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VPLIV 6,7-DIHIDROKSI SKUPINE PRI FLAVONIH NA *IN VITRO* SPOSOBNOST KELIRANJA ŽELEZA

THE EFFECT OF 6,7-DIHYDROXYGROUP IN FLAVONES ON *IN VITRO* IRON CHELATING ACTIVITY

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Statement

I declare that I have carried out my diploma work independently under the mentorship of Assist. Prof. Mojca Kerec Kos, M. Pharm., Ph. D. and the co-mentorship of Assoc. Prof. Přemysl Mladěnka, Pharm. D., Ph.D.

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TABLE OF CONTENTS

List of tables and figures

POVZETEK

Železo je v človeškem organizmu nepogrešljivo za normalen potek širokega spektra različnih celičnih procesov. V vodnem mediju se železo pojavlja v dveh oblikah: kot Fe(II) in kot Fe(III). Kemizem, visoka afiniteta do kisika in sposobnost, da privzame vlogo tako akceptorja kot donorja elektronov, so lastnosti, ki železu zagotavljajo pomembno vlogo v mnogih fizioloških in metabolnih poteh - v organizmu je prisotno predvsem v številnih kofaktorjih, ki vsebujejo železo in so vključeni v biološke procese, kot so celična proliferacija, transport kisika ter produkcija energije.

Čeprav je koncentracija železa v organizmu strogo uravnavana z različnimi specifičnimi proteini, ki so odgovorni za ustrezno regulacijo transporta in shranjevanja železa, lahko različni patofiziološki procesi vodijo do prekomernega nalaganja v organizmu in toksičnosti. »Prosto« železo je oblika železa, ki ni vezana na noben plazemski protein in je pri normalnih fizioloških pogojih v zanemarljivo majhni koncentraciji. Povišana koncentracija prostega železa se lahko pojavi v določenih patoloških pogojih, predvsem pri ishemiji. Ker je v prisotnosti molekularnega kisika železo sposobno prehajati med dvema oksidacijskima stanjema, je soodgovorno tudi za neželen nastanek radikalov. »Prosto« železo lahko namreč v organizmu s pomočjo Fenton/Haber-Weissove reakcije katalizira nastanek visoko toksičnih hidroksilnih radikalov, zaradi česar presežek železa pomembno prispeva k nastanku oksidativnega stresa. Preko oksidativnega stresa je železo posledično soodgovorno tudi za neželene celične procese, ki lahko povzročijo poškodbo molekule DNA in celično smrt. Omenjeni procesi lahko vodijo do pojava nevrodegenerativnih bolezni, kot sta Parksinsonova in Alzheimerjeva bolezen ter do nastanka kardiovaskularnih bolezni.

Flavonoidi so ubikvitarni sekundarni rastlinski metaboliti, ki jih najdemo v skoraj vseh delih rastlin. Osnovni skelet je sestavljen iz flavanskega jedra, ki ga tvorita dva benzenska obroča, povezana z piranskim obročem, ki vsebuje kisik. Flavonoidi se razlikujejo v stopnji alkiliranja in/ali glikozilacije, številu in razporeditvi hidroksilnih skupin ter konjugaciji med A in B obročem. Flavonoidi so znani po svojem antioksidantivnem delovanju, ki lahko poteka na različne načine - predvsem s pomočjo lovljenja prostih radikalov ali s pomočjo keliranja kovin. Posledično je različna tudi zveza med strukturo in

aktivnostjo , kar je pomemben aspekt pri določanju funkcionalnih skupin, ki so odgovorne za aktivnost testirane spojine.

V diplomski nalogi smo ugotavljali vpliv 6,7-dihidroksi skupine pri flavonoidih na sposobnost keliranja železa pri različnih (pato)fiziološko relevantnih pH vrednostih. Kot bioaktivni spojini smo uporabili baikalein in baikalin, ki imata osnovni skelet flavonoidov in ju najdemo v tradicionalni kitajski rastlini *Scullateria baicalensis*. Azijske kulture jo že dalj časa uporabljajo za zdravljenje simptomov, kot so vročina, prekomerno potenje in vnetje, medtem ko so se zahodne kulture za njene potencialno blagodejne učinke začele zanimati šele pred nedavnim. Najbolj kisel pH medij smo uporabili za prikaz pogojev ki so prisotni v organizmu v gastro-duodenalnem traktu, ter pri tumorjih in v lizosomih, za katere je bil dokazan pomemben vpliv pri metabolizmu železa. pH vrednosti medija 5,5 in 6,8 smo uporabili za prikaz patofizioloških pogojev, ki so prisotni pri ishemiji, in kjer je vloga železa pri poškodbi tkiva dobro dokazana.

Za določitev nastanka kompleksov med testirano spojino in železom smo uporabili UV-Vis spektroskopijo, dobljene absorpcijske spektre pa smo ustrezno ovrednotili z matematično analizo. Za samo določitev nastanka kompleksa smo pri absorpcijskih spektrih čiste spojine z dodatkom železa v presežku opazovali spremembe spektra. Pri določanju stehiometrijskega razmerja med spojino in železom, smo uporabili komplementarni pristop, pri katerem smo med posamezno meritvijo spreminjali koncentracijo testirane spojine, koncentracijo železa pa ohranjali konstantno. Matematično analizo podatkov smo izvedli po 6 različnih metodah, tako da je končna ocena določitve stehiometrijskega razmerja med substanco in železom povzetek in najboljši približek rezultatov vseh uporabljenih matematičnih metod.

Obe testirani spojini sta tvorili komplekse z obema oksidacijskima stanjema železa pri vseh testiranih pH vrednostih. Edina izjema je bil baikalin z Fe(II) pri pH vrednosti 4,5, kjer do nastanka kompleksa ni prišlo. Rezultati študije so pokazali, da je pri vseh pH vrednostih najpogostejše stehiometrijsko razmerje med spojino in železom 1:1, kar nakazuje, da sta testirani spojini dobra kelatorja železa. V nekaterih primerih je bila določitev stehiometrijskega razmerja zaradi nejasnosti spektrov in/ali izjemno majhne količine nastalega kompleksa večkrat otežena oziroma v redkih primerih povsem onemogočena.

V naši študiji smo torej ugotovili, da je baikalein dober kelator železa pri različnih pH vrednostih medija. Baikalin, ki je glikozid baikaleina in ima na mestu 7' pripeto sladkorno komponento, ima v primerjavi z baikaleinom nižjo afiniteto do železa v obeh oksidacijskih stanjih. Prav tako le pri baikalinu pri pH vrednosti medija 4,5 ni prišlo do tvorbe kompleksa. To je v skladu s teorijo, da imajo glikozilirane oblike flavonoidov značilno nižjo sposobnost keliranja z železom. Z izvedeno eksperimentalno primerjavo med testiranima spojinama smo potrdili pomembnost 6,7-dihidroksilacije za učinkovitost keliranja železa pri flavonih.

ABSTRACT

In human organism, iron plays a crucial role in a wide spectrum of different cellular activities. In aqueous media, iron appears in two different forms: as Fe(II) and as Fe(III). Chemical properties of iron are vital for its participation in different physiological and metabolic pathways. Although the concentration of iron is kept under strict control in the body, certain (patho)physiological conditions might lead to excessive release of "free" iron, which is not bound to plasma proteins and its concentration is negligible at physiological conditions and can catalyse the formation of highly toxic hydroxyl radicals. Flavonoids are secondary plant metabolites known for their potent antioxidative effects. Their precise mechanism of action in humans is still not known.

Baicalein and baicalin that were used in our research work are substances of flavonoid skeleton and are found in the traditional Chinese herb *Scullateria baicalensis*. The aim of the present study was to determine the effect of the 6,7-dihydroxygroup in flavonoids on the ability of iron chelation at different (patho)physiological pH values. A numerous studies emphasised that in group of flavonoids, baicalein represents the most active iron chelator and that its activity can be compared with the activity of deferoxamine, chelating agent, which is used in treatment of diseases and pathological conditions linked with iron overload and toxicity.

In order to determine the complex formation rate, the UV-Vis spectroscopy was used, and the resulting absorption spectra were then subjected to precise mathematical data analysis. In laboratory measurements, a complementary approach was used, in which the concentration of the tested substance was kept constant during each measurement, while the concentration of iron was altering. Mathematical data analysis was performed with 6 different methods, and the final assessment of the determination of the stoichiometric substance-iron ratio, was the summary and the best approximation of all methods. Results showed that for both substances the most frequent stoichiometric substance-iron ratio at each tested pH is 1:1. This indicates that the tested substances are good iron chelators and could thus possess a therapeutic potential toward pathologies associated with iron dysbalance. In agreement with theoretical assumption, baicalin, a glycoside of baicalein with the sugar moiety attached at the site 7', had a reduced ability of iron chelation, thereby confirming the importance of 6,7-dihydroxygroups in flavones on iron chelation.

KEY WORDS

Flavonoids, iron, Fenton chemistry, antioxidant, stoichiometry, complementary approach

LIST OF ABBREVATIONS

- **ArOH** – flavonoid molecule
- **BAI –** baicalein
- **BAIN –** baicalin
- **COX** – cyclooxygenase
- **GPx** – glutathione peroxidise
- **GSH** – glutathione
- **HA** – hydroxylamine
- **LDL** – low density lipoprotein
- **LIP** – labile iron pool
- **LOX** – lipooxygenase
- **PhOH** – phenolic antioxidant
- **RNS** – reactive nitrogen species
- **RONS** – reactive oxygen and nitrogen species
- **ROS** – reactive oxygen species
- **SAR** – structure activity relationship
- **SOD** – superoxide oxidoreductase dismutase
- **Tf** – plasma transferrin
- **TR** – transferrin receptor
- **XO –** xanthine oxidase

1. INTRODUCTION

1.1 Iron and its pathophysiological role

Iron is one of the most abundant transition metals in human organism: it plays a central role in different cellular activities and biochemical processes that are essential for normal functioning of the body. The two main features that make iron so important are its high affinity for oxygen and its plain redox chemistry. Iron is characterized by incompletely filled d-orbitals, and can therefore take on different valences: its most common forms in aqueous solutions are Fe(II) and Fe(III). Because of the redox potential between these two oxidation states, oxidation processes involving iron are involved in a wide spectrum of different physiological and metabolic pathways (1, 2).

Another reason why iron is indispensable for the normal functioning of the body is its ability to act both as an electron acceptor and as donor. In human organism there are various iron-containing cofactors (e.g. iron-sulphur clusters and heme) that are involved in vital biological processes, such as cellular production, oxygen transport, and energy production. The major obstacle in the iron assimilation process is its bioavailability. In aqueous solutions, Fe(II) can be readily oxidized to ferric hydroxide, which is insoluble at neutral pH (2, 3).

