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CONTENT

Editorial

The scientific significance of holding competitions in science for primary and secondary school students cannot be overstated. These competitions serve as vital platforms for nurturing a deep interest in the sciences among young learners. They offer students the opportunity to apply theoretical knowledge in practical settings, encouraging a hands-on approach to learning that is both engaging and educational. In the context of Bosnia and Herzegovina, where student performance in international assessments such as PISA and TIMSS has been below average, the reintroduction of such competitions is particularly timely and beneficial.

For many years, Bosnia and Herzegovina did not hold chemistry competitions for its students. However, after significant effort and dedication, the first competition in many years was successfully held in Sarajevo Canton in May. This event marked a pivotal moment in the educational landscape. The competition not only rekindled a spirit of chemistry inquiry among students but also highlighted the importance of practical, competitive learning experiences. The positive aspects of the competition are manifold. Firstly, it provided students with a platform to showcase their chemistry knowledge and skills, boosting their confidence and motivating them to pursue further studies in chemistry. Secondly, it fostered a sense of community and collaboration among students and educators alike. Teachers were able to exchange best practices and innovative teaching methods, which can be incorporated into their regular curricula. Additionally, the competition drew attention to the importance of chemistry education, potentially influencing educational policy and resource allocation in the future.

Competitions can inspire a cultural shift towards valuing chemistry and education. When students see their peers succeeding and being celebrated for their achievements, it can create a ripple effect, encouraging more students to take an interest in this subject. This increased interest can lead to a more scientifically literate population, better prepared to tackle the challenges of the modern world. Looking ahead, there are plans to extend this competition to all cantons, ultimately establishing it at the national level. This expansion is crucial for ensuring that all students across Bosnia and Herzegovina have the opportunity to benefit from these enriching experiences. By making the competition national, organizers can foster a more unified and robust approach to science education throughout the country. This nationwide initiative can also help to address the disparities in educational resources and opportunities between different regions.

Editors

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Evaluation of Antioxidant, Antibacterial and Cytotoxic Effects of Pink Pepper Fruit Essential Oil

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Abstract: Pink pepper is classified into the Californian/Peruvian type (*Schinus molle*) and the Brazilian type (*S. terebinthifolia*). According to studies, pink pepper extracts and essential oils have shown anti-inflammatory, antioxidant, genotoxic and antidiabetic properties in *in vitro* and *in vivo* studies. The results of bioactivity tests vary depending on the geographical origin of the pepper. In this paper, the cytotoxic, antioxidant and anti-bacterial effects of the essential oil of the commercial pink pepper fruit from the Tuzla market were investigated. To assess the cytotoxic potential, a tetrazolium salt reduction (MTT) viability assay was performed. The antioxidant potential was examined spectrophotometrically, using DPPH and FRAP methods. Diffusion techniques were used to evaluate the antibacterial activity of the essential oil. Using GC/MS, 24 components of red pepper essential oil were identified, of which α-pinene, α-phellandrene, δ-3-carene and D-limonene dominate. The studied pink pepper essential oil inhibited the cell proliferation in the HeLa cell line, causing a dose-dependent cytotoxic effect (IC_{50}=389.46 µg/mL). The essential oil inhibits DPPH radicals. The reducing ability is relatively weak. For the essential oil, an extremely good ability to inhibit the growth of the bacteria used in this study was confirmed. These results indicate a very high potential of essential oil of pink pepper fruit as an inhibitor of pathogenic organisms.

INTRODUCTION

Essential oils (EOs) are natural aromatic products with volatile and lipophilic properties. They can be isolated from leaves, flowers, seeds and other plant parts. As secondary plant metabolites, they are important in the resistance of plants to adverse external conditions such as climatic variations, mechanical damage, insects, etc. (Da Silva Dannenberg et al., 2016; Fonseca et al., 2021). EOs are widely used in the pharmaceutical, cosmetic and other industries, but also proved to be promising for use in the food industry. The use and studies of EOs are based on their diverse medicinal properties, primarily antimicrobial, anti-inflammatory and antioxidant.

Several studies have shown adequate antimicrobial function of EOs (Fonseca et al., 2021; Locali-Pereira et al., 2022). The medicinal properties of EOs are attributed to biologically active substances. EOs can induce pore formation in bacterial cell walls, resulting in disruption of bacterial cell walls and changes in permeability, thereby allowing the release of cellular components. Other modes of action include a decrease in intracellular pH and changes in the intracellular concentration of adenosine triphosphate (ATP) (Da Silva Dannenberg et al., 2016).

The biological function of these molecules in plants indicates that there is a possibility of applying EOs in different systems, with the aim of achieving effects similar to those that the compounds have in the plant (Dannenberg et al., 2019). Essential oils act as natural preservatives, as they have shown positive inhibitory results against some microorganisms in *in vitro* assays (Kavoosi and Rowshan, 2013). The antimicrobial activity of an EO can be affected by several factors, causing changes to occur. Some of them include the type and strain of microorganisms used, the chemical composition of the oil, the concentration of specific molecules, the climatic and soil conditions in which the plant was grown, etc (Fonseca et al., 2021).

The pink pepper (PP), *Schinus terebinthifolius* Raddi is a plant native to Brazil, Paraguay, Argentina. This species belongs to the Anacardiaceae family (Bittencourt Fagundes et al., 2020; Figueiredo et al., 2021; Locali-Pereira et al., 2022; Merlo et al., 2019; Silva et al., 2017). It is used as a sophisticated condiment in the international cuisine. Recent research has shown that pink pepper extracts are an interesting alternative as natural food preservatives and aromatic spices (Almeida et al., 2022; Bittencourt Fagundes et al., 2020; Figueiredo et al., 2021). The pharmaceutical industry uses PP essential oil (PPEO) in cosmetics for its aroma, with the advantage of being classified as GRAS (generally recognized as safe) (Ghabraie et al., 2016; Uliana et al., 2009). However, to ensure safety for effective commercial use in the food industry, detailed toxicological testing is required. Thanks to its medicinal, cosmetic, and pharmaceutical properties, pink pepper has great commercial potential. The astringent, antidiarrheal, depurative, diuretic and febrifugal properties of this species are attributed to various chemical compounds (Bittencourt Fagundes et al., 2020; Figueiredo et al., 2021; Fonseca et al., 2021; Locali-Pereira et al., 2022; Merlo et al., 2019).

Several bioactive compounds have been extracted from these plants, many of which are the main compounds of PP essential oil (PPEO). PPEO can be isolated by different extraction methods, but hydrodistillation is widely used due to its simplicity (Almeida et al., 2022; Magalhaes et al., 2021). The most commonly used plant parts for PPEO isolation are green and ripe fruits, seeds and leaves. Some studies have shown that the PP peel also has a high bioactive potential (Soares Carneiro et al., 2022). The reddish fruit is small and round and has sparked the interest of researchers due to the properties of its essential oil. The results of previous studies indicate antimicrobial, antifungal and antitumor effects. Antimicrobial activity has been studied against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens that can cause outbreaks of foodborne diseases (Da Silva Dannenberg et al., 2016; Dannenberg et al., 2017, 2019; Fonseca et al., 2021).

Studies on the PPEO are limited, but various chemical compounds, such as terpenes, alcohols, ketones, ascorbic acid, phenols, flavonoids and carotenoids are present in the fruit, stem and leaves. Antioxidant, antibacterial, antitumor and anti-inflammatory activity can be related to the content of flavonoids, anthocyanins and carotenoids. Antibacterial activity is mainly attributed to terpenes, terpenoids and phenylpropenes. Regarding the cytotoxic activities on tumor cells, some studies have shown that the essential oil collected from the fruits of PP is more effective than the oil from the leaves. The presence of α-pinene is associated with apoptosis of cancer cells in melanoma therapy, while germacrene D from PP has a potential effect against prostate and ovarian cancer (Figueiredo et al., 2021; Merlo et al., 2019; Soares Carneiro et al., 2022).

MATERIAL AND METHODS

The pepper sample was obtained commercially. Pink pepper originates from Vietnam. The sample was crushed using an electric mill and kept in a dark and dry place until distillation. Double-distilled deionized water or culture medium were used for solution preparations and dilutions. Thiazolyl Blue Tetrazolium Bromide (MTT) cell viability reagent and methanol used for the DPPH radical neutralization assay were obtained from Sigma-Aldrich. 2,4,6-Tripyridyl-s-triazine, iron(III) chloride, hydrochloric acid, sodium acetate (for preparation of FRAP reagent) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA). Spectroscopic measurements were performed on a Perkin Elmer Lambda 25 spectrophotometer. The HeLa (Sigma-Aldrich) cell line was cultured in Minimum Essential Medium Eagle medium (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma Aldrich), 1% non-essential Amino Acids (Sigma- Aldrich), 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin antibiotics (Sigma-Aldrich).

Hydrodistillation

The crushed pink pepper fruit was subjected to hydrodistillation for four hours on a Clevenger apparatus. The obtained essential oil was separated, dried on anhydrous sodium sulfate and stored at -20°C until analysis.

GC/MS analysis

The essential oil of the red pepper fruit was analyzed by the gas chromatography with mass detector (GC-MSD) technique. This method was used to determine the composition of the EO, in which individual peaks of the chemical components within the obtained chromatogram were identified by comparing their retention indices with the indices of the compounds in the databases, and by matching the mass spectrum of the compounds in the sample with the mass spectrum in the databases. GC/MS analysis of the essential oil sample of pink pepper fruit was performed at Agilent Technologies, Inc. gas chromatograph (7820A) with capillary HP5-ms ultra-inert column (-60 to 325°C, 30m × 250µm, film thickness 0.25µm). The gas chromatograph was equipped with an Agilent mass selective detector (MSD-5977E). Helium gas (purity 5.0) was used as carrier gas at a constant flow rate of 1.0 mL/min. The sample was injected in a volume of 1 µL. The oven temperature was programmed from 60°C (hold 1 min) to 246 C (hold 0 min) at a rate of 3°C/min and then to 280°C at a rate of 10°C/min., Three washes of the needle with solvent (*n*-hexane) were used before and after each injection. The program resulted in a total duration of 86.40 minutes. The mass detector (MSD)

was operated in the 40-400 m/z range scan mode. The MSD transfer line temperature was 250°C, and the ion source temperature was 230°C. ChemStation software was used for instrument control and data analysis. The results are expressed as a percentage concentration (% (V/V)) of each component in relation to the entire area of the obtained chromatogram.

Examination of the reducing ability (FRAP method)
The test of the reducing ability of the pepper oil was tested using the FRAP (ferric reducing antioxidant power) method, according to the published procedure (Benzie and Strain, 1999). 3 mL of the prepared FRAP reagent (a mixture of acetate buffer, iron(III) chloride hexahydrate and TPTZ reagent in a volume ratio of 10:1:1) was mixed with 0.1 mL of EO. Absorbance at 593 nm was recorded after 30 min of incubation at 37°C.

Inhibition of DPPH radicals
The DPPH radical inhibition assay was performed according to the published method (Horozić et al., 2019). Pink pepper fruit oil was mixed with absolute methanol and then mixed with a DPPH radical solution. Absorbance measurements were performed at 517 nm, after which DPPH radical inhibition was calculated according to the equation:

$$I = Ac - As / Ac \times 100 \ [\%]$$

where As is the absorbance of the solution containing the sample at 517 nm, and Ac is the absorbance of the DPPH solution.Results are expressed as IC_{50} value. Vitamin C was used as a positive control.

In vitro culture of the cell lines
Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. For each experiment, cells were grown to % confluence in cell culture flasks.

Analysis of Cell cytotoxicity by MTT
The cytotoxic effects of pink pepper fruit oil were assessed using the MTT assay. For each experiment, cells were seeded ($2x10^4$ cells/well) in 96 well plates and incubated overnight. The following day, cells were treated with increasing final concentrations of essential oil (80-800 µg/mL) and incubated for an additional 48 hours. After incubation, 10% MTT solution 5 mg/mL was added to each well, and the plates were incubated for another 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, resulting MTT-formazone crystals were dissolved by adding 200 µl of DMSO to each well with continuous shaking for 15 minutes. The absorbance was read using a microplate reader (Tecan, Sunrise) at a wavelength of 570 nm. The concentration of the essential oil leading to 50% inhibition of viability (IC_{50}) was assessed from the dose response curve plot. The experiment was repeated twice and each experiment was performed in triplicate. Untreated cells were used as a negative control, and cells treated with 30% DMSO in the culture medium were used as a positive control. The prepared stock solutions of essential oil were sterilized by filtration through 0.2 µm sterile syringe filters.

In vitro antibacterial activity
The antibacterial activity was investigated by the diffusion method on reference strains *E. faecalis* (ATCC 51299), *S. aureus* (ATCC 25923), *L. monocytogenes*(ATCC 19118), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. enterica* (ATCC 13076) and *B. subtilis* (ATCC 6633). Sterile drill-shaped holes (diameter 6 mm) were made in the agar into which 50 and 100 µL of EO were added. The plates were incubated at 37°C for 24 hours. After the incubation period, the size of the inhibitory zone was measured.

RESULTS AND DISCUSSION

Chemical composition
Essential oil was isolated from red pepper by hydrodistillation with a yield of 1.77%. The chemical composition of the essential oil obtained from the pink pepper is presented in Table 1. The identification of 24 components by GC/MS enabled for 97.1% of the total pink pepper fruit oil to be identified. Monoterpenes constitute 80.1% of the essential oil, while identified sesquiterpenes correspond to 17.1%. These percentages are close to the values reported for mono and sesquiterpenes fractions by other authors (Locali-Pereira et al., 2020; Dannenberg et al., 2019). The most abundant components were four monoterpenes δ-3-carene (22.0%), D-limonene (16.5%), α-phellandrene (16.1%) and α-pinene (13.4%) followed by two sesquiterpenes germacrene D (4.9%) and caryophyllene (4.1%). These components were also identified by other authors, but with different ratios. The results of the essential oil profile revealed the dominance of β-myrcene (Dannenberg et al., 2019), α-pinene (Cavalcanti et al., 2015) or α-phelandrene (Danila et al., 2019). The differences compared to data reported in the literature for pink pepper fruit oil can be attributed to location, climatic conditions, genetic variability (chemotype), pre-treatment of the raw material and extraction process.

Antioxidant activity
The results of the analysis of polyphenol content and antioxidant activity in *in vitro* conditions are shown in Table 2. Red pepper fruit oil showed effectiveness in inhibiting DPPH radicals, with an IC_{50} value of 41 mg/mL. The EO showed extremely weak reducing ability. Vitamin C was used as a control, which was found to have a significantly higher reducing capacity, as well as a higher efficiency in neutralizing DPPH radicals.The mechanism of the antioxidant effect is associated with the presence of oxygen-containing groups. The antioxidant capacity also depends on the presence of an aromatic core in the structure of the compounds rich in essential oil, whereby the free radical can be neutralized by direct reduction by electron transfer or radical quenching by hydrogen atom transfer.
Using the FRAP method, an extremely low conversion efficiency of Fe(III) ions into Fe(II) ions using red pepper essential oil was determined. The essential oil showed low reducing ability compared to the positive control.

Cytotoxic Activity

To evaluate the cytotoxic effect of pink pepper fruit oil, HeLa cells were treated for 24 hours with increasing concentrations of the essential oil (80-800 µg/mL). The obtained results indicate a dose-dependent cytotoxic effect at all tested concentrations (Figure 1).

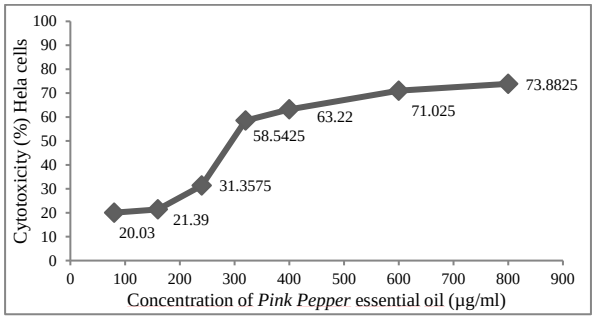

Figure 1: Cytotoxic effects on the HeLa cancer cell line, assessed by MTT after 24 hours exposure to increasing concentrations of pink pepper fruit essential oil

The highest cytotoxic effect was observed in the sample treated with 800 µg/mL essential oil, where 73.88% cytotoxicity was measured. The lowest cytotoxic effect of 20.03% was measured in cells treated with 80 µg/mL essential oil. The calculated IC_{50} value is 389.46 µg/mL. The obtained results are in accordance with previously published results in which the cytotoxic activity of the essential oil extracted from the leaves and fruits of the pink pepper on lung, breast and melanoma cancer cells was determined (Magalhaes et al., 2021). Anti-proliferative effects of *Schinus mole* L. extracts rich in sesquiterpene hydrocarbons and elemol were confirmed on neuroblastoma SH-SY5Y cells and leukemia HL60 cells (Ovidi et al., 2021).

