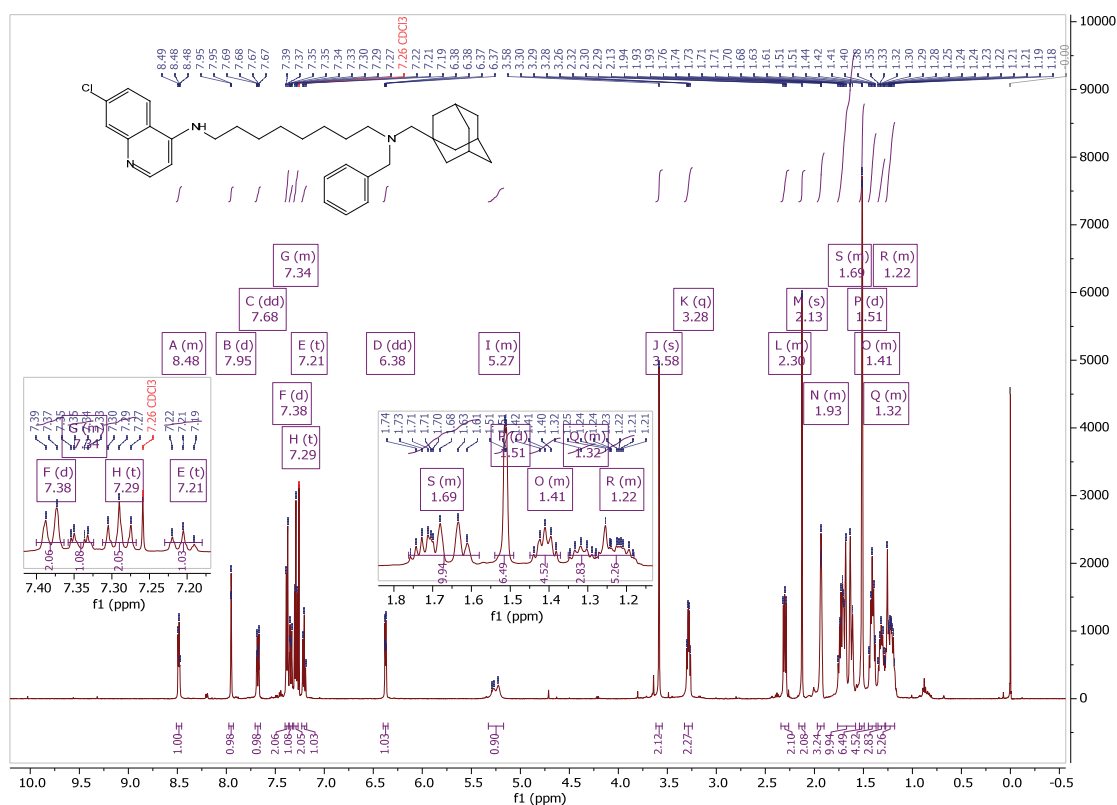
^{13}C NMR spectrum of N^1 -(2-(adamantan-1-yl)ethyl)- N^1 -benzyl- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (5)



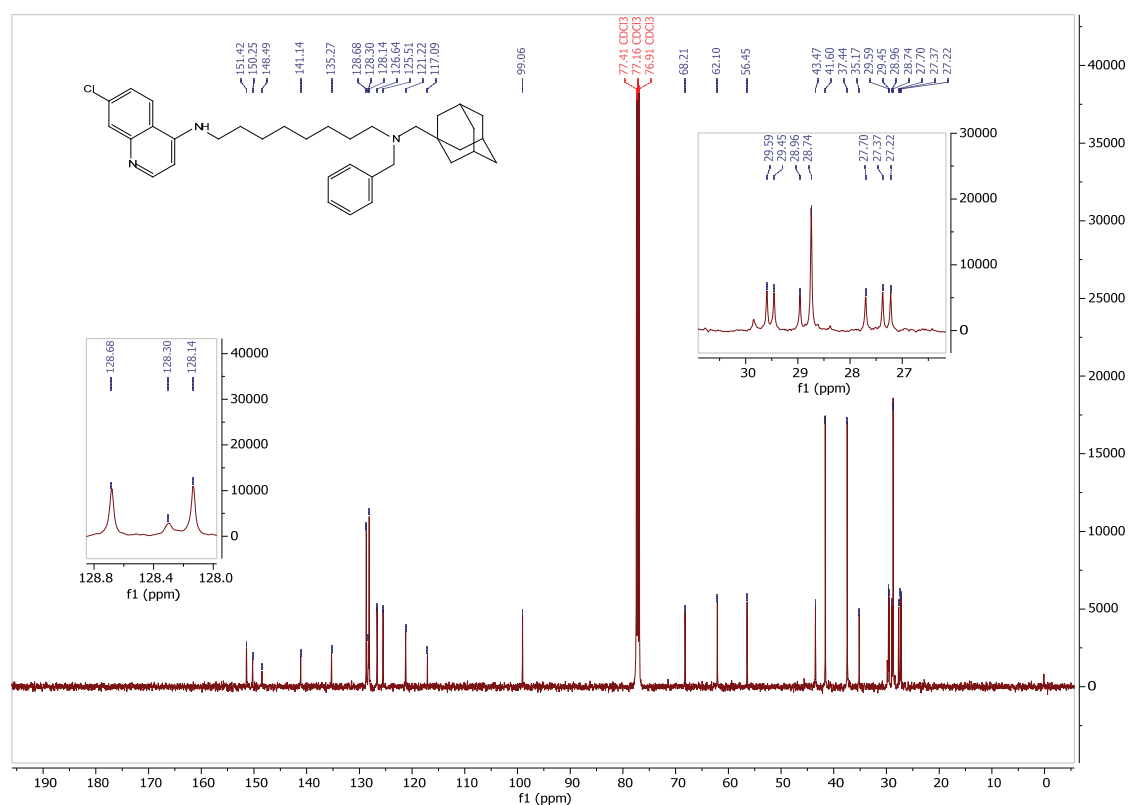
^1H NMR spectrum of *N*-(7-chloroquinolin-4-yl)octane-1,8-diamine (9)

¹H NMR spectrum of *N*¹-((adamantan-1-yl)methyl)-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (1)

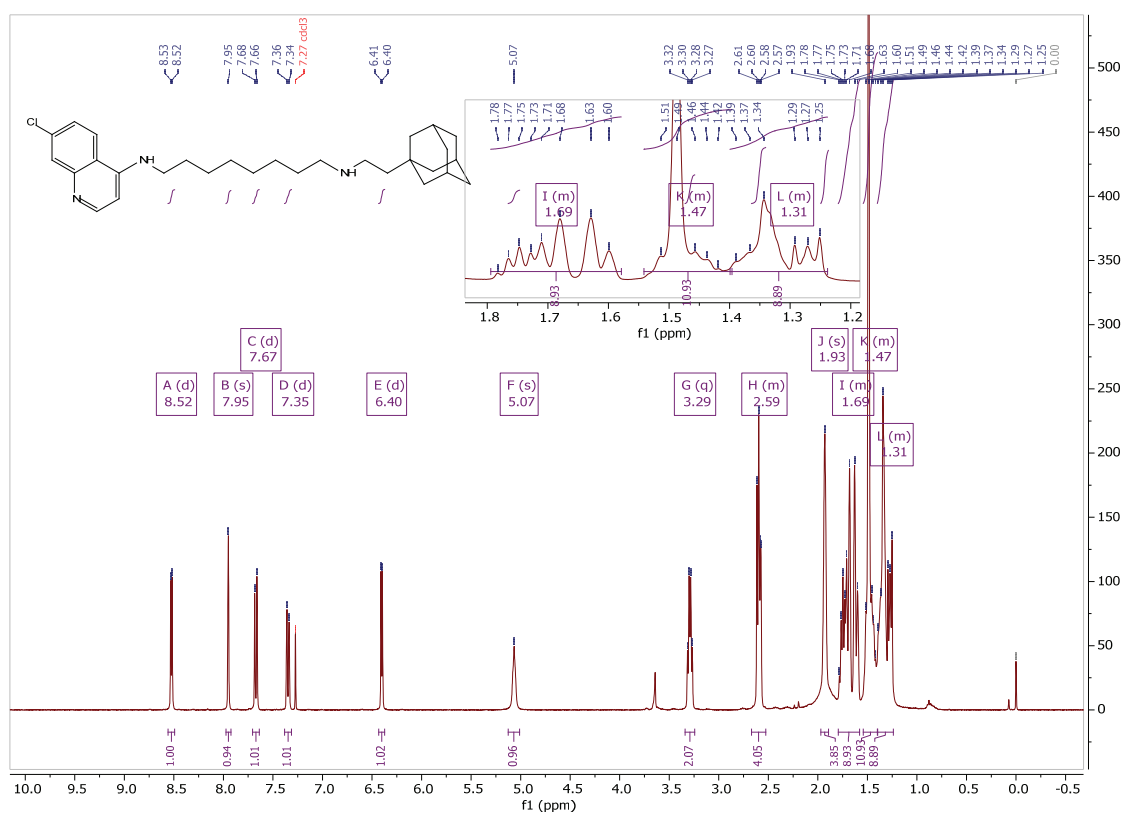


¹H NMR spectrum of *N*'-((adamantan-1-yl)methyl)-*N*-benzyl-*N*⁸-(7-chloroquinolin-4-yl)octane-1,8-diamine (4)

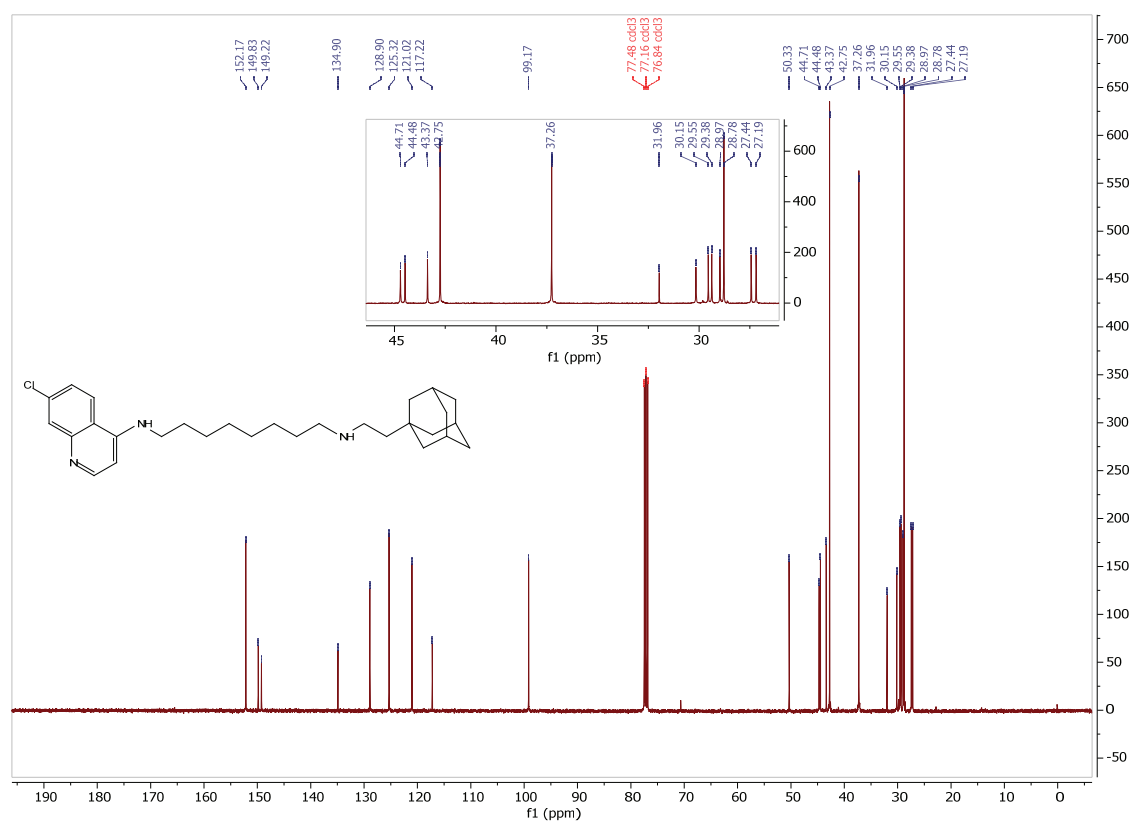
^{13}C NMR spectrum of N^1 -((adamantan-1-yl)methyl)- N^1 -benzyl- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (4)

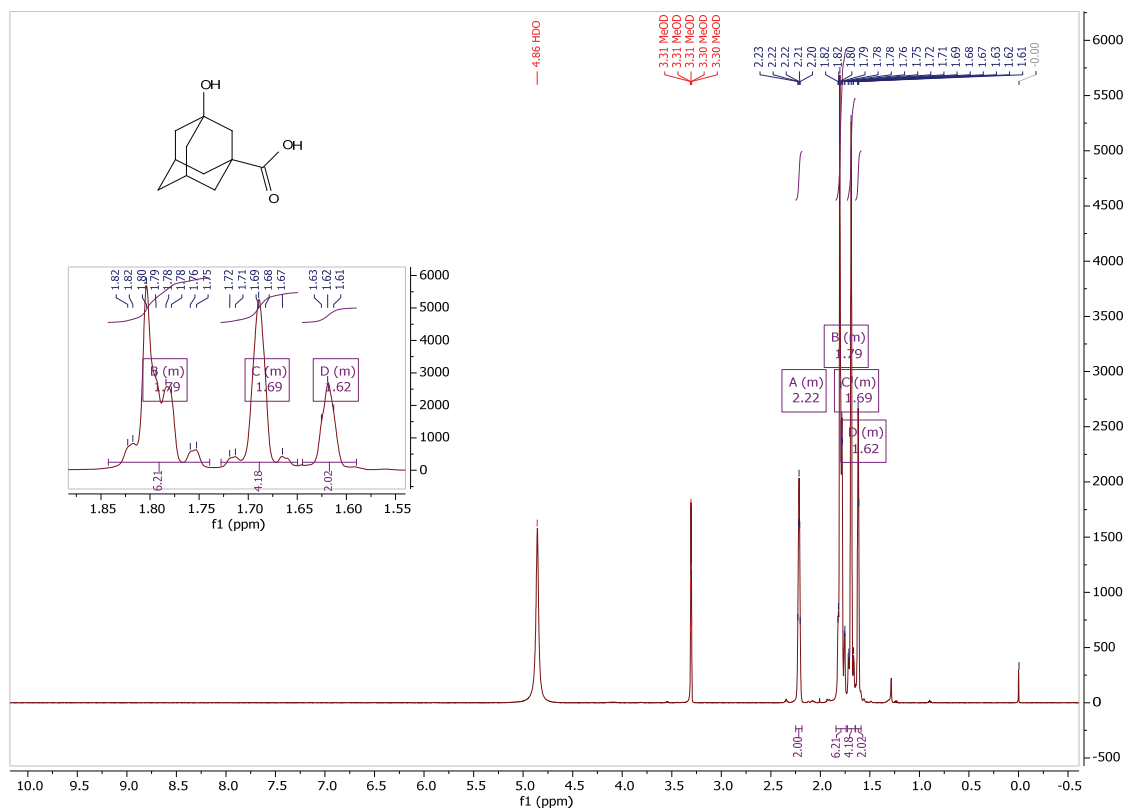


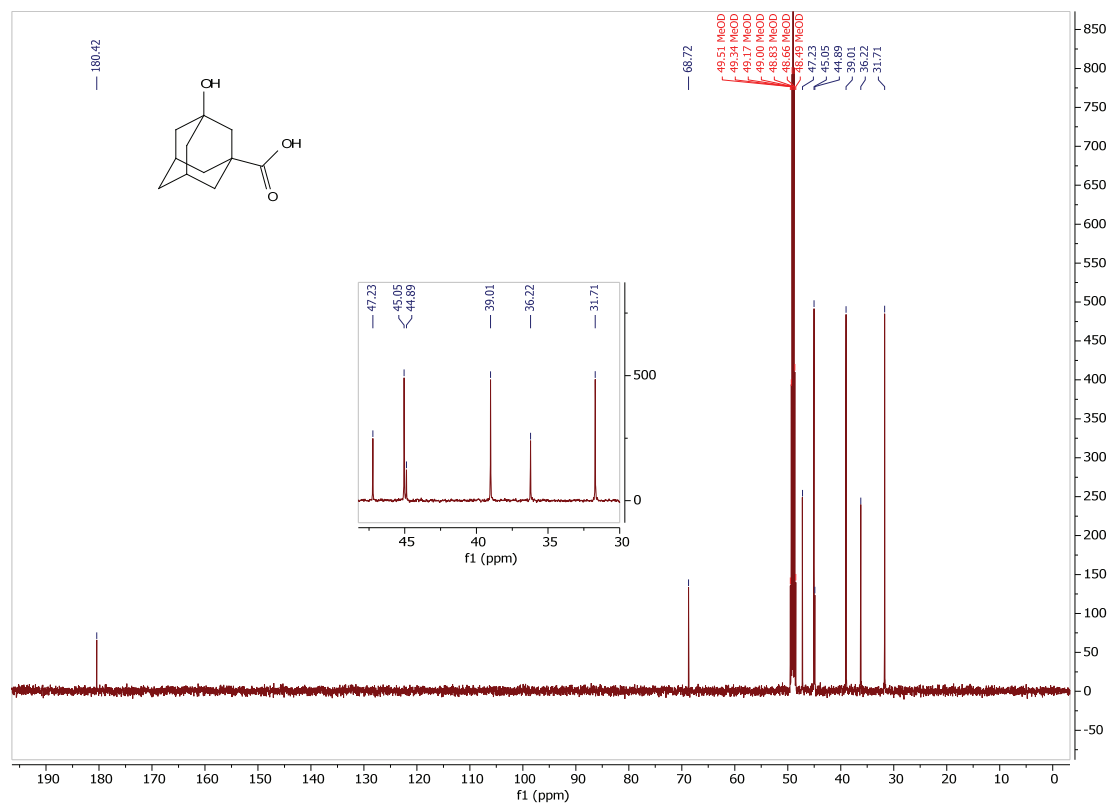
^1H NMR spectrum of N^1 -(2-(adamantan-1-yl)ethyl)- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (3)

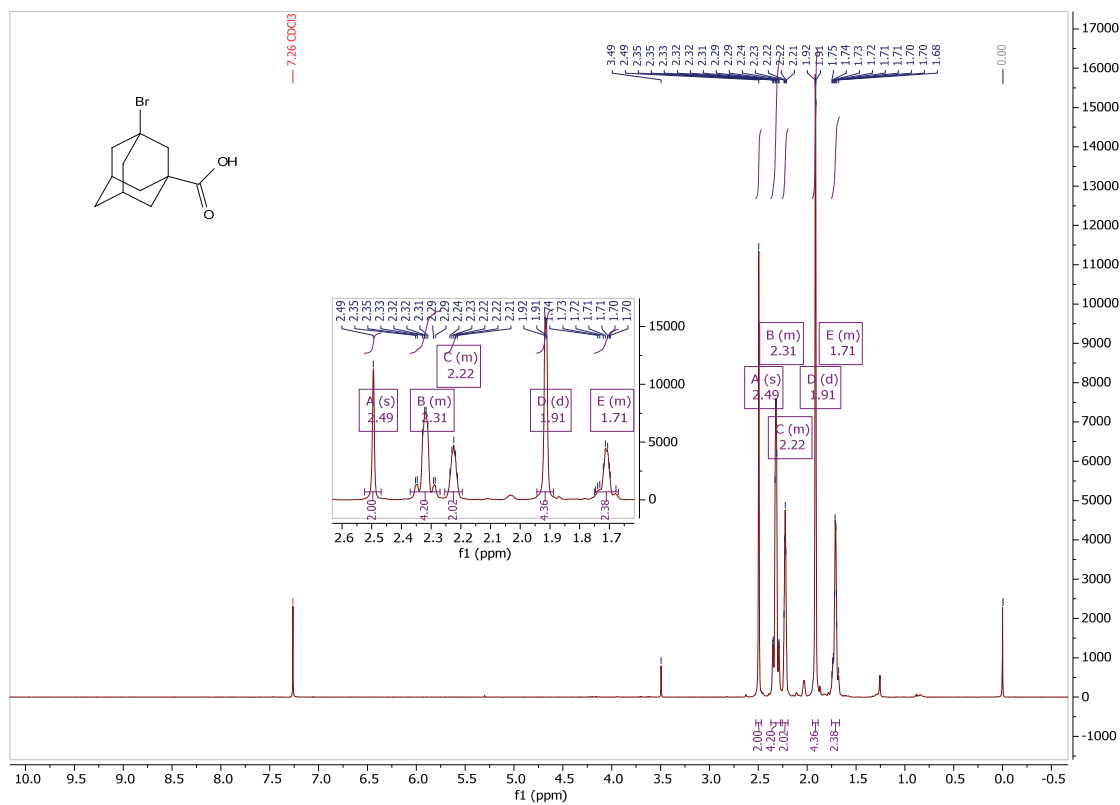


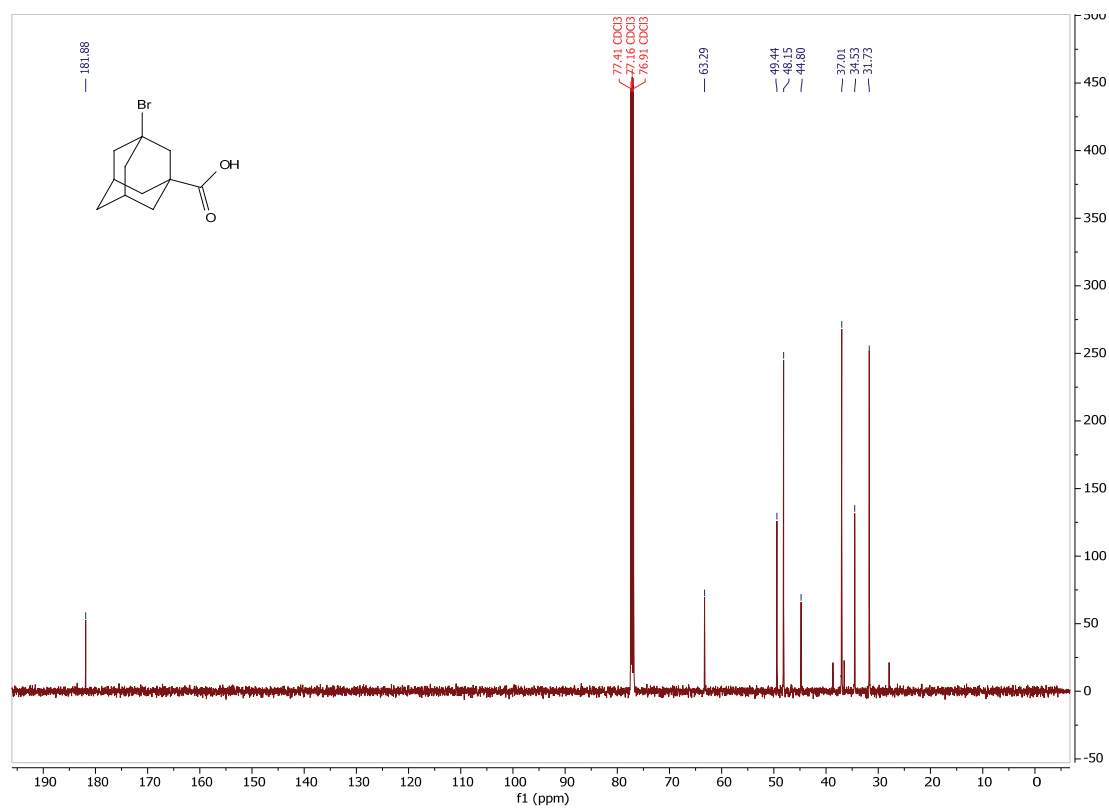
^{13}C NMR spectrum of N^1 -(2-(adamantan-1-yl)ethyl)- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (3)

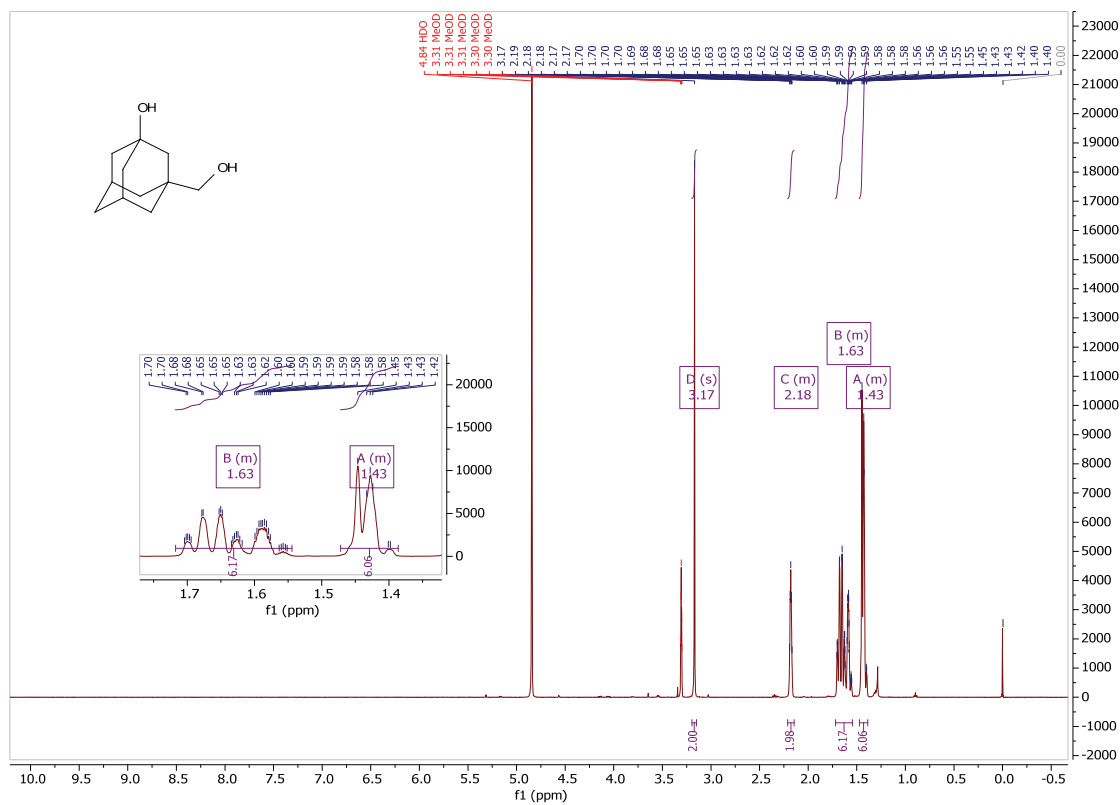


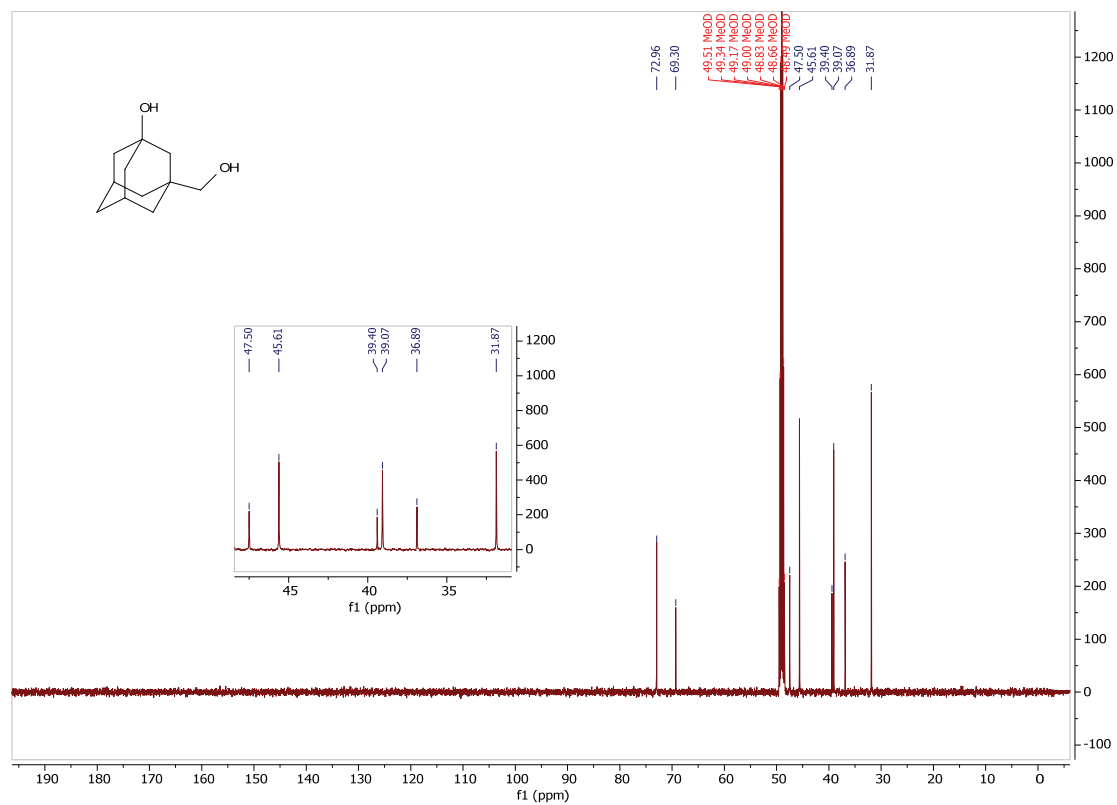
¹H NMR spectrum of 3-hydroxyadamantane-1-carboxylic acid (11)

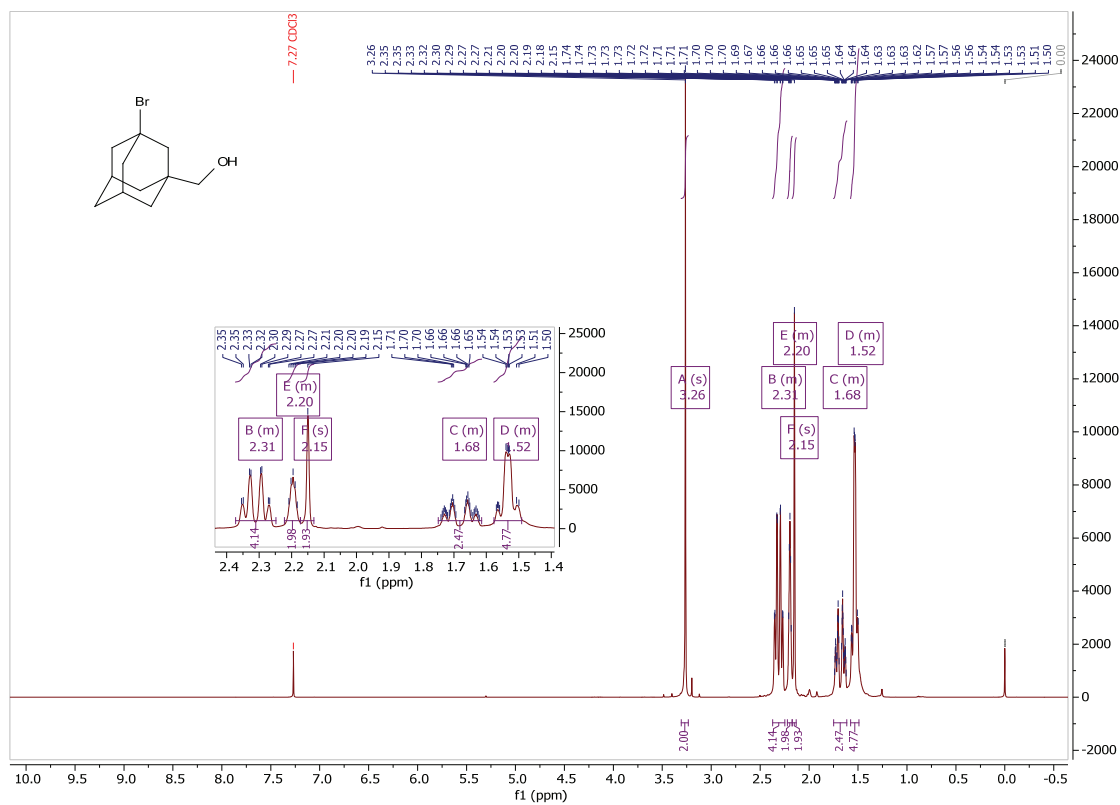
^{13}C NMR spectrum of 3-hydroxyadamantane-1-carboxylic acid (11)

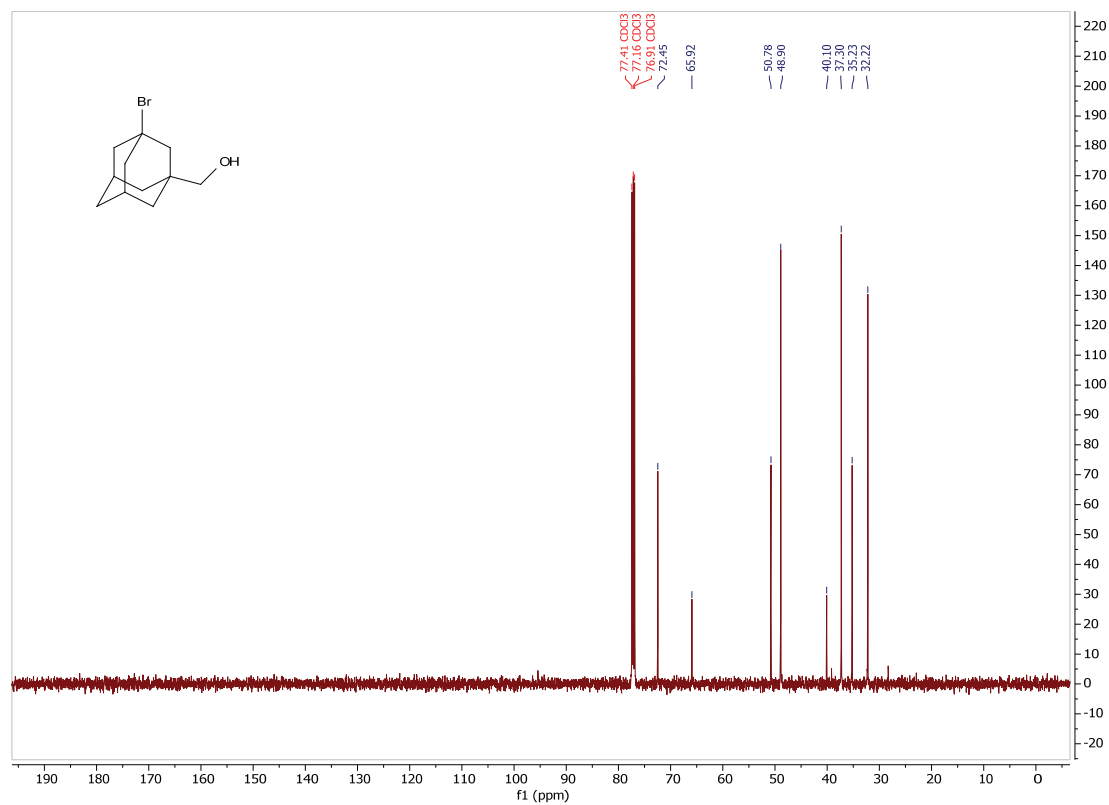
¹H NMR spectrum of 3-bromoadamantane-1-carboxylic acid (12)

^{13}C NMR spectrum of 3-bromoadamantane-1-carboxylic acid (12)

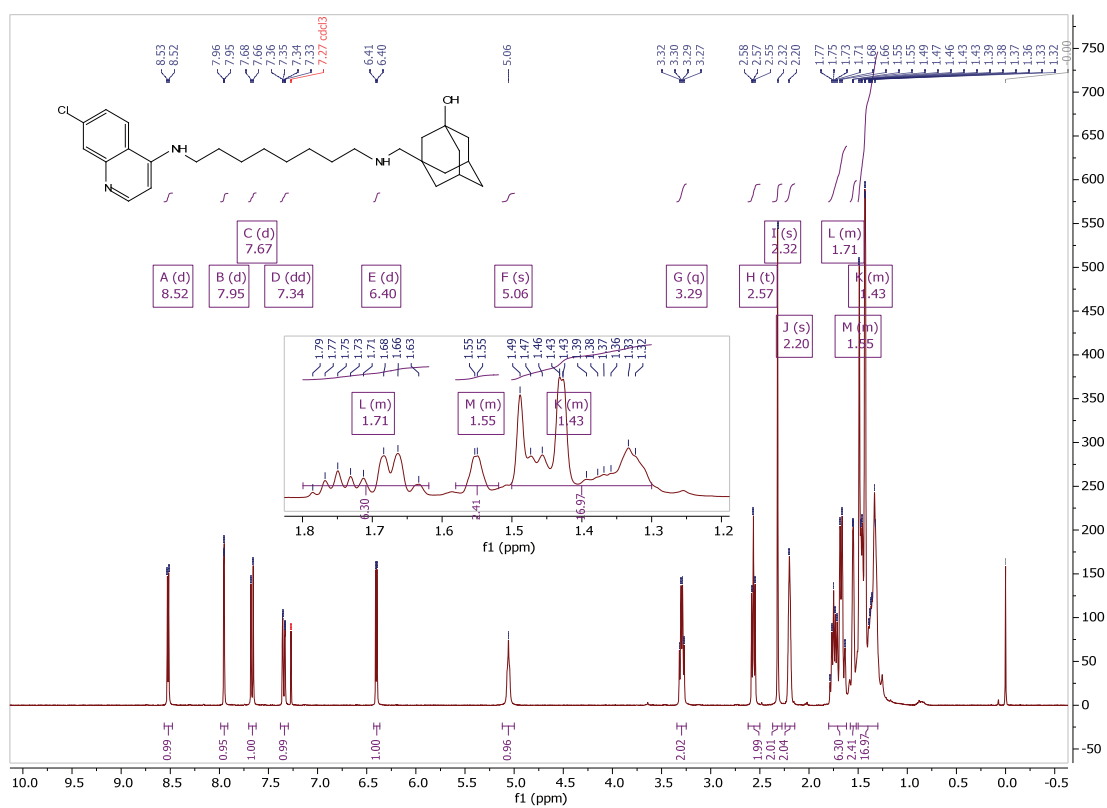
¹H NMR spectrum of 3-(hydroxymethyl)adamantan-1-ol (13)

¹³C NMR spectrum of 3-(hydroxymethyl)adamantan-1-ol (13)

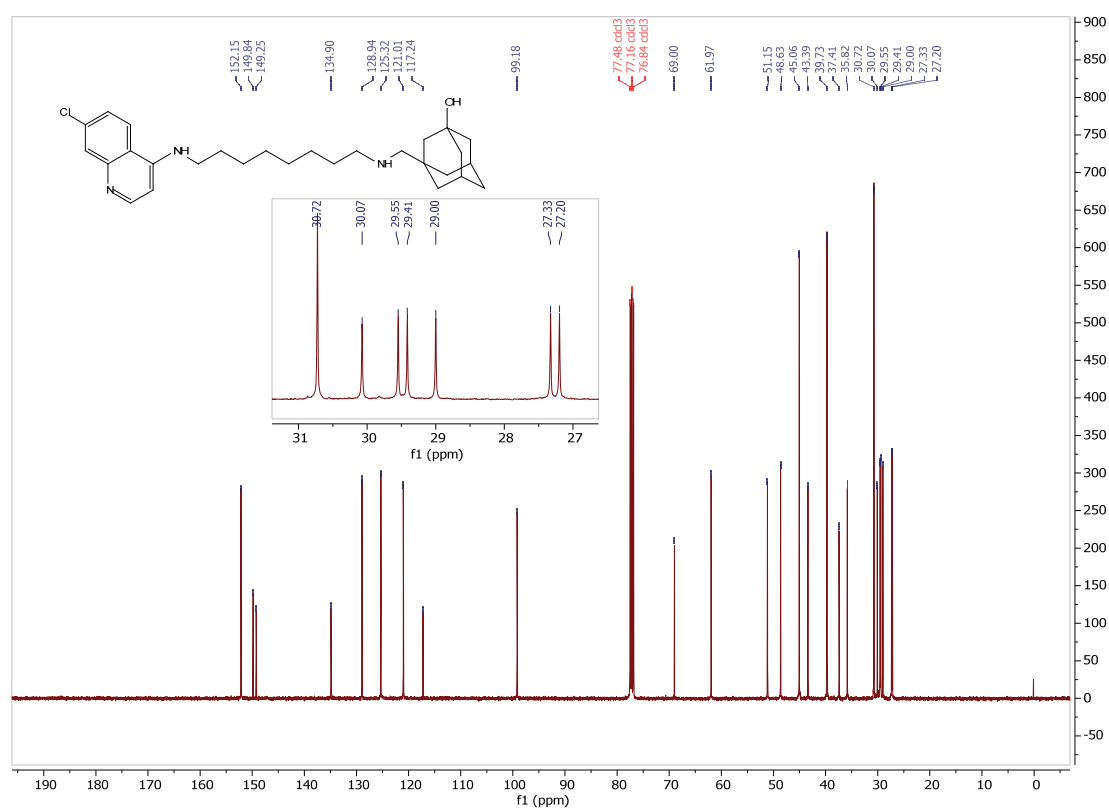
¹H NMR spectrum of (3-bromoadamantan-1-yl)methanol (14)

¹³C NMR spectrum of (3-bromoadamantan-1-yl)methanol (14)

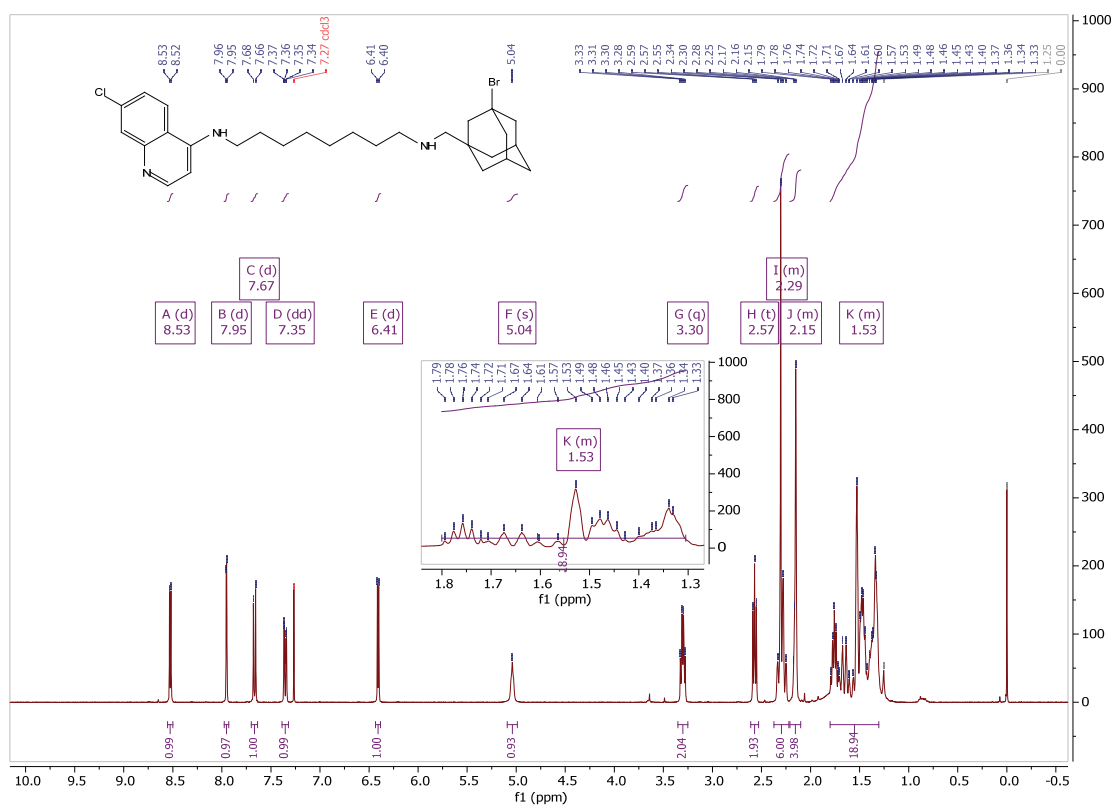
^1H NMR spectrum of 3-(((8-((7-chloroquinolin-4-yl)amino)octyl)amino)methyl)adamantan-1-ol (6)



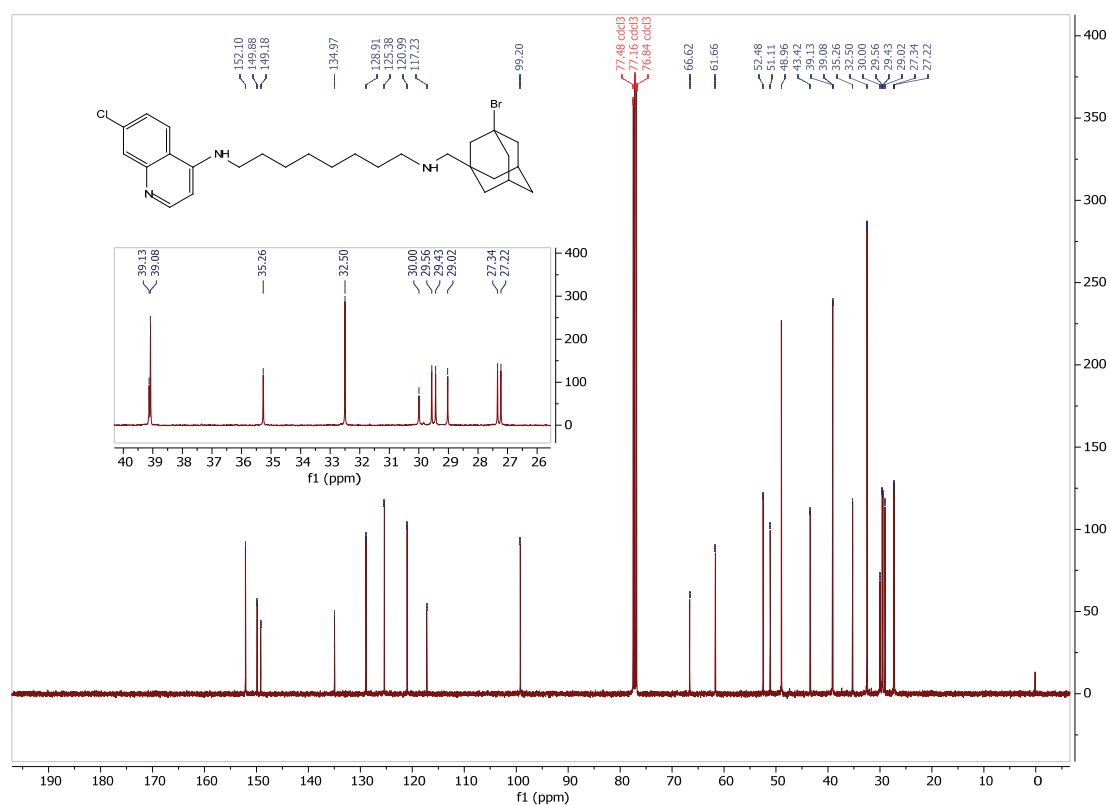
^{13}C NMR spectrum of 3-(((8-((7-chloroquinolin-4-yl)amino)octyl)amino)methyl)adamantan-1-ol (6)

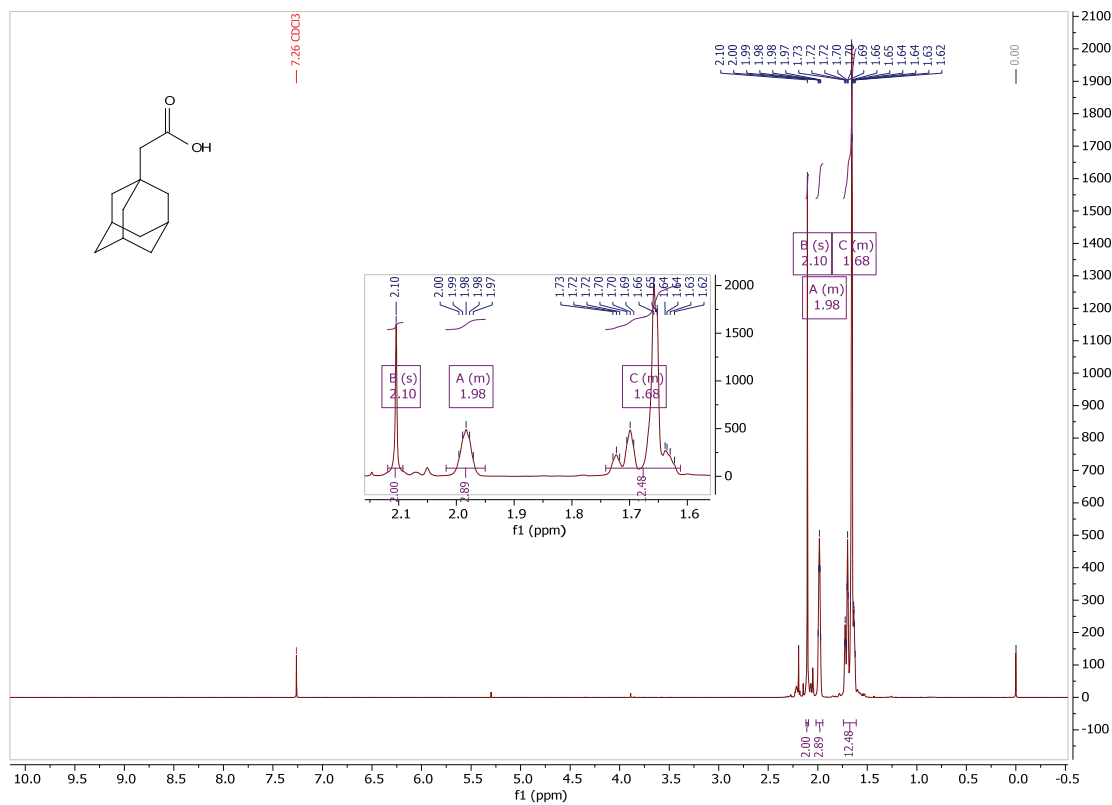


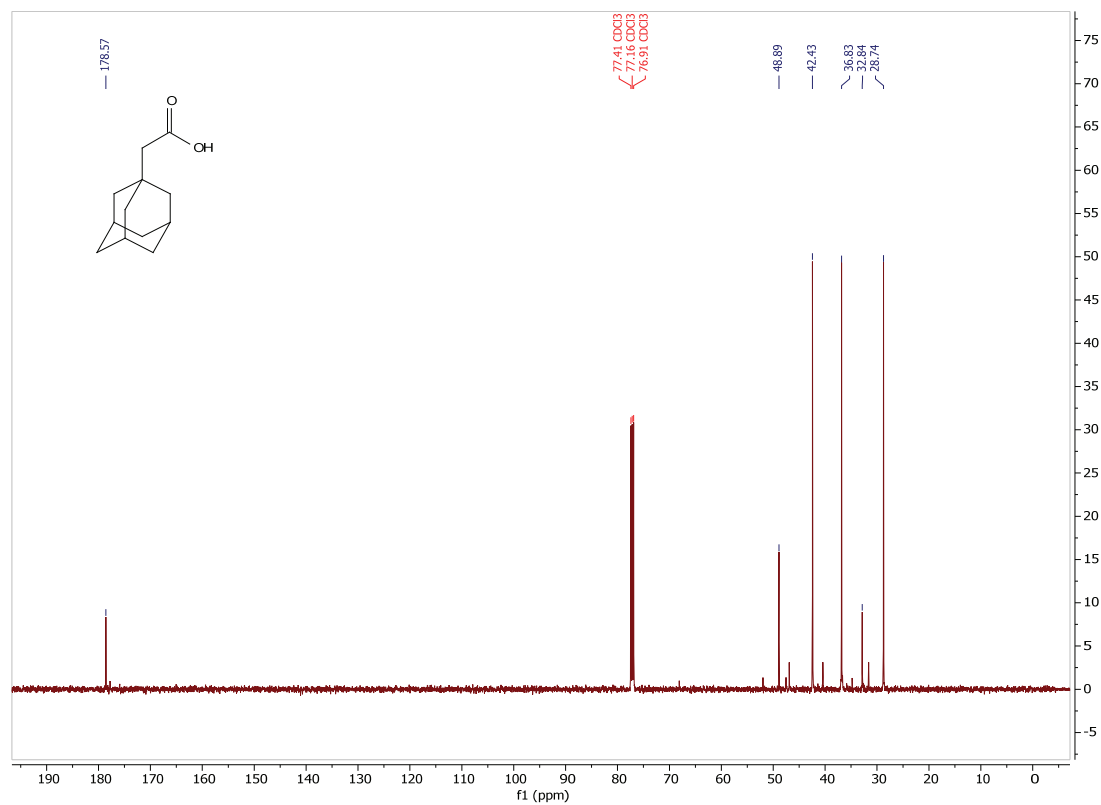
^1H NMR spectrum of N^1 -((3-bromoadamantan-1-yl)methyl)- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (7)

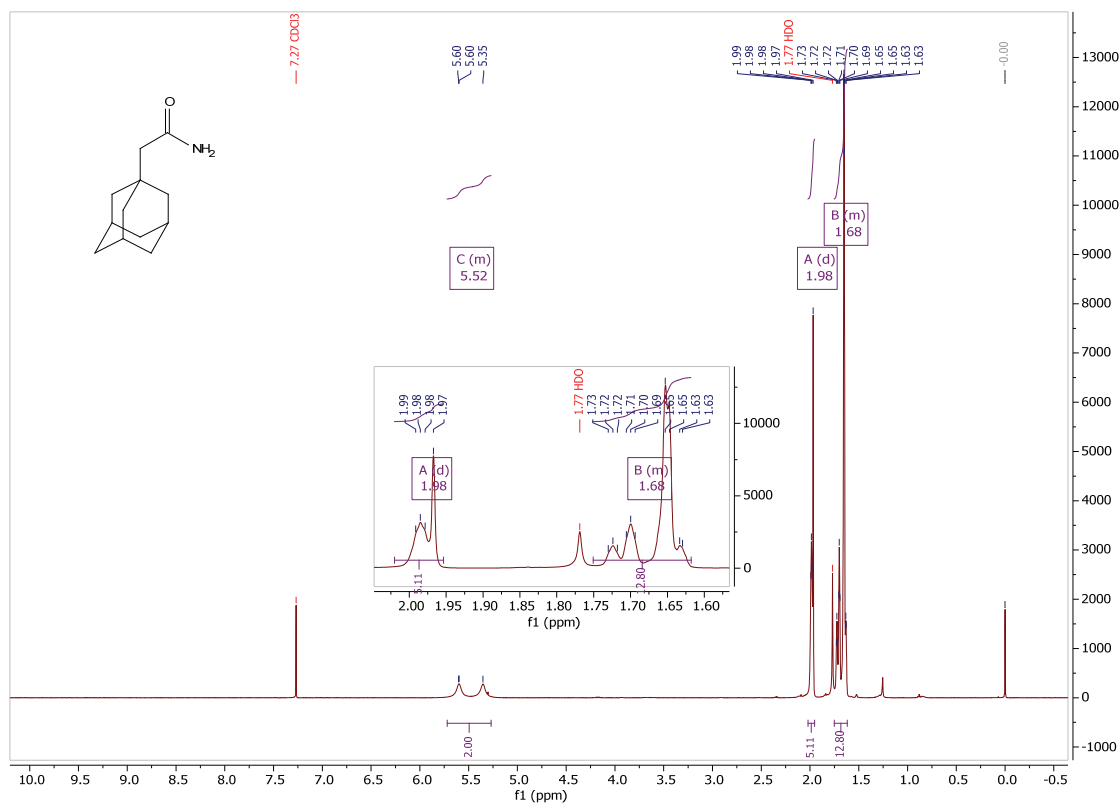


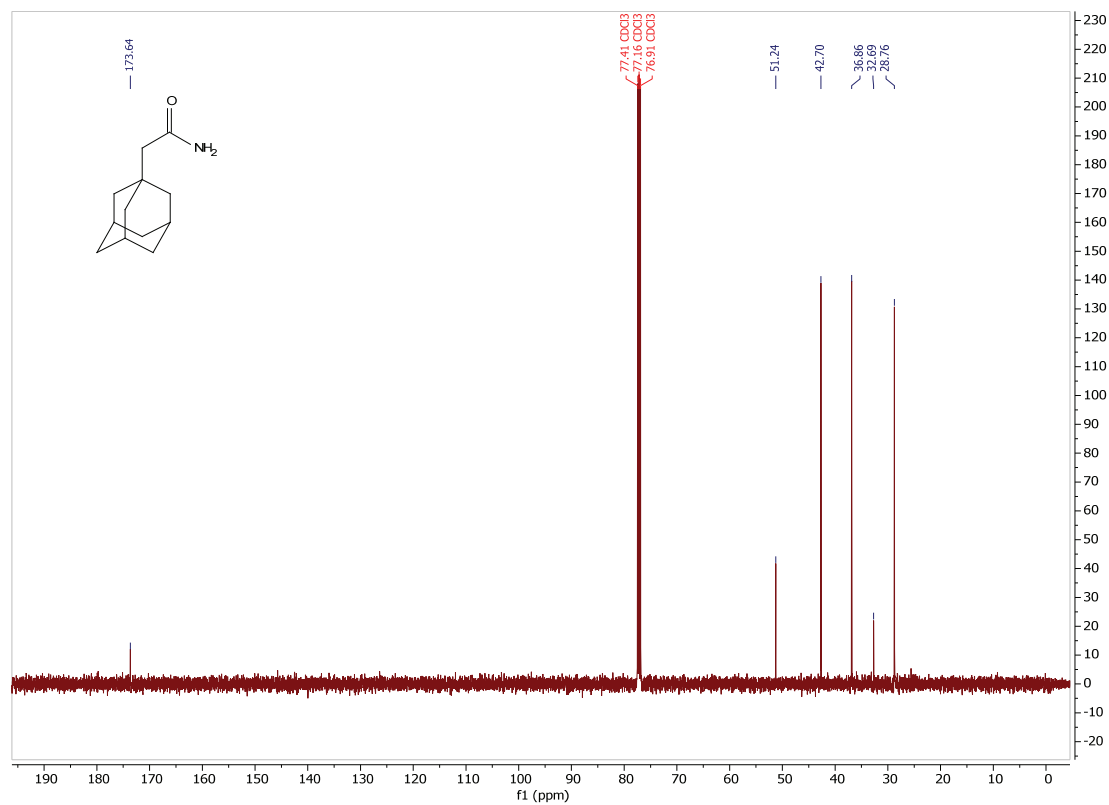
^{13}C NMR spectrum of N^1 -((3-bromoadamantan-1-yl)methyl)- N^8 -(7-chloroquinolin-4-yl)octane-1,8-diamine (7)