The amount of iron in average adult human body is between 3 and 5 g: approximately 55 mg/per kg of body weight for males, and 44 mg/per kg for females. Iron is mostly stored in liver, spleen, and bone marrow (2). The haemoglobin of developing erythroid precursors and mature red blood cells contains more than two thirds of iron (3), and the daily requirements of iron for erythropoiesis amount to approximately 20 mg (4). The remaining iron is found in body mostly in a transit pool in reticuloendothelial macrophages or stored in hepatocytes within iron storage proteins, ferritin. 90% of dietary iron consists of inorganic (non-heme) iron, which is most commonly found in the intestinal lumen in the form of Fe(III) (3). For purposes of homeostasis, the body content of iron is kept in a relatively good balance by means of different functional compounds, such as transport chelates, storage complexes, ingestion, and excretion (2).

Specific proteins are responsible for the properly regulation of iron transport and storage. Plasma transferrin (Tf) is a monomeric glycoprotein with the atomic mass 80 kDa; Tf is known to be a powerful chelator with the ability to bind two Fe(III) ions with high affinity and is capable of binding iron tightly, but reversibly. Plasma membrane protein transferrin receptor (TR) is responsible for the iron uptake mechanism. Iron is transported into the plasma in the form of a diferric-transferrin complex. Approximately 30% of Tf is saturated with iron and the concentration of »free« iron, which in physiological conditions is not bound to plasma proteins, is virtually zero, since the amount of Tf greatly exceeds that of iron. Binding affinity is strongly correlated with pH of medium – at extracellular pH of 7.4 it is significantly higher, while in endosomes, which are known to be more acidic, it tends to decrease significantly. Under acidic conditions, the dissociation of Fe(III) ensues. Iron chelation by Tf serves 3 principal purposes: (i) it facilitates regulated iron transport and cellular uptake, (ii) it maintains Fe(III) in a redox-inert state, preventing the formation of toxic free radicals, and (iii) it maintains Fe(III) in a soluble form under physiological conditions (2, 3, 5).

In order to maintain adequate levels and prevent excessive concentrations of the micronutrient, the amount of intracellular iron is meticulously regulated. Excessive iron is stored in the cytoplasm bound to ferritin and in lysosomes bound to haemosiderin (the latter especially in cases of significant iron loading). Both iron storage proteins keep the metal within the cell in a bioavailable, soluble and non-toxic form. Ferritin represents the major intracellular iron storage protein, and because of its ability to store up to 4500 atoms of iron, it has a major role in preventing iron toxicity. Ferritin is found in the neutral cytosol environment, with pH around 7, while haemosiderin is placed in lysosomal or siderosomal compartments, where the environment is considerably more acidic. Presuming that chelators have access to both cellular compartments (cytosol and lysosomes), it seems safe to conclude that, intracellularly, the rate of chelation from both storage proteins is probably quite similar. Another route of exportation of the excessive amounts of intracellular iron is through ferroportin, a transmembrane protein, known for its ability to transport iron from the inside to the outside of the cell (2, 3, 6, 7).

Yet even though it has been ascertained that iron is indispensable for the normal function of all living cells, it has also been shown that in excessive amounts it has deleterious

effects on the human organism. The meticulous regulation of iron absorption therefore plays an essential role in preventing systemic iron excess or deficiency. The main reasons for iron overload are excessive intestinal absorption of iron due to hereditary disorders and frequent parental blood transfusions (3).

The highly toxic hydroxyl radicals can be produced through the Fenton/Haber-Weiss reactions. The latter are catalysed by the trace amounts of "free" iron (8), which is why iron excess is believed to contribute significantly to oxidative stress. "Loosely-bound" iron has the capability of redox-cycling between oxidation states and may be the main catalyst for the production of free radicals, when oxygen is present. Iron status in the cell is characterized by "free intracellular iron", the so-called "labile iron pool" (LIP). LIP is a pool of chelatable iron, which is transitory in nature and serves as a crossroads of cell iron metabolism (9). Some other authors define LIP as a low-molecular weight pool, where iron is weakly chelated and is able to pass through the cell rapidly. LIP seems to consist of both forms of iron ions (i.e. Fe(II) and Fe(III)) bound to different endogenous compounds, such as phosphate, citrate, nucleotides, carboxylates, etc. (1, 2, 7).

Iron is permanently required for protein synthesis which is assured with its constant flux from extracellular environment to the cytoplasm. This process of iron uptake may be the leading source of LIP. It was also stated that LIP is in the midway stage in absorption of iron and its sequestering with proteins. The availability and abundance of LIP seems to be the critical factor in different metabolic pathways dependent on the iron-containing compound. In order to avoid the damaging effects of excessive "free" iron, the LIP level is kept as low as possible. This is achieved by means of proteins that are involved in iron homeostasis, utilizing the mechanism of transcriptional and posttranscriptional control of the expression. However, it should be emphasized that LIP forms a negligible fraction of the overall cellular iron – between 3 and 5% (8).

It is also important to point out that the lysosomes can be particularly susceptible to oxidative damage: it has been observed that, within normal cells, these organelles contain LIP as a result of the digestion of iron-containing metalloproteins. In the presence of oxidants, lysosomal membranes tend to be destabilized, resulting in the release of hydrolytic enzymes into the cell cytoplasm. As a consequence, a cascade of physiological

events might follow, leading to cell death (apoptotic or necrotic). The main function of lysosome is autophagocytotic degradation, resulting in the breakdown of biological polymers, long-lived proteins included. Iron-containing metalloproteins are decomposed within the acidic environment of these organelles; the overall process is coupled with the release of redox-active iron, which may represent a major intracellular source of "free" iron. This "free" iron can be then used in the synthesis of new iron-containing proteins. It thus seems that the intralysosomal redox-active iron might pose a serious threat when cells are exposed to oxidative stress (10).

Redox-active metals have a very significant impact on the generation of free radicals. The cellular redox state is closely dependent on an iron redox-couple and is maintained in the strict physiological limits. The LIP levels are somewhere in the middle between the cellular iron requirements and the haphazard excessive generation of the hydroxyl radicals. For this reason, it has been suggested that LIP plays a significant role as a source of iron ions, which participate in the Fenton reaction. Strict iron regulation ensures that there is almost no "free" intracellular iron. However, *in vivo*, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. The released iron can enter into the **Fenton reaction** (equation *1*), producing highly reactive hydroxyl radicals:

$$\mathbf{Fe(II) + H_2O_2 \rightarrow Fe(III) + \bullet OH + OH^-} \qquad (1)$$

$$\mathbf{Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2} \qquad (2)$$

$$\mathbf{\bullet O_2 + H_2O_2 \xrightarrow{Fe} O_2 + \bullet OH + OH^-} \qquad (3)$$

In the next step of the reaction cycle, cellular reducing equivalents (e.g. superoxide ($\bullet O_2^-$)) carry out the reduction of the oxidised metal (equation *2*). The reaction products are Fe(II) and oxygen. **Haber-Weiss reaction** (equation *3*) is the summary reaction, combining the Fenton reaction and the reduction of Fe(III) by $O_2^{\bullet -}$. Haber-Weiss reaction can also occur in the absence of transition metal ions; however, in the presence of iron, the reaction rate

is considerably enhanced. Hydroxyl radicals are highly reactive species. Their half-life in aqueous media is less than 1 nanosecond. When produced *in vivo*, they react close to the formation site. The formation of such highly reactive species is potentially deleterious, and for this reason, a plethora of protective intracellular means have been developed. One of the most important damage-preventing strategies is the meticulous control of iron storage, transport, and distribution. In human organism the physiological mechanism with ability of elimination iron is absent and extensive majority of iron is recycled within the organism; for purposes of homeostasis the regulation of iron absorption is vital. In healthy individuals, iron levels are rigorously controlled, so there are only a few opportunities where iron-catalysed free radicals could be produced. However, in some circumstances, the iron status can be modified - either locally (as in ischemic tissue) or systematically (as with transfusion-induced iron overload or genetic haemochromatosis) (1, 7, 8).

Iron has an important impact in the reactive oxygen species generation (ROS) *in vivo,* and in undesirable processes, such as oxidative stress, DNA damage, and cell death. A radical-induced DNA damage can occur at the phosphate backbone or at nucleotide bases (20). Because of this, iron has been the main target of various antioxidant therapies. Oxidative stress is responsible for the release of iron from proteins, resulting in elevated non-protein-bound iron concentration and consequently it is involved in neurodegenerative diseases (e.g. Alzheimer's and Parkinson's) and cardiovascular diseases. Additionally, even slight excess of iron levels has been associated with increased cancer incidence (11, 12).

1.2 Oxidative stress

Oxidative stress can be defined as a condition where the balanced concentrations of reactive-oxygen and nitrogen species (RONS) are elevated. RONS species include the already-mentioned oxygen-free radicals (ROS) and reactive nitrogen species (RNS) (2, 13). In human organism, oxidative stress thus represents an imbalance between endogenous antioxidants and RONS, with the predominance of the latter (15). Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are toxic species, and their generation is the result of iron-dependent conversion into the highly reactive hydroxyl radicals ($HO^\bullet$) (2). Hydroxyl radicals are known to damage proteins, lipids, and DNA in cells. It is possible that the redox imbalance is implicated in the oncogenic stimulation, mutagenesis, cellular

aging, and carcinogenesis; it may be a contributing factor in neurodegenerative diseases and in certain cardiovascular diseases, such as arteriosclerosis (11).

Free radicals are atoms, molecules or molecular fragments containing unpaired electrons, which are the main cause of a substantial degree of reactivity. ROS include the following species: singlet oxygen (O_2), superoxide ($O_2^{\bullet -}$), alkyl peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), and hydroxyl radicals ($HO^{\bullet}$). An example of RONS is peroxynitrite, a potent oxidant, known for its ability to induce LDL oxidation and proinflammatory cytokine-mediated myocardial dysfunction (14). It appears that RNS also contribute significantly to the pathology of cardiovascular diseases, which is why effective prevention of oxidative stress generated by RONS seems to be of utmost concern in the prevention and treatment of the above-mentioned ailments (11).

RONS are produced (i) by neutrophils and macrophages during inflammation, (ii) by the UV light, X-rays, and gamma-rays during irradiation; (iii) are present as pollutants in the atmosphere; (iv) are by-products of mitochondria-catalyzed electron transport reactions, and (v) are products of metal-catalysed reactions. The production of RONS can be initiated by endogenous (cytochrome P450 metabolism, mitochondria, peroxisomes, and inflammatory cell activation) and exogenous structures and processes. RONS can also be produced by a plethora of different enzymes (most commonly NADPH oxidase, xanthine oxidase (XO), and lipoxygenases (LOX)), capitalizing on the catalytic capacities of iron (13, 15).