Table 1: Chemical composition of pink pepper fruit essential oil

Test parameter / Component	Retention index	Retention time	Result v/v (%)
α-thujene	929	5.639	0.3
α-pinene	937	5.842	13.4
sabinene	974	6.971	1.6
β-pinene	979	7.077	2.6
β-myrcene	991	7.500	3.0
α-phellandrene	1005	7.973	16.1
δ-3-carene	1011	8.176	22.0
o-cymene	1022	8.650	2.9
D-limonene	1030	8.819	16.5
α-terpinolene	1088	11.043	1.4
5-isopropenyl-2-methyl-7-oxabicyclo[4.1.0]heptan-2-ol	1169	17.915	0.3
δ-elemene	1338	21.577	0.7
β-elemene	1391	23.877	0.3
caryophyllene	1419	24.968	4.0
trans-α-bergamotene	1435	25.658	0.2
humulene	1454	26.351	0.4
germacrene D	1481	27.484	4.9
bicyclogermacrene	1495	28.098	0.4
aciphyllene	1499	28.212	0.3
δ-cadinene	1524	29.184	0.7
elemol	1549	30.186	3.5
germacrene B	1557	30.444	0.2
caryophyllene oxide	1581	31.442	0.3
rosifoliol	1600	32.432	0.3

Table 2: Antioxidant activity of pink pepper fruit essential oil

Sample	FRAP [µmol/g of EO]	DPPH IC_{50} value [mg/mL]
Pink pepper fruit essential oil	0.99	41.2
Vitamin C	14 250	0.03

Antibacterial activity

The results of the antibacterial screening of pink pepper fruit EO for volumes of 50 and 100 µL are presented in Table 3. The EO showed significant antibacterial potential against gram positive and gram negative bacteria. The zones of inhibition are in the range of 12-17 mm for a volume of EO of 50 uL, or 16-23 mm for 100 uL of EO.

Table 3: Results of the antibacterial effect of pink pepper fruit EO

Bacterial strain	Inhibition zone [mm]	
	50 µL	100 µL
E. faecalis	13	16
L. monocytogenes	17	23
S. aureus	14	18
B. subtilis	15	20
E. coli	13	16
P. aeruginosa	-	-
S. enterica	12	17

The complete absence of antibacterial activity was recorded in *P. aeruginosa*. The highest efficiency of bacterial growth inhibition was recorded with *L. monocytogenes*, *B. subtilis* and *S. aureus*. In general, better inhibition results were reported for Gram positive bacteria. The better efficiency of EO in inhibiting the growth of Gram-positive bacteria can be related to the hydrophobicity of EO, whereby it easily penetrates into bacterial cells, leading to lysis and cell death (Sikkema et al., 1994; Kumar Patra and Baek, 2016). Ciprofloxacin at a concentration of 0.5 mg/mL, which was used as a control, showed a higher efficiency of inhibiting the growth of bacterial strains, with inhibition zones greater than 20 mm.

CONCLUSIONS

Commercial essential oil of pink pepper fruit, bought in a market in Tuzla, shows a cytotoxic effect on the HeLa cell line. The antibacterial potential of the oil is extremely high, and the mechanism of the inhibitory effect is connected to its hydrophobicity, which enables it to bind to cell membranes, which leads to disruption of cell integrity and cell death. The antioxidant potential of EO of pink pepper fruit is extremely weak compared to the results of antioxidant capacity obtained for vitamin C. The mentioned *in vitro* studies need to be further expanded in order to gain a better insight into the biological action of this essential oil.

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Summary/Sažetak

Ružičasti biber se svrstava u kalifornijski/peruanski tip (*Schinus molle*) i brazilski tip (*S. terebinthifolia*). Prema studijama, ekstrakti i eterična ulja ružičastog bibera pokazali su protuupalna, antioksidativna, genotoksična i antidijabetička svojstva u *in vitro* i *in vivo* studijama. Rezultati testova bioaktivnosti variraju u zavisnosti od geografskog porijekla ružičastog bibera. U ovom radu istraženo je citotoksično, antioksidativno i antibakterijsko djelovanje ploda eteričnog ulja komercijalnog ružičastog bibera sa tuzlanskog tržišta. Da bi se procijenio citotoksični potencijal, korišten je MTT test. Antioksidativni potencijal ispitan je spektrofotometrijski, DPPH i FRAP metodom. Za procjenu antibakterijske aktivnosti eteričnog ulja korištena je difuziona tehnika. Pomoću GC/MS identifikovane su 24 komponente eteričnog ulja crvenog bibera, od kojih dominiraju α-pinen, α-felandren, δ-3-karen i D-limonen. Proučavano eterično ulje ploda ružičastog bibera inhibira ćelijsku proliferaciju u HeLa ćelijskoj liniji, uzrokujući citotoksični efekat ovisan o dozi (IC_{50}=389,46 μg/mL). Eterično ulje inhibira DPPH radikale. Sposobnost redukcije Fe(III) iona je također visoka. Za eterično ulje potvrđena je izuzetno dobra sposobnost inhibicije rasta bakterija korištenih u ovoj studiji. Ovi rezultati ukazuju na vrlo visok potencijal eteričnog ulja ploda ružičastog bibera kao inhibitora patogenih organizama.

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The inhibitory properties of the boiling extracts from *Malus sylvestris* and *Syringa vulgaris* flowers on the corrosion of stainless steel in sulphuric acid medium

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Abstract: The inhibitory properties of the *Malus sylvestris* flower and *Syringa vulgaris* flower boiling extracts against the stainless steel EN Fe37-3FN corrosion in 0.5 M sulphuric acid medium were studied using electrochemical methods, including open circuit potential measurement, potentiodynamic polarisation and EIS. The addition of 10 mg/l of the *Malus sylvestris* flower extract slows the corrosion by 15%, and that of of 10 g/l – by 65%, while the addition of 10 mg/l of the *Syringa vulgaris* flower extract slows the corrosion by 30%, and that of of 1 g/l and more – by 65%. The Langmuir absorption model describes the adsorption of the components of the extracts on a surface of the steel, and the adsorption is physical in its nature. The *Malus sylvestris* flower and *Syringa vulgaris* flower extracts reveal themselves as interesting and environmentally safe substances for the steel corrosion rate reduction in acidic environments.

INTRODUCTION

In recent years, researchers have become increasingly interested in the use of chemicals derived from natural products as green corrosion inhibitors (Raja, Ghoreishiamiri, Ismail 2015; Nasab et al. 2022). The use of natural chemicals, which are typically eco-friendly, lowers the expenses associated with the search for and special manufacturing of inhibitors. Typically, plant extracts from the roots, leaves, flowers, fruits, and seeds are employed (Alrefaee et al. 2021; Umoren et al. 2019). Additionally, it creates a new opportunity for the utilisation of food waste and products made from biomass (Marzorati, Verotta, Trasatti 2018).

The wild crabapple (*Malus sylvestris*) and the common lilac (*Syringa vulgaris*) are very popular ornamental plants in gardens and parks both in Europe and Asia because of their attractive flowers. Their flowers contain several flavonoids (Mustafa, Nebija, Hajdari 2018) and phenols (Mustafa, Nebija, Hajdari 2018; Hanganu et al. 2021). However, both flavonoids (Bhardwaj, Sharma, Kumar 2021; Kadhim et al. 2021) and phenols (Kadhim et al. 2021) exhibit inhibitory properties on metal oxidation, and therefore, the boiling extracts of both *Malus sylvestris* and *Syringa vulgaris* flowers might be useful corrosion inhibitors safe for the environment. However, the inhibitory properties of flower extracts of the lilac or the wild crabapple on the metal corrosion were never examined, and there are no published studies in the literature.

Therefore, in the present study, the inhibitory ability of the boiling extracts of the *Malus sylvestris* and *Syringa vulgaris* flowers against the corrosion of stainless steel EN Fe37-3FN in 0.5 M sulphuric acid medium are aimed to be investigated.

MATERIAL AND METHODS

Reagents and Equipment

Ethanol (analytical grade) and sulphuric acid (pure grade) were purchased from LLC “Lenreaktiv”. Steel electrodes were manufactured from cylindrical ingots made of stainless steel EN Fe37-3FN (containing no more than 0.14% C, 0.3% Ni, Cu, and Cr, 0.05% Si, 0.4% Mn, 0.05% P and 0.04% S). The unused flat end surface of the ingots was sealed by the epoxy resin, and the cylindrical

working surface immersed in the solution was equal to 6.3 cm^2.

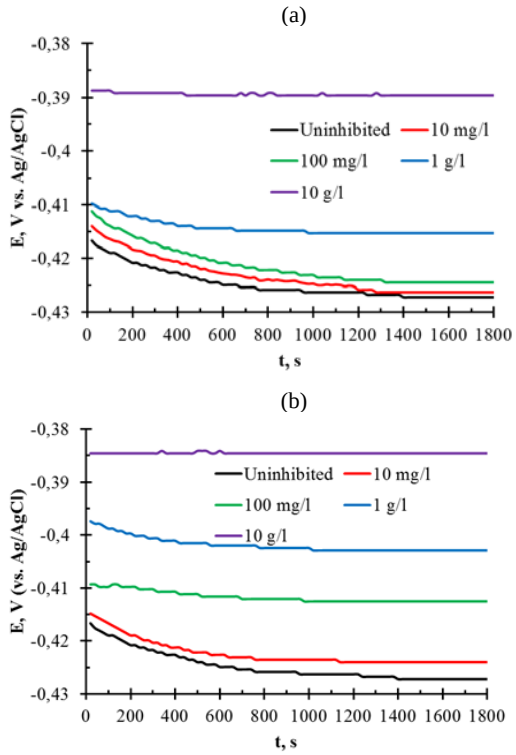

Figure 1: The open circuit potential of steel in 0.5 M H_2SO_4 with the different additions of (a) the *Malus sylvestris* flower extract, (b) the *Syringa vulgaris* flower extract after 30 min of exposure.

Weighting of the samples was performed using the analytical balance *HT-224RCE* (Vibra). Electrochemical and EIS measurements were conducted using the potentiostat-galvanostat *P-45X* with the frequency response analyser *FRA-24M* (LLC "Electrochemical Instruments"). The Faraday shield cell *SH-3M* (LLC "Electrochemical Instruments") was used for shielding the electrochemical cell. The distilled water for solution preparation was produced using the aquadistiller *Liston A1204* (LLC "Liston"). The magnetic stirrer *RET control-visc* (IKA) was used for stirring and heating the solutions. A laboratory glassware of 2nd grade was used.

Preparation of the Extracts

The flowers of *Malus sylvestris* and *Syringa vulgaris* were harvested during the blooming period in mid-May from the several wild trees growing on the streets of Kurgan, Russia. The flowers were air-dried during three months.

A total of 100 g of dried *Malus sylvestris* flowers and of 100 g of dried *Syringa vulgaris* flowers were weighted, immersed into a liter of the distilled water, heated and boiled under the reflux condenser during 3 h. The boiling extracts were cooled, the flowers were removed, and the solid residues were filtered off through the filter paper with the pore diameter of 12 μm. A total of 10 ml of each extract were taken, placed in a beaker and heated to dryness in order to determine the masses of the dissolved substances and the initial concentrations of the extract solutions. Then the working solutions of the *Malus sylvestris* flower extract and those of the *Syringa vulgaris* flower extract with the concentrations ranging from 0.02 to 20 g/l were prepared by the appropriate dilutions. The solutions were then equally diluted by 1 M sulphuric acid to finally produce a series of acidic solutions of *Malus sylvestris* flower extract and of *Syringa vulgaris* flower extract in 0.5 M H_2SO_4 with concentrations ranging from 0.01 to 10 g/l.

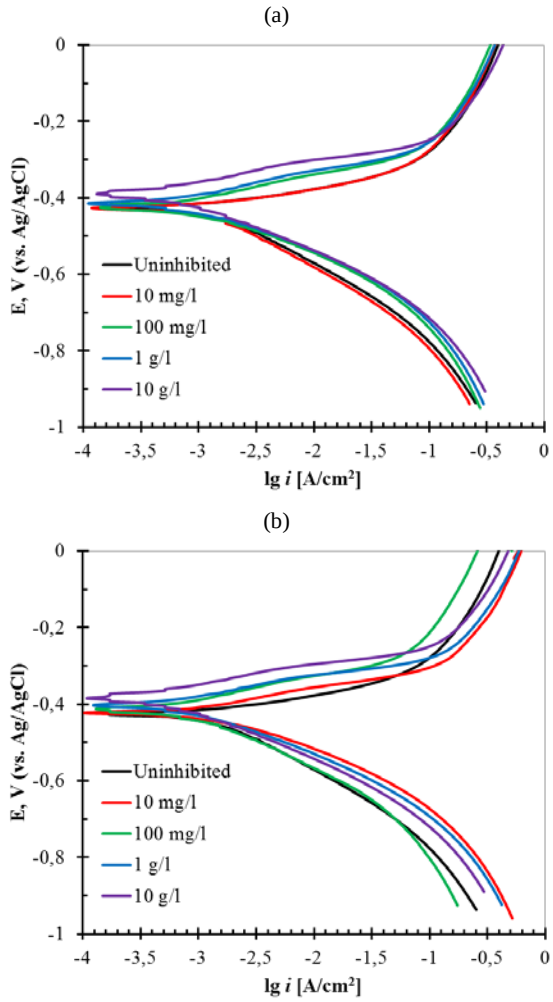

Figure 2: The polarisation curves of steel in 0.5 M H_2SO_4 with the different additions of (a) the *Malus sylvestris* flower extract, (b) the *Syringa vulgaris* flower extract after 30 min of exposure.

RESULTS

Polarisation studies

For polarisation tests, solutions of 0.5 M sulphuric acid and 0.5 M sulphuric acid with the addition of different concentrations of *Malus sylvestris* flower or *Syringa vulgaris* flower extract ranging from 0.01 to 10 g/L were prepared. Electrodes made of EN Fe37-3FN stainless steel and sealed with the epoxy resin with the working surface of 6.3 cm^2 were polished using the P2500 emery paper and degreased by ethanol. The measurements were conducted in a standard three-electrode electrochemical cell, consisting from the working electrode (steel sample), auxiliary electrode from the porous graphite, and the silver-silver chloride reference electrode. The cell was placed into the Faraday shield cell. An open circuit potential was recorded during 30 min. The results are presented in Figure 1. Polarisation curves were recorded

in the potential range from –500 to +500 mV relatively to the measured open circuit potential with the potential sweep rate of 10 mV/s. Each experiment was performed in triplicate. The obtained polarisation curves were presented in the coordinates E(lg i), and the Tafel slopes, the corrosion current density, and the polarisation resistance were evaluated from them (Kadhim et al. 2021). The inhibitory ability of the compound was estimated from the ratio of the corrosion current densities in the absence (i_0) and in the presence (i) of the inhibitor: IE = ($i_0 - i$) / i_0 · 100%, and also from the ratio of the polarisation resistances in the presence (R) and in the absence (R_0) of the inhibitor: IE = ($R - R_0$) / R · 100% (Kadhim et al. 2021). The results are presented in Figure 2 and in Table 1.

Table 1: The results of the electrochemical measurement of the corrosion rates.

c_{inh}, g/l	E_{cor}, mV	b_a, mV/dec	b_c, mV/dec	R_p, Ohm ·cm^2	IE, %	i_{cor}, mA/cm^2	IE, %
0	–427	73.9	–179.6	13.2	–	1.72	–
***Malus sylvestris* flower extract**							
0.01	–426	69.9	–175.8	15.0	11.8	1.45	16.1
0.1	–424	60.7	–157.8	16.6	20.1	1.15	33.5
1	–415	55.6	–148.5	18.9	30.0	0.93	46.0
10	–390	42.6	–150.2	24.4	45.9	0.59	65.6
***Syringa vulgaris* flower extract**							
0.01	–424	59.3	–159.5	16.5	19.7	1.14	33.8
0.1	–412	47.3	–123.8	19.6	32.4	0.76	55.9
1	–402	40.1	–132.3	22.3	40.6	0.60	65.0
10	–385	39.7	–141.1	32.8	59.7	0.41	76.1

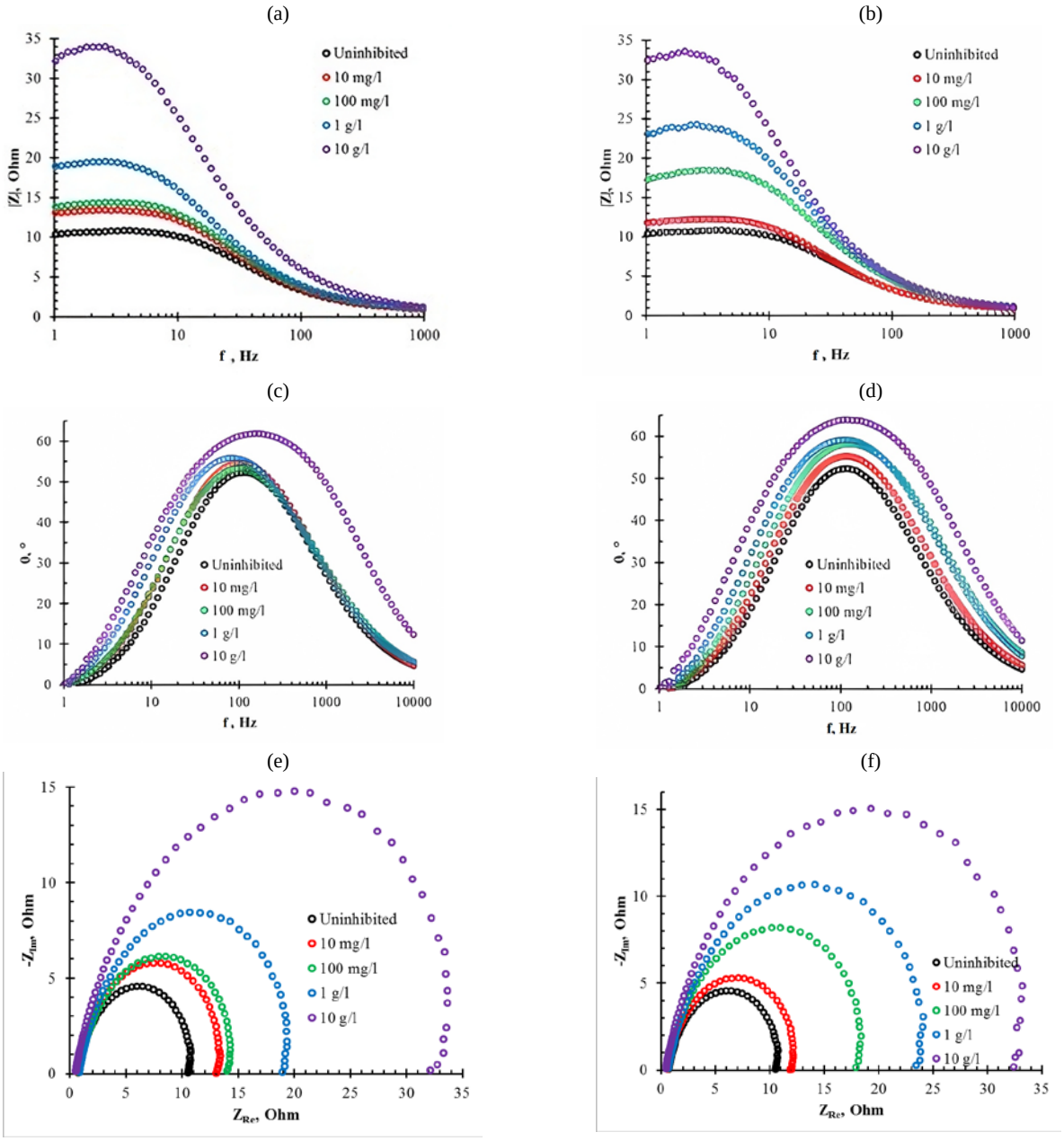

Figure 3: The Bode plots of steel in 0.5 M H_2SO_4 with the different additions of (a), (c) the *Malus sylvestris* flower extract, (b), (d) the *Syringa vulgaris* flower extract, and the Nyquist plots of steel in 0.5 M H_2SO_4 with the different additions of (e) the *Malus sylvestris* flower extract, (f) the *Syringa vulgaris* flower extract after 30 min of exposure.