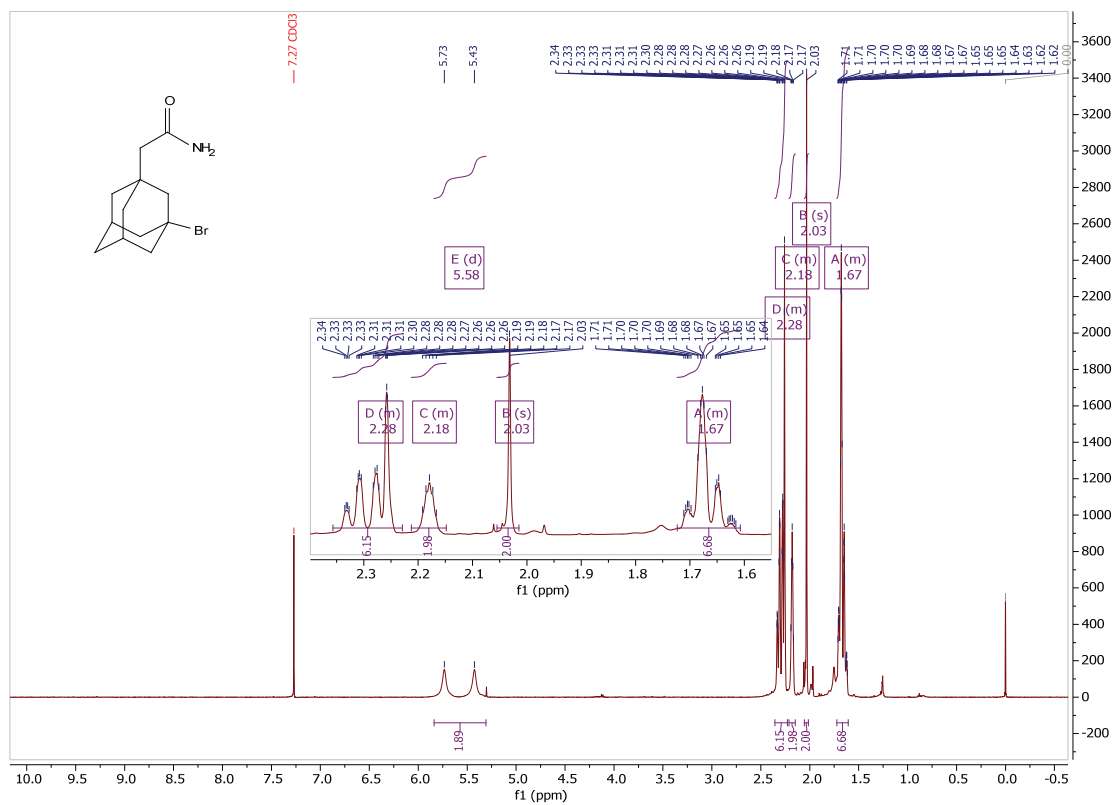


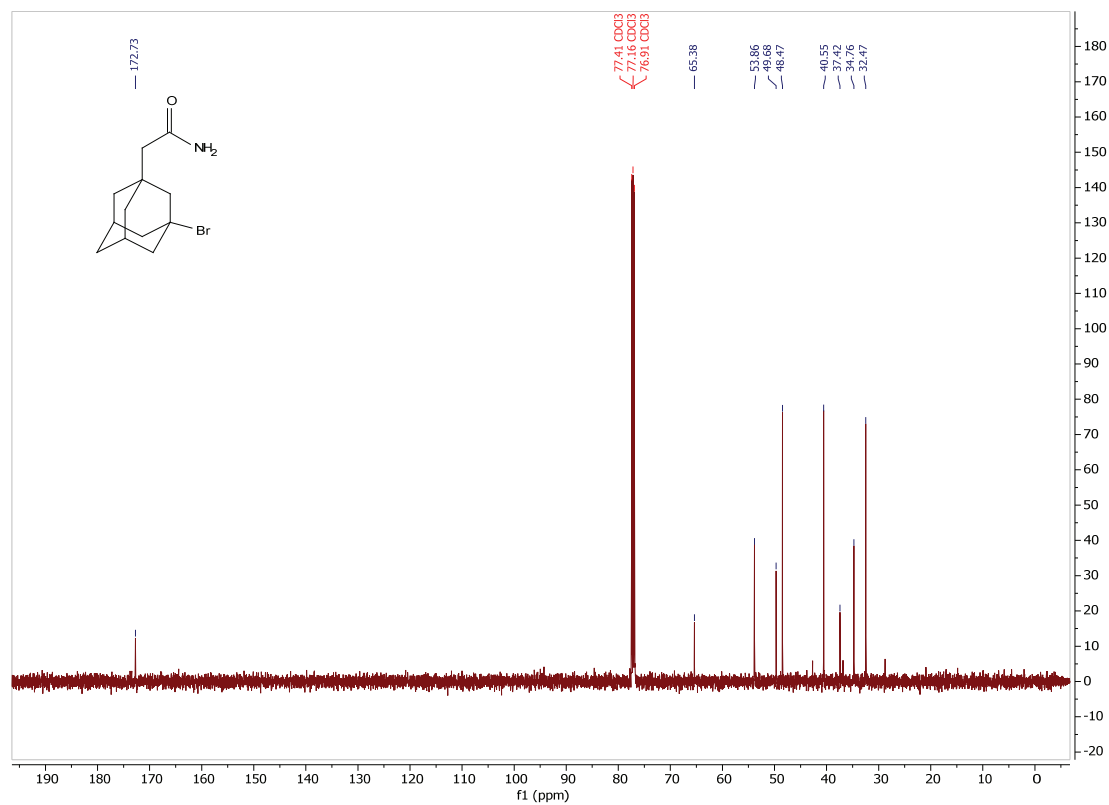
¹H NMR spectrum of 1-adamantaneacetic acid (18)

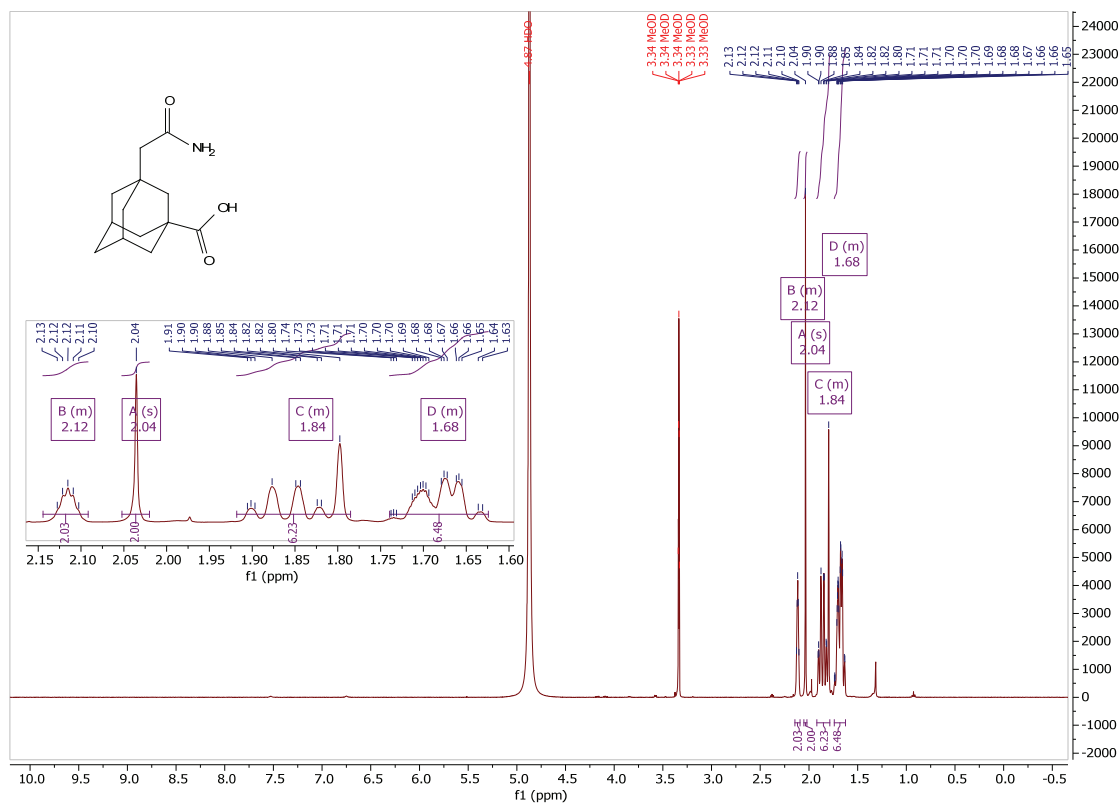
^{13}C NMR spectrum of 1-adamantaneacetic acid (18)

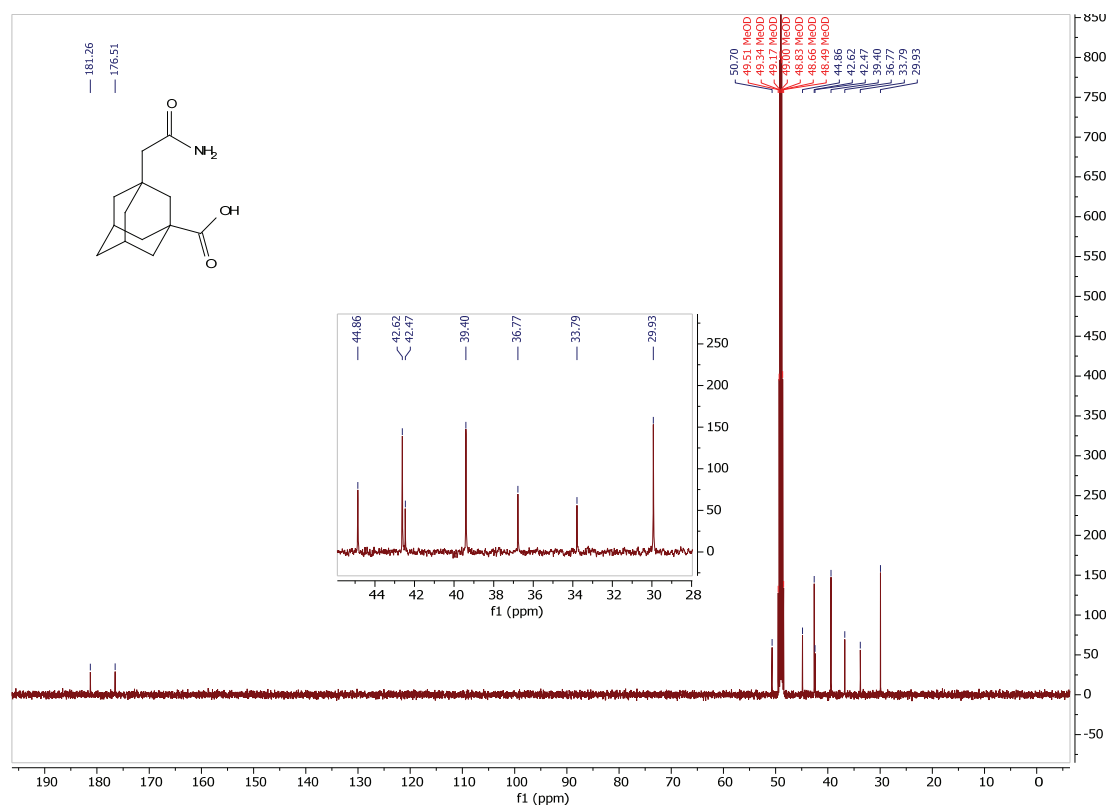
NC(=O)CC12CC3CC4C1CC5C(C2)CC6C4C(C3)CC7C5C(C6)CC8C7C(C8)C12

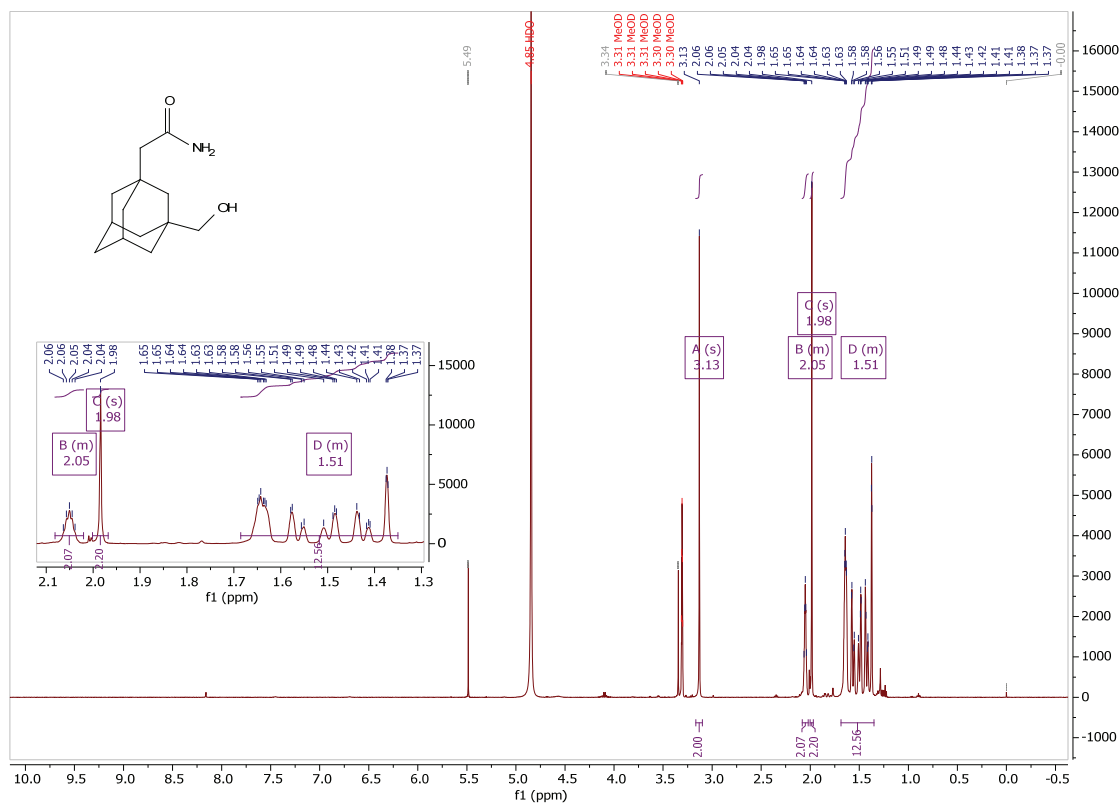
¹³C NMR spectrum of 1-adamantaneacetamide (19)

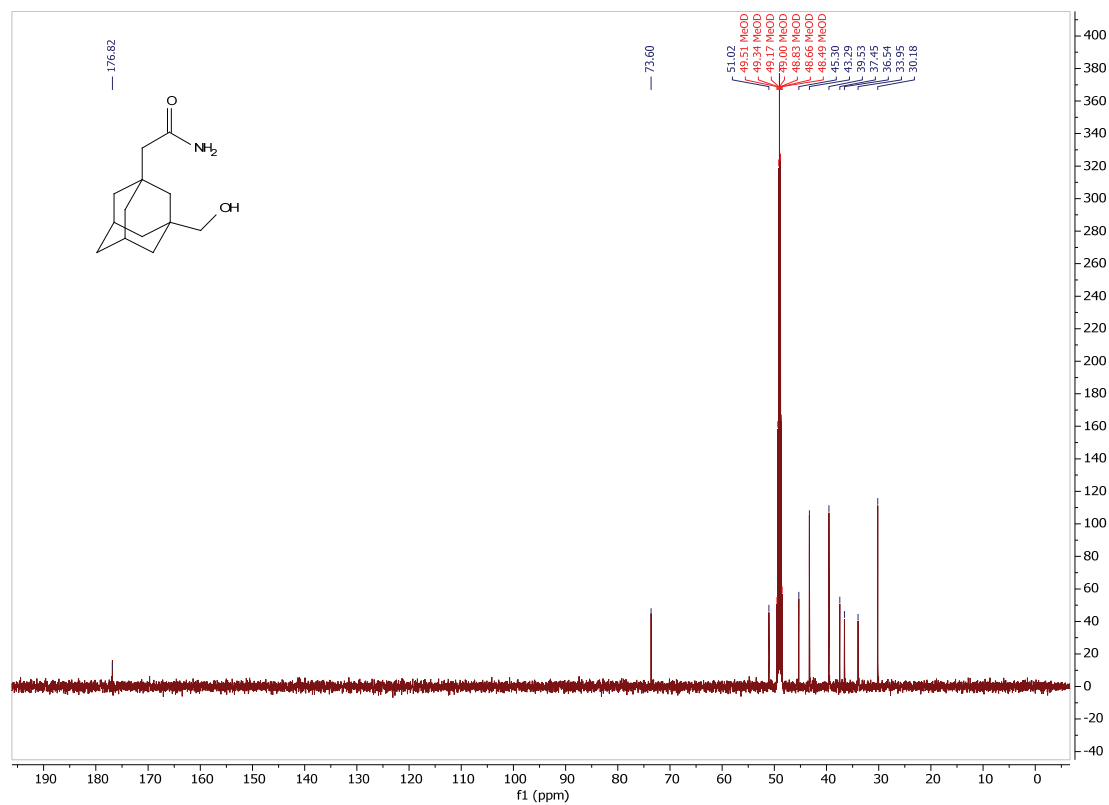
¹H NMR spectrum of 3-bromo-(1-adamantaneacetamide) (20)

¹³C NMR spectrum of 3-bromo-(1-adamantaneacetamide) (20)

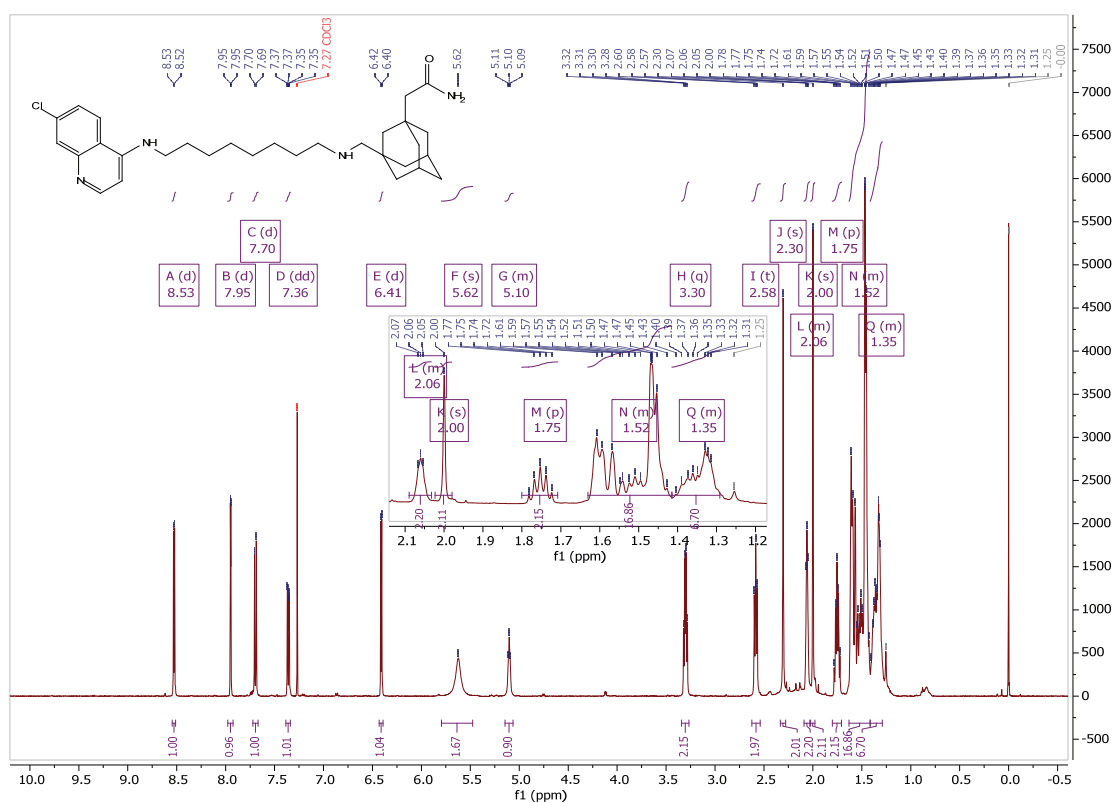
¹H NMR spectrum of 3-(2-amino-2-oxoethyl)adamantane-1-carboxylic acid (21)

^{13}C NMR spectrum of 3-(2-amino-2-oxoethyl)adamantane-1-carboxylic acid (21)

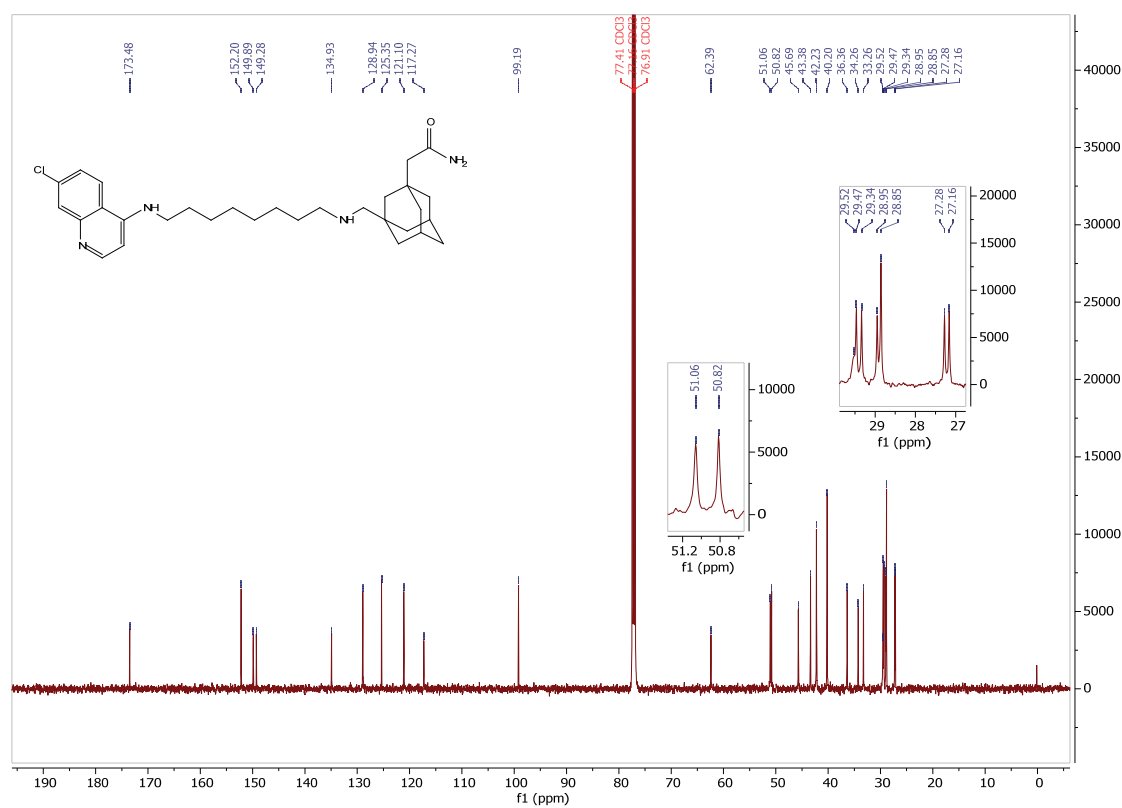
¹H NMR spectrum of 2-(3-(hydroxymethyl)adamantan-1-yl)acetamide (22)

^{13}C NMR spectrum of 2-(3-(hydroxymethyl)adamantan-1-yl)acetamide (22)

¹H NMR spectrum of 2-(3-(((8-((7-chloroquinolin-4-yl)amino)octyl)amino)methyl)adamantan-1-yl)acetamide (8)



¹³C NMR spectrum of 2-(3-(((8-((7-chloroquinolin-4-yl)amino)octyl)amino)methyl)adamantan-1-yl)acetamide (8)



5. HPLC PURITY CHROMATOGRAMS

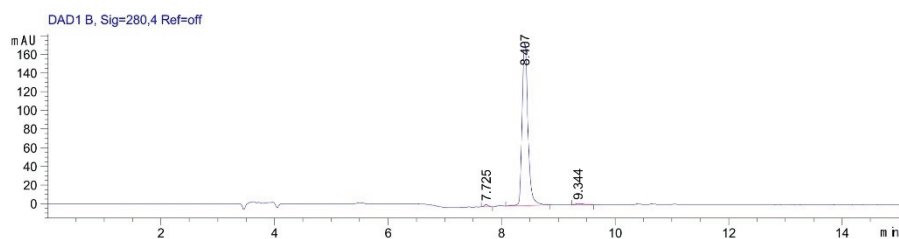
HPLC chromatogram of compound 3, method I

Sample Name: KBK45 Method I Compound 3
=====

Acq. Operator : SYSTEM	Seq. Line : 18
Sample Operator : SYSTEM	
Acq. Instrument : HPLC1260	Location : 48

Inj Volume : 2.000 µl

Method
metoda 2.AC.N.M (Sequence Method)



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Fraction Information

=====

No Fractions found.

=====

=====

Area Percent Report

=====

Sorted By : Signa
Multiplier : 1.000
Dilution : 1.000

Use Multiplier & Dilution Factor with ISTDs

Signal 2: DAD1 B, Sig=280,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.725	BB	0.0717	12.56595	2.73035	0.9881
2	8.407	BB	0.1113	1247.28442	174.62508	98.0745
3	9.344	BB	0.1150	11.92265	1.40566	0.9375

Totals : 1271.77302 178.76108

=====

*** End of Report ***

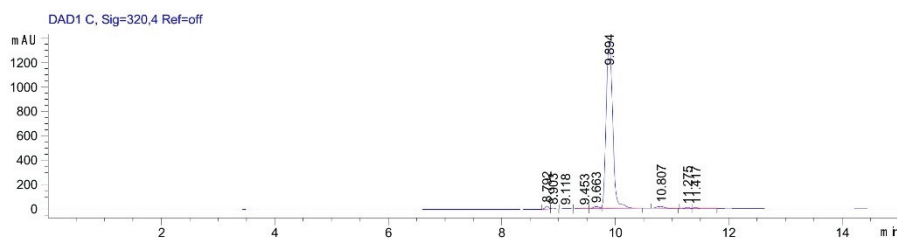
```

Sample Name: KBK45          Method II          Compound 3

=====
Acq. Operator   : SYSTEM                      Seq. Line :    2
Sample Operator : SYSTEM
Acq. Instrument : HPLC1260                    Location  :   48
                                           Inj Volume: 3.000 µl

Method
      metoda 1.M (Sequence Method)

Method Info      : Metoda 1 MeOH
  
```



No Fractions found.

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs
Signal 3: DAD1 C, Sig=320,4 Ref=off

Peak	RetTime	Type	Width	Area	Height	Area
#	[min]		[min]	[mAU*s]	[mAU]	%
1	8.792	BV	0.0698	100.77717	22.67707	0.8539
2	8.903	VB	0.0697	30.38930	6.59882	0.2575
3	9.118	BB	0.0939	22.18881	3.70206	0.1880
4	9.453	BV E	0.1123	11.00179	1.48812	0.0932
5	9.663	VV E	0.1145	118.22020	16.72628	1.0017
6	9.894	VB R	0.1342	1.12518e4	1364.23877	95.3386
7	10.807	BB	0.1291	160.95866	16.31722	1.3638
8	11.275	VB	0.1085	54.36814	8.08267	0.4607
9	11.417	VB	0.1095	52.25653	7.29542	0.4425

Totals : 1.18019e4 1447.12644

*** End of Report ***

HPLC chromatogram of compound 4, method III

Sample Name: KBK47 Method III Compound 4

=====

Acq. Operator : SYSTEM Seq. Line : 7

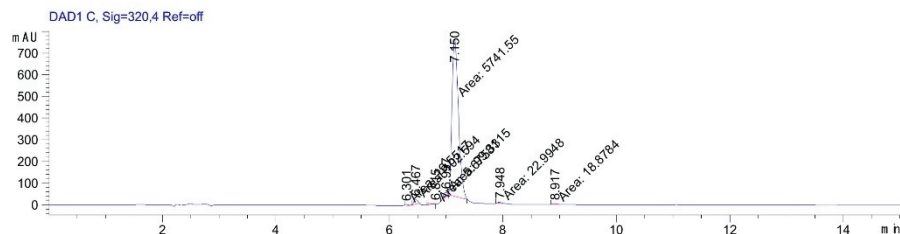
Sample Operator : SYSTEM

Acq. Instrument : HPLC1260 Location : 26

Inj Volume : 3.000 µl

Method

metoda 2.ACN.M (Sequence Method)



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Fraction Information

=====

No Fractions found.

=====

=====

Area Percent Report

=====

Sorted By : Signal

Multiplier : 1.0000

Dilution : 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 3: DAD1 C, Sig=320,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.301	MM	0.0656	19.55173	4.96475	0.3253
2	6.467	MM	0.0816	102.59441	20.94633	1.7069
3	6.815	MM	0.0360	5.67581	2.62664	0.0944
4	6.997	MM	0.0815	99.33145	20.30647	1.6526
5	7.150	MM	0.1318	5741.55273	726.23987	95.5241
6	7.948	MM	0.0742	22.99480	5.16225	0.3826
7	8.917	MM	0.0927	18.87844	3.39475	0.3141

Totals : 6010.57937 783.64105

=====

*** End of Report ***

HPLC chromatogram of compound 4, method IV

Sample Name: KBK47 Method IV Compound 4

=====

Acq. Operator : SYSTEM Seq. Line : 41

Sample Operator : SYSTEM

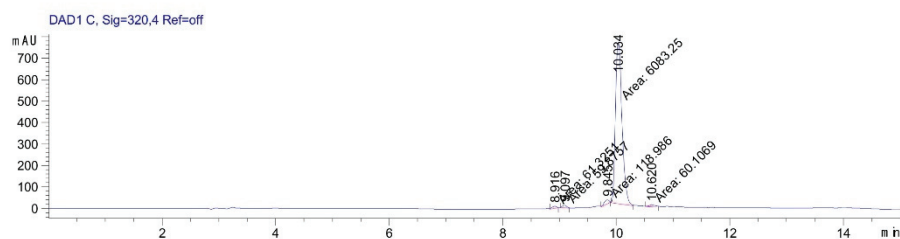
Acq. Instrument : HPLC1260 Location : 26

Inj Volume : 3.000 µl

Method

metoda 1.M (Sequence Method)

Method Info : Metoda 1 MeOH



=====

Fraction Information

=====

No Fractions found.

=====

=====

Area Percent Report

=====

Sorted By : Signa

Multiplier : 1.000

Dilution : 1.000

Use Multiplier & Dilution Factor with ISTDs

Signal 3: DAD1 C, Sig=320,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.916	MM	0.0993	61.32508	10.29342	0.9607
2	9.097	MM	0.0926	59.87572	10.78159	0.9380
3	9.845	MM	0.1056	118.98644	18.78357	1.8640
4	10.034	MM	0.1347	6083.24805	752.96307	95.2958
5	10.620	MM	0.1203	60.10685	8.32531	0.9416

Totals : 6383.54214 801.14698

=====

*** End of Report ***

HPLC chromatogram of compound 5, method I

Sample Name: KBK48 Methoda I Compound 5

=====

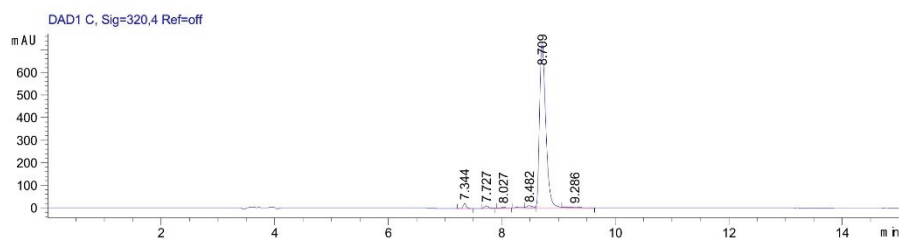
Acq. Operator : SYSTEM Seq. Line : 12

Sample Operator : SYSTEM Location : 42

Acq. Instrument : HPLC1260 Inj Volume : 2.000 µl

Method

metoda 2.AC.N.M (Sequence Method)



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Fraction Information

=====

No Fractions found.

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=====

Area Percent Report

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Sorted By : Signal

Multiplier : 1.0000

Dilution : 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 3: DAD1 C, Sig=320,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.344	BB	0.0593	87.20990	22.47513	1.4465
2	7.727	BB	0.0777	52.47802	10.61152	0.8704
3	8.027	BB	0.0868	25.87752	4.51626	0.4292
4	8.482	VV E	0.1078	67.10393	9.34007	1.1130
5	8.709	VV R	0.1229	5759.69629	735.13507	95.5346
6	9.286	VB E	0.1541	36.54370	3.08680	0.6061

Totals : 6028.90936 785.16485

=====

*** End of Report ***

HPLC chromatogram of compound 5, method II

Sample Name: KBK48 Methoda II Compound 5

=====

Acq. Operator : SYSTEM Seq. Line : 34

Sample Operator : SYSTEM

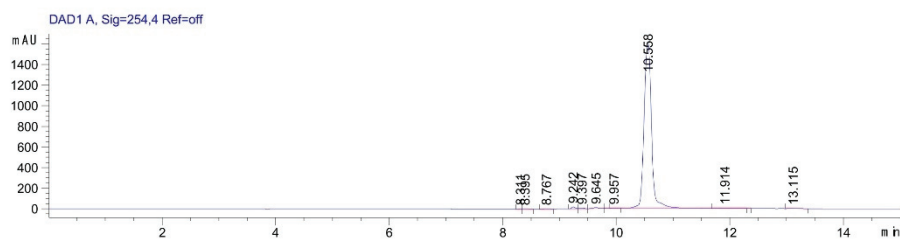
Acq. Instrument : HPLC1260 Location : 42

Inj Volume : 3.000 µl

Method

metoda 1.M (Sequence Method)

Method Info : Metoda 1 MeOH



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Fraction Information

=====

No Fractions found.

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Area Percent Report

=====

Sorted By : Signal

Multiplier : 1.0000

Dilution : 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.311	BV	0.0524	5.83001	1.77242	0.0388
2	8.395	VB	0.0748	25.67034	5.08937	0.1706
3	8.767	BB	0.0746	14.68180	2.92145	0.0976
4	9.242	BV	0.0710	86.24202	18.30317	0.5733
5	9.397	VB	0.0727	42.73519	9.10699	0.2841
6	9.645	BB	0.0963	66.82705	9.95200	0.4442
7	9.957	BV E	0.0935	13.95853	2.41147	0.0928
8	10.558	VV R	0.1402	1.47068e4	1615.26794	97.7614
9	11.914	VB E	0.1537	60.21075	5.25358	0.4002
10	13.115	BB	0.1438	20.61539	2.00828	0.1370

Totals : 1.50436e4 1672.08669

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*** End of Report ***

HPLC chromatogram of compound 6, method III

Sample Name: KBK64 Method III Compound 6

=====

Acq. Operator : SYSTEM Seq. Line : 14

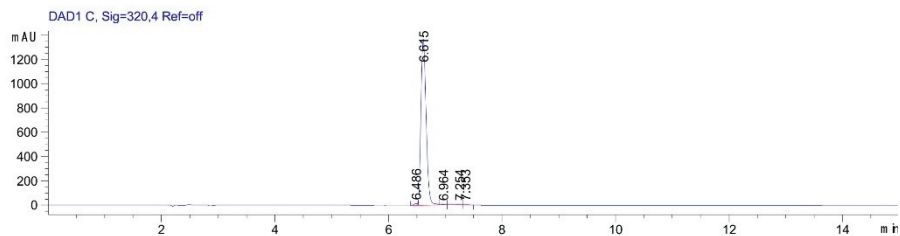
Sample Operator : SYSTEM

Acq. Instrument : HPLC1260 Location : 33

Inj Volume : 3.000 µl

Method

metoda 2.ACN.M (Sequence Method)



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Fraction Information

=====

No Fractions found.

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Area Percent Report

=====

Sorted By : Signal

Multiplier : 1.0000

Dilution : 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 3: DAD1 C, Sig=320,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.486	BV E	0.0613	59.80951	15.40364	0.6924
2	6.615	VV R	0.0962	8491.18359	1372.08960	98.3058
3	6.964	VB E	0.0625	9.22906	2.31716	0.1068
4	7.254	VV R	0.1028	39.71161	5.59855	0.4598
5	7.353	VB	0.0866	37.58918	6.38948	0.4352

Totals : 8637.52296 1401.79843

=====

*** End of Report ***

HPLC chromatogram of compound 6, method IV

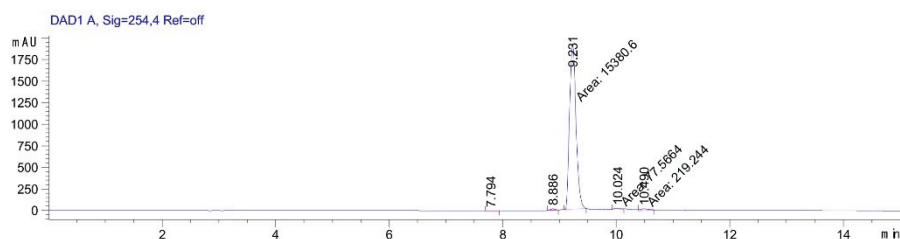
Sample Name: KBK64 Method IV Compound 6

=====

Acq. Operator : SYSTEM	Seq. Line : 48
Sample Operator : SYSTEM	
Acq. Instrument : HPLC1260	Location : 33
Inj Volume : 3.000 µl	

Method
metoda 1.M (Sequence Method)

Method Info : Metoda 1 MeOH



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Fraction Information

=====

No Fractions found.

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Area Percent Report

=====

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.794	BB	0.0972	6.76740	1.04925	0.0429
2	8.886	BB	0.0930	89.83051	15.64876	0.5695
3	9.231	MM	0.1336	1.53806e4	1918.15881	97.5060
4	10.024	MM	0.1306	77.56643	9.89686	0.4917
5	10.490	MM	0.1917	219.24388	19.06480	1.3899

Totals : 1.57741e4 1963.81848

=====

*** End of Report ***

HPLC chromatogram of compound 7, method III

Sample Name: KBK63b

Method III

Compound 7

Acq. Operator : SYSTEM

Sample Operator : SYSTEM

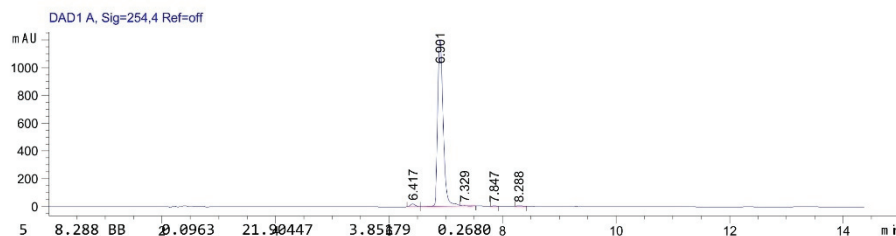
Acq. Instrument : HPLC1260

Location : 24

Inj Volume : 3.000 µl

Acq. Method : C:\ChemStation\1\Methods\HPLC cistoca metoda 2.ACN.M

Analysis Method : C:\ChemStation\1\Methods\DEF_LC.M



Fraction Information

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.417	BV	0.0911	120.12967	20.90011	1.4696
2	6.901	VV R	0.1040	8007.90674	1197.37476	97.9653
3	7.329	VB E	0.1134	16.89939	2.15666	0.2067
4	7.847	BV	0.0733	7.38698	1.55718	0.0904
5	8.288	BB	0.0963	21.90447	3.85179	0.2680

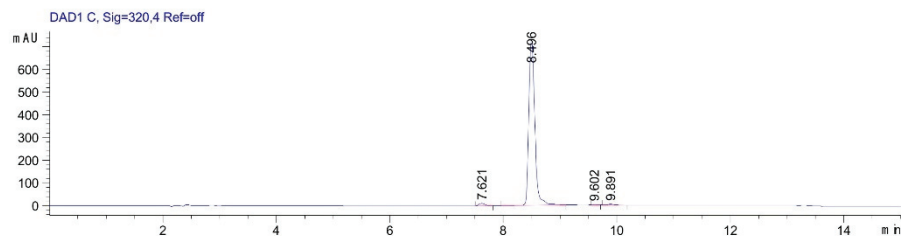
*** End of Report ***

HPLC chromatogram of compound 7, method IV

Sample Name: KBK63b Method IV Compound 7

```
=====
Acq. Operator   : SYSTEM
Sample Operator : SYSTEM
Acq. Instrument : HPLC1260                      Location :   24

                                           Inj Volume : 3.000 µl
Acq. Method    : C:\ChemStation\1\Methods\HPLC cistoca metoda 1.M
                                           (modified after loading)
Analysis Method : C:\ChemStation\1\Methods\DEF_LC.M
```



```
=====
Fraction Information
=====
```