Cells continuously generate ROS as normal by-products of the aerobic metabolism and have therefore developed a protective physiological response to this – the deleterious effects of ROS are stabilized with the antioxidant activity of non-enzymatic antioxidants, coupled with antioxidant enzymes. Antioxidant defences are not always impeccable, which is why the cells also utilize the intracellular repair enzymes that are able to destroy proteins damaged by free radicals, repair the DNA damage, and eliminate oxidized fatty acids from cellular membranes. Mitochondria is the main organelle where free radicals are generated, and for this reason, it is exceedingly enriched by antioxidants, such as GSH and enzymes (particularly superoxide oxidoreductase dismutase (SOD)), and glutathione peroxidase (GPx). In order to minimize the oxidative stress in this organelle, antioxidants

can be found on both sides of membranes. Other antioxidant defences, such as lactoferrin, ceruloplasmin or transferrin, are largely extracellular (13, 16).

1.3 Polyphenols

In 1930, a new substance with positive effects on the capillary permeability reduction was isolated from oranges. At first it was believed that the substance belongs to a new class of vitamins and was therefore designated as vitamin P; afterwards, however, it was identified as a flavonoid (rutin). Because of their central role in plant physiology, plant polyphenols have been one of the major focal points of study in the last few decades. Polyphenols participate in various physiological functions, including growth, reproduction, and defence against pathogens and predators. The polyphenolic profiles of plants are quite different, and depend heavily on varieties of the same species. They can be found in green and black tea, coffee, different fruits, fruit juices, vegetables, olive oil, chocolate, and in red and white wines. Their quantities vary, the precise concentrations are dependent on the specific food type. According to one study, (17), people consume an average 23 mg of polyphenols per day, and according to another study (18), the consumption varies from several hundred mg to 1–2 g/day. At the moment, the number of identified polyphenolic compounds exceeds 8000, and they can be further subdivided into ten different subclasses. One of the most important among these is the flavonoid (sub)class, and thus far, more than 4000 members of this (sub)class have been identified. Many polyphenols, including flavonoids, possess the ability to chelate potentially toxic transition metal ions (Fe(II), Fe(III), Cu(II), etc.), and thus belong to a large class of potent antioxidants. Due to their chelation ability and due to the promising results in the treatment and prevention of certain medical conditions that seem to be correlated with iron-generated ROS and oxidative stress, they are subject to intense investigation (11, 19, 20).

1.3.1 Flavonoids

Flavonoids are ubiquitous plant secondary metabolites found in almost all plant parts (21) and they constitute one of the most widespread groups in plant phenolics (19). The nucleus of all flavonoids consists of flavane (2-phenyl-bezo-γ-pyrane), which provides the basis of their overall chemical structure: two benzene rings (A and B) connected by an oxygen-containing pyrane ring (C) (Figure 1). According to the hydroxylation pattern of the

nucleus, the substituent at carbon 3, and the degree of oxidation of the C-ring, the flavonoids can be categorized into several subclasses: flavanols, flavones, flavonols, isoflavones, flavanones, anthocyanins, and flavans (21, 22).

Numerous studies indicate that flavonoids exert a positive impact on human health. Scientific interest in flavonoid chemistry has therefore increased significantly, and recent *in vitro* studies suggest that flavonoids have potent RONS scavenging properties (13). One of the arguments that speak in favour of this observation is the fact that polyphenols, including flavonoids, poses antioxidant activities that are significantly greater than those of vitamins C and E (18).

Dietary flavonoids differ in their structural properties, the extent of alkylation and/or glycosylation, the number and/or arrangement of the hydroxyl groups, and the conjugation between the A- and B-rings. In food, they are normally found as 3-O-glycosides and polymers. During metabolism, the so-called functionalization reactions take place, in which the -OH groups are added, methylated, glucuronidated, or sulfated. When different sugar residues are added to flavonoid skeleton, the glycosides are produced. Glucose represents the majority in sugar residues occurring; others residues may be galactose, arabinose, rhamnose or xylose. The preferred glycosylation sites on the flavonol molecule are at the position 7 and at the position 3, with the predominance of the latter. Plants normally contain glycoside derivates, and are therefore very unlikely to contain the aglycone form (with the exception of catehines) (14, 19, 21, 22, 23).

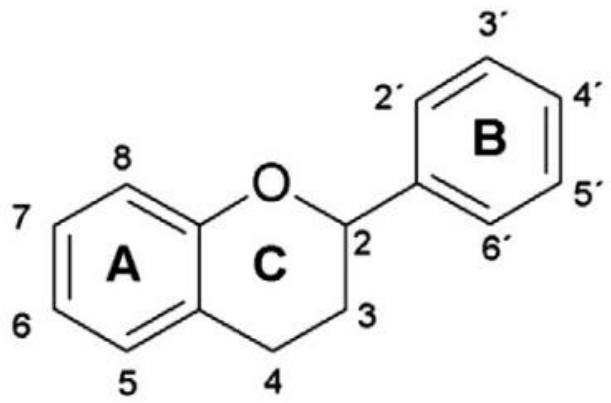


Figure 1: General chemical structure of flavonoids (31)

The interest in flavonoids has been on the increase ever since the discovery of the so-called »French Paradox«. This dietary anomaly was first observed in the French population and was followed by other Mediterranean nations. The French are known for their fat-rich diet and high smoking rates; these habits are unhealthy, yet the French have reduced rates of coronary heart disease when compared to the northern European nations. Epidemiological studies have revealed flavonoid-fortified diets to be in positive correlation with the decreased incidence of cardiovascular diseases and the increased life expectancy. The most plausible explanation for this extraordinary phenomenon is provided by epidemiological studies, emphasising a possible correlation between relatively high daily consumption of red wines, which are known for their high levels of phenolic compounds, and the protection against heart disease (22, 24).

Flavonoids are known to possess a wide spectrum of pharmacological, medicinal and biochemical features, including antioxidant, immune-stimulating, anti-inflammatory, antiallergic, antibacterial, antiviral, antimutagenic, and anti-cancer characteristics, as well as vasoprotective, antiangiogenic, vasodilatory, anti-ischemic, estrogenic effects and antidiabetic properties. They function as modulators of enzymatic activities, thereby contributing significantly to the inhibition of phospholipase A2, cyclooxygenase 2 (COX-2), LOX, glutathione reductase, and XO enzymes. The anti-cancer properties are the result of their ability to induce apoptosis, disrupt mitotic spindle formation, inhibit angiogenesis, or block the cell cycle. At the molecular level, flavonoids showed the ability to modulate platelet-derived growth factor receptors, certain types of protein kinase (e.g. serine-tyrosine kinase and protein kinase-C), cyclin-dependent kinases, epidermal growth factor receptors, and also vascular endothelial growth factor receptors; all these factors contribute to cancer pathology. Flavonoids are also able to inhibit platelet aggregation and lipid peroxidation, and improve increased capillary permeability and fragility (21, 22, 24, 25).

1.4 Antioxidant properties

The term “antioxidant” looms large in scientific literature; however, since there are numerous methods that can be used to measure their activity, there is currently no universally accepted definition of what the term actually denotes. According to one of the most prevalent definitions, antioxidant is any substance that has the ability to prevent,

delay, or remove oxidative damage in living cells. As suggested by the provided definition, the main role of antioxidants in human organism is to prevent the damage to cellular components, induced by free radicals (24, 26).

Flavonoids belong to the category of phenolic antioxidants (PhOH), known for their capability to scavenge free radicals and prevent the damage caused by ROS in one of the following manners: (i) activation of antioxidant enzymes, (ii) direct scavenging of ROS, (iii) transient metal (iron/copper) chelating activity, (iv) reduction of α-tocopheryl radicals, (v) inhibition of oxidases (in particular XO, NADPH oxidase and LOX), and (vi) increase in uric acid levels (22, 24).

In order to be labelled as efficient antioxidants, polyphenols need to possess some basic properties: when they are present in low concentrations, they have to be capable of delaying, retarding, or preventing the oxidation mediated by free radicals or autooxidation. Another requirement is that formed radicals must be stabilised through intermolecular hydrogen bonding to prevent further oxidation processes (23). In order to provide efficient protection against DNA damage caused by ROS species, PhOH also have to possess the following two properties: (i) the ability to remove loosely bound redox-active iron from specific intracellular locations, and (ii) the ability to pass through the plasma membrane (27).

1.5 Structure-activity relationship (SAR) of flavonoids

The activity of flavonoids is strongly correlated with their structure. Carbon atoms of the basic flavonoid skeleton have different substitutions and hence flavonoids have different lipid solubility; for this reason, they are not equally physiologically active. Flavonoids generally occur in the form of glycosides, but their bioactivity normally depends on the aglycone structure – the latter seems to have greater antioxidant property than the corresponding glycosides. It is also important to note that their bioavailability is increased by glucose moiety in molecule (14, 18).

Flavonoids are characterized by their highly conjugated and electron-rich chemical structure, so they generally act as very good electron and hydrogen donors and these properties are highly important determinants of their antioxidant activity (12). The free

radical scavenging ability is also greatly affected by the torsion angle of the B-ring with respect to the rest of the molecule, which gives the planar properties to molecule. Flavones lack this feature and are slightly twisted. Planarity permits conjugation, electron dislocation, and a corresponding increase in flavonoid phenoxyl radical stability. The removal of the 3-OH group abrogates coplanarity and conjugation, thereby compromising scavenging ability (14).

Another possible mechanism of the protective role in polyphenols is associated with their ability to sequester transition metal ions by chelation. Iron binding with PhOH can reduce the accessibility of the iron for the molecular oxygen and thus reduce its toxicity. Under physiologically relevant conditions, iron chelation can be an effective tool in modulating cellular iron homeostasis. Determining the acidity of PhOH is a critical parameter that needs to be taken into consideration: the process of iron chelation usually occurs through deprotonated -OH groups in the PhOH, and the process of metal chelation is supposed to be easier, if the energy required to deprotonate the -OH groups (acidity) is correlatively smaller (22).

The SAR for radical scavenging and iron chelating is different. Favourable structural requirements for effective RONS scavenging are:

1. the 3-OH and 5-OH groups (required for the maximal radical scavenging capacity);
2. the o-dihydroxy structure in the B-ring (required for high stability of the flavonoid phenoxyl radicals via hydrogen bonding or by expanded electron delocalization);
3. the C2–C3 double bond in conjugation with the 4-oxo group (required for the determination of the coplanarity of the heteroring and the radical stabilization via electron delocalization over all three ring systems) (18, 23).

A detailed statistical analysis of flavonoids (28) established no direct correlation between the number of -OH groups and reduction activity. From this, it can be further hypothesized that the localization of the -OH groups plays a major role in the reduction activity. Another study (15) found the presence of two -OH groups in an ortho position in the B-ring to be of the most importance in the reduction abilities. However, it was also

suggested that the adjacent -OH groups at positions 5, 6 and 7 in ring A may replace ring B -OH groups scavenging function (15, 28).