Table 2: The results of the EIS measurement of the corrosion rates.

c_{inh}, g/l	R_s, Ohm	P, mOhm^{-1} · s^n	n	C_{dl}, µF	d, nm	R_{ct}, Ohm	IE, %
0	0.7	1.3	0.85	374	7.4	11.7	–
***Malus sylvestris* flower extract**							
0.01	0.7	1.25	0.85	357	7.8	12.6	7.1
0.1	0.8	1.2	0.84	332	8.3	15.8	25.9
1	0.8	1.15	0.85	316	8.7	21.5	45.6
10	0.6	0.75	0.84	172	16.1	37.8	69
***Syringa vulgaris* flower extract**							
0.01	0.6	1.2	0.85	332	8.3	13.6	13.9
0.1	0.6	0.95	0.85	253	10.9	20.9	44.0
1	0.6	0.9	0.84	214	13.1	27.5	57.4
10	0.4	0.85	0.84	185	15.0	38.7	69.8

EIS Studies

For EIS tests, solutions of 0.5 M sulphuric acid and 0.5 M sulphuric acid with the addition of different concentrations of *Malus sylvestris* flower or *Syringa vulgaris* flower extract ranging from 0.01 to 10 g/L were prepared. Electrodes made of EN Fe37-3FN stainless steel and sealed with the epoxy resin with the working surface of 6.3 cm^2 were polished using the P2500 emery paper, and degreased by ethanol. The measurements were conducted in a standard three-electrode electrochemical cell, consisting from the working electrode (steel sample), auxiliary electrode from the porous graphite, and the silver-silver chloride reference electrode. The cell was placed into the Faraday shield cell. An open circuit potential was recorded during 30 min. An impedance values were recorded at the open circuit potential value in the alternate current frequency interval from 1 Hz to 10 kHz with the potential amplitude of 10 mV. Each experiment was performed in triplicate. The obtained results were presented in the form of Bode and Nyquist plots (Yuan et al 2010). For the estimation of the impedance parameters, a simplified Randles equivalent electrical circuit (Yuan et al 2010), containing the solution resistance R_s, the consecutive charge transfer resistance R_{ct} of the passivation layer, and the parallel constant-phase element representing the double electric layer, was employed. The imaginary resistance of the constant-phase element is represented by the equation $1 / Z = P \cdot (i \cdot f)^n$, where f is the alternate current frequency, P and n are the adjustable parameters. The fitting of the equivalent circuit parameters to the experimental impedance values was performed using the free software EIS Spectrum Analyser (Bondarenko, Ragoisha 2005). In addition, the capacitance and the thickness of the double electric layer were estimated. The inhibitory ability of the compound was estimated from the ratio of the charge transfer resistances in the presence (R) and in the absence (R_0) of the inhibitor: $IE = (R - R_0) / R \cdot 100\%$ (Kadhim et al. 2021). The results are presented in Figure 3 and in Table 2.

Langmuir Adsorption Model

The description of the adsorption of the flower extract components on the steel surface was performed in terms of the Langmuir adsorption model. The Langmuir adsorption isotherm equation was linearised in the form $c_{inh}/\theta = 1/K_{ads} + c_{inh}$, where c_{inh} is the concentration of the *Malus sylvestris* flower or *Syringa vulgaris* flower extract solution (g/l), K_{ads} is the adsorption-desorption equilibrium constant (l/g), and θ is the percentage of the surface covered by the inhibitor, which assumed to be equal to the inhibition efficiency. The dependencies of c_{inh}/θ on c_{inh} are presented in Figure 4 and in Table 3. The data were processed using the least squares technique, and the equilibrium constants K_{ads} were estimated as the intercepts of the regression equations. The Gibbs energy changes of the sorption were estimated from the equation $\Delta_{ads}G = -RT \ln (K_{ads} \cdot c_{water})$, where $c_{water} = 10^3$ g/l is the water concentration in the extracts. The results are presented in Table 3.

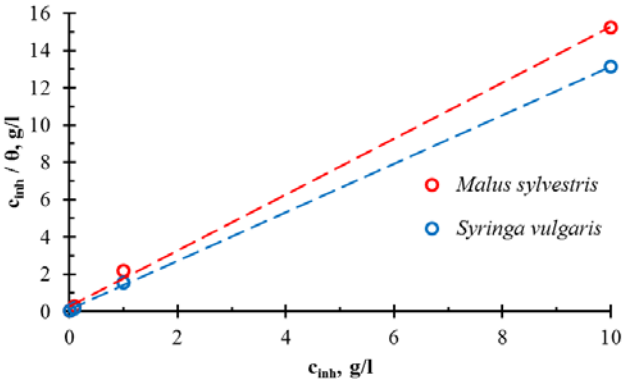

Figure 4: The plots of c_{inh} / θ vs. c_{inh} for the adsorption of the *Malus sylvestris* flower extract and the *Syringa vulgaris* flower extract on the steel surface.

DISCUSSION

From the results of the electrochemical and EIS measurements it is evident that the concentration of the extract from *Malus sylvestris* flowers equal to 10 mg/l in the sulphuric acid solution leads to the inhibition efficiency ~15% on the corrosion of stainless steel EN Fe37-3FN, and at theconcentration of 10 g/l the inhibition efficiency increases up to ~65%.

Table 3: The parameters of the Langmuir adsorption model

c_{inh}, g/l	θ	c_{inh} / θ, g/l	Regression equation	K_{ads}, l/g	$\Delta_{ads}G$, kJ/mol
***Malus sylvestris* flower extract**					
0.01	0.161	0.062	$c_{inh} / \theta = (1.50 \pm 0.04) \cdot c_{inh} + (0.3 \pm 0.2)$; $R^2 = 0.998$	3 ± 2	–20 ± 5
0.1	0.335	0.298			
1	0.460	2.171			
10	0.656	15.236			
***Syringa vulgaris* flower extract**					
0.01	0.338	0.029	$c_{inh} / \theta = (1.30 \pm 0.01) \cdot c_{inh} + (0.09 \pm 0.07)$; $R^2 = 0.999$	11 ± 4	–23 ± 8
0.1	0.559	0.179			
1	0.650	1.537			
10	0.761	13.131			

In addition, the extract from *Syringa vulgaris* flowers has even stronger corrosion inhibition properties, and its inhibition efficiency is ~30% at the concentration level of 10 mg/l, and ~65% at the concentrations greater than 1 g/l. The Langmuir adsorption model fairly describes the adsorption of the components from the extracts on the steel surface. The estimated Gibbs energies of sorption for

both *Malus sylvestris* flower and *Syringa vulgaris* flower extracts are in the range ~ –20 kJ/mol, and it characterises the physical nature of the adsorption. In any case, *Malus sylvestris* flowers and *Syringa vulgaris* flowers are widely cultivated in several countries, and the raw material are easily available. This study reveals a possible new usage of the boiling extracts of these flowers as environmentally benign corrosion inhibitors.

CONCLUSIONS

Commercial essential oil of pink pepper fruit, bought in a market in Tuzla, shows a cytotoxic effect on the HeLa cell line. The antibacterial potential of the oil is extremely high, and the mechanism of the inhibitory effect is connected to its hydrophobicity, which enables it to bind to cell membranes, which leads to disruption of cell integrity and cell death. The antioxidant potential of EO of pink pepper fruit is extremely weak compared to the results of antioxidant capacity obtained for vitamin C. The mentioned *in vitro* studies need to be further expanded in order to gain a better insight into the biological action of this essential oil.

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Summary/Sažetak

Inhibicijska svojstva vodenih ekstrakata cvijeta *Malus sylvestris* i cvijeta *Syringa vulgaris* protiv korozije nehrđajućeg čelika EN Fe37-3FN u 0,5 M sumpornoj kiselini proučavana su korištenjem elektrokemijskih metoda, uključujući mjerenje potencijala otvorenog strujnog kruga, potenciodinamičku polarizaciju i EIS. Dodavanje 10 mg/l ekstrakta cvijeta *Malus sylvestris* usporava koroziju za 15%, a dodavanje 10 g/l usporava koroziju za 65%, dok dodavanje 10 mg/l ekstrakta cvijeta *Syringa vulgaris* usporava koroziju za 30%, a dodavanje 1 g/l i više usporava koroziju za 65%. Langmuirov model adsorpcije opisuje adsorpciju komponenti ekstrakata na površini čelika, a adsorpcija je fizičke prirode. Ekstrakti cvijeta *Malus sylvestris* i *Syringa vulgaris* su zanimljivi i ekološki sigurne supstance za smanjenje stope korozije čelika u kiselim sredinama.

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Binding constants and *in silico* analysis of albumin interaction with phenolic acids and flavonoids

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Abstract: In this study, fluorescence techniques were utilized to investigate the interactions of selected phenolic acids (PAs) and flavonoids (FLs) with bovine serum albumin (BSA) under physiological conditions. The binding of PAs/FLs with BSA was investigated at three temperatures: 292, 303 and 310 K. From the obtained spectra, the Stern-Volmer constant (K_{sv}), bimolecular quenching constant (k_q), binding constants (K_b), and binding site number (n) constants were calculated. Presented results indicate that fluorescence quenching of BSA in the presence of phenolic acids/flavonoids is a static quenching process. The strongest static binding occurs during the formation of the BSA-pHBA (*p*-hydroxybenzoic acid) complex (k_q = 57.1×10^{12} M^{-1} s^{-1} at 292 K), and BSA-Que (quercetin) complex (k_q = 42.8×10^{12} M^{-1} s^{-1} at 292 K). The structure of PAs/FLs was revealed to significantly affect the binding/quenching process and additionaly, fluorescence resonance energy transfer studies confirmed the static nature of this process. The results of synchronous fluorescence spectra suggest changes in the microenvironment of tyrosine. Three-dimensional spectra showed changes related to the backbone structures of the protein chain (caused by the π-π* transition of the carbonyl group). Furthermore, thermal denaturation was performed by nano differential scanning fluorimetry (nanoDSF) and transition temperature (T_m) values for BSA complexes with PAs/FLs are slightly lower than T_m for BSA, except T_m for BSA complexes with kaempferol and chrysine. According to *in silico* analysis, theoretically, caffeic acid and quercetin showed the best binding position with albumin (4F5S).

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INTRODUCTION

Serum albumins, the most abundant soluble proteins, are capable of reversibly binding with large relatively insoluble endogenous and exogenous ligands. They are responsible for the transport of metabolites such as nutrients, hormones, fatty acids, metals, and various pharmaceuticals (Naveenraj and Anandan, 2013). Albumins comprise multiple binding sites, i.e. seven binding sites for long and medium-chain fatty acids (Zhu, Zhang, Luo, *et al.,* 2018), four additional sites for short-chain fatty acids (Bhattacharya, Grüne and Curry, 2000), and two sites for drugs (Sudlow, Birkett and Wade, 1975). The intrinsic fluorescence of serum albumins appears at 340 nm when excited at 280 nm, which is mainly contributed by the aromatic amino acids tryptophan (Trp) and tyrosine (Tyr). Hence, fluorescence spectroscopy plays a pivotal role in the investigation of interactions between ligands and protein. In particular, fluorescence quenching studies are utilized for revealing the accessibility of a ligand to the fluorophore moiety in a protein, which in turn helps us to understand the nature and the underlying mechanism of ligand-protein interactions (Lakowicz, 2013). The fluorescence

characteristics are very sensitive to the microenvironment of the fluorescent amino acid residues or changes in the local surroundings of serum albumins, such as conformational transition, biomolecular binding, and denaturation. Bovine serum albumin (BSA) is one of the most extensively utilized protein in laboratory practice due to its similarity with human serum albumin (HSA), (Bujacz, 2012; He and Carter, 1992). Both HSA and BSA display approximately 80% sequence identity and a repeating pattern of disulfides. BSA is composed of 583 amino acid residues with 20 Tyr and two Trp, Trp134 and Trp212 residues. Trp134 is located at the surface of the molecule, and Trp213 is buried in a hydrophobic pocket (Peters, 1995). Phenolic acids (PAs) belongs to the large class of plant secondary metabolites, known as phenylpropanoids. There are two subgroups of phenolics acid, the hydroxybenzoic acid derivatives (HBAs) with a general structure of C_6-C_1 and variations in their basic structure being hydroxylations and methoxylations of the aromatic ring, and the hydroxycinnamic acid derivatives (HCAs) with a general structure of C_6-C_3, and representing a series of (*E*)-3-phenylpropenoic acids differing in their ring substitution. They are usually found as esters or glycosides rather than as free compounds and are usually present in the diet, e.g. in fruits, vegetables, nuts, coffee, and teas (Vermerris and Nicholson, 2006). Due to their active phenolic groups, they exhibit a broad spectrum of biological and pharmacological properties, have good antioxidant, anti-microbial, and anti-cancerogenic effects (Cui, Yan, Cai, *et al*., 2002; Kacem, Kacem, Simon, *et al*., 2015; Zhao, Chen, Zhao, *et al*., 2015). Flavonoids are compounds derived from 2-phenylbenz-γ-piron, 2-phenylchromom, flavonon (dihydroflavon) and flavonol (3-hydroxyflavon). They are common in a great variety of fruits, vegetables, and beverages. (Ng, Lyu, Mark, *et al*., 2019; Mabry, Markham and Thomas, 2012). Because of their high reactivity with reactive oxygen species such as hydroxyl, alkoxyl, or peroxyl radicals as well as their efficient inhibition of lipid peroxidation in micelle systems, flavonoids are thought to be associated with antiaging, antifungal, anti-inflammatory, and especially anticancer activities (Tamba and Torreggiani, 2004; Zheng, Song, Zhang, *et al.,* 2020). Owing to their phenolic nature, flavonoids are quite polar but poorly water-soluble and their absorption could be scarce.

In the present study, four PAs and four FLs were chosen to assess their binding affinities to BSA, i.e. salicylic acid (SA), *p*-hydroxybenzoic acid (*p*HBA), caffeic acid (CA), ferulic acid (FA), chrysin (Chr), naringenin (Nar), kaemferol (Kae) and quercetin (Que). Their structural formulas are presented in Figure 1. The fluorescence quenching method and the molecular docking were carried out to interpret the interactions under physiological conditions. From the fluorescence spectra the Stern-Volmer constant (K_{sv}), bimolecular quenching constant (k_q), binding constant (K_b), binding site number (*n)*, and thermodynamic parameters (Gibbs energy, Δ*G*, enthalpy, Δ*H*, and entropy, Δ*S*) were calculated. Synchronous fluorescence and 3D fluorescence were used to determine conformational changes in the secondary structure of BSA influenced by PAs and FLs. The binding distance (*r*) between BSA and selected phenolic compounds were calculated by Förster theory (fluorescence resonance energy transfer-FRET). Furthermore, thermal stability was performed by nano differential scanning fluorimetry (nanoDSF) and analyzed by monitoring the change in transition temperature. The molecular docking was performed to determine the geometrical binding structure of BSA and selected phenolics using AutoVina and PyMOL software.

EXPERIMENTAL

Chemicals

Bovine serum albumin (BSA), caffeic acid (≥98%, HPLC), chrysin (≥ 98%, HPLC), (*E*)-ferulic acid (99%), *p*-hydroxybenzoic acid (≥99%), kaemferol (≥98%, HPLC), naringenine (≥98%, HPLC), quercetin (≥98%, HPLC), and salicylic acid (≥99%, ACS reagent) were of the highest purity available and purchased from the Sigma-Aldrich. Ethanol, hydrochloric acid, and sodium chloride were obtained from Semikem, while tris(hydroxymethyl)aminomethane was from Kemika.