No Fractions found.

```
=====
Area Percent Report
=====
```

```
Sorted By      :      Signal
Multiplier     :      1.0000
Dilution       :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 3: DAD1 C, Sig=320,4 Ref=off
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.621	BB	0.1206	76.21922	10.51346	1.4169
2	8.496	BB	0.1123	5272.46436	729.43896	98.0116
3	9.602	BB	0.0836	5.84354	1.18304	0.1086
4	9.891	BB	0.0962	24.90395	4.02220	0.4629

Totals : 5379.43106 745.15767

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*** End of Report ***
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HPLC chromatogram of compound 8, method III

Sample Name: KBK68 Method III Compound 8

=====

Acq. Operator : SYSTEM Seq. Line : 18

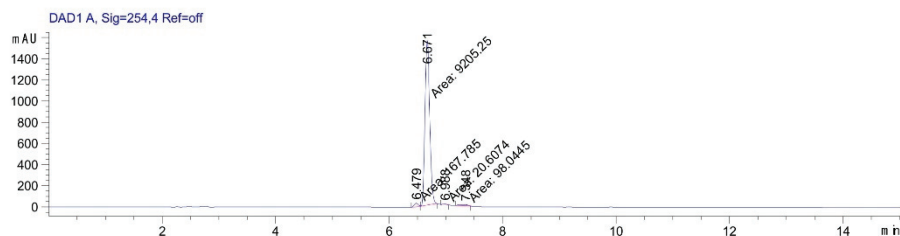
Sample Operator : SYSTEM

Acq. Instrument : HPLC1260 Location : 37

Inj Volume : 3.000 µl

Method

metoda 2.AC.N.M (Sequence Method)



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Fraction Information

=====

No Fractions found.

=====

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Area Percent Report

=====

Sorted By : Signal

Multiplier : 1.0000

Dilution : 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.479	MM	0.0823	167.78525	33.99279	1.7677
2	6.671	MM	0.0984	9205.25488	1558.82935	96.9822
3	6.988	MM	0.0570	20.60745	6.02871	0.2171
4	7.348	MM	0.1513	98.04446	10.80194	1.0330

Totals : 9491.69204 1609.65278

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*** End of Report ***

HPLC chromatogram of compound 8, method IV

Sample Name: KBK68 Method IV Compound 8

=====

Acq. Operator : SYSTEM Seq. Line : 52

Sample Operator : SYSTEM

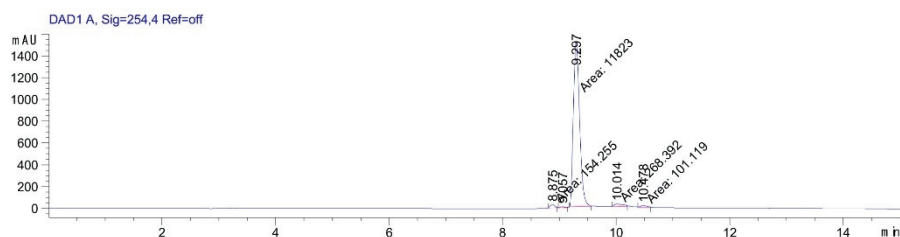
Acq. Instrument : HPLC1260 Location : 37

Inj Volume : 3.000 µl

Method

metoda 1.M (Sequence Method)

Method Info : Metoda 1 MeOH



=====

Fraction Information

=====

No Fractions found.

=====

=====

Area Percent Report

=====

Sorted By : Signal

Multiplier : 1.0000

Dilution : 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.875	MM	0.0941	154.25528	27.31053	1.2453
2	9.057	VB	0.1015	40.69626	6.29104	0.3285
3	9.297	MM	0.1302	1.18230e4	1513.76807	95.4433
4	10.014	MM	0.1856	268.39194	24.10622	2.1666
5	10.478	MM	0.1172	101.11902	14.38363	0.8163

Totals : 1.23874e4 1585.85949

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*** End of Report ***

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JSCS–5406

Evaluation of antimicrobial, anticancer and neuroprotective activities of silver nanoparticles (AgNPs) green-synthesized using a red pigment produced by *Streptomyces* sp. A23 strain isolated from Algerian bee pollen

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Abstract: In this work, the red pigment of *Streptomyces* sp. A23 strain isolated from Algerian bee pollen was used for the green synthesis of silver nanoparticles (AgNPs) as well as for evaluating their antimicrobial, anticancer and neuroprotective activities. AgNPs were synthesized as a result of the reduction of 1 and 5 mM silver nitrate solutions at various pH values (5, 7 and 9) and were subsequently characterized. AgNPs (5 mM, pH 9) exhibited a maximum UV–Vis absorbance at 433 nm. Dynamic light scattering revealed that the average diameter was 112 nm. A zeta potential peak was found at –33 mV corresponding to the increased stability. XRD analysis confirmed the crystallization nature of the material. Furthermore, FT-IR analysis revealed the specific functional groups at 3471 to 478 cm^{–1}. In addition, FE-SEM showed that the mean size of the spherical AgNPs was 54.5 nm in diameter. The presence of Ag was revealed by EDX analysis. Additionally, good antimicrobial activity was observed against *Enterococcus faecalis* ATCC 19433, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883 and *Escherichia coli* ATCC 7839, with inhibition zones of 32, 30, 30, 27, 25, 20 and 19 mm, respectively. The lowest minimum inhibitory concentration and minimum bactericidal concentration were recorded against *B. subtilis* ATCC 6633, with a value of 62.5 µg mL^{–1}. Intriguingly, all the synthesized

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<https://doi.org/10.2298/JSC240915008M>

AgNPs at concentrations of 2, 4 and 8 $\mu\text{g mL}^{-1}$ had cytotoxic effects on SH-SY5Y neuroblastoma cell lines. In addition, AgNPs (1 mM, pH 7) exhibited the significant neuroprotective activity at the lowest tested concentration. Finally, the AgNPs synthesized using the red pigment of *Streptomyces* sp. strain A23 can be considered as promising therapeutic agents.

Keywords: actinomycetes; microbial pigment; green synthesis; antimicrobial activity; cancer; neuroprotective activity.

INTRODUCTION

Recently, the use of nanomaterials in the medical field has attracted increased attention, especially as novel and alternative therapeutic agents with multifaceted applications.¹ One of these nanomaterials are silver nanoparticles (AgNPs), which are emerging as possible hopeful alternatives due to their beneficial features.² Two conventional methods were applied in order to synthesize AgNPs and they are chemical and physical methods. Due to their undesirable disadvantages, some suitable and green alternative methods must be found. Among these methods, the biological methods which use bacteria, fungi, algae, plants, and others have gained the substantial prominence due to their high safety.³ Many researchers have used Actinomycetes, including *Streptomyces* bacteria to synthesize AgNPs.^{4,5} The genus *Streptomyces* is known as a source of valuable and variety of secondary metabolites, which are excellent tools for the green synthesis of nanoparticles.^{6,7} One such secondary metabolite is pigments which not only serve as an identifier product for *Streptomyces*, but also demonstrate a prominent potential for synthesizing AgNPs.⁸ This process of synthesis provides a fast, safe and eco-friendly approach, and imparts significant biological properties like antioxidant, antimicrobial, and antiproliferative activities, to the resulting nanoparticles, which hold promise as multifaceted therapeutic agents capable of treating both communicable and noncommunicable diseases.^{9,10} Regarding communicable diseases, the growth of antibiotic-resistant microorganisms is one of the most important public health problems, which have emerged as a global crisis, underscoring the urgent need to discover novel and alternative antimicrobial agents to the conventional antibiotics.¹¹ AgNPs synthesized using secondary metabolites produced by *Streptomyces* exhibit good antimicrobial effects against a wide range of pathogenic microorganisms, including bacteria and fungi.¹² Moreover, when AgNPs are combined with conventional antibiotics, they can have synergistic effects that may overcome antibiotic resistance.¹³ For noncommunicable diseases, cancer and neurodegenerative disorders remain major health concerns, necessitating the discovery of new effective therapeutic agents.^{14,15} AgNPs synthesized using the microbial pigments have shown promising anticancer and neuroprotective effects.^{12,16} These nanoparticles are promising agents in cancer therapy because they can target the cancer cells, while

avoiding the cells that are normal.¹⁷ Furthermore, the distinctive physicochemical properties of these nanoparticles allow them to exhibit antioxidant activities, reducing the oxidative stress that might lead to neurodegenerative disorders. Additionally, these nanoparticles can modulate inflammatory responses, further contributing to their neuroprotective effect.¹⁸

Therefore, this study aims to synthesize AgNPs using a red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen as a new biological reducing agent to evaluate their antimicrobial, anticancer and neuroprotective activities.

EXPERIMENTAL

Materials

Silver nitrate (AgNO₃), hydrogen peroxide (H₂O₂) and dimethyl sulfoxide (DMSO) were purchased from Sigma. Muller-Hinton broth was obtained from Conda and calcium carbonate (CaCO₃) was obtained from Merck. The GF-1 nucleic acid extraction kit, universal primers (27F and 1492R) and clean-up kit were from Vivantis Technologies. Gentamicin discs were provided from Oxoid™. *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 7839, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883 and *Candida albicans* ATCC 10231 were obtained from the applied microbiology laboratory, Ferhat Abbas University, Algeria. SHSY-5Y human neuroblastoma cells (ATCC®, CRL-2266) were obtained from Erfarma Center, Erciyes University, Turkey.

Bee pollen sampling

The bee pollen samples were provided by the International Api-Phyto Therapy Center, Setif, Algeria, from different Algerian clean lands during the spring of 2019, in sterile plastic containers. The samples were subsequently transported to the laboratory and stored at 4 °C until use.

Isolation, purification and conservation of the A23 strain

All the samples were crushed, air dried at room temperature and heated at 50 °C for 30 min in a water bath (Memmert, Germany). One gram from all bee pollen samples was mixed with 0.1 g of calcium carbonate and incubated at 28 °C for one week.¹⁹ Then, 1 g of each pre-treated sample was suspended in 9 mL of sterile saline water and diluted to 10⁻², after which 100 µL of the diluted sample was plated on different media for Actinomycetes isolation and incubated at 28 °C for approximately 10 days. During the period of incubation, the selected strain was purified using yeast extract-malt extract agar (ISP 2 medium), labelled by the A23 strain and conserved at 4 °C.²⁰

Phenotypic identification of the A23 strain

The primary phenotypic characteristics of the A23 strain were determined *via* Gram staining, catalase and oxidase tests.^{21,22} In addition, the culture characteristics were described based on the growth intensity, aerial and substrate mycelia and the pigment production on the ISP 2 agar medium.²³ Also, the carbohydrate assimilation was assessed on minimal growth medium supplemented with the carbon source at a final concentration of 1 %, as described by Shirling and Gottlieb (1966).²⁴ The production of extracellular hydrolytic enzymes (starch hydrolysis, protease and lipolysis) was studied on the minimum agar medium added to the

substrates at a concentration of 1 %.²⁵ The assignments of the A23 strain at least to the genus level were performed by comparing the results of the phenotypic characterization to those reported in Bergey's Manual of Systematic Bacteriology.²⁶

Genotypic identification of the A23 strain

DNA extraction, 16S rRNA amplification and electrophoresis. The DNA extraction of the selected strain was carried out by using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia). The 16S rRNA amplification was performed by PCR using thermocycler (iCycler Bio-Rad, USA) with the help of two universal primers, 27F and 1492R. This process was repeated for 30 cycles,²⁷ after which the products of PCR were subjected to electrophoresis and subsequently purified by a Clean-Up Kit (Vivantis Technologies, Malaysia).²²

DNA sequencing and bioinformatics analysis. The PCR products were purified and subsequently sequenced using 3130 Genetic Analyzer (Applied Biosystems®, Waltham, MA, USA).²² BLAST was used to compare the obtained sequence of the studied strain with 16S rRNA gene sequences of other Actinomycetes in GenBank. MEGA 11 software was used to construct the phylogenetic tree.²⁸

Green synthesis, purification and characterization of silver nanoparticles (AgNPs)

Production and purification of red pigment. Red pigment was produced from the A23 strain by the submerged fermentation on ISP 2 broth using an orbital shaker at 180 rpm and 28 °C. A week later, the culture broth was centrifuged at 4000 rpm and 4 °C for 10 min to remove the mycelia. The supernatant that contained the produced pigment was collected and stored at 4 °C.²⁹

Synthesis and purification of AgNPs. Two mL of the obtained pigment were filtered through a 0.45 µm syringe filter and mixed with 18 mL of 1 or 5 mM silver nitrate solutions at pH 5, 7 and 9 using a magnetic hotplate stirrer at 1500 rpm and room temperature for 24 h in dark conditions.^{9,30} The first part of the nanoparticles suspension was obtained directly and kept at 4 °C for UV–Vis spectrophotometry, zeta potential and DLS analyses and further experiments. The second part was centrifuged and washed two times with distilled water at 15000 rpm for 10 min, after which the precipitates were dried at 70 °C for 2 days and subjected to the characterization using XRD, FT-IR, FE-SEM and EDX analyses.³¹

Characterization of synthesized AgNPs. Several techniques have been used to characterize the synthesized AgNPs. The formation of AgNPs was confirmed by the visual observation and UV–Vis (Perkin Elmer).⁹ The hydrodynamic diameter was determined *via* dynamic light scattering (DLS, Malvern Panalytical, Malvern, UK). The surface charge was examined by zeta potential analyser. The presence of functional groups, playing a role in the synthesis, was determined by FT-IR (Perkin Elmer Spectrum 400) analysis. The crystallization was determined by XRD (Bruker AXS D8, Ettlingen, Germany) analysis. The morphology and the elemental presence was determined by the field emission scanning electron microscopy (FE-SEM) and energy-dispersive (EDX) analyses (Zeiss Gemini SEM 500, Aalen, Germany).³²

Biological activities of synthesized AgNPs

Antimicrobial activity. The first test of antimicrobial activity of the synthesized AgNPs was evaluated using the agar well diffusion method, as described by Balouiri *et al.* (2016)³³ against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. faecalis* ATCC 19433, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 and *C. albicans* ATCC 10231. The fresh microbial suspensions (10^7 cfu mL⁻¹) were prepared and swabbed

using sterile cotton swabs on Muller–Hinton agar plates. After that, the wells were made using sterilized micropipette tips and 100 μL of synthesized AgNPs were poured into each well. Finally, all plates were kept at room temperature for 30 min to diffusion and subsequently incubated at 37 °C for 24 h. After incubation, the inhibition zone diameter observed around each well was measured and is expressed in mm. The gentamicin discs (Oxoid™) and silver nitrate solutions were used as positive controls; pigment alone and distilled water were used as negative controls. After that the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of AgNPs (5 mM, pH 9) were determined by the micro-broth dilution method using tetrazolium salts (TTC) in 96 well microplates in triplicate and on Mueller–Hinton agar plates, respectively.⁹

Anticancer and neuroprotective activities. The final concentrations of 2, 4, 8, 12 and 16 $\mu\text{g mL}^{-1}$ of the synthesized AgNPs were prepared to be used in the evaluation of the cytotoxicity and the neuroprotective effects on the SHSY-5Y neuroblastoma cells by using the MTT test.

Cytotoxicity assay. After the preparation of SHSY-5Y cells, they were exposed to all prepared concentrations of AgNPs for 24 h in 96 well plates contains approximately 10^4 cells per well. Then, 100 μL of the prepared MTT solution was added to each well and incubated at 37 °C for 4 h. Afterwards, the supernatant portion was subsequently removed from the wells, followed by the addition of 100 μL of DMSO to each well in order to dissolve the MTT salts. Finally, the microplate reader (BioTek, Synergy HT, Winooski, Vt, USA) was used to measure the absorbances at a wavelength of 560 nm.³¹

Neuroprotective activity. To evaluate the potential neuroprotective effects of the synthesized nanoparticles on neuroblastoma cells, the prepared concentrations of AgNPs compounds were added 1 h after the treatment of cells by H_2O_2 (300 μM) in 96 well plates. Cell viability was measured by the MTT test after 24 h of incubation. The same volume of medium without AgNPs or H_2O_2 was used as a control.³¹

Statistical analysis. Data analysis was performed using the GraphPad Prism 8.0 software by using ANOVA one-way and Dunnett's post-hoc tests. All differences were considered significant when $P < 0.05$. The n value was four for the cell culture studies

RESULTS AND DISCUSSION

Phenotypic and genotypic identification of the isolated strain (A23)

As shown in Fig. 1, the isolated strain in our study was characterized by earthy odour and powdery colonies with a grey white colour, and it was a filamentous, branching and gram-positive bacterium. The morphological and the cultural properties have shown abundant growth with the variations in the colour of aerial and substrate mycelia. Additionally, it has the potential to produce a diffusible red pigment. Actinomycetes can produce visible powdery colonies with different colours from the 3rd to 7th day, with an earthy odour as a result of these bacteria's ability to produce various secondary metabolites, including pigments.³⁴ Furthermore, the strain can use different carbon sources for growth, and it is also positive for catalase and negative for the oxidase reactions. Protease, lipase, and amylase are produced by the described strain (Table I). With the help of Bergey's manual of systematic bacteriology, these phenotypic properties are consistent with the typical characteristics described for the genus *Streptomyces*.²⁶

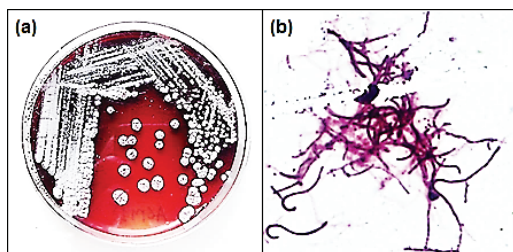


Fig. 1. *Streptomyces* sp. A23 strain a) Colony morphology b) Gram staining.

TABLE I. The phenotypic characteristics of the isolated strain (*Streptomyces* sp. A23); +) positive reaction; –) negative reaction; +++) elevated production

Growth intensity on ISP2 agar	+++
Aerial mycelium colour on ISP2 agar	Grey white
Substrate mycelium colour on ISP2 agar	Dark red
Diffusible pigment	+++
Gram staining	Positive
Carbon sources assimilation	
Glucose	+
Fructose	+
Lactose	+
Maltose	+
Galactose	+
Mannitol	+
Enzymes production	
Catalase	+
Oxidase	–
Starch hydrolysis	+
Casein hydrolysis	+
Lipolysis	+

According to the obtained 16S rRNA sequence (1096 bp) and the phylogenetic tree analyses (Fig. 2), the A23 strain belongs to the genus *Streptomyces*. It was labelled *Streptomyces* sp. A23 strain and registered in GenBank under the accession number OR236137.1.

Green synthesis and characterization of AgNPs

AgNPs were successfully synthesized via a rapid, easy, and eco-friendly procedure, using the red pigment of the *Streptomyces* sp. A23 strain that isolated from bee pollen as a bio-reducing agent source. Silver nitrate (AgNO_3) is a colourless solution, and the pigment is red colour. When the pigment was mixed with silver nitrate solution, the mixture slowly turned to the brown colour after 24 hours in the dark (Fig. 3). The colour change was due to silver nitrate interacting with the pigment compounds and being bio-reduced from silver nitrate (AgNO_3) to elemental silver. These elementals aggregate to form stable AgNPs.³⁵ A previous study indicated the formation of AgNPs using a pink pigment

produced from *Streptomyces* sp. NS-05 isolated from a rhizospheric soil by observing the change in colour to brown.⁸

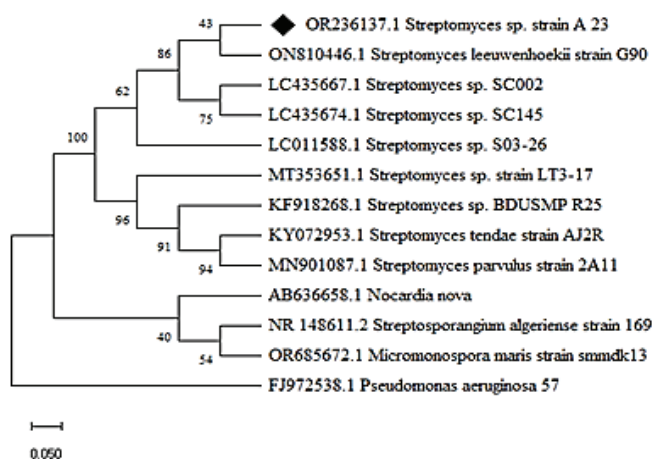


Fig. 2. Phylogenetic tree of the *Streptomyces* sp. A23 strain.

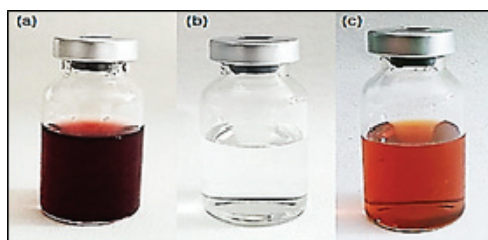


Fig. 3. Visual observation of synthesized AgNPs: a) pigment, b) AgNO_3 solution and c) AgNPs suspension.

In this study, the light absorption points, the hydrodynamic diameter, the surface charge, the crystallization, the phytochemical composition, the morphology and the elemental presence of synthesized AgNPs (5 mM, pH 9) were determined by UV-Vis, DLS, zeta potential, XRD, FT-IR, FE-SEM and EDX analyses, respectively. The optical and electrical properties of nanoparticles are interrelated, and unlike those of their bulk forms, they have a UV-Vis absorption band called surface plasmon resonance (SPR).

UV-Vis spectroscopy was used for characterizing the nanoparticles synthesized by various methods. The diffractions observed by analysis can be controlled by many factors, such as reducing agents and solvents.³⁶ According to the obtained results, the characteristic light absorption bands detected at 278 and 433 nm by UV-Vis analysis were associated with the presence of biomolecules in the pigment and AgNPs, respectively (Fig. 4). The characteristic light absorption bands of AgNPs synthesized by the secondary metabolites of *Streptomyces rochei* and marine *Streptomyces* sp. were measured at 413 and 434 nm, respectively.^{37,38}

The structural properties of nanoparticles which depend on the synthesis conditions, also affected the UV–Vis characterization bands.³⁹

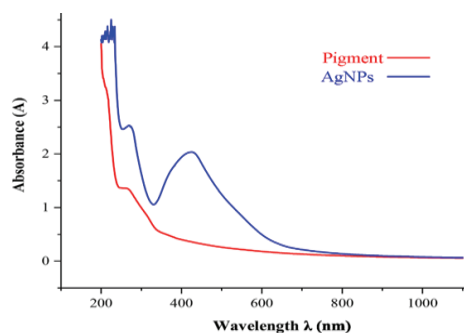


Fig. 4. UV–Vis spectrum of AgNPs (5 mM, pH 9).

The surface charge of nanoparticles determines the distribution properties of particles, the adsorption properties of ions, and molecules, and it is an important parameter for the characterization of nanomaterials.⁴⁰ The surface charge of the AgNPs synthesized in our study was determined to be -33 mV (Fig. 5). A relatively low zeta potential increases the electrostatic repulsion force between nanoparticles, which prevents their aggregation and ensures their stabilization.⁴¹ The surface charge (negative, positive or neutral) of nanoparticles is related to the functional groups on the surface structure.⁴²

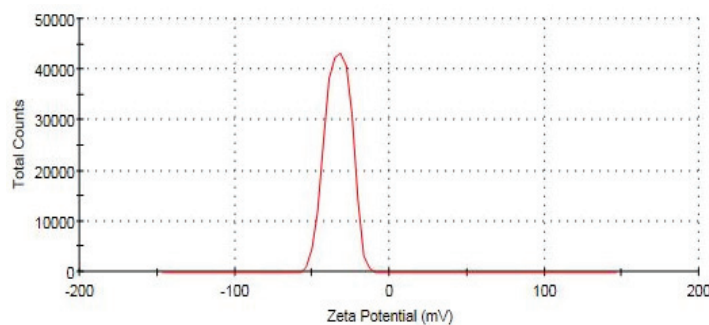


Fig. 5. Zeta potential analysis of AgNPs (5 mM, pH 9).

The dynamic light scattering (DLS) is a spectroscopic analysis technique used to determine the size of particles suspended in a liquid from 1 to 1000 nm.⁴³ The average hydrodynamic size of our synthesized AgNPs was 112 nm (Fig. 6). The DLS measurements are expected to yield more intense results than microscopic analyses, which can be explained by the presence of ions or molecules bound, and the increase in the thickness of the hydration shell around nanoparticles in solution.^{44,45}

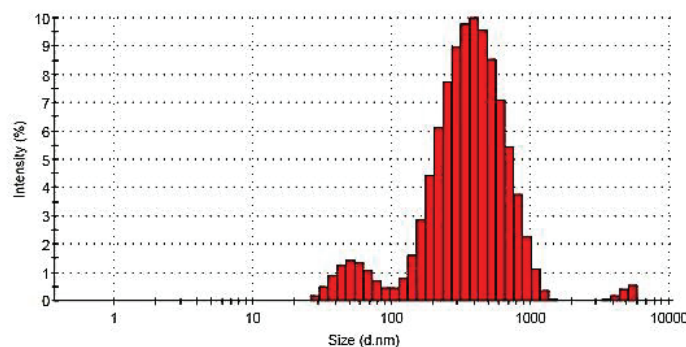


Fig. 6. DLS analysis of AgNPs (5 mM, pH 9).

According to the FE-SEM image, our synthesized spherical AgNPs tend to form agglomerations and appear to be monodispersed with a mean particle size of 54.5 nm (Fig. 7). The average size of the spherical AgNPs synthesized, using the pink pigment of *Streptomyces* sp. NS-05 was 42.5 nm.⁸ The size, shape, and structural properties of NPs synthesized by biological methods can vary depending on the biological agent used and the synthesis conditions, such as the concentration and type of metal salt used and the bio-reducing agent, temperature and reaction time.⁴⁶

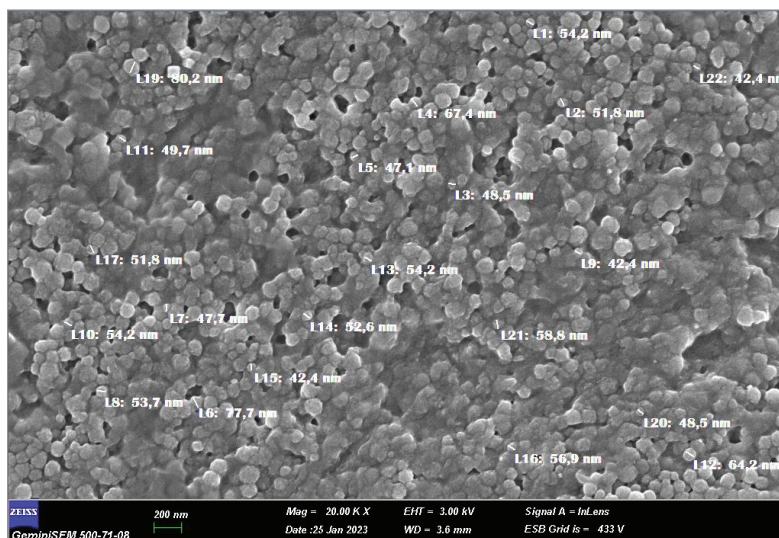


Fig. 7. FE-SEM image of AgNPs (5 mM, pH 9).

EDX and XRD were performed to determine the elemental composition and crystal structure of the AgNPs. The presence of Ag was revealed by EDX analysis (Fig. 8).

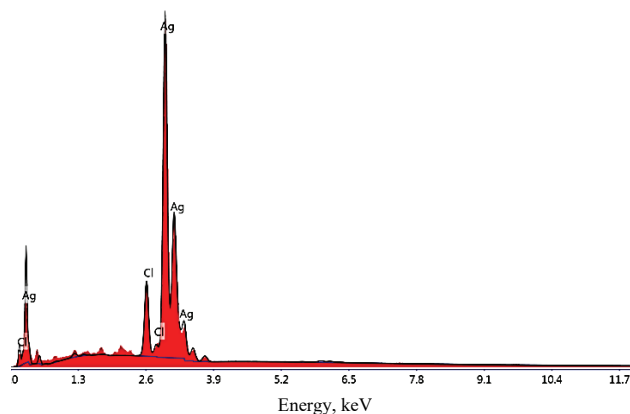


Fig. 8. EDX analysis of AgNPs (5 mM, pH 9).

According to the XRD analysis of the AgNPs, the 27.7, 32.3, 38.4, 44.1, 46.2, 54.5, 57.6, 64.3 and 77.2° peaks observed at 2 theta corresponded to the (1,0,0), (1,2,2), (1,1,1), (2,0,0), (2,0,0), (2,2,0), (2,2,0), (2,2,0) and (3,1,1) planes, respectively (Fig. 9). The obtained peaks at 27.7, 32.3 and 54.5° indicate AgNPs (JCPDS (01-076-1489)). The peaks at 32.3, 38.4, 46.2, 54.5, 57.6, 64.3 and 77.2° reflect the face-centered cubic Ag phase (JCPDS (00-001-1167)).⁴⁷⁻⁴⁹

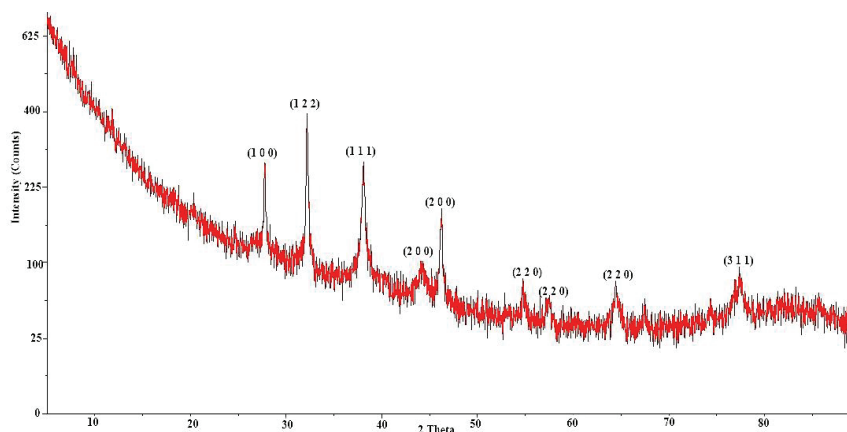


Fig. 9. XRD pattern of AgNPs (5 mM, pH 9).

FT-IR can provides the information about the bioactive molecules that may be responsible for reducing silver ions into AgNPs.¹² The functional groups that play a role in the green synthesis of our AgNPs were revealed by the peaks observed in the FT-IR spectrum with diffractions at 3471, 2918, 1620, 1409, 1321 and 478 cm^{-1} , which indicate the presence of alcohol (O-H), alkane (C-H), amine (N-H), fluoro compound (C-F), aromatic amine (C-N) and metal (Ag),

respectively (Fig. 10). Similar to our findings, previous studies reported that alcohol, alkane, and amine groups were responsible for the reduction and capping of Ag ions for the synthesis of NPs.^{11,38,41}

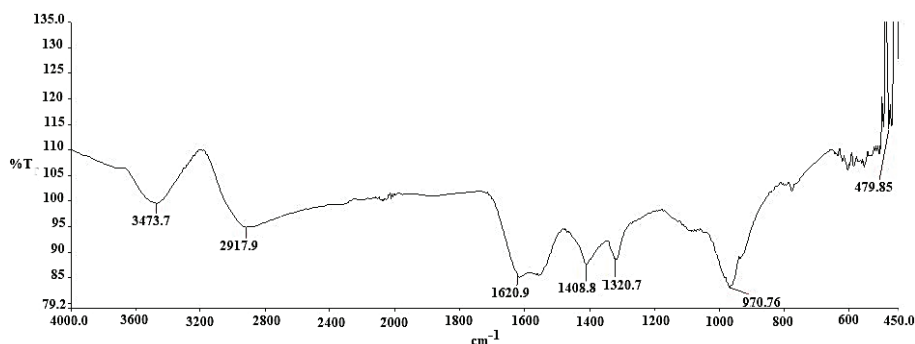


Fig. 10. FT-IR spectrum of AgNPs (5 mM, pH 9).

Antimicrobial activity

Firstly, the antimicrobial activity of synthesized nanoparticles (AgNPs) by using the red pigment produced from *Streptomyces* sp. A23 strain was evaluated using an agar well diffusion assay as illustrated in Fig. 11.

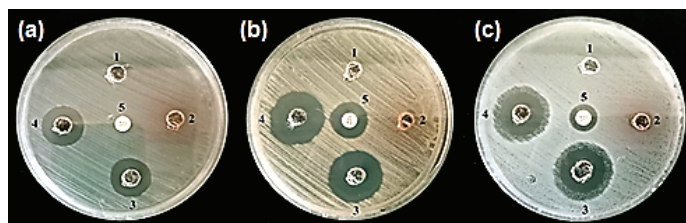


Fig. 11. Antimicrobial activity of AgNPs (1 mM, pH 7) against: a) *E. faecalis*, b) *S. aureus* and c) *P. aeruginosa*; 1) H₂O, 2) pigment, 3) AgNO₃, 4) AgNPs and 5) gentamicin.

AgNPs (5 mM, pH 9) are more effective because of their spherical shape and smaller size (54.5 nm), and their contact with microbial surfaces is good and easy due to their high negative surface charge (−33 mV). According to several reports, physical properties such as size, shape, and surface charge can influence the antimicrobial activity.^{50–52} In addition, many previous studies have shown that one of the critical characteristics of nanoparticles is size, and a smaller size has a higher effect on the ability of nanoparticles to penetrate into microbial cells.⁵² Additionally, the shape of AgNPs plays an important role in the antimicrobial activity, and it was decided that a spherical shape would result in excellent activity.⁵³ Furthermore, the surface charge plays a crucial role in the initial adsorption of nanoparticles to cell membranes.⁵¹

For the *MICs* and *MBCs* of AgNPs (5 mM, pH 9), we recorded the lowest *MIC* against *S. aureus* ATCC 6538P and *B. subtilis* ATCC 6633 with a value amounted by $62.5 \mu\text{g mL}^{-1}$, while the highest *MIC* was against *P. aeruginosa* ATCC 27853 ($250 \mu\text{g mL}^{-1}$). The lowest *MBC* ($62.5 \mu\text{g mL}^{-1}$) was recorded against *B. subtilis* ATCC 6633, while the highest *MBC* ($> 1000 \mu\text{g mL}^{-1}$) was against *P. aeruginosa* ATCC 27853 (Table II). *MIC* value is the lowest concentration of the tested substance as an antibiotic that inhibits the growth of the test microorganisms after an overnight of incubation, while *MBC* value is the lowest concentration required to kill 99.9 % of the test microorganisms.^{7,9}

TABLE II. *MICs* and *MBCs* results of AgNPs (5 mM, pH 9)

Pathogenic microorganism	<i>MIC</i> / $\mu\text{g mL}^{-1}$	<i>MBC</i> / $\mu\text{g mL}^{-1}$
<i>S. aureus</i> ATCC 6538P	62.5	125
<i>B. subtilis</i> ATCC 6633	62.5	62.5
<i>E. coli</i> ATCC 7839	125	125
<i>P. aeruginosa</i> ATCC 27853	250	> 1000
<i>K. pneumoniae</i> ATCC 13883	125	125

There is no clear or exact mechanism for the antimicrobial effect of AgNPs, but many existing studies have reported that the most common and prominent mechanisms are as follows. a) AgNPs can adhere to the cell wall and membrane of microbial cells, causing cell rupture and leading to cell lysis. b) AgNPs can penetrate the inside of microbial cells and damage biomolecules (DNA and enzymes) and intracellular structures (mitochondria, vacuoles and ribosomes). c) AgNPs have the potential to increase ROS production, which can cause oxidative stress and damage to microbial cells.^{54,55}

Anticancer and neuroprotective activities

In the MTT test, SH-SY5Y cells were treated with various concentrations of AgNPs to determine their cytotoxic effects (Fig. 12). The compound-1 (AgNPs, 1 mM, pH 5) did not alter cell viability at concentrations of 2 or $4 \mu\text{g mL}^{-1}$, but at concentrations of 8, 12 and $16 \mu\text{g mL}^{-1}$ ($p < 0.001$), it significantly the reduced cell viability compared to that of the control group. For the compound-2 (AgNPs, 1 mM, pH 7), the concentration of $2 \mu\text{g mL}^{-1}$ tended to increase the cell viability, but this increase was not statistically significant. Additionally, there was no change in the cell viability at $4 \mu\text{g mL}^{-1}$. Also, at the concentrations of 8, 12 and $16 \mu\text{g mL}^{-1}$ ($p < 0.001$), the cell viability was markedly lower than that in the control group. Compound-3 (AgNPs, 5 mM, pH 9) significantly decreased ($p < 0.001$) cell viability at concentrations of 2, 4, 8, 12 and $16 \mu\text{g mL}^{-1}$ compared to that of the control.

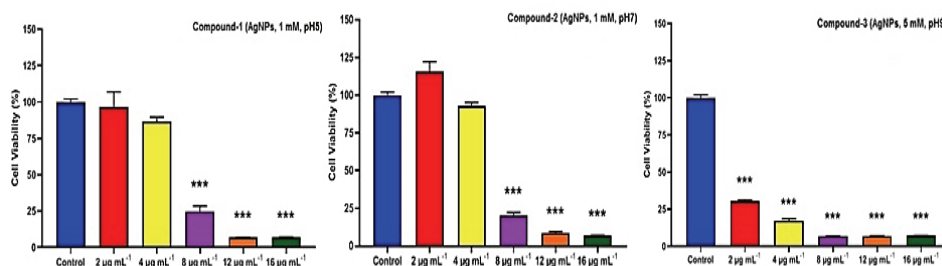


Fig. 12. Effects of synthesized AgNPs on the viability of SH-SY5Y cells;
***: $p < 0.001$ compared to the control.

For the neuroprotective activity, MTT assay was performed to determine the neurotoxic dose of H_2O_2 as shown in Fig. 13. The treatment with 100, 150, 200, 300, 400 and 600 μM of H_2O_2 reduced ($p < 0.001$) the cell viability compared to that of the control group. To evaluate the neuroprotective effect of synthesized nanoparticles, 300 μM H_2O_2 was added one hour before the AgNPs were added, and the cell viability was investigated via the MTT test at the 24th hour of AgNPs treatment. When the neuroprotective effects of the AgNPs were examined, the compound-1 (AgNPs, 1 mM, pH 5) at concentrations of 2, 4, 8, 12 and 16 $\mu\text{g mL}^{-1}$ ($p < 0.001$) significantly reduced the viability of the SH-SY5Y cells, when compared to that of the H_2O_2 group. The compound-2 (AgNPs, 1 mM, pH 7) at a concentration of 2 $\mu\text{g mL}^{-1}$ ($p < 0.001$) significantly increased the cell viability with the presence of H_2O_2 . In addition, at the concentrations of 8, 12 and 16 $\mu\text{g mL}^{-1}$ ($p < 0.001$), the cell viability was markedly lower than that in the H_2O_2 group. The treatment with the compound-3 (AgNPs, 5 mM, pH 9) at the concentrations of 2, 4, 8, 12 and 16 $\mu\text{g mL}^{-1}$ ($p < 0.001$) significantly reduced the viability of the SH-SY5Y cells compared to that of the H_2O_2 group.

In this study, the cell culture experiments were conducted to address two primary objectives concerning SH-SY5Y neuroblastoma cells. First, the anticancer activity of the synthesized AgNPs was investigated by assessing their cytotoxic effects on neuroblastoma cells. The determination of the optimal cytotoxic dose was crucial for the evaluation of the anticancer potency of the synthesized materials. Notably, Compound-1, Compound-2, and Compound-3 exhibited cytotoxic effects at concentrations of 2, 4 and 8 $\mu\text{g mL}^{-1}$, respectively. Comparative analysis revealed the superior anticancer activity of compound-3 against neuroblastoma cells. This observation aligns with the recent studies, confirming the anticancer efficacy of silver nanoparticles synthesized using the bio-active molecules produced by some *Streptomyces* strains on different cancer cell lines.^{7,39} Additionally, our study was the first to show that AgNPs synthesized using a red pigment of *Streptomyces* sp. had anticancer activities on human

neuroblastoma cells, although there are several studies showing anticancer and cytotoxic effects of different source-based AgNPs.^{56,57}

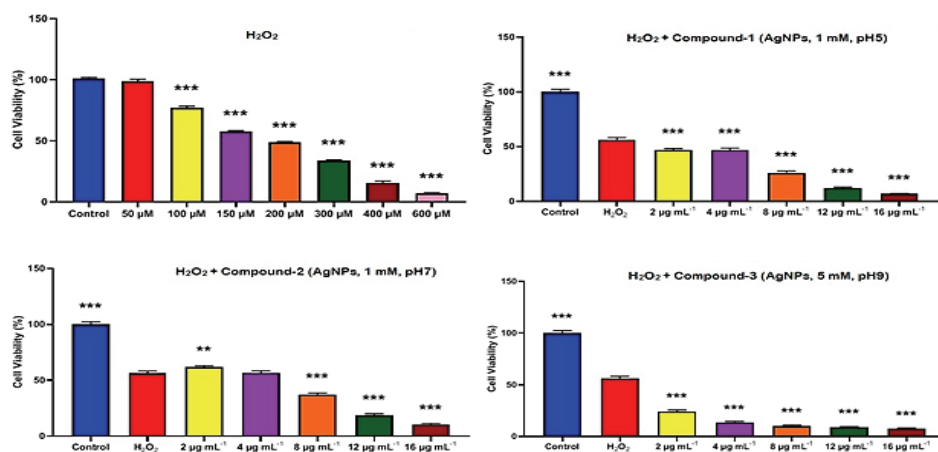


Fig. 13. Neuroprotective activity of synthesized AgNPs; *: $p < 0.01$; **: $p < 0.001$ compared to the control group.

The second objective focused on elucidating the potential neuroprotective effects of AgNPs against hydrogen peroxide-induced cytotoxicity, a common mechanism underlying neurodegenerative diseases. Hydrogen peroxide (H₂O₂) at a concentration of 100 μ M demonstrated cytotoxic effects, and a concentration of 300 μ M was selected for its moderate cytotoxic effects, as suggested by prior study.⁵⁸ Intriguingly, compound-2 exhibited some neuroprotective effects at the lowest concentration tested, distinguishing it from the other compounds. The observed neuroprotective effects of compound-2 were hypothesized to be associated with its potential antioxidant properties, drawing parallels with findings of Alkhalaf *et al.* in 2020,⁵⁹ who demonstrated that the antioxidant and anti-inflammatory effects of AgNPs synthesized using *Nigella sativa* extract in rodents. Controversially, Zhai *et al.* (2022) showed the cytotoxic effects of AgNPs *via* oxidative stress mechanism in SH-SY5Y cells. It has been seen that AgNPs causes oxidative stress at very high doses (40 and 60 μ g mL⁻¹), whereas we noticed the neuroprotective effect of low doses of AgNPs (2 and 4 μ g mL⁻¹), against a well-known oxidative stressor, H₂O₂, in our study. These differences suggest a dose-dependent antioxidant or oxidant effect of AgNPs synthesized, using the red pigment produced by the *Streptomyces* sp. A23 strain.⁵⁷ These findings suggested a nuanced interplay between the antioxidant effects at lower concentrations and antiproliferative effects at higher concentrations for the compound-2.

CONCLUSION

In conclusion, the green synthesis of silver nanoparticles (AgNPs) using the red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen represents a new approach with the remarkable neuroprotective, anticancer and antimicrobial activities. This synthesis method not only harnesses the inherent capabilities of the *Streptomyces* sp. A23 strain, but also produces the silver nanoparticles with unique biological properties, paving the way for their application in diverse therapeutic avenues. The future studies should address the molecular mechanisms underlying the therapeutic properties of these nanoparticles, including the molecular interactions between the AgNPs and biological targets, which will also provide deeper insight into their mode of action. Additionally, the exploration of synergistic effects with other therapeutic agents could open new possibilities for the enhanced treatment strategies, especially in the multidrug-resistant infections and the complex diseases like cancer and neurodegeneration.

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ИЗВОД

ОДРЕЂИВАЊЕ АНТИМИКРОБНЕ, АНТИКАНЦЕРОГЕНЕ И НЕУРОПРОТЕКТИВНЕ АКТИВНОСТИ НАНОЧЕСТИЦА СРЕБРА (AgNP), СИНТЕТИСАНИХ ПРИМЕНОМ ЗЕЛЕНЕ ХЕМИЈЕ, КОРИШЋЕЊЕМ ЦРВЕНОГ ПИГМЕНТА СОЈА *Streptomyces* sp. A23 ИЗОЛОВАНОГ ИЗ АЛЖИРСКОГ ПЧЕЛИЊЕГ ПОЛЕНА

МОКХНАЧЕ МОХАМЕД¹, БЕЛХАДЖ ХАНИ¹, ФАТИХ ДОГАН КОЧА², ГОКХАН УНАЛ³, НАСРАТ АБДУЛ РАХМАН⁴, АЈСЕГУЛ БАСМА², НУХ МЕХМЕТ БОЗКУРТ³, АХМЕД АЛИЕН МОХАМЕД БАЧИР¹ И ХАРЗАЛЛАХ ДАОУД¹

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Црвени пигмент из соја *Streptomyces* sp. A23 изолован из алжирског пчелињег полена је коришћен за синтезу наночестица сребра (AgNP) применом зелене хемије, а затим је одређивана њихова антимикробна, антиканцерска и неуропротективна активност. AgNP су синтетисане редукцијом 1 mM и 5 mM раствора сребро нитрата на различитим pH (5, 7 и 9), а затим окарактерисане. AgNP (5 mM, pH 9) су испојиле максимум UV-Vis апсорбације на 433 nm. Методом DLS је утврђено да је просечни пречник честица 112 nm. Максимум зета потенцијала је утврђен на -33 mV, потврђујући повећану стабилност. XRD анализом је констатована кристална природа материјала. FT-IR анализом су утврђене специфичне функционалне групе на положају од 3471 до 478 cm⁻¹. FE-SEM методом је показано да је средња вредност сферних пречника AgNP 54,5 nm. Присуство Ag је потврђено EDX анализом. Наночестице су испојиле значајну антимикробну активност спрам *Enterococcus faecalis* ATCC 19433, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883 и *Escherichia coli* ATCC 7839, уз зону инхибиције од 32, 30, 30, 27,

25, 20, односно 19 mm. Најниже вредности MIC и MBC су измерене спрам *B. subtilis* ATCC 6633 – 62,5 $\mu\text{g mL}^{-1}$. Занимљиво је да су све синтетисане AgNP, у концентрацијама 2, 4 и 8 $\mu\text{g mL}^{-1}$, испољиле цитотоксични ефекат према SH-SY5Y ћелијској линији неуробластома. Додатно, AgNP (1 mM, pH 7) су испољиле значајну неуропротективну активност при најнижим тестираним концентрацијама. Из свега наведеног произилази да се AgNP синтетисане применом црвеног пигмента *Streptomyces* sp. сој A23 могу сматрати за обећавајуће терапеутске агенсе.

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Optimizing alginate immobilization of food-derived C-phycocyanin: Structural and functional characterization

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Abstract: C-phycocyanin (C-PC) represents a significant component of the cyanobacteria *Arthrospira platensis* (*Spirulina*) biomass. Beyond its nutritional value, this protein exhibits numerous beneficial biological activities. A covalently attached chromophore, phycocyanobilin, gives C-PC a blue colour, enabling its use as a natural food colorant. Additionally, phycocyanobilin exhibits various bioactive properties, including metal-binding activities. A key drawback to the broader industrial application of C-PC is its poor stability. Alternative food formulations using natural polymers as carriers and active components have recently gained considerable scientific attention. This paper describes the optimized conditions for C-PC immobilization using alginate. The structural stabilization of immobilized C-PC was analysed under high temperature (60 °C) and high pressure (450 MPa). The storage stability of immobilized C-PC in dried alginate beads was tested by keeping the samples at 4 °C for one month. The potential application of immobilized C-PC for the removal of mercury ions was also investigated. Alginate immobilization proved effective in stabilizing C-PC, significantly preserving its structure during prolonged storage, thermal treatment and high-pressure exposure. Under the tested conditions, 97 % of Hg²⁺ were removed by immobilized C-PC. Overall, this study optimized the procedure for enhancing C-PC stability through alginate immobilization and broadened its potential applications in food and bioremediation industries.

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INTRODUCTION

Cyanobacteria *Arthrospira platensis*, commonly known as *Spirulina*, is a popular alternative food source considered a “superfood”. The annual production of *Spirulina* reaches about 50,000 tons, with the majority produced in China.¹ *Spirulina* biomass is rich in proteins (over 50 %), lipids, vitamins and minerals.² The major protein of *Spirulina* is the intensively blue-coloured C-phycocyanin (C-PC).³ This protein is part of multi-protein complexes known as phycobilisomes, which play a role in light-harvesting during photosynthesis.⁴

In its native state, C-PC is a hexamer with a $\alpha_6\beta_6$ structure. The building block of this hexamer is the $\alpha\beta$ monomer, consisting of two subunits with molecular masses of 17.6 kDa for the α subunit and 18.1 kDa for the β subunit.⁵ During assembly, three monomers form a trimer, which then combines with another trimer to form a hexamer in a face-to-face orientation.⁶ The isolation of this protein yields a mixture of monomers, trimers and hexamers, depending on the applied conditions and C-PC concentration.⁷ The blue colour of C-PC originates from the covalently attached tetrapyrrole chromophore phycocyanobilin (PCB). Each monomer of C-PC contains three PCB molecules – one in the α subunit and two in the β subunit – attached *via* thioether bond to cysteine (Cys) residues.⁸ Besides its direct role in light harvesting,⁹ numerous beneficial biological activities of C-PC are associated with PCB, including antioxidant, anticancer and anti-inflammatory properties.¹⁰ Additionally, due to the presence of PCB, C-PC is capable of binding heavy metals, exhibiting dissociation constants in the nM range for Hg^{2+} .¹¹

The potential for industrial use of C-PC is significant. In addition to its nutritional value, its blue colour makes it an attractive natural food colorant, especially as consumers increasingly seek natural ingredients in their diets.¹² Blue colorants are rare in nature and synthetic alternatives are primarily used.¹³ Both the Food and Drug Administration (FDA) and the European Union accept the usage of C-PC as a food colorant. A limiting factor for the broader use of C-PC lies in its poor stability.¹⁴ C-PC from *Spirulina* is most stable in a pH range of 5.5–6.0. Its half-life rapidly decreases at temperatures between 47 and 64 °C.¹⁵ Numerous approaches for C-PC stabilization have been reported so far in the literature and are covered in several review papers, which describe the addition of stabilizers in the form of small molecules like sugars, the presence of proteins and encapsulation methods.¹⁶ Encapsulation methods not only stabilize C-PC, but they are also potential approaches for creating novel, attractive types of food consisting of hydrogels packed with higher concentrations of nutritive proteins. Due to its vivid colour, C-PC is particularly interesting in this regard.¹⁷ The controlled release of proteins from encapsulating material is also an important benefit.

The alginate immobilization of C-PC has been reported to be cheap, efficient and easy to fabricate and manipulate and it also enables the controlled pH release. While several papers on C-PC immobilization using alginate exist, they either involve combinations with other components besides alginate^{18–20} or lack details about immobilization optimization regarding pH, protein structure after immobilization and physical and chemical treatments, as well as the potential for prolonged storage without prior lyophilization of the alginate beads.^{20–24} This paper aims to fill these gaps by fabricating the alginate beads containing a higher concentration of C-PC and structurally characterized by absorption spectrometry after thermal and high-pressure treatments and prolonged storage at 4 °C. Their potential application in bioremediation is also explored by testing their ability to bind mercury ions.

EXPERIMENTAL

Materials

All reagents were of analytical quality and were purchased from Sigma (Darmstadt, Germany). Commercial *Spirulina* powder was obtained from Nutrex (Hawaii, USA). C-PC was partially purified using the same procedure described in our previous paper.²⁵ Absorbance spectra of partially purified C-PC were recorded using LLG-uniSPEC 4 spectrophotometer (LLG labware, Meckenheim, Germany). Based on the absorbance spectra, the concentration of C-PC was determined to be 8.36 mg mL⁻¹ using a published equation.²⁶ Its purity was measured to be 1.04 by the absorbance ratio at 620 and 280 nm,²⁷ indicating the food-grade purity. Food-grade sodium alginate was purchased from Health Leads (Llandysul, United Kingdom).

Alginate immobilization of C-PC

The alginate immobilization of C-PC followed similar published procedures,^{20,22} with slight modifications. The final concentration of C-PC used for immobilization was 4.18 mg mL⁻¹. The alginate concentrations tested were 0.5, 1 and 2 %, while the concentration of CaCl₂ solution was 2.5 %. The CaCl₂ solution was prepared in distilled water. Equal volumes of alginate and protein solutions were mixed and then added dropwise to the CaCl₂ solution using a syringe with an 18G needle. The resulting beads were kept in CaCl₂ solution for 5 h, then washed with the same CaCl₂ solution on a filter paper until no further blue colour leached from the beads. The beads were stored at 4 °C overnight in CaCl₂ solutions of different pH values: 1.35 (45 mM HCl), 2.75 (1.78 mM HCl), 4 and 5.5 (50 mM acetate buffer) and 7 (50 mM HEPES buffer). Beads were photographed the next day and those with minimal C-PC leakage and no observable colour change were selected for further analysis. For subsequent experiments, unless otherwise stated, the beads were prepared with 1% alginate, 4.18 mg mL⁻¹ of C-PC and 2.5 % of CaCl₂ in 50 mM acetate buffer, pH 4, except for Hg²⁺ binding experiments, where 0.5 % alginate was used. The fabricated beads were stored in solution at 4 °C for no longer than 7 days until all experiments were conducted.

Temperature stability

For the temperature stability test, the alginate beads were placed in four separate microcentrifuge tubes with 1 ml of 50 mM acetate buffer, pH 4. Tubes were placed in a thermoshaker and incubated at 60 °C under 200 rpm for 30 min. Every 10 min, one microcentrifuge tube was removed. The control tube was incubated for 30 min at room temperature. After the incubation,

beads were placed in 1 mL 50 mM phosphate buffer, pH 7, and incubated at room temperature for 3 h, enabling swelling of alginate beads and C-PC leakage into the solution, with concomitant recording of absorbance spectra of obtained solutions from 300 to 750 nm.

High-pressure stability

Alginate beads were placed in a plastic tube filled with 50 mM acetate buffer, pH 4, and sealed with parafilm to separate the sample from the water used as the pressure-transmitting liquid. The sample was placed in a custom-built stainless steel high-pressure cell at the Laboratoire Léon-Brillouin (Saclay, France) and connected to a pressure generator. The pressure was increased to 4,500 bar at 200 bar/min. After reaching the desired pressure, the sample was incubated for 30 min and then returned to atmospheric pressure. The beads were transferred to 10 mL of 50 mM phosphate buffer, pH 7, and incubated at room temperature for 3 h. The absorbance spectra were obtained as described in the temperature stability section.

Storage stability

The alginate beads containing C-PC were removed from the 50 mM acetate buffer and placed in a glass beaker without any solution. The beaker was covered with stretch film and stored in a fridge at 4 °C for one month. The beads were then swollen by adding 20 mL of 50 mM phosphate buffer, pH 7, and after 3 h, absorbance spectra were recorded as in the temperature stability section. A control sample was made by placing a portion of alginate beads containing immobilized C-PC immediately into the same phosphate buffer for 3 h and recording the absorbance spectra of leaked C-PC as described in the temperature stability section.

Removal of Hg²⁺ by alginate beads containing C-PC

The ability of C-PC in solution to bind Hg²⁺ at pH 4 was first tested by determining the affinity constant using a FluoroMax spectrofluorimeter (Horiba Scientific, Japan). The increasing concentrations of HgCl₂, from 50 to 700 nM, were added to 2.45 nM C-PC and the emission spectra of C-PC were recorded from 600 to 750 nm, with the excitation at 590 nm (PCB chromophore excitation). The buffer (50 mM acetate) spectrum was subtracted from the sample spectra. In this setup, no inner filter effect was observed and the affinity constant was calculated using the following equation:

$$\log \frac{F_0 - F}{F} = n \log [L] + n \log K_a \quad (1)$$

where F_0 is the fluorescence of C-PC without Hg²⁺, F is the fluorescence of C-PC in the presence of C-PC, $[L]$ is the concentration of Hg²⁺ in mol dm⁻³, n is the Hill coefficient and K_a is the affinity constant in M⁻¹.

The potential of mercury removal by C-PC immobilized into alginate beads was tested by adding 40 alginate beads to 15 mL of 1.75 ppm HgCl₂ solution in ultrapure water at pH 4. The control experiments were performed with the same number of alginate beads without C-PC and an additional control solution of HgCl₂ was incubated without beads. The incubation lasted 48 h at room temperature under constant shaking at 200 rpm. Hg²⁺ concentration in the samples was determined using an ICP-OES iCap 6500 Duo (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

All experimental results represent the averages of triplicates, with standard deviations not exceeding 5 %. The statistical significance for Hg²⁺ binding was confirmed using one-way ANOVA with Tukey's multiple comparison test, with p -value < 0.05 being considered statistically significant. The test was performed using OriginLab software (Northampton, MA, USA),

version 8.5.1. The absorbance spectra were first normalized to their maximum absorption values and then to the baseline.

RESULTS AND DISCUSSION

Optimization of C-PC immobilization into alginate beads

Using a straightforward procedure, the alginate beads containing C-PC were successfully created. The beads exhibited an intense blue colour due to the presence of native C-PC and maintained a spherical shape (Fig. 1).



Fig. 1. Alginate beads with encapsulated C-PC.

By washing off the unbound C-PC and calculating the concentration of both the unbound C-PC and that released in the CaCl_2 solution, it was estimated that more than 50 % of the initial C-PC was successfully immobilized into the alginate beads. This level of efficiency aligns with previously published results, indicating that the alginate entrapment is an effective method for immobilizing substantial amounts of C-PC.^{21,23,24} Interestingly, to the best of our knowledge, no published work has provided a detailed analysis of the pH conditions necessary for fabricating stable alginate/C-PC beads in solution.

When the alginate/C-PC beads were incubated overnight at five different pH values, significant C-PC leakage was observed at pH 5.5 and 7 (Fig. 2D–E), regardless of the alginate concentration. In contrast, no leakage occurred at acidic pH values of 1.35 and 2.75. However, the beads appeared green (Fig. 2A–B), suggesting that the immobilized C-PC underwent structural alterations and protonation of the pyrrole ring in the PCB chromophore. At pH 4, the beads retained their intense blue colour, with no detectable C-PC leakage under any of the tested alginate concentrations (Fig. 2C).