The metal-flavonoid complex stoichiometry varies in accordance with structural differences and with pH value of environmental media; pH has great impact on complex-formation processes as well as on basic structural features of flavonoids. The optimal pH for the complex formation is around 6, since flavonoids tend to be weakly polybasic; it should be noted that the precise optimal pH value is different for different metal ions. At pH less than 3.0, flavonoids stay undissociated, which has adverse effects on complex formation. At high pH deprotonation of flavonoids occurs, which enables easier formation of complexes. However, under strong basic conditions, reactions of hydrolysis occur, thus assisting in the formation of hydroxo-complexes (12, 29).

As mentioned before, SAR for iron chelation is different from that of free radical scavenging. The following metal-complexing domains seem to be prevalent: (i) between the 3', 4'-dihydroxyl group (located on the B-ring), (ii) between the 3-hydroxyl and 4 carbonly group, and (iii) between the 5-hydroxyl and 4-carbonyl group (located on the A-ring) (15). It was observed that the first mentioned chelation domain is preferable, but in contrast to the other two domains, it is not available under more acidic conditions. The reason for this can be found in the fact that under neutral conditions, the process of dissociation of both catechol groups in the B-ring is facilitated, while in the presence of acidic conditions, the dissociation is limited. Flavonoids with free 6-OH and 7-OH groups may also avail themselves with this alternative site for iron chelation and it seems to be generally preferred to the 5-hydroxyl and 4-keto site. Under neutral conditions, most flavonoid-iron complexes are typically formed in the ratio 2:1 or 1:1. In conditions of iron overload or impaired sequestering of iron by storage or transport proteins, the oxidation process caused by the Fenton chemistry tends to be strongly inhibited by the PhOH with 3'4'-catechol, 4-oxo, and 5-OH arrangements. Iron can also bind to 5-hydroxy-4-keto site. However, it was shown that its importance is lower compared to the previously mentioned sites. Moreover, the complex formed at this site is less stable and iron can be much more easily released at more acidic conditions (14, 15, 30, 31). It was also observed in another study (28) that the presence of the 5,6-dihydroxy group is linked with a significantly lower ferric ions reducing potential than the catecholic B-ring in flavones, and that the three adjacent hydroxyl groups in the A-ring did not improve the ferric ion-reducing potential.

As mentioned previously, flavonoids can also use the 6,7-dihydroxy conformation in the A-ring, which was shown to be the most efficient iron chelating site, followed by the 3-hydroxy-4-keto site with the 2,3-double bond and the catecholic B-ring (31).

It can thus be concluded that different proton dissociations of -OH groups are the main cause of differences in iron chelation among flavonoids and of their specific iron chelating behaviour under different pH conditions. The dissociation occurring under acidic conditions is favoured more in the 7-OH group than in the 4'-OH group, and is less probable in the 5-OH group. The efficiency of the catecholic B-ring is more pronounced when pH conditions are neutral, and the activity is significantly decreased under the acidic conditions. The chelation potential can be additionally improved by increasing the number of -OH groups in the A-ring (31).

2 RESEARCH OBJECTIVE

Iron has an important impact on a wide spectrum of cellular processes and plays a crucial role in human organism. Different biochemical activities, such as energy production, cellular proliferation and oxygen transport, are dependent on iron-containing cofactors. However, too much iron in the organism can have undesirable effect, as it leads to oxidative stress. When oxygen is present, iron redox chemistry is responsible for generating free radicals. Flavonoids are one of the most promising and effective antioxidants. Numerous studies have been carried out to determine the crucial functional groups of flavonoids that are responsible for free radical scavenging and iron chelation.

The present study will try to determine the role of hydroxyl groups at the position 6 and 7 of the A-ring in flavones on the iron chelating capacity and the stability of the newly formed complexes. Baicalein with the 5,6-dihydroxyflavone core is the most active iron chelator among flavonoids, with the activity comparable to that of the standard iron chelator deferoxamine. To better elucidate the interactions of the 6,7-dihydroxygroup with iron, a close baicalein congener, baicalin, with a blocked 7-hydroxygroup will be tested. The *in vitro* formation of the flavonoid-iron complexes will be determined by means of the UV-Vis spectrophotometry. A complementary method will be used, in which the concentration of iron will stay the same throughout the measurement, while the concentration of flavonoids will vary. The experiments will be performed at 4 different (patho)physiological relevant pH conditions (4.5, 5.5, 6.8, 7.5). The experimental data will be then mathematically analysed to provide a strong basis for an adequate interpretation of results.

3. MATERIALS AND METHODS

3.1 Reagents

- baicalein (5,6,7-trihyroxyflavone); M = 270.24 g/mol (Sigma-Aldrich, Germany)
- baicalin (7-glucuronic acid, 5,6-dihydroxy flavone); M = 446.36 g/mol (Sigma-Aldrich, Germany)
- ferrozine; M = 492.46 g/mol (Sigma-Aldrich, Germany)
- ferrous sulphate heptahydrate; M = 278.01 g/mol (Sigma-Aldrich, Germany)
- ferric chloride hexahydrate; M = 270.30 g/mol (Sigma-Aldrich, Germany)
- ferric tartrate; M = 555.90 g/mol (Sigma-Aldrich, Germany)
- hydroxylamine hydrochloride (HA); M = 69.49 g/mol (Sigma-Aldrich, Germany)
- acetic acid; M = 60.05 g/mol (Sigma-Aldrich, Germany)
- HEPES – sodium salt; M = 260.29 g/mol (Sigma-Aldrich, Germany)
- sodium acetate; M = 82,03 g/mol (Sigma-Aldrich, Germany)
- HEPES (Sigma-Aldrich, Germany)
- methanol (J.T. Baker (Avantor Performance Materials, Inc., USA))
- ultrapure water, produced by Milli-Q RG (Merck Millipore, USA)

3.2 Equipments and spectral measurements

- semi-micro polystyrene and ultraviolet-transparent cuvettes (BrandTech Scientific Inc., The United Kingdom)
- spectrophotometer Helios Gama (ThermoFisher Scientific Inc., USA), equipped with VisionLite Software 2.2
- analytical balance (Kern Alt-220 4NM, Kern, Germany)

- ultrasound bath (Bandelin Sonorex, Bandelin electronic, Germany)
- pipettes (Eppendorf Research Plus, Eppendorf, Germany)
- microtubes (1.5 ml - microtube Eppendorf PP 1.5 ml, Boettger, Germany)
- tubes (15 ml - Centrifuge tube 15 ml PP, Thermo Scientific)

Buffers were prepared as 15 mM solutions – for pH 4.5 and 5.5 acetate buffers were used and for pH 6.8 and 7.5 HEPES buffers were used. All spectrophotometric measurements were performed in semi-micro polystyrene or ultraviolet-transparent cuvettes. Absorbance was measured at room temperature (25°C).

3.3 Assessment of iron concentration in stock solutions

3.3.1 Stock solutions of Fe(II) and Fe(III)

Every day before the experiment, a fresh 5 mM stock solution of iron was prepared. As a source of ferrous irons, ferrous sulphate heptahydrate was used. For the preparation of a 5 mM stock solution of iron, 208.51 mg of ferrous sulphate heptahydrate was weighed with an analytical balance and dissolved in 15 mL of ultrapure water. Due to its low stability, the stock solution was prepared fresh every day.

Because of the low solubility of Fe(III) at higher pH levels two different ferric ion sources were used in the measurement of the Fe(III) chelation – ferric chloride hexahydrate and ferric tartrate. The stock solution of 5 mM was used for ferric chloride and 1.5 mM for ferric tartrate (Figure 2). 202.73 mg of ferric chloride or 416.93 mg of ferric tartrate was weighed and dissolved in 15 mL of ultrapure water. The stock solution of ferric tartrate was stored in the freezer and thawed prior to use; solutions of ferric chloride ($FeCl_3$), on the other hand, were prepared fresh each day.

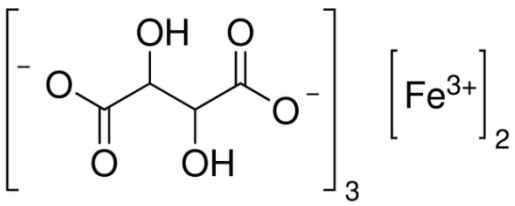


Figure 2: Chemical structure of Fe(III) tartrate

3.3.2 Calibration of iron

First, the blank sample was measured, in which ultrapure water was used. For the purposes of the Fe(II)- and Fe(III)-calibration, a 10 mM stock solution of hydroxylamine (HA) was prepared, which was diluted from a 100 mM stock solution of HA. For the preparation of a 100 mM stock solution of HA, 104.24 mg of HA was weighted with an analytical balance and dissolved in 15 mL of ultrapure water. After this, it was diluted to a 10 mM stock solution. The latter was stable and could be stored in the fridge for future use. We also had to prepare a 5 mM stock solution of ferrozine in ultrapure water. To this end, 369.35 mg of ferrozine was weighed and dissolved in 15 ml of ultrapure water. Thus acquired stock solution was stable and could be stored in the fridge. Ferrozine is a specific spectrophotometric reagent which forms a magenta coloured complex (absorption maximum at 562 nm) with ferrous ions (32).

- **Calibration of Fe(II):**

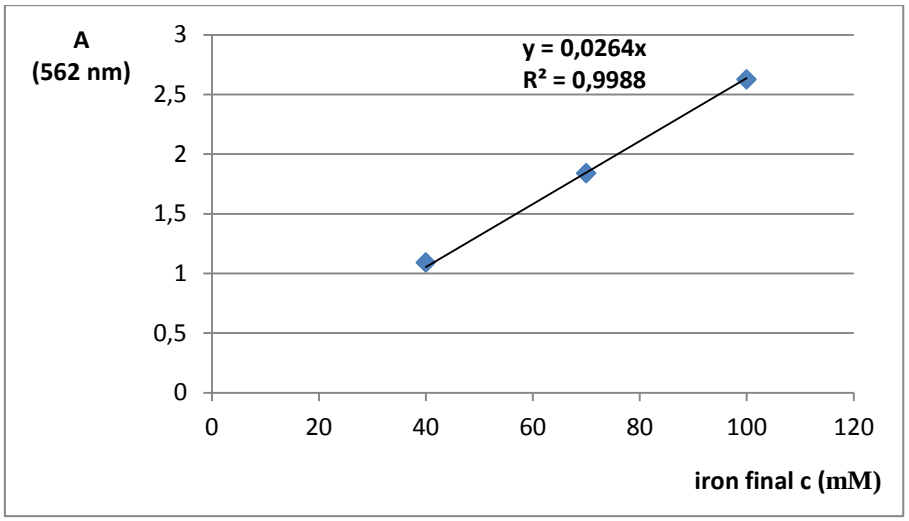
The chemicals (10 mM solution of HA, 5 mM stock solution of Fe(II) salt, 5 mM ferrozine) were pipetted directly into the Eppendorf tubes for the preparation of 3 different molar concentrations of the final Fe (II). They were pipetted in the following order (Table I):

Table I: Preparation of solutions for calibration of Fe(II) stock solutions

final c Fe(II)	V (Fe(II))	V (ferrozine)	V (HA)
mM	ml	µl	µl
100	30.0	500.0	970.0
70	21.0	500.0	979.0
40	12.0	500.0	988.0

c – concentration, V – volume, HA – hydroxylamine

The mixtures in the Eppendorf tubes were stirred and their absorbances were measured – the spectra ranged from 450 to 650 nm. With the measured absorbances at λ 562 nm the calibration curve (Figure 3) was calculated ($y = kx + n$). The determination of the range for the ***k*** constant had been performed with several experiments and they were confronted with ferrozine assay and values lead to non-significant differences in the results of the latter assay (28). For **Fe(II)** ***k*** constant should be between **0.023** and **0.028**. In the case of other values, a new calibration with the new solution of the Fe(II) salt was conducted.