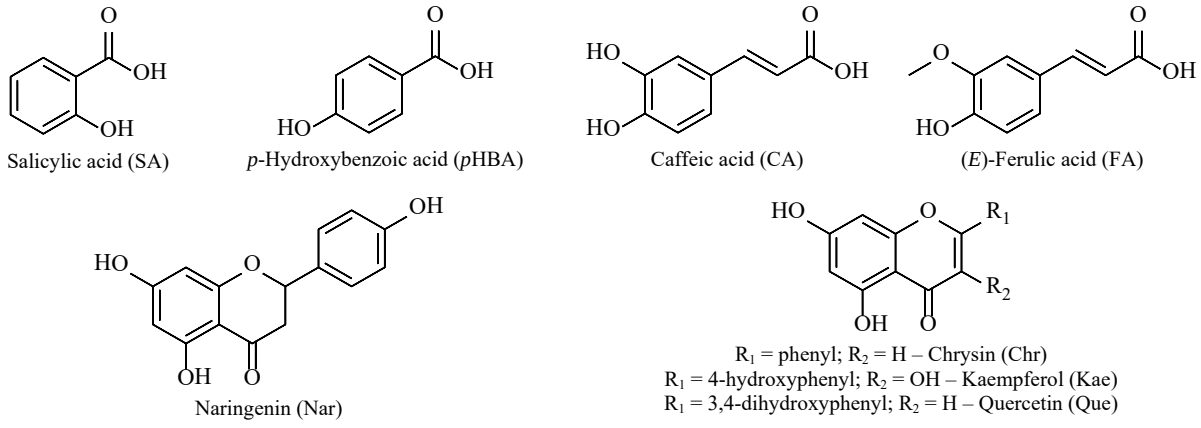


Figure 1: The structural formulas of selected Pas and FLs

Solution preparation

Tris–HCl buffer (10 mM, pH 7.4) containing 0.1 M NaCl was selected to keep the pH value and maintain the ionic strength of the solution. BSA solution (0.029 mM) was prepared in Tris-HCl buffer and stored in a refrigerator. Standardization of the BSA solution was done spectrophotometrically at 279 nm using the extinction coefficient (ε = 43824 M^{-1} cm^{-1}). The stock solution of PAs and FLs (1.5 mM) were prepared by directly dissolving them in ethanol.

Fluorimetric titration

The fluorescence quenching method was used to investigate the interactions of PAs and FLs with BSA. All spectra measurements were obtained using a luminescent spectrometer (LS-55, Perkin Elmer) at three different temperatures (292, 303, and 310 K). The solution of BSA (2 mL, 0.029 mM) was titrated in cuvette by successive addition of individual phenolics solution aliquots (10μL) from a stock of 1.5 mM. The emission spectra were recorded in the wavelength range of 300–400 nm, while a fixed wavelength of 279 nm was used for the excitation. The excitation and emission slit widths were set to 10 nm and the scanning speed was 500 nm/min (Jin, Wei, Qi, *et al*., 2012). The synchronous fluorescence spectra were obtained by synchronous scanning at the wavelength range of 250–350 nm, with the wavelength interval ($\Delta\lambda$) at 15 and 60 nm, at which the spectrum only shows the spectroscopic behavior of Tyr and Trp residues of BSA, respectively (Lloyd and Evett, 1977). The three-dimensional (3D) fluorescence spectrum of BSA and the BSA–PA/FL (60 μL) complexes at 292 K were performed under the following conditions: the excitation scan range of 200–350 nm in 10 nm increments, and the emission spectrum was set as between 200 and 500 nm (Zahirović, Žilić, Pavelić *et al*., 2019). The number of scanning curves was 16, and other scanning parameters were the same as the fluorescence quenching spectra.

Thermal denaturation

Simultaneous monitoring of the PAs and FLs at 330 nm and 350 nm during the thermal conversion of purified albumin was carried out using nanoscale differential scanning fluorimetry (nanoDSF) with an excitation wavelength of 280 nm. The capillaries were filled with albumin (2 μM) and PA/FL (7.8 μM), placed into the sample holder and the temperature was increased from 297 to 368 K with a temperature gradient of 1K/min, with one fluorescence measurement per 0.2 K (Magnusson *et al*, 2019). The ratio of the recorded emission intensities (Em_{350nm}/Em_{330nm}), which represents the change in Trp fluorescence intensity, was plotted as a function of the temperature. Additionally, their first derivative was calculated with the manufacturer's software, displaying as the peak at the point of the maximal slope, which corresponds to the unfolding transition temperature (T_m) which is defined as the temperature at which half of the protein is unfolded and acts as an important parameter for the conformational stability of a protein.

Computational studies

Density function theory (DFT)

Density function theory (DFT) is a computational modeling study used to investigate the electronic structure of molecules. Quantum chemical calculations were performed at the B3LYP level with a 3-21G basis set using ORCA 5.0. software (Neese, 2022). All compounds were first optimized using Avogadro software. Relevant energetic properties such as the dipole moment and energy were calculated for each compound. Frontier molecular orbitals (FMO) studies can be used to predict the chemical reactivity of compounds and identify their most likely reactive sites. Two important parameters that quantitatively describe these interactions are the energy of HOMO and LUMO. The calculated EHOMO and ELUMO energies of compounds help to explain global reactivity descriptors that influence the nature of the interaction. Additionally, the chemical reactivity parameters such as electronegativity (χ), chemical potential (μ), global electrophilicity index (ω), global hardness (η) and global softness (*S*) were calculated.

Docking study

Docking process were carried out using Autodock Vina (Trott and Olson, 2010). Phenolic acids and flavonoids have been optimized and exported to a pdf file. Non-polar hydrogens were merged, rotatable bonds were defined and torsional bonds of ligand were set free. Protein 3D structure of bovine albumin serum (BSA) was acquired form Protein Data Bank (PDB code 4F5S) (Bujacz, 2012). Polar hydrogens, Kollman charges and solvent parameters were added. The binding site were defined using grid size coordinates of 94x67x90 and grid center coordinates of x=72, y=27, z=92 with a grid space of 0.375 Å (Bautista-Aguilera *et al.,* 2014). For the visualization of the docking results was used PyMOL software (DeLano, 2002).

RESULTS AND DISCUSSION

Quenching constant and bonding parameters

Fluorescence quenching has proven to be a very sensitive technique with many capabilities to analyze the interaction between ligands and proteins (local changes in the polarity, conformation, and/or exposure to the solvent). The interaction will lead to modifications in fluorescence intensity-decrease ('quenching') or an increase ('enhancement') of protein. Bovine serum albumin has an emission maximum at 348 nm under excitation at 279 nm mainly because of the presence of Trp residue. It was found that the fluorescence intensity of BSA is gradually decreasing with increasing concentration of selected PAs and FLs as a function of the level of BSA modification by the attachment of the ligands (Figure 2). SA with BSA shows *isosbestic point* which indicates the formation of a stable protein-ligand complex. The *isosbestic point* shows the equilibrium dissociation reaction of the complex and is independent of the reactant concentration.

The quenching mechanism for interacted molecule was analyzed according to the Stern-Volmer equation (1):

$$\frac{F_0}{F} = 1 + k_q\tau_0 = 1 + K_{SV}[Q] \qquad (1)$$

where F_0 is the fluorescence intensity in the absence of the quencher, F is the fluorescence intensity in the presence of the quencher, [Q] is the ligand concentration, and k_q the quenching constant for a bimolecular reaction, K_{SV} the Stern-Volmer constant, and τ_0 the average lifetime for fluorophore without a quencher. The above equation is applied to determine quenching constants (k_q and K_{SV}) by linear regression of a plot of F_0/F against [Q]. The binding constant for ligand-protein interaction (K_b) and the binding site number for BSA (n) were calculated by equation (2) for the equilibrium between free and bound molecules.

$$\log\frac{F_0 - F}{F} = \log K_b + n\log[Q] \qquad (2)$$

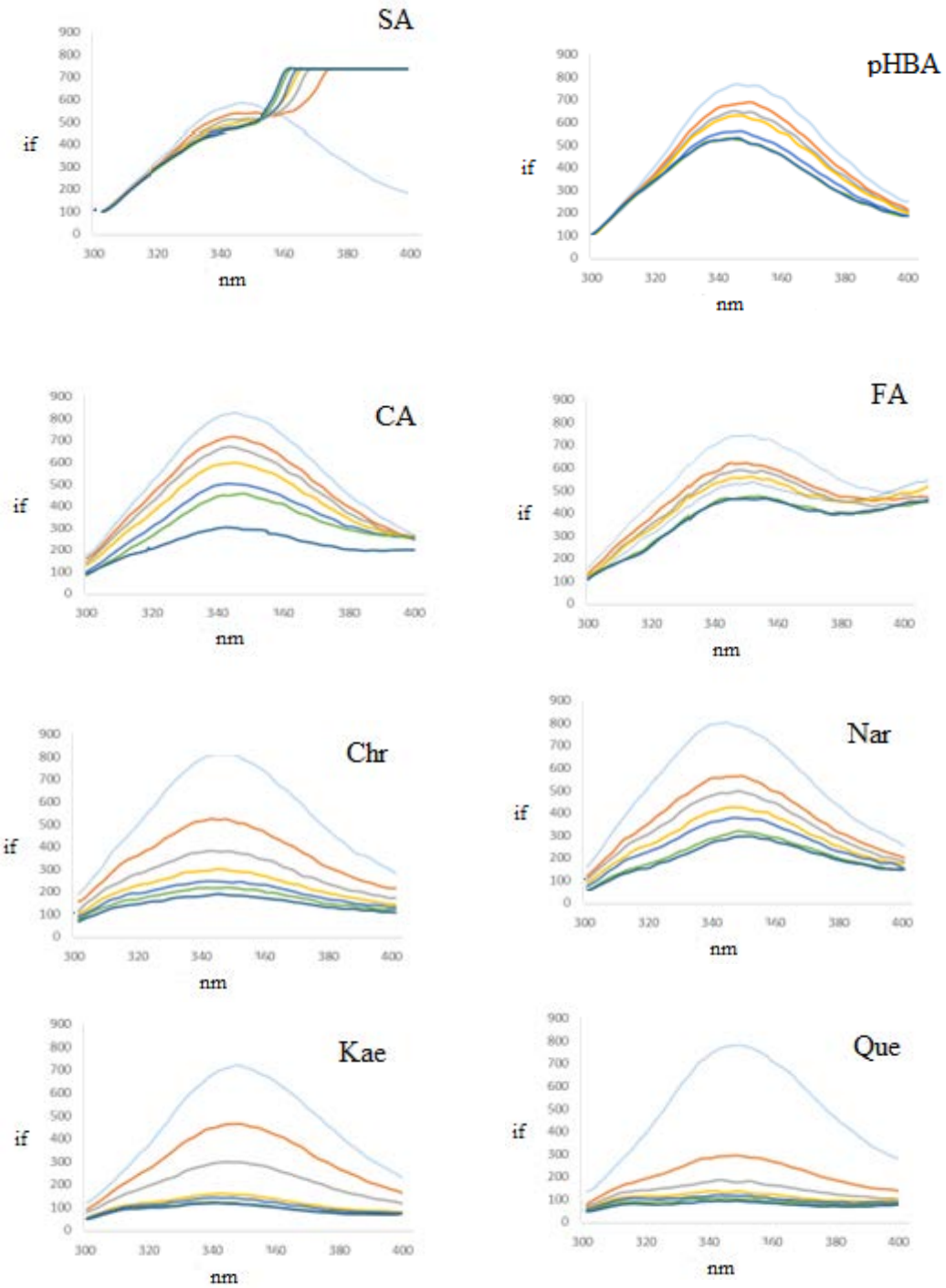


Figure 2: Fluorescence quenching of BSA by selected PAs and FLs. From the top to bottom the values of Pas/FLs concentration were 0, 2.5, 5.0, 7.4, 9.9, 12.2, and 14.6 μM, respectively, T = 292 K

The values of K_b and n could be determined from the intercept and slope by plotting log $(F_0\text{-}F)/F$ against log[Q]. The quenching constants and binding parameters for BSA by four different PAs are summarized in Table 1. Values for bimolecular quenching constants (k_q) reflect quenching or the accessibility of the fluorophores to the quencher. Fluorescence quenching mechanism may result from a variety of processes such as excited state reactions, molecular rearrangements, energy transfer, ground-state complex formation (static quenching), or collisional interactions (dynamic quenching), (Lakowicz, 2013). Static quenching refers to the formation of the fluorophore quencher complex in the ground state; whereas dynamic quenching refers to a process where the fluorophore and the quencher interact during the excited-state lifetime of the fluorophore. The values of k_q are two orders of magnitude greater than the maximum diffusion collision quenching constant (~10^{10} M^{-1} s^{-1}) and can be assumed that the quenching mechanism was due to complex formation between BSA and PAs/FLs (a static mechanism), rather than dynamic collision (Bose, 2016).

Table 1: Quenching constants and binding parameters for BSA-PAs/FLs complexes

BSA-PAs/FLs	T (K)	$K_{SV}\times10^4$ (M^{-1})	k_q*$\times10^{12}$ (M^{-1} s^{-1})	K_b (M^{-1})	n
BSA-SA	292	1.07	2.14	9.52×10^1	0.53
	303	3.46	6.93	7.04×10^3	0.83
	310	4.70	9.41	1.58×10^3	0.70
BSA-pHBA	292	2.85	57.10	2.50×10^3	0.77
	303	6.59	13.10	3.96×10^3	0.78
	310	4.11	8.22	2.74×10^2	0.55
BSA-CA	292	3.45	6.91	1.83×10^6	1.28
	303	3.87	7.74	4.99×10^2	0.53
	310	7.33	14.60	2.13×10^4	0.85
BSA-FA	292	10.15	23.00	7.17×10^2	0.64
	303	9.14	18.20	9.53×10^2	1.02
	310	1.26	25.20	8.51×10^2	1.21
BSA-Chr	292	46.00	23.00	$2.59\text{x}10^5$	1.00
	303	24.90	14.10	$5.78\text{x}10^3$	0.73
	310	16.66	8.32	$1.14\text{x}10^4$	0.81
BSA-Nar	292	21.20	10.60	$1.22\text{x}10^4$	0.80
	303	42.30	21.10	$1.69\text{x}10^6$	1.21
	310	33.70	16.80	$2.56\text{x}10^5$	1.05
BSA-Kae	292	79.30	39.60	$1.66\text{x}10^7$	1.32
	303	3.27	16.30	$1.80\text{x}10^6$	1.26
	310	2.82	14.10	$1.47\text{x}10^4$	0.82
BSA-Que	292	85.60	42.80	$6.27\text{x}10^4$	0.81
	303	39.30	19.60	$4.35\text{x}10^4$	1.30
	310	57.00	28.50	$1.28\text{x}10^4$	0.93

*The quenching constant (k_q) were calculated using equation $k_q = K_{SV}/\tau_0$, τ_0 is taken as 5×10^{-9} s

BSA-Que complex and BSA-*p*HBA showed stronger quenching constants (k_q) at 292 K (42.80×10^{12} M^{-1} s^{-1} and 57.10×10^{12} M^{-1} s^{-1}, respectively). This effect was probably dependent upon the position and number of the hydroxy group. The intensive quenching ability has Kae to the BSA. Also, the presence of the methoxy group seems to be important for quenching activity too. The k_q value for BSA-FA was slightly lower than the k_q of BSA-*p*HBA.

The temperature rises could decrease the quenching constant because of the lower stability of the ligand-BSA complex, while they could increase the dynamic quenching constant due to the increased possibility of diffusivity of the molecules and molecular collision. The values of the Stern-Volmer constant *(K_{SV})* at different temperatures were shown in Table 1. The results for BSA-PAs interaction showed that the K_{SV} will increase with increasing temperature, aside from the *K*sv values for the BSA-FA decrease with the increasing temperature which coincides with the static form of the hardening mechanism. Data obtained for hydroxycinnamic acids systems in this study corresponded with these findings by other authors (Trnkova, Bousova, Kubicek, *et al*, 2010). The results for the interaction between BSA and FLs showed an increase in the BSA-Nar complex and a decrease in the BSA-Chr and BSA-Kae complexes, while the BSA-Que complex showed an irregular change in Ksv values. These values are consistent with the results in the literature (Wang, Qin, Chang, *et al,* 2018).

The binding constant K_b reflects the power of ligand-protein association and thus can be used for comparison of binding affinities of structurally-related ligands to protein molecule connected with alteration of its secondary structure. It was demonstrated that the interaction of PAs/FLs with protein molecule depends mainly on the size and structure of the ligand, especially on the number and position of hydroxy groups on the aromatic ring (Bartolomé, Estrella, and Hernandez, 2000). The binding constant (K_b) for BSA-PAs interaction was ranked in the order BSA-CA > BSA-*p*HBA > BSA-FA > BSA-SA. BSA-CA system showed a significant binding constant which confirms the significance of hydroxy groups in the process of binding. The same constant for the interaction of FLs with BSA was ranked in the order BSA-Kae > BSA-Nar > BSA-Que > BSA-Chr. Also, in these interactions with BSA there was a significant impact of hydroxy groups in ligands.
The binding site number shown in Table 1 ranged between 0.53 and 1.32 (at 292 K) suggesting that nearly one molecule of tested phenolics was associated with BSA. Kaemferol, with four hydroxy groups on rings has the highest value.

Thermodynamic parameters

Thermodynamic parameters are important for the noncovalent acting forces and they are used to determine the type of interaction between ligand and protein. Utilizing the binding constant K_b, the free energy change (ΔG) enthalpy (ΔH), and entropy (ΔS) values can be estimated from the van't Hoff and thermodynamic equations:

$$\ln\frac{K_{b1}}{K_{b2}} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\left(-\frac{\Delta H}{R}\right) \quad (3)$$

$$\Delta G = -RT\ln K_b \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

where T is the temperature and R the universal gas constant. The equation (4) is applied to determine the value of ΔG, while ΔS and ΔH could be determination from the intercept and slope by plotting ΔG against T. Hydrophobic (ΔH >0 and ΔS>0), electrostatic (ΔH<0 and

$\Delta S>0$), Van der Waals and hydrogen bonds ($\Delta H<0$ and $\Delta S<0$) interaction are the main forces. The negative values of ΔG were indicating a spontaneous process of binding for interaction between BSA and PAs/FLs. Hydrogen bonding and Van der Waals forces played a major role in the interaction of BSA with *p*BHA, CA, Chr and Kae while hydrophobic bonds were found in the interaction of BSA with SA, FA, Nar and Que. (Table 2).