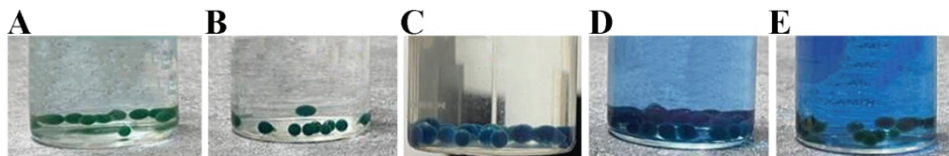


Fig. 2. Alginate beads containing encapsulated C-PC incubated for 24 h at 4 °C in 2.5 % CaCl_2 solutions at pH: 1.35 (A), 2.75 (B), 4 (C), 5.5 (D) and 7 (E).

It can be concluded that at pH 4, no significant structural alteration of the immobilized C-PC within the alginate beads, if any, occurred at all. Additionally,

when these beads were transferred to a 50 mM phosphate buffer, pH 7, C-PC began to leak, turning the solution an intense blue, with no precipitation observed. The native state of C-PC was further confirmed through absorbance spectra (see Fig. 4, for example). Interestingly, when the alginate/C-PC beads incubated in solutions of pH 1.35 and 2.75 were placed in a neutral buffer, the blue colour appeared in a solution containing beads made in pH 2.75, suggesting that the structural alterations of immobilized C-PC under these conditions are partially reversible (Fig. 3).

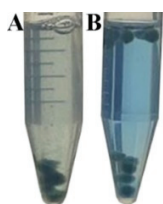


Fig. 3. Alginate beads with encapsulated C-PC incubated at pH 1.35 (A) and 2.75 (B) and subsequently placed in 50 mM phosphate buffer at pH 7.

These results demonstrate that alginate immobilization can provide partial structural stabilization to C-PC under acidic conditions. This is noteworthy because soluble C-PC is unstable at pH levels below 5 and tends to aggregate.²⁸

Interestingly, another study investigating C-PC encapsulation into alginate beads reported stability at pH 7, which we could not replicate due to significant leakage of C-PC.²² The reason for this discrepancy remains unclear, especially since the preparation of alginate beads in both studies was almost identical. Possible factors could include differences in the starting materials (C-PC and alginate) or variations in the initial pH encapsulation and buffer compositions, which were not fully detailed in the other study.

The pH conditions for fabricating alginate beads play a critical role, as alginate is a pH-sensitive polymer. At higher pH values, alginate swells due to the numerous negatively charged carboxyl groups within its structure. Conversely, at lower pH values, these groups become protonated, causing the alginate gels to shrink. Since C-PC is physically entrapped within alginate gel formed by Ca^{2+} cross-linking, the porosity of the alginate gel significantly impacts the system's stability. Additionally, C-PC's structural characteristics are influenced by pH. At higher pH, C-PC tends to dissociate from hexamers into trimers, while at lower pH, it adopts higher oligomeric states and tends to aggregate.²⁹ The higher oligomeric state of C-PC and the partial shrinkage of alginate likely prevented the C-PC leakage from beads formed at pH 4 and lower.

Structural stabilization of C-PC by alginate immobilization

The stabilization of C-PC entrapped in alginate beads was tested under three different experiments: increased temperature, high pressure and prolonged storage. Tested beads were incubated in 50 mM acetate buffer, pH 4.

The appearance of alginate beads with encapsulated C-PC remained unchanged after thermal treatment (Fig. 4A). Moreover, spectral analysis of C-PC released after incubating alginate beads at 60 °C for 30 min showed a significant increase in C-PC's thermal stability under these conditions (Fig. 4B).

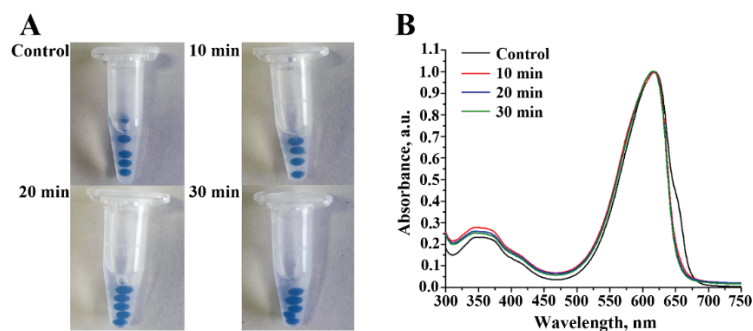


Fig. 4. Alginate beads with encapsulated C-PC during thermal treatment at 60 °C, photographed at 10, 20 and 30 min and after 30 min without thermal treatment, control sample (A) and absorbance spectra of control C-PC sample and C-PC samples after thermal treatment (B).

The A_{620}/A_{280} ratio remained nearly the same and the spectral shape showed minimal alteration compared to the non-heated control sample. The slight increase in absorbance around 350 nm suggests that the chromophore's conformation remained virtually unchanged during the incubation. The melting temperature (t_m) of C-PC is reported to range from 50 to 60 °C,^{30,31} and it depends on several factors, some of which are the purity and pH of the solution. The increased purity of C-PC reduces its thermal stability,³¹ while its stability is greater at pH 5 compared to neutral and alkaline conditions.³² At higher temperatures, C-PC undergoes structural degradation, destabilizing the chromophore and altering its colour properties.³³

Our findings suggest that the simple physical entrapment of C-PC within alginate beads provides both pH and thermal stability, thus expanding the potential industrial applications of C-PC. Two previous studies have also investigated the stability of encapsulated C-PC in alginate beads. One study dried the beads at 4 °C and incubated them for three days at 35, 45 and 55 °C. C-PC's stability was evaluated using the DPPH antioxidant test, concluding that alginate immobilization enhanced thermal stability.²⁴ However, this study did not directly assess C-PC's structural characteristics, so its conclusions are limited. In another study, the authors incubated alginate beads with encapsulated C-PC at different temperatures, demonstrating similar stabilization at 60 °C after 30 min by recording the absorbance spectra in the visible range.²² However, the pH conditions of their experiment were unclear.

In our study, we analysed the structural characteristics of encapsulated C-PC by recording absorbance spectra from 300 to 750 nm, offering more detailed

insights into C-PC and the attached PCB behaviour under specific treatments. During the C-PC denaturation, PCB undergoes a conformational shift from linear to cyclic, evidenced by an increase in absorbance at 360 nm and a corresponding decrease at 620 nm.³⁴

The high-pressure (HP) food treatment is an environmentally friendly alternative to thermal treatment, offering superior preservation of nutritive components in treated foods.³⁵ In this study, alginate beads containing C-PC were subjected to high-pressure treatment at 4,500 bar for 30 min, resulting in a visible colour change from blue to turquoise. This suggests that the encapsulated C-PC underwent structural alterations (Fig. 5A), a conclusion supported by the recorded absorbance spectra (Fig. 5B).

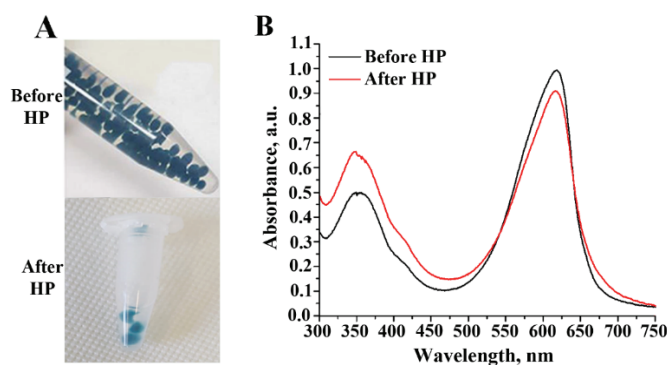


Fig. 5. Alginate beads with encapsulated C-PC photographed before and after HP treatment (A) and C-PC absorbance spectra recorded before and after HP treatment (B).

The absorbance at 620 nm slightly decreased, while there was a parallel increase in absorbance around 350 nm. Additionally, the baseline of the post-treatment C-PC spectra showed a continual increase from higher to lower wavelengths. These spectral changes indicate the partial denaturation of C-PC, with the PCB chromophore transitioning from a linear to a more cyclic conformation. Similar findings were reported when HP treatment was used to extract C-PC from *Spirulina* biomass, where C-PC absorbance at 620 nm significantly decreased under 4,000 and 6,000 bar after only 3.5 min.³⁶ Other studies indicate that, at pH 7, C-PC's colour is better preserved during HP treatment than during thermal treatment, even when C-PC is in solution.^{37,38}

It is important to note that, in our study, HP treatment was conducted at pH 4, as this pH prevents leakage of encapsulated C-PC. Given C-PC's lower stability at this pH and the prolonged duration of HP treatment, our results imply that alginate encapsulation provides substantial stabilization for C-PC during HP processing.

Interestingly, after thermal treatment, the alginate beads appeared more compact (less swollen) than after pressure treatment. This could be due to the increased

storage modulus of calcium alginate gels at higher temperatures, which suggests greater gel stiffness.³⁹ In contrast, the electrostatic interactions that dominate alginate gels made by Ca^{2+} may be disrupted under HP conditions. The pressure release could induce gel network restructuring, leading to a higher degree of swelling compared to the beads before pressure treatment.

Storage stability of C-PC encapsulated in alginate beads

To test storage stability, alginate beads with encapsulated C-PC were placed in a glass beaker without buffer and left to dry at 4 °C for one month. After a month, the beads appeared flattened and adhered to the bottom of the beaker, with a dark blue appearance. Upon adding the phosphate buffer (pH 7), the beads quickly swelled, releasing the encapsulated C-PC. This rapid release may be due to structural damage and surface cracks in the alginate caused by drying at low temperatures.²⁴

The absorbance spectrum of the released C-PC was nearly identical to that of the control sample (Fig. 6), with only a minor increase in absorbance around 350 nm, indicating that the structure of the encapsulated C-PC remained largely intact during prolonged storage. These results suggest that in addition to freeze-drying,²⁰ alginate encapsulation is effective for stabilizing C-PC when dried at 4 °C for at least a month.

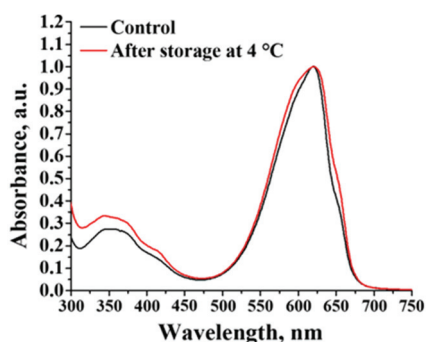


Fig. 6. Absorbance spectra of encapsulated C-PC obtained before (control sample) and after incubation of dried alginate beads at 4 °C for one month.

Mercury removal by alginate beads containing C-PC

C-PC has been shown to bind mercury ions with high affinity in the pH range from 5 to 10.¹¹ Considering that, in our study, alginate encapsulation of C-PC was most efficient at pH 4, we tested the potential for mercury removal by the immobilized C-PC at this pH. The fluorescence intensity of free C-PC in solution decreased as increasing concentrations of Hg^{2+} were added, indicating an interaction between C-PC and Hg^{2+} (Fig. 7A). The affinity constant was calculated from the obtained fluorescence spectra, yielding a value of $4 \times 10^6 \text{ M}^{-1}$ (Fig. 7B), confirming that free C-PC binds Hg^{2+} with high affinity. When encapsulated C-PC was incubated with Hg^{2+} solution for two days at room temperature with

constant shaking, more than 97 % of the mercury was removed from the solution (Fig. 7C). The control samples consisting of empty alginate beads had almost no effect on mercury binding, demonstrating that the presence of C-PC is crucial for mercury removal.

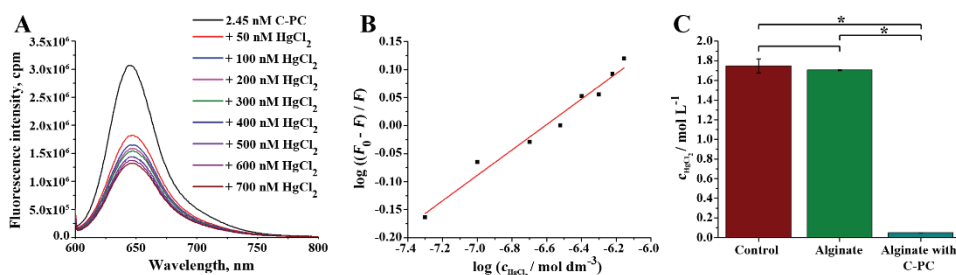


Fig. 7. A) Fluorescence spectra of C-PC in the presence of increasing concentrations of Hg²⁺. B) Plot used to calculate the affinity constant (K_a) from Eq. (1). C) Concentration of Hg²⁺ in solution without alginate beads (control), after incubation with empty alginate beads and with alginate beads containing encapsulated C-PC. Statistically significant differences between groups, $p < 0.05$, are marked with the “*” symbol.

In a previous study, C-PC immobilized onto the surface of chitosan was also effective for mercury removal, with binding occurring within 1 h.²⁵ Since alginate encapsulation entraps C-PC inside the alginate matrix, without direct exposure to metal ions, Hg²⁺ must diffuse into the alginate beads for binding to occur, which prolonged the process. This is why we chose a 2 day-incubation period in this study. Additionally, we tested the beads made with 0.5 % alginate, which created a more porous structure and sped up the diffusion process.

Adsorption as a method for heavy metals removal is widely studied due to its operational simplicity, reusability potential and efficiency.⁴⁰ Choosing an efficient, low-cost system is critical. Thus, only partial purification of C-PC was performed in this study. Further purification of C-PC could enhance the system's mercury removal efficiency, though it would also significantly increase the cost of the encapsulated material. While using encapsulated C-PC in alginate for heavy metal removal shows promise, further research is needed to optimize this approach.

CONCLUSION

In this study, the immobilization of C-PC through alginate entrapment was optimized. The primary factor controlling C-PC leakage from alginate beads was pH, with pH of 4 being the most favourable, showing almost no C-PC leakage. Under these conditions, the beads retained an intense blue colour, indicating that the structure of C-PC is preserved, as confirmed by absorption spectroscopy. The alginate immobilization provided significant stabilization for C-PC during both high-temperature and high-pressure treatments. Additionally, the stabilization at

low pH was observed, as beads prepared in acidic conditions released blue C-PC when placed in a neutral buffer, suggesting the preservation of C-PC structure to some extent. The shelf life of the immobilized C-PC was also enhanced and the alginate/C-PC beads demonstrated efficient mercury ion removal at pH 4, achieving the 97 % removal rate under tested conditions. The results suggest that alginate is an effective medium for immobilizing C-PC at pH 4, enhancing its stability and expanding its potential applications in the food industry and bioremediation efforts.

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ИЗВОД

ОПТИМИЗАЦИЈА АЛГИНАТНЕ ИМОБИЛИЗАЦИЈЕ С-ФИКОЦИЈАНИНА ДОБИЈЕНОГ ИЗ ХРАНЕ: СТРУКТУРНА И ФУНКЦИОНАЛНА КАРАКТЕРИЗАЦИЈА

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С-фикоцијанин (С-РС) представља значајну компоненту биомасе цијанобактерије *Arthrospira platensis* (*Spirulina*). Осим своје нутритивне вредности, овај протеин испољава бројне корисне биолошке активности. Ковалентно везана хромофора, фикоцијанолин, даје С-РС плаву боју, што омогућава његову употребу као природне боје за храну. Поред тога, фикоцијанолин испољава различита биоактивна својства, укључујући активности везивања метала. Кључни недостатак шире индустријске примене С-РС је његова лоша стабилност. Недавно су алтернативне формулације хране које користе природне полимере као носаче и активне компоненте привукле значајну научну пажњу. Овај рад описује оптимизоване услове за имобилизацију С-РС коришћењем алгината. Структурна стабилизација имобилизованог С-РС је испитана под високом температуром (60 °C) и високим притиском (450 МПа). Стабилност складиштења имобилизованог С-РС у осушеним куглицама алгината је тестирана држањем узорка на 4 °C током једног месеца. Такође је испитана потенцијална примена имобилизованог С-РС за уклањање јона живе. Имобилизација алгината се показала ефикасном у стабилизацији С-РС, значајно чувајући његову структуру током дужег складиштења, термичке обраде и излагања високом притиску. У тестирањима условима, 97 % Hg²⁺ је уклоњено имобилизованим С-РС. Свеобухватно, ова студија је оптимизовала процедуру за побољшање стабилности С-РС алгинатном имобилизацијом и проширила његову потенцијалну примену у индустрији хране и биоремедијације.

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Electrochemical synthesis and anticancer inhibitory effect of copper(II)–diclofenac/decanoic acid complexes on MCF 7 breast cancer cells

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Abstract: In this study, the Cu(II)–diclofenac/decanoic acid (Cu(II)–DF/DA) (copper(II) 2-[2-(2,6-dichloroanilino)phenyl]acetamide-decanoate) complex was synthesised using the electrochemical method by oxidising a Cu anode to release Cu²⁺, with graphite and KNO₃ serving as the cathode and supporting electrolyte, respectively. The synthesised Cu(II)–DF/DA complex underwent characterisation using ATR-FTIR, NMR, XRD and UV–Vis, confirming the success of the electrochemical synthesis. Surface morphology and particle size analyses using FESEM and TEM revealed that the synthesised Cu(II)–DF/DA complex possesses a thread-like structure with an average particle size of 4.77±1.77 nm. Subsequently, the synthesised complex was used to assess the anticancer inhibitory effects on human breast cancer (MCF 7) and normal human breast epithelial (MCF 10A) cells. The treatment of MCF 7 cancer cells with Cu(II)–DF/DA at concentrations of 25 and 100 µmol L^{−1} resulted in a significant reduction in cell viability, with only 18 and 7 % of cells remaining viable after 72 h, respectively. In contrast, nearly 90 % of MCF 10A cells remained viable at comparable concentrations. This suggests that the synthesised Cu(II)–DF/DA shows potential as an effective and selective anticancer agent, being toxic to cancer cells while displaying lower toxicity to normal cells.

Keywords: copper(II) complex; diclofenac; electrochemical synthesis; fatty acid; nanoparticle; anticancer.

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INTRODUCTION

In recent years, the focus of chemotherapeutic research has increasingly shifted towards the investigation of non-platinum-based compounds as potential alternatives. This shift stems from the need to develop new metal-based anticancer drugs with lower toxicity. Cu has attracted considerable interest as a viable candidate due to its expected lower toxicity relative to platinum compounds. Moreover, copper complexes are compelling because of their ability to induce DNA damage, a property linked to their biologically accessible redox potential and strong affinity for nucleobases. Since the 1970s, a variety of therapeutic ligands – including thiosemicarbazones (TSCs), imidazoles and phosphines – have been incorporated into copper complexes to assess their anticancer potential.¹ According to Renfrew, the effectiveness of metal-based anticancer agents hinges on both the central metal atom and the bioactive ligand within the complex.² This is because the chemical and biological properties of the complex are significantly influenced by the donor atoms of the bioactive ligands and their characteristics.³

Diclofenac (DF, Fig. 1), also known by its IUPAC name, 2-[2-(2,6-dichloro-anilino)phenyl] acetic acid, a widely used nonsteroidal anti-inflammatory drug (NSAID), is primarily known for its efficacy in treating pain and inflammation. Chemically, DF is a phenylacetic acid derivative, characterised by a benzene ring substituted with two chlorine atoms and an acetic acid moiety. Beyond its conventional use as an anti-inflammatory agent, recent research has highlighted DF's potential anticancer properties.^{4–6} The ability of DF to induce apoptosis and inhibit cell proliferation in various cancer cell lines has garnered significant attention. The anticancer effects of DF are believed to stem from its interference with the COX-2 enzyme, which is often over expressed in tumour cells and is associated with cancer progression and metastasis. According to a study conducted by Poku *et al.* (2020),⁷ the complex of DF with docosahexaenoic acid (DHA), a type of polyunsaturated fatty acid, has greater anticancer activity than their parent compound alone and the complex can be further explored for the complementary treatment of lung cancer.

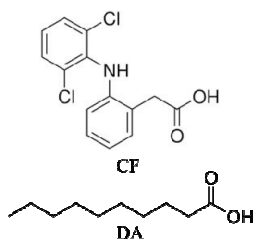


Fig. 1. Structure of DF and DA.

Fatty acids are naturally occurring monocarboxylic acids characterised by long hydrocarbon chains, which can be either saturated or unsaturated. Research has so far shown that fatty acids themselves possess antibacterial, antifungal and

anticancer properties.^{8–10} These effects are likely due to the physical interaction of fatty acids with cell membranes, where they may alter membrane permeability by interacting with the phospholipid bilayer.¹¹ The already published studies have also demonstrated that modifying fatty acids with existing anticancer drugs can enhance tissue selectivity, potentially increasing the effectiveness of chemotherapy while reducing toxicity to normal cells.^{12,13} In this study, decanoic acid (DA, Fig. 1) will be used as the fatty acid component for chemical modification with an anticancer drug. The extensive literature exists on the *in vitro* cytotoxicity of DA. Narayanan *et al.* reported that DA inhibits the proliferation of human colorectal carcinoma (HTC-116), human skin epidermoid carcinoma (A-431) and human mammary gland adenocarcinoma (MDA-MB-231).¹⁴ Additionally, Nordin *et al.* found that incorporating DA as a ligand in Cu(II) complexes produced moderate cytotoxic activity against A549 and HeLa cell lines, with IC_{50} values of 15.85 and 20.89 μ M, respectively.¹⁵

Despite the promising cytotoxic activity of DF and fatty acids, there are relatively few studies on the chemical modification of DF with fatty acids available in the literature. This gap in research gave the idea for the present study, where DF was selected as the non-steroidal anti-inflammatory drug component for conjugation with decanoic acid (DA). The synthesised DF/DA was used as a ligand in the direct electrochemical synthesis of a Cu(II)–DF/DA complex. The synthesised complexes were characterised using various techniques, including ATR-FTIR, UV–Vis, NMR, XRD, FESEM-EDX and TEM. The synthesised complexes were then evaluated for their anticancer inhibitory effects on MCF 7 and MCF 10A cell lines.

EXPERIMENTAL

Chemicals and cell culture

All chemicals used were analytical grade and used without further purification. Diclofenac (DF) with 98 % purity, methanol and decanoic acid (DA) with 99 % purity were purchased from Alfa Aesar Company. Cu foil (99 % purity), ethanol (95 % purity), benzotriazole-1-yl-oxy tris (dimethylamino) (BOP reagent) with 97 % purity, dimethyl-*d*₆-sulfoxide, potassium nitrate (KNO₃), methanol and sulphuric acid were supplied from Sigma Aldrich. Acetone was purchased from EMSURE(R) ACS. Triethylamine (Et₃N) and anhydrous magnesium sulphate (MgSO₄) were supplied from Acros Organics. Dichloromethane (CH₂Cl₂), dichloromethane (extra dry), diethyl ether (99 % purity), silica chromatography and sodium hydroxide were purchased from Fisher Chemical. Human breast epithelial cell lines (MCF 10A) and human breast cancer cell lines (MCF 7) were obtained from the American Type Culture Collection (ATCC). The MCF 10A and MCF 7 cell lines were prepared in Dulbecco's modified eagle medium: nutrient mixture F-12 (D-MEM/F-12). The D-MEM/F-12 medium solution was supplemented with 1 % antibiotic (100 units mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin) and 5 % of fetal bovine serum (FBS). All cell culture reagents were purchased from Thermo Fisher Scientific.

Synthesis of DF/DA compound

The stock solution of DF and DA was prepared separately by dissolving DF (0.0740 g) and DA (0.0431 g) in 24 °C ethanol to prepare 0.01 mol L⁻¹ of concentration each. Then, a 25 mL of each stock solution was added to a 100 mL beaker. After that, 25 mL of dried CH₂Cl₂, 0.27 g of BOP reagent and 15 mL of Et₃N were added into the mixed solution. At 22 °C, the resulting solution was mixed for 2 h. After obtaining the reaction mixture, 15 mL of 0.67 % HCl_{aq} solution was added into 100 mL beaker. Then, it was extracted using CH₂Cl₂ (2×25 mL). To dry the combined organic layers, a half spatula of anhydrous MgSO₄ was added after the combined organic layers had been rinsed with 1 % HCl_{aq} solution (4×25 mL) and ultrapure water (2×25 mL). The product was then extracted using column chromatography on silica gel with a mixture of 95 % CH₂Cl₂ and 5 % methanol as the eluent. The final product was rinsed with 2 mL of diethyl ether.

Electrochemical synthesis of Cu(II)–DF/DA

An electrochemical synthesis technique was used to produce the Cu(II)–DF/DA complex. As indicated in Fig. 2, the electrolysis used a Cu foil (3×2 cm, 0.1 cm thickness) as the anode and a graphite rod as the cathode with a supporting electrolyte solution of 0.01 mol L⁻¹ KNO₃. The synthesis was carried out for 6 h using an applied voltage of 1 V. Prior to the synthesis, both the anode and the cathode were rinsed with a small amount of acetone and ultrapure water. The electrochemical synthesis was conducted using a Twintex (TP-2305TK) direct current (DC) power source. The stock solutions of previously prepared DF/DA compound were prepared by dissolving a certain amount of the compound in methanol while KNO₃ solution was prepared by dissolving the salt in ultrapure water. Then, both solutions were mixed with the volume ratio of 1:1 into the simple and undivided electrolysis cell in the 100 mL of beaker. The reaction was carried out at a temperature of 22–23 °C, with a speed of 500 rpm and initial pH 4.5. The synthesis of Cu(II)–DF/DA required 6 h to be completed. As a result, the Cu(II)–DF/DA complex will formed as a blue precipitates and will be filtered using filter paper and washed with ultrapure water and acetone several times to remove the unreacted compound, electrolyte and impurities. The precipitate was then dried in oven at 80 °C for 1 h. Melting point of DF/DA ranges 145–150 °C and Cu(II)–DF/DA 220–225 °C.

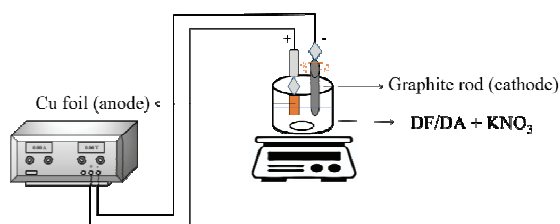


Fig. 2. Schematic diagram of the experimental set-up.

Characterisation of Cu(II)–DF/DA and DF/DA compounds

The electrochemically synthesised Cu(II)–DF/DA complex was then characterised using attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-FTIR, Perkin Elmer 1310) in the range of 4000 to 600 cm⁻¹ and UV–Vis spectrophotometer (Perkin Elmer Lambda 35) in the wavelength range of 200 to 800 nm in a 10 mm quartz cuvette in ultrapure water as a reference solvent at room temperature. In addition, proton and carbon-13 nuclear magnetic resonance (¹H- and ¹³C-NMR) were carried out using the Bruker-Avance III 500

with a 500 MHz adjustment while the elemental composition of the synthesised complex was determined with an energy dispersive X-ray (EDX, Hitachi-Regulus 8220, Tokyo, Japan) with. Transmission electron microscopy (TEM, Zeiss-Libra 120, Oberkochen, Germany) with a 100 kV accelerating voltage was used to observe the nanoparticles' size and shape. Applying a drop to the grid and letting the solvent (ethanol) evaporate in the environment, the samples were prepared on carbon-coated copper grids covered with a polyvinyl formal polymer. Furthermore, an X-ray diffractometer (XRD, D8 Advance, Bruker, Billerica, MA, USA) was used to confirm the crystalline structures of the pure DF, DA and synthesised Cu(II)-DF/DA complex.

Inhibition studies

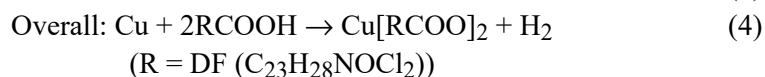
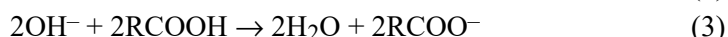
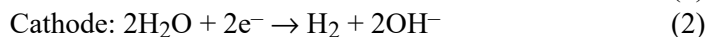
To prepare a stock solution of tested drugs (Cu(II)-DF/DA complex, DF/DA and DF (control)), the tested compounds were dissolved separately in a certain volume of dimethyl sulfoxide (DMSO). Then, mixed the 10 % of drugs and 90 % of media into the each well plate. The cells were seeded at a density of 1×10^5 cells per mL in a 96-well flat-bottomed microplate. After that, the cells were incubated at 37 °C in a 5 % CO₂ environment at different hours (24, 48 and 72 h). The cell viability assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used to examine the inhibition effect on MCF 10A and MCF 7 cells upon treatment with three different compounds (DF/DA, Cu(II)-DF/DA and DF (control)) at two different concentrations (25 and 100 $\mu\text{mol L}^{-1}$). The medium in each well was removed when the cells were 70 to 80 % confluent and it was replaced with a medium containing the drugs above with two different concentrations, which are 25 and 100 $\mu\text{mol L}^{-1}$. When the treatment period was over, the medium that contained the drugs was discarded and a fresh medium that included 10 μL of MTT reagent was added to each well. The described method was performed three more times. After that, the optical density (OD) of the cells was determined using the FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) at wavelengths from 570 to 620 nm.

RESULTS AND DISCUSSION

Synthesis of DF/DA and Cu(II)-DF/DA

Fig. 3 shows the proposed chemical reactions used in the synthesis of DF/DA compound. According to Chrzanowska et al., the interaction of DF drug and DA can only occur on the NH moiety of drug molecules by forming an amide bond with the carbonyl group of DA.¹³

Eqs. (1)–(4) describe the mechanism responsible for the electrochemical technique of Cu(II)-DF/DA:



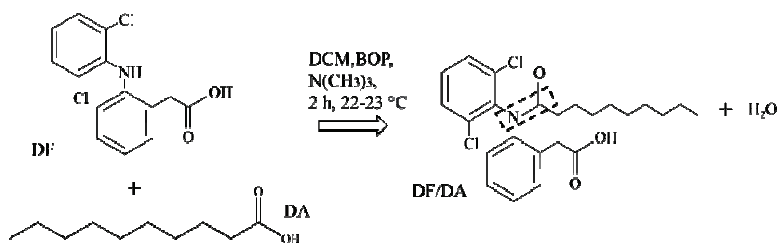


Fig. 3. Proposed chemical reaction of DF/DA synthesis.

According to the mechanism in Eq. (1), the release of Cu^{2+} from electrochemical oxidation of the Cu anode is required for the formation of Cu(II)–DF/DA complex. The electrolysis conditions should be optimised in order to get the optimal conditions for the synthesis of Cu(II)–DF/DA complex. The electrolysis conditions investigated included the type of the applied voltage, the supporting electrolyte concentration and the pH solutions. Therefore, it is expected that this optimisation research will produce the most Cu(II)–DF/DA and the least amount of Cu anode waste.

The positively charged Cu anode was electrochemically oxidised in the aqueous phase to generate Cu^{2+} (Eq. (1)). At the negatively charged graphite cathode, OH^- was formed by water reduction (Eq. (2)) and immediately interacted with DF and DA to produce $\text{C}_{23}\text{H}_{28}\text{NOCl}_2\text{COO}^-$ (Eq. (3)) in aqueous phase. The interaction of Cu^{2+} with DF/DA ions led to the formation of a blue precipitate of Cu(II)–DF/DA complex in the organic phase (Eq. (4), Fig. 4).

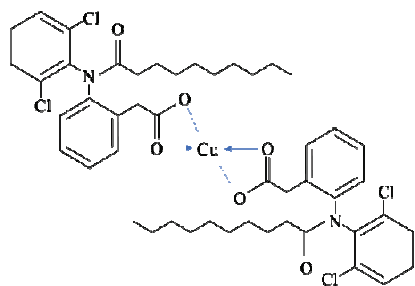


Fig. 4. Proposed structure of Cu(II)–DF/DA.

Characterisation of DF/DA and Cu(II)–DF/DA

ATR-FTIR spectroscopy. The ATR-FTIR spectrum of DF (Fig. 5) revealed an absorption peak at 3400 cm^{-1} , corresponding to the secondary amino group.¹⁶ Additionally, strong peaks observed at 1610 and 1570 cm^{-1} were attributed to the $\text{C}=\text{O}$ stretching and the $\text{C}=\text{C}$ stretching in the aromatic ring, respectively, within the DF structure.¹⁷ In the ATR-FTIR spectrum of DA, a prominent peak at 1698 cm^{-1} was identified, representing the stretching vibration of the carboxyl group in the fatty acid.¹⁵ Moreover, several peaks in the $2800\text{--}3000\text{ cm}^{-1}$ range indi-

cated the asymmetrical (ν_{as}) and symmetrical (ν_s) stretching vibrations of the methyl and methylene groups in the DA carbon chain, respectively.¹⁸ Similar peaks present in both the DF and DA spectra were also observed in the DF/DA spectrum (Fig. 5), confirming the formation of the DF/DA conjugate. The disappearance of the secondary amino group peak at 3400 cm^{-1} in the DF spectrum suggested the successful formation of an amide bond between the secondary amine in DF and the carbonyl group in DA, indicating that the DF/DA conjugate was successfully synthesised. After the formation of the Cu(II)–DF/DA complex, the ATR-FTIR spectra still exhibited several characteristic peaks of the DF/DA compound.

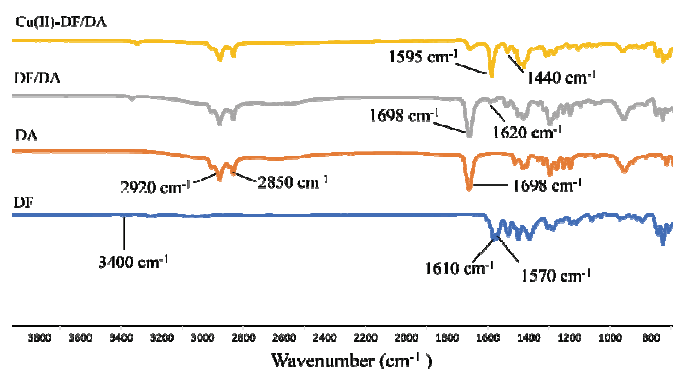


Fig. 5. ATR-FTIR spectra of: a) DF, b) DA, c) DF/DA and d) Cu(II)–DF/DA.

NMR spectroscopy. The ^1H -NMR spectrum of DF/DA (Fig. 6) revealed that all of the proton peaks could be observed in both the DF (Fig. S-1 of the Supplementary material to this paper) and the DA (Fig. S-2 of the Supplementary material) spectrum. Furthermore, this study observed the same proton peaks as those found in earlier studies by Yoko *et al.*¹⁹ (^1H -NMR spectrum of DA) and Suhara *et al.*²⁰ (^1H -NMR spectrum of DF) when compared. The proton that connects to the nitrogen atom in the DF structure, established as “a” in Fig. S-1 at δ_{H} of 12 ppm, was not identified in the DF/DA compound’s NMR spectrum. This is due to the fact that a proton has been substituted by the carboxyl group of DA. The OH group from the carboxyl group of DA generated water molecules with the proton that linked to the nitrogen atom in DF. As a result, at the end of the reaction, the carboxyl group of DA is attached to the nitrogen atom, forming a tertiary amide in the DF/DA compound and this reaction agrees with the proposed structural formula of the DF/DA compound that is shown in Fig. 6.

The presence of carbon in the carbonyl group of the fatty acid group was revealed by the results from the ^{13}C -NMR spectrum of DF (Fig. S-3 of the Supplementary material) and DA (Fig. S-4 of the Supplementary material), which were presented at δ_{C} of 172 and 176 ppm, respectively. These two peaks show

simultaneously in the spectrum of the DF/DA compound (Fig. 7), indicating that the complex contains two carbonyl groups. The carbon signals appear before δ_c of 40 ppm in the DF/DA spectrum, indicating the presence of different methyl groups, as shown in the DA spectrum, whereas carbon signals appear after δ_c of 110 ppm in the DF spectrum, indicating the presence of carbon in aromatic rings. Therefore, based on the ^1H - and ^{13}C -NMR spectra, it can be concluded that the DF/DA molecule has been successfully synthesised.

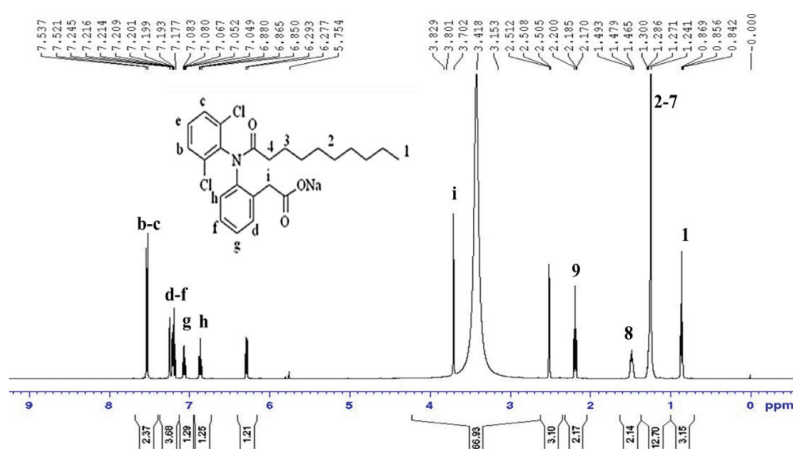


Fig. 6. ^1H -NMR spectrum of DF/DA.

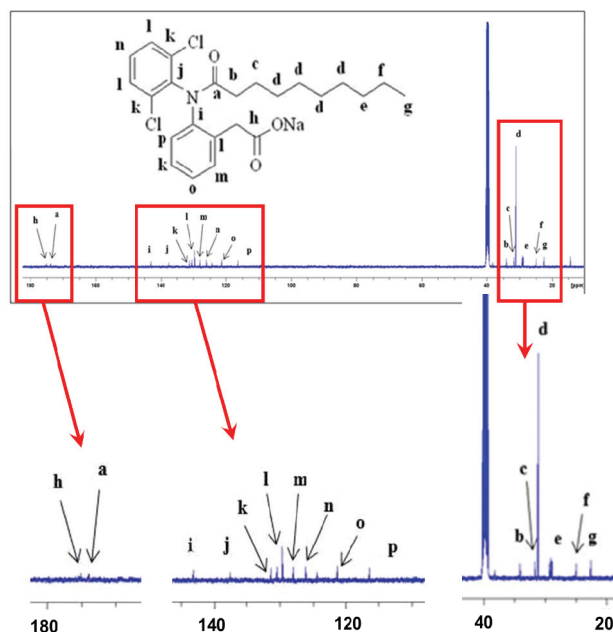


Fig. 7. ^{13}C -NMR spectrum of DF/DA compound.

UV-Vis spectroscopy. The UV-Vis spectrum of the Cu(II)-DF/DA complex shows the presence of two different absorption peaks (Fig. 8). A prominent signal in the UV region at 300 nm indicates the $n-\pi^*$ transition of the carbonyl group in the DF structure.²¹ Additionally, an intense peak that can be seen in the visible region at 674 nm (Cu(II)-DF/DA) indicates the d-d transition of Cu^{2+} , as a result of a transition from the filled d level (z^2 , xy , xz/yz) to the half-full d level (x^2-y^2).¹⁸ Without the complexation with the Cu metal centre, the UV-Vis spectrum of the DF/DA ligand displays a single absorption peak at a wavelength of 277 nm, corresponding to the $n-\pi^*$ transition of the carbonyl group in the DF structure. The shift of the UV-Vis absorption peaks of a ligand to longer wavelengths after the complexation with a transition metal centre is due to the electronic interactions between the ligand and the metal ion.¹⁸ The desired Cu(II)-DF/DA complexes have been successfully formed by the electrochemical technique that relies on the obtained spectra.

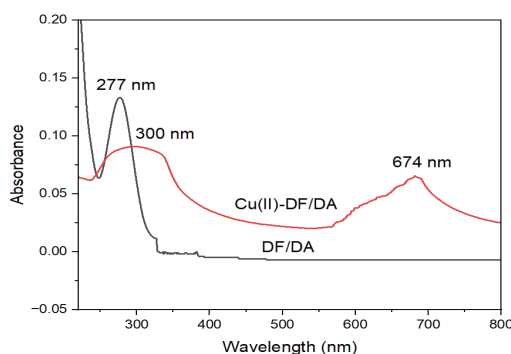


Fig. 8. UV-Vis spectra of Cu(II)-DF/DA and DF/DA compounds.

XRD. Fig. 9 shows the powder XRD patterns of DF, DA and electrochemically synthesized Cu(II)-DF/DA. All samples in Fig. 9 showed well-defined

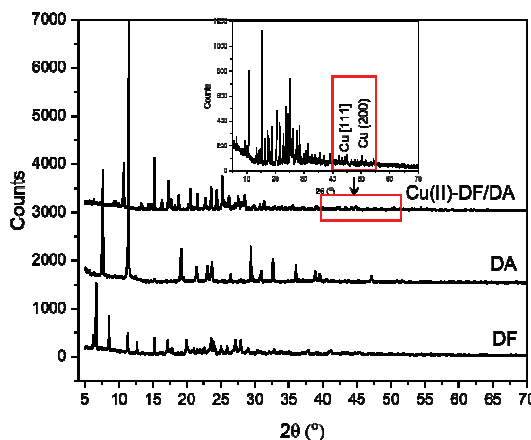


Fig. 9. XRD patterns of DF, DA and Cu(II)-DF/DA.

diffraction peaks thus proving the samples crystallized into layered structures. The main peaks indicated by the DA (at 7 and 11° at a lower angle)²² and DF (at 6, 8 and 15° at a higher angle)²³ diffractograms in this study are consistent with those reported in previous studies. Interestingly, the main peaks that exhibited the characteristic features of DF and DA are present in the Cu(II)–DF/DA diffractogram, indicating the presence of both compounds in the synthesised complex. Meanwhile, the presence of Cu in the synthesised complex is confirmed by the weak peaks observed at 44 and 50° at higher angle, corresponding to the (111) and (200) planes, respectively.

FESEM-EDX analysis. FESEM analysis was employed to investigate the surface morphology of synthesised Cu(II)–DF/DA complex. The micrograph obtained in Fig. 10a reveals that the synthesised complex exhibits a thin, thread-like structure with varying thicknesses. Meanwhile, EDX analysis was used to determine the elemental composition of the synthesised Cu(II)–DF/DA complex. The EDX spectrum in Fig. 10b demonstrated that Cu(II)–DF/DA complexes contain C, N, O, Cl and Cu. This corroborates the findings from the spectroscopy-based characterization described earlier, confirming the successful synthesis of the Cu(II)–DF/DA complex.

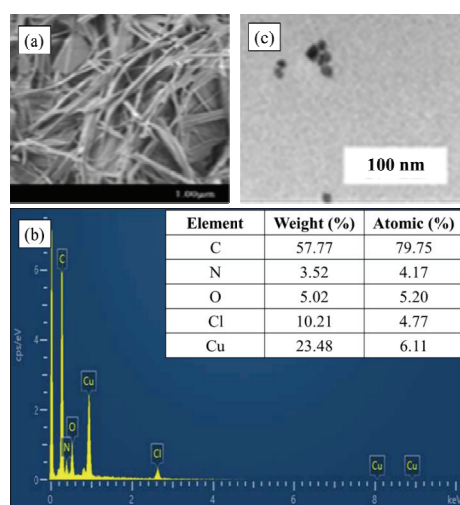


Fig. 10. a) FESEM micrograph, b) elemental composition using EDX and c) TEM micrograph of the synthesised Cu(II)–DF/DA.

Particle size. Fig. 10c illustrates the size of Cu particles in Cu(II)–DF/DA complex. When applying a low voltage (1 V) and supporting electrolyte concentration (0.01 mol L^{−1} KNO₃) during the electrochemical synthesis of Cu(II)–DF/DA, it yields smaller CuNPs with an average size of 4.77±1.77 nm. This phenomenon can be attributed to the slow release of Cu²⁺ from the electrochemical oxidation of Cu anode into the bulk solution containing DF/DA ligand when using a low applied voltage and supporting electrolyte concentration thereby

resulting in smaller Cu particle sizes. This study has shown that the particle size of the synthesised product can be controlled by controlling the applied voltage and supporting electrolyte concentration during the synthesis process as reported by previous researchers.^{24–27}

Anticancer inhibitory effect of Cu(II)–DF/DA on MCF 7 and MCF 10A cells

The purpose of this study is to assess the inhibitory effect of Cu(II)–DF/DA on MCF 7 cells (tumour cells) and MCF 10A cells (normal cells) using the MTT assay. It was conducted to evaluate the efficacy of suppressing the tumour cell proliferation while simultaneously minimizing toxicity on normal cells following treatment with Cu(II)–DF/DA. Nevertheless, it should be highlighted here that this study focuses on the inhibitory effects of the synthesised Cu(II)–DF/DA complexes on selected cell lines at concentrations of 25 and 100 $\mu\text{mol L}^{-1}$ to assess how these concentrations affect cellular growth rather than their cytotoxic effects. The inhibitory effects of the Cu(II)–DF/DA on both cell types were depicted in Fig. 11. As a control, the inhibitory effects of the commercial DF sample and DF/DA ligand were also examined to compare the inhibitory efficiency of synthesised Cu(II)–DF/DA with that of the commercial DF and the DF/DA ligand only. Based on Fig. 10a, there was a greater inhibitory effects of Cu(II)–DF/DA on MCF 7 cells when the incubation period was extended from 24 to 72 h. The following treatment with Cu(II)–DF/DA at a concentration of 25 $\mu\text{mol L}^{-1}$, the percentage of viable cells are reduced. After 72 h of treatment, Cu(II)–DF/DA showed the strongest inhibitory effect among the samples, with significantly lower percentage of viable cells (18 %) than DF and DF/DA molecules. This suggests that the formation of complexes between Cu and the DF/DA ligand increased the toxicity of the synthesised complexes against selected cancer cells. Interestingly, after 72 h of incubation, approximately 90 % of the MCF 10A normal cells remained alive upon treatment with the tested samples, indicating low inhibitory effects and less toxicity towards normal MCF 10A cells (Fig. 11a).

The results presented in Fig. 10b demonstrate that the inhibitory effects on the selected cancer cells are enhanced when the tested sample concentration increases from 25 to 100 $\mu\text{mol L}^{-1}$. In addition, low percentage of viable cells was detected when compared to the treatment utilising a 25 $\mu\text{mol L}^{-1}$ of Cu(II)–DF/DA sample. When treated with 100 $\mu\text{mol L}^{-1}$ of Cu(II)–DF/DA, the percentage of viable cells reduced from 18 (Fig. 10a) to 7 % (Fig. 11b), indicating that synthesised complex had the most inhibitory impact among the samples on MCF 7 cells. Notably, following a 72 h treatment with 100 $\mu\text{mol L}^{-1}$ of all evaluated drugs, the vitality of normal MCF 10A cells continued to be above 90 %. Thus, there was no toxicity observed towards MCF 10A cells when the sample concentration was increased to 100 $\mu\text{mol L}^{-1}$. Importantly, the Cu(II)–DF/DA complex

demonstrated selective anticancer properties, significantly reducing cell viability in MCF7 cancer cells while showing minimal toxicity to normal MCF10A cells, suggesting its potential as an effective and targeted anticancer agent. These findings open the door for further research into copper-based complexes as selective cancer therapies with reduced side effects on healthy cells.

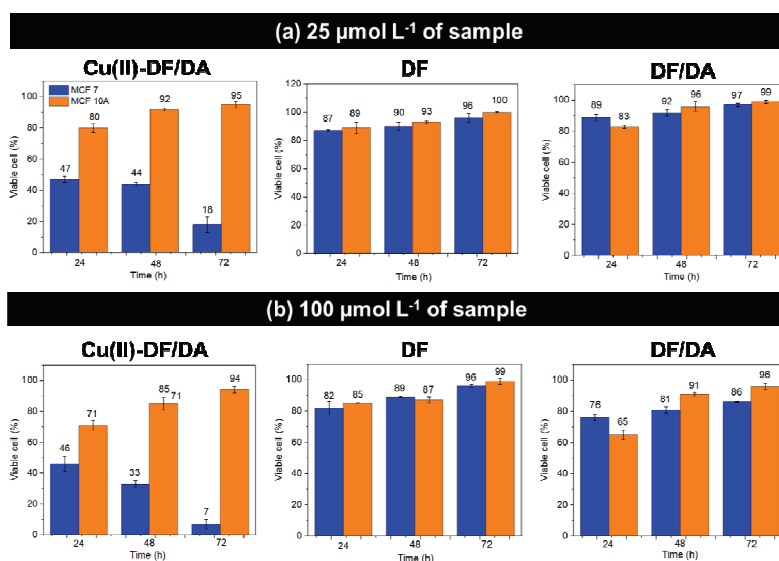


Fig. 11. Percentage of viable cells at different incubation times upon treatment with Cu(II)-DF/DA, DF and DF/DA at different concentrations.

CONCLUSION

The Cu(II)-DF/DA complex was successfully synthesised using an electrochemical approach, with the DF/DA combination serving as the ligand and Cu^{2+} as the metal centre. To confirm the formation of the desired complex, both the DF/DA compound and the Cu(II)-DF/DA complex were characterised using ATR-FTIR, NMR, XRD and UV-Vis spectroscopy. The ATR-FTIR spectra of both the DF/DA compound and the Cu(II)-DF/DA complex exhibited the presence of all functional group peaks from the raw materials. NMR analysis further confirmed that the chemical shifts of hydrogen and carbon peaks in both the compound and the complex were consistent with those of DF and DA, respectively. Additionally, EDX analysis verified the presence of Cu in the complex, indicating that Cu served as the metal centre, forming a chemical bond with the ligand. Surface morphology and particle size analyses using FESEM and TEM revealed that the synthesised Cu(II)-DF/DA complex possesses the thread-like structure with an average particle size of 4.77 ± 1.77 nm. Regarding the anticancer activity, treatment of MCF 7 cancer cells with Cu(II)-DF/DA at concentrations

of 25 and 100 $\mu\text{mol L}^{-1}$ resulted in a significant reduction in cell viability, with only 18 and 7 % of cells remaining viable after 72 h, respectively. Contrary to the given facts, nearly 90 % of normal MCF 10A cells remained viable at comparable concentrations. These findings suggest that the synthesised Cu(II)–DF/DA complex, with its small particle size of 4.77 ± 1.77 nm, is less toxic to certain normal cells, while effectively inhibiting the proliferation of selected cancer cells. Future studies could explore a wider range of Cu(II)–DF/DA concentrations to calculate the IC_{50} values and selectivity index for a more comprehensive understanding of the Cu(II)–DF/DA complexes' potency and selectivity, which would provide a more detailed assessment of their potential for targeted cancer therapy.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13059>, or from the corresponding author on request.

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ИЗВОД

ЕЛЕКТРОХЕМИЈСКА СИНТЕЗА И АНТИКАНЦЕРОГЕНИ ИНХИБИТОРНИ ЕФЕКАТ КОМПЛЕКСА БАКАР(II)–ДИКЛОФЕНАК/ДЕКАНСКА КИСЕЛИНА НА ЋЕЛИЈЕ КАРЦИНОМА ДОЈКЕ MCF-7

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У овој студији, комплекс бакар(II)–диклофенак/деканска киселина (Cu(II)–DF/DA) (бакар(II) 2-[2-(2,6-дихлороанилино)фенил]ацетамид-деканат) синтетисана је електрохемијском методом оксидацијом бакарне аноде ради ослобађања Cu^{2+} , док су графит и KNO_3 коришћени као катода, односно помоћни електролит. Синтетисани Cu(II)–DF/DA комплекс је окарактерисан техником ATR-FTIR, NMR, XRD и UV–Vis, чиме је потврђен успех електрохемијске синтезе. Анализа морфологије површине и величине честица помоћу FESEM и TEM показала је да синтетисани Cu(II)–DF/DA комплекс има структуру налик нитима, са просечном величином честица од 4.77 ± 1.77 nm. Након тога, испитан је антиканцерогени инхибиторни ефекат синтетисаног комплекса на ћелије карцинома дојке MCF-7 и нормалне епителне ћелије дојке MCF-10A. Третман MCF-7 ћелија са Cu(II)–DF/DA у концентрацијама од 25 и 100 $\mu\text{mol L}^{-1}$ довео је до значајног смањења ћелијске виталности, при чему је након 72 h преживело само 18, односно 7 % ћелија. Насупрот томе, готово 90 % MCF-10A ћелија остало је витално при истим концентрацијама. Ови

резултати указују да синтетисани Cu(II)–DF/DA комплекс има потенцијал као ефикасан и селективан антиканцерогени агенс, који показује токсичност према ћелијама карцинома, уз значајно мању према нормалним ћелијама.

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SUPPLEMENTARY MATERIAL TO
**Electrochemical synthesis and anticancer inhibitory effect of
copper(II)–diclofenac/decanoic acid complexes on
MCF 7 breast cancer cells**

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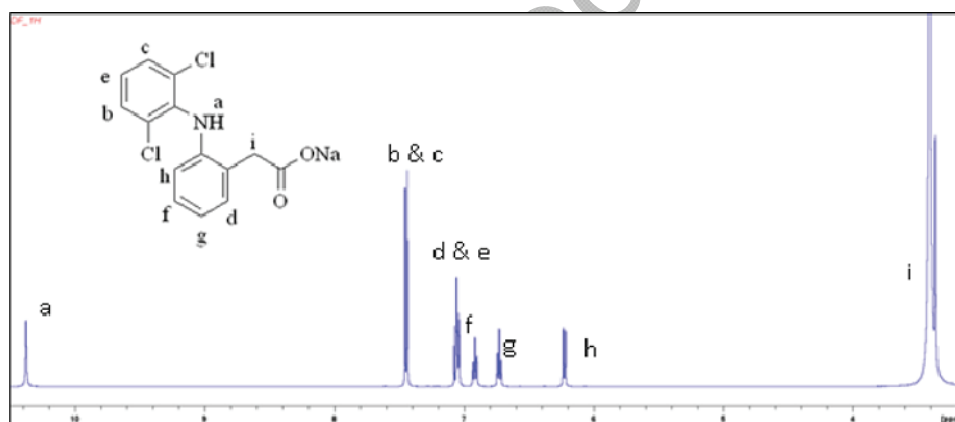
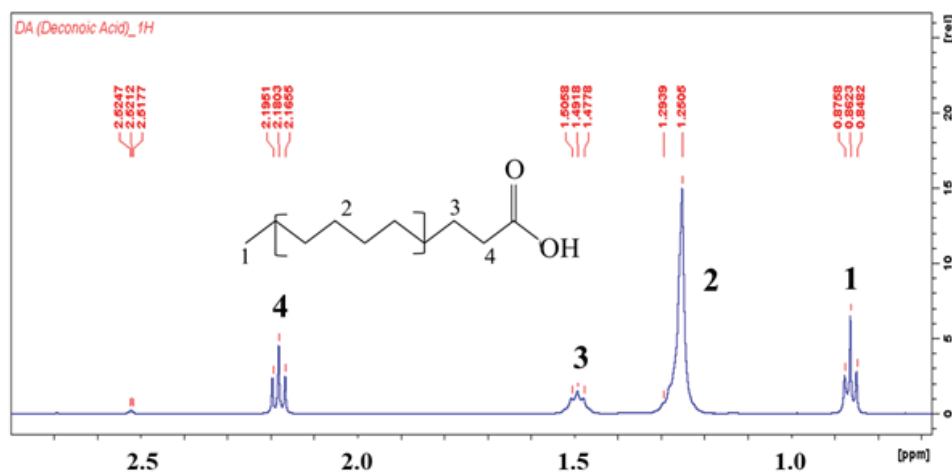
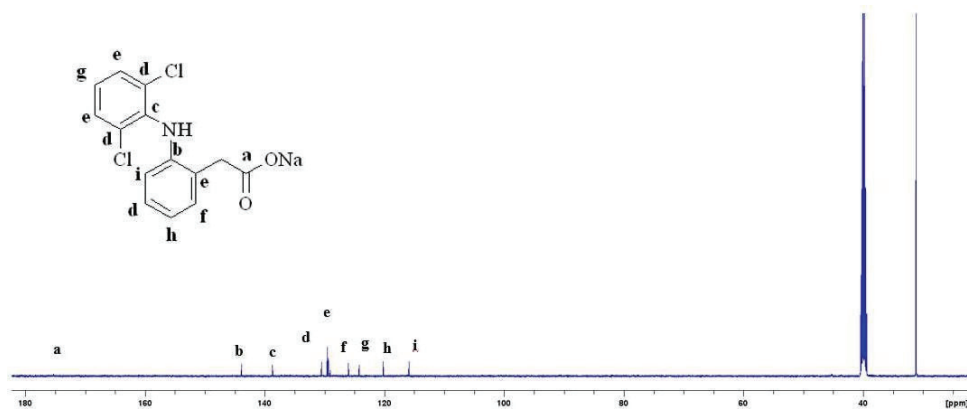
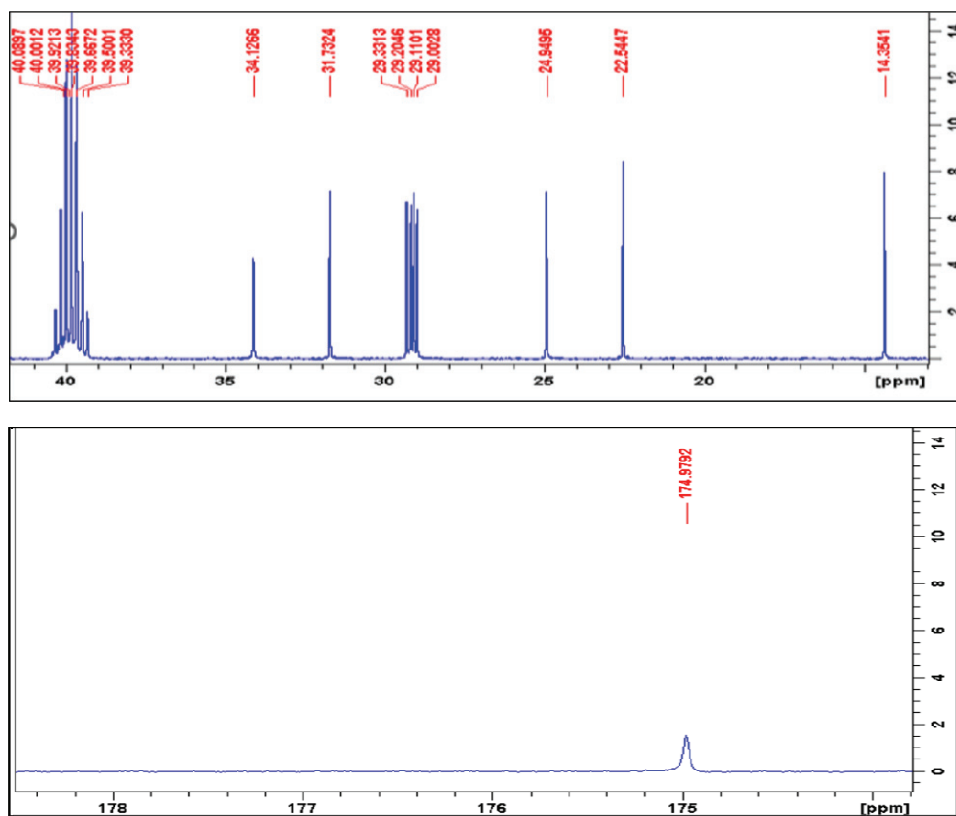


Fig. S-1. ¹H-NMR spectrum of DF.

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Fig. S-2. ^1H -NMR spectrum of DA.Fig. S-3. ^{13}C -NMR spectrum of DF.

Fig. S-4. ^{13}C -NMR spectrum of DA.



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Enhancing hydrogen evolution reaction using transition metal atoms on 6,6,12-graphyne: A DFT study

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Abstract: The escalating global population and the urgent need for sustainable energy solutions with minimal environmental impact have underscored the significance of developing renewable energy sources. Hydrogen, as an energy carrier and fuel, presents substantial advantages over other energy forms, alongside diverse applications in medical treatments and the production of critical chemicals like methane and methanol. Thus, hydrogen emerges as a potential alternative to fossil fuel, offering a stable and clean energy solution. In this study, we present a theoretical design of transition metal (TM) anchored 6,6,12-graphyne (GY) based catalysts for the hydrogen evolution reaction (HER) using density functional theory (DFT). Our findings reveal that among all evaluated systems, cobalt single-atom catalyst (SAC) anchored on 6,6,12-graphyne exhibit the highest thermodynamic stability and superior HER catalytic performance, with a remarkably low ΔG_{H^*} value of 0.042 eV. We have investigated the density of states, HOMOs, LUMOs, electron density and band structures of our designed SACs. This work provides a practical strategy for experimental groups to effectively tune the electronic structures of catalysts and enhance their catalytic activity.

Keywords: single-atom catalysts; density functional theory; HER.

INTRODUCTION

The global population has surpassed seven billion and is projected to exceed nine billion by the mid-21st century.¹ Correspondingly, energy consumption is anticipated to rise from 16 TW in 2010 to 23 TW by 2030 and potentially 30 TW by 2050.² The critical challenges posed by environmental issues and the extensive consumption of fossil fuels necessitate the development of high-efficiency, green, and sustainable energy technologies.³ Consequently, the demand for renewable energy sources has surged, as conventional non-renewable energy sources are finite. Renewable energy sources such as solar, wind, and thermal energy offer

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alternatives to traditional fossil fuels.⁴ However, the uneven distribution of these energy sources limits their large-scale applications.

Hydrogen is a clean and versatile energy carrier with significant potential as a sustainable alternative to fossil fuels in future energy systems.⁵ Hydrogen is particularly attractive due to its high gravimetric energy density (the amount of energy released per kilogram of fuel) and its environmentally friendly combustion, which produces only water. This makes hydrogen an ideal candidate for low-carbon energy applications.⁶

Currently, most H₂ is produced *via* steam reforming of fossil fuels. However, it can also be generated from renewable sources such as water and biomass, as well as non-renewable sources like hydrogen sulfide. Water, one of the most abundant resources on Earth, is a primary candidate for hydrogen production through water-splitting methods.^{1,7}

Hydrogen can be stored in various materials, including metal organic frameworks, owing to their high surface area, customizable structures, and versatile applications.⁸

The fundamental reactions in water splitting include the hydrogen evolution reaction (HER) and the oxygen evolution reaction (OER). The production and storage of hydrogen *via* electrochemical water splitting have garnered significant attention from scientific communities, industries, and governments worldwide. Under suitable conditions, water molecules can be decomposed into hydrogen and oxygen through various methods and energy inputs, including electrolysis powered by renewable energy sources.

One major challenge in this field is the design of electrocatalysts for HER that offer appropriate efficiency at a reasonable cost. Precious metals and their oxides have traditionally been used as water-splitting catalysts, but their high costs and scarcity hinder widespread commercialization.⁹ To overcome the high energy barrier of HER, an efficient catalyst is required.¹⁰ Single-atom catalysts (SACs) are gaining increasing attention due to their unique properties, including maximum atom utilization efficiency, low cost, high activity and high selectivity.^{11–15}

A novel class of 2D graphyne nanosheets, featuring suitable porous carbon structures with sp-hybridized carbon atoms, has been synthesized and proven effective for supporting single atoms.^{16–18} Graphdiyne, an allotrope of graphyne, has demonstrated promise as a nanomaterial for electrolysis^{12,19,20} and was synthesized by Lee's group.²¹ One type of graphyne, GY, exhibits remarkable properties that have led to various applications and considerable research interest. Numerous studies have sought cost-effective electrocatalysts based on non-precious transition metals, including Mo, Co, Ni, Fe and their complexes.^{22,23}