A (562 nm)
2,309
1,737
1,045

Figure 3: Example of calibration curve for determination of Fe(II) concentration

- **Calibration of Fe(III):**

The chemicals (10 mM solution of HA, super distilled water, stock solution of Fe(III) salt, 5 mM ferrozine) were pipetted into the Eppendorf tubes for the preparation of 3 different molar concentrations of the final Fe(III). They were pipetted in the following order (Tables II and III):

Table II: Preparation of solutions for calibration of ferric tartrate

final c Fe(III)	V Fe(III)	V HA	V ferrozine	V H_2O
µM	**ml**	**µl**	**µl**	**µl**
100	30.0	500.0	500.0	470.0
70	21.0	500.0	500.0	479.0
40	12.0	500.0	500.0	488.0

c – concentration, V – volume, HA – hydroxylamine, H_2O – ultrapure water

Table III: Preparation of solutions for calibration of ferric chloride

final c Fe(III)	V Fe(III)	V HA	V ferrozine	V H_2O
µM	**ml**	**µl**	**µl**	**µl**
100	30.0	500.0	500.0	470.0
70	21.0	500.0	500.0	479.0
40	12.0	500.0	500.0	488.0

c – concentration, V – volume, HA – hydroxylamine, H_2O – ultrapure water

The mixtures in the Eppendorf tubes were stirred and their absorbances were measured in the spectra range from 450 to 650 nm. With the measured absorbances at λ 562 nm the

calibration curve was calculated. Range of the ***k*** constant for **Fe(III) tartrate** is supposed to be between **0.018** and **0.022**, and for **Fe(III) chloride** between **0.024** and **0.028**. In the case of other values, a new calibration with the new solution of the Fe(III) salt was conducted.

3.4 Experiment preparation for the determination of the chelating capacity

Having provided a successful control of the iron concentration in stock solutions, experiments for the determination of the chelating capacity of baicalein (BAI) (Figure 4) and baicalin (BAIN) (Figure 5) with Fe(II)/Fe(III) at 4 different (patho)physiologically relevant pH values were performed. Both baicalein and baicalin were weighed with an analytical balance, dissolved in methanol, and prepared as 5 mM solutions.

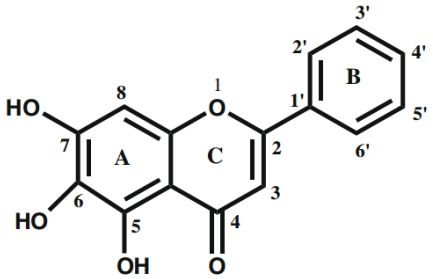


Figure 4: Chemical structure of baicalein (34)

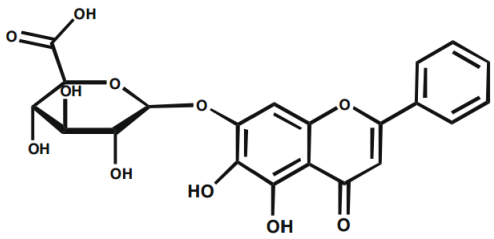


Figure 5: Chemical structure of baicalin (34)

The chelating capacity was determined in buffers. 15 mM sodium acetate buffer was used for pH 4.5 and 5.5, and HEPES for pH 6.8 and 7.5. At pH 7.5, the oxidation of Fe(II) is significantly higher, and for this reason, HA was added in the final concentration of 5 mM to the HEPES buffer with the aim of inhibiting ferrous oxidation. In order to determine the Fe(III) chelation at pH 7.5, the HEPES buffer without HA was used.

Measurements of absorption spectra of tested compounds (baicalein and baicalin with the excess of iron) were performed as shown in Table IV. At first, blank sample was prepared from 500 µl of the solvent (methanol) and 1000 µl of the suitable buffer for the appropriate pH value, and measured with spectrophotometer. Samples were prepared sequentially, in the order depicted in Table IV (at first molar ratio 6:0, then 4:0, etc.). Having prepared a suitable dilution of stock solution of tested substance (it was diluted from 5 mM to 0.25 mM), the prescribed volume of the solvent (methanol) was pipetted into the Eppendorf tubes, followed by the prescribed volume of the tested compounds (0.25 mM stock solution of baicalein/baicalin), adequate amount of buffer, and the 5 mM stock solution of iron (Fe(II)/Fe(III)). The samples were stirred for 1 minute and their absorbance was then measured with spectrophotometer. Iron was not added in the first 3 examples, where only the pure compound was measured. The aim of these measurements was to assess the characteristics of pure compound first and then that of the complex (absorbance maxima and molar absorption coefficients at these maxima).

Table IV: Measurement of absorption spectrum of baicalein/baicalin (substance) in the presence of excess of iron

molar ratio substance : iron			**substance final c**	**iron final c**	**solvent (methanol) volume**	**0,25 mM solution of substance volume**	**buffer volume**	**5 mM iron stock solution volume**
			mM	mM	µl	µl	µl	µl
6	**:**	**0**	0.060	0	140.0	360.0	1000.0	0
4	**:**	**0**	0.040	0	260.0	240.0	1000.0	0
2	**:**	**0**	0.020	0	380.0	120.0	1000.0	0
1	**:**	**6**	0.042	0.25	250.0	250.0	925.0	75.0
1	**:**	**10**	0.025	0.25	350.0	150.0	925.0	75.0
1	**:**	**15**	0.017	0.25	400.0	100.0	925.0	75.0

Experiments aimed at determining complex stoichiometries were performed by means of a complementary approach that had been developed in the laboratory at an earlier date (10). For this experiment, a dilution of the 5 mM iron stock solution was prepared (the final concentration has to be 0.25 mM). Methanolic solutions of the tested substance were mixed with a constant iron concentration in the adequate buffer. After mixing for 1 minute, the absorption spectra were recorded with the UV-Vis spectrophotometer. The performed measurement molar ratios ranged from 0.25:1 to 3:1 (substance:iron) (Table V).

Throughout the measurement, the concentration of the iron was constant during the measurement (0.02 mM), while the concentrations of tested substances increased (the approach is characterized as complementary because its methodological design differs significantly from the standard Job's method).

Table V: Measurements determining the chelation capacity by means of the complementary method

molar ratio substance : iron			substance final c	iron final c	solvent (methanol) added volume	substance (baicalein/baicalin) added volume	buffer added volume	iron stock solution added volume
			mM	mM	µl	µl	µl	µl
0.25	**:**	**1**	0.005	0.020	470.0	30.0	880.0	120.0
0.5	**:**	**1**	0.010		440.0	60.0		
0.75	**:**	**1**	0.015		410.0	90.0		
1	**:**	**1**	0.020		380.0	120.0		
1.25	**:**	**1**	0.025		350.0	150.0		
1.5	**:**	**1**	0.030		320.0	180.0		
1.75	**:**	**1**	0.035		290.0	210.0		
2	**:**	**1**	0.040		260.0	240.0		
2.5	**:**	**1**	0.050		200.0	300.0		
3	**:**	**1**	0.060		140.0	360.0		

3.5 Mathematical data analysis

Having performed all measurements, we were equipped with the absorption spectra of the iron-containing compounds at different chelation ratios. In order to determine the stoichiometry of chelates, the acquired experimental data was analyzed, using mathematical stoichiometrical calculations.

Method I – absorbance at the absorption maximum of the complex

The first method determines the stoichiometry by evaluating the absorbance values of a series of samples at the wavelength of the absorption maximum of the complex λ_{cmax}.

Method II – symmetry of the absorption maximum of the complex

The second method is based on the theoretical fact that the absorption spectrum of a given complex is symmetric if there are no interfering local absorption maxima nearby. In other

words, the absorbance of the complex ($A_{C\lambda sym1}$ and $A_{C\lambda sym2}$) (Eq. 3) at a similar distance (d) from the maximum λ_{Cmax} (Figure 6) is identical on both sides ($\lambda sym1$ – to the left or $\lambda sym2$ – to the right) (Eq. (1) and (2)).

$$\lambda_{sym2} = \lambda_{Cmax} - d \quad (1)$$

$$\lambda_{sym2} = \lambda_{Cmax} + d \quad (2)$$

$$A_{C\lambda sym1} = A_{C\lambda sym2} \quad (3)$$

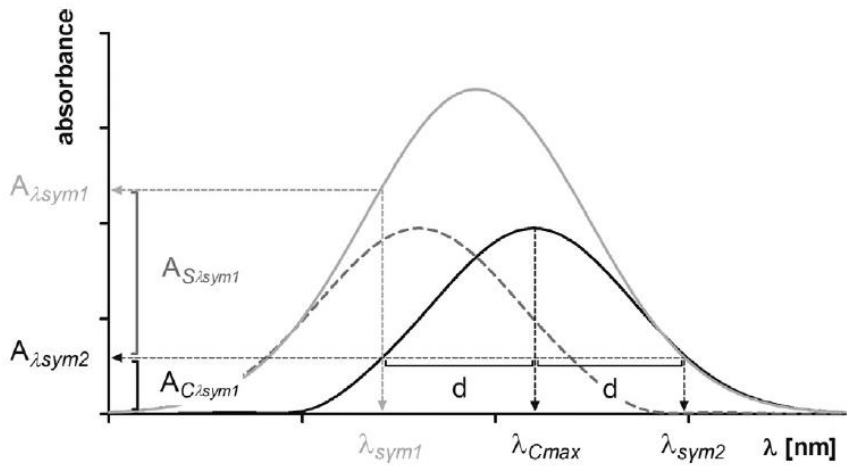


Figure 6: The method of the symmetry of absorption maximum of the complex (33)

The measured absorption spectrum is the sum of the absorbance of the complex and the absorbance of the unreacted substance. It was assumed that the measured absorbance $A_{\lambda sym2}$ is equal to the absorbance of the complex $A_{\lambda Csym2}$, as long as $A_{\lambda Csym2}$ is sufficiently high (>0.1) and the absorbance of the substance $A_{S\lambda sym2}$ is zero. The measured absorbance of $A_{\lambda sym1}$ was thus used to directly assess the molar concentration of the unreacted substance:

$$A_{\lambda sym2} = A_{\lambda Ssym2} + A_{C\lambda sym2} \quad (4)$$

$$A_{\lambda sym2} = A_{c\lambda sym2} \quad (5)$$

Using Eqs. (*3*) and (*5*) gives us:

$$A_{\lambda sym1} = A_{S\lambda sym1} + A_{C\lambda sym1} \quad (6)$$

$$A_{\lambda sym1} = A_{S\lambda sym1} + A_{\lambda sym2} \quad (7)$$

The molar concentration of the unreacted substance c_s (Eq. 9) can then be determined using the Lambert-Beer law (Eq. 8):

$$A_{S\lambda sym1} = c_S \times \varepsilon_{S\lambda sym1} \times \mathrm{l} \quad (8)$$

$$c_S = \frac{A_{\lambda sym1} - A_{\lambda sym2}}{\varepsilon_{S\lambda sym1} \times l}, \quad (9)$$

where l is the width of the cuvette and $\varepsilon_{S\lambda sym1}$ is the molar absorption coefficient of the substance at λ_{sym1}.