Table 2: Thermodynamic parameters for BSA-PAs/FLs complexes

#	ΔG (kJ mol^{-1}) (T (K))	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
BSA-SA	-11.06 (292 K) -22.32 (303 K) -18.99 (310 K)	135,06	505,38
BSA-*p*HBA	-19.00 (292 K) -20.88 (303 K) -14.48 (310 K)	-79.82	-204.43
BSA-CA	-35.03 (292 K) -15.65 (303 K) -25.70 (310 K)	-233.48	-656.10
BSA-FA	-15.97 (292 K) -28.89 (303 K) -35.20 (310 K)	299.42	1080.51
BSA-Chr	-30.28 (292 K) -21.83 (303 K) -24.10 (310 K)	-143.21	-390.30
BSA-Nar	-22.87 (292 K) -36.14 (303 K) -32.11 (310 K)	147.81	590.42
BSA-Kae	-40.38 (292 K) -36.30 (303 K) -24.74 (310 K)	-279.34	-813.51
BSA-Que	-26.83 (292 K) -38.52 (303 K) -32.11 (310K)	55.98	291.17

Energy transfer from BSA to PAs

Fluorescence resonance energy transfer (FRET) is a mechanism related to the transfer of energy between two chromophores that depends on their mutual distance. During protein-ligand interactions, the excitation energy is transferred from the donor/protein (BSA) to the acceptor/ligand (PAs or FLs), and the necessary conditions for this are: the donor molecule can produce fluorescence; the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor; and the distance between the donor and the acceptor is less than 8 nm (Zhang, Zhou, Liu, *et al*., 2008). Based on FRET, the energy transfer efficiency (E) can be expressed as:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \qquad (6)$$

where F and F_0 are the fluorescence intensities of the donor after and before acceptor binding, r is the distance of the acceptor from the donor, R_0 is the critical distance at E = 50% which can be calculated as:

$$R_0^6 = 8.8\times10^{23}\ [\kappa^2 n^{-4} \Phi J(\lambda)] \qquad (7)$$

where κ^2 is the orientation factor between donor and acceptor (2/3), n is the refractive index of the medium (1.334) and Φ is the quantum yield of the donor (0.15). The spectral overlap integral (J) of the donor emission spectrum and the acceptor absorption spectrum is given as:

$$J(\lambda) = \int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4\, d\lambda \qquad (8)$$

where $F(\lambda)$ and $\varepsilon(\lambda)$ are the fluorescence intensity of the donor and the molar absorption coefficient of the acceptor at the wavelength λ, respectively (Jayabharathi, Thanikachalam and Perumal, 2012). The overlap of the BSA fluorescence emission and *p*HBA absorption spectra are represented in Figure 3, while the summarized FRET results for all BSA-PAs/FLs pairs are given in Table 3. In all cases, the distance of BSA from the PAs/FLs is less than 8 nm, which indicates a high possibility of energy transfer from BSA to ligands how the resulting distance was obtained with great accuracy using FRET theory. Additionaly, it could be further confirmed PAs quenched BSA fluorescence in the manner of the static quenching due to $r > R_0$ (Phopin Ruankham, Prachayasittikul *et al*., 2020).

Synchronous and three-dimensional fluorescence spectroscopic studies

To explain the structural changes to BSA resulting from the addition of selected PAs or FLs, synchronous and 3D fluorescent spectra were recorded. These spectra provide information about the molecular environment near the chromophore. When the $\Delta\lambda$ value is stabilized at 15 and 60 nm, the synchronous fluorescence spectra give characteristic information of Tyr and Trp residues (Liu, Huang, Zhong, *et al*., 2018). The effect of selected PAs/FLs is shown in Table 4.

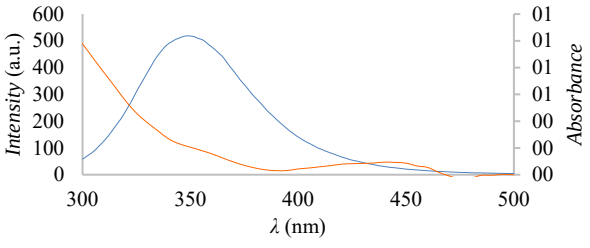


Figure 3: Overlap between emission spectrum of BSA and absorption spectrum of *p*HBA.

Table 3: BSA-PAs/FLs energy transfer parameters at 292 K.

#	J (cm^3 M^{-1})	R_0 (nm)	E	r
BSA-SA	3.347E-15	2.129	0.170	2.773
BSA-*p*HBA	3.021E-15	2.093	0.259	2.493
BSA-CA	8.133E-15	2.468	0.391	2.657
BSA-FA	5.652E-15	2.323	0.279	2.721
BSA-Chr	9.372E-15	2.527	0.701	2.193
BSA-Nar	5.309E-15	2.299	0.514	2.277
BSA-Kae	3.132E-14	3.090	0.802	2.448
BSA-Que	2.526E+14	2.981	0.844	2.250

Table 4: Shifts in synchronous fluorescence spectra

PAs	Δλ		FLs	Δλ	
	15 nm	60 nm		15 nm	60 nm
SA	2 nm (284→286)	1 nm (279→278)	**Chr**	5 nm (284→289)	2 nm (279→281)
***p*HBA**	3 nm (284→287)	0 nm	**Nar**	2 nm (284→282)	0 nm
CA	3 nm (284→287)	1 nm (279→278)	**Kae**	2 nm (284→282)	0 nm
FA	3 nm (284→287)	0 nm	**Que**	2 nm (284→282)	2 nm (279→281)

The emission wavelength of the Tyr residue is red-shifted in all cases for PAs and for Chr. This red shift indicates that the conformation of BSA was changed and it suggests a more polar (or less hydrophobic) environment for Tyr residue. At the same time, all additions of selected PAs/FLs cause a minor significat change shift in the fluorescence intensity of Trp residues in the position of the maximum (a minor blue shift was observed in the cases of CA and SA and a minor red shift was observed in the cases of Chr and Que). This suggests that the samples do not cause significant changes in the microenvironment of Trp residues. A possible explanation for the above lies in the fact that Tyr, unlike Trp, contains an aromatic OH group that can dissociate in the excited state, allowing easier binding and quenching of fluorescence (Jayabharathi *et al.*, 2012).

Three-dimensional spectra for BSA were also obtained in the absence/presence of selected PAs/FLs, and there two peaks were observed (Peak I, $\lambda_{ex}/\lambda_{em}$: 225/340 nm and Peak II, $\lambda_{ex}/\lambda_{em}$: 275/340 nm). While Peak I denotes the fluorescence spectral features of the polypeptides present in BSA and are due to π–π* transition of the polypeptide structures, Peak II is because of the existence of Tyr and Trp residues (Zhang *et al*., 2008). Except for SA for which the results of conformational changes are not clear, according to contour plots (Figure 4) in all cases, there is an increase in polarity in the Tyr and Trp microenvironment. Besides, a decrease and displacement of the Peak I suggest that PA addition to BSA might have decreased its diameter by interacting with the polypeptide residues, reflecting a conformational change in BSA (Wani, AlRabiah, Bakheit, *et al*., 2017). The changes in Peak I were also observed in the interaction of albumin with flavonoids, in particular with quercetin and kaempferol. The obtained results of conformational changes of BSA are in agreement with already published studies on structurally similar phenolics such as cinnamic acid, ferulic acid, caffeic acid, and chlorogenic acid (He, Liang, Luo, *et al*., 2010; Li, *et al*., 2010).

Thermal denaturation

The measuring principle of advanced differential scanning fluorimetry (nanoDSF) is an increasing temperature profile followed by changes in the intrinsic fluorescence of a protein. Destabilizing chemical or thermal influences might lead to changes in a protein and hence to changes in fluorescence intensities as well as shifts in unfolding transition temperature (T_m). In most cases, the loss of protein stability correlates with a reversible or irreversible unfolding often followed by an aggregation process. According to Figure 5, T_m values for BSA complexes with PAs, Nar and Que are slightly lower than T_m for BSA. The complexes BSA with Kae and Chr showed higher Tm values than BSA.

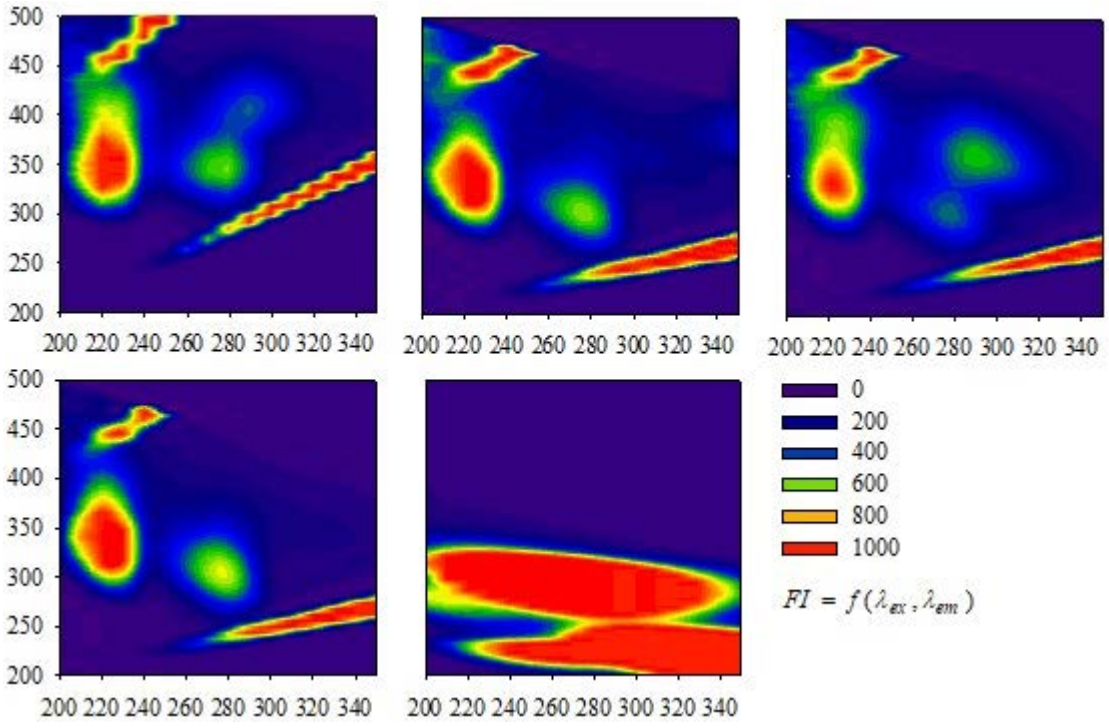


Figure 4: 3D fluorescence spectra. (a) BSA, (b) BSA-CA, (c) BSA-FA, (d) BSA-*p*HBA, and (e) BSA-SA

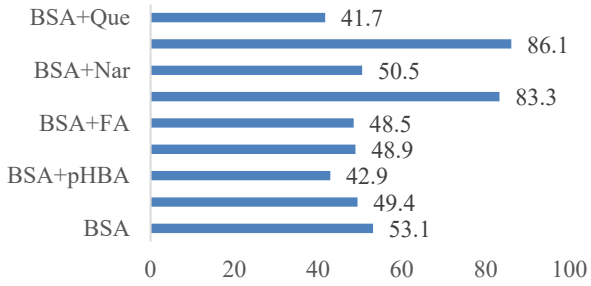


Figure 5: nanoDSF measurement. Unfolding transition temperature (*T*m, °C) values for BSA and BSA with PAs.

It should also be kept in mind that ligands can interplay with both the folded and unfolded states of target proteins, and a negative shift in melting temperature does not exclude binding to the native state (Gao, Oerlemans, Groves, 2020).

Molecular docking

Molecular docking is a widely used approach for modeling interactions between small compounds and macromolecules, including BSA, at the atomic level, which enables the characterization of the behavior of compounds at the binding site of target macromolecules (Cheng, Wang, Tang, et al., 2019).

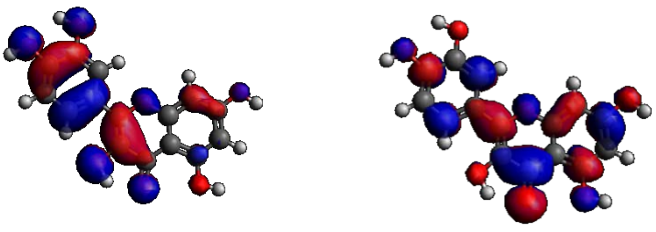
The electronic structure of the ligand was investigated using a DFT modeling study. Relevant energetic properties such as the dipole moment (D) and energy were calculated for each compound (Table 5). Frontier molecular orbitals (FMO) predict the chemical reactivity of the lignand and identify the most likely reactive sites. The calculated energies of HOMO and LUMO help to explain the global reactivity descriptors (chemical hardness, chemical potential, and electrophilicity). The stability of the studied ligands was confirmed by the negative values obtained for their EHOMO and ELUMO (Yousef, El-Reash, El Morshedy, 2013).

The band energy gap correlates with the chemical reactivity and chemical stability of molecules. It was found that the energy difference [EHOMO-ELUMO] for Que is smaller than the band energy gap observed for other ligands, indicating greater reactivity. In contrast, the ligand *p*HBSA exhibited greater stability. An important parameter is the electrophilicity (ω) of the ligand, which evaluates its ability to accept electrons from its environment. The BSA selected for docking has an amino acid chain consisting of three homologous but structurally different domains (I, II and III), which are subdivided into nine loops by disulfide bonds and arranged in a heart-shaped molecule. Each of these domains consists of two subdomains, A and B. Molecular docking calculations were performed to determine the most probable binding site for the individual PAs and FLs in BSA and to identify the major amino acid residues and intermolecular forces involved in the interaction. The best-bound compound (with higher binding affinity for the protein) was revealed to be lidands Chr and Que. The docking pose for the best-bound ligand from PAs (BSA-SA) and from FLs (BSA-Que) is illustrated in Figure 6.

Table 5: Shifts in synchronous fluorescence spectra.

	E_{HOMO}	E_{LUMO}	*I*	*A*	Δ*E*	η	χ	μ	σ	ω	*D*	*E* (kJ/mol)
CA	-5.575	-1.568	5.575	1.568	4.007	2.004	3.572	-3.572	0.499	12.778	2.274	-2549.817
FA	-5.545	-1.449	5.545	1.449	4.096	2.048	3.497	-3.497	0.488	12.523	1.891	-5862.934
*p*HBA	-6.276	-0.902	6.276	0.902	5.374	2.687	3.589	-3.589	0.372	17.306	2.388	-2064.243
SA	-6.230	-1.013	6.230	1.013	5.217	2.609	3.622	-3.622	0.383	17.106	4.628	-2064.212
Kae	-4.954	-1.003	4.954	1.003	3.951	1.976	2.979	-2.979	0.506	8.763	-	-4281.861
Chr	-5.883	-1.456	5.883	1.456	4.427	2.214	3.670	-3.670	0.452	14.903	5.561	-3665.790
Que	-5.075	-1.417	5.075	1.417	3.658	1.829	3.246	-3.246	0.547	9.636	3.814	-4594.991
Nar	-5.734	-0.916	5.734	0.916	4.818	2.409	3.325	-3.325	0.415	13.317	3.391	-3973.851

I-ionization potential; *A*-electron affinity; Δ*E*-energy gap; η-global hardness; χ -electronegativity; μ-chemical potential; σ-global softness; ω-electrophilicity, *D*-dipole moment; *E*-energy



HOMO orbitals LUMO orbitals

Figure 5: Optimized structure of quercetin.

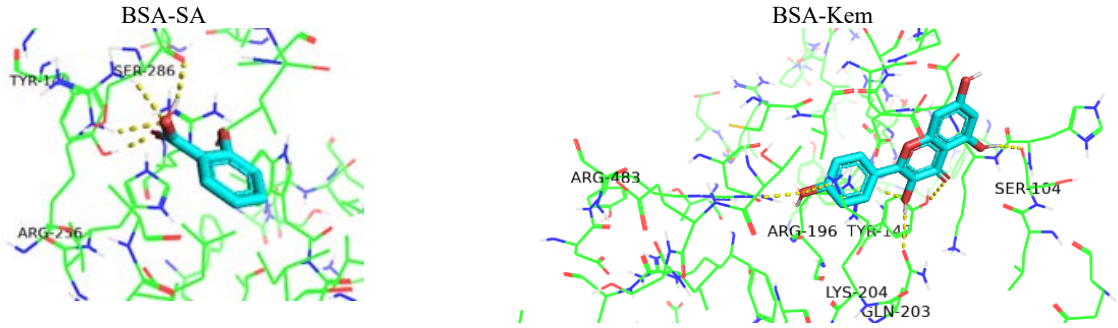


Figure 6: The complex BSA-SA and BSA-Kae as viewed in PyMOL.

The results obtained are summarized in Table 6, where a more negative affinity value indicates a stronger binding of the tested PAs/FLs to BSA and correlates with the H-bonds obtained.

Table 6: Hydrogen bonds in BSA (4F5S) interaction with selected PAs and FLs.