Tianwei *et al.* decorated graphdiyne with first-row transition metals (from Sc to Zn) and Pt, investigating their efficacy as electrocatalysts for water splitting. Their results showed that graphdiyne decorated with scandium, titanium, vana-

dium, iron and platinum exhibited high catalytic activity towards HER. Additionally, calculations indicated that graphdiyne decorated with platinum and nickel are promising bifunctional electrocatalysts for water splitting.¹² Faizan *et al.* used density functional theory to design and investigate first-row transition metal monoatomic catalysts supported on graphyne, concluding that Ni SAC supported on graphyne showed the highest thermodynamic stability and best HER catalytic performance with a ΔG_{H^*} value of 0.08 eV.²⁴ Uwamungu *et al.* studied the stability and electronic properties of iron-doped graphyne-like BN-yne nanosheets, finding that HER is significantly influenced by charge redistribution caused by iron on the surface, increasing the number of active sites. Thus, iron-doped graphyne-like BN-yne can be considered a promising electrocatalyst for HER.²³

Among the different types of graphyne structures, 6,6,12-graphyne (GY) stands out due to its significant thermal conductivity and directional anisotropy, attributed to its non-sixfold symmetric structure. Despite the promising characteristics of graphynes as electrocatalysts for water splitting reactions, GY has not yet been explored as an electrocatalyst for the HER. This research aims to fill this gap by employing density functional theory (DFT) computations to investigate the potential of GY, with single transition metal atoms (Fe, Co, Ni, Cu) anchored on its structure, to enhance HER catalytic activities. This study highlights the novelty of utilizing GY for HER, showcasing its unique properties and significant potential as a high-performance electrocatalyst.

COMPUTATIONAL METHOD

In this research, all calculations were performed using density functional theory (DFT) *via* the Dmol3 code (version 17.1.0.48).²⁵ The generalized gradient approximation (GGA) with the Perdew–Burke–Ernzerhof (PBE) functional was employed to describe exchange–correlation interactions.²⁶ The numerical double-numerical plus polarization (DNP) basis set, comparable in quality to 6-31G sets, was used for all atoms. The long-range van der Waals interactions were described using the empirical correction in Grimme’s scheme.²⁷ A convergence threshold of 10^{-6} Hartree* for electronic self-consistent field (SCF) calculations and 10^{-5} Hartree for total energy, 0.002 Hartree/Å for maximum force, and 0.005 Å for maximum displacement for geometry optimization were adopted. A $7 \times 7 \times 1$ Monkhorst–Pack k-mesh was used for sampling the Brillouin zone during the structural optimization of the supercell (2×2 unit cell) and for electronic structure calculations. To simulate the H₂O solvent environment with a dielectric constant of 78.54, a conductor-like screening model (COSMO) was employed in all calculations.²⁸

The intermediate states in HER (H^*) were investigated by exploring multiple initial configurations for hydrogen adsorption on both GY and TM@GY electrocatalysts. The subsequent analysis focused on the most energetically favorable adsorption structures, determined based on their minimum adsorption energies (ΔE_{ads}). These ΔE_{ads} values were calculated using the following equation:²⁹

$$\Delta E_{ads} = E_{\text{substrate+adsorbent}} - E_{\text{substrate}} - \Delta E_{\text{adsorbent}} \quad (1)$$

* 1 Hartree = 2625.5 kJ/mol

where $E_{\text{adsorbent}}$, $E_{\text{substrate}}$ and $E_{\text{substrate} + \text{adsorbent}}$ are the total energies of the adsorbent, the substrate, and the substrate-adsorbent composites, respectively. More negative ΔE_{ads} values indicate stronger binding between the catalyst and the intermediate, as well as better thermodynamic stability.

Hydrogen production from water splitting involves two steps: a) atomic H adsorption on the catalysts, and b) formation and release of H_2 molecules.³⁰ When one H atom is adsorbed on the catalyst, the calculated intermediate state energy determines the HER reaction barrier (overpotential for the electron). Consequently, HER performance can be assessed through ΔG_{H^*} . The following equation describes the production of H_2 :³¹



where * and H^* represent the active site and the adsorbed intermediate, respectively. Under standard conditions (pH 0, $U = 0$ and 298.15 K), the chemical potential of $\text{H}^+ + \text{e}^-$ ($\mu_{(\text{H}^+ + \text{e}^-)}$) is equivalent to that of $1/2\text{H}_2$ ($1/2\mu_{\text{H}_2}$). In other words, $\mu_{(\text{H}^+ + \text{e}^-)} = 1/2\mu_{\text{H}_2}$, based on the calculation hydrogen electrode model.³¹ The ideal ΔG_{H^*} value for HER is zero. Both weak and strong binding energy of the intermediate state can lead to poor HER efficiency. Therefore, ΔG_{H^*} is obtained by the following equation:²⁹

$$\Delta G_{\text{H}^*} = \Delta E_{\text{H}^*} - \Delta E_{\text{ZPE}} - T\Delta S_{\text{H}^*} \quad (3)$$

where ΔE_{H^*} is the energy of hydrogen adsorption, obtained from Eq. (1). ΔS_{H^*} is the entropy difference between the gas phase and the adsorbed state, and T is the temperature at 298 K. ΔE_{ZPE} is the zero-point energy difference between the H_2 gas phase and the adsorbed H, given by:³²

$$\Delta E_{\text{ZPE}} = E_{\text{ZPE}}^{\text{H}^*} - E_{\text{ZPE}}^* - \frac{1}{2}E_{\text{ZPE}}^{\text{H}_2} \quad (4)$$

Where ΔE_{ZPE} is the zero-point energy of the pristine substrate, $E_{\text{ZPE}}^{\text{H}^*}$ is the zero-point energy of adsorbed hydrogen on the substrate, and $E_{\text{ZPE}}^{\text{H}_2}$ is the zero-point energy of gas-phase H_2 . Due to the small calculated vibrational entropy of the adsorbed state H^* , the adsorption entropy of $1/2\text{H}_2$ is $\Delta S_{\text{H}} \approx -0.5 S_{\text{H}_2}^0$, where $S_{\text{H}_2}^0$ is the entropy of gas-phase H_2 , approximately $130 \text{ J mol}^{-1} \text{ K}^{-1}$ under standard conditions.^{33,34} According to Nørskov's hypothesis,²⁸ the overpotential of the hydrogen evolution reaction (η^{HER}) is obtained using the following equation:

$$\eta^{\text{HER}} = \frac{|\Delta G_{\text{H}^*}|}{e} \quad (5)$$

To explain the HER kinetics under standard conditions, $|\Delta G_{\text{H}^*}|$ is used to derive the volcano curve for the theoretical exchange current (i_0):³⁴

$$i_0 = -ek_0 \frac{1}{1 + \exp(|\Delta G_{\text{H}^*}| / k_{\text{b}}T)} \quad (6)$$

where k_{b} , k_0 and T are the Boltzmann constant, the reaction rate constant at zero overpotential, and the temperature, respectively. For illustrative purposes, k_0 is set to 1. Additionally, molecular dynamics (MD) simulations with an NVT ensemble were used to evaluate the thermodynamic stability of the catalysts. The MD simulation lasted for 2000 steps with a time step of 2.0 fs, and the temperature was controlled at 300 K using the Nosé–Hoover chains method.³⁵

RESULTS AND DISCUSSION

HER performance of pure GY

In this section, we present our findings on the performance of GY-based TM as a HER catalyst. We began our investigation by examining the GY monolayer (Fig. 1). To ensure accurate results, we used a supercell large enough to prevent interactions between adsorbates in two periodic units. Fig. 2 illustrates the density of states (DOS) and the band structure of the supercell. Our calculations revealed that the band gap of 6,6,12-graphyne is 0.061 eV. Following this initial characterization, we proceeded to evaluate the HER performance of various TM atoms anchored on the GY structure. Our aim was to identify promising electrocatalysts for the HER based on their performance at different active sites.

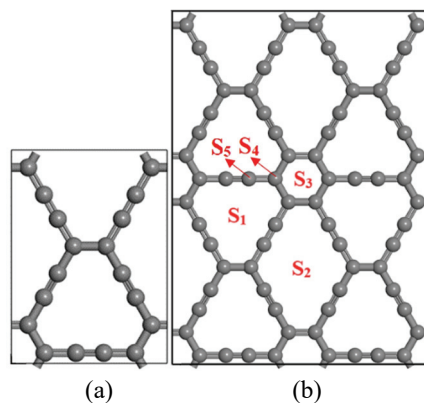


Fig. 1. a) The GY unit cell; b) the (2x2) GY supercell, showing the possible binding sites (S_1 , S_2 , S_3 , S_4 and S_5) for single TM atoms and the hydrogen intermediate.

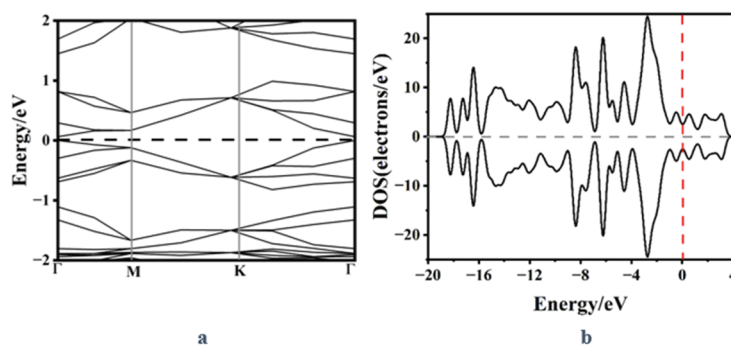


Fig. 2. a) The band structures of the 2×2 supercell GY; b) DOS of the 2×2 supercell GY, with the Fermi level indicated by the red dashed line.

We further decorated GY with various TMs (TM = Fe, Co, Ni, Cu) at the five introduced sites. The binding energies were negative for all structures. According

to the results, when the metal atom is anchored at S_1 , the binding energies are much larger than at other sites, indicating that S_1 is the most energetically stable site. The binding energies for Fe@GY, Co@GY, Ni@GY and Cu@GY were -5.76 , -5.72 , -5.92 and -3.76 eV, respectively. Additionally, the most negative absorption energy is related to nickel. These large binding energies indicate strong chemisorption between the GY monolayer and TM atoms, which is favorable for catalytic processes.

To evaluate pure GY as an electrocatalyst for hydrogen production, the adsorption of intermediate H at five different sites with varying distances on the GY substrate was investigated. The results are listed in Table I. According to Table I, the energy of hydrogen adsorption at the S_5 site (sp-hybridized C) is thermodynamically favorable, with a Gibbs energy change and overpotential of 0.15 eV and 0.15 V, respectively.

TABLE I. Final distance of H intermediate from the substrate, total energy (E_{total}) and absorption energy (E_{ad}) at different sites

Site	Distance / Å	E_{total} / eV	E_{ad} / eV
S_1	1.10	-74582.70	0.15
S_2	2.02	-74580.63	2.22
S_3	2.23	-74579.90	2.95
S_4	1.10	-74582.51	0.35
S_5	1.10	-74582.83	0.02

The formation energies of TM@GY were further calculated using the expression $E_f = E_{\text{total}} + \mu_C - (E_{\text{GY}} + E_{\text{TM}})$, where E_{total} is the total energy of TM@GY, E_{GY} is the energy of pristine GY, E_{TM} is the energy per atom of the transition metal and μ_C is the chemical potential of a single C atom in GY. The formation energies for Co@GY, Ni@GY, Fe@GY and Cu@GY were -5.72 , -5.93 , -5.76 and -3.76 eV, respectively. These low formation energies suggest that TM@GY catalysts can be easily synthesized experimentally.³⁶ The thermodynamic stability of the Co@GY catalyst was tested under standard conditions. The results indicated that the energies oscillate near the equilibrium state, with no significant structural reconstruction at the end of the MD simulation (Fig. 3), demonstrating the good thermodynamic stability of Co@GY in the HER catalytic process.

Stability, and electronic properties of structures

The band structures of Fe@GY, Co@GY, Ni@GY and Cu@GY were computed to assess their electrical conductivities. As shown in Fig. 4, there is no effect of spin polarization for Co@GY, Ni@GY and Cu@GY, with no difference between the states related to alpha and beta spins, indicating no magnetic moment creation. For Fe@GY, the alpha and beta spins do not align, creating a local magnetic moment. The energy gap for Ni@GY is 0.024 eV, while it is zero for the

other structures. HOMOs and LUMOs are shown in Fig. S-1 of the Supplementary material to this paper.

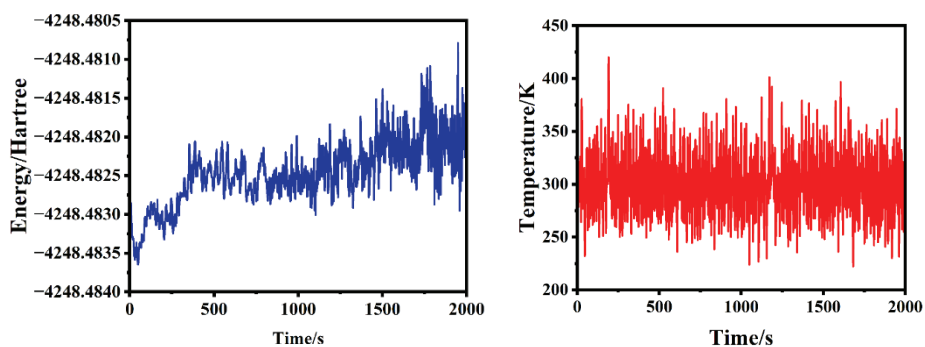


Fig. 3. Fluctuations of energy and temperature during MD simulations of Co@GY catalyst.

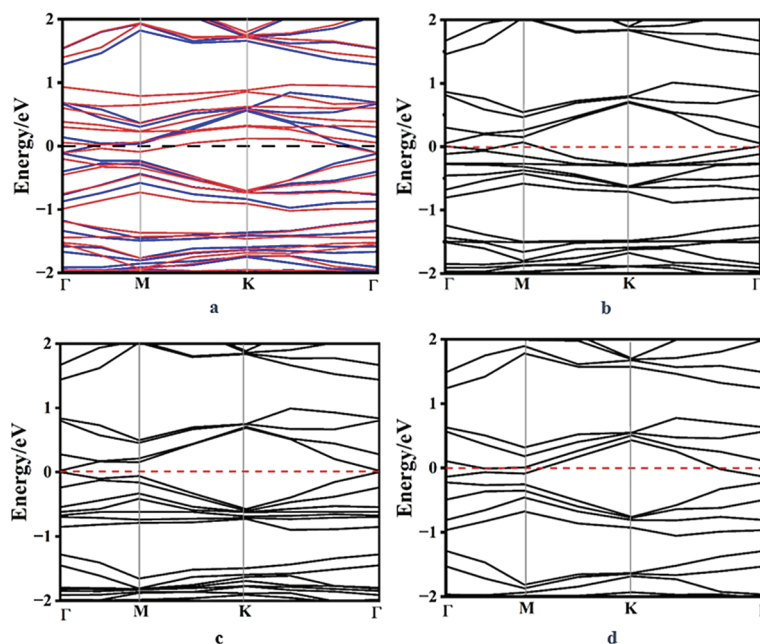


Fig. 4. Band structures (at GGA-PBE level) of: a) Fe@GY, b) Co@GY, c) Ni@GY and d) Cu@GY, with the zero line indicating the Fermi level.

The density of states (DOS) in Fig. 2 shows the semimetallic property of the pristine GY monolayer. Importantly, all TM@GY nanosheets present decreased band gaps and most exhibit metallic properties after decorating with TM atoms. The PDOS for TM@GY is presented in Fig. 5. The predominant peaks of TM-d states crossing the Fermi level imply high carrier density, enhancing electronic

conductivity of TM@GY monolayers and favoring electrocatalytic processes. The difference electron density of TM@GY are also calculated (Fig. 6), showing Co@GY and Ni@GY catalysts display more charge density, especially over the acetylenic ring.

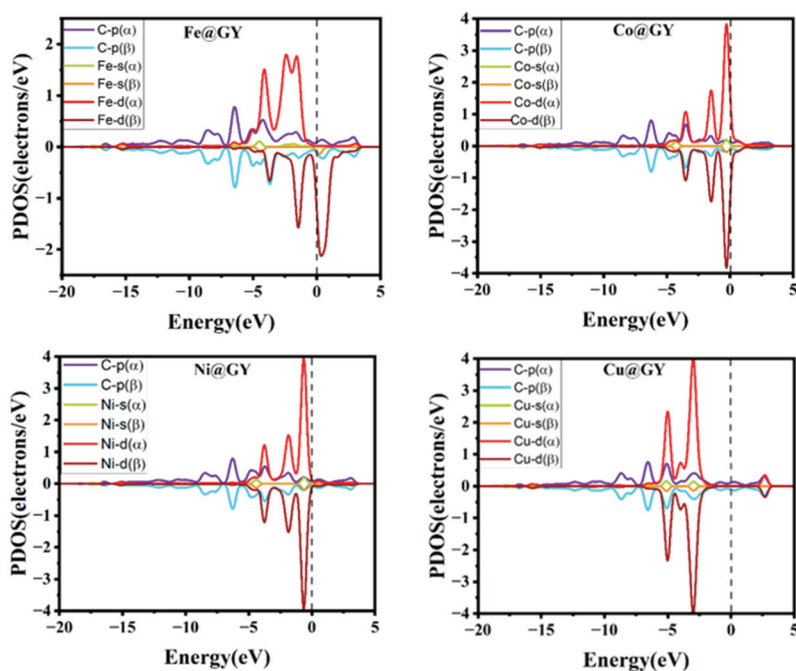


Fig. 5. Partial density of states (PDOS) of TM@GY. The Fermi level is set to zero.

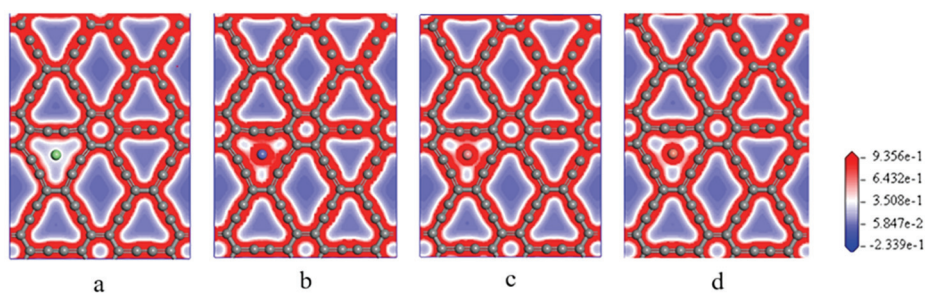


Fig. 6. Difference electron density images of: a) Fe@GY, b) Co@GY, c) Ni@GY and d) Cu@GY; blue and red colors represent charge depletion and accumulation, respectively.

HER performance of TM@GY

The origin of the electrocatalytic activity of TM@GY catalysts for HER is explored. Table II presents the ΔG_{H^*} values for H^* adsorption on pristine GY and on 6,6,12-graphyne decorated with various TMs. For the S_5 site of pristine GY,

the ΔG_{H^*} value is 0.15 eV, indicating a weak binding strength between C and H atoms. However, after decorating the GY monolayer with TM atoms, the acetylenic C and sp and sp^2 hybridized carbon atoms of TM@GY samples can become active sites (Fig. 7). This modification significantly alters the hydrogen adsorption properties of the material, potentially enhancing its catalytic activity for HER.

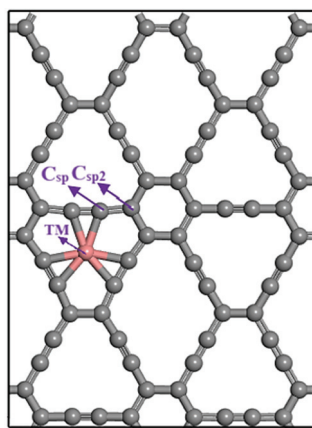


Fig. 7. Possible active sites on TM@GY for the hydrogen evolution reaction.

The TM@GY catalyst shows an increased number of active sites and enhanced catalytic performance, particularly at the Co, Fe (C_{sp}) and Co (C_{sp}) sites, with ΔG_{H^*} values of 0.042, 0.044, and 0.060 eV, respectively. These values are comparable to the state-of-the-art Pt catalysts (-0.09 eV)³⁷ and close to the ideal value ($\Delta G_{H^*} = 0$ eV), indicating that decorating TM atoms at the acetylenic ring is an effective strategy for increasing active sites and boosting catalytic activity for HER. The electron transfer from TM atoms to acetylenic carbon atoms improves the interaction between H and acetylenic C atoms (Table S-I of the Supplementary material), moving ΔG_{H^*} closer to zero. These changes are attributed to charge redistribution introduced by TM atom decoration.³⁸ According to Table II, Ni@GY and Cu@GY are unsuitable catalysts for hydrogen production, as all values are positive. The most stable adsorption configurations are shown in Fig. S-2 of the Supplementary material.

TABLE II. Calculated Gibbs energy changes (eV) of the H atom binding on GY and TM ($\Delta G_H(TM)$) and carbon atoms ($\Delta G_H(C_{sp})$ and ($\Delta G_H(C_{sp2})$)) for different TMs supported on GY

TM@GY	$\Delta G_H(TM)$	$\Delta G_H(C_{sp2})$	$\Delta G_H(C_{sp})$
Fe@GY	0.453	0.601	0.044
Co@GY	0.042	0.624	0.060
Ni@GY	0.954	0.189	0.590
Cu@GY	1.810	0.178	0.501
GY	—	—	0.15

Figure 1 consists of two plots. Plot (a) is a scatter plot showing the logarithmic pre-exponential factor $\text{Log}(t_0/A)/\text{cm}^{-2}$ on the y-axis (ranging from -35 to 0) versus the activation energy $\Delta G_{H^0}/\text{eV}$ on the x-axis (ranging from 0.0 to 1.8). Data points are labeled with metal@carbon nanotube systems: Cu@Fe(C₉₂), Cu@Fe(C₅₄), Cu@Fe(C₂₄), Cu@Fe(C₁₈), Cu@Fe(C₁₂), Cu@Fe(C₆), Cu@Fe(C₃), Cu@Fe(C₂), Cu@Fe(C₁), Cu@Fe(C₀), Cu@Fe(C₋₁), Cu@Fe(C₋₂), Cu@Fe(C₋₃), Cu@Fe(C₋₄), Cu@Fe(C₋₅), Cu@Fe(C₋₆), Cu@Fe(C₋₇), Cu@Fe(C₋₈), Cu@Fe(C₋₉), Cu@Fe(C₋₁₀), Cu@Fe(C₋₁₁), Cu@Fe(C₋₁₂), Cu@Fe(C₋₁₃), Cu@Fe(C₋₁₄), Cu@Fe(C₋₁₅), Cu@Fe(C₋₁₆), Cu@Fe(C₋₁₇), Cu@Fe(C₋₁₈), Cu@Fe(C₋₁₉), Cu@Fe(C₋₂₀), Cu@Fe(C₋₂₁), Cu@Fe(C₋₂₂), Cu@Fe(C₋₂₃), Cu@Fe(C₋₂₄), Cu@Fe(C₋₂₅), Cu@Fe(C₋₂₆), Cu@Fe(C₋₂₇), Cu@Fe(C₋₂₈), Cu@Fe(C₋₂₉), Cu@Fe(C₋₃₀), Cu@Fe(C₋₃₁), Cu@Fe(C₋₃₂), Cu@Fe(C₋₃₃), Cu@Fe(C₋₃₄), Cu@Fe(C₋₃₅), Cu@Fe(C₋₃₆), Cu@Fe(C₋₃₇), Cu@Fe(C₋₃₈), Cu@Fe(C₋₃₉), Cu@Fe(C₋₄₀), Cu@Fe(C₋₄₁), Cu@Fe(C₋₄₂), Cu@Fe(C₋₄₃), Cu@Fe(C₋₄₄), Cu@Fe(C₋₄₅), Cu@Fe(C₋₄₆), Cu@Fe(C₋₄₇), Cu@Fe(C₋₄₈), Cu@Fe(C₋₄₉), Cu@Fe(C₋₅₀), Cu@Fe(C₋₅₁), Cu@Fe(C₋₅₂), Cu@Fe(C₋₅₃), Cu@Fe(C₋₅₄), Cu@Fe(C₋₅₅), Cu@Fe(C₋₅₆), Cu@Fe(C₋₅₇), Cu@Fe(C₋₅₈), Cu@Fe(C₋₅₉), Cu@Fe(C₋₆₀), Cu@Fe(C₋₆₁), Cu@Fe(C₋₆₂), Cu@Fe(C₋₆₃), Cu@Fe(C₋₆₄), Cu@Fe(C₋₆₅), Cu@Fe(C₋₆₆), Cu@Fe(C₋₆₇), Cu@Fe(C₋₆₈), Cu@Fe(C₋₆₉), Cu@Fe(C₋₇₀), Cu@Fe(C₋₇₁), Cu@Fe(C₋₇₂), Cu@Fe(C₋₇₃), Cu@Fe(C₋₇₄), Cu@Fe(C₋₇₅), Cu@Fe(C₋₇₆), Cu@Fe(C₋₇₇), Cu@Fe(C₋₇₈), Cu@Fe(C₋₇₉), Cu@Fe(C₋₈₀), Cu@Fe(C₋₈₁), Cu@Fe(C₋₈₂), Cu@Fe(C₋₈₃), Cu@Fe(C₋₈₄), Cu@Fe(C₋₈₅), Cu@Fe(C₋₈₆), Cu@Fe(C₋₈₇), Cu@Fe(C₋₈₈), Cu@Fe(C₋₈₉), Cu@Fe(C₋₉₀), Cu@Fe(C₋₉₁), Cu@Fe(C₋₉₂), Cu@Fe(C₋₉₃), Cu@Fe(C₋₉₄), Cu@Fe(C₋₉₅), Cu@Fe(C₋₉₆), Cu@Fe(C₋₉₇), Cu@Fe(C₋₉₈), Cu@Fe(C₋₉₉), Cu@Fe(C₋₁₀₀). Plot (b) shows the free energy profile $\Delta G/\text{eV}$ on the y-axis (ranging from 0.00 to 0.20) versus reaction coordinates on the x-axis. The profile shows the energy barrier for the hydrogen evolution reaction on GY nanotubes, with the highest energy state labeled *H and the lowest energy state labeled H⁺ + e⁻. The energy barrier is highest for Cu@GY(C₉₂) and lowest for Cu@GY(C₀).

CONCLUSION

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SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13006>, or from the corresponding author on request.

ИЗВОД

ПОБОЉШАЊЕ РЕАКЦИЈЕ ИЗДВАЈАЊА ВОДОНИКА КОРИШЋЕЊЕМ АТОМА МЕТАЛА НА 6,6,12-ГРАФИНУ: DFT СТУДИЈА

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Нагли раст глобалног становништва и хитна потреба за одрживим решењима за енергију са минималним утицајем на животну околину, истакли су значај развијања обновљивих извора енергије. Водоник, као носилац енергије и гориво, представља битну предност над другим облицима енергије, поред осталог има примену у медицинским поступцима и производњи критичних хемикалија, попут метана и метанола. Тако се водоник јавља као потенцијална алтернатива фосилним горивима, нудећи стабилно и чисто решење за енергију. У овој студији представљамо теоријски дизајн катализатора заснованог на прелазном металу (ТМ) закаченом на 6,6,12-графин (GY) за реакцију издвајања водоника (HER) користећи теорију функционала густине (DFT). Наши налази откривају да од свих испробаних система, кобалтов једноатомски катализатор (SAC) закачен за 6,6,12-графин испољава највишу термодинамичку стабилност, испољавајући највишу термодинамичку стабилност и надмоћно HER каталитичко дејство, са уочљиво ниском вредношћу ΔG_{H^*} од 0,042 eV. Испитивали смо густину стања, НОМО, ЛУМО, електронску густину и структуру трака наших дизајнираних SAC. Овај рад обезбеђује практичну стратегију за експериментаторске групе да ефикасно подесе електронске структуре катализатора и повећају њихову каталитичку активност.

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SUPPLEMENTARY MATERIAL TO

**Enhancing hydrogen evolution reaction using transition metal atoms
on 6,6,12-graphyne: A DFT study**

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TABLE S-I. Detailed parameters for the optimized TM@GY structures. $d_{\text{TM-C}}$ is the distance between the TM atom and its nearest acetylenic C atoms. Q refers to charges transferred from TM atoms to the substrate obtained by Hirshfeld charge analysis

TM@GY	$d_{\text{TM-C}}$ (Å)	Q (e)
Fe@GY	1.99	0.24
Co@GY	1.94	0.04
Ni@GY	1.95	0.03
Cu@GY	2.04	0.38

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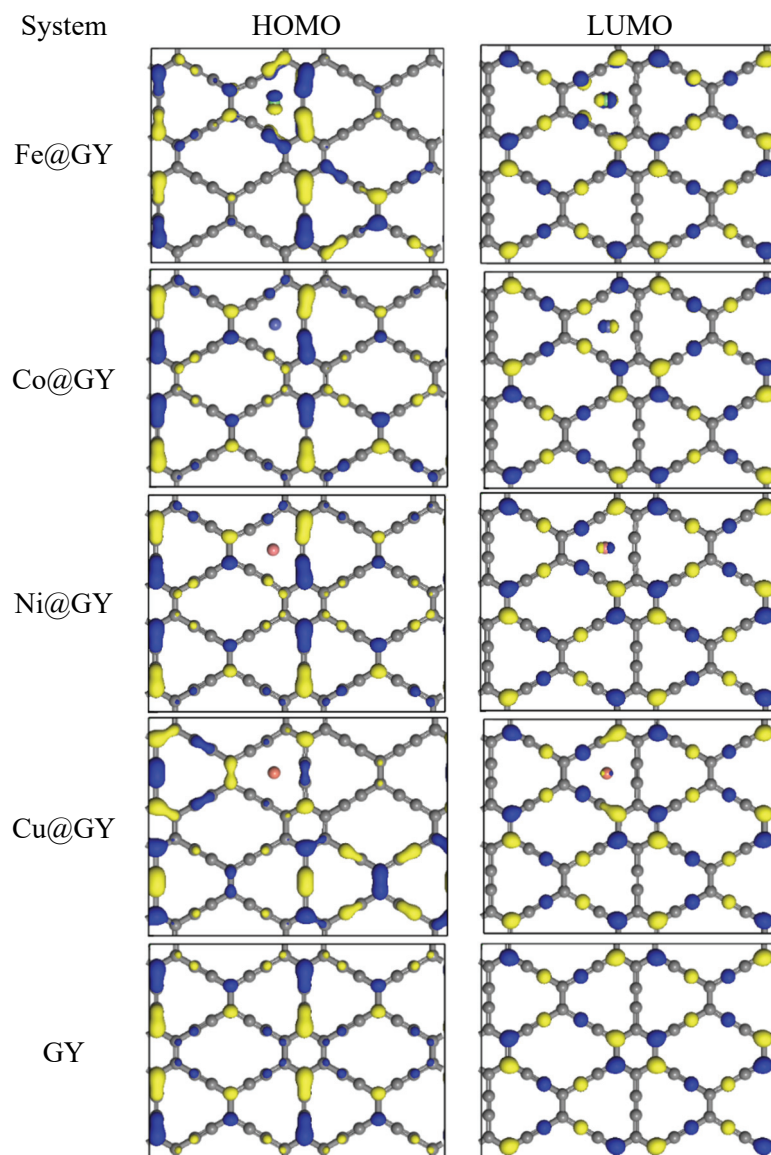


Fig. S-1. Graphical representation of HOMOs and LUMOs of pristine and TM-decorated 6,6,12-graphyne nanosheets

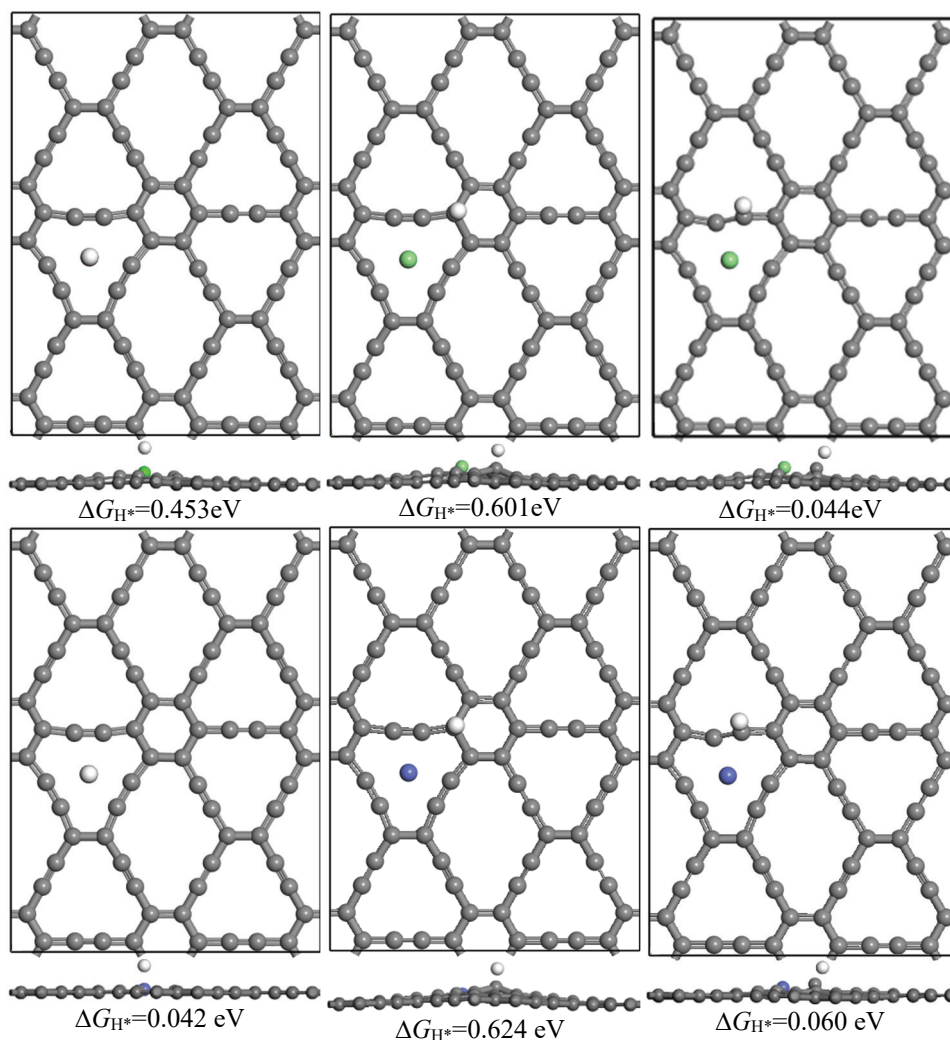


Fig. S-2. Most stable adsorption configurations and corresponding adsorption free energies of H^* on different sites of Fe@GY, Co@GY, Ni@GY, and Cu@GY. Grey, blue, green, yellow, orange, and white balls represent C, Co, Fe, Ni, Cu, and H, respectively

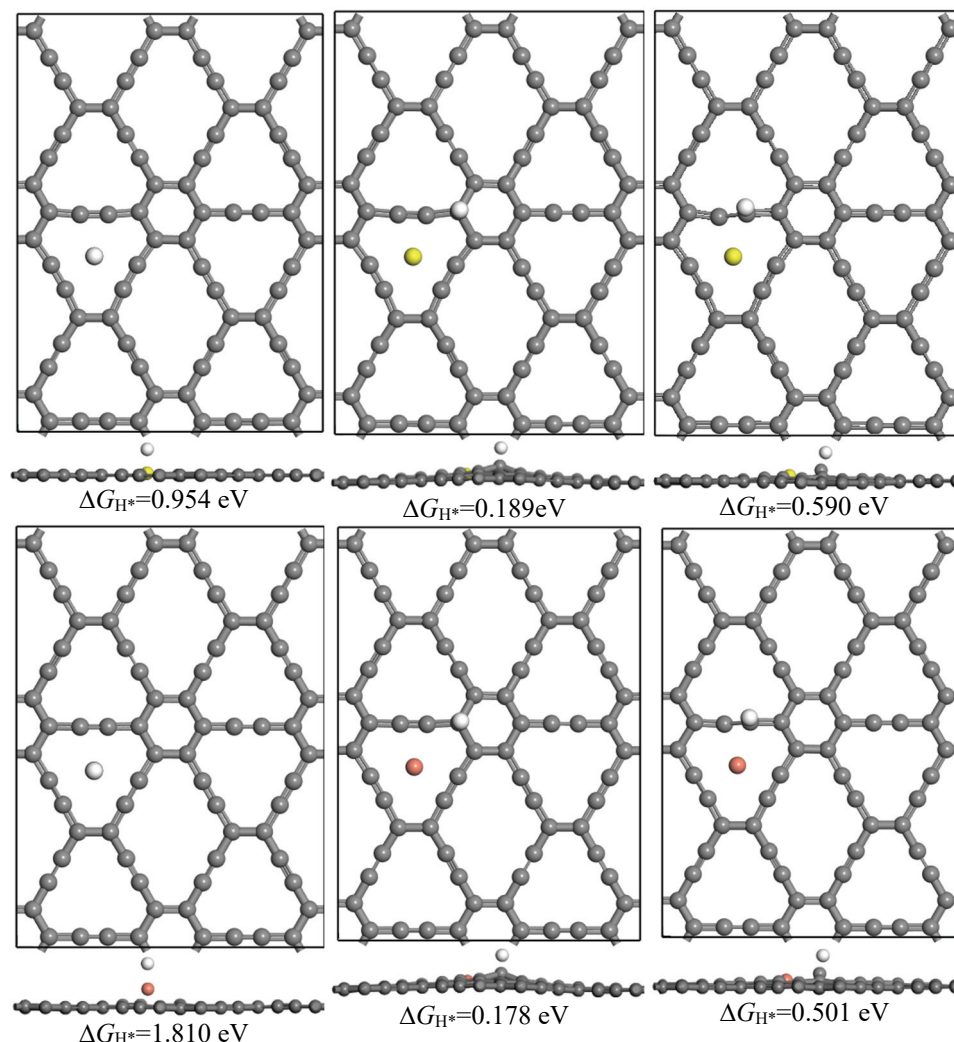


Fig. S-2. Cont. Most stable adsorption configurations and corresponding adsorption free energies of H^* on different sites of Fe@GY, Co@GY, Ni@GY, and Cu@GY. Grey, blue, green, yellow, orange, and white balls represent C, Co, Fe, Ni, Cu, and H, respectively

TABLE S-II. Zero-point energy (ZPE) and entropic corrections (TS) in determining the free energy of reactants, intermediates, and products adsorbed on catalysts at 298 K. For the adsorbate, the ZPE value is not sensitive to the metal and coordination since they have close values.

Species	ZPE(eV)	TS(eV)
H ₂	0.27	0.41
H*	0.16	0



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Synthesis and characterization of nano $\text{Fe}_2\text{CuAl}_2\text{O}_7$ as a reusable catalyst for Biginelli reaction

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Abstract: In this research, the novel mixed metal oxide nanoparticles (NPs) $\text{Fe}_2\text{CuAl}_2\text{O}_7$ were synthesized by applying sol-gel auto-combustion method. The $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs were identified by XRD, FT-IR, Mapping and EDS. The XRD pattern showed that $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs contain a crystalline structure and have just one phase, and types of crystals are FCC. The size distribution of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs was determined by FESEM to be about 41.44 nm. Using the BET equation, the surface area of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs was calculated as $26.174 \text{ m}^2 \text{ g}^{-1}$. $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs were used as a catalyst for the Biginelli reaction. 3,4-Dihydropyrimidine-2(1*H*)-one/thione derivatives were prepared in the presence of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst with short time and 75–97 % efficiency in water. In addition, the recovery and the reuse of the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst was done up to five times without significant change in catalytic ability.

Keywords: sol-gel auto-combustion; mixed metal oxides; BET; nanocatalyst; one-pot synthesis.

INTRODUCTION

Mixed metal nano-oxides have attracted special attention of researchers in the nano field due to their unique attributes in terms of properties, structure, and applications.¹ Properties such as electrical,² dielectric,³ magnetic,⁴ optical,⁵ redox⁶ and Lewis acid base behaviour⁷ that are of interest. The most widely used fields of these materials are targeted drug delivery,⁸ productions of antibacterial,^{9,10} superhydrophobic textiles,¹¹ insecticides,¹² gas storage,¹³ batteries,¹⁴ sensors¹⁵ and nanocatalysts.^{16–20} Aligning the synthesis of nanocatalysts and green chemistry is a big challenge that chemical scientists have managed well and achieved good success in recent years. Mixed metal nano oxides are one of the types of nanocatalysts that are used as support phase²¹ or active phase²² in

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various organic reactions. Mixed metal nano oxides can be used for the oxidation²³ and reduction²⁴ of organic compounds, the combustion of hydrocarbons,^{25,26} the synthesis of widely used chemicals in the industry,²⁷ biodiesel production²⁸ and Claisen–Schmidt condensation.²⁹ They attributed the catalytic properties of oxide surfaces to Lewis acid and base in such a way that Lewis bases are the same oxygens that gather electrons around them, and Lewis acids are metal cations. Also, the defects created on the surface of oxides have a decisive task in their catalytic performance. In general, mixed metal oxides have a crystal structure that depends on the calcination temperature and the synthesis method used for their preparation.^{30,31}

The basic structure of many molecules that have medicinal activity such as anticancer, antibacterial, antiviral, antipyretic, and anti-inflammatory as well as fungicidal are heterocyclic compounds.³² Among numerous heterocyclic compounds, dihydropyrimidines are the most demanding fields of research due to their extraordinary therapeutic and medicinal properties such as cardiovascular drugs,³³ anti-cancers,³⁴ blood pressure drugs³⁵ and anti-inflammatory agents.³⁶ The first compound was synthesized by Biginelli from the three-component one-pot reaction of urea, aldehyde and ketoester in the presence of acid but with low efficiency. During the last decades, various types of catalysts have been reported for this reaction. Among the catalysts can be mentioned such as polymer-immobilized reagents,³⁷ caffeine,³⁸ silicotungstic acid supported on Ambelyst-15,³⁹ mesoporous $\text{NH}_4\text{H}_2\text{PO}_4/\text{MCM-4}$,⁴⁰ $\text{Fe}_3\text{O}_4/\text{SiO}_2$,⁴¹ NiO nanocatalyst⁴² and nano-ZrO₂ sulfuric acid.⁴³ In addition to the advantages, these catalysts suffer from a series of negative features such as low efficiency, use of expensive and toxic solvents, complex conditions for separation, harsh reaction conditions, or sometimes long reaction times. Therefore, it is very important to use a catalyst that performs this profitable reaction with greater efficiency, shorter time, less cost, and less damage to the environment.

In this research, 3,4-dihydropyrimidine-2(1*H*)-one/thione derivatives were synthesized from the one-pot reaction of ethyl acetoacetate, urea or thiourea, and aromatic aldehyde in the presence of the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ heterogeneous nanocatalysts under green and easy conditions.

Integrating atoms or molecules, which is considered a kind of bottom-up approach and is also known as molecular technology, is possible by sol-gel auto-combustion method.⁴⁴ To synthesis of pure $\text{Fe}_2\text{CuAl}_2\text{O}_7$ with high level of homogeneity and appropriate size, sol-gel auto-combustion method is a technique that has the advantages of sol-gel and combustion together. This process starts by preparing a solution of metal nitrates in water and sol particles are formed by adding a fuel source such as citric acid. After a heating step, the sol turns into a gel which is a foam-shaped and bulky powder. $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs are synthesized by the combustion process with homogenous particles, high purity

percentage and larger surface area. This procedure is an environmentally friendly method, due to the release of CO₂, H₂O and N₂ gases.⁴⁵ An uncomplicated preparation procedure, cheap, and commercially available raw materials for the synthesis of Fe₂CuAl₂O₇ nanocatalysts are the advantages of this method. The Fe₂CuAl₂O₇ nanocatalysts can be recycled up to five stages without reducing its efficiency. The reaction yield and the purity of the products were excellent without the need for complicated separation methods. Researches in various literature showed that the synthesis of the Fe₂CuAl₂O₇ nanocatalysts and its application in the preparation of dihydropyrimidines are reported for the first time. The synthesized catalyst has a high stability for a long time, and it has a good durability in the conditions of humidity, heat, and in the open air.

EXPERIMENTAL

Materials

Benzaldehyde derivatives, ethyl acetoacetate, urea, thiourea, citric acid, Al(NO₃)₃·9H₂O, Cu(NO₃)₂·3H₂O, Fe(NO₃)₃·9H₂O and the solvents were purchased from reputable companies, and were used without the need for purification.

Synthesis of Fe₂CuAl₂O₇ NPs

At first, in 1000 ml of distilled water 20.20 g (50 mmol) of Fe(NO₃)₃·9H₂O, 18.75 g (50 mmol) of Al(NO₃)₃·9H₂O and 6.05 g (25 mmol) of Cu(NO₃)₂·3H₂O were dissolved. Then, in 250 ml of distilled water 47.13 g (245 mmol) of Citric acid was dissolved and added to the previous mixture. After 3 h stirring the reaction mixture, a sol was formed. A gel formation occurred by water evaporation. The obtained gel was heated overnight at 120 °C by an oven and completely dried. The formed xerogel was calcined in air at 700 °C for 1 h.

Preparation of 3,4-dihydropyrimidine-2(1H)-ones/thiones

A mixture of ethyl acetoacetate (2.5 mmol, 325 mg), benzaldehyde derivatives (2.5 mmol), urea or thiourea (3.75 mmol), and Fe₂CuAl₂O₇ nanocatalyst (0.5 mmol, 170.6 mg) in distilled water (20 mL) was heated under 100 °C. After the accomplishment of the reaction time, as observed by TLC (ether/*n*-hexane: 1/4), the reaction temperature was down to 25 °C, and to the reaction mixture 25 mL of distilled water was added. The catalyst was detached for use in the subsequent reaction by ordinary centrifugation. The 3,4-dihydropyrimidine-2(1H)-ones/thiones were extracted with ethyl acetate (3×30 mL), and the organic phase was dried with anhydrous Na₂SO₄ (5 g). Ethyl acetate was separated from the product under reduced pressure, and 3,4-dihydropyrimidine-2(1H)-ones/thiones with 75–97 % yields were afforded by recrystallization of residue from ethanol.

Characterization

D500 diffractometer (Siemens) was employed for the pattern X-ray diffraction (XRD) of particles. The XRD pattern of Fe₂CuAl₂O₇ NPs at 25 °C was obtained using CuKα radiation (λ = 0.154 nm) and with a scanning speed of 2°/min. Data were recorded in the range of 10 to 80° with a scan step of 0.5°/s. The dimensions of Fe₂CuAl₂O₇ NPs were determined to be 45.045 nm based on the Debye–Scherrer equation (Table I):

$$D = 0.9\lambda/\beta\cos\theta \quad (1)$$

In the Debye–Scherrer equation, θ is the Bragg angle in $^{\circ}$, β is the full width at half maximum peak intensity in rad and $\lambda = 1.54060 \text{ \AA}$ (in the case of $\text{CuK}\alpha$).

TABLE I. Crystallographic data of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs; crystal system: cubic; space group: Fd-3m; space group number: 227

Peak No.	Peak position, $2\theta / ^{\circ}$	<i>FWHM</i>	Crystallite size, <i>D</i> / nm	Avg. <i>D</i> / nm
1	30.8955	0.2460	33.49	45.045
2	36.2296	0.1476	56.60	–

The information about the size of the particles and morphology was obtained with the device FESEM, Tescan Mira III (field emission scanning electron microscopy) with an accelerating voltage of 20 kV. X-ray electron dispersive spectroscopy (EDS) using a SAMX detector was used to analyse and determine the relative abundance of elements. Spectroscopy Fourier transforms infrared (FT-IR) in the range of $400\text{--}4000 \text{ cm}^{-1}$ with a KBr disk was used on a Thermo Avatar spectrometer. The adsorption–desorption isotherms, the surface area, the total pore volume and the mean pore diameter were obtained with a Bel Sorp Mini II device on a Bel Prep Vac II analyser by the multipoint Bronauer–Emmett–Teller (BET) method. The completion of the reaction was observed by thin-layer chromatography (TLC) on precoated Merck silica gel 60 F254 and RP-18 F254 plates. The melting points were measured by open capillary with an Electro thermal 9200 device. The reaction yields were calculated after product isolation.

RESULTS AND DISCUSSION

XRD analysis

Fig. 1 shows the XRD pattern for $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs as a function of angle. Table II lists the $2\theta (^{\circ})$ and relative intensity of each peak.

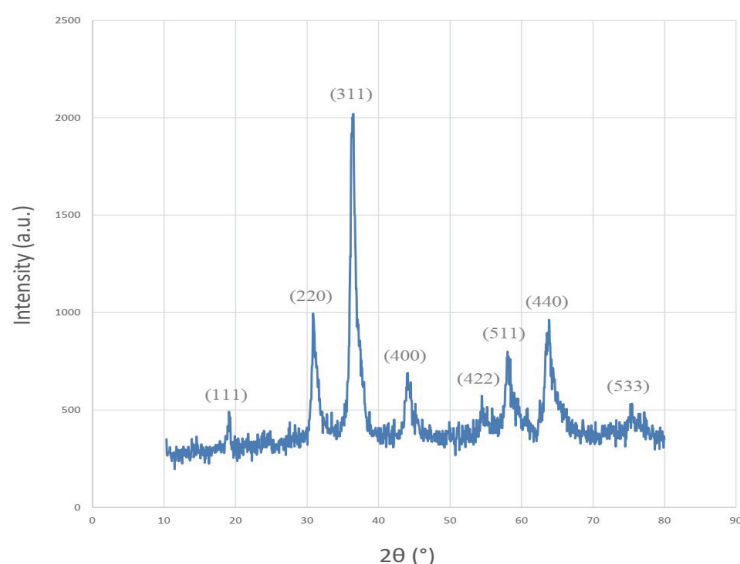
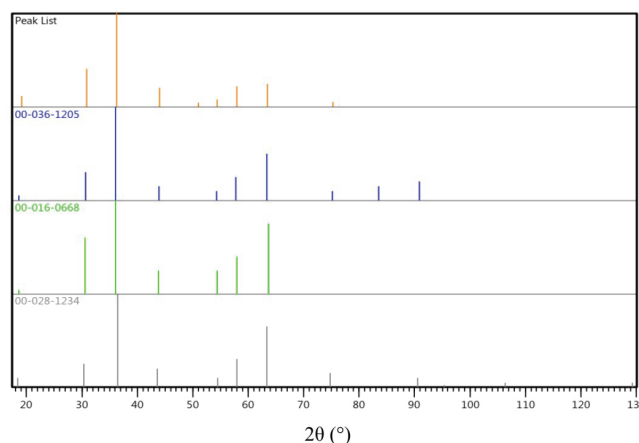


Fig. 1. XRD pattern of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs.

TABLE II. Peak list of Fe₂CuAl₂O₇ NPs

Pos. (2θ / °)	Height, cts	FWHM Left (2θ / °)	D-Spacing, Å	Rel. int., %	Tip width
19.1406	161.58	0.2952	4.63700	11.61	0.3542
30.8955	565.78	0.2460	2.89435	40.67	0.2952
36.2296	1391.21	0.1476	2.47952	100.00	0.1771
44.0461	283.22	0.4920	2.05595	20.36	0.5904
51.0711	56.77	0.2952	1.78842	4.08	0.3542
54.4426	105.93	0.9840	1.68537	7.61	1.1808
57.9885	304.09	0.4920	1.59047	21.86	0.5904
63.4387	335.83	0.3936	1.46633	24.14	0.4723
75.2882	74.89	0.5904	1.26227	5.38	0.7085

The phase determination was done by comparison of the location and intensity of the XRD pattern of Fe₂CuAl₂O₇ NPs with references peaks (Fig. 2). The location of the peaks and the relative intensities of the XRD pattern of Fe₂CuAl₂O₇ NPs match with the references (Table III).

Fig. 2. Plot of identified phases of Fe₂CuAl₂O₇ NPs.TABLE III. Identified patterns list of Fe₂CuAl₂O₇ NPs

Ref. code	Score	Compound name	Scale factor	Chemical formula
00-036-1205	65	Cadmium gallium manganese oxide	0.521	CdMnGaO ₄
00-016-0668	55	Lithium cobalt vanadium oxide	0.420	LiCoVO ₄
00-028-1234	38	Cobalt chromium iron oxide	0.759	FeCoCrO ₄

According to the crystal structure of the references mentioned in Table III, the crystal structure of Fe₂CuAl₂O₇ NPs is FCC type.

FESEM analysis

FESEM analysis is a microscopic method with high magnification, which was used to investigate the morphology, shape, the surface structure, and the size

distribution of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs in nanometer dimensions (Fig. 3). FESEM image shows that $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs are crystalline and homogeneous. According to the FESEM image, the average size of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs is about 41.443 nm.

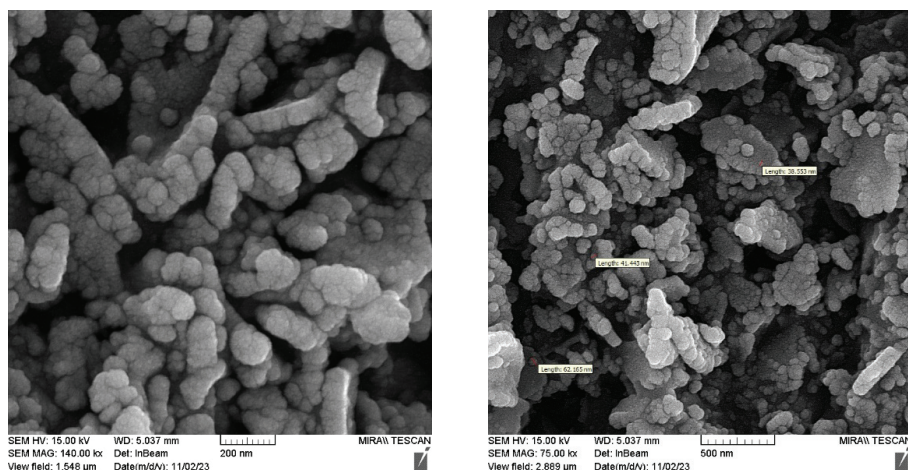


Fig. 3. FESEM images of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs.

EDS analysis

EDS was used to specify the stoichiometry (chemical composition of nanoparticles) and to determine the chemical purity of them (Figs. 4 and 5). The results showed that the only main elements were Fe, Cu, Al and O, respectively, with wt. % of 35.37, 17.39, 18 and 29.24 as well as by the ratio of the number of atoms 18.62, 8.05, 19.61 and 53.72. According to the obtained percentages, the molecular formula of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ was confirmed.

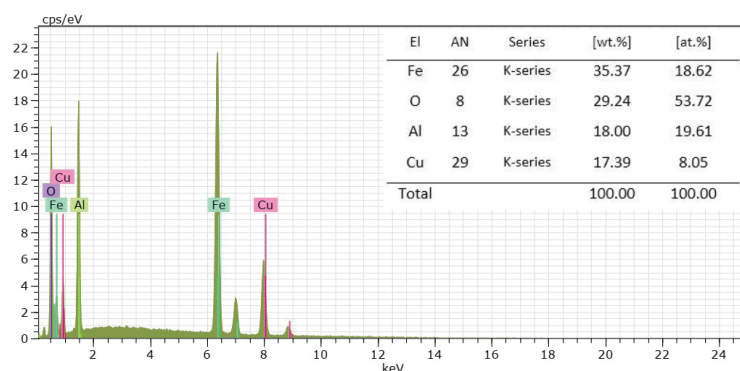


Fig. 4. EDS spectrum of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs.

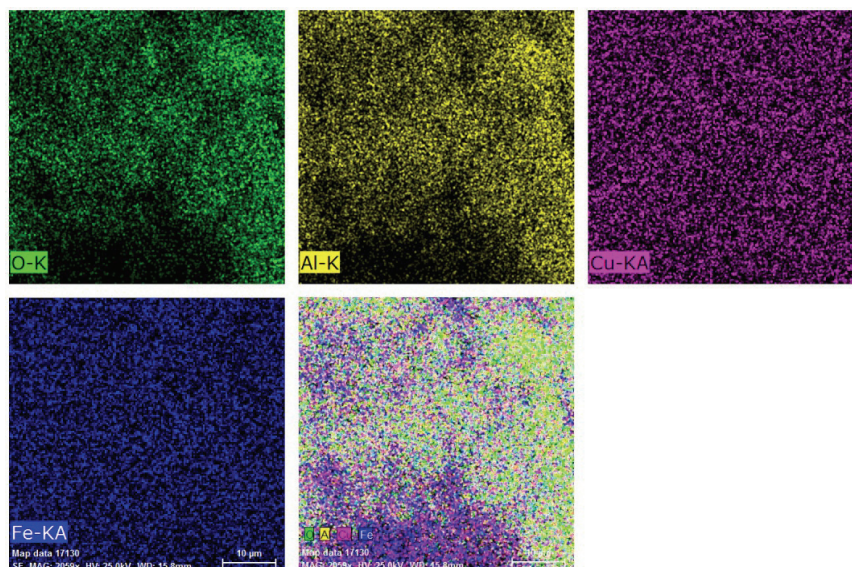


Fig. 5. Mapping data of Fe₂CuAl₂O₇ NPs.

Mapping analysis

In concentrated mapping analysis, a particular region of Fe₂CuAl₂O₇ NPs was investigated. In mapping analysis was taken from many points in a particular region, and the results of this analysis were represented as a series of coloured points, where each colour represents an element (Fig. 5). Considering that there was a strong bond between the elements, which stayed together and clearly showed the homogeneous distribution of Fe, Cu, Al and O elements according to the molar ratios throughout the structure.

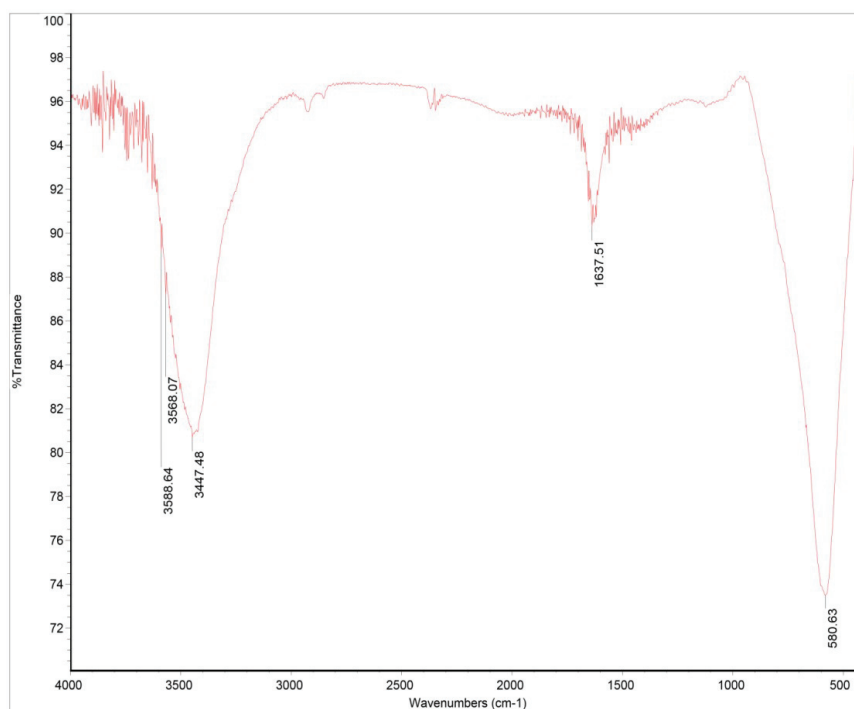
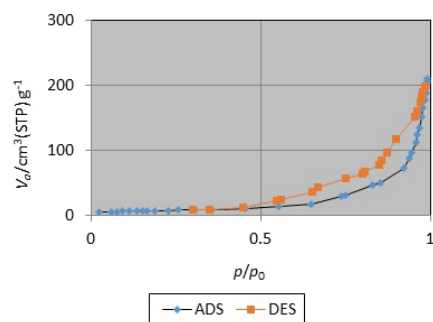
FT-IR analysis

The FT-IR spectrum of Fe₂CuAl₂O₇ NPs is shown in Fig. 6. In this spectrum, the broad peak that appeared in the bounds of 3447 cm⁻¹ is related to the absorbed water in the stretching state of it. The stretching vibration of the M–O band is at 1637 cm⁻¹, whenever the bending vibration is at 580 cm⁻¹.

Textural analysis

N₂ adsorption–desorption isotherms were used to measure the size distribution, surface area and pore volume of Fe₂CuAl₂O₇ NPs at 77 K (Fig. 7). The shape of N₂ adsorption–desorption isotherms according to IUPAC classification is type IV, that specifies the mesopore structure of Fe₂CuAl₂O₇ NPs. Based on the hysteresis loop (H3), Fe₂CuAl₂O₇ NPs have wedge-shaped pores.

Fe₂CuAl₂O₇ NPs capacity was determined by the volumetric gas absorption method with Bel Sorp Mini II absorption device. The total volume of pores was cal-

Fig. 6. FT-IR spectrum of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs.Fig. 7. N_2 adsorption-desorption isotherms of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs.

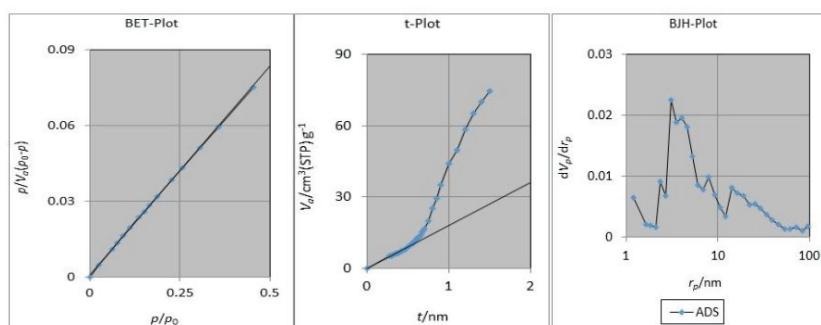
culated using the amount of nitrogen adsorbed at the highest operating pressure of the experiment. The total surface area was calculated using the BET equation in the initial part of the isotherm (Table IV).

t-Plot (Fig. 8) was used to measure the surface area and volume of pores according to the thickness of gas adsorbed on the sample. t-Plot diagram indicating that $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs are no micropores because it has no intercept elevation. The mesoporous distribution of the adsorption branch data was performed according to the BJH method. The pore size distribution diagram shows that the pore size is in the range of 2 to 50 nm and the structure of the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs

is completely mesoporous. The pores size distribution curve obtained from the adsorption branch by the BJH method shows that pores with a size of 3.09 nm have the maximum abundance.

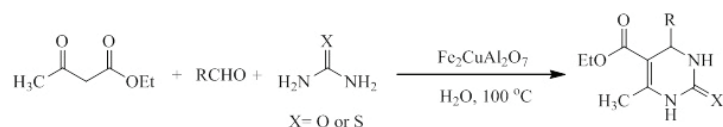
TABLE IV. The calculated data of BET, t and BJH plots

Parameter	Value	Unit
BET-plot		
V_m	6.0135	cm ³ g ⁻¹
$a_{s,BET}$	26.174	m ² g ⁻¹
C	167.72	
Total pore volume ($p/p_0 = 0.990$)	0.3241	cm ³ g ⁻¹
Mean pore diameter	49.527	nm
t-Plot, adsorption branch		
a_1	27.834	m ² g ⁻¹
V_1	0	cm ³ g ⁻¹
BJH-Plot, adsorption branch		
V_p	0.3316	cm ³ g ⁻¹
$r_{p,peak}$	3.09	nm
a_p	49.485	m ² g ⁻¹

Fig. 8. a) BET plot, b) t-plot and c) BJH plot of Fe₂CuAl₂O₇ NPs.

Fe₂CuAl₂O₇ nanocatalyst activity

In the present study, Fe₂CuAl₂O₇ NPs were used as a catalyst to produce the 3,4-dihydropyrimidine-2(1H)-ones/thiones in excellent yield (Scheme 1). Due to the hydrophobicity of the catalyst and organic reactants, water as a solvent was used in the reactions at a temperature of 100 °C.



Scheme 1. Synthesis of the 3,4-dihydropyrimidin-2(1H)-ones/thiones.

At first, the progress of the reaction was investigated without the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst. Then, in the presence of different amounts of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst the reaction efficiency was calculated at the same temperature and time (Table V). The best yield was obtained in the case of using 20 mol % of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst.

TABLE V. Optimization of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst; 2.5 mmol benzaldehyde, 2.5 mmol ethyl acetoacetate, 3.75 mmol urea under 100 °C for 90 min

Entry	Catalyst content, mol %	Yield, %
1	–	34
2	10	52
3	15	79
4	20	91
5	25	90
6	30	88

The reaction of other benzaldehydes with electron-withdrawing and electron-donating functional groups were investigated with urea or thiourea and ethyl acetoacetate in order to prepare of the 3,4-dihydropyrimidine-2(1*H*)-ones/thiones. All reactions were performed in the presence of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst using a catalytic amount (20 mol %) in water at 100 °C. The preparation of the 3,4-dihydropyrimidine-2(1*H*)-ones/thiones were obtained in 75–97 % yield (Table VI). According to the information in Table VI, the electron-withdrawing functional groups are effective in the activity of benzaldehyde, however spatial hindrance can reduce the speed and efficiency of the reaction.

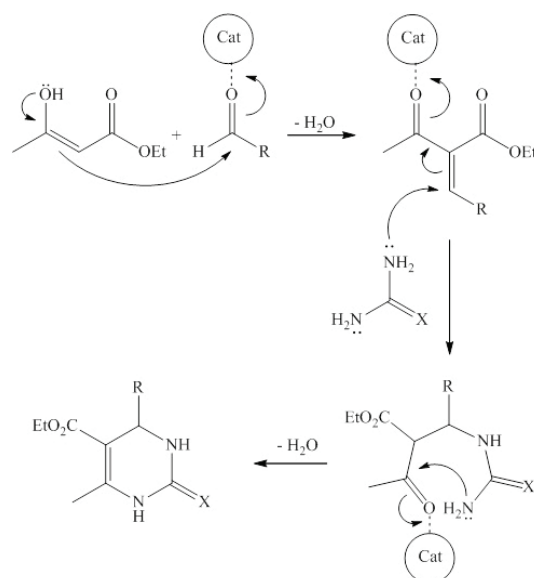
A proposed mechanism is presented based on the reactivity of benzaldehyde derivatives for the preparation of the 3,4-dihydropyrimidine-2(1*H*)-ones/thiones (Scheme 2). In the first step, $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst leads to the activation of aldehyde and then ethyl acetoacetate and aldehyde produce intermediate chalcone during aldol condensation in the presence of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst. Finally, the 3,4-dihydropyrimidine-2(1*H*)-ones/thiones are produced by Michael addition and removal of water by urea or thiourea.

Reusability of the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst

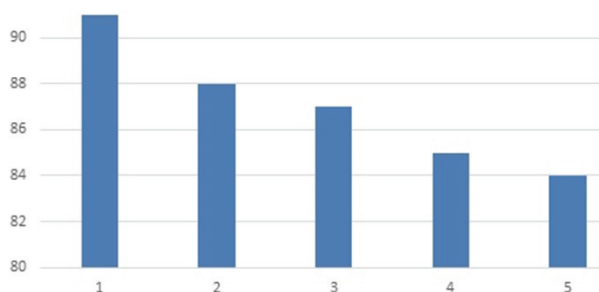
It was possible to separate and purify the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst, which is done by filtration or centrifugation. $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst was reused after washing with water, ethanol and ethyl acetate and then drying at 80 °C for 2 h. This process was carried out in five cycles and the reduction of yield was observed from 91 to 84 (7 % reduction of yield during 5 cycles of reaction, Fig. 9). $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst showed excellent stability and activity during 5 consecutive reaction periods. The reaction efficiency shows that the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst maintains its activity and durability during recycling.

TABLE VI. Synthesis of the 3,4-dihydropyrimidine-2(1*H*)-ones/thiones; 2.5 mmol aldehyde, 2.5 mmol ethyl acetoacetate, 3.75 mmol urea or thiourea and 170.6 mg of Fe₂CuAl₂O₇ nano-catalyst in water at 100 °C for 90 min

Entry	R	X	Yield, %	MP / °C	
				Found	Reported ⁴⁶⁻⁵¹
1	Ph	O	91	202–204	204–206
2	4-Me-Ph	O	81	215–217	214–216
3	4-MeO-Ph	O	85	203–205	201–203
4	2-HO-Ph	O	84	209–211	210–212
5	3-HO-Ph	O	82	170–172	167–170
6	4-HO-Ph	O	75	227–229	230–232
7	2-O ₂ N-Ph	O	90	220–222	221–222
8	3-O ₂ N-Ph	O	92	226–228	229–231
9	4-O ₂ N-Ph	O	94	213–215	211–213
10	4-Cl-Ph	O	92	214–216	215–216
11	Ph	S	92	207–209	205–207
12	4-Me-Ph	S	77	192–194	191–193
13	4-MeO-Ph	S	80	150–152	153–155
14	2-HO-Ph	S	84	203–205	206–208
15	3-HO-Ph	S	93	181–183	183–185
16	4-HO-Ph	S	79	193–195	193–194
17	2-O ₂ N-Ph	S	95	213–215	215.4–216.4
18	3-O ₂ N-Ph	S	90	217–219	214–216
19	4-O ₂ N-Ph	S	97	191–193	190–192
20	4-Cl-Ph	S	91	188–190	192–195



Scheme 2. The suggested mechanism for synthesis of the 3,4-dihydropyrimidin-2(1*H*)-ones/thiones.

Fig. 9. Reusability of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst.

CONCLUSION

In this paper, $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs were synthesized by sol-gel auto-combustion method. The XRD analysis showed that the crystalline structure of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NPs is the FCC type. According to the FESEM imaging, the particle size was determined to be about 41.443 nm. Fe, Cu, Al and O elements with ratio 2:1:2:7 were identified in the EDS analysis. The BET analysis showed the surface area of $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst to be $26.174 \text{ m}^2 \text{ g}^{-1}$. The Biginelli's multicomponent reaction was carried out in the presence of 20 mol % $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst at 100°C in water as a solvent. The $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst can be recovered and used for at least five periods without a significant decrease in activity. The use of water as the solvent, the short reaction time, the high efficiency and the easy separation of the $\text{Fe}_2\text{CuAl}_2\text{O}_7$ nanocatalyst are the advantages of this method.

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ИЗВОД

СИНТЕЗА И КАРАКТЕРИЗАЦИЈА НАНО $\text{Fe}_2\text{CuAl}_2\text{O}_7$ КАТАЛИЗАТОРА ЗА ВИШЕСТРУКУ УПОТРЕБУ ЗА БИГИНЕЛИЈЕВУ РЕАКЦИЈУMARZIEH M. KESHTIBAN¹, ABBAS NIKOO² и BAKHSHALI MASSOUMI¹

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У овом раду, нове наночестице мешаних металних оксида (NP) $\text{Fe}_2\text{CuAl}_2\text{O}_7$ су синтетисане применом методе сол-гел ауто-сагоревања. $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NP су идентификоване применом следећих техника: XRD, FTIR спектроскопије, мапирања и EDS. Дифрактограми су показали да $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NP имају кристалну структуру и само једну фазу, а кристали површински центрирану кубну решетку. Расподела величина $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NP, одређена FESEM техником, је око 41,44 nm. Применом BET једначине, одређена је специфична површина $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NP: $26,174 \text{ m}^2 \text{ g}^{-1}$. Синтетисане $\text{Fe}_2\text{CuAl}_2\text{O}_7$ NP су коришћене за Бигинелијеву реакцију. Деривати 3,4-дихидропиримидин-2(1H)-он/тиона су добијени у присуству $\text{Fe}_2\text{CuAl}_2\text{O}_7$ нанокатализатора за кратко време и 75–97 % ефикасности у води. Додатно, $\text{Fe}_2\text{CuAl}_2\text{O}_7$ нанокатализатор је успешно коришћен у пет циклуса без значајније промене каталитичких карактеристика.

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Application of principal component and hierarchical cluster analysis to classify different edible *Cucurbita* based on *in vitro* antioxidant activity

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Abstract: This study investigated the variations in antioxidant profiles between nine edible *Cucurbita* species (pericarp and seed), using pattern recognition tools; classification was achieved based on the results of global antioxidant activity assays (DPPH, ABTS, FRAP, CUPRAC, total reducing of power, levels of total phenolics and flavonoids compounds). The pericarp samples showed significantly lower total phenol values than the seed samples. The spaghetti squash shows the highest ability to neutralize DPPH radicals among seed and pericarp extracts. This extract also shows the highest FRAP and CUPRAC values. The tested samples were grouped into pericarp extract groups and seed extract groups based on the principal component analysis of antioxidative profiles. Hierarchical cluster analysis confirmed the PCA analysis. The presented results can guide breeders in selecting *Cucurbita* varieties with enhanced antioxidant traits, contributing to the development of nutritionally superior crops.

Keywords: *Cucurbita* genus; total phenolic content; total flavonoid content; antioxidant activity; chemometric approach.

INTRODUCTION

Cucurbitaceae is a large family of plants, also known as cucurbits, with 130 genera and 800 species, mainly tropical or subtropical, with a few species extending into temperate climate.¹ The name of the *Cucurbitaceae* family came from Latin, where the word *corbis* means bottle or basket. *Cucurbitaceae* is the most diverse plant family and is cultivated worldwide in various environmental conditions. Humans utilize over 300 plant species, with 150 being extensively

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cultivated and 30 of these are essential for global food production. The edible plants from this family that are frequently consumed for nutrition are mainly found in the genera *Cucurbita* (squash, pumpkin, zucchini), genus *Citrullus* (watermelon) and genus *Cucumis* (cucumber, various melons).² In the *Cucurbitaceae* family, pumpkin seeds are the only ones not discarded as waste, while melon and watermelon seeds are mostly thrown away despite being abundant and edible. The *Cucurbita* genus comprises 14 species with six subspecies and two wild varieties.³ The cultivated cucurbits are quite similar in their requirements for growth and development but their fruit morphology (sizes, shapes, colours, pulp structure) is highly variable. The original *Cucurbita* genus has been subject to intensive breeding that yielded the varieties and their hundreds of hybrids, we know today.

The most popular vegetables from this family are cucumber and pumpkin, but squash and zucchini are increasingly used in the kitchen. Pumpkin is one of the first domesticated plants and has been grown worldwide for several hundred years, thus giving farmers enough time to obtain and introduce cultivars with unique characteristics. Courgette, classified as a summer squash, belongs to *Cucurbita pepo*.^{4,5} Today, courgette is called summer squash, marrow, or zucchini, depending on the place of its cultivation.⁶ Summer squash (*Cucurbita pepo* subsp. *pepo*) is frequently consumed worldwide. Its shape resembles a ridged cucumber, but zucchini is available in yellow and green colors. *Cucurbita* fruits can often be used when they are not yet fully ripe. Botanically, this vegetable is considered as fruit, but in gastronomic terms, this is a vegetable. Zucchini has a firm texture with ripened fruit and a characteristic flower. It contains several beneficial micronutrients such as minerals (potassium, magnesium, phosphorus and calcium), carotenoids, vitamin C, phenolic compounds, *etc.*^{7–10} This fruit contains approximately 93.5–95 % of water, fiber (1.1 g/100 g f.w.), proteins (1–2.5 g/100 g f.w.), carbohydrates (2.3–4.2 g/100 g f.w.).^{11,12} Fruits of the *Cucurbita* genus are also sources of functional compounds with nutraceutical and preventive potential against cardiovascular diseases and diseases derived from eating disorders. A characteristic feature of the *Cucurbita* genus is its low-fat content and low glycemic index, attributed to its high dietary fiber content, particularly pectin.¹³ *Cucurbitaceae* plants are grown for their seeds, flowers, roots, leaves and fruits, all of which are edible.¹⁴

Certain seeds from the *Cucurbitaceae* family have been traditionally utilized in folkloric medicine.^{15,16} Due to bioactive nutrients, species from the *Cucurbita* genus are assumed to be used as a remedy for lowering blood cholesterol or depression.^{17,18} The folk medicinal properties attributed to zucchini are diverse and include its use in the treatment of benign prostatic hyperplasia and irritable bladder conditions.^{19,20}