Finally, the chelation ratio (X) is calculated as

$$x = \frac{c_{S0} - c_s}{c_{Fe}}, \quad (10)$$

where c_{So} is the initial molar concentration of the substance and c_{Fe} is the final molar concentration of iron in the sample.

Method III – calculation using the absorption maximum of the substance

As in the previous method, the calculation of stoichiometry is based on the molar concentration of the unreacted substance c_s. The difference is that the absorbance at the wavelength of the absorption maximum of the substance ($A_{\lambda Smax}$) is used. As before, $A_{\lambda Smax}$ is the sum of the absorbances of the unreacted substance and the formed complex. Again, by applying the Lambert-Beer law, c_s can be determined from the equation

$$A_{\lambda Smax} = c_S \times \varepsilon_{\lambda Smax} \times l + c_c \times \varepsilon_{c\lambda Smax} \times l, \quad (11)$$

where $\varepsilon_{S\lambda smax}$ and $\varepsilon_{c\lambda smax}$ stand for molar absorption coefficients of the substance and the complex at the wavelength of the absorption maximum of the substance λ_{Smax}. Taking into account that

$$c_c + c_s = c_{So}, \quad (12)$$

the concentration of the unreacted substance c_s can be calculated as

$$c_S = \frac{\frac{A_{\lambda Smax}}{l} - \varepsilon_{Csmax} \times c_{So}}{\varepsilon_{S\lambda Smax} - \varepsilon_{C\lambda Smax}}. \quad (13)$$

The chelation ratio is then calculated as in the Method II using the Eq. 10.

Method IV – calculation using the absorption maximum of the complex

This method is the same as the Method III, but with the absorbance measured at the wavelength of the absorption maximum of the complex (λ_{cmax}).

Method V – theoretical means of determining the absorbance of the complex at the wavelength of its absorption maximum

In the next method, theoretical lines are constructed, approximating the absorbance of the most probable stoichiometries. Since the molar concentration of iron was kept constant throughout the experiment, the absorbance initially increased in proportion with the increasing concentration of the complex.

$$A_{\lambda cmax} = A_{C\lambda max} \quad (14)$$

$$A_{C\lambda max} = c_{So} \times \varepsilon_{C\lambda cmax} \times l \quad (15)$$

After all iron was used, the increase in the absorbance of $A_{\lambda Cmax}$ was due to the absorbance of the subsequently added (unreacted) substance $A_{S\lambda cmax}$ (Eqs. (16) and (17)):

$$A_{\lambda Cmax} = A_{\lambda Ssym1} + A_{S\lambda Cmax} \quad (16)$$

$$A_{C\lambda max} = c_{eq} \times \varepsilon_{C\lambda cmax} \times l + (c_{So} - c_{eq}) \times \varepsilon_{S\lambda cmax} \times l \quad (17)$$

The molar concentrations reach the equilibrium (c_{eq}) at $c_{so} = c_{Fe}$ for stoichiometry 1:1, $c_{So} = 2 \times c_{Fe}$ for stoichiometry 2:1, etc. By comparing the measured absorbance with the constructed theoretical lines we were able to find the appropriate stoichiometry, or even the reaction stoichiometry, at different molar concentration ratios. The same approach was used with the absorption maximum wavelength of the substance.

Method VI – theoretical means of determining the sum of the absorbance of the unreacted tested substance and the complex at the absorption maximum of the tested substance

As in the previous method, theoretical lines are constructed, approximating the absorbance of the most probable stoichiometries. The underlying idea is the same – in the presence of iron, the absorbance depends on the formation of a specific complex; and when the iron has been consumed for the complex formation, the absorbance depends only on the subsequently added substance (Eqs. (14)–(16)). In contrast to the Method V, it can be assumed that different complexes with different molar absorption coefficients could be formed, so the absorbance might not increase in proportion to the concentration equilibrium. Thus, those lines that were dependent solely on the unreacted substance were constructed in view of the measured absorbance at the most probable chelation ratios (1:1, 2:1, 3:1, etc.). The same approach was used at the wavelength of the absorption maximum of the complex (33).

4. RESULTS

4.1 Measuring absorbance of baicalein/baicalin in the presence of iron

4.1.1 Baicalein: experimental results

The following absorption spectra (Figs. 7, 9, 11, 13, 15, 17, 19, 21) depicts various spectra of baicalein at different pH values (4.5, 5.5, 6.8 and 7.5) and with both Fe oxidation states /Fe(II) and Fe(III)/.

At each pH the absorption spectra of the substance with the excess of iron are presented. Red, green and dark blue lines represent the pure tested substance in 3 different molar concentrations (0.06 mM, 0.04 mM and 0.02 mM, respectively), while pink, light blue and yellow lines depict the mixtures of the substance with the excess of iron. The concentration of baicalein was 0.017 mM (yellow), 0.025 mM (light blue) and 0.042 mM (pink), and the concentration of iron was 0.25 mM. This correspond to 3 different molar ratios (substance:iron) 1:6, 1:10 and 1:15, respectively.

In the UV-Vis absorption spectra used in the complementary approach, the final concentration of iron was 20 µM, while that of baicalein changed from 5 µM (molar ratio 0.25:1) to 60 µM (molar ratio 3:1).

Fe(II), pH 4.5

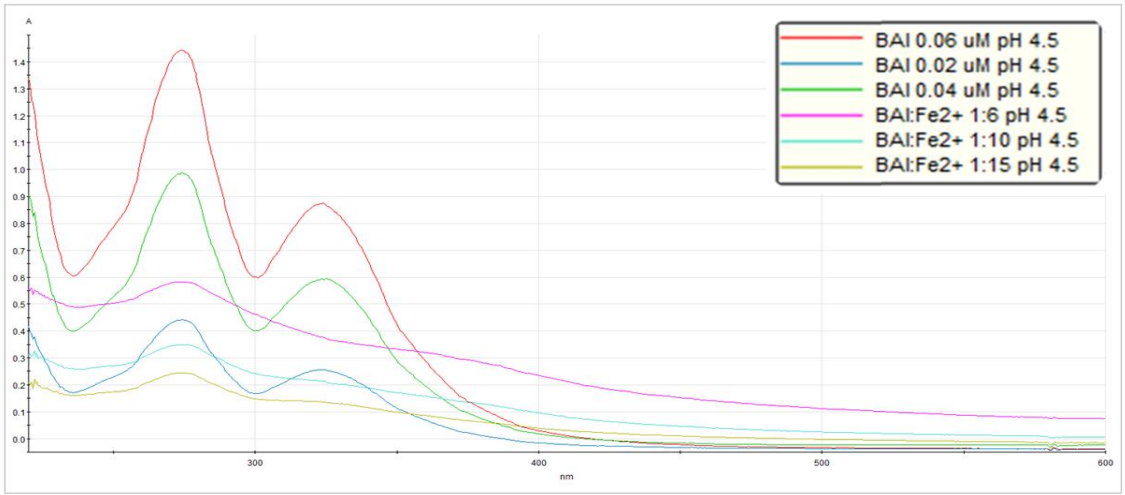


Figure 7 - Absorption spectra of baicalein (BAI) and its complex with Fe(II) at pH 4.5

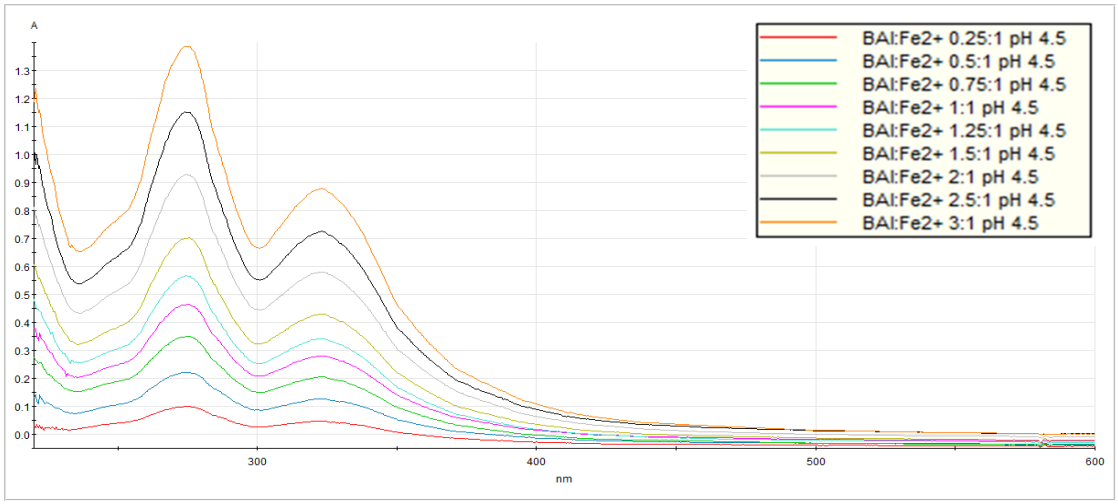


Figure 8: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(II) at pH 4.5

Fe(II), pH 5.5

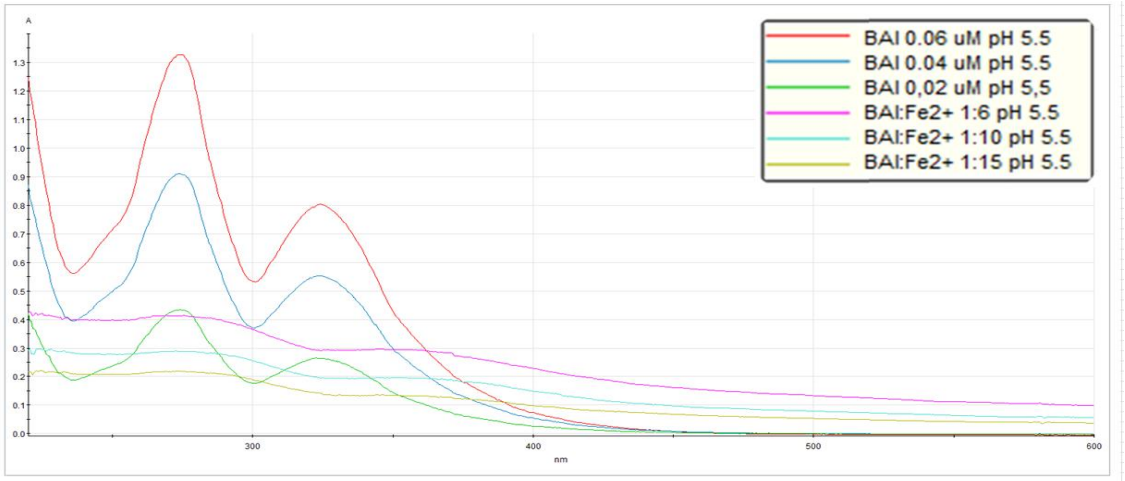


Figure 9: Absorption spectra of baicalein (BAI) and its complex with Fe(II) at pH 5.5