Sample	Affinity (kcal/mol)	Bonding in AChE/Distance (Å)
CA	-6.4	***H***-Lys132 → ***O***-[C4-OH] / 2.4 Å
FA	-6.3	***H***-Tyr137 → ***O***-[C1-O] / 2.0 Å
*p*HBA	-5.7	***H***-Arg208 → ***O***-[C1-OH] / 2.4 Å
SA	-5.6	***H***-[C1-OH] → ***O***-Ser286 / 2.1 Å ***H***-Tyr149 → ***O***-[C1-O] / 2.1 Å ***H***-Arg256 → ***O***-[C1-OH] / 2.2 Å ***H***-Arg256 → ***O***-[C1-OH] / 2.4 Å
Kem	-7.4	***H***-Arg196 → ***O***-[C3-OH] / 1.9 Å ***H***-Arg483 → ***O***-[C4'-OH] / 2.3 Å ***H*** [C5-OH] → ***O***-Ser104 / 2.3 Å ***H*** [C3-OH] → ***O***-Gln203 / 2.4 Å ***H***-Lys204 → ***O***-[C3-OH] / 2.3 Å ***H***-Tyr147 → ***O***-[C4-O] / 3.2 Å
Chr	-8.2	***H***-Tyr137 → ***O***-[C7-OH] / 2.8 Å
Nar	-7.9	***H***-[C7-OH] → ***O***-Glu125 / 2.3 Å ***H***-Lys116 → ***O***-[C4-O] / 2.7 Å
Que	-8.2	***H***-Lys136 → ***O***-[C4'-OH] / 2.2 Å ***H***-Lys132 → ***O***-[C3'-O] / 2.7 Å ***H***-Tyr160 → ***O***-[C4-O] / 3.1 Å

According to the results obtained, CA shows the strongest binding (affinity -6.4 kcalmol^{-1}) and binds with one H-bond (Lys132) and Que (affinity -8.2 kcalmol^{-1}) with three H-bonds (Lys132, Lys136 and Tyr160). All predicted binding sites are already proven sites of excellent binding and transport of bioactive compounds such as (*S*)-ibuprofen and (*S*)-ketoprofen (subdomain IA), while it is known that the active metabolite of nabumetone, 6-methoxy-2-naphtylacetic acid, binds similarly as CA in subdomain IIIA (Czub, Handing, Venkataramany, *et al*., 2020).

CONCLUSIONS

All PAs and FLs quenched the Trp fluorescence of BSA mainly by static quenching mechanism and thus showed the formation of non-fluorescent BSA-PAs/FLs complexes. The binding constant and binding site number depend on the number and position of hydroxyl groups in the molecules of phenolics. All interactions between PAs/FLs and BSA were spontaneous processes, hydrogen and hydrophobic bonds were the main acting force. The results of synchronous and 3D fluorescence spectroscopy indicate conformational changes in the structure of BSA in all BSA-phenolics systems, while the results of molecular docking support and correlate well with *in vitro* assays. Overall, the presented results imply that PAs/FLs could be stored and transported by BSA which may influence their biological and pharmacological activities in organisms.

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Summary/Sažetak

U ovoj studiji korištene su tehnike fluorescencije za ispitivanja interakcija odabranih fenolnih kiselina (PA) i flavonoida (FL) s albuminom goveđeg seruma (BSA) pri fiziološkim uvjetima. Vezanje PA/FL s BSA ispitivano je na tri temperature: 292, 303 i 310 K. Iz dobivenih spektara nađene su: Stern-Volmerova konstanta (Ksv), bimolekularna konstanta gašenja (kq), konstanta vezanja (Kb) i broj vezivnih mjesta (n). Predstavljeni rezultati pokazuju da je gašenje fluorescencije BSA u prisutnosti fenolnih kiselina/flavonoida statički proces gašenja. Najjače statičko vezanje događa se tijekom stvaranja kompleksa BSA-*p*HBA (*p*-hidroksibenzojeva kiselina) (kq = 57,1×1012 M-1 s-1 pri 292 K), i BSA-Que (kvercetin) kompleksa (kq = 42,8× 1012 M-1 s-1 na 292 K). Otkriveno je da struktura PA/FL značajno utječe na proces vezanja/gašenja, a dodatno su studije prijenosa energije fluorescentne rezonancije potvrdile statičku prirodu ovog procesa. Rezultati spektra sinkrone fluorescencije ukazuju na promjene u mikrookruženju tirozina. Trodimenzionalni spektri pokazali su promjene povezane sa strukturama okosnice proteinskog lanca (uzrokovane prijelazom π-π∗ karbonilne skupine). Nadalje, toplinska denaturacija je provedena nano diferencijalnom skenirajućom fluorimetrijom (nanoDSF), a vrijednosti prijelazne temperature (T*m*) za BSA komplekse s PAs/FL su nešto niže od T*m* za BSA, osim T*m* za BSA komplekse s kamferolom i krizinom. Rezultati *in silico* analize pokazuju da kafena kiselina i kvercetin imaju najbolje vezivanje s albuminom (4F5S).

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Application of hydrophobic solvents based on L-menthol, as greener alternatives to classical solvents for Pb(II) ions extraction

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Abstract: Deep eutectic solvents (DESs) as a new-generation of solvents are receiving increasing attention as environmentally friendly solvents in various analytical techniques. These solvents are new generation solvents, and based upon what they are derived from, they can be the safest, cheapest, and most effective extraction methods available. With DES, the extraction efficiency and metal ion recycling are significantly improved. In this work, the potential application of these solvents for the extraction of Pb(II) ions was investigated. For that purpose, hydrophobic DESs (HDESs), based on L-menthol as H-acceptor and decanoic acid as H-donor, were prepared at molar ratios of acceptor to donor of 1:1 and 1:2. In the optimized extraction procedure, the Pb(II) ions were extracted into the organic phase with the efficiency of 94.3% and 97.3% for 1:1Men:DecA and 1:2 Men: Dec A, respectively. The results also showed that unlike of classical liquid-liquid extraction methods, in the original solutions, counterions are not required to transfer the analyte to the hydrophobic phase. Furthermore, no ligands were required to transfer the analyte ions to the HDES phase: the results show that the extraction efficiency of 1:1 HDES decreased to 88.65% in the presence of 18C6, and to 96.5% for 1:2 HDES. Comparable results for HDES (1:1 Men: DecA) extraction efficiency in the proposed method with the efficiency of 1,2-dichloroethane and chloroform in classical methods (96.7% and 92%, respectively), without counterions and without the requirement for ligands as carriers, make this HDES-based extraction method simpler, less expensive, and most importantly, more environmentally friendly.

INTRODUCTION

Deep eutectic solvents (DESs) as a new generation of solvents are receiving increasing attention as environmentally friendly solvents in various analytical techniques. The concept of a "deep" eutectic solvent (DES) first appeared in the scientific world in 2003. when it was announced that a group of designed solvents could meet the principles of "green" chemistry, unlike the ionic liquids used at the time(Anastas and Eghbali, 2010).
Moreover, the possibility of synthesis of these solvents from non-toxic ingredients, as well as components of natural origin (NADES, Natural DES) allows overcoming the limitation of ionic liquids, such as toxicity and poor biodegradability (Tuzen et al., 2016). Hydrophobic deep eutectic solvents (HDES) prepared from terpenes (menthol, thymol) and fatty acids are considered relatively non-toxic, less volatile, more environmentally friendly, and renewable (Abbot et al., 2004), (Florindo et al., 2014). Their hydrophobicity makes them promising alternatives to traditional organic solvents used in sample preparation, as well as solvents used in the field of LLE of non-polar analytes and transition metals from aqueous environments.

In this paper, hydrophobic "deep" eutectic solvents based on natural neutral ingredients (L-menthol and natural organic acids) were prepared and their effect on the extraction of metal cations was studied. Only chemically stable DESs were selected to be used as solvents in the extraction. Practical applications of HDES in a sample preparation include conventional liquid-liquid extraction

(Zhao et al., 2015). The aim of this work is to examine all factors that affect the efficiency of Pb(II) ions removal, i.e. defining the conditions for the extraction of cations from the initial aqueous solution into a hydrophobic solution, all using a menthol-based solvent. Considering the low viscosity of the prepared menthol-based HDES, which makes them suitable for use in extraction techniques, L-menthol was also chosen in this work as an H-bond acceptor in the synthesis of HDES solvents (Ribeiro et al., 2015).
The final result is compared with the results of classical extraction of Pb(II) ions with hydrophobic organic solvents. Considering the relevance of such research and the still insufficiently researched area of application of hydrophobic eutectic solvents as alternative extracts for heavy metal ions as pollutants, the concept of this research was created. The obtained results will make a significant contribution to the expansion of knowledge in the field of application of hydrophobic eutectic solvents in liquid-liquid extraction.

MATERIALS AND METHODS

Chemicals:

- Standard Pb(II), solution (1000 mg/L), Merck
- Picric acid ($C_6H_3N_3O_7$), 99%, Kemika

Hydrophobic deep eutectic solvents prepared from:

- $C_{10}H_{20}O$, L(-)-mentol, 99,5%; Acros Organic
- $C_8H_{16}O_2$, octanoic acid; 99%, Acros Organics
- $C_{10}H_{20}O_2$, decanoic acid; 99%, Alfa Aesar
- $C_{12}H_{24}O_2$,dodecanoic acid ; 99%, Acros Organics

Macrocyclic ligands:

- $C_{12}H_{24}O_6$ (18-crown-6); 99%, ACROS OGANICS,

Stripping solution:
Triton X-100 surfactant; purrum.p.a. Sigma-Aldrich
Disodium-EDTA, > 99%; Sigma-Aldrich

Acetic acid buffer solution (pH=5), prepared from:
CH_3COOH (purris. p.a., Fluka) NaOH (g.r., Merck)

DES preparation
The preparation of hydrophobic DESs, as homogeneous liquids immiscible with water, was done by mixing two solid components (L-menthol as HBA and different HBDs) in different molar ratios, e.g. 2:1, 1:1 and 1:2. Different molar ratios were chosen to test whether DES solvents could be prepared in a wide or small range of compositions. The first component was weighed directly in the flask, and the second component was first weighed on a scale, after which the entire amount was transferred to the flask. (Rajabi et al., 2018).
The components in the flask were previously mixed with a glass rod and heated in a metal heating block. The formation of hydrophobic DESs was investigated using a standard procedure. After preparation and mixing, the flasks were heated and mixed at a temperature of approximately 40°C until the melting of the solid components was achieved and stability of the resulting solvent was determined (Cao et al., 2017). For mixtures that have not turned into a liquid state, the temperature is first increased to 60°C, and if (according to the previously explained procedure) this is not enough, the mixture is further heated up to 80°C (Phelps et al., 2018). During the experiments, in some cases after 24 h at room temperature, crystals were visible in the solvent, so these solvents were discarded from the study and not further analyzed (SalA:Men; tDecA:Men).

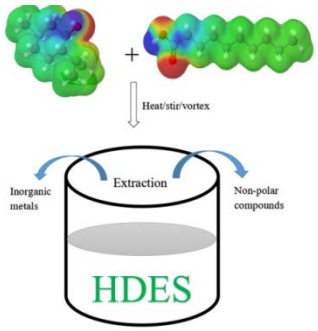

Figure 1. Synthesis of hydrophobic deep eutectic solvents (HDES) from DL-Menthol (HBA) and decanoic acid (HBD) at 1:1 molar ratio (Dwamen, 2019)

Extraction procedure
DES hydrophobicity was introduced in 2015 by van Osch and co-workers, although the authors acknowledged earlier work involving menthol-based hydrophobic eutectic mixtures. For the optimized liquid-liquid extraction procedure, 5 mL of standard feed solutions containing the analyte (standard metal solutions ($1\cdot10^{-4}$mol/L) and counterions (picrates, $1\cdot10^{-3}$mol/L) were mixed with a hydrophobic organic phase (volume of 3-5 mL) which represents the solvent used for the extraction. 5 mL of a buffered aqueous solution of "stripping agent" (thiosulfate, concentration 0.10 mol/L or EDTA concentration $1\cdot10^{-3}$ and $1\cdot10^{-2}$ mol/L) represents the final water phase - RP (eng. receiving phase). The aqueous phase and the organic hydrophobic phase (HDES solvent) were mixed for different periods of time (15 min to 2 h) on an automatic shaker (rotation: 300 rpm), after which the two phases of different polarity were physically separated. In the aqueous phase, the ion concentration of the analyte is then measured by AAS.

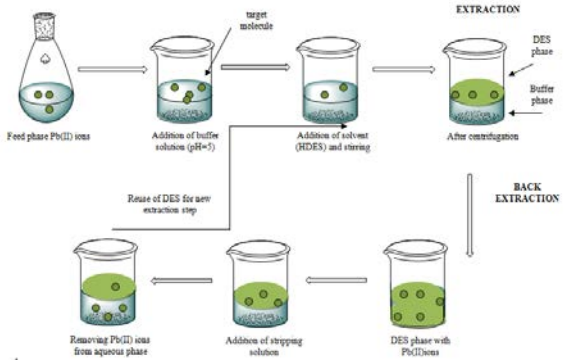

Figure 2. Extraction procedure

The efficiency of extraction was calculated:

$$\%\ of E = \frac{(analyte\ conc.\ before\ extraction) - (analyte\ conc.\ after\ extraction)}{analyte\ conc.\ before\ extraction} . 100$$

Instruments

The pH of the aqueous solutions was measured using a pH meter (GLP31 Crison Instruments).

Quantification of metal ions removed during the transport experiments was obtained by the Flame Atomic Absorption Spectrometry technique, using a Perkin Elmer AAnalyst 200 instrument.

RESULTS AND DISCUSSION

Various parameters were studied in order to optimize the conditions for selective extraction and the most efficient removal of Pb(II) ions. Factors affecting the selective extraction of Pb(II) ions were analyzed: the type of solvent used and its volume, the counterion concentration in the feed solution, the pH value of the feed and stripping solutions, and the extraction equilibrium time,.

The influence of analyte concentration on extraction efficiency was investigated.

Given that the increase in lead concentration has no significant effect on the extraction efficiency, the analyte concentration of 20 mg/L ($1 \cdot 10^{-4}$mol/L) was taken as optimal for further research. A study by Zolgharnein, Hosseini, Sangi et al. (2002) also confirmed the significant effect of different concentrations of counterions in the original solution on the efficiency of ion removal during transport. In this paper, the results showed that there is no significant effect of counter-ions on the extraction of Pb(II) ions. Namely, even in the absence of counter-ions, a satisfactory extraction efficiency is achieved (96.7%). It can be concluded that it is possible to achieve a high extraction efficiency even without the use of picric acid.

Since the amount of solvent used directly affects the cost of the extraction process, the effect of HDES volume on the removal of ions from the source phase was also investigated. Initially a volume of 5 mL was used, but smaller volumes (1 to 4 mL) were also tested. Treatment of samples with HDES volumes of 1 and 2 mL resulted in significantly reduced extraction efficiency compared to treatment of samples with DES volumes of 5 mL, performed under the same conditions. These results are consistent with the principles of mass transfer, since the driving force is the concentration gradient between the aqueous and organic phases.

However, using a volume of 3 mL, as well as 4 mL of solvent, achieves extraction efficiency comparable to the results of using 5 mL of solvent. Considering that reducing the amount of solvent did not significantly change the amount of ions removed, 3 mL was chosen as the optimal parameter for further experiments.

The effect of pH was tested by extraction experiments with Men/OctA (1:1) solvent, in the pH range between 3 and 6. At pH=3, there is no significant removal of Pb(II) ions in the HDES phase, which is probably due to the greater stability of menthol-based solvents in the pH 4-6 range.

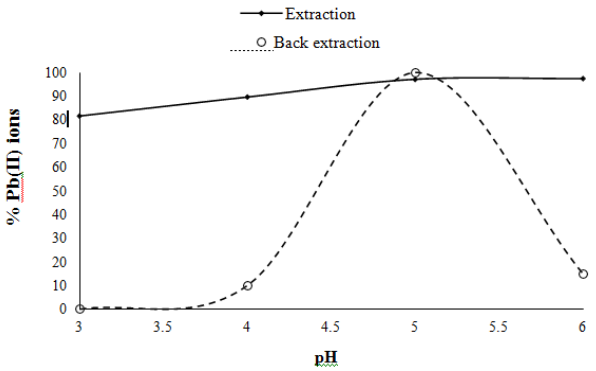

Figure 3. Dependence of extraction/back extraction efficiency on the pH value of aqueous solutions
SP contains: $[Pb^{2+}] = 1 \cdot 10^{-4}$ mol/L and $[Pic^{-}] = 4 \cdot 10^{-3}$ mol/L; RP contains: $[EDTA] = 1 \cdot 10^{-3}$ mol/L; time of mixing : 2h; HDES : Men:OctA; V(HDES) = 3 mL; V(SP)= 5 mL

Although experiments at pH 6 resulted in the highest extraction of analyte ions, the efficiency of the back extraction procedure (15%) does not justify working at this pH value. Therefore, pH=5 was chosen as the optimal pH value, for the proposed extraction procedure. In this work, it was also examined whether the use of macrocyclic ligands has an effect on the extraction of Pb(II) ions (Figure 4).