Due to its antioxidant, anti-inflammatory, antimicrobial, anti-carcinogenic, antiviral and analgesic activities^{21–24} summer squash has been used in traditional folk medicine to treat colds and to alleviate aches. The natural plant components found in pumpkin could recover the liver against alcohol-induced liver toxicity and oxidative stress in rats.²⁵ The presence of bioactive nutrients suggests it could be used to lower blood cholesterol or treat depression. Pumpkin flesh exhibits hypoglycemic, *in vitro* antioxidant, cholinesterase and tyrosinase inhibitory effects, along with hypolipidemic activity.^{26–30} The cell growth inhibition of prostate, breast and colon cancer cells by ethanolic seed extract authenticates the ethnomedical use of pumpkin seeds for the treating of benign prostate hyperplasia urinary dysfunction.¹⁵ Pumpkin also exhibits various pharmacological actions, such as anticancer, antihypertension, antioxidant, anti-inflammation, anti-hyperlipidemic, antimicrobial, hypoglycemic and immunomodulation.^{31–36}

Natural antioxidants have been demonstrated to be more effective and devoid of the adverse health effects associated with synthetic antioxidants.^{37,38} Considerable attention has been given to the potential applications of natural antioxidants found in vegetables, fruits and grains, such as carotenoids, tocopherols, flavonoids, phenolic acids, vitamin C and minerals, in the prevention of numerous diseases.

The purpose of this study was to examine extracts from pericarp and seed of nine varieties of *Cucurbita* genus: (*Curcubita pepo* Ghost rider, *Curcubita maxima* Roter zentner, Spaghetti squash, Marrow Long Green Bush, Sweet gourmet zucchini, Summer Green Tiger Zucchini, Round Zucchini green, Green zucchini), concerning their content of total phenolics, flavonoids and antioxidant activity. As a result of the large amount of data that analytical techniques generate, the use of advanced multivariate chemometric tools is mandatory for extracting as much valuable information as possible.^{39–41} Obtained data were processed using principal component analysis (PCA) and hierarchical cluster analysis (HCA). The findings of this study may allow the identification of *Cucurbita* species with desired phenolic and flavonoid levels, aiding in the selection of antioxidant-rich varieties.

EXPERIMENTAL

Sample collection

Nine varieties of *Cucurbita* genus were acquired from local supermarkets in the Niš region, Southern Serbia. Examined species were cultivated at multiple locations within the Niš region. The fruits of nine varieties of *Cucurbita* genus underwent a rapid cold-water wash, the pericarps (*Curcubita pepo* Ghost rider (P1), *Curcubita maxima* Roter zentner (P2), Spaghetti squash (P3), Marrow Long Green Bush (P4), Sweet gourmet zucchini (P5), Summer Green Tiger Zucchini (P6), Round Zucchini green (P7), Round Zucchini yellow (P8), Zucchini green (P9)) and seeds (*Curcubita pepo* Ghost rider (S1), *Curcubita maxima* Roter zentner (S2), Spaghetti squash (S3), Marrow Long Green Bush (S4), Sweet gourmet zucchini (S5), Summer Green Tiger Zucchini (S6), Round Zucchini green (S7), Round Zucchini yellow (S8),

Zuchinni green (S9)) of each sample were separated. Fresh samples were pulverized with an electric blender and immediately analysed.

Preparation of extracts

The protocol used for extracting polyphenolic compounds from pericarp and seed of different edible *Cucurbita* varieties was based on a previously employed procedure.⁴² The pericarp and seed extracts, obtained from grinding 4 g of fresh samples, were extracted four times by stirring with 40 mL of 80 vol. % methanol in an ultrasonic bath (power supply: 220 V / 50 Hz, 10 A) at 25°C for 15 min. Samples were left in the solvent for 15 min, filtered and diluted to a final concentration of 100 mg mL⁻¹.

Chemicals and instruments

Except 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), FeCl₃·6H₂O, AlCl₃·6H₂O, CuCl₂, Na₂CO₃, Folin–Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and methanol were purchased from Sigma; all chemicals were purchased from Merck. Spectrophotometric assays were performed on a double-beam UV–Vis spectrophotometer Perkin Elmer lambda 15 (Massachusetts, USA).

Total phenolic content (TPC)

The Folin–Ciocalteu redox method is commonly used to analyse total phenolic content, as phenolics are a major group of bioactive plant compounds that function as primary antioxidants or free radical scavengers. This assay is particularly important for evaluating total antioxidant capacity, as a high phenolic content is strongly associated with enhanced antioxidant activity. This assay is significant in measuring total antioxidant capacity since high phenolic content has been linked to high antioxidant capacity. Total phenolic content was determined using the Folin–Ciocalteu reagent as described by Mitic *et al.*⁴² The reaction mixture consisted of 0.05 mL of extract, 2 mL of sodium carbonate solution, 5.0 mL of distilled water and 0.5 mL of the Folin–Ciocalteu reagent. The reaction was carried out in the dark for 30 min and then the absorbance was measured at 750 nm. Results were expressed as mg gallic acid equivalents per 100 g of fresh weight (mg GAE 100 g⁻¹ f.w.) since gallic acid was used to calculate the standard curve ($y = 0.0163x + 0.0121$, $R^2 = 0.9751$).

Total flavonoid content (TFC)

The total flavonoid content was determined using the aluminium chloride method, a widely used assay for quantifying flavonoids in plant extracts. In this method, aluminium chloride reacts with the carbonyl group at position C4 and the hydroxyl groups at positions C3 and C5 in flavonols and flavones, forming a stable complex. Additionally, it can form labile acid complexes with ortho-dihydroxyl groups (catechol groups) present in the flavonoid structure. Total flavonoid content of analysed samples was determined by a method described by Dimitrijević *et al.*⁴³ Extract aliquot (50 µL) was mixed with 0.15 mL of a NaNO₂ solution. After 5 min, 0.75 mL of AlCl₃ solution was added and the solution was kept 5 min at room temperature. Then, 1 mL of NaOH solution was added to the mixture and water was added to a final volume of 5 mL. The absorbance was measured at 510 nm. Rutin solution was used for calibration curve construction ($y = 0.0356x + 0.0214$, $R^2 = 0.9907$) and results were expressed as mg rutin equivalents (RE) per 100 g of fresh weight (mg RE 100 g⁻¹ f.w.).

DPPH free radical scavenging assay

Diphenyl-1-picrylhydrazyl (DPPH) is a popular, quick, easy and affordable approach for the measurement of antioxidant properties. This test measures the scavenging capacity of anti-

oxidants against the free radical DPPH. The advantage of this method lies in its ability to allow DPPH to interact with the entire sample, providing sufficient time for the reaction to occur, even with weak antioxidants that react more slowly. Quantitative tests of methanol extracts for DPPH radicals were performed according to the method of Mitic *et al.*⁴² The reagent was prepared 2 h before use to ensure all the DPPH had dissolved. The flask containing DPPH solution was covered with aluminium foil to protect it from light. 1.5 mL of methanol solution of DPPH radical at the concentration of 100 mmol L⁻¹, 0.1 mL of extract concentration of 100 mg mL⁻¹ and methanol to a total volume of 4 mL were added to a test tube. The mixture was shaken vigorously and left in the dark at room temperature for 60 min. The reduction in the colour of the solution caused by free radicals (DPPH) was measured at 515 nm. Trolox dissolved in methanol in appropriate dilution was used as a standard. A linear calibration curve was obtained in the range of 0.5–4 µg mL⁻¹ Trolox, with regression equation $y = 0.0405x - 0.0495$ and linear regression coefficient $R^2 = 0.9963$. The antioxidant capacities of the samples were expressed as mg Trolox equivalents per 100 g of fresh weight g (mg TE 100 g⁻¹ f.w.).

ABTS cation radical decolorization assay

ABTS cation radical “scavenging” activity assay is based on the ability of antioxidant molecules to quench ABTS radical, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water-soluble vitamin E analogue. The ABTS^{•+} cation radical was prepared by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1). The flask containing this solution was covered with aluminium foil to protect it from light and it was stored at room temperature for 12–16 h before use as described by Mitic *et al.*⁴² The prepared solution was then diluted with methanol to obtain an absorbance of 0.7±0.02 units at 734 nm. An aliquot of each extract, concentration 100 mg mL⁻¹ was mixed with 1.8 mL of diluted ABTS solution and diluted with methanol to a total volume of 4 mL, absorbance was measured 6 min after initial mixing. An appropriate solvent blank was used in each assay. The same procedure was repeated for all standard Trolox solutions (0.1–2.5 µg mL⁻¹) and a standard curve ($y = 0.0322x - 0.0141$, $R^2 = 0.9997$) was constructed. Results were expressed as mg Trolox equivalents per 100 g of fresh weight (mg TE 100 g⁻¹ f.w.).

Ferric reducing antioxidant power (FRAP) assay

The FRAP analysis (ferric reducing antioxidant power) indicates the reducing ability of the extract based on the reduction of colourless Fe³⁺–TPTZ to blue Fe²⁺–TPTZ complex. This assay was performed according to the protocol described.⁴² The FRAP reagent was prepared by mixing a freshly prepared acetate buffer (pH 3.6), the 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution and ferric chloride (20 mM), (10 mM in 40 mM HCl) in a 10:1:1 ratio. An aliquot of the extract (10 µL) was mixed with 3 mL of the freshly prepared FRAP solution and diluted with water to a final volume of 4 mL. After incubation of the mixture at 37 °C (for 5 min) the absorbance at 595 nm was recorded. The value of FRAP was determined by plotting on a standard curve ($y = 0.0955x - 0.0185$, $R^2 = 0.9941$) prepared by adding ferrous sulphate (0.7–7 µg mL⁻¹) to the FRAP reagent and the results were expressed as mg Fe(II) per 100 g of fresh weight (mg Fe(II) 100 g⁻¹ f.w.). The control solution was prepared as above but contained distilled water instead of extract samples.

Cupric reducing antioxidant capacity (CUPRAC) assay

Cupric reducing antioxidant capacity (CUPRAC) spectrophotometric method is based on the absorbance measurement of Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction of chain-breaking antioxidants with the CUPRAC reagent, Cu(II)-Nc, where

absorbance is recorded at the maximal light absorption wavelength of 450 nm; thus this is an electron-transfer (ET)-based method. In a test tube, the following were added in sequence: 0.05 mL of the extract, 1 mL of phosphate buffer (pH 7.0), neocuproine, copper(II) chloride. The mixture was then diluted with water to a total volume of 4.1 mL.⁴² The reaction mixtures were incubated at room temperature for 30 min. Trolox dissolved in methanol in appropriate dilution (5–25 $\mu\text{g mL}^{-1}$) was used as the calibration curve's standard under the same parameters. The stability of the Trolox standards was monitored over a range of concentrations (in triplicate) by measuring their absorbance values and plotting their average against a range of time points. It was confirmed that the working Trolox standards were stable for the first three hours of the assay thereby removing any absorbance measurement inaccuracies, due to the decomposition of standards in the assay system. Cupric reducing antioxidant capacity was expressed as mg Trolox equivalents per 100 g of fresh weight (mg TE 100 g^{-1} f.w.).

Total reducing power (TRP) assay

The reducing power of the extracts was determined according to the potassium ferricyanide-ferric trichloroacetic acid method based on the ability of antioxidants to reduce Fe(III) hexacyanate to Fe(II) hexacyanate, resulting in an increase in the absorbance in the absorbance of the reaction mixtures. The higher absorbance of the extracts indicated greater ferric reducing capacity. The reaction mixtures were prepared by mixing 1 mL of 1 % solution $\text{K}_3[\text{Fe}(\text{CN})_6]$, 1 mL of phosphate buffer (pH 6.6), 0.01 mL of the extract and diluted with water to 3.7 mL. The mixtures were incubated at 50 °C for 30 min and then 1 mL of 10 % solution of trichloroacetic acid and 0.6 mL of FeCl_3 were added.⁴² The absorbance was measured at 700 nm against the blank sample. A standard curve ($y = 0.1056x - 0.0452$, $R^2 = 0.9855$) was plotted using different concentrations of ascorbic acid (2–50 $\mu\text{g mL}^{-1}$), so that the results were expressed as mg ascorbic acid equivalents per 100 g of fresh weight (mg AAE 100 g^{-1} f.w.). Working ascorbic acid standards were stable for the first three hours of the assay, thereby avoiding the absorbance measurement inaccuracies due to the decomposition of standards in the assay system.⁴⁴

Statistical analysis

Statistical analyses were performed using the Statistica version 12.0 software package. All the experiments were conducted in triplicate. To eliminate uncertain data, the Q-Dixon test was performed. All data were checked for normality using the Shapiro–Wilk test. ANOVA's Tukey multiple comparison test was used to compare the variance in the response variables: total phenolic concentration (TPC), total flavonoids content (TFC) and the antioxidant capacity: DPPH, ABTS, CUPRAC, TRP of nine varieties of *Cucurbita* genus. Differences were considered statistically significant at a level of 0.05 (that is, $p < 0.05$). Agglomerative hierarchical clustering was used as a multivariate analysis tool to cluster samples based on similar characteristics and then plotted as a dendrogram. The distance matrix was calculated using the Euclidean Pythagorean distance differences and hierarchical cluster analysis was performed using Ward's method. Pattern recognition methods were applied to the data collection; these were principal component analysis (PCA). PCA facilitated the identification of similarities among samples and revealed the primary associations between variables contributing to the total variability in the analysed data.

RESULTS AND DISCUSSION

Total phenolic content (TPC)

Polyphenols include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, condensed tannins, lignans and lignins. According to the human physiological standpoint, phenolic compounds play a significant role in defence mechanisms, such as antioxidant, anti-inflammatory, anti-aging and anti-proliferative activities. Quantifying polyphenols is a standard practice for identifying foods and food products with high potential to combat free radicals. In this process, polyphenols in extracts react with a specific redox reagent, the Folin–Ciocalteu reagent, forming a blue complex, that can be measured using visible-light spectrophotometry. For an approximate estimation of the TPC of *Cucurbita* species extracts the Folin–Ciocalteu phenol reagent assay is used. The mean content values of the phenolics in the pericarp and seeds of the nine *Cucurbita* species are listed in Fig. 1a. TPC values for pericarp and seed samples were in the intervals 8.88–19.76 mg GAE 100 g⁻¹ f.w. and 20.21–48.71 mg GAE 100 g⁻¹, respectively.

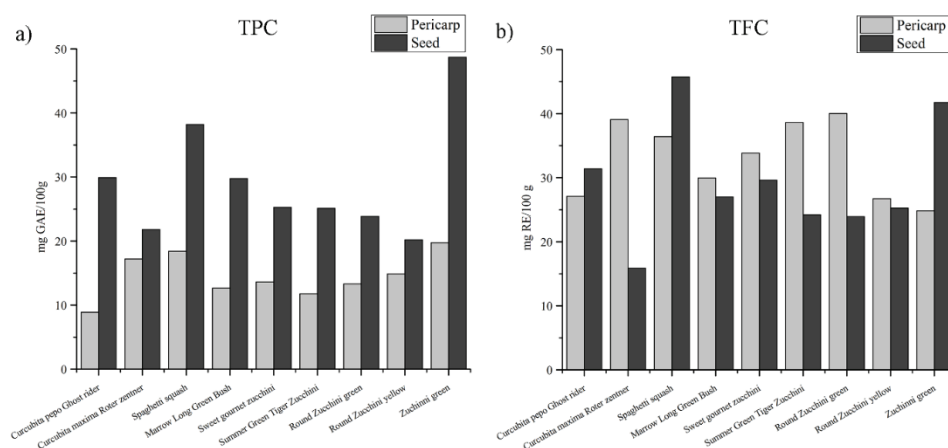


Fig. 1. Results of: a) total phenolic contents (TPC) and b) total flavonoid content (TFC) in the pericarp and seed of nine varieties of *Cucurbita* genus.

The pericarp samples showed significantly lower TPC values than the seed samples. The TPC in four seed samples (*Zucchini green*, *Spaghetti squash*, *Cucurbita pepo* Ghost rider, *Marrow Long Green Bush*) were the highest (48.71, 38.20, 29.89 and 29.75 mg GAE 100 g⁻¹ f.w., respectively). The pericarp samples *Cucurbita pepo* Ghost rider and *Summer Green Tiger Zucchini* have the lowest values (8.88 and 11.78 mg GAE 100 g⁻¹ f.w., respectively). Among the nine *Cucurbita* species examined, seeds and pericarp of *Zucchini green* stands out with notable higher TPC, when compared to the other tested varieties.

As Tejada *et al.* (2020) indicated, factors such as variety, harvest period, growth conditions and ripening stage significantly influence the antioxidant profiles of courgette fruits. For example, the frozen raw zucchini showed reduced phenolic content ($0.12 \mu\text{g GAE g}^{-1}$ dry extract) compared to the fresh samples ($0.15 \mu\text{g GAE g}^{-1}$). Stir-frying increased the phenolic content ($0.14 \mu\text{g GAE g}^{-1}$) compared between steaming ($0.12 \mu\text{g GAE g}^{-1}$) and raw samples. Other studies revealed varying TPC levels across *Cucurbita* species and parts, influenced by genetic, environmental and extraction factors. In the frozen raw zucchini ($0.12 \mu\text{g GAE g}^{-1}$ dry extract) a decrease in the content of phenolic compounds is observed compared to the fresh samples ($0.15 \mu\text{g GAE g}^{-1}$ dry extract).⁴⁵ For *Cucurbita pepo*, the highest levels of total phenolics were noted in the fruits of “Kamo Kamo” (51.5 mg chlorogenic equivalents per 100 g of fresh weight) and the “Sweet Dumpling” (48.1 mg chlorogenic equivalents per 100 g of fresh weight) cultivars.¹⁴ The total phenolic content of aqueous extracts from seed, peel, leaf and flower powder of *C. maxima* was found to be as follows: seed – $0.79 \text{ mg GAE g}^{-1}$, peel – $2.46 \text{ mg GAE g}^{-1}$, leaf – $0.27 \text{ mg GAE g}^{-1}$, flower – $15.73 \text{ mg GAE g}^{-1}$. The isopropanol extracts from the same plant parts obtained the following TPC values: seed $41.05 \text{ mg GAE g}^{-1}$, peel $0.20 \text{ mg GAE g}^{-1}$, leaf $0.66 \text{ mg GAE g}^{-1}$ and flower $33.27 \text{ mg GAE g}^{-1}$.⁴⁶ In an India-based study, TPC of pumpkin flowers was found to be $49.6 \text{ mg GAE } 100 \text{ g}^{-1}$ dried powder.⁴⁷ In contrast, the Ethiopia-based research reported that the total phenolic content in pumpkin peel and seed ranged from 354 to 380 $\text{mg GAE } 100 \text{ g}^{-1}$ and 80 to 102 $\text{mg GAE } 100 \text{ g}^{-1}$ powder, respectively.⁴⁸

Other studies revealed varying TPC levels across *Cucurbita* species and parts, influenced by genetic, environmental and extraction factors. The total phenolic content of pumpkin peel and pulp extracts was comparable, measuring $5.21 \text{ mg GAE g}^{-1}$ for the peel and $5.19 \text{ mg GAE g}^{-1}$ for the pulp.⁴⁹ The content of total phenolic compounds in the analyzed *Cucurbita* spp. peel extracts varied from 17.599 ± 0.124 to $4.623 \pm 0.082 \text{ mg GAE g}^{-1}$ d.w. as published by Gawel-Beben *et al.*⁵⁰ The average total phenolic compounds recorded in zucchini was 8.67 GAE g^{-1} f.w. reported by Hamissou *et al.*⁵¹

The variation in phenolic content is known to be due to genetic factors, degree of maturity and environmental conditions. Secondly, the extractability of phenolic compounds is governed by the type of solvent (polarity) used, the degree of polymerization of phenolics, the interaction of phenolics with other constituents, as well as the extraction time and temperature. The complete extraction of all phenolic compounds from plant materials cannot be achieved using a single, universally effective method. In addition, different ways of expressing the content of polyphenolic components (concerning the equivalents of gallic acid, equivalents of pyrocatechol, equivalents of quercetin according to fresh sample,

according to dry sample, according to extract) make it difficult to compare all the results.

Total flavonoids content (TFC)

Flavonoids are a diverse group of natural compounds with the ability to scavenge free radicals, chelate metal ions and modulate enzymatic activities. This antioxidant activity is primarily attributed to their phenolic hydroxyl groups, which can donate hydrogen atoms or electrons to neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS). As a basis for the quantitative determination, the flavonoid contents in selected plant extracts were determined using aluminium chloride in a colorimetric method. The basic principle involved in this method is that AlCl_3 forms acid-stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. The TFC values of the samples assayed in this study differed according to pericarp and seed of the *Cucurbita* species (Fig. 1b). The TFC values for pericarp samples were in the intervals 24.82 (Zucchini green) – 40.02 (Round Zucchini green) mg RE 100 g⁻¹ f.w.), while the values 15.88 (*Curcubita maxima* Roter zentner) – 45.73 (Spaghetti squash) mg RE 100 g⁻¹ f.w. were found in seed samples. Extract of the seed of the *Curcubita maxima* Roter zentner showed the lowest values in this test, also this sample has one of the lowest values of the content of total phenolic compounds (21.81 mg GAE 100 g⁻¹ f.w.). At the same time, the content of flavonoids (39.09 mg RE 100 g⁻¹ f.w.) in the pericarp of *Curcubita maxima* Roter zentner is one of the highest. Although less abundant in *Cucurbita* species, flavonoids are highly effective as antioxidants, even at low concentrations.⁵² A survey of past literature reports found that Mokhtar *et al.* analysed the flavonoids of *Curcubita moschata* at various stages of ripening (unripe, mature, ripened) and determined antioxidant activity. The content of the flavonoids compound in ripe pumpkin was 28.6 mg QE g⁻¹.⁵³ The total flavonoid content in pumpkin peel and seeds was found to range from 130 to 153 mg QE per 100 g⁻¹ in the peel and from 51 to 67 mg QE per 100 g⁻¹ in the seed powder, as reported by Hagos *et al.*⁴⁸ Kar *et al.* reported that the aqueous extracts from the seed and peel of *C. maxima* contain 71.06 mg QE g⁻¹ and 63.80 mg QE g⁻¹, respectively.⁴⁶ The total flavonoid content in the analysed *Cucurbita* spp. peel extracts, varied from 2.598±0.127 (*C. moschata* “Muscat”) to 7.108±0.120 (*C. maxima* “Hokkaido”) mg QE g⁻¹ d.w.⁴⁶ Rutin was the flavonol found in all analyzed cultivars (the content from 51.92±0.03 to 5.09±0.01 mg 100 g⁻¹). Kaempferol, quercetin, isoquercetin, astragalin and myricetin were detected only in some of the varieties. No significant differences in the content of the listed flavonols were found between the *C. pepo* and *C. moschata* cultivars, except for quercetin, which was most abundant in *C. pepo* (3.29±3.43

vs. $1.07 \pm 1.74 \text{ mg } 100 \text{ g}^{-1}$) as publishes by Gawel-Beben *et al.*⁵⁰ Saha *et al.* found that 1 g of dry extract *C. maxima* contained $26.50 \pm 1.40 \text{ mg}$ equivalent of quercetin.⁵⁴ The results of the total flavonoid content obtained in previous studies by Astutik and Yanti were $0.146 \text{ mg QE } 100 \text{ g}^{-1}$ extract of unripe pumpkin, $0.221 \text{ mg QE } 100 \text{ g}^{-1}$ extract of mature pumpkin and $0.191 \text{ mg QE } 100 \text{ g}^{-1}$ extract of ripened pumpkin.⁵⁵ Previous research highlighted significant variability in flavonoid content across *Cucurbita* species, influenced by extraction methods, solvent types and environmental factors. Such differences underscore the importance of standardizing extraction and measurement methods for reliable comparisons. No direct comparisons were possible between the results of our study and most of the previously published assay values, due to the vast differences in the published literature in terms of extracting solvents, assay incubation time, selection of different reference standards, presentation of results in various units, etc.

Antioxidant capacity of Cucurbita species

Due to their complex mechanisms, antioxidants exhibit diverse responses to different radicals or oxidants. Consequently, various assay systems are required to comprehensively and accurately evaluate antioxidant activity. *In vitro* methods for assessing antioxidant activity are notably diverse – some include a distinct oxidation step followed by measurement, while others do not delineate these procedural stages.⁵⁶

In this study, five spectrophotometric methods were used to monitor variations in antioxidant capacity: DPPH free radical scavenging assay, ABTS cation radical decolorization assay, cupric reducing antioxidant capacity (CUPRAC) ferric reducing antioxidant power assay (FRAP) and total reducing power (TRP). The findings are summarized in Figs. 2a and b and 3a–c.

DPPH free radical scavenging assay

The radical scavenging activity (RSA) of extracts from nine *Cucurbita* species was assessed using the DPPH free radical scavenging assay, which primarily operates through electron transfer and hydrogen atom abstraction mechanisms. As shown in Fig. 2a, Spaghetti Squash demonstrated the highest ability to neutralize DPPH radicals among both seed extracts ($5.29 \text{ mg TE } 100 \text{ g}^{-1} \text{ f.w.}$) and pericarp extracts ($5.18 \text{ mg TE } 100 \text{ g}^{-1} \text{ f.w.}$). The other seed extracts, including those from Marrow Long Green Bush, *Cucurbita pepo* Ghost Rider and Sweet Gourmet Zucchini, also exhibited intense DPPH radical-scavenging activities, with values of 5.23, 5.19 and $5.16 \text{ mg TE } 100 \text{ g}^{-1} \text{ f.w.}$, respectively. The DPPH activity values ranged from $1.12 \text{ mg TE } 100 \text{ g}^{-1} \text{ f.w.}$ for pericarp extracts. (Summer Green Tiger Zucchini) to $5.18 \text{ mg TE } 100 \text{ g}^{-1} \text{ f.w.}$ (Spaghetti Squash). Notably, the pericarp of *Cucurbita maxima* Roter Zentner showed vigorous scav-

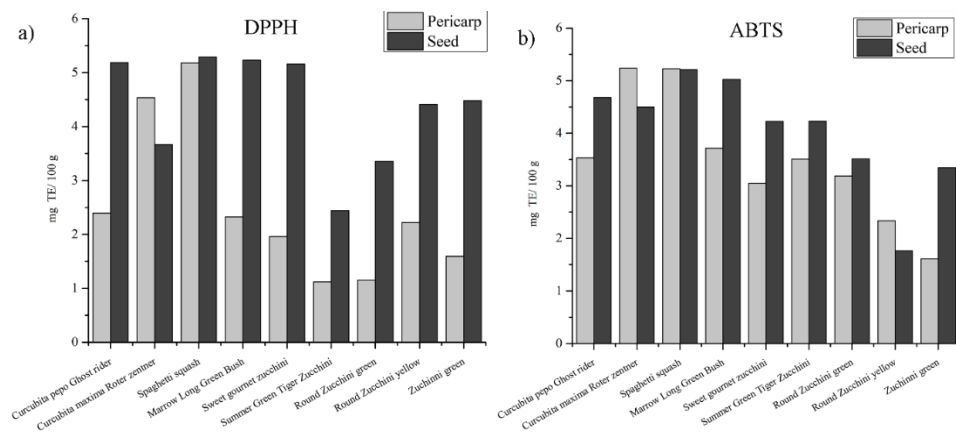


Fig. 2. Results of: a) DPPH free radical scavenging assay and b) ABTS cation radical decolorization assay in the pericarp and seed of nine varieties of *Cucurbita* genus.

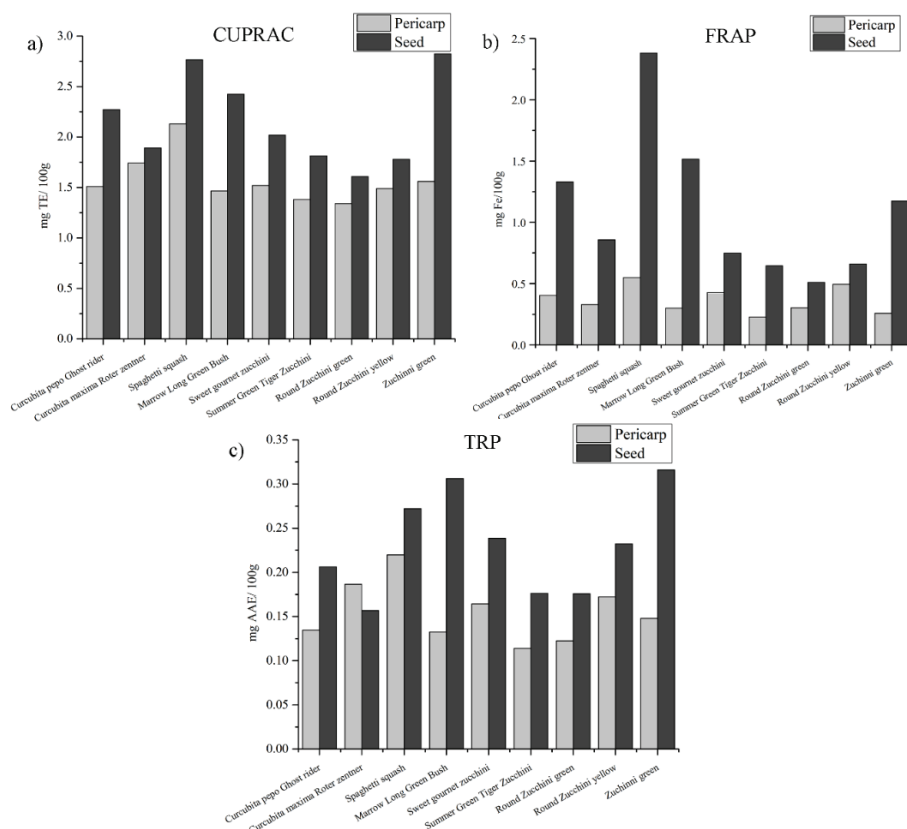


Fig. 3. Results of: a) ferric reducing antioxidant power (FRAP), b) total reducing power (TRP) and c) cupric reducing antioxidant capacity (CUPRAC) in the pericarp and seed of nine varieties of *Cucurbita* genus.

enging activity (4.53 mg TE 100 g⁻¹ f.w.). In comparison, Round Zucchini Green and Zucchini Green exhibited minimal activity (1.15 and 1.59 mg TE 100 g⁻¹ f.w., respectively).

Several studies have explored the antioxidant activity of *Cucurbita* species using the DPPH assay. Kostecka-Gugała *et al.* reported DPPH values ranging from 3.31–32.1 μmol TE 100 g⁻¹ f.w. for *C. pepo*, 1.01–13.08 μmol TE 100 g⁻¹ f.w. for *C. maxima* and 2.45–16.08 μmol TE 100 g⁻¹ f.w. for *C. moschata*.¹⁴ In some research, DPPH results are expressed as the amount of antioxidants required to reduce the initial DPPH concentration by 50 % (EC₅₀). For example, Mala and Kurian (2016) found that methanol extracts of pumpkin peel and pulp achieved 50 % inhibition (IC₅₀) at a concentration of 18 mg mL⁻¹, while at 50 mg mL⁻¹, scavenging activity reached approximately 80 %.⁴⁹ Kar *et al.* (2023) reported that the aqueous extracts of dried *C. maxima* seeds, peels, leaves and flowers showed DPPH scavenging activities of 48.41, 55.71, 83.91 and 58.28 %, respectively. Interestingly, the isopropanol extracts exhibited higher scavenging activities of 67.99, 90.35, 49.80 and 74.60%, respectively.⁴⁶ Various methods exist for expressing the antioxidant activity results, which can be as diverse as the measurement techniques. For instance, while some results are expressed regarding EC₅₀ or Trolox equivalents, others report percent scavenging activity, complicating direct comparisons between studies.

ABTS cation radical decolorization assay

The ABTS cation radical decolorization assay, also known as the trolox equivalent antioxidant capacity (TEAC) assay, evaluates the ability of antioxidants to donate electrons or hydrogen atoms to neutralize radical species. The reduction of the ABTS radical cation (ABTS•⁺) to ABTS serves as an indicator of antioxidant effectiveness. All extracts from *Cucurbita* species demonstrated their capacity to decolorize ABTS solution, underscoring their efficacy in scavenging organic radicals. The trends observed in the ABTS and DPPH assays were generally consistent. The ABTS scavenging activity of all examined seed extracts ranged from 1.76 to 5.21 mg TE 100 g⁻¹ f.w. The highest scavenging activity was observed in the pericarp of *Cucurbita maxima* Roter Zentner (5.24 mg TE 100 g⁻¹ f.w.) and Spaghetti squash (5.23 mg TE 100 g⁻¹ f.w.). In comparison, the lowest activities were recorded in Zucchini Green pericarp (1.61 mg TE 100 g⁻¹ f.w.) and Round Zucchini Yellow seed (1.76 mg TE 100 g⁻¹ f.w.). Seed extracts of *Curcubita pepo* Ghost rider and *Curcubita maxima* Roter Zentner also showed high ABTS scavenging activity 4.68 mg TE 100 g⁻¹ f.w. and 4.49 mg TE 100 g⁻¹ f.w., respectively. In this assay, the pericarp extracts of *Curcubita maxima* Roter Zentner, Spaghetti squash and Round zucchini yellow displayed greater ABTS binding capacity (5.24, 5.23, 2.34 mg TE 100 g⁻¹ f.w.) than their corresponding seed extracts (4.49, 5.21, 1.76 mg TE 100 g⁻¹ f.w.).

Additionally, a study by Mala and Kurian reported ABTS radical scavenging activity for pumpkin peel and pulp extracts, with IC_{50} values around 6 and 10 mg mL⁻¹, respectively.⁴⁹

Comparing antioxidant results across assays remains challenging due to differences in mechanisms, solvents and reaction conditions, as well as the variability in how results are expressed (*e.g.*, in μ mol, mmol, or mg of standard equivalents per g, 100 g or kg of fresh/dry weight).⁴⁴

CUPRAC

The CUPRAC method assesses antioxidant activity at near-physiological pH, contrasting with the basic (pH 10) or acidic conditions required for Folin or FRAP assays. Among the tested extracts, *Spaghetti squash* showed the highest antioxidant activity, with values of 2.77 mg TE 100 g⁻¹ f.w. in seeds and 2.13 mg TE 100 g⁻¹ f.w. in the pericarp. In contrast, Round Zucchini Green displayed the lowest values, measuring 1.61 mg TE 100 g⁻¹ f.w. in seeds and 2.77 mg TE 100 g⁻¹ f.w. in the pericarp. Notably, the seed extracts of Zucchini Green and Marrow Long Green Bush also demonstrated elevated CUPRAC values, recording 2.77 and 2.42 mg TE 100 g⁻¹ f.w., respectively. The cupric-reducing antioxidant capacity of extracts can be attributed to the content of phenolics, since the phenolic content in seed samples is higher than in pericarp samples. Comparative data on ferric-reducing antioxidant power can be found in the study by Kostecka-Gugała *et al.* (2020). The published FRAP values for different species are as follows: *Cucurbita pepo*: 11.7–57.8 μ mol TE 100 g⁻¹ f.w., *Cucurbita maxima*: 21.7–47.9 μ mol TE 100 g⁻¹ f.w., *Cucurbita moschata*: 21.2–139.9 μ mol TE 100 g⁻¹ f.w.¹⁴

Ferric-reducing antioxidant power (FRAP)

The determination of ferric-reducing antioxidant power (FRAP) is a typically simple, rapid and cost-effective ET-based method performed under the acidic pH conditions. The transformation ability of compounds from Fe³⁺/ferricyanide complex to Fe²⁺/ferrous form serves as a reliable indicator for antioxidant activity. The FRAP assay of antioxidants is convenient, reproducible and linearly concentration-dependent.⁵⁷ The results (Fig. 3b) indicated that Spaghetti squash showed the highest FRAP activity in seeds (2.38 mg Fe 100 g⁻¹ f.w.) and pericarp extracts (0.55 mg Fe 100 g⁻¹ f.w.), correlating with its high polyphenol content (seeds – 18.42 mg GAE 100 g⁻¹ f.w., pericarp – 38.20 mg GAE 100 g⁻¹ f.w.). The seed extract of Marrow Long Green Bush also shows high activity in this test 1.52 mg Fe 100 g⁻¹ f.w..

The available data for the ferric-reducing antioxidant power can be seen in the Kostecka-Gugała *et al.* paper¹⁴ where published values in the interval 11.7–

–57.8 $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ f.w.}$ (*C. pepo*) 21.7–47.9 $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ f.w.}$ (*C. maxima*) 21.2–139.9 $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ f.w.}$ (*C. moschata*).

Our results cannot be directly compared with the previously published findings, due to differences in testing methodologies and the presentation of results.

The reducing power assay (TRP)

The reducing power assay is a convenient and rapid screening method for measuring the antioxidant potential. The reducing power of extracts is related to their electron transfer ability and may serve as a significant indicator of potential antioxidant activity. The results of the reducing power assay of the tested extracts are summarized in Fig. 3c. The seed extract of Marrow Long Green Bush exhibited the highest reducing power (0.31 mg AAE $100 \text{ g}^{-1} \text{ f.w.}$), correlating with its phenolics (29.75 mg GAE $100 \text{ g}^{-1} \text{ f.w.}$) and flavonoids (27.00 mg QE $100 \text{ g}^{-1} \text{ f.w.}$) content. Among the pericarp extracts, Spaghetti squash showed the highest reducing power (0.22 mg AAE $100 \text{ g}^{-1} \text{ f.w.}$). In comparison, Summer Green Tiger Zucchini had the weakest activity (0.11 mg AAE $100 \text{ g}^{-1} \text{ f.w.}$), aligning with its low DPPH activity. To the best of our knowledge, there are no available studies in the literature on the antioxidant activity of the *Cucurbita* species extracts determined by this method.

The analytical findings of antioxidant profiles from *Cucurbita* species assist in identifying varieties with high antioxidant activity, providing a valuable benchmark for breeding programs. The detailed analysis of antioxidant profiles allows breeders to pinpoint which specific compounds (*e.g.*, specific phenols or flavonoids) are most responsible for high antioxidant activity. This understanding enables targeted breeding for varieties enriched in those compounds rather than merely increasing overall antioxidant levels. Varieties with high antioxidant activity are attractive for their potential health benefits, including reducing oxidative stress and lowering the risk of chronic diseases.

Statistic analysis

The interpretation of the chemical data from phenol and flavonoid content and antioxidant activity has recently gained importance through chemometrics. These methods facilitate the evaluation of similarities and differences between various extracts or project samples onto a two-dimensional factorial plane based on distinct characteristics using diverse mathematical and statistical approaches. Multivariate statistical techniques, particularly chemometrics, are increasingly applied in science and technology due to their ability to extract comprehensive information from chemical data, including chemical composition and antioxidant activity. This study employed pattern recognition techniques such as principal component analysis (PCA) and hierarchical cluster analysis (HCA). PCA, an unsupervised classification approach and HCA, an unsupervised learning

method, effectively identified patterns and grouped species with high antioxidant content. These analyses identify patterns and groupings of species with high antioxidant content, enabling the targeted selection of varieties for specific purposes and can guide breeders in selecting *Cucurbita* varieties with enhanced antioxidant traits, contributing to the development of nutritionally superior crops, so PCA and HCA streamline the selection process, saving time and resources in breeding programs.

Before the chemometrics application, all variables were autoscaled (transformation into *z*-scores) to standardize the statistical importance of all responses. After this mathematical operation, each parameter contributes equally to the data set variance and carries equal weight in the principal component calculation. Then, the matrix of samples ($n = 18$) and the response variables ($n = 7$) was built, in which the samples were adopted as lines and variables as rows, totaling 126 data points. The correlation matrix was constructed to analyse the relation between the values of antioxidant capacities for all the analysed cultivars and the content of phenolics and flavonoids. (Table I). A correlation matrix provides quantitative insights into the relationships between multiple variables in a dataset: values of Pearson's correlation coefficients indicate the strength and direction of the relationship between each pair of variables. Also, it highlights how one variable changes in response to another, which can help identify trends, dependencies, or redundancies within the data.

TABLE I. The Pearson's correlation coefficients for selected antioxidant parameters of nine varieties of *Cucurbita* genus; abbreviations: ABTS-2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay, DPPH-diphenyl picrylhydrazyl free radical scavenging assay, TRP-total reducing power assay CUPRAC-cupric reducing antioxidant capacity assay, FRAP-ferric reducing antioxidant power assay TPC-total phenolic content, TFC-total flavonoids content, *values of Pearson's correlation coefficients significant at $p < 0.05$, **values of Pearson's correlation coefficients significant at $p < 0.001$

	ABTS	DPPH	TRP	CUPRAC	FRAP	TPC	TFC
ABTS	1.000000	0.65**	0.33	0.51*	0.48*	0.28	0.28
DPPH		1.00	0.82**	0.81**	0.68**	0.64**	0.13
TRP			1.00	0.91**	0.75**	0.84**	0.25
CUPRAC				1.00	0.87**	0.91**	0.33
FRAP					1.00	0.77**	0.27
TPC						1.00	0.25
TFC							1.00

The correlation analysis revealed numerous statistically significant connections between the contents of bioactive compounds with antioxidative properties. A significant positive correlation was observed between the total phenolic compound content and antioxidant capacity, as measured by the CUPRAC ($r = 0.91$ **) and TRP ($r = 0.84$ **) methods. The content of phenolics was also posi-

tively correlated with FRAP ($r = 0.77^{**}$) and DPPH ($r = 0.64^{**}$) assays. These results emphasized the importance of phenolic compounds in the antioxidant behaviour of the examined extracts and indicated that the phenolic compounds contributed significantly to the total antioxidant activity. The high correlation coefficient for antioxidant capacities measured by FRAP and CUPRAC ($r = 0.87^{**}$) confirms the similarity of the two methods in terms of their mechanisms of action. The antioxidant capacity as measured by CUPRAC and by DPPH was positively correlated ($r = 0.81^{**}$). There were also positive correlations between the content of total phenolic compounds and FRAP ($r = 0.77^{**}$), the content of total phenolic compounds and DPPH ($r = 0.64^{**}$) and between the FRAP and TRP ($r = 0.75^{**}$). No negative correlation was observed between the investigated variables. The principal components (PCs) were determined from the eigenvalues of the correlation matrix of observations. The eigenvalues were calculated as 4.5926 for PC1 and 0.9723 for PC2. The contribution of the variables to the PCs is shown in Fig. 4 and as can be seen, the first two PCs explain 79.50 % of the total variance (65.61 % (PC1) and 13.89 % (PC2) of the total variance, respectively.)

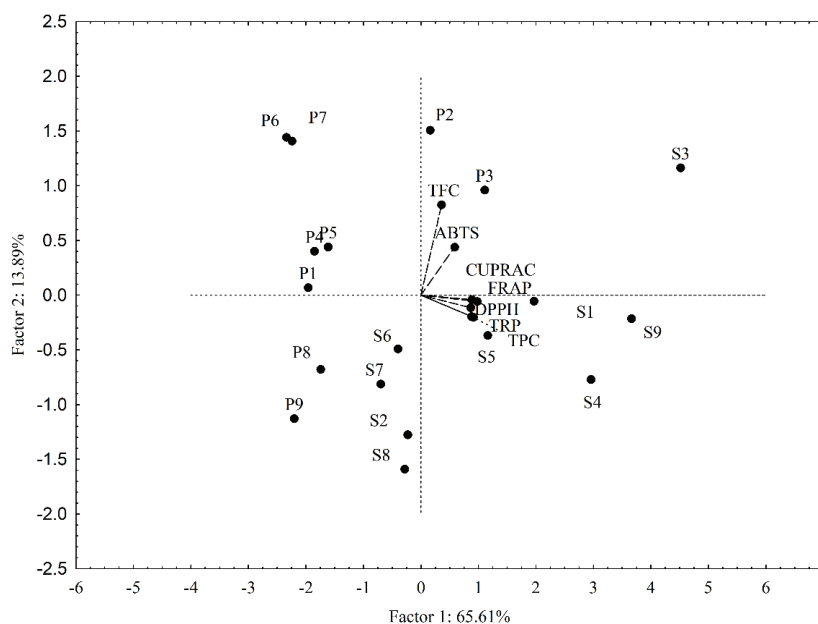


Fig. 4. Principal component analysis (PCA) of total phenolic content (TPC), total flavonoid content (TFC), DPPH free radical scavenging assay, ABTS cation radical decolorization assay, ferric reducing antioxidant power (FRAP), total reducing power (TRP) and cupric reducing antioxidant capacity (CUPRAC) in the pericarp and seed of nine varieties of *Cucurbita* genus. The first principal component (PC1 – 65.61 %) and the second principal component (PC2 – 13.89 %) represent the total variance of the data (79.50 %).

PC1 is generally better correlated with the variables than PC2. This is expected, as the principal components (PCs) are extracted sequentially, with each one accounting for the maximum possible remaining variance. The CUPRAC and TRP values strongly correlate with PC1 in the negative direction (0.9819, 0.9152), while the ABTS values show a positive correlation with PC2 (0.4384). Based on the principal component analysis, all tested samples were clustered into two groups: one comprising seed extracts and the other comprising pericarp extracts. These parameters are significantly correlated as evidenced by their Pearson correlation coefficients. TPC and ABTS are found in opposition to FRAP, CUPRAC, DPPH, TRP and TFC. Among the tested extracts, the Spaghetti squash seed, has the highest loading (4.5177) on PC1. High loadings on PC1 are also shown by the samples of the Marrow Long Green Bush seed (2.9591) Summer Green Tiger Zucchini pericarp (−2.3387), the Round Zucchini green seed (−2.2413). The pericarp extract of *Curcubita maxima* Roter Zentner, Sweet gourmet zucchini, Round Zucchini green and Summer Green Tiger Zucchini have the highest loading on PC2 (1.5061, 1.4409, 0.4402 1.4080, respectively). Using the plots in Fig. 4, it is possible to suggest reasons for the location of the varieties based on their antioxidant activity. The location of *Spaghetti squash pericarp* and *Curcubita maxima Roter Zentner pericarp* in the first quadrant of Fig. 4 may be explained by their high values of TPC and ABTS which are co-located in this region of the PC space.

The same data matrix used in the principal component analysis was applied for cluster analysis, utilizing Euclidean distance and Ward's method. The results obtained the following HCA are shown as a dendrogram (Fig. 5) in which three well-defined clusters are visible. Samples will be grouped in clusters in terms of their nearness or similarity. The seed extracts of Zucchini green and Spaghetti squash have a strong relationship with each other, forming a distinct cluster separate from all other groups. These species are associated in the first cluster with high antioxidant activity measured by ABTS, DPPH, CUPRAC, FRAP and TRP. The third cluster contains pericarp samples from all tested extracts except Zucchini green. The second cluster includes samples of seven seed extracts and the pericarp extract of Zucchini green. The high flavonoid concentration in the pericarp of Zucchini Green plays a key role in this clustering.

The high phenolic and flavonoids content in Green zucchini seed suggests a notable antioxidant potential, as phenolic compounds are commonly linked to such activity. However, not all phenolic compounds possess equivalent antioxidant efficacy. While the seed of Zucchini green has a high total phenol concentration and exhibits significant antioxidant activity, as determined by the CUPRAC and TRP methods, the specific phenolic compounds it contains may be less effective at scavenging free radicals. Antioxidant efficiency often relies on the synergistic interaction between phenolics and other bioactive compounds,

such as carotenoids, vitamins, tannins. Furthermore, the bioavailability and solubility of certain phenols in the seed of Green zucchini may limit their functionality in biochemical assays, resulting in an underutilization of their antioxidant potential. Consequently, despite its elevated phenol levels, the seed of Green zucchini demonstrates suboptimal performance in DPPH, ABTS and FRAP antioxidant activity. In the second cluster (seed cluster), the greatest similarity was observed between Summer Green Tiger Zucchini and Round Zucchini green – Euclidean distance 1.8 and between *Curcubita pepo* Ghost rider and Marrow Long Green Bush Euclidean distance of 4.4. In the third cluster, the pericarp cluster, varieties Summer Green Tiger Zucchini and Round Zucchini green also exhibit the highest similarity, forming a subcluster with the smallest Euclidean distance of 2.1. Additionally, a high similarity (Euclidean distance of 3) is observed between the samples *Curcubita maxima* Roter Zentner and Spaghetti squash.

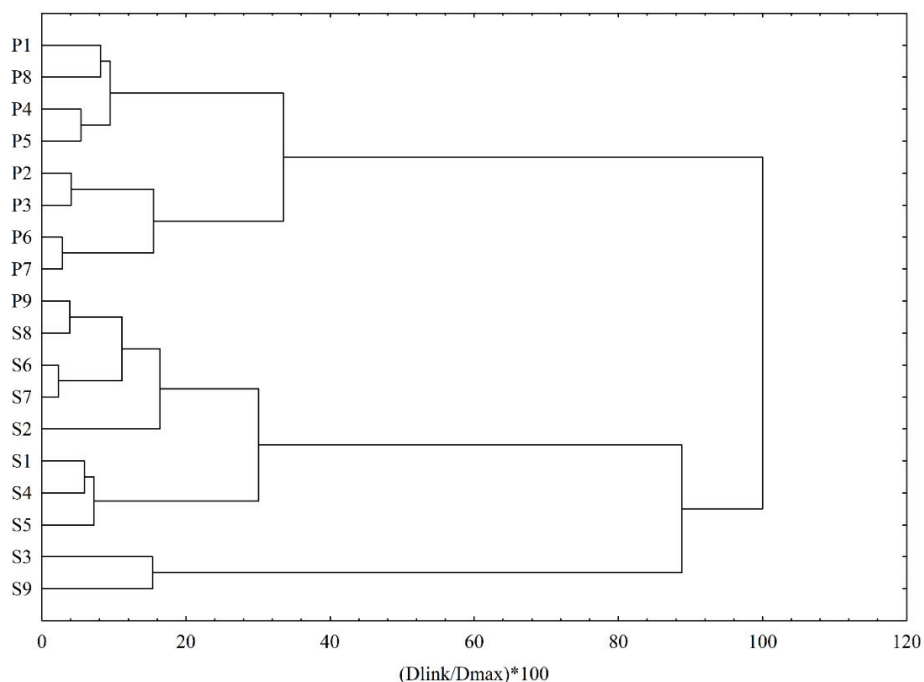


Fig. 5. Hierarchical Cluster Analysis (HCA) of total phenolic content (TPC), total flavonoid content (TFC), DPPH free radical scavenging assay, ABTS cation radical decolorization assay, ferric reducing antioxidant power (FRAP), total reducing power (TRP) and cupric reducing antioxidant capacity (CUPRAC) in the pericarp and seed of nine varieties of *Cucurbita* genus. *Dlink/Dmax* represents the quotient between the linkage distances for a particular case divided by the maximal linkage distance. The dendrogram was generated using Ward's method, with Euclidean distance applied to assess similarity.

CONCLUSION

In the present study analysis of free radical scavenging activity and total phenolics and flavonoid content showed that the methanol extract from pericarp and seed of nine varieties of *Cucurbita* genus can be a potent source of natural antioxidants. Some considerable variations were observed between the different examined varieties in terms of total antioxidant activity and different antioxidant polyphenolic compounds. The Spaghetti squash is the species whose pericarp extract showed the best antioxidant characteristics in all methods (ABTS, DPPH, TRP, CUPRAC, FRAP). Also, the *Cucurbita maxima* Roter zentner pericarp extract shows significant antioxidant characteristics in the method. The Spaghetti squash seed extract also has the highest results in four (ABTS, DPPH, CUPRAC, FRAP) of the five applied methods.

The principal component analysis obtained from antioxidative profiles grouped the tested samples into two groups, a group of pericarp extracts and a group of seed extracts. The hierarchical cluster analysis confirmed the PCA analysis, with one small difference. In addition to the two clusters containing only seed samples (Cluster 1) and only pericarp samples (Cluster 3), the hierarchical analysis identified a mixed cluster, which includes one pericarp sample (Green zucchini) alongside seven seed samples. The combined use of PCA and HCA demonstrated clear differentiation among *Cucurbita* extracts, based on their antioxidant profiles. These insights are instrumental for breeding programs to enhance the specific antioxidant traits in *Cucurbita* varieties, enabling the development of nutritionally superior crops.

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ИЗВОД

ПРИМЕНА АНАЛИЗЕ ГЛАВНИХ КОМПОНЕНАТА И ХИЈЕРАРХИЈСКЕ КЛАСТЕР
АНАЛИЗЕ ЗА КЛАСИФИКАЦИЈУ РАЗЛИЧИТИХ ЈЕСТИВИХ *CUCURBITA* НА ОСНОВУ
ИН ВИТРО АНТИОКСИДАТИВНЕ АКТИВНОСТИ

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Ова студија је истраживала варијације у антиоксидативним профилима девет јестивих *Cucurbita* врста (перикарп и семе), користећи алате за препознавање образаца; класификација је постигнута на основу резултата тестова антиоксидативне активности (DPPH, ABTS, FRAP, CUPRAC, укупна редукујућа моћ, нивои укупних фенолних једињења и флавоноида). Узорци перикарпа су имали значајно ниже вредности укупних фенола од узорака семена. Екстракти семена и перикарпа врсте *Spaghetti squash* показују највећу способност неутралисања DPPH радикала. Ови екстракти такође показују и највеће вредности у FRAP и CUPRAC тестовима. Анализа главних компоненти вредности антиоксидативних профила испитиваних екстраката груписала је испитиване

узорке у две групе: екстракти перикарпа и екстракти семена. Хијерархијска кластер анализа потврдила је резултате анализе главних компоненти. Добијени резултати могу да усмере узгајиваче у одабиру *Cucurbita* врста са побољшаним антиоксидативним особинама, доприносећи развоју нутритивно супериорних усева.

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Analysis and statistical modeling of copper ions biosorption onto calcined chicken eggshell

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Abstract: The study on the possible use of calcined chicken eggshells as a biosorbent for copper ions removal from aqueous solutions, as well as some comparisons between raw and calcined eggshells, are presented in this paper. SEM-EDS and FTIR analysis of the calcined chicken eggshell samples were performed. In addition, the DTA-TGA analysis on raw chicken eggshells was performed. The influence of various process parameters, such as solution pH, stirring rate, biosorbent mass and Cu²⁺ concentration, was investigated. The kinetic analysis using four different empirical kinetic models was performed. The equilibrium analysis was done using the Langmuir, Freundlich and Temkin isotherm models. The process was optimized using the response surface methodology based on the Box–Behnken design (RSM-BBD). The obtained results are compared to our previous study on raw eggshells as a biosorbent for Cu²⁺ removal, in order to determine the justification for biosorbent modification (*i.e.*, the calcination of raw eggshells).

Keywords: biosorption; calcined chicken eggshell; equilibrium; kinetics; Box–Behnken design.

INTRODUCTION

Water is a crucial resource that occupies an indispensable position in earth's environment. Its main purpose is to support life. Availability of clean drinking water is of vital importance for human welfare. Unfortunately, in many parts of the world, we face the challenge of low quality water sources, which leads to health concerns. The quality of water has a significant effect on the biodiversity in the aquatic ecosystems as well. Lastly, water is an important constituent in numerous industrial processes.¹

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Heavy metal pollution originating from wastewater generated by industrial activity is a serious global problem. The importance of removing pollutants, including hazardous heavy metals, is increasing with time, as a consequence of growing demands for clean water all around the world.^{2,3}

One of the widespread water contaminants among heavy metals is copper (Cu^{2+}). Main sources of copper wastewater pollution include mining, metal, electroplating, textile and leather industry. The reported copper concentrations in wastewater from different sources range from 2.5 to 10000 mg dm^{-3} . According to the WHO, maximum Cu^{2+} content in drinking water is 2 mg dm^{-3} . Hence, wastewater treatment in order to remove this pollutant is crucial.^{3,4}