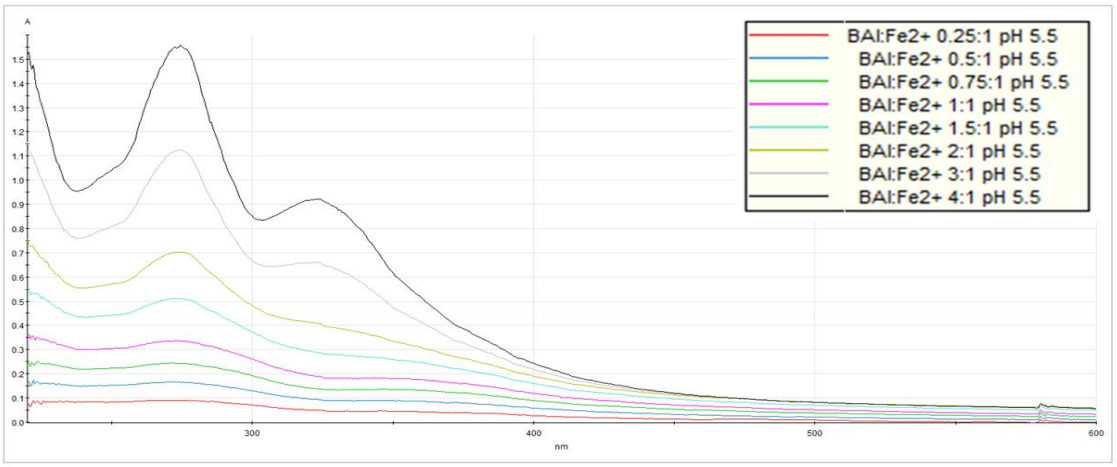


Figure 10: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(II) at pH 5.5

Fe(II), pH 6.8

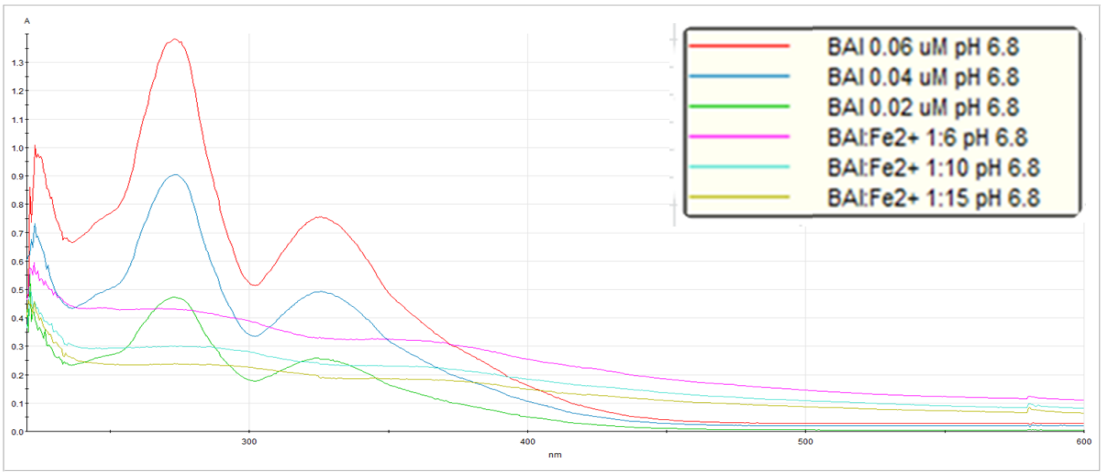


Figure 11: Absorption spectra of baicalein (BAI) and its complex with Fe(II) at pH 6.8

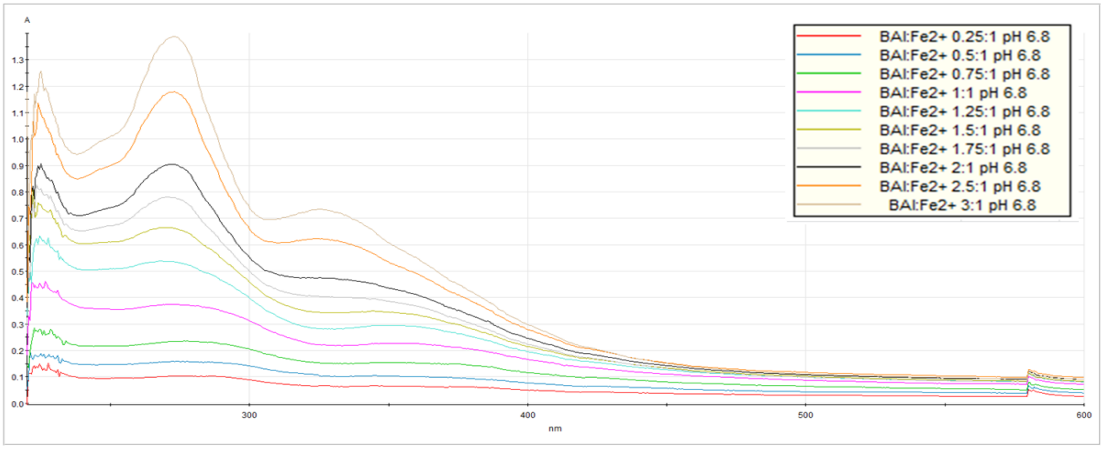


Figure 12: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(II) at pH 6.8

Fe(II), pH 7.5

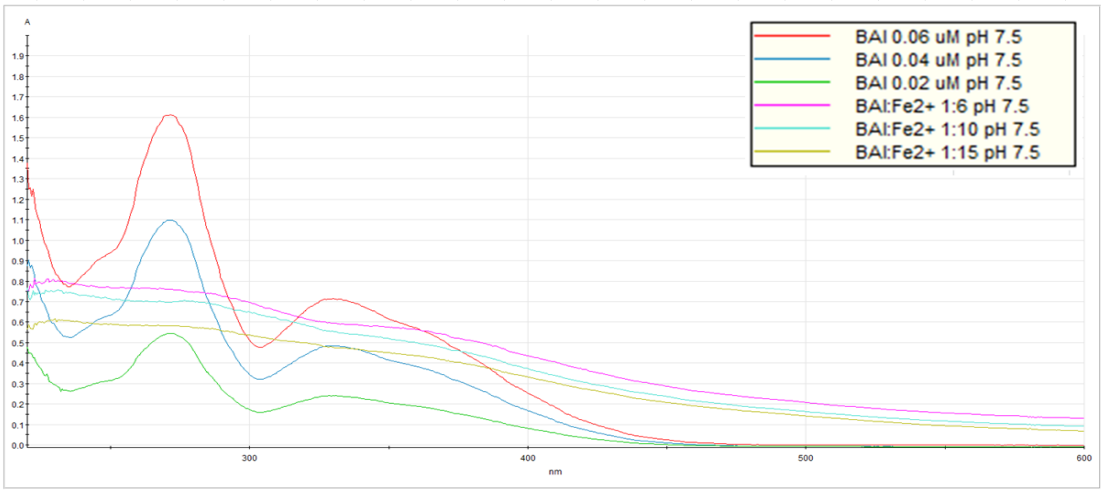


Figure 13: Absorption spectra of baicalein (BAI) and its complex with Fe(II) at pH 7.5