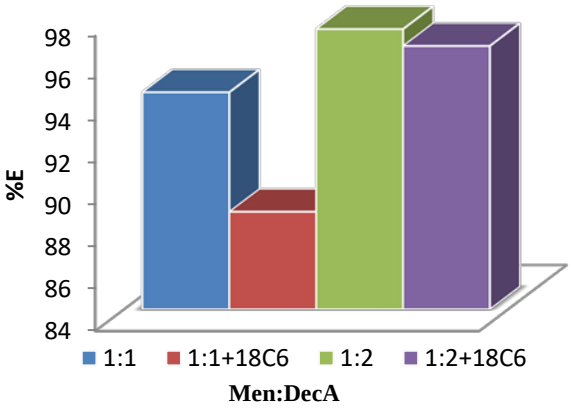

Figure 4. Comparison of Pb(II) ion extraction efficiency using Men:DecA solvent, with and without macrocyclic ligand 18C6 (SP contains: $[Pb^{2+}] = 1 \cdot 10^{-4}$ mol/L and $[Pic-] = 4 \cdot 10^{-3}$ mol/L; pH=5; mixing time: 2h; used V(HDES)=5 mL, V (SP)=5 mL)

In the classic LLE process, ligands are necessary as "carriers" of metal ions. In this work, the use of macrocyclic ligands is not necessary, it even has a negative effect on the removal of analyte ions, based on which we can assume that analyte ions enter into direct interactions with the hydrophobic solvent, without ligands as mediators. The results showed, that Pb(II) ions were extracted into the organic phase with the efficiency of 94.3% and 97.3% for 1:1 Men:DecA and 1:2 Men:DecA, respectively. For the HDES solvent Men:DecA (1:1) the extraction efficiency of 94.3% was reduced in the presence of 18C6 (88.65%), while in the case of the HDES solvent Men:DecA (1:2) the extraction

efficiency of 97.3% decreased to 96.5% in the presence of 18C6. Therefore, it did not make sense to the macrocyclic ligand 18C6 for further research. It is also evident that the greater hydrophobicity of the solvent Men:DecA (1:2) enables a higher extraction efficiency (97.3% > 94.3%) compared to Men:DecA (1:1).

In order to emphasize the advantage of the proposed method for the extraction of Pb(II) ions, we make a comparison with the results of classical liquid-liquid extraction with chloroform and 1,2-dichloroethane as solvents. The results obtained from this research are shown in Figure 5. A comparison was made between the results obtained using the HDES solvent Men-OctA (1:1), without the use of ligands, at pH=5 (for both aqueous phases SP and RP), and with equimolar concentrations of analyte ions and counterions within the SP, as well as the stripping agent in the RP. The mixing time was the same for the compared techniques (120 min), as was the mixing speed (300 rpm). It should be noted that for classical extraction in chloroform and 1,2-DCE, 18-crown-6 was used as a macrocyclic ligand for complexation of analyte ions (extraction is not possible without a ligand).

The results show a higher extraction efficiency (97%) for the procedure with HDES solvent (Men:OctA) even without ligand, and compared to chloroform (92%) and dichloroethane (96.7%) as classical solvents, under the same experimental conditions (120 min stirring at 300 rpm). In particular, a higher back-extraction efficiency (98%) from HDES compared to chloroform (55%) and dichloroethane (16.7%) is evident.

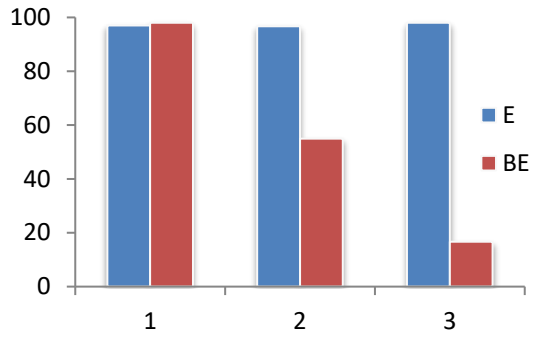

Figure 5. Comparison of extraction/back-extraction efficiency in procedures with HDES solvents and classic chlorinated organic solvents (1-HDES, 2-$CHCl_3$, 3-1,2-DCE)

CONCLUSIONS

The water-immiscible nature of HDES solvents makes them promising in the liquid-liquid extraction of non-polar analytes and transition metals from the aqueous phase.

Since the most HDES solvents are synthesized from natural raw materials, the solvent is considered relatively non-toxic, environmentally friendly and sustainable.

The use of menthol-based HDES solvent in the technique of liquid-liquid extraction of Pb(II) ions has a number of advantages: simplicity and shorter duration of the procedure, non-toxicity of the solvent and high efficiency of analyte ion removal.

In the series of H-donors used for the preparation of HDES with L-menthol, octanoic acid showed the best results, both for the extraction efficiency (97%) and for the back-extraction procedure (98%).

The most efficient extraction of Pb(II) ions is achieved using 3 mL of HDES solvent Men:OctA (1:1), without the use of ligands, without counterions in the original solution, at pH 5.

The higher efficiency of HDES solvent extraction, even without ligand, gives this procedure an advantage over the classical one.

Considering that the presence of other ions does not affect the Pb(II) removal efficiency , the proposed procedure can be used in Pb(II) removal procedures from real samples.

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Summary/Sažetak

U posljednjoj deceniji „duboki“ eutektički rastvarači (DES) naširoko su proučavani i primijenjivani u tehnikama pripreme uzoraka. Donedavno je većina sintetiziranih DES rastvarača bila hidrofilna, što je sprječavalo njihovu upotrebu u ekstrakciji vodenih uzoraka. HDES (hidrofobni DES) su obećavajuće alternative tradicionalnim organskim rastvaračima koji se koriste u pripremi uzoraka. Mogućnost HDES sinteze od netoksičnih sastojaka čini HDES da zadovolji sve standarde zelene analitičke hemije. U ovom radu pripremljeni su hidrofobni „duboki eutektički rastvarači“ na bazi prirodnih neutralnih sastojaka (L-mentol i prirodne organske kiseline), te je ispitan njihov uticaj na ekstrakciju metalnih kationa. Samo su hemijski stabilni DES-ovi odabrani da se koriste kao rastvarači u ekstrakciji. Praktične primjene HDES-ova u pripremi uzoraka uključuju konvencionalnu tečno-tečnu ekstrakciju. Cilj ovog rada je ispitati sve faktore koji utiču na efikasnost uklanjanja Pb(II) iona, tj. definisanje uslova za ekstrakciju kationa iz polaznog vodenog rastvora u hidrofobni rastvor, a sve to pomoću rastvarača na bazi mentola. Kvantitativno određivanje kationa u ovim slučajevima se uglavnom vrši nekom od spektrometrijskih metoda, a najčešće je to atomska apsorpciona spektroskopija (AAS). Konačni rezultat je kompariran sa rezultatima klasične ekstrakcije Pb(II) iona hidrofobnim organskim rastvaračima. S obzirom na aktuelnost ovakvih istraživanja i još uvijek nedovoljno istraženo područje primjene hidrofobnih eutektičkih rastvarača kao alternativnih ekstraktanata za ione teških metala kao polutante, napravljen je koncept ovog istraživanja. Dobijeni rezultati će dati značajan doprinos proširivanju znanja iz područja primjene hidrofobnih eutektičkih rastvarača u tečno-tečnoj ekstrakciji.

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The relationship between the physical and lipid characteristics of eggs from hens that were fed a diet supplemented with fat

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Abstract: The aim of this study was to investigate the potential correlations between certain physical and lipid characteristics of eggs from hens fed diets supplemented with three different types of fat. A six-week-last experiment was conducted on 45 Brown Lohman laying hens, 56 weeks of age. Animals were randomly divided into three groups of 15 and fed one of three experimental diets supplemented with either 3% of fish oil, palm olein, and lard. Samples of 10 eggs per group were collected at the start and at the end of the experiment to determine four physical and six lipid characteristics in boiled eggs. The supplementation of the hens' diet with fat at significantly affected the physical characteristics of the The biggest impact on such finding for total sample of investigated eggs had palm olein as a supplement in hen diet. Among the three investigated fat supplements, the addition of 3% lard to the laying hen diet resulted in the smallest total number of significant correlations between investigated physical and lipid traits. Comparing the end to the start of our experiment, supplementation of hen diet with fat decreases the number of correlations among egg physical and lipid characteristics, what can be considered as a positive result from both egg producer and consumer interests.

INTRODUCTION

Eggs are often considered a nutritional marvel due to their rich composition, encompassing around 40 proteins, among which are antihypertensive and bactericidal proteins. Additionally, eggs boast 18 different amino acids, including nine essential amino acids, stable amino acid composition, an optimal balance of saturated and unsaturated fatty acids, and the absence of carbohydrates or trans fats. As a result, eggs have gained recognition as a benchmark protein source for humans (Damaziak, Riedel, Gozdowski, et al., 2017), paralleling the biological value of breast milk (Molnar and Szollosi, 2020).

The global production of table eggs has surged by 24.4% in the past decade, reaching 76.7 million tons in 2018, with expectations of further growth owing to the escalating demand for animal-originated protein (Dilawar, Mun, Rathnayake, et al., 2021). Egg quality parameters play a pivotal role in the egg industry, influencing egg classification, pricing, consumer preferences, hatchability, chick weight, and overall quality (Kumar, Dahiya, Ratwan, et al., 2022). Egg quality in poultry is influenced by a variety of factors such as breed (genotype), age, health status, type of production system, number of birds per space (stocking density), diet, conditions of storage, and how long the eggs are stored.Previous studies have established connections between external and internal egg quality characteristics, with these relationships being influenced by factors such as the type of rearing system (Yang HM, Yang Z, Wang W, et al., 2014), the age and genotype of the hen, and nutritional considerations (Ahmadi and Rahimi, 2011). Furthermore, the environmental conditions of poultry facilities indirectly impact egg weight (Freitas, Tinoco, Baeta, et al., 2017). Lipid components in eggs are primarily concentrated in the egg yolk, with particular attention paid to the cholesterol content. Cholesterol, a vital component present in every cell of living organisms, plays a crucial role in cellular functions. The cholesterol level in eggs exhibits

variability based on factors such as the species, breed, lines, and age of the animal (Yenilmez and Atay, 2023). Notably, consumer preferences can be influenced by the cholesterol content in egg yolks, leading to misconceptions and a potential reduction in egg consumption. This reduction poses a significant loss in terms of protein intake in human nutrition. Nutrition also plays a role in cholesterol deposition in egg yolks. Commercial layer diets often incorporate specific feedstuffs, such as vegetable oils rich in unsaturated fatty acids, to alter egg yolk lipid profile and decrease egg cholesterol content. Enhancing the nutritional quality of eggs from laying hens can be achieved by incorporating diverse fatty acid compositions into their diets. This approach holds promise as a valuable functional feed ingredient in poultry nutrition, aiming to produce eggs with enhanced functional food properties (Vlaicu PA, Panaite TD, Turcu RP, 2021). Balanced oil diets from various sources have demonstrated significant potential in enhancing the performance of laying hens and the quality of eggs (Gao, Zhang, Li et al., 2021), especially from the consumer's health perspective. Fatty acid composition and various microconstituents of added fats are the main factors affecting egg production and quality traits. Results from the literature on the potential impact of diet on egg cholesterol levels are inconsistent (Faitarone, Garcia, Roça, et al., 2013). Numerous researchers have reported that the inclusion of polyunsaturated fatty acid (PUFA)-rich oils in the diet leads to a reduction in egg cholesterol concentrations. However, other studies suggest that yolk cholesterol content remains constant, independent of dietary factors (Bertechini, 2003).

Taking into account all mentioned above, this study aimed to investigate possible correlations of some egg physical and lipid characteristics from hens fed a diet supplemented with three types of fat – fish oil, palm olein, and lard, which has significance to both egg producers and egg consumers. The investigated fats differ in their fatty acid composition and their utilization as a diet supplement in conventional egg production.

Table 1. Correlations of egg physical and lipid characeristics from hens fed diets supplemented with fish oil (n=10), palm olein (n=10) or lard (n=10) at the start and at the end of experimental feeding (total n=30)

Start End	**Egg weight (g)**	**Egg yolk weight (g)**	**Egg white weight (g)**	**Egg shell weight (g)**	**Egg total lipids (g/egg)**	**Egg TG (g/egg)**	**Egg TC (mg/egg)**	**Egg yolk total lipids (mg/g)**	**Egg yolk TG (mg/g)**	**Egg yolk TC (mg/g)**
Egg weight (g)	-	**r=0,666 P=0,000**	**r=0,971 P=0,000**	**r=0,646 P=0,000**	**r=0,624 P=0,000**	r=0,335 P=0,070	**r=0,437 P=0,016**	r=0,289 P=0,122	r=0,184 P=0,331	r=0,061 P=0,747
Egg yolk weight (g)	**r=0,486 P=0,007**	-	**r=0,506 P=0,004**	r=0,330 P=0,075	**r=0,802 P=0,000**	r=0,288 P=0,123	**r=0,401 P=0,028**	r=0,219 P=0,246	r=0,037 P=0,845	r= - 0,168 P=0,375
Egg white weight (g)	**r=0,938 P=0,000**	r=0,185 P=0,327	-	**r=0,550 P=0,002**	**r=0,533 P=0,002**	r=0,292 P=0,117	**r=0,400 P=0,029**	r=0,316 P=0,089	r=0,183 P=0,332	r=0,118 P=0,535
Egg shell weight (g)	**r=0,718 P=0,000**	r=0,312 P=0,094	**r=0,595 P=0,001**	-	r=0,220 P=0,243	r=0,288 P=0,123	r=0,289 P=0,121	r= -0,004 P=0,984	r=0,217 P=0,250	r=0,100 P=0,599
Egg total lipids (g/egg)	**r=0,404 P=0,027**	**r=0,887 P=0,000**	r=0,152 P=0,422	r=0,187 P=0,321	-	r=0,240 P=0,202	**r=0,403 P=0,027**	**r=0,757 P=0,000**	r=0,039 P=0,838	r= - 0,049 P=0,797
Egg TG (g/egg)	r=0,147 P=0,439	**r=0,398 P=0,029**	r=0,037 P=0,846	r= - 0,065 P=0,733	r=0,247 P=0,188	-	r= - 0,294 P=0,115	r=0,076 P=0,691	**r=0,966 P=0,000**	**r= - 0,480 P=0,007**
Egg TC (mg/egg)	r=0,293 P=0,116	r=0,353 P=0,056	r=0,186 P=0,326	r=0,297 P=0,111	r=0,352 P=0,056	r= -0,048 P=0,800	-	r=0,227 P=0,228	**r= - 0,394 P=0,031**	**r=0,834 P=0,000**
Egg yolk total lipids (mg/g)	r= -0,028 P=0,882	r=0,073 P=0,703	r= - 0,015 P=0,938	r= - 0,184 P=0,331	**r=0,524 P=0,003**	r= -0,163 P=0,338	r=0,119 P=0,532	-	r=0,022 P=0,906	r=0,110 P=0,561
Egg yolk TG (mg/g)	r= -0,068 P=0,723	r=0,027 P=0,888	r= - 0,070 P=0,712	r= - 0,214 P=0,257	r= - 0,075 P=0,694	**r=0,925 P=0,000**	r= 0,199 P=0,291	r= - 0,171 P=0,367	-	**r= - 0,437 P=0,016**
Egg yolk TC (mg/g)	r= -0,002 P=0,993	r= - 0,292 P=0,117	r=0,086 P=0,651	r=0,110 P=0,564	r= - 0,215 P=0,254	r= -0,301 P=0,107	**r=0,789 P=0,000**	r=0,085 P=0,657	r= - 0,212 P=0,262	-

r - the Pearson correlation coefficient
P<0,05 is considered statistically significant

EXPERIMENTAL

A six-week lasting experiment was conducted on 45 Brown Lohman laying hens, 56 weeks of age, and in the 34th week of production. Animals were randomly assigned to three groups of 15 birds each and fed one of three experimental diets supplemented with either 3% fish oil, palm olein, and lard. Samples of 10 eggs per group were collected at the start and at the end of the experiment to determine the following boiled egg physical characteristics: (egg weight (g), egg yolk weight (g), egg white weight (g), eggshell weight (g)), and lipid characteristics (egg total lipids (g/egg), egg triglycerides/egg TG (g/egg), egg total cholesterol/egg TC (mg/egg), egg yolk total lipids (mg/g), egg yolk triglycerides/egg/yolk TG (mg/g), egg yolk total cholesterol/egg yolk TC (mg/g)). Experimental design, the nutritional, chemical, and fatty-acid composition of experimental diets, as well as methods of preparation of egg and hard-boiled yolk and determination of egg physical and lipid characteristics, were in detail described and published in our earlier paper (Hodzic, Hamamdzic, Gagic, et al., 2008). Egg physical characteristics were determined by weighing. The preparation of eggs and yolk for laboratory processing was done according to Berrio and Hebert (1990), and the fat of the boiled egg yolk was extracted according to Folch, Lees, and Stanley (1957). The egg yolk total lipids were determined gravimetrically. Egg yolk TG was determined by the GPO-PAP method, using an enzymatic colorimetric assay with a lipid scavenging factor ("Human", Wiesbaden, Germany). Egg yolk TC was determined spectrophotometrically by the Liebermann-Burchard method with commercial tests ("Semikem", Sarajevo, B&H). Egg total lipids, egg TG, and egg TC were calculated from the respective yolk concentrations and weights.

To examine the relationship between the analyzed parameters, Pearson correlation coefficients (r) were calculated using Minitab statistical software (Minitab, Inc. 2014.). Results with P values less than 0,05 were considered statistically significant.

RESULTS AND DISCUSSION

The results are presented in Tables 1-4, and statistically significant results (P<0,05) are marked in bold.