Various processes are used for wastewater treatment on an industrial scale, including chemical precipitation, ion exchange, reverse osmosis, ultrafiltration, coagulation and flocculation, *etc.* However, these processes are not suitable for treating wastewater contaminated with heavy metals in low concentrations. Adsorption is considered a more suitable treatment method for wastewater with low concentrations of heavy metals. Adsorption can be described as a process of mass transfer, which consists of transferring the adsorbate from the solution to the surface of the adsorbent, where it is bound by physical and/or chemical interactions.^{5,6}

The removal of heavy metals from wastewater by inexpensive biosorbents has been the focus of numerous scientific studies in recent years. This process is called biosorption. The biosorption process involves the use of inactive biomass as an adsorbent. Therefore, the mechanism of removal of the pollutants is complex and is usually based on mechanisms such as ion exchange, adsorption, chelation, chemisorption, precipitation or their combination.^{6,7}

Eggshells are considered waste, which is a product of the food industry that can be a potential biosorbent due to its high calcium content. Eggshell biomass has no utility value, no specific application and in most cases is disposed of as biowaste in landfills. The possible use of eggshells in the biosorption process thus solves several problems. First of all, the use of eggshells as a biosorbent would lead to a reduction in the amount of waste in landfills, thus helping industries which use eggs by reducing the cost of disposing of their waste. On the other hand, the use of eggshells as a biosorbent for treating wastewater contaminated with heavy metals would help solve the problem of environmental pollution.⁷

The main purpose of this research is the evaluation of the possibility of using a modified form of chicken eggshell (calcined chicken eggshell) as a biosorbent for copper ions removal from aqueous solutions. This work is a continuation of a previous study on the use of raw chicken eggshells for Cu^{2+} removal from aqueous solutions.⁸ Therefore, it analyses the difference in the efficiency in Cu^{2+} removal of the modified eggshell in comparison to the raw eggshell, as well as the other differences between the two processes. This analysis serves as an ans-

wer to the question whether the use of additional resources and energy for the modification of the eggshells is justified. Additionally, the potential use of eggshells (raw or calcined) as a biosorbent could prove to be very important from more than one point of view, as mentioned above. Firstly, the removal of Cu^{2+} from wastewaters would have a significant effect on environmental pollution. Secondly, the use of a waste product, such as eggshells, would help solving the landfill problem.

EXPERIMENTAL

Biosorbent

Raw chicken eggshell (RCE, Fig. 1a) were crushed, sieved through a set of laboratory sieves, and the fraction $(-1 + 0.4)$ was used for further preparation of the biosorbent. The eggshells were rinsed with distilled water, dried, and then calcined in a laboratory furnace, at $900\text{ }^{\circ}\text{C}$, for one hour. The obtained calcined chicken eggshell (CCE, Fig. 2a) sample was used as a biosorbent for the Cu^{2+} biosorption experiments.⁹

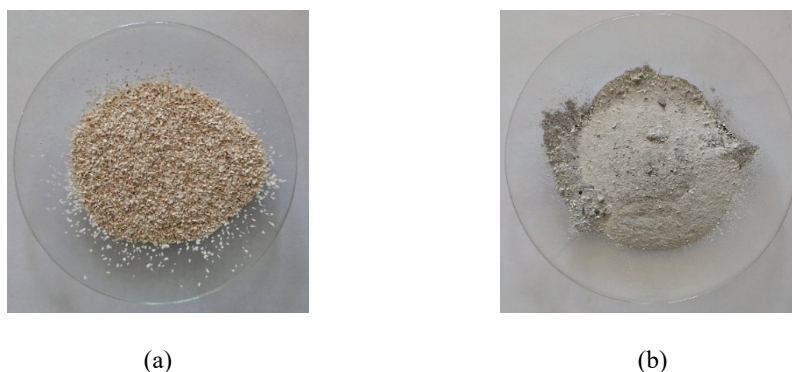


Fig. 1. Raw chicken eggshell (RCE) sample (a), and calcined chicken eggshell (CCE) sample (b).

Materials and methods

The biosorption experiments were performed with synthetic copper ions solutions prepared with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (p.a. purity). The pH of the solutions was adjusted using 0.1 M KOH, and 0.1 M HNO_3 solutions. The chemicals used were manufactured by Lachema (Czech Republic).

The concentration of Cu^{2+} in the solutions was determined on a Spectroquant Pharo 300 spectrophotometer (Merck). The pH value of the solution was measured using a pH meter Jenco VisionPlus, while the conductivity of the solution was measured by the WTW inoLab cond – 720 conductometer. The DTA-TGA analysis was performed on a simultaneous DSC-DTA-TGA device SDT Q600 (TA Instruments). FTIR analysis of the samples was performed on a Smart iTR Nicolet iS50 spectrophotometer (Thermo Fisher Scientific). The surface morphology of the samples was recorded using the SEM-EDS method on a Vega 3 LMU Tescan scanning electron microscope with X-act SDD 10 mm^2 energy-dispersive X-ray analysis (Oxford Instruments).

The biosorption capacity and metal removal degree were calculated using as:

$$q_t = \frac{C_i - C_t}{m} V \quad (1)$$

$$RD\% = 100 \left(1 - \frac{C_t}{C_i} \right) \quad (2)$$

where: q_t is the biosorption capacity defined as mass of the adsorbed metal per unit mass of the biosorbent (mg g^{-1}) at time t ; C_i and C_t are the initial and final concentrations of metal ions (g dm^{-3}) at time t ; V is the volume of the solution used in the biosorption experiments (dm^3); and m is the mass of the biosorbent (g); $RD\%$ is the removal degree (%).

All the experiments, bar the thermodynamic analysis, were performed at room temperature.

Calcined eggshells characterization

The DTA-TGA of the eggshell sample was performed in order to obtain information about the thermal stability and degradation of this material. The eggshell sample was heated in an inert atmosphere from 20 to 900 °C. The SEM-EDS analysis was performed on CCE samples before and after the Cu^{2+} biosorption process. FTIR analysis was performed on CCE samples taken before the biosorption of copper, and after, to determine which functional groups interact with Cu^{2+} in the process of binding these ions to the structure of the adsorbent. The FTIR spectrum of the calcined chicken eggshell samples was recorded in the range from 4000 to 400 cm^{-1} . The sample was placed on a diamond plate and fixed using a pressure tower. Prior to the measurements, a signal of the background was recorded and automatically subtracted by an accompanying Omnic™ software. The copper-loaded CCE was prepared for the characterization analysis by bringing into contact 1 g of the samples with Cu^{2+} solutions (initial concentration 5 g dm^{-3} , and the pH value of the solutions 4.5) for 90 min.

The influence of different process parameters on biosorption efficiency

A series of experiments were carried out to determine the influence of pH on biosorption capacity. 50 cm^3 Cu^{2+} solutions (initial concentration 5 g dm^{-3}) with pH values ranging from 2 to 5 were brought into contact with 1 g CCE samples, for 60 min, and the biosorption capacity was analyzed. The pH values were adjusted using 0.1 M KOH and 0.1 M HNO_3 .

The effect of initial copper ions concentration on the biosorption capacity was analyzed by bringing into contact 1 g of CCE samples with 50 cm^3 solutions of different Cu^{2+} concentrations, in the range from 0.5 to 20 g dm^{-3} . The pH value of the solutions was 4.5. The suspensions were stirred for 60 min and the biosorption capacity was analyzed.

The influence of the mass of the biosorbent on the biosorption degree was analyzed by bringing into contact 50 cm^3 of Cu^{2+} solutions (2 g dm^{-3} initial concentration) with different amounts of CCE samples, ranging from 0.1 to 2 g, and stirring the suspensions for 60 min. The pH value of the solutions was 4.5.

The effect of stirring rate on the biosorption degree was evaluated by bringing into contact 1 g of CCE with 50 cm^3 copper ions solutions (initial Cu^{2+} concentration 5 g dm^{-3} , and the pH value of the solutions 4.5) at different stirring rates, ranging from 100 to 600 rpm. The contact between phases was sustained for 60 min, after which the suspension was filtered and the filtrate analyzed.

Biosorption kinetic study and determination of activation energy

Kinetic models can be used to analyze the experimental data, to obtain information about the biosorption rate and its mechanism. To obtain the experimental kinetic data, CCE (1 g) were brought into contact with 50 cm³ of Cu²⁺ solutions (initial concentration 5 g dm⁻³ and the pH value of the solutions 4.5) at different contact times, ranging from 1 to 90 min. The obtained experimental data were analyzed using four kinetic models: the pseudo-first order kinetic model (Eq. (3)), the pseudo-second order kinetic model (Eq. (4)), the Weber–Morris kinetic model (Eq. (5)) and the Elovich kinetic model (Eq. (6)). These models can be expressed as follows:^{9–11}

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \quad (3)$$

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad (4)$$

$$q_t = k_i t^{1/2} + C_i \quad (5)$$

$$\frac{dq_t}{dt} = \alpha e^{-\beta q_t} \quad (6)$$

where: q_t is the biosorbent capacity defined as the mass of the adsorbed metal per unit mass of the biosorbent (mg g⁻¹) at time t ; q_e is the biosorbent capacity defined as the mass of the adsorbed metal per unit mass of the adsorbent (mg g⁻¹) at equilibrium; and k_1 is the biosorption rate constant for the pseudo-first order kinetic model (min⁻¹); k_2 is the biosorption rate constant for the pseudo-second order kinetic model (g mg⁻¹ min⁻¹); k_i is the internal particle diffusion rate constant (mg g⁻¹ min^{-0.5}); and C_i is a constant that provides insight into the thickness of the boundary layer; α is the starting biosorption rate (mg g⁻¹ min⁻¹); β is the parameter that expresses the degree of surface coverage and activation energy for chemisorption (g mg⁻¹).

Biosorption equilibrium study

The equilibrium study of the biosorption process provides insight into the interaction between metal ions and biosorbents, which helps to determine the effectiveness and the mechanism of the process.¹²

The biosorption isotherm data was obtained by bringing into contact 1 g of CCE samples with 50 cm³ Cu²⁺ solutions of different initial concentrations, in the range from 0.5 to 10 g dm⁻³. The pH value of the solutions was 4.5. The suspension was stirred for 60 min, filtered and the filtrate analyzed. The obtained experimental data were fitted with three different isotherm models. The used models were: the Langmuir isotherm model (Eq. (8)), the Freundlich isotherm model (Eq. (9)) and the Temkin isotherm model (Eq. (10)).⁸

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad (7)$$

$$q_e = K_L C_e^{1/n} \quad (8)$$

$$q_e = B \ln(K_T C_e) \quad (9)$$

where: C_e is the equilibrium concentration of metal ions (mg dm⁻³); q_e is the equilibrium adsorption capacity (mg g⁻¹); q_m is the maximum adsorption capacity (mg g⁻¹); and K_L is the Langmuir equilibrium constant (dm³ g⁻¹); K_F is the Freundlich equilibrium constant ((mg g⁻¹) (dm³ mg⁻¹)^{1/n}); $B = RT/b$ is the Temkin constant, which refers to the adsorption heat (J mol⁻¹);

b is the variation of adsorption energy (J mol^{-1}); R is the universal gas constant ($\text{J mol}^{-1} \text{K}^{-1}$); T is the temperature (K); K_T is the Temkin equilibrium constant ($\text{dm}^3 \text{g}^{-1}$).

Biosorption thermodynamic study

The thermodynamic parameters were determined by bringing into contact 1 g of CCE with 50 cm^3 of Cu^{2+} solutions (initial concentration 5 g dm^{-3}) for 60 min, at different temperatures, in the range from 25 to 45 °C. The obtained results were used to calculate the thermodynamic parameters using the following equations:⁸

$$K_d = \frac{C_A}{C_S} \quad (10)$$

$$\Delta G = -RT \ln K_d \quad (11)$$

$$\ln K_d = \left(\frac{\Delta S}{R} \right) - \left(\frac{\Delta H}{RT} \right) \quad (12)$$

where: K_d is the thermodynamic equilibrium constant; C_A is the concentration of the adsorbed adsorbate (mg dm^{-3}); C_S is the equilibrium concentration of the adsorbate in the solution (mg dm^{-3}); ΔG is the Gibbs energy change (kJ mol^{-1}); R is the universal gas constant ($\text{J mol}^{-1} \text{K}^{-1}$); T is the temperature (K); ΔS is the entropy change ($\text{J mol}^{-1} \text{K}^{-1}$); ΔH is the enthalpy change (kJ mol^{-1}).

Biosorption optimization study

Copper ions biosorption using CCE as a biosorbent was optimized using the Box–Behnken experimental design in order to determine the effects of three selected variables on the efficiency of Cu^{2+} removal. The response surface methodology (RSM) is an experimental design used to predict and model relationships between independent and dependent factors and one or more responses.¹³ The Box–Behnken factor experimental design, which consists of 17 experiments, was applied to optimize the biosorption process by analyzing and comparing the three selected factors: adsorbent mass (A), initial copper ion concentration (B) and contact time (C). The test series and their values are listed in Table I.

TABLE I. Experimental design (experimental ranges and their levels)

Factor	Range level		
	−1	0	1
A – Adsorbent mass, g	0.5	1	1.5
B – Initial metal ion concentration, g dm^{-3}	5	7	10
C – Contact time, min	10	60	90

RESULTS AND DISCUSSION

DTA-TGA analysis of chicken eggshells

Thermal analysis of the chicken eggshells sample was performed in order to determine the changes that occur during the calcination process. The obtained results are shown on Fig. 2.

The TGA curve, given in Fig. 2, shows two stages of weight loss of the sample during the heating/calcination process. In the range from 20 to 600 °C, a weight loss of 3.59 % was recorded, corresponding to the decomposition of org-

anic matter (proteins), as well as the release of the structurally bound water (moisture). In the range from 600 to 900 °C, a weight loss of 43.26 % was observed, which can be attributed to the decomposition of calcium carbonate, during which CO₂ gas is released. This process is accompanied by an endothermic peak on the DTA curve, with a maximum at 771.54 °C. This stage marks the transformation of the chicken eggshell sample into stable CaO. The total weight loss was 46.85 %. Similar findings were reported in the work of Witton.¹⁴

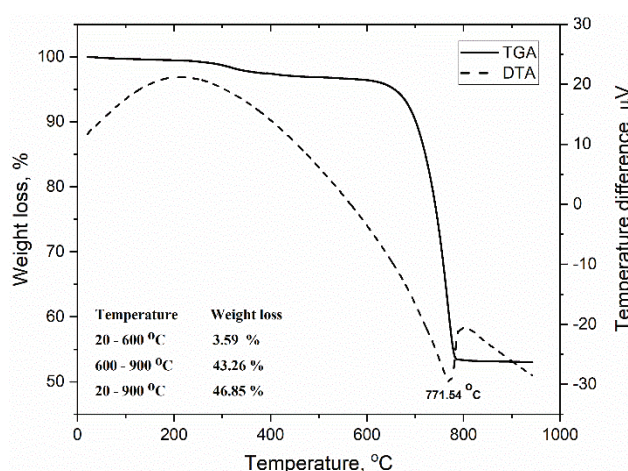


Fig. 2. DTA-TGA analysis of the RCE sample.

SEM-EDS analysis of CCE before and after the Cu²⁺ biosorption

The results of the SEM analysis of the CCE sample before and after the Cu²⁺ biosorption are shown on Fig. 3. The obtained EDS analysis is given in Table II.

The morphology of the RCE sample, was examined in the previous work. The obtained results given in the previously published work suggested that the surface morphology of the RCE sample was mainly porous.⁸ A change in the surface morphology of the eggshell samples can be noted after calcination (Fig. 3a and 3b), as the calcination process led to the forming of an irregular structure, consisting of agglomerates and flakes of different sizes (from 1 up to 500 μm).^{15,16} The SEM image of the CCE sample after the biosorption is given on Fig. 3c and d. It can be noted that the particles were partially agglomerated after the interaction between the Cu²⁺ solution and the biosorbent. The irregular structure is still present, but the particles are somewhat larger in size, with the present flakes partially agglomerated.

The EDS analysis of the CCE samples before the biosorption process shows that Ca, Mg and K are present. The EDS analysis after Cu²⁺ biosorption showed a significant decrease in the Ca content, while the Mg and K disappeared completely, as well as an appearance of Cu on the surface of the analyzed sample.

The changes showed by the EDS analysis indicate that Ca, Mg and K could potentially be involved in the biosorption process. The RCE EDS analysis obtained in the previous research showed similar results.⁸

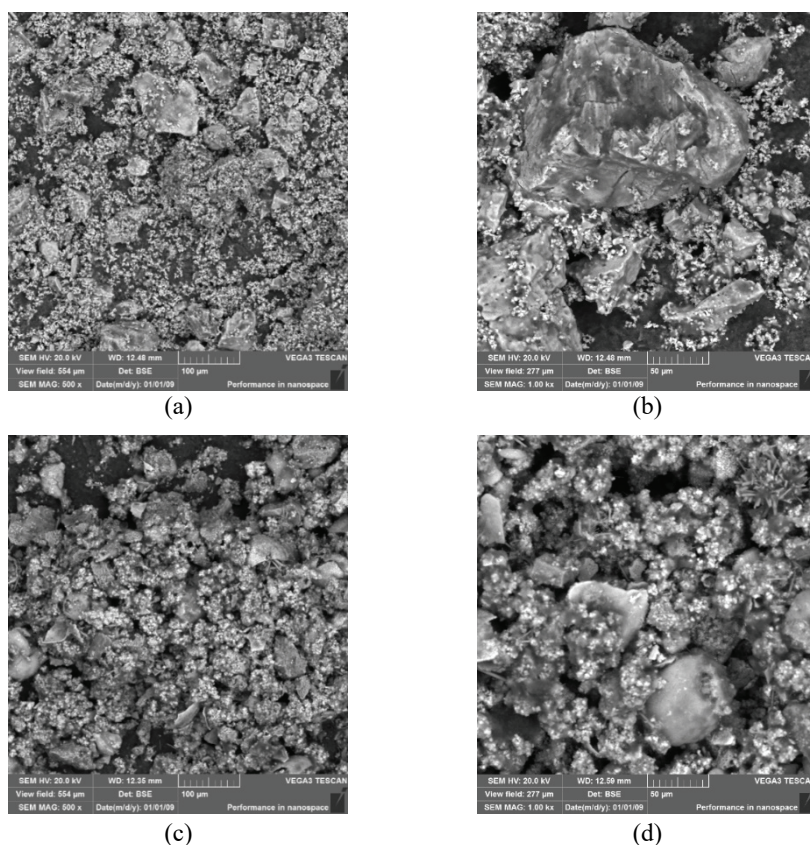


Fig. 3. SEM micrograph of the CCE before at 500× (a) and 1000× (b) and after the biosorption process at 500× (c) and 1000× (d).

TABLE II. The EDS analysis of the CCE samples before and after the biosorption process

Before Cu ²⁺ biosorption	Element	O	Mg	K	Ca	Cu	Total
	Content, wt. %	58.7	0.67	0.53	40.1	–	100
After Cu ²⁺ biosorption	Element	O	Mg	K	Ca	Cu	Total
	Content, wt. %	49.19	–	–	14.89	35.92	100

FTIR analysis of the raw RCE sample and CCE sample before and after Cu²⁺ biosorption

The results of the FTIR analysis of the raw RCE and CCE sample before and after Cu²⁺ biosorption are shown in Fig. 4.

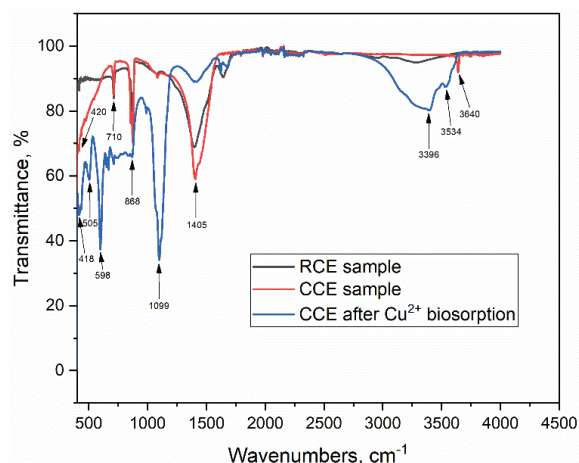


Fig. 4. FTIR spectra of the raw RCE sample, as well as CCE samples, before and after copper ions biosorption.

The tested samples exhibited, as expected for this type of analyte, all characteristic peaks present. The peaks appearing at the low-end of the spectrum of the CCE sample, below 500 cm^{-1} as well as the peak at 710 cm^{-1} , can be attributed to the Ca-O bond, which indicates to the conversion of CaCO_3 to CaO . The obtained FTIR spectra of the raw RCE sample and the CCE sample before the biosorption process shows peaks at around 710 and 869 cm^{-1} indicate to the out-plane and in-plane deformation of $\text{C}=\text{O}$, respectively. These peaks, and the peak observed at 1405 cm^{-1} can be strongly associated with the presence of CaCO_3 in the samples.^{17–19} These results indicate that the decarbonization and transformation of CaCO_3 to CaO did occur during the calcination process, but it was not complete, as the presence of both the calcium oxide and carbonate was confirmed by the FTIR analysis of the CCE sample.

A sharp peak is observed at 3640 cm^{-1} before the biosorption process, which is contributed to the O-H stretching vibration and the bending hydroxyl groups, which indicate to the presence of Ca(OH)_2 . The Ca(OH)_2 was formed as CaO adsorbed water.^{20,21}

The spectra recorded after the Cu^{2+} biosorption shows bands at 418 and 505 cm^{-1} , that can be attributed to the metal-oxygen bonds. The peaks between 650 and 868 cm^{-1} correspond to the hydrogen bonding frequencies originating from the bending vibrations of Cu-O-H . The band recorded at 1100 cm^{-1} , which appears after the biosorption process, is attributed to the sulfate group, originating from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ used for the preparation of the solutions. The broad peaks recorded between 3300 and 3500 cm^{-1} are connected to the stretching of the O-H originating from molecular water.^{21,22}

The influence of different process parameters on biosorption efficiency

The influence of pH on the biosorption efficiency. The influence of the pH value of the solution on the biosorption capacity is shown on Fig. 5a. The obtained results indicate that the biosorption capacity increases with the increase in the pH of the solution. As the pH increased from 2 to 5, the biosorption capacity rose from 148 (pH 2) to 225 mg g⁻¹ (pH 5). The pH value influence is in accordance with the results showed by RCE, however the biosorption capacity by CCE was recorded to be over 10 times higher (10.82 at pH 2 and 21.62 at pH 5 reported for RCE).⁸ With the pH value increase, the concentration of OH⁻ also increases, which leads to an overall negative charge of the biomass. This leads to higher biosorption capacities, as the Cu ions in the solution are positively charged. It is considered that, at higher pH values, the precipitation of Cu²⁺ in the form of Cu(OH)₂ also occurs. This phenomenon could be another reason for the big difference between the biosorption capacities of CCE and RCE.^{8,23,24}

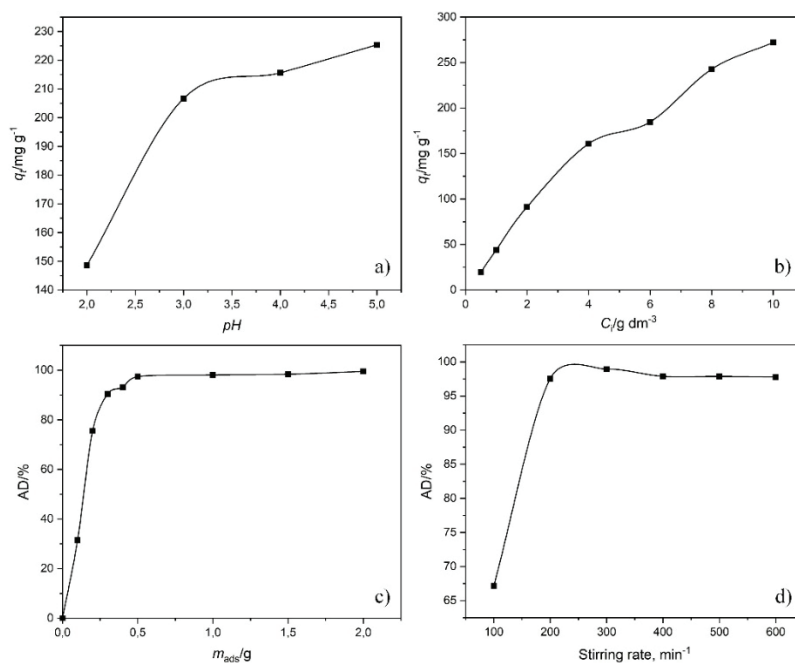


Fig. 5. The influence of pH value (a), initial copper ions concentration (b), biosorbent mass (c) and stirring rate (d) on the biosorption efficiency.

The effect of the initial Cu²⁺ concentration on the biosorption efficiency. The effect of the initial copper ions concentration on the biosorption capacity is shown on Fig. 5b. As can be seen, the biosorption capacity increases with the increase in the initial Cu²⁺ concentration, up to 272 mg g⁻¹ at 10 g dm⁻³ Cu²⁺.

The increase in the biosorption capacity with higher initial metal ions concentration can be attributed to the higher probability of collision between the copper ions and the biosorbent as a larger amount of metal ions are available for biosorption.²⁵

The influence of biosorbent mass on the biosorption efficiency. The analysis of the influence of biosorbent mass on the efficiency of the process is shown on Fig. 5c. The obtained results indicate that the biosorption degree rises rapidly with increasing mass of biosorbent. The maximum biosorption degree was obtained at 0.5 g of CCE (98 %). As the Fig. 5c shows, the biosorption degree remained constant with further increase in the biosorption mass.

The effect of stirring rate on the biosorption degree. The experimental results shown on Fig. 5d show that the biosorption degree rises from 67 to 98 % between 100 and 300 rpm. A slight decrease in the biosorption degree was noted between 300 and 400 rpm, after which there is no significant change in the biosorption degree.

Biosorption kinetic study

The kinetic analysis of the obtained experimental data, using the pseudo-first order, pseudo-second order, intraparticle diffusion and Elovich kinetic models (Eq. (3)–(6)) is shown on the Fig. 6. The obtained kinetic parameters are given in Table III.

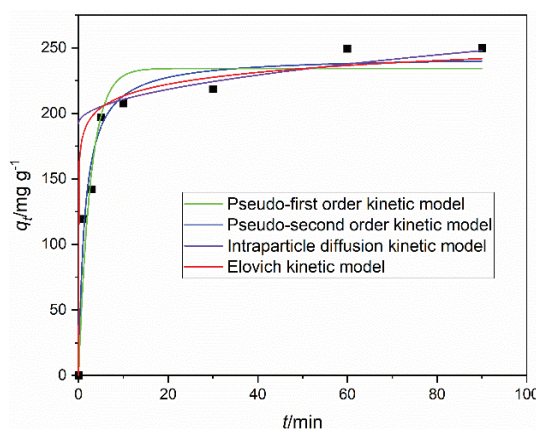


Fig. 6. Kinetic data for copper ions biosorption onto CCE.

The obtained kinetic data, given in Table III, indicates that the pseudo-second order kinetic model is the best fit for the Cu^{2+} biosorption onto CCE. This model suggests that the chemical interaction between the CCE samples and the copper ions from the solution was the rate-determining phase of this process, *i.e.*, that chemisorption is the predominant mechanism of binding of copper ions to CCE.¹⁰ Comparing the obtained results to the work on the RCE,⁸ it can be

noted that the process of binding copper ions onto CCE is significantly faster, with the experimentally obtained biosorption capacity being 10 times higher than the one recorded in the experimental analysis of Cu^{2+} biosorption onto RCE. The two processes do not share the same binding mechanisms, as they follow different kinetic models.

TABLE III. The values of kinetic parameters for copper ions biosorption onto CCE

Model	Parameter	Value
Pseudo-first order kinetic model	k_1 / min^{-1}	0.371
	$q_{e,\text{exp}} / \text{mg g}^{-1}$	234.36
	$q_{e,\text{cal}} / \text{mg g}^{-1}$	249.68
	R^2	0.913
Pseudo-second order kinetic model	$k_2 / \text{g mg}^{-1} \text{min}^{-1}$	0.003
	$q_{e,\text{exp}} / \text{mg g}^{-1}$	234.47
	$q_{e,\text{cal}} / \text{mg g}^{-1}$	249.68
	R^2	0.971
Weber–Morris kinetic model	$k_i / \text{g mg}^{-1} \text{min}^{-0.5}$	5.88
	$C_i / \text{mg g}^{-1}$	192.11
	R^2	0.247
Elovich kinetic model	$\alpha / \text{mg g}^{-1} \text{min}^{-1}$	1.74×10^7
	$\beta / \text{g mg}^{-1}$	0.077
	R^2	0.698

Biosorption equilibrium study

The biosorption experimental data were fitted using the Langmuir, Freundlich and Temkin isotherm models (Eqs. (7)–(9)), and the obtained results are shown on Fig. 7.

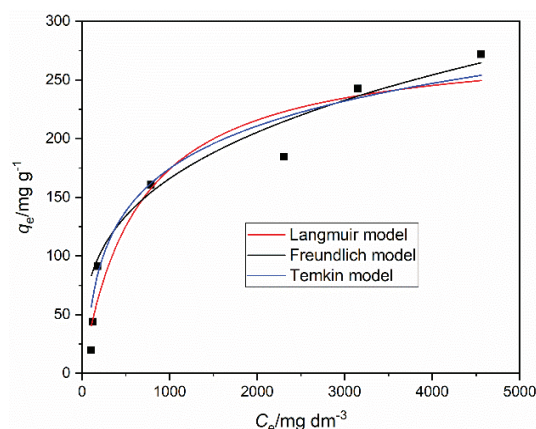


Fig. 7. Isotherm data for copper ions biosorption onto CCE.

The obtained isotherm parameters (Table IV) indicate that the Temkin isotherm model is the best fit for the experimental data ($R^2 = 0.981$). This model is

based on the assumption that the heat of the adsorption of all molecules in the layer decreases linearly with the coverage of the molecules and that the binding energies are uniformly distributed, up to a maximum binding energy.²⁶

TABLE IV. Isotherm parameters for copper ions biosorption onto CCE

Model	Parameter	Value
Langmuir isotherm model	$K_L / \text{dm}^3 \text{mg}^{-1}$	0.002
	$q_{e,\text{exp}} / \text{mg g}^{-1}$	234.36
	$q_m / \text{mg g}^{-1}$	284.12
	R^2	0.941
Freundlich isotherm model	K_F	19.79
	$1/n$	0.308
	R^2	0.944
Temkin isotherm model	$B / \text{J mol}^{-1}$	52.39
	$K_T / \text{dm}^3 \text{mg}^{-1}$	0.028
	R^2	0.981

The obtained Freundlich constant n indicates that the process of binding Cu^{2+} onto CCE is a favorable process ($1/n$ is lower than 1).²⁷

The maximum biosorption capacity is a parameter which is used to analyze the performance of a biosorbent. The maximum theoretical biosorption capacity for Cu^{2+} biosorption onto RCE was 94.59 mg g^{-1} , as reported in the previous work.⁸ In comparison, the maximum biosorption capacity for Cu^{2+} biosorption onto CCE (Table IV) is 284.12 mg g^{-1} . It can be concluded that the calcination of the eggshells leads to a significant increase in its biosorption capacity.

Biosorption thermodynamics

The Gibbs energy change for copper ions biosorption on CCE was calculated using the Eq. (11). The ΔH and ΔS parameters were determined from the plot $\ln K_d$ vs. $1/T$ (Fig. 8). The obtained results are given in Table V.

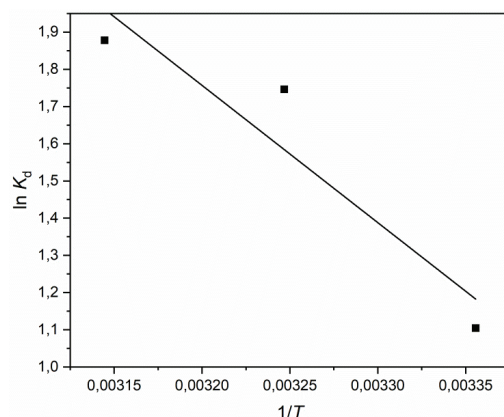


Fig. 8. Thermodynamic plot $\ln K_d$ vs. $1/T$ for copper ions biosorption onto CCE.

The negative ΔG values indicate that the process of biosorption of Cu^{2+} on CCE is spontaneous and more favorable at higher temperatures.²⁸

Positive ΔH and ΔS values indicate that this process is endothermic and that increased randomness is present at the contact interface between Cu^{2+} and CCE. The positive enthalpy also confirmed that increasing the system temperature would lead to an increase in the efficiency of copper removal.²⁹

TABLE V. Thermodynamic parameters for copper ions biosorption onto CCE

T / K	$\Delta G / \text{kJ mol}^{-1}$	$\Delta H / \text{kJ mol}^{-1}$	$\Delta S / \text{J mol}^{-1} \text{K}^{-1}$
298	-2.74	3.69	13.56
303	-4.47		
313	-4.96		

Biosorption optimization study

The process of copper ions biosorption onto CCE was optimized using response surface methodology, with the help of the Box–Behnken design. The experimental design, given in Table I, was applied to optimize the analyzed process. The matrix of the experimental design and the reaction R (biosorption degree) are shown in Table VI.

TABLE VI. Box–Behnken design matrix for three factors along with observed response for Cu^{2+} biosorption onto the CCE

Run	A: Adsorbent mass g	B: Initial Cu^{2+} concentration g L^{-1}	C: Contact time min	R: Adsorption degree %
1	1	0	1	98.785
2	0	0	0	98.391
3	-1	-1	0	82.168
4	0	0	0	94.055
5	0	-1	-1	31.45
6	0	1	1	72.555
7	1	1	0	77.127
8	0	1	-1	11.003
9	0	0	0	97.499
10	0	-1	1	98.482
11	1	-1	0	97.901
12	-1	1	0	38.526
13	1	0	-1	24.243
14	0	0	0	91.56
15	-1	0	1	50.614
16	-1	0	-1	13.818
17	0	0	0	98.42

A polynomial Eq. (13) was fitted in order to describe the correlation between the independent variables: linear ($\beta_1, \beta_2, \beta_3$), quadratic ($\beta_{11}, \beta_{22}, \beta_{33}$), interaction terms ($\beta_{12}, \beta_{13}, \beta_{23}$) and the response (R):³⁰

$$R = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} AA + \beta_{22} BB + \beta_{33} CC + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (14)$$

The results obtained are shown in Table VI. The copper ions biosorption onto CCE can be expressed as:

$$R = 95.99 + 14.12A - 13.85B + 29.99C + 5.72AB + 9.44AC - 1.37BC - 14.28A^2 - 7.77B^2 - 34.84C^2 \quad (15)$$

The ANOVA analysis was used to assess the statistical significance of the applied model, and the results are given in Table VII. The significance of the individual coefficients was analyzed by the *F*-values and *P*-values. The coefficient is more significant as the corresponding *F*-value is larger and *P*-value lower. *P*-values below 0.0500 indicate a highly significant regression at a confidence level of 95 %.¹³

TABLE VII. ANOVA analysis for response surface model for Cu²⁺ biosorption onto CCE

Source	Sum of squares	df	Mean square	<i>F</i> -value	<i>P</i> -value	Significance
Model	17492.34	9	1943.59	115.83	< 0.0001	Significant
A–A	1594.15	1	1594.15	95.01	< 0.0001	
B–B	1534.30	1	1534.30	91.44	< 0.0001	
C–C	7195.32	1	7195.32	428.83	< 0.0001	
AB	130.74	1	130.74	7.79	0.0269	
AC	356.19	1	356.19	21.23	0.0025	
BC	7.51	1	7.51	0.4474	0.5250	
A ²	858.72	1	858.72	51.18	0.0002	
B ²	254.43	1	254.43	15.16	0.0059	
C ²	5110.55	1	5110.55	304.58	< 0.0001	
Residual	117.45	7	16.78			
Lack of fit	80.14	3	26.71	2.86	0.1678	Not significant
Pure error	37.32	4	9.33			
Cor total	17609.80	16				

The obtained *F*-value of 115.83 means that the model is significant. This further indicates that there is only a 0.01 % chance that a *F*-value of this magnitude can appear due to noise.

P-values of less than 0.0500 provide further proof to the significance of the used model. In this research, *A*, *B*, *C*, *AB*, *AC*, *A*², *B*², *C*² are considered significant terms. *P*-values above 0.1000 indicate that the model terms are not significant.

The 2.86 lack of fit *F*-value indicates that the lack of fit is not significant. There is a probability of 16.78 % that such a large lack of fit *F*-value can occur due to noise.

The obtained F - and P -values of the model (115.83 and 0.0001, respectively) show that the model is significant. P -values below 0.05 indicate that the model terms are significant. The model fit was also confirmed by the regression coefficients of the predicted and experimental values ($R^2 = 0.993$ and $\text{adj-}R^2 = 0.985$).

The diagram of actual *versus* predicted responses is shown in Fig. 9. The obtained results show a very good agreement between the predicted and experimental data.

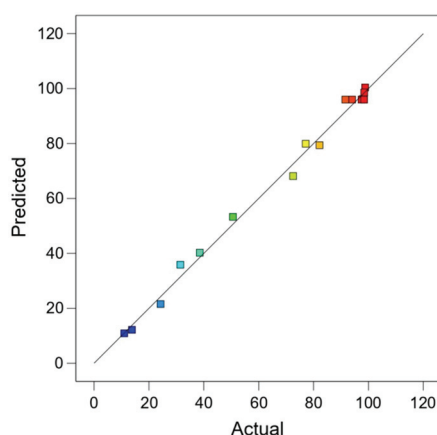


Fig. 9. Actual *vs* predicted responses.

Fig. 10 shows 3D surface plots that illustrate the influence of the selected parameters on the response (R). Fig. 10a shows that a lower initial metal ion concentration in combination with a higher adsorbent mass leads to a very high biosorption efficiency (ANOVA analysis indicated to a significant combination of these two factors (A and B)). Fig. 10b shows the interaction between the adsorbent mass and the contact, indicating that higher values of these factors lead to a high percentage of metal removal, and the ANOVA analysis also confirms that the combination of these factors is significant. Fig. 10c shows that lower initial metal ion concentrations and longer contact time lead to a high metal removal percentage.

The optimization of the biosorption process of copper ions onto CCE was performed using the RSM-BBD method. The influence of the mass of the biosorbent, the initial metal ion concentration and the contact time was evaluated. The model used proved to be statistically significant. The analysis showed that all model terms and most of their interactions were significant. The model suggests that the optimal biosorption conditions are: 90 min contact time, 1 g biosorbent mass and 5 g dm^{-3} initial Cu^{2+} concentration.

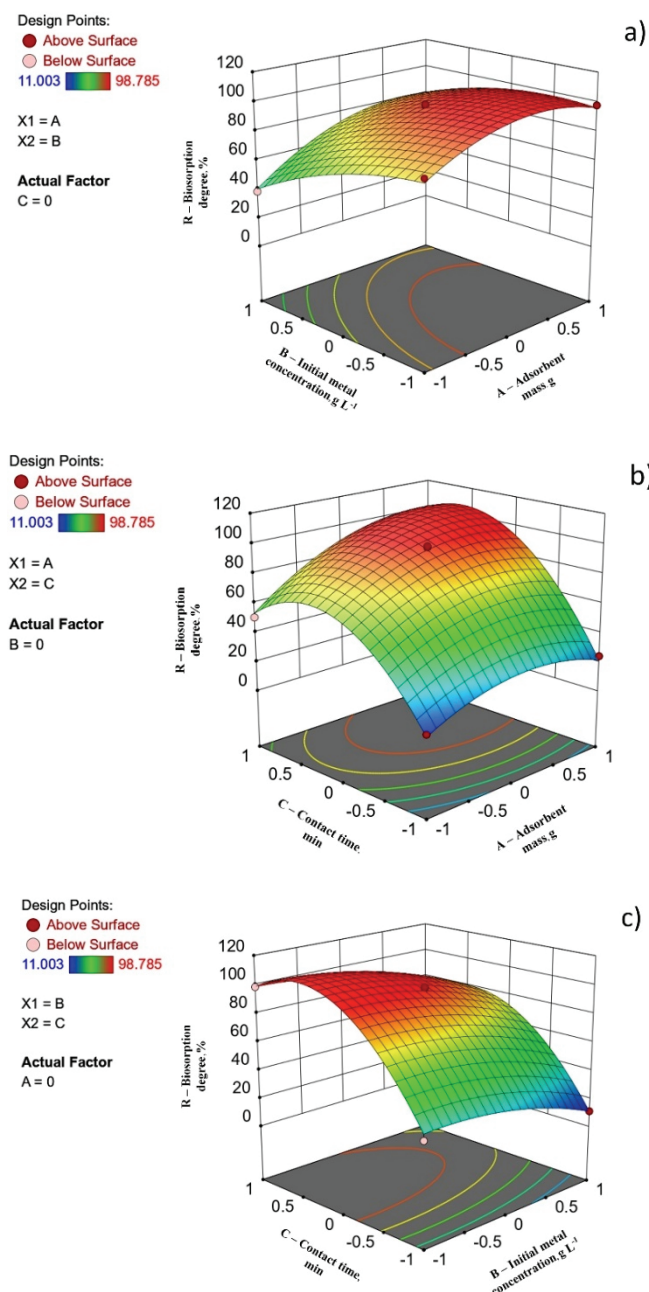


Fig. 10. Response surface plots showing the interaction and influence on the biosorption rate (R) of: a) adsorbent mass (A) and initial copper ions concentration (B); b) adsorbent mass (A) and contact time (C); c) initial copper ions concentration (B) and contact time (C).

CONCLUSIONS

The DTA-TGA analysis of the eggshells sample indicated to the formation of stable CaO during the calcination process. These findings were confirmed by the FTIR analysis, which showed the presence of both CaO and CaCO₃.

The SEM analysis of the CCE showed that an irregular structure consisting of agglomerates and flakes of different sizes formed during the calcination process.

The kinetic analysis showed that the pseudo-second order kinetic model is the best fit for the analyzed data, indicating that chemisorption is the predominant mechanism for the binding of copper ions onto CCE.

The equilibrium analysis indicated that the Temkin model is in the best agreement with the experimental data. This analysis also showed that the calcinations of the eggshells leads to a significant increase in their maximum biosorption capacity.

The thermodynamic analysis showed that this process is beneficial and endothermic at higher temperatures and that there is an increased randomness at the interface between Cu²⁺ and CCE.

The process of biosorption of copper ions onto CCE was optimized using response surface methodology based on the Box–Behnken design. The influence of biosorbent mass, initial Cu²⁺ concentration and contact time on the degree of biosorption was analyzed and modeled. ANOVA analysis showed that the model used and all three parameters analyzed were significant. The optimal parameter values were determined (90 min contact time, 1 g of biosorbent and 5 g dm⁻³ Cu²⁺ concentration).

The maximum experimentally obtained biosorption capacity for the biosorption of copper ions on CCE was 284.12 mg g⁻¹. Comparing this result to the work on RCE,⁸ it can be concluded that the modification of the eggshells (calcinations) increases their biosorption capacity around 10 times.

ИЗВОД

АНАЛИЗА И СТАТИСТИЧКО МОДЕЛИРАЊЕ БИОСОРПЦИЈЕ ЈОНА БАКРА НА
КАЛЦИНИСАНОЈ ЉУСКИ КОКОШИЈЕГ ЈАЈЕТА

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У овом раду представљена је студија о могућој употреби калцинисаних љуски кокошијих јаја као биосорбента за уклањање јона бакра из водених раствора, као и поређење између сирових и калцинисаних љуски јаја. Извршене су SEM-EDS и FTIR анализе узорака калцинисаних љуски кокошијих јаја. Поред тога, спроведена је DTA-TGA анализа сирових љуски јаја. Испитиван је утицај различитих параметара процеса, као што су pH вредност раствора, брзина мешања, маса биосорбента и концентрација Cu²⁺. Кинетичка анализа је извршена коришћењем четири различита емпијска кинетичка модела. Анализа равно-

теже је спроведена применом Лангмировог, Фројндлиховог и Темкиновог модела изотерми. Процес је оптимизован коришћењем методологије површине одзива на основу Вох–Бећнкен дизајна (RSM-BBD). Добијени резултати су упоређени са претходном студијом о употреби сирових љуски јаја као биосорбента за уклањање Cu^{2+} , како би се утврдила оправданост модификације биосорбента (односно калцинације сирових љуски јаја).

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Assessment of water quality in Yapialtın Dam Lake (Sivas, Turkey) during dry and rainy seasons using various parameters and water quality indices

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Abstract: There are many dam lakes in the Şarkışla district of Sivas, where agricultural activities are intense. Yapialtın Dam Lake, which constitutes our study area, is one of them. The research was conducted at three stations in Yapialtın Dam Lake during the dry season in August 2023 and the rainy season in May 2024. Eighteen physicochemical variables of lake water were measured. The quality of dam lake water was assessed using indices such as the eutrophication index (*EI*), organic pollution index (*OPI*), nutrient pollution index (*NPI*) and water quality index (*WQI*). The similarity of the stations in terms of physicochemical parameters was determined using Bray–Curtis similarity analysis. Additionally, Pearson and Spearman correlation analyses were employed to examine the relationships among the physicochemical data. The analyses revealed seasonal variations in all water quality indices. In conclusion, the study provides recommendations for the sustainable use of the dam lake.

Keywords: environmental variables; reservoir; multivariate analyses.

INTRODUCTION

Dam lakes provide numerous benefits to people, including sources of drinking water, irrigation and hydroelectric power generation. They also play a crucial role in managing river water flow and protecting communities from flood hazards. Additionally, dam lakes offer various recreational opportunities, such as birdwatching, hiking and fishing. Modern dams are strategically important, playing significant roles in energy production, particularly in developing countries.

Eutrophication, acidification and various changes in hydrology and geomorphology are major pressures affecting the integrity of lakes.¹ The excessive input of plant nutrients, particularly nitrogen and phosphorus, into lakes leads to the growth of organic matter, algae, periphyton and macrophytes, resulting in eutrophication. This growth induces changes in aquatic organisms and water quality.²

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Eutrophication can lead to decreased dissolved oxygen levels in the water, which may cause hypoxia and toxic algal blooms.² Human activities, including domestic and industrial wastewater discharge and agricultural runoff, contribute to physical, chemical and biological pollution of freshwater resources, resulting in a deterioration of water quality. This situation also limits the use of freshwater resources for various purposes.³

However, intensive and excessive use of these areas can harm both the environment and the living organisms that inhabit them.

This study aimed to calculate the water quality indices, such as the eutrophication index (*EI*), organic pollution index (*OPI*), nutrient pollution index (*NPI*) and water quality index (*WQI*), for Yapıaltın Dam Lake, which is used for irrigation purposes. It also sought to evaluate the relationships between these parameters using various statistical methods, identify the factors contributing to the lake's pollution and compare the findings with similar studies conducted in Turkey.

EXPERIMENTAL

The study was conducted at three stations (ST1, ST2 and ST3) during both the dry season 2023 and the rainy season 2024. ST1 represents the relatively clean part of the lake, ST2 is surrounded by pastures where livestock graze and ST3 is situated near agricultural fields. During sampling, water temperature (WT) was measured using a thermometer. Electrical conductivity (*EC*), pH levels and total dissolved solids (TDS) were measured in the field using a portable Hanna HI 98129 instrument. For other physicochemical parameters, water samples were collected with a Nansen water sampler and stored in dark-colored glass bottles with a 1-L capacity.

The water samples brought to the laboratory were immediately prepared for analysis. Dissolved oxygen (DO), calcium and magnesium levels were determined using standard methods.⁴ Chlorides, total hardness (*TH*) and salinity values were measured using conventional titrimetric methods as described in the literature.⁵ Nitrite nitrogen ($\text{NO}_2\text{-N}$), nitrate nitrogen ($\text{NO}_3\text{-N}$), sulfates, phosphates, ammonium nitrogen ($\text{NH}_4\text{-N}$), biochemical oxygen demand (*BOD*₅) and chemical oxygen demand (*COD*) values were also measured according to standards.⁴

The physicochemical parameters of the sampling stations in Yapıaltın Dam Lake were compared using Bray–Curtis cluster analysis in the Biodiversity Pro 2.0 program during both the dry and rainy seasons.⁶ The relationship between physicochemical parameters was examined using Spearman and Pearson correlation analyses conducted in IBM SPSS Statistics version 27.⁷

Water quality indices

The *EI* is employed to assess the trophic condition of a surface water body. *EI* values were calculated using the formula below: If the calculated *EI* value is less than 1, it indicates absence of eutrophication. A value of 1 or greater suggests the presence of eutrophication.^{2,8} *DIN*; dissolved inorganic N (mg L^{-1}), *DIP*; dissolved inorganic P (mg L^{-1}).⁸

$$EI = \frac{COD \times DIN \times DIP}{4500} 10^6 \quad (1)$$

The *OPI* is a tool used to evaluate water pollution, specifically focusing on the organic pollution status of surface water resources. *OPI* values were calculated using the formula below. The value obtained from the *OPI* formula categorizes water quality as follows: <0: excellent water quality; 0–1: good water quality; 1–2: water starting to be polluted; 2–3: slightly polluted water; 3–4: moderately polluted water; >4: heavily polluted water.^{8,9}

COD, *DIN*, *DIP* and *DO* standard concentration values were used for these calculations.^{8,10}

$$OPI = \frac{COD}{COD_S} + \frac{DIN}{DIN_S} + \frac{DIP}{DIP_S} + \frac{DO}{DO_S} \quad (2)$$

The *NPI* is derived from concentrations of NO_3 and PO_4 in surface water sources. It is calculated using the following formula: The resulting *NPI* value categorizes pollution levels as follows: <1 no pollution, 1–3 moderately polluted, 3–6 significantly polluted, >6 very high pollution.^{8,11} NO_3 -N maximum limit ($mg\ L^{-1}$) is named as MAC_N , PO_4 -P maximum limit ($mg\ L^{-1}$) is named as MAC_P , values were taken:^{8,10}

$$NPI = \frac{C_N}{MAC_N} + \frac{C_P}{MAC_P} \quad (3)$$

The *WQI* is a crucial metric for assessing the overall quality of surface water resources. It integrates multiple water quality parameters into a single value.^{8,12} In this study, 10 parameters including *EC*, pH, *DO*, *BOD*₅, *COD*, Cl, NO_3 -N, NO_2 -N, NH_4 -N and SO_4 were used to calculate *WQI* values. It is calculated using the following formula; *n*: total number of selected parameters, C_i the value assigned to *i* parameter; P_i : the relative weight of parameter (1–4).⁸ The values are classified as follows: 0–25 indicates very poor water quality, 26–50 indicates poor water quality, 51–70 indicates moderate water quality, 71–90 indicates good water quality and 91–100 indicates excellent water quality:^{8,12}

$$WQI = \frac{\sum_{i=1}^n C_i P_i}{\sum_{i=1}^n P_i} \quad (4)$$

RESULTS AND DISCUSSION

The study area is discussed, in details, in Supplementary material to this paper.

The station codes, coordinates and information on stations are presented in Supplementary material (Table S-I).

Physicochemical parameters, units, average values and quality classes of the dam lake water according to¹³ and Turkey surface water quality regulation¹⁴ protocols are presented in Supplementary material (Table S-II).

The lowest *WT* measured in the lake was 18 °C (rainy season, ST3), with the highest recorded at 35 °C (dry season, ST2) and an average of 26.4 °C. The lowest *EC* value was 307 $\mu S\ cm^{-1}$ (rainy season, ST3), the highest was 584 $\mu S\ cm^{-1}$ (rainy season, ST2) and the average was 424 $\mu S\ cm^{-1}$. The lowest pH value observed was 7.91 (rainy season, ST1), the highest was 8.20 (dry season, ST1) and the average was 8.05. *BOD*₅ ranged from a minimum of 9.6 $mg\ L^{-1}$ (rainy season, ST2) to a maximum of 30.3 $mg\ L^{-1}$ (dry season, ST3), with an average of

18.15 mg L⁻¹. *COD* values ranged from a minimum of 18 mg L⁻¹ (rainy season, ST3) to a maximum of 64.6 mg L⁻¹ (dry season, ST3), with an average of 35.66 mg L⁻¹. *TDS* ranged from a minimum of 176 ppm (dry season, ST3) to a maximum of 307 ppm (rainy season, ST3), with an average of 238 ppm. *TSS* ranged from a minimum of 85 mg L⁻¹ (rainy season, ST3) to a maximum of 150 mg L⁻¹ (dry season, ST1), with an average of 113 mg L⁻¹. The lowest *Cl* measured in the lake was 15.99 mg L⁻¹ (dry season, ST3), with the highest observed 20.99 mg L⁻¹ (dry season, ST1) and the average 18.49 mg L⁻¹. Salinity ranged from a minimum of 0.01 ‰ (dry season, ST2) to a maximum of 0.05 ‰ (rainy season, ST2 and ST3), with an average of 0.03 ‰. *Ca* levels ranged from a minimum of 18.84 mg L⁻¹ (dry season, ST3) to a maximum of 44.88 mg L⁻¹ (rainy season, ST3), with an average of 29.72 mg L⁻¹. *Mg* levels ranged from a minimum of 0.58 mg L⁻¹ (rainy season, ST2) to a maximum of 3.80 mg L⁻¹ (dry season, ST2), with an average of 1.88 mg L⁻¹.

TH ranged from a minimum of 1 FS° (rainy season, ST3) to a maximum of 11.6 FS° (dry season, ST2), with an average of 6.26 FS°. *NO₃-N* ranged from a minimum of 3.13 mg L⁻¹ (dry season, ST3) to a maximum of 20.31 mg L⁻¹ (rainy season, ST2), with a mean value of 7.50 mg L⁻¹. *NO₂-N* was not detected in all three stations during the dry season and the highest concentration found was 0.021 mg L⁻¹ (rainy season, ST1), with an average of 0.0065 mg L⁻¹. The lowest *NH₄-N* measured in the lake 0.010 mg L⁻¹ (rainy season, ST3), with the highest recorded 0.034 mg L⁻¹ (rainy season, ST1) and an average was 0.021 mg L⁻¹. *PO₄* ranged from a minimum of 0.79 mg L⁻¹ (dry season, ST1) to a maximum of 1.80 mg L⁻¹ (dry season, ST3), with an average of 1.22 mg L⁻¹. *SO₄* ranged from a minimum of 9.89 mg L⁻¹ (rainy season, ST1) to a maximum of 17.51 mg L⁻¹ (dry season, ST1), with an average of 13.53 mg L⁻¹.

When evaluating the dam lake in terms of water quality indices, the highest *EI* was found at ST3 during the rainy season as 32 and the lowest was at ST1 during the dry season as 8. The *OPI* was also highest at ST3 during the rainy season as 15.09 and lowest at ST1 during the dry season as 5.97. The *NPI* was lowest at ST1 during the dry season as 6.53 and highest at ST3 during the rainy season as 15.8. The *WQI* was lowest at ST2 during the dry season as 10.19 and highest at ST3 during the rainy season as 48.83. The change in water quality index values across the stations for dry and rainy seasons is illustrated in Fig. 1.

Pearson correlation analysis was applied to normally distributed variables among the physicochemical parameters of lake water. According to this analysis, strong positive correlations were found between *WT* and *SO₄*, *TDS* and *NO₃-N*. Strong negative correlations were found between *WT* and *TDS*, *NO₃-N* and *TDS*, *WT* and *NO₃-N*, *TDS* and *SO₄* and *NO₃-N* and *TDS*. The table showing the results of the Pearson correlation analysis and the coefficients of variation is provided in Table S-III of the Supplementary material. Values in the table are

denoted as follows: * indicates that the correlation is significant at the 0.05 level ($p < 0.05$); ** indicates that the correlation is significant at the 0.01 level ($p < 0.01$); and “–” indicates that no statistically significant correlation was detected.

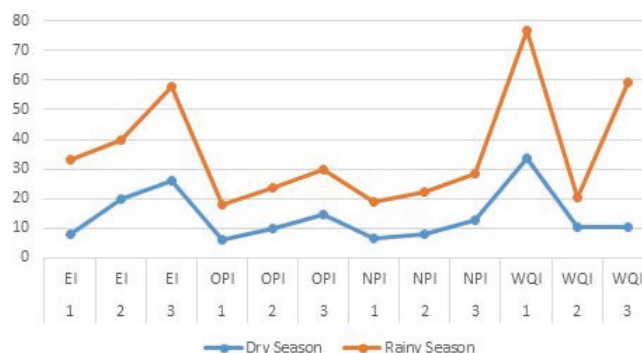


Fig 1. Variation graph of water quality indices.

Pearson correlation analysis was applied to normally distributed variables among the physicochemical parameters of lake water. According to this analysis, strong positive correlations were found between *WT* and SO_4 , *TDS* and $\text{NO}_3\text{-N}$. Strong negative correlations were found between *WT* and *TDS*, $\text{NO}_3\text{-N}$ and *TDS*, *WT* and $\text{NO}_3\text{-N}$, *TDS* and SO_4 and $\text{NO}_3\text{-N}$ and *TDS*. The results of the Pearson correlation analysis and the coefficients of variation is provided in Table S-III. Values in the table are denoted as follows: * indicates that the correlation is significant at the 0.05 level ($p < 0.05$); ** indicates that the correlation is significant at the 0.01 level ($p < 0.01$); and “–” indicates that no statistically significant correlation was detected.

Spearman correlation analysis was applied to the variables that did not show normal distribution among the physicochemical parameters of water. A strong positive correlation was found between *BOD* and *COD*. A negative correlation was found between salinity and *BOD*, as well as between salinity and *COD*, *TH* and *BOD*. The results of the Spearman correlation analysis and the coefficients of variation is provided in Table S-IV. Values in the table are denoted as follows: * indicates that the correlation is significant at the 0.05 level ($p < 0.05$); ** indicates that the correlation is significant at the 0.01 level ($p < 0.01$); and “–” indicates that no statistically significant correlation was detected.

The similarity of the stations in terms of physicochemical parameters was analysed using Bray–Curtis similarity analysis. It was observed that the similarity between the stations was quite high. In the dry season, ST2 and ST3 showed 99.83 % similarity, with station 1 also clustering with them. The Bray–Curtis similarity dendrogram for the stations is presented in Fig. 2.

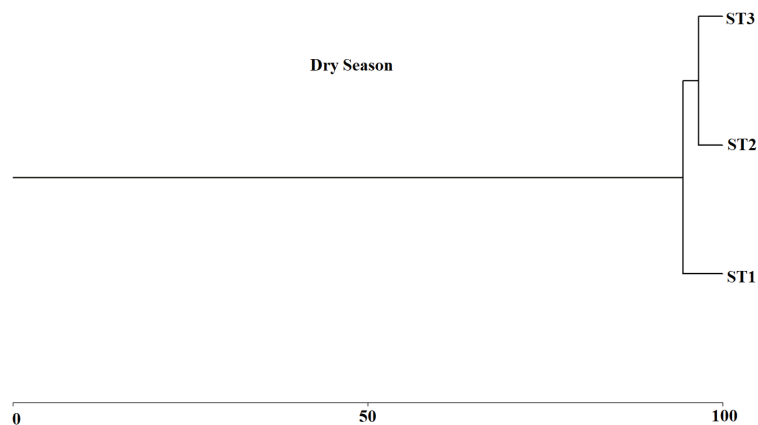


Fig. 2. Bray-Curtis similarity dendrogram of stations during the dry season.

In the rainy season, the similarity between the stations was quite high. The ST1 and ST2 showed a similarity of 99.92 %, with ST3 clustering with them. The Bray-Curtis similarity dendrogram for the rainy season is shown in Fig. 3.

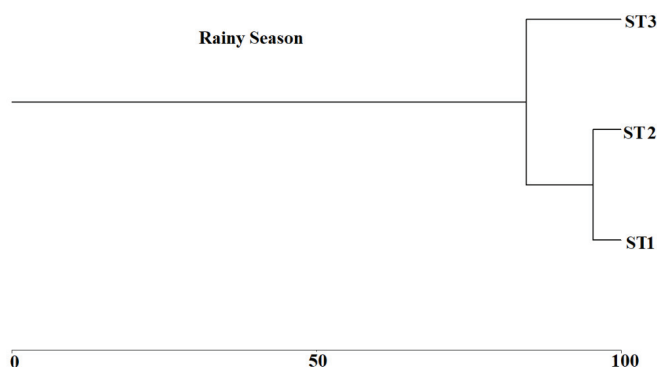


Fig. 3. Bray-Curtis similarity dendrogram of stations during the rainy season.

In this paper, the analyses conducted at three selected stations in the Yapi-altın dam lake during both dry and rainy seasons are discussed in detail. It was found that the *WT*, *pH*, *COD*, *TDS*, *Cl*, *NO₂-N*, *NH₄-N* and *SO₄* values of the dam lake were within the first class water quality standards.^{13,14} The *EC* value was determined to fall between class I and II water quality standards.^{13,14} The *DO* and *NO₃-N* values of the dam lake water were found to range between class II and III water quality.^{13,14} Additionally, the *BOD₅* value was identified to be within the III class quality range.^{13,14}

It is observed that the water temperature values of the dam lake are higher in summer (dry season). This variation may be attributed to seasonal temperature