Table 2. Correlations of egg physical and lipid characteristics from hens fed diet supplemented with fish oil at the start and at the end of experimental feeding (n=10)

Start End	Egg weight (g)	Egg yolk weight (g)	Egg white weight (g)	Egg shell weight (g)	Egg total lipids (g/egg)	Egg TG (g/egg)	Egg TC (mg/egg)	Egg yolk total lipids (mg/g)	Egg yolk TG (mg/g)	Egg yolk TC (mg/g)
Egg weight (g)	-	r=0,489 P=0,151	**r=0,975** **P=0,000**	r=0,611 P=0,060	r=0,539 P=0,108	r=0,367 P=0,297	r=0,413 P=0,236	r=0,395 P=0,259	r=0,218 P=0,544	r= - 0,214 P=0,554
Egg yolk weight (g)	r=0,477 P=0,163	-	r=0,299 P=0,401	r= - 0,002 P=0,995	**r=0,841** **P=0,002**	r=0,576 P=0,081	r=0,331 P=0,350	r=0,374 P=0,286	r=0,207 P=0,566	**r= - 0,745** **P=0,013**
Egg white weight (g)	**r=0,951** **P=0,000**	r=0,260 P=0,469	-	r=0,608 P=0,062	r=0,383 P=0,274	r=0,297 P=0,404	r=0,417 P=0,230	r=0,335 P=0,344	r=0,207 P=0,566	r= - 0,023 P=0,949
Egg shell weight (g)	r=0,574 P=0,083	r= - 0,118 P=0,746	r=0,537 P=0,110	-	r=0,210 P=0,560	r= -0,071 P=0,846	r= .0,136 P=0,707	r=0,333 P00,347	r= - 0,113 P=0,755	r= - 0,148 P=0,684
Egg total lipids (g/egg)	r=0,404 P=0,247	**r=0,830** **P=0,003**	r=0,315 P=0,376	r= - 0,361 P=0,306	-	**r=0,704** **P=0,023**	r=0,345 P=0,329	**r=0,815** **P=0,004**	r=0,438 P=0,205	r= - 0,587 P=0,075
Egg TG (g/egg)	r=0,395 P=0,259	r=0,542 P=0,105	r=0,418 P=0,229	r= - 0,248 P=0,490	r=0,631 P=0,050	-	r=0,295 P=0,408	r=0,617 P=0,058	**r=0,916** **P=0,000**	r= - 0,361 P=0,306
Egg TC (mg/egg)	**r=0,779** **P=0,008**	r=0,429 P=0,217	**r=0,799** **P=0,006**	r=0,404 P=0,247	r=0,584 P=0,076	r=0,443 P=0,200	-	r=0,263 P=0,462	r=0,218 P=0,544	r=0,378 P=0,281
Egg yolk total lipids (mg/g)	r=0,124 P=0,732	r=0,272 P=0,447	r=0,231 P=0,521	r= - 0,508 P=0,134	**r=0,760** **P=0,011**	r=0,493 P=0,148	r=0,497 P=0,144	-	r=0,564 P=0,089	r= - 0,191 P=0,597
Egg yolk TG (mg/g)	r=0,165 P=0,650	r=0,069 P=0,850	r=0,324 P=0,361	r= - 0,283 P=0,428	r=0,298 P=0,403	**r=0,873** **P=0,001**	r=0,270 P=0,451	r=0,476 P=0,165	-	r= - 0,051 P=0,888
Egg yolk TC (mg/g)	r=0,376 P=0,285	r= - 0,425 P=0,221	r=0,584 P=0,076	r=0,497 P=0,144	r= - 0,123 P=0,735	r= - 0,023 P=0,950	**r=0,635** **P=0,048**	r=0,268 P=0,454	r=0,210 P=0,561	-

r - the Pearson correlation coefficient
P<0,05 is considered statistically significant

Considering a total number of samples (n=30), and when laying hens were fed a diet without added fat – at the start of the experiment, 18 statistically significant correlations among investigated parameters were found (Table 1). At the end of the experiment, and after six weeks of feeding with dietary fat, the number of statistically significant correlations was 10 (Table 1). The number of statistically significant correlations among investigated egg parameters was higher at the end compared to the start of the experiment (7 vs 6) in the group of hens fed a diet supplemented with fish oil only (Table 2). A six-week lasting hen feeding with a diet supplemented with 3% palm olein resulted in the loss of statistically significant correlations of egg TC with other investigated physical and lipid parameters (Table 3). On the other hand, the addition of lard in hen diet had the biggest impact on egg total lipids (Table 4).Hen diet supplementation with investigated types of fats resulted in a few negative correlations among egg physical and lipid components, but none statistically significant. In general, a hen diet supplemented with fat significantly affected relationships among egg physical characteristics in our experimental design (Table 1). The most impact on such findings for a total sample of investigated eggs (Table 1) had palm olein as a supplement in hen diet (Table 3). In their study on 85-week-old laying hens, Inca, Martinez and Vilchez, (2020) identified statistically significant phenotypic correlations among external egg quality characteristics, including egg, yolk, albumen, and shell weight. Similarly, Mitrovic, Pandurevic, Milic, et al. (2010) observed statistically significant phenotypic correlation coefficients between egg weight and the weight of individual egg components. Notably, the study by Ketta and Tumova (2018) found that egg weight exhibited a significant increase as eggs became thicker. This suggests a potential relationship between increased egg weight and higher eggshell weight, which also demonstrated an increase in the eggshell thickness category. Comparing the end to the start of our experiment, it seems that supplementation of the hen diet with fat, regardless of the type of added fat, decreases the number of correlations among egg physical and lipid characteristics (Tables 1, 2, 3, and 4), and the smallest number of significant correlations among investigated egg parameters is found in diet supplemented with lard (Table 4).

Table 3. Correlations of egg physical and lipid characteristics from hens fed diet supplemented with palm olein at the start and at the end of experimental feeding (n=10)

Start **End**	**Egg weight (g)**	**Egg yolk weight (g)**	**Egg white weight (g)**	**Egg shell weight (g)**	**Egg total lipids (g/egg)**	**Egg TG (g/egg)**	**Egg TC (mg/egg)**	**Egg yolk total lipids (mg/g)**	**Egg yolk TG (mg/g)**	**Egg yolk TC (mg/g)**
Egg weight (g)	-	r=0,483 P=0,158	**r=0,979** **P=0,000**	r=0,503 P=0,138	r=0,460 P=0,181	r= -0,075 P=0,836	**r=0,721** **P=0,019**	r=0,192 P=0,594	r= - 0,252 P=0,482	r=0,559 P=0,093
Egg yolk weight (g)	**r=0,657** **P=0,039**	-	r=0,403 P=0,248	r= - 0,152 P=0,675	**r=0,746** **P=0,013**	r=0,468 P=0,173	r=0,223 P=0,536	r=0,105 P=0,772	r=0,167 P=0,646	r= - 0,123 P=0,735
Egg white weight (g)	**r=0,966** **P=0,000**	r=0,440 P=0,193	-	r=0,392 P=0,262	r=0,409 P=0,240	r= -0,184 P=0,610	**r=0,672** **P=0,033**	r=0,198 P=0,583	r= - 0,349 P=0,324	r=0,537 P=0,110
Egg shell weight (g)	**r=0,854** **P=0,002**	r=0,418 P=0,229	**r=0,840** **P=0,002**	-	r= - 0,082 P=0,823	r= -0,073 P=0,841	**r=0,648** **P=0,043**	r=0,022 P=0,952	r= - 0,015 P=0,967	**r=0,706** **P=0,022**
Egg total lipids (g/egg)	r=0,490 P=0,150	**r=0,944** **P=0,000**	r=0,262 P=0,465	r=0,310 P=0,383	-	r=0,243 P=0,499	r=0,187 P=0,604	**r=0,740** **P=0,014**	r=0,012 P=0,975	r= - 0,069 P=0,850
Egg TG (g/egg)	r=0,420 P=0,226	r=0,526 P=0,118	r=0,323 P=0,363	r=0,136 P=0,708	r=0,337 P=0,341	-	r= 0,276 P=0,440	r= - 0,109 P=0,765	**r=0,949** **P=0,000**	r= - 0,446 P=0,197
Egg TC (mg/egg)	r=0,124 P=0,733	r=0,282 P=0,428	r=0,035 P=0,923	r=0,136 P=0,708	r=0,193 P=0,594	r=0,560 P=0,092	-	r=0,056 P=0,879	r= - 0,382 P=0,275	**r=0,940** **P=0,000**
Egg yolk total lipids (mg/g)	r= -0,498 P=0,143	r= - 0,167 P=0,646	r= - 0,562 P=0,091	r= - 0,313 P=0,379	r=0,167 P=0,645	r= -0,509 P=0,133	r= 0,255 P=0,477	-	r= - 0,150 P=0,679	r=0,022 P=0,951
Egg yolk TG (mg/g)	r=0,140 P=0,700	r=0,126 P=0,728	r=0,119 P=0,744	r= - 0,063 P=0,864	r= - 0,054 P=0,883	**r=0,908** **P=0,000**	r=0,523 P=0,121	r= - 0,469 P=0,172	-	r= - 0,449 P=0,193
Egg yolk TC (mg/g)	r= -0,357 P=0,312	r= - 0,435 P=0,209	r= - 0,299 P=0,401	r= - 0,171 P=0,637	r= - 0,479 P=0,161	r=0,173 P=0,633	**r=0,737** **P=0,015**	r= - 0,112 P=0,759	r=0,425 P=0,221	-

r - the Pearson correlation coefficient
P<0,05 is considered statistically significant

Actually, at the start of the experiment, there were several significant correlations among different egg and egg yolk physical and lipid characteristics, while at the end of the experiment, the only consistent and significantly positive correlation among egg physical and lipid characteristics was this one between egg total lipids and egg yolk weight found in all tested groups as well as in the total sample of eggs. In the study by Inca et al. (2020), it was observed that phenotypic correlations between egg quality characteristics in older laying hens remained unaffected by yolk quality characteristics. Similarly, Shafey (1996) found that changes in the proportion of egg components associated with egg size did not have an impact on yolk lipid and fatty acid concentrations. Interestingly, within a specific age group, no significant differences were noted in yolk lipid and fatty acid concentrations. Shafey (1996) also reported that egg yolk lipid produced by 1-year-old hens exhibited 2.5% lower lipid and 4.3% higher linoleic acid (LA) concentrations compared to those produced by 6-month-old hens. The reduction in yolk total lipid and the increase in the unsaturated fatty acid, LA, along with a decrease in saturated fatty acids in eggs from older hens, may be of interest to consumers. However, Fennema (1993) proposes that variations in total yolk lipid content are more influenced by bird genetic strain than diet.Significant positive correlations between the same egg and egg yolk lipid components are expected, taking into account that egg lipid components are mainly contained in egg yolk. The only one such significant correlation was lacking – between egg and egg yolk total lipid, and when palm olein (Table 3) and lard (Table 4) were added to hen diets. Considering egg/egg yolk cholesterol as the most interesting lipid parameter, the only significant correlation we found between egg TC and egg weight was when hens were fed a diet supplemented with fish oil rich in PUFAs (Table 2). Excessive energy intake, surpassing maintenance and production requirements, leads to increased body weight and cholesterol synthesis. Consequently, an excess of cholesterol may be transferred to the egg yolk (Faitarone et al., 2013). In Cornell random-bred Leghorn (LC) and Athens-Canadian random-bred (AC) hen populations, correlations between yolk cholesterol concentration and other egg traits, such as proportions of the egg, percentage yolk dry matter, and percentage yolk fat, were found to be low (Washburn and Marks, 1985), consistent with our study.

Table 4. Correlations of physical and lipid characteristics from hens fed diet supplemented with lard at the start and at the end of experimental feeding (n=10)

Start End	**Egg weight (g)**	**Egg yolk weight (g)**	**Egg white weight (g)**	**Egg shell weight (g)**	**Egg total lipids (g/egg)**	**Egg TG (g/egg)**	**Egg TC (mg/egg)**	**Egg yolk total lipids (mg/g)**	**Egg yolk TG (mg/g)**	**Egg yolk TC (mg/g)**
Egg weight (g)	-	**r=0,901 P=0,000**	**r=0,978 P=0,000**	**r=0,846 P=0,002**	**r=0,885 P=0,001**	r=0,606 P=0,063	r=0,558 P=0,093	r=0,153 P=0,673	r=0,392 P=0,263	r= - 0,137 P=0,706
Egg yolk weight (g)	r=0,036 P=0,922	-	**r=0,806 P=0,005**	**r=0,844 P=0,002**	**r=0,864 P=0,001**	r=0,377 P=0,283	**r=0,699 P=0,024**	r= - 0,065 P=0,858	r=0,107 P=0,768	r= - 0,037 P=0,919
Egg white weight (g)	**r=0,891 P=0,001**	r= - 0,408 P=0,241	-	**r=0,742 P=0,014**	**r=0,855 P=0,002**	r=0,620 P=0,056	r=0,455 P=0,186	r=0,262 P=0,465	r=0,440 P=0,203	r= - 0,181 P=0,617
Egg shell weight (g)	**r=0,680 P=0,031**	r=0,393 P=0,261	r=0,372 P=0,289	-	**r=0,687 P=0,028**	r=0,611 P=0,061	r=0,605 P=0,064	r= - 0,133 P=0,714	r=0,403 P=0,248	r= - 0,034 P=0,926
Egg total lipids (g/egg)	r=0,103 P=0,777	**r=0,906 P=0,000**	r= - 0,302 P=0,397	r=0,397 P=0,257	-	r=0,446 P=0,197	r=0,382 P=0,277	r=0,445 P=0,197	r=0,213 P=0,556	r= - 0,339 P=0,338
Egg TG (g/egg)	r= -0,113 P=0,756	r=0,504 P=0,137	r= - 0,345 P=0,329	r=0,159 P=0,660	r=0,382 P=0,277	-	r=0,185 P=0,609	r=0,185 P=0,608	**r=0,960 P=0,000**	r= - 0,112 P=0,758
Egg TC (mg/egg)	r= -0,095 P=0,794	r=0,409 P=0,240	r= - 0,256 P=0,475	r=0,037 P=0,919	r=0,124 P=0,732	r= -0,089 P=0,806	-	r= - 0,476 P=0,164	r= - 0,002 P=0,996	**r=0,686 P=0,028**
Egg yolk total lipids (mg/g)	r=0,135 P=0,711	r= - 0,034 P=0,926	r=0,146 P=0,687	r=0,064 P=0,861	r=0,393 P=0,261	r= -0,182 P=0,615	r= 0,593 P=0,071	-	r=0,201 P=0,578	r= - 0,595 P=0,069
Egg yolk TG (mg/g)	r= - ,132 P=0,717	r=0,081 P=0,825	r= - 0,177 P=0,625	r= - 0,012 P=0,973	r= - 0,006 P=0,987	**r=0,901 P=0,000**	r= 0,314 P=0,377	r= - 0,175 P=0,629	-	r= - 0,098 P=0,787
Egg yolk TC (mg/g)	r= -0,097 P=0,790	r= - 0,418 P=0,229	r=0,104 P=0,775	r= - 0,261 P=0,466	r= - 0,630 P=0,051	r= -0,510 P=0,132	**r=0,657 P=0,039**	r= - 0,578 P=0,080	r= - 0,385 P=0,272	-

r - the Pearson correlation coefficient
P<0,05 is considered statistically significant

Despite supplementing layer diets with vegetable oils rich in PUFAs, such as rapeseed, canola and soybean oils, at various levels in commercial diets, there were no significant changes observed in the nutritional composition of egg yolks. Additionally, the inclusion of these PUFAs did not result in reduction in yolk cholesterol content (Faitarone et al., 2013). According to Bertechini (2003), chickens can produce 10 times more cholesterol per kilogram of liver compared to humans. Consequently, manipulating layer diets to decrease egg cholesterol levels is not highly effective, as chickens can maintain the egg cholesterol levels that are considered essential for egg composition. Yolk cholesterol concentration exhibits resistance to change due to the required level of yolk cholesterol level essential for embryo development (Shafey and Cham, 1994). However, hens can adjust yolk PUFAs content in response to dietary lipid sources.

This capability stems from the fact that, unlike mammals, poultry absorb dietary fat through the portal system as portomicrons, directly entering the bloodstream and being transported to the liver – the primary site of lipogenesis - allowing for direct fat absorption by the liver (Hodzic, Hamamdzic, Gagic, et al, 2012).

CONCLUSION

Among the three investigated fat supplements, the addition of 3% lard to the laying hen diet resulted in the smallest total number of significant correlations between investigated physical and lipid traits. Moreover, lard supplementation also resulted in the biggest decrease in statistically significant correlations at the end of the experiment. In general, comparing the end to the start of our experiment, supplementation of hen diet with fat decreases the number of correlations among egg physical and lipid characteristics, which can be considered as a positive result for both egg producer and consumer interests.

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Summary/Sažetak

Cilj ovog istraživanja je bio istražiti moguće korelacije pojedinih fizičkih i lipidnih karakteristika jaja kokoši hranjenih sa tri vrste masti – ribljim uljem, palminim oleinom i svinjskom mašću, što ima značaja kako za proizvođače tako i za potrošače jaja. Eksperiment u trajanju od šest sedmica je proveden na 45 kokoši nosilja Brown Lohman, starosti 56 sedmica, odnosno u 34. sedmici proizvodnje. Životinje su nasumično raspoređene u tri grupe od po 15 ptica i hranjene jednom od tri eksperimentalne smješe sa dodatkom 3% ribljeg ulja, palminog oleina ili svinjske masti. Uzorci od po 10 jaja po grupi prikupljani su na početku i na kraju eksperimenta kako bi se odredile fizičke karakteristike jaja (težina jaja, težina žumanjka, težina bjelanjka, težina ljuske jajeta) i lipidne komponente (ukupni lipidi jajeta, trigliceridi jajeta, ukupni holesterol jajeta, ukupni lipidi žumanjka, trigliceridi žumanjka, ukupni holesterol žumanjka).

Ishrana kokoši s dodatkom masti značajno je utjecala na fizičke karakteristike jaja. Najveći utjecaj na takav nalaz ukupnog uzorka ispitivanih jaja imao je palmin olein kao dodatak u hrani kokoši. Uspoređujući kraj i početak našeg eksperimenta, čini se da dodavanje masti u hranu kokoši, bez obzira na vrstu dodane masti, smanjuje broj korelacija između fizičkih i lipidnih karakteristika jaja, što se može smatrati pozitivnim nalazom i za proizvođače i za potrošače jaja.

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