changes. The *WT* is a critical climatic factor that enhances biological activity rates and reduces oxygen saturation.¹⁵ The *DO* values of the lake also exhibited significant differences between dry and rainy seasons. The solubility of oxygen in water increases as temperature decreases.¹⁶ In this study, it can be observed that the lowest *DO* values were recorded during the dry season when temperatures were highest, whereas the highest *DO* values were observed in the rainy season when temperatures were lowest (Table S-II).

The pH value of the lake was determined to be alkaline. It was observed that *TDS* levels were low during the dry season and high during the rainy season (Table S-II). Precipitation is likely the primary reason for this variation, as rain-water runoff carries dissolved solids into the lake. It has been noted that water conductivity values vary based on geological structure and precipitation levels, rather than nutrient salts in the water.¹⁷ Specifically, conductivity in the dam lake was lowest only in ST3 during the rainy season. It is recognized that regions with high rainfall generally have less saline surface waters due to continuous soil washing.¹⁵ The salinity values were found to be higher in the rainy season compared to the dry season, with minimal difference in Cl values.

The Ca levels in the water were higher than the Mg levels, which may be attributed to the geological composition of the lake. During the rainy season, Ca levels peaked in ST1 and ST3 (Table S-II). In terms of $\text{NO}_3\text{-N}$ values, they were classified as water quality class I and II during the dry season, while in ST2 they reached class III during the rainy season. This variation could be linked to livestock grazing near ST2, with animal feces potentially contributing to increased $\text{NO}_3\text{-N}$ levels in that area. $\text{NH}_4\text{-N}$ values were found in first class water quality. PO_4 content of the lake was found to be quite low.

SO_4 levels were higher during the dry season and lower during the rainy season. This seasonal variation is expected and can be attributed to the dilution effect of rainfall during the rainy season.

The *BOD* represents the amount of oxygen consumed by bacteria and other microorganisms when decomposing organic matter under aerobic conditions at a given temperature.¹⁸ The *COD* is the amount of oxygen required for the oxidation of organic matter and inorganic chemicals. The *COD* value is higher than the *BOD* value.¹⁹ In the study, *COD* values were higher than *BOD* values.

In this study, it was found that the water quality level indices obtained from the results of *EI*, *OPI*, *NPI* and *WQI* calculated using parameters other than *WT*, *TDS*, *TSS*, Cl, salinity, Ca, Mg and *TH* were very similar to each other. When evaluated in terms of indices, since *EI* values ≥ 1 in all stations, it is seen that there is eutrophication in the lake both in dry and rainy seasons. Since the *OPI* values were > 4 , the lake was found to be extremely organically polluted. Since the *NPI* values were > 6 in the seasons and all stations examined in the lake, it was found that there was very high pollution. ST1 in dry season and ST1 and

ST3 in rainy season were found to have poor water quality, while the other stations were found to have very poor water quality in dry and rainy seasons. During the dry season, all three stations were found to have extremely poor water quality. An increase in dam lake water temperature leads to higher evaporation from the dam lake's surface, which lowers the lake level and alters the water quality.²⁰ It was determined that ST2 had very poor water quality during the rainy season. During this period, ST1 and ST3 were found to have poor water quality. According to all index results, eutrophication was observed in the lake and overall, the lake had poor water quality. During the rainy season, the increase in rainfall on the lake's surface can lead to changes in the amount of water in the lake, which may affect the water quality of the dam lake.

According to the Bray–Curtis dendrogram, the highest similarity rates between ST1 and ST2 occurred during the rainy season. The primary reason for this could be the absence of factors affecting these stations during this season. In contrast, ST3, situated near agricultural areas, experiences heavy pesticide use during May fertilization period. These pesticides can be transported via irrigation or rainwater into the lake, altering its physicochemical parameters and distinguishing it from other stations. In the dry season, ST2 and ST3 exhibited the highest similarity rates. The primary reason for this could be attributed to several factors: firstly, the high rate of water evaporation from the lake due to warming weather conditions, leading to a decrease in lake water levels. Additionally, water withdrawal from the lake for irrigation purposes in agricultural areas near the ST3 further reduces the lake's water volume, thereby increasing the concentration of physicochemical substances in the remaining water. Furthermore, around the ST2, grazing of animals may contribute to the transport of animal feces into the water. This, coupled with pesticides applied to nearby agricultural areas, can lead to changes in the physical, chemical and biological composition of the lake water.

When comparing this study with previous research conducted in lakes²¹ we utilized the *WQI* based on 13 water quality parameters in the Kastamonu Karaçomak dam lake. They found that *WQI* values varied from poor to excellent water quality, attributing these variations to seasonal changes and surface runoff in the downstream area, which contributed to water quality degradation. It is noted that the *EC*, *BOD*, *COD*, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ values measured in this current study are consistent with their findings. When comparing this study with the findings of ²² on four dam lakes in Poland, several similarities and differences are noted. In their study,²² it was found that *WQI* values ranged between moderate and poor. Specifically DO ratios were higher in our current study compared to their findings. *EC* values were similar between both studies. $\text{NO}_3\text{-N}$ values were lower in this current study. SO_4 and Ca values were higher in this current study. Mg values were comparable between the two studies. These com-

parisons suggest both similarities and variations in water quality parameters between the two studies conducted in different dam lakes.

Researchers have conducted an analysis of 17 water quality parameters by sampling water from a dam lake in Mexico across different months.²³ The study revealed several key findings: they found no significant difference in *WQI* values between the selected stations within the dam. They observed seasonal variations in *WQI*, indicating that water quality varied throughout the year. The categorization of water quality varied from poor in some months to moderate and to good in others, also moderate to poor in other months. These findings underscore the dynamic nature of water quality in dam lake ecosystems, influenced by seasonal changes and possibly local environmental conditions. Comparisons with their study can provide valuable insights into the variability and factors affecting water quality assessments in different geographical contexts.

Also, a research conducted an assessment of water quality by calculating the *WQI* using 22 physicochemical parameters from waters sampled from Mumcular and Geyik dam lakes, as well as Çamköy underground wells.²⁴ The study reported finding very low *WQI* values, indicating poor water quality. Specifically, the dam lakes evaluated, which are used as sources of drinking water, were identified as requiring treatment before use to meet quality standards. This highlights the importance of ongoing monitoring and treatment processes to ensure safe drinking water supply from these sources.

The studies provide various information to examine whether the dam lakes are suitable for the purpose for which they will be used. The common point seen in all studies is that water quality parameters vary seasonally. In the studies, various suggestions are also given about the purpose for which the dam water can be used or not. In this study, various recommendations on the sustainable use of the dam lake were summarised in the conclusion section.

CONCLUSION

In conclusion, this study analysed water samples collected from three stations in Yapialtın Dam Lake, during both dry and rainy seasons. Significant relationships were identified regarding seasonal variations in water quality indices. It was observed that agricultural activities and livestock breeding near water sources could lead to reductions in water quality parameters, potentially shifting from class I to class III water quality standards.

To ensure the sustainable use of the dam lake, regular monitoring of water quality is crucial. Additionally, assessments for toxic metals, microbiological contaminants, pesticides and algal biodiversity are recommended. These measures are essential for maintaining and improving water quality standards, safeguarding both ecological health and the suitability of the dam lake as a water resource.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13049>, or from the corresponding author on request.

ИЗВОД

ПРОЦЕНА КВАЛИТЕТА ВОДЕ У ЈЕЗЕРУ YAPIALTIN DAM (СИВАС, ТУРСКА) ТОКОМ
СУШНИХ И КИШНИХ СЕЗОНА, КОРИШЋЕЊЕМ РАЗЛИЧИТИХ ПАРАМЕТАРА И
ИНДЕКСА КВАЛИТЕТА ВОДЕ

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Постоји много језера насталих изградњом брана у округу Şarkışla у Сивасу, где су пољопривредне активности интензивне. Језеро Yarıaltın Dam, које чини подручје нашег истраживања, је једно од њих. Истраживање је спроведено на три станице у језеру Yarıaltın Dam током сушне сезоне у августу 2023. и кишне сезоне у мају 2024. Измерено је осамнаест физичко–хемијских варијабли језерске воде. Квалитет воде у језерским бранама је процењен коришћењем индекса као што су индекс еутрофикације (EI), индекс органског загађења (OPI), индекс загађења нутријентима (NPI) и индекс квалитета воде (WQI). Сличност станица у погледу физичко–хемијских параметара утврђена је помоћу Bray–Curtis анализе сличности. Поред тога, коришћене су Пирсонове и Спирманове корелационе анализе да би се испитале везе између физичко–хемијских података. Анализе су откриле сезонске варијације у свим индексима квалитета воде. У закључку, студија даје препоруке за одрживо коришћење језера насталих изградњом бране.

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SUPPLEMENTARY MATERIAL TO
**Assessment of water quality in Yapıaltın Dam Lake (Sivas,
Turkey) during dry and rainy seasons using various
parameters and water quality indices**

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STUDY AREA

Yapıaltın Dam is a dam lake constructed on Çaylak Stream for irrigation purposes in 1977. The dam lake has a capacity of 1,000,000 m³ and stands 37 meters above the river bed. At normal water level, the dam lake holds 17 hm³ of water and covers an area of 1.42 km². It serves an irrigation area spanning 2,894 hectares. The area surrounding Yapıaltın Dam, near Şarkışla, has been developed by Şarkışla Municipality, featuring recreational and picnic areas. Particularly in summer, it is frequented by the public for recreational purposes (<https://www.dsi.gov.tr/>). A map of the study area and sampling stations is provided in Fig. 1.

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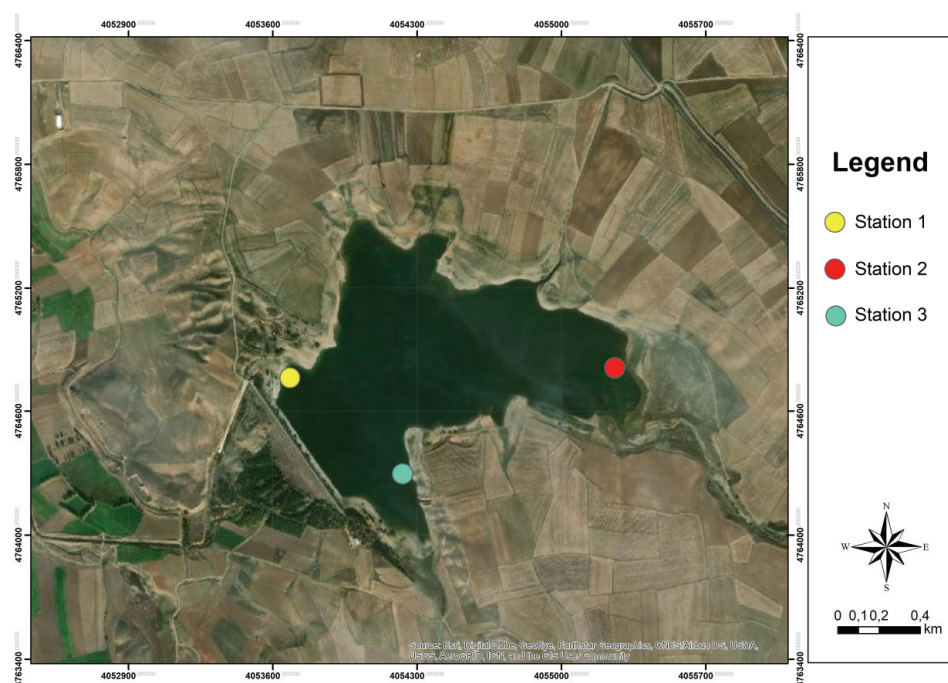


Fig. S-1. The study area map and sampling stations

Table S- I. Information about stations

Station Code	Coordinates	Information on Stations
ST1	39°18'01"N 36° 24' 52"E	Behind the set of dam
ST2	39°18'01"N 36° 25' 47"E	Opposite the set of dam
ST3	39°17'47"N 36 25' 12"E	In front of agricultural areas

ST1:First Station; ST2: Second station; ST3:Third station

Table S- II. Physicochemical parameters, mean values, and quality classes measured during dry and rainy seasons

Parameters	Unit	Dry Season			Rainy Season			Average	13, 14
		ST1	ST2	ST3	ST1	ST2	ST3		
W.T.	°C	33	35	34.3	19.3	18.3	18	26.4	I ¹³
EC	µscm ⁻¹	384	361	354	554	584	307	424	I and II ¹⁴
pH		8.20	8.08	8	7.91	8.11	8.03	8.05	I ¹⁴
DO	mgL ⁻¹	4.95	4.37	4.56	7.42	5.90	5.52	5.45	II and III ¹⁴
BOD ₅	mgL ⁻¹	18.6	28.4	30.3	11.6	9.6	10.4	18.15	III ¹⁴
COD	mgL ⁻¹	35.7	56.2	64.6	21.2	18.3	18	35.66	I ¹⁴
TDS	ppm	192	180	176	277	297	307	238	I ¹³
TSS	mgL ⁻¹	150	130	110	95	113	85	113	
Cl	mgL ⁻¹	20.99	17.99	15.99	18.99	16.99	19.99	18.49	I ¹³
Salinity	‰	0.03	0.01	0.02	0.04	0.05	0.05	0.03	
Ca	mgL ⁻¹	21.64	27.25	18.84	41.68	24.04	44.88	29.72	
Mg	mgL ⁻¹	2.54	3.80	2.13	1.02	0.58	1.24	1.88	
T.H.	FS°	11.2	11.6	10.6	2	1.2	1	6.26	
NO ₃ -N	mgL ⁻¹	4.16	4.84	3.13	16.12	20.31	16.20	10.79	II and III ¹³
NO ₂ -N	mgL ⁻¹	0	0	0	0.021	0.006	0.012	0.0065	I ¹³
NH ₄ -N	mgL ⁻¹	0.021	0.020	0.017	0.034	0.028	0.010	0.021	I ¹⁴
PO ₄	mgL ⁻¹	0.79	1.04	1.80	1.06	1.11	1.54	1.22	
SO ₄	mgL ⁻¹	17.51	16.74	16.37	9.89	10.33	10.39	13.53	I ¹³

WT:water temperature; EC:electrical conductivity; DO:dissolved oxygen; BOD₅: biological oxygen demand; COD: chemical oxygen demand; TDS: total dissolved solids; TSS: total suspended solid; Cl: chloride; Ca: Calcium; Mg:magnesium; T.H: total hardness; NO₃-N:nitrate nitrogen; NO₂-N: nitrite nitrogen; NH₄-N:Ammonium nitrogen; PO₄:phosphate; SO₄:sulphate. ST1: First station; ST2: Second station; ST3: Third station.

Table S- III. Pearson correlation analysis between physicochemical variables

	WT	pH	EC	TDS	TSS	NO ₃ -N	SO ₄
WT	1						
pH	,360	1					
EC	-,525	-,186	1				
TDS	-,993**	-,288	,456	1			
TSS	,725	,792	-,103	-,706	1		
NO ₃ -N	-,975**	-,286	,632	,970**	-,641	1	
SO ₄	,982**	,510	-,555	-,962**	,801	-,964**	1

WT: water temperature; EC: electrical conductivity; TDS: total dissolved solids; TSS: total suspended solid NO₃-N:nitrate nitrogen; NO₂-N: nitrite nitrogen; NH₄-N:Ammonium nitrogen; SO₄:sulphate.

Table S- IV. Spearman Correlation Analysis between Physicochemical Variables

	BOD	COD	TH	Salinity
BOD	1			
COD	,943**	1		
TH	,771	,829*	1	
Salinity	-,928**	-,928**	-,928**	1

BODs: biological oxygen demand; COD: chemical oxygen demand; T.H: total hardness



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Amino-starch derivates for adsorption of specific pharmaceuticals and pesticides in contaminated water: Examination in both spiked and real water samples

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Abstract: In this study, the possibility of using modified potato starch, with nitrogen-containing chemical agents (melamine, cysteine and histidine) as green adsorbents for removing pharmaceuticals and pesticides from water has been investigated. The influence of additional modification of amino-starch with clay and diatomaceous earth was examined. The effect of the applied modification on the structural, surface and morphological properties was determined by FTIR, XRD and SEM analysis, while the adsorption properties were determined through the effectiveness of prepared materials to remove selected pollutants from spiked and real water samples. The efficiency of investigated amino-starches for the adsorption of pharmaceuticals and pesticides decreases in order: starch–histidine > starch–cysteine > starch–melamine, with a slightly better efficiency for pharmaceuticals adsorption. Additional modification of amino-starches with clay/diatomaceous earth did not contribute to the increase in adsorption efficiency. It was found that the influence of the matrix of real water samples on the adsorption efficiency is up to 10%, which represents a promising potential for applying amino-starch as a cheap and effective adsorbent for wastewater treatment. Also, starch–histidine and starch–cysteine showed the possibility of reusing up to three cycles of adsorption.

Keywords: potato starch; modification; adsorption; organic pollutants; reuse.

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INTRODUCTION

Growing world population, increased investment in medical research, innovations in the field of medicine, ubiquitous availability of the global market, increase in chronic diseases, as well as the prevention and treatment of animal diseases in intensive livestock have led to a significant increase in the consumption of pharmaceuticals in the last few decades.^{1,2} Exposure of non-target organisms to even low concentrations of pharmaceutical products can have negative effects reflected in ecotoxicity (acute and chronic toxicity, genotoxicity and carcinogenicity); pharmacological effects (hormone and immune system interference) and the development of microorganism resistance.³ Another category of pollutants frequently identified in water is pesticides. About 85 % of the world's pesticide production is spent on agricultural purposes, crop protection and chemical control of various pests, such as insects, bacteria, fungi and algae in electrical equipment, refrigerators, paints, paper, cardboard and food packaging materials. Adverse effects on human health have been observed even at very low concentrations of pesticides. Pesticide poisoning can lead to various diseases, such as cancer, hormonal disorders, asthma, various allergies, birth defects and fetal death.⁴

In the field of environmental engineering, adsorption plays an integral role among methods to treat wastewater because of its robustness in handling a range of pollutants, simplicity in operation, low operating costs, simplicity of the process and variety of materials that can be used as adsorbents.⁵ Natural material, starch, is an attractive material for adsorption application, due to its low cost, availability in nature in large amounts, biorenewability, non-toxicity and good adsorption characteristics. Previous studies have shown that chemical modification, *i.e.*, the inclusion of functional groups in the backbone of starch, improves the efficiency of modified starch for the adsorption of various organic and inorganic pollutants from water.^{5,6}

Following the goals of sustainable development, this research brings a novel approach to the modification of natural materials and the preparation of a green adsorbent that can be used for wastewater treatment. The chemical modification of starch with melamine and amino acids was carried out to increase the number of active sites for adsorption, while the physicochemical modification with clay/diatomaceous earth was carried out to increase the stability of starch in the aqueous environment. It is also expected that modification with clay and diatomaceous earth will increase adsorption efficiency, due to their good adsorption properties.

In this work, the adsorption of selected pharmaceuticals (erythromycin, lorazepam, diazepam and clopidogrel) and pesticides (atrazine, propazine, malathion and tebufenozide) from spiked water and real water samples (surface water, groundwater and wastewater) was examined. Chemically modified starch with nitrogen-rich compounds (melamine, cysteine, histidine) and materials based on

amino-starch derivates additionally modified by adding bentonite clay and diatomaceous earth were used as adsorption materials. The applied adsorption process using modified starch can be considered ecologically sustainable and economically acceptable, due to the use of natural materials modified by a simple and safe method, solving the extremely important problem concerning the presence of pharmaceutical products and pesticides in the environment. In order to assess the cost-effectiveness and sustainability of the application of amino-starch derivates as adsorbents for the removal of selected pharmaceuticals and pesticides in real systems, their reuse was examined.

EXPERIMENTAL

Materials and chemicals

Details of materials and chemicals are given in the Supplementary material to this paper.

Preparation of amino-modified starch

Amino-modified starch (AMS) was synthesized according to our previous reports.⁵ The synthesis of the material with melamine was carried out at a temperature of 80 °C until the melamine was dissolved in the presence of acetic acid and distilled water. After the addition of oxidized starch, the reaction was conducted at 30 °C for another 2 h with stirring at 400 rpm. After washing and filtering, the obtained material was dried at 50–60 °C to a constant mass. Modification with cysteine and histidine was performed by mixing oxidized starch with distilled water for 30 min at 40 °C to obtain a starch suspension to which the amino acid was then added and stirring at 400 rpm was continued at 40 °C for another 3 h. The resulting mixture was filtered and washed several times and then dried at 50 °C to constant weight.

Preparation of biocomposites

The preparation of starch-based biocomposites was carried out according to methods described in the literature with some changes.^{7,8} AMS (5 g) was suspended in 30 mL of deionized water and kept in a water bath at 70 °C with continuous stirring at 400 rpm for 1 h. The clay/diatomaceous earth (0.5 g) was dispersed in 30 mL of deionized water and the dispersion was poured into the gelatinized starch/AMS solution. The reaction was continued at 60 °C with constant stirring on a magnetic stirrer at 400 rpm for 3 h. The sample was dried at 50 °C in an oven and finally, the dry sample was ground into a powder. Samples with native starch and clay/diatomaceous earth were prepared in the same way. Obtained starch-based biocomposites were labeled according to Table S-I of the Supplementary material.

Characterization methods

The determination of the surface, structural and morphological characteristics of the material was carried out using Fourier-transform infrared spectroscopy (FTIR), a scanning electron microscope (SEM) and X-ray diffraction (XRD). Details of these characterization methods are given in the Supplementary material.

Adsorption performance and regeneration studies

The adsorption efficiency of examined samples was investigated in a batch system with constant mixing (180 rpm) at room temperature. The adsorption of selected pharmaceuticals (erythromycin, lorazepam, diazepam and clopidogrel) and pesticides (atrazine, propazine, malathion, tebufenozide) from multi-component spiked water samples, surface water, ground-water and wastewaters was investigated. Adsorption tests were performed at constant adsorption

orbent dosage (0.05 g), initial concentration (500 mg dm⁻³) and solution volume (10 mL) for 180 min, in the batch system with constant shaking at room temperature. The initial pH of the pesticides/pharmaceuticals solution and adsorbent was about 5, for all samples. After adsorption, all samples were filtered through the PVDF 0.45 µm filters.

The adsorption efficiency (%) of selected pharmaceuticals and pesticides from aqueous solutions was calculated according to the equation:

$$\text{Adsorption efficiency, \%} = 100 \left(\frac{C_0 - C_t}{C_0} \right) \quad (1)$$

where C_0 and C_t (mg dm⁻³) are the concentrations of a pollutant at the initial time and after time t (min).⁵

For regeneration investigation, the dried adsorbents were immersed in 0.05 mol L⁻¹ HNO₃ solution for 120 min with constant stirring at room temperature. After that, adsorbents were washed with deionized water and dried at 50 °C for the next adsorption cycle.

The concentration of selected pesticides and pharmaceuticals was analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Details related to this instrumental technique are given in the Supplementary material.

RESULTS AND DISCUSSION

FTIR analysis

FTIR spectra of all examined samples are presented in Fig. 1. The spectra of all modified starch/AMS samples exhibit a broad absorption band at approximately 3330 and 3335 cm⁻¹, which can be attributed to the stretching vibration of O–H groups from the glucose unit. A small peak at 2993, 2930 and 2928 cm⁻¹, present in FTIR spectra of all tested samples, can be assigned to symmetrical and asymmetric C–H vibrations in methyl or methylene groups. An intense absorption band at 1001 and 1004 cm⁻¹ is typical in the spectra of starch and its derivative and attributed to the CH₂–O–CH₂ stretching vibrations of anhydroglucose unit.⁹ The weaker absorption peak at 1710, 1720 and 1725 cm⁻¹ in the spectra of the modified AMS samples can be attributed to –C=O stretching vibrations.¹⁰ The absorption peak at 3121, 3124 and 3127 cm⁻¹ in the FTIR spectrum of SM–clay, SM–d.e and SM is assigned to –NH₂ stretching vibration; whereas those at 1542, 1540, 808 and 764 cm⁻¹ are assigned to the triazine ring.¹¹ In the FTIR spectrum of SC, SC–clay and SC–d.e, the absorption peak at 1583, 1580 and 1565 cm⁻¹ can be attributed to the presence of the –C=N group, while the peak at 2553, 2530 and 2536 cm⁻¹ corresponds to the S–H stretching vibrations.¹² In the FTIR spectrum of SH, SH–clay and SH–d.e, the peak at 1483 and 1480 cm⁻¹ corresponds to the bending vibration of the –NH₂ group, while the ring vibration appears at 635, 630 and 623 cm⁻¹.¹² The characteristic peaks for bentonite clay corresponding to the Si–O and Al–O vibrations (3691, 3620, 832 and 693 cm⁻¹)¹³ can be seen in Fig. 1c. The absorption bands corresponding to Si–O, O–Si–O and Si–O–Si vibrations in the diatomaceous earth structure are observed at 1076, 812 and 703 cm⁻¹ (Fig. 1d).^{13,14}

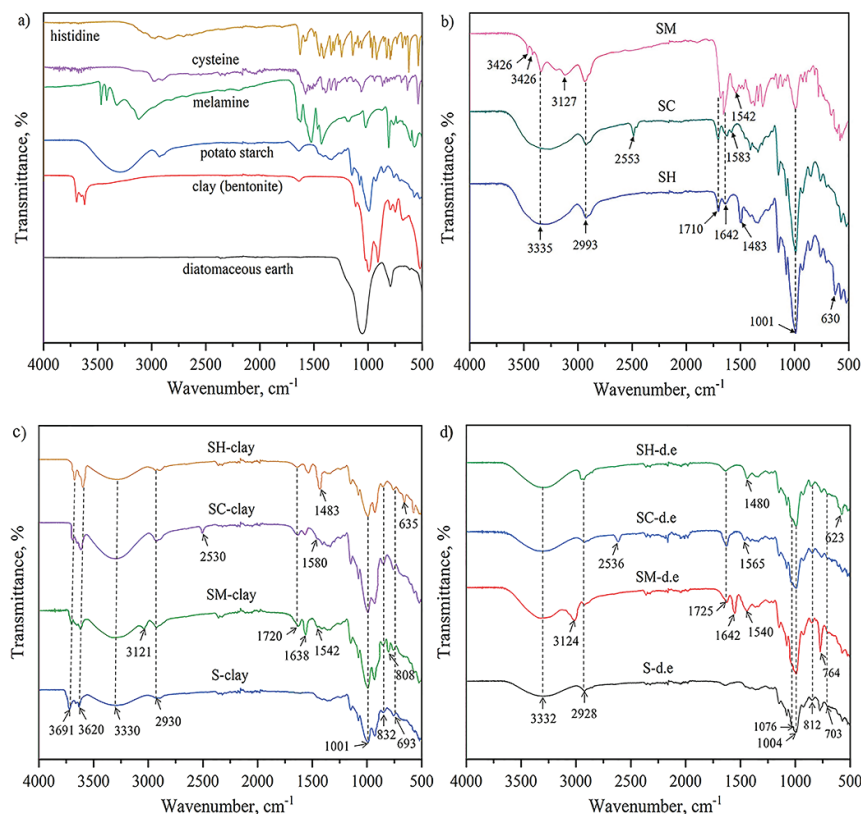


Fig. 1. FTIR spectra of examined starch-based adsorbents.

SEM analysis

Fig. 2 shows SEM micrographs of all tested samples. The micrographs of bentonite clay (Fig. 2a) indicate the existence of a roughed matrix, *i.e.*, a crystalline morphology with the dominance of platelet shapes in the section and a certain degree of porosity.¹⁵ Fig. 2b shows the smooth surface of natural potato starch granules, typically elliptical and spherical shapes without any cavities or cracks. SEM micrographs of diatomaceous earth (Fig. 2c) show the presence of a porous structure in the form of a honeycomb with impurities that can be incorporated into the new material during modification.¹⁶

Fig. 2d–f show SEM micrographs of amino-modified starches, with melamine, cysteine and histidine, respectively. Since there was no harsh physical treatment or application of high temperatures during the modification process, the starch granules retained their original shape. The success of modification was confirmed by the surface roughness, *i.e.*, the presence of unevenness and impurities in the structure of the new materials originating from amino derivatives.

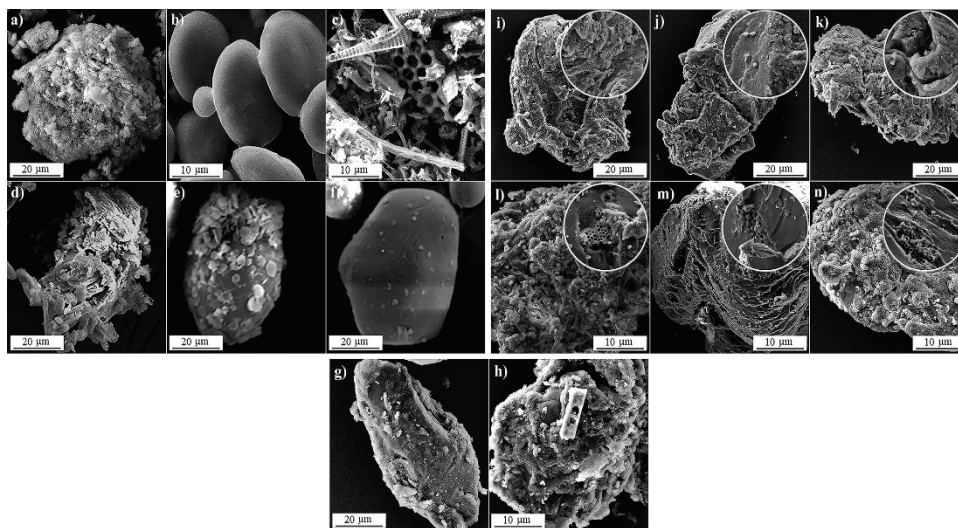


Fig. 2. SEM micrographs of clay (bentonite) (a); potato starch (b); diatomaceous earth (c); SM (d); SC (e); SH (f); S-clay (g); S-d.e. (h); SM-clay (i); SC-clay (j); SH-clay (k); SM-d.e (l); SC-d.e (m); and SH-d.e (n).

Fig. 2d shows the presence of melamine as crystalline segments, not only on the surface of the starch granules but even within the inner parts of the starch granules. Fig. 2e shows the presence of melamine as crystalline segments, not only on the surface of the starch granules but even within the inner parts of the starch granules. In the case of starch modified with cysteine (Fig. 2e), the presence of cysteine is observed on the surface of the starch, in the form of stuck parts, while in the case of starch modified with histidine, a more homogeneous structure is observed, *i.e.*, fewer parts on the surface, which means that histidine coats the starch granules to a greater extent and sticks less to the surface (Fig. 2d). In the second part of the modification, the possibility of reacting starch/ amino-modified starch with clay/diatomaceous earth was examined. The reaction is simply carried out by adding clay, or diatomaceous earth, to a gelatinized starch solution or amino-modified starch. All SEM micrographs (Fig. 2g–n) clearly show that the form of starch granules has been changed, but that the shape is not completely destroyed. The edges have lost clear boundaries, the surface has become rough, with protrusions and cracks, while in the cross-section of the starch granules, parts originating from the initial components, amino derivatives, clay and diatomaceous earth can be observed.

XRD analysis

Comparative XRD diagrams for the investigated samples are presented in Fig. 3. Native potato starch showed the typical B-type X-ray pattern with reflection diffraction peaks (2θ) at 5.3, 15.1, 17.5, 19.5, 22.5, 24.1 and 26.4°. ¹⁷ The

characteristic XRD peaks for bentonite clay (Fig. 3b) at the diffraction angle 2θ 12.5, 20.8, 26.5, 35.3, 36.7, 38.9 and 55.2° can be associated with the presence of the smectite and impurities such as opal, quartz and calcite in the structure of pure bentonite clay.¹⁸ The XRD pattern of diatomaceous earth (Fig. 3c) revealed the main phase of non-crystalline opal-A with the characteristic broad peak centered at 21.3° .¹⁹

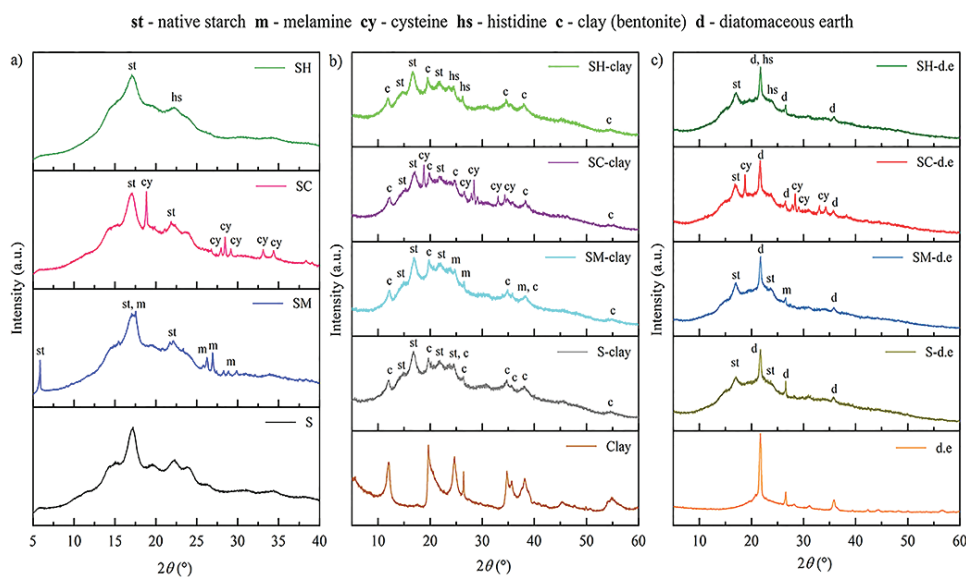


Fig. 3. XRD patterns of examined starch-based adsorbents.

Compared to native starch (Fig. 3a), additional peaks for SM appearing at 2θ 5.92, 17.5 and 22.7° and ranging from 25.5 to 30.2° originate from melamine, while the peaks for SC that are characteristic of the cysteine structure were observed at 2θ 18.8° and in the range from 27.8 to 34.4° .^{20,21} A significant decrease in crystalline domains was observed for SH, where a decrease in peak intensity is observed in the interval from 19.6 to 24.1° compared to native starch, while the characteristic peaks for the structure of pure histidine, ranging from 16.2 to 28.3° were not visible, as a consequence of their lower intensity and overlap with the characteristic peak of the starch structure.²² Fig. 3b and c show that, in addition to the characteristic XRD peaks originating from the structure of starch and amino derivatives, XRD peaks characteristic for bentonite clay (Fig. 3b) and diatomaceous earth (Fig. 3c) are also present.²³ Based on the XRD patterns of the tested samples, it can be concluded that the additional modification of the samples with bentonite clay (Fig. 3b) and diatomaceous earth (Fig. 3c) contributes to the increase of crystalline domains in the materials compared to the native starch and amino-modified samples (Fig. 3a).

Adsorption study

The adsorption efficiency values of examined materials to adsorb selected pharmaceuticals and pesticides from spiked deionized water are presented in Fig. 4 and Table S-IV of the Supplementary material.

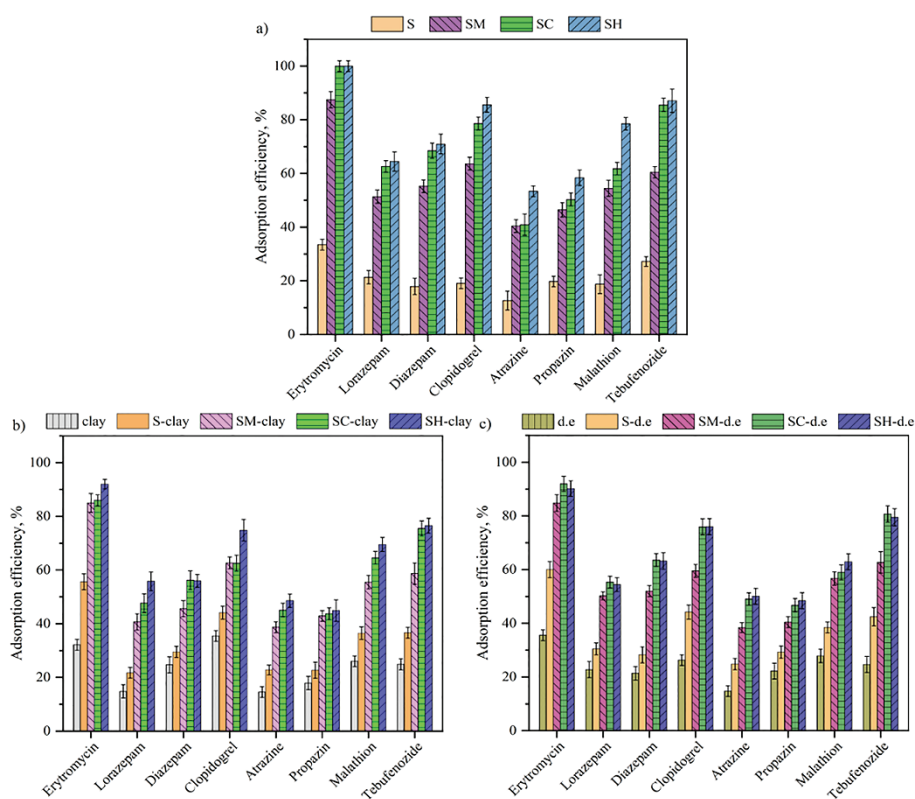


Fig. 4. Adsorption efficiency of examined starch-based adsorbents.

It can be noticed that the modification of starch contributed to an increase in the efficiency of pharmaceutical and pesticide adsorption two to three times compared to the adsorption on unmodified starch. Applied modifications significantly contributed to the adsorption efficiency and the modification of starch with amino acids, especially histidine, contributed the most to the high percentage of all selected pollutants adsorption. Also, the same adsorption trend for pharmaceuticals (erythromycin > clopidogrel > diazepam > lorazepam) and pesticides (tebufenozide > malathion > propazine > atrazine) has been observed for all examined materials. The order of the affinity obtained can be explained by the different intensities of electrostatic forces acting between the positively charged surface of all amino-modified starch samples, on one hand and molecules of

pharmaceuticals and pesticides, on the other. All tested materials showed a slightly better efficiency for the removal of pharmaceuticals, than for pesticides. The order of the adsorption efficiency for clopidogrel > diazepam > lorazepam indicates that the adsorption efficiency decreases with increased polarity of the pharmaceutical (SRM chromatograms, Fig. S-1 of the Supplementary material). The exception is erythromycin, possibly as a consequence of its large molecular mass, as well as of the highest electronegativity of this molecule compared to other pollutants. This could also explain, why the erythromycin molecules bind to the surface of the adsorbent first and then the molecules of other pollutants. The same explanation applies to the adsorption efficiency of pesticides; with increasing polarity of pesticide molecules (SRM chromatograms, Fig. S-1) the adsorption efficiency decreases (tebufenozide > malathion > propazine > atrazine). Based on all of the above, it can be stated that the investigated materials are more suitable for the adsorption of less polar compounds.

Additional modification of starch with clay and diatomaceous earth had no significant influence on the adsorption properties of amino materials. As a result of the starch surface modification with clay and diatomaceous earth (Fig. 2), the surface of the starch granules was coated with parts of clay/diatomaceous earth, which reduced its porosity and the number of available sites for adsorption.

Adsorption study in real water samples

Following the results obtained in the previous part, amino-starch samples modified with melamine and amino acids were selected for testing the adsorption of pharmaceuticals and pesticides from spiked real waters. Three samples of real water, a sample of surface water collected from river Pek, Serbia; a sample of groundwater, collected from Belgrade Ranney wells, Serbia; and a sample of wastewater from the entrance to the water purification plant Arandelovac, Serbia; were analyzed (Fig. 5, Table S-V). The results obtained for spiked deionized water were compared to the results for real water samples to examine the matrix effect, *i.e.*, the influence of components present in water matrix on the adsorption efficiency of used adsorbents. One of the most important factors that can affect the efficiency of adsorption is the presence of dissolved organic matter (DOM), which consists of different size fractions (*i.e.*, building blocks, humic and fulvic acids, biopolymers and low molecular weight organic matter) that can interfere with the adsorption process by blocking the pores of the adsorbent or competing with the contaminants of interest for adsorption sites.²⁴ According to the literature, the effect of adsorbent saturation is more pronounced when dealing with the adsorption of anionic compounds because DOM is negatively charged at the overall pH of the wastewater and interferes with the adsorption of anionic compounds through electrostatic repulsion.²⁵ However, many studies show that DOM does not have a significant effect or can even have a positive effect on the adsorption.

orption of some organic products, depending on the experimental conditions, which has also been confirmed in this research.^{26,27}

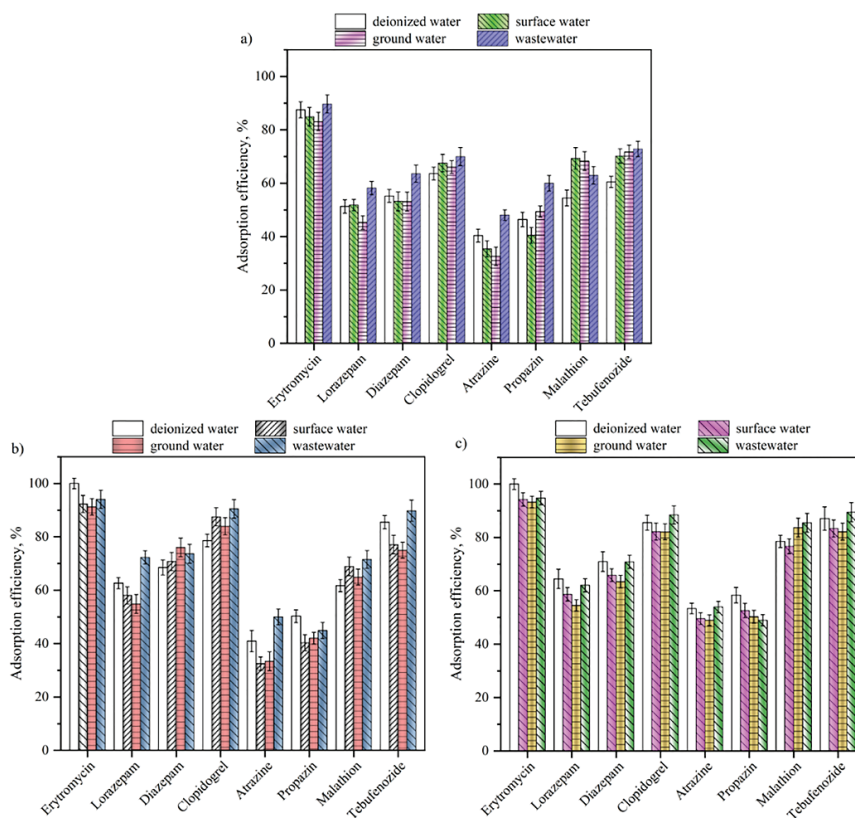


Fig. 5. Adsorption efficiency of amino-modified starches, SM (a), SC (b) and SH (c) for the removal of selected pharmaceuticals and pesticides from real water samples.

Changes in adsorption efficiencies of up to 10 % were observed compared to the adsorption from deionized water. A particularly significant result was the increase in the removal efficiency of almost all pharmaceuticals and pesticides from wastewater suggesting that the matrix of real water samples either has no significant influence or has a positive influence on the adsorption efficiency.

Comparative study of different adsorbents – literature review

Table I shows data from the literature on the use of different types of modified starch for the removal of different pharmaceuticals and pesticides from water. By comparing the adsorption efficiency, the modified starch obtained in this study gives comparable results with literature data, bearing in mind that it is a different type of starch modification, type of pollutant and different exper-

ental conditions that significantly affect the adsorption efficiency. Also, it is important to point out that the adsorption of selected pharmaceuticals and pesticides in this work was performed from a mixture of several pharmaceuticals and pesticides, which helps to evaluate the efficiency of these adsorbents for application in the treatment of real wastewater because wastewater often contains more than one pollutant.

TABLE I. Modified starches for the adsorption of different pharmaceuticals and pesticides

Adsorbent	Type of pollutant	Adsorption efficiency	Ref.
Pharmaceuticals			
Starch/porous carbon	Tetracycline	67.16 %	28
Grafted starch with octenyl succinic anhydride	Cephalexin	0.92 mg g ⁻¹	32
Starch-g-P(AM-co-AMPS)	Tetracycline	95.7 %	33
Crosslinked starch/chitosan	Tetracycline	99.4 %	34
Carboxymethyl starch-modified magnetic bentonite clay	Ciprofloxacin		
	Tetracycline	169.7 mg g ⁻¹	29
Pesticides			
Starch/porous carbon	Atrazine	80%	28
	Acetamiprid		
	Difenoconazole		
P-doped biochar from corn straw	Triazine	79.6 mg g ⁻¹	35
Corn/starch-based mesoporous activated carbon (ACS)	Pyraclostrobin	66.2 mg g ⁻¹	36
Microporous starch immobilized laccase	Atrazine	0.2527 mg g ⁻¹	37
	Prometryn	0.1323 mg g ⁻¹	
Iron-starch modified with 3,5-diaminobenzidine and (3-aminopropyl) triethoxysilane	Isobenzan	0.00075 mg g ⁻¹	31
	Endosulfan	0.002 mg g ⁻¹	
	Chlordane	0.001 mg g ⁻¹	

In most of the studies from Table I for obtaining starch-based adsorbents for the removal of pharmaceuticals and pesticides, complex procedures lasting several hours, at high temperatures, with the use of expensive and toxic chemicals were used.^{28–31} The main advantage of the amino starch derivatives obtained in this work is the method of their preparing which is based on a simple and quick procedure, without the use of expensive and toxic solvents, or by-products, with minimal consumption of electricity and the use of starch modifiers that are environmentally acceptable and safe for use (amino acids and melamine), which is in accordance with the principles of green chemistry and sustainable development.

Regeneration and reuse study

The results of the reuse of amino-starch derivatives synthesized in this study are shown in Fig. 6. Desorption and reuse were investigated for the two adsor-

bents that showed the highest adsorption efficiency for all tested pollutants, that is starch modified with cysteine (Fig. 6a) and starch modified with histidine (Fig. 6b). The adsorption efficiency of the tested adsorbents decreases significantly, by 12–15 %, after the first cycle. The reason for this may be the disruption of the functional structure due to treatment with a desorption agent, *i.e.*, nitric acid.²⁹ After the second cycle, the efficiency decrease is smaller than the first, so three adsorption cycles are optimal for both investigated adsorbents.

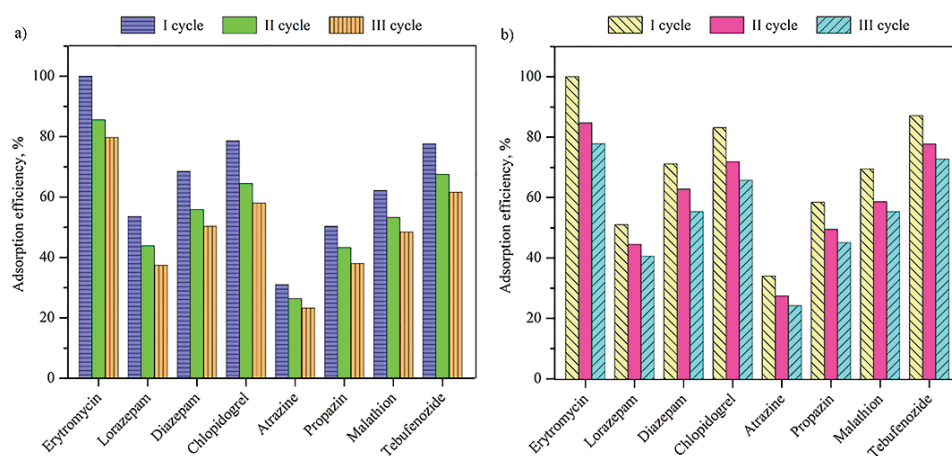


Fig. 6. Adsorption efficiency of amino-starch derivatives a) SC and b) SH for the removal of selected pharmaceuticals and pesticides through three reuse cycles.

CONCLUSION

In this study, an environmentally acceptable and economically viable method was developed for the modification of starch by introducing nitrogen-rich compounds (melamine, cysteine and histidine). Obtained amino-starch derivatives were used as adsorbents for the removal of selected pharmaceuticals and pesticides from aqueous solutions. Structural, morphological and surface analysis have confirmed the success of the modification and an increase in the adsorption efficiency of amino-starch derivatives compared to unmodified starch has been achieved. Additional modification of amino-starch with clay and diatomaceous earth did not have a significant impact, compared to the basic modification, probably due to the coating of the starch surface with parts of clay or diatomaceous earth, *i.e.*, reduced porosity of the material and less available sites for adsorption. Starch modified with histidine proved to be the most efficient material for the adsorption of investigated pharmaceuticals and pesticides, with the possibility of reuse in three adsorption cycles. The application of newly prepared adsorbents in real water samples (surface, ground and wastewater) has shown that the matrix of real water had no significant effect on the adsorption efficiency, indicating that

amino-starch derivatives, as a cheap and effective adsorbent could be considered as promising materials for wastewater treatment.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13054>, or from the corresponding author on request.

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ИЗВОД

АМИНО–СКРОБНИ ДЕРИВАТИ ЗА АДОРПЦИЈУ СПЕЦИФИЧНИХ ФАРМАЦЕУТИКА И ПЕСТИЦИДА ИЗ ЗАГАЂЕНИХ ВОДА: ИСПИТИВАЊЕ ВЕШТАЧКИ ОНЕЧИШЋЕНИХ И РЕАЛНИХ УЗОРАКА ВОДА

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У овој студији испитана је могућност употребе модификованог кромпировог скроба, са хемијским агенсима који садрже азот (меламин, цистеин и хистидин) као зеленог адсорбента за уклањање лекова и пестицида из воде. Испитан је и утицај додатне модификације аминокроба глином и дијатомејском земљом. Ефекат примењене модификације на структурна, површинска и морфолошка својства одређен је FTIR, XRD и SEM анализом, док су адсорпциона својства припремљених адсорбента одређена кроз ефикасност уклањања изабраних загађујућих материја из спајкованих и реалних узорка воде. Ефикасност припремљених аминокробова за адсорпцију лекова и пестицида смањује се по следећем редоследу: скроб–хистидин > скроб–цистеин > скроб–меламин, уз нешто бољу ефикасност свих материјала за адсорпцију лекова. Додатна модификација аминокробова глином/дијатомејском земљом није допринела повећању ефикасности адсорпције. Утврђено је да матрица реалних узорка воде утиче на ефикасност адсорпције до 10 %, што указује на обећавајући потенцијал за примену аминокроба као јефтиног и ефикасног адсорбента за третман отпадних вода. Такође, адсорбенти скроб–хистидин и скроб–цистеин су показали могућност поновне употребе кроз три циклуса адсорпције.

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SUPPLEMENTARY MATERIAL TO

Amino-starch derivates for adsorption of specific pharmaceuticals and pesticides in contaminated water: Examination in both spiked and real water samples

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EXPERIMENTAL

Materials and chemicals

The following materials and chemicals were used to prepare the starch samples: potato starch (loss on drying at 105 °C < 10%, sulfured ash < 0.5%, SuperLab, Serbia), melamine (2,4,6-triamino-1,3,5-triazine, 99%, Thermo Fisher), L(–)-cysteine ((R)-2-amino-3-mercaptopropionic acid, ≥ 97%, Thermo Fisher), L-histidine ((S)-2-amino-3-(4-imidazolyl)propionic acid, ≥ 98.5%, Carl Roth), bentonite clay (nanoclay, Sigma-Aldrich) and diatomaceous earth (SiO₂ 95%, Sigma-Aldrich).

Pharmaceuticals and pesticides selected for the study were the most commonly used and frequently detected in the investigated area (Table 1). High purity (> 95%) analytical standards of four chosen pharmaceuticals: erythromycin, lorazepam, diazepam and clopidogrel were provided by national pharmaceutical companies (Hemofarm, STADA Group, Vršac, Serbia and Zorka-Pharma, Šabac, Serbia). The analytical standards of four selected pesticides: atrazine, propazine, malathion and tebufenozide were supplied from Riedel-de Haën (Seelze, Germany).

The stock standard solutions were prepared in methanol at the concentration of 100 µg mL⁻¹. The working standard solutions were prepared by mixing the appropriate amounts of the stock standard solutions and diluting them with methanol. All solutions were preserved at -4 °C. All solvents used were HPLC grade from J.T. Baker (Center Valley, US) or Sigma-Aldrich (St. Louis, US) and all reagents were of analytical grade. Deionized water was obtained by passing the distilled water through a GenPure ultrapure water system (TKA, Niederelbert, Germany).

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TABLE S-I. Labels of samples

	Modification	Biocomposite abbreviation
Native starch	+ Melamine	= SM
	+ Cysteine	= SC
	+ Histidine	= SH
	+ Clay (bentonite)	= S-clay
	+ Diatomaceous earth	= S-d.e
Starch-Melamine (SM)	+ Clay (bentonite)	= SM-clay
	+ Diatomaceous earth	= SM-d.e
Starch-Cysteine (SC)	+ Clay (bentonite)	= SC-clay
	+ Diatomaceous earth	= SC-d.e
Starch-Histidine (SH)	+ Clay (bentonite)	= SH-clay
	+ Diatomaceous earth	= SH-d.e

Characterization methods

Fourier-transform infrared spectroscopy (FTIR) was performed using a Nicolet iS10 spectrometer (Thermo Scientific) in the attenuated total reflectance (ATR) mode with a single bounce 45 °F Golden Gate ATR accessory with a diamond crystal and DTGS detector. FTIR spectra were obtained at 4 cm⁻¹ resolution with ATR correction. The FTIR spectrometer was equipped with OMNIC software and the spectra were recorded in the wavelength range from 2.5 μm to 20 μm (*i.e.*, 4000-500 cm⁻¹).

The morphology of samples was examined using a scanning electron microscope (SEM) (type of instrument – FE-SEM, TESCAN Mira3 XMU) operating at 10 kV. Before analysis, samples were coated with gold to reduce the charging effect and improve the image quality.

X-ray diffraction (XRD) was performed using an Ultima IV Rigaku diffractometer, equipped with CuKα_{1,2} radiations, using a generator voltage (40.0 kV) and a generator current (40.0 mA). The range of 5–40° 2θ was used for all powders in a continuous scan mode with a scanning step size of 0.02° and at a scan rate of 2° min⁻¹, using D/TeX Ultra high-speed detector. A monocrystalline silicon sample carrier for sample preparation was used.

Adsorption performance and regeneration studies

The separation of pesticides and pharmaceuticals was conducted using a Dionex UltiMate 3000® LC system (Thermo Scientific, USA). For detection and quantification of pesticides and pharmaceuticals, LTQ XL (Thermo Scientific, USA) mass spectrometer was used with an electrospray ion source and linear ion trap mass analyzer. The gradient of the mobile phase consisting of methanol (A), water (B) and 10% acetic acid (C) is shown in Table S-I. Selected reaction monitoring (SRM) chromatograms of investigated pharmaceuticals and pesticides are given in Fig. S-1 and LC/MS-MS quantification parameters are presented in Table S-II.

TABLE S-II. Gradient and flow rate of the mobile phase

Time (min)	Flow rate (cm ³ min ⁻¹)	Content, %		
		A	B	C
0	0.5	49	50	1
0	0.5	49	50	1
15.00	1	0	100	0
18.00	1	0	100	0
18.01	0.5	49	50	1
23.00	0.5	49	50	1

TABLE S-III. LC/MS and MSⁿ optimized parameters for identification of the selected pharmaceuticals and pesticides

Pollutant	Retention time, min	<i>m/z</i>	Collision energy	<i>m/z</i>
		Precursor ion	a. u.*	Product ion
Erythromycin	4.72	734.1	28	576.1
Lorazepam	6.49	321.0	32	302.9
Diazepam	8.23	285.2	40	257.2
Clopidogrel	10.72	321.9	28	211.8
Atrazine	6.50	216.0	38	174.0
Propazine	7.91	230.0	36	188.0
Malathion	8.62	331.0	28	284.7
Tebufenozide	10.01	375.0	34	225.0

* arbitrary units defined by LCQ system

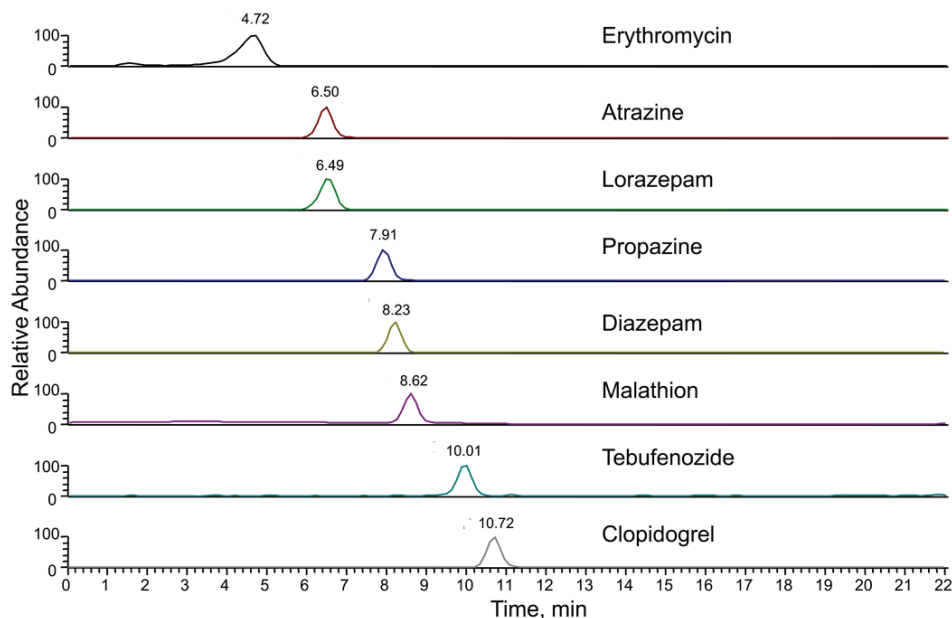


Fig. S-1. SRM chromatograms of the selected pharmaceuticals and pesticides

TABLE S-IV. Adsorption efficiency of tested materials for the removal of selected pharmaceuticals and pesticides

Sample	Pollutant							
	Erytrom.	Atraz.	Loraz.	Propaz.	Diazep.	Malath.	Tebuf.	Chlop.
Adsorption efficiency, %								
S	33.44	12.64	21.34	19.71	17.86	18.73	19.15	19.07
SM	87.5	29.39	41.32	39.41	63.21	39.46	35.47	39.62
SC	100	30.93	51.64	50.27	68.51	61.75	77.52	78.62
SH	100	33.36	51.44	58.39	70.92	69.51	87.06	82.56
Clay	32.12	14.53	16.78	17.89	24.66	28.97	32.85	35.46
S-clay	59.6	22.77	26.69	34.65	34.49	51.45	46.66	49.11
SM-clay	94	28.72	35.72	36.9	52.57	65.45	70.66	75.67
SC-clay	100	30.05	43.67	43.75	53.19	78.58	80.54	82.57
SH-clay	100	32.58	50.78	44.91	55.97	81.53	82.54	86.86
d.e	35.58	14.76	22.72	22.21	25.37	27.85	24.62	26.29
S-d.e	83	29.79	34.45	29.27	28.27	38.49	47.44	44.19
SM-d.e	94.82	38.38	37.22	40.27	41.99	56.7	66.69	71.51
SC-d.e	98	44.02	39.33	39.74	41.6	59.03	72.75	75.97
SH-d.e	98.21	45.04	41.41	28.39	45.24	62.89	79.49	86.03

TABLE S-V. Adsorption efficiency of tested materials for the removal of selected pharmaceuticals and pesticides from real water samples

Sample	Pollutant							
	Erytrom.	Atraz.	Loraz.	Propaz.	Diazep.	Malath.	Tebuf.	Chlop.
Adsorption efficiency, % (distilled water)								
SM	87.5	29.39	41.32	39.41	63.21	39.46	35.47	39.62
SC	100	30.93	51.64	50.27	68.51	61.75	77.52	78.62
SH	100	33.36	51.45	58.39	70.92	69.51	87.07	82.56
Adsorption efficiency, % (surface water)								
SM	96.94	12.39	21.83	19.45	63.18	91.28	80.19	92.53
SC	92.29	14.48	30.04	16.34	70.76	89.88	77.09	90.52
SH	69.22	32.59	29.68	32.62	32.89	76.67	83.33	82.18
Adsorption efficiency, % (groundwater)								
SM	96.13	14.62	18.28	18.4	77.11	95.29	81.75	88.08
SC	91.26	18.39	32.87	22.05	76.06	84.92	74.99	83.99
SH	64.31	31.98	24.55	32.31	22.45	83.71	82.2	82.11
Adsorption efficiency, % (wastewater)								
SM	98.7	48.05	62.19	69.99	79.57	96.6	90.86	95.15
SC	94.05	57.98	72.31	25.91	73.65	91.59	89.79	92.49
SH	53.78	38.93	42.08	28.97	44.84	85.51	89.46	88.44