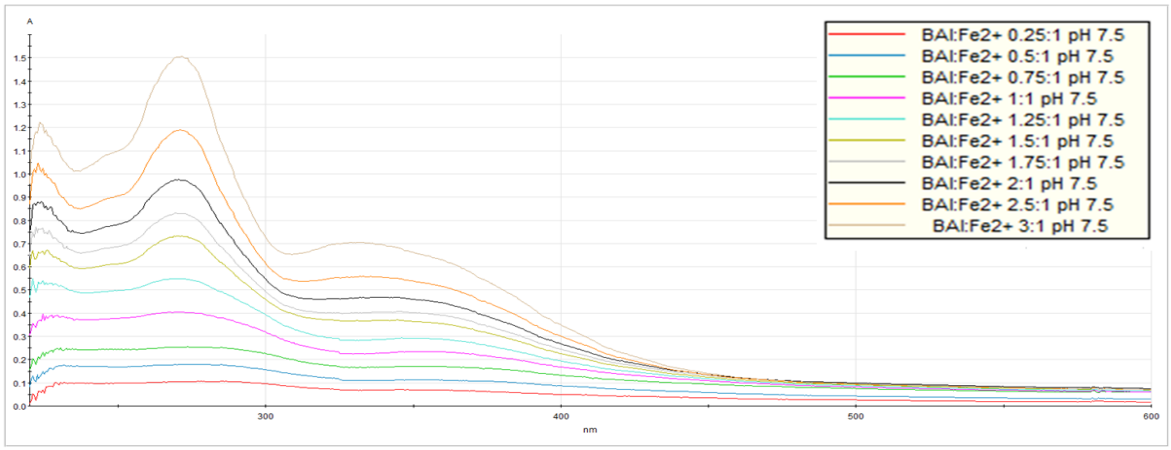


Figure 14: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(II) at pH 7.5

Fe(III), pH 4.5

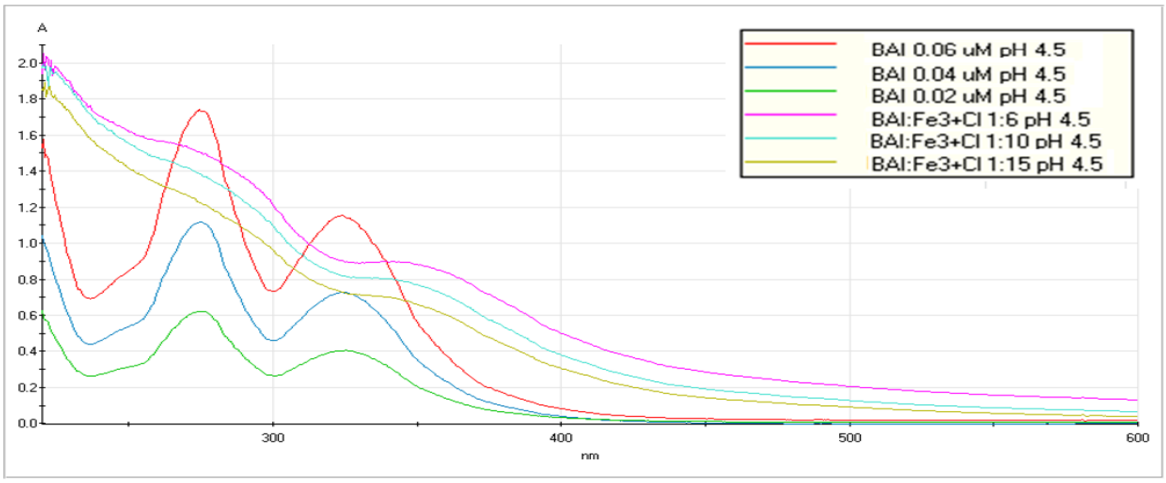


Figure 15: Absorption spectra of baicalein (BAI) and its complex with Fe(III) at pH 4.5

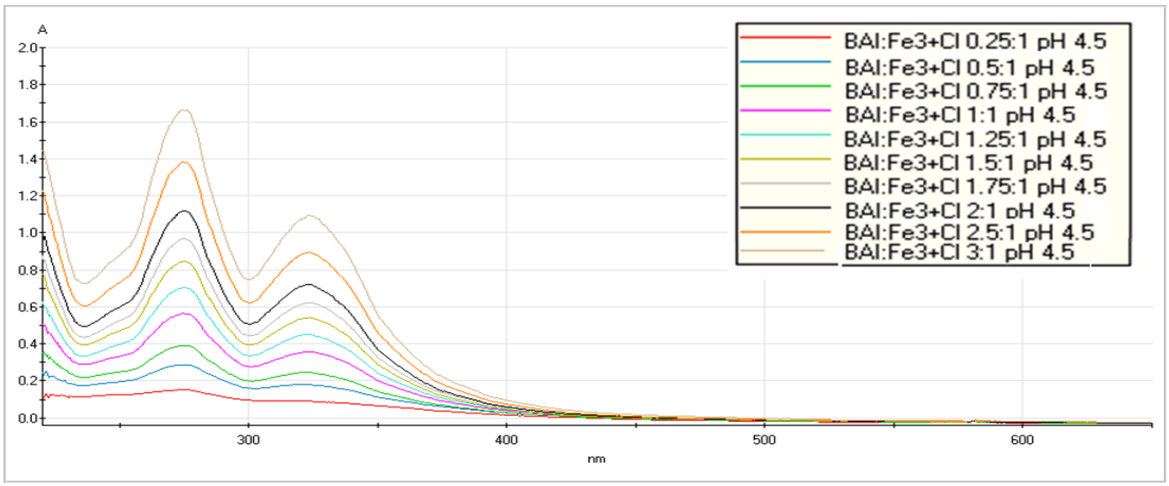


Figure 16: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(III) at pH 4.5

Fe (III), pH 5.5

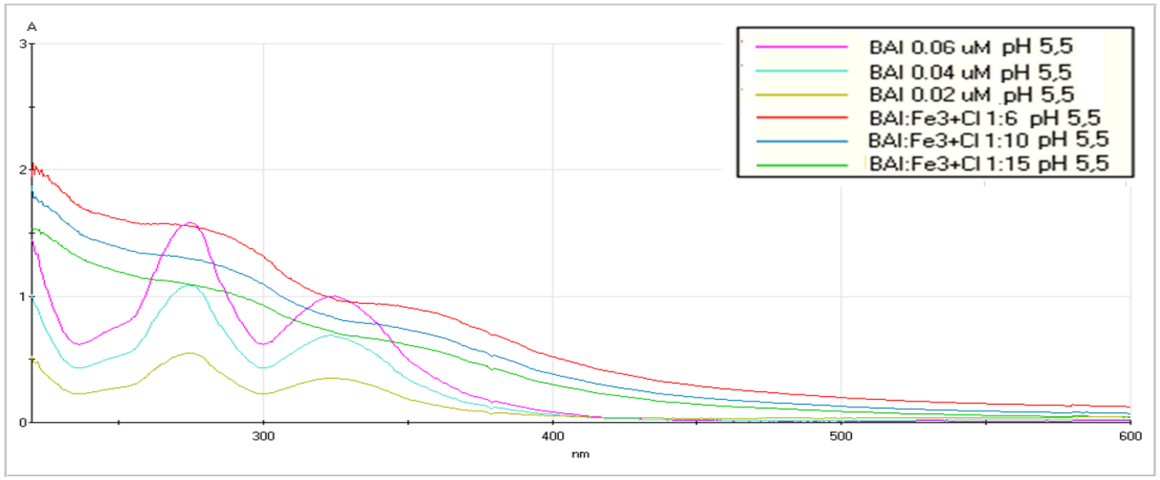


Figure 17: Absorption spectra of baicalein (BAI) and its complex with Fe(III) at pH 5.5

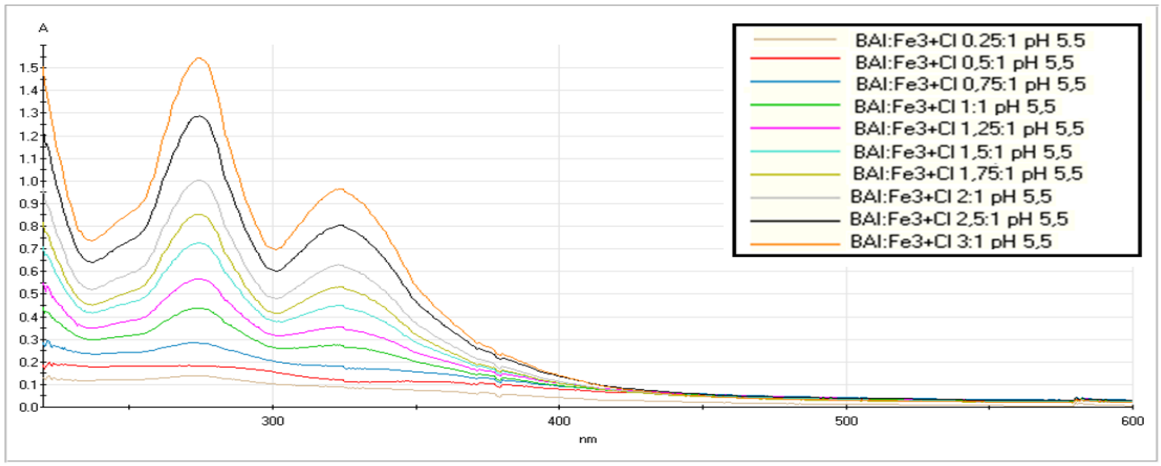


Figure 18: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(III) at pH 5.5

Fe(III), pH 6.8

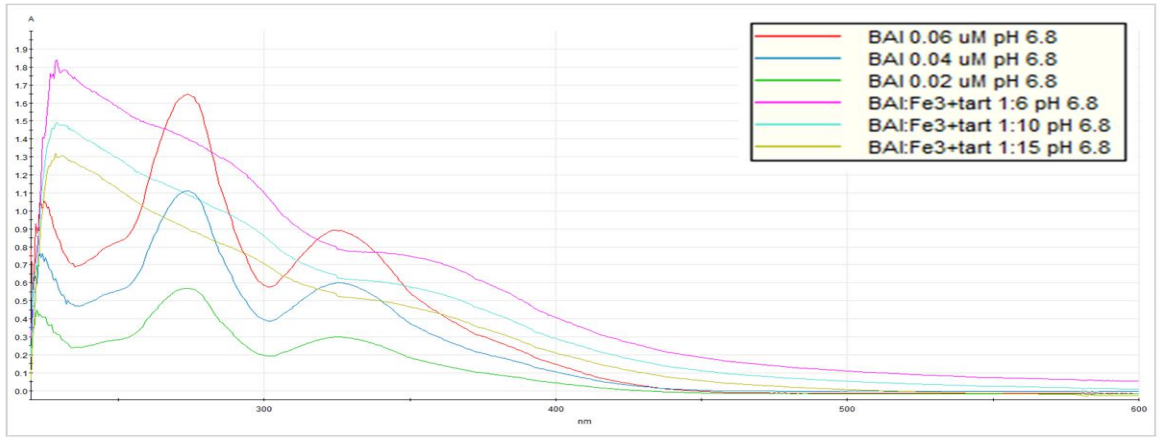


Figure 19: Absorption spectra of baicalein (BAI) and its complex with Fe(III) at pH 6.8

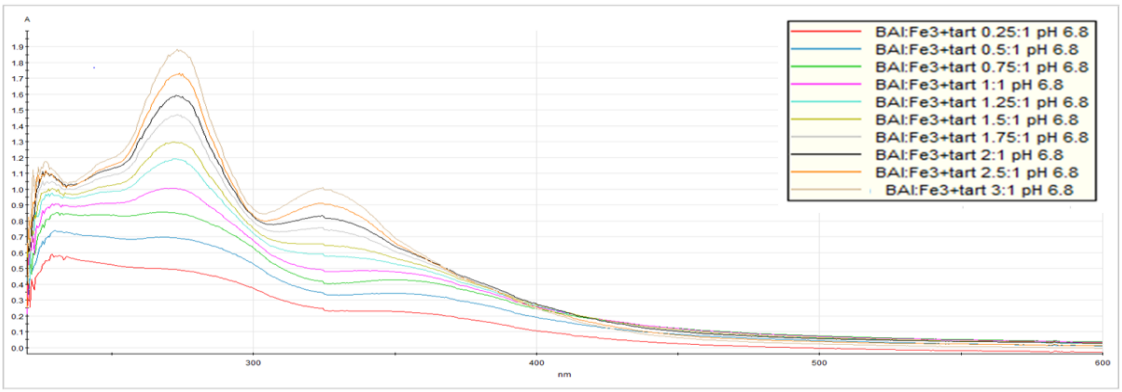


Figure 20: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(III) at pH 6.8

Fe(III), pH 7.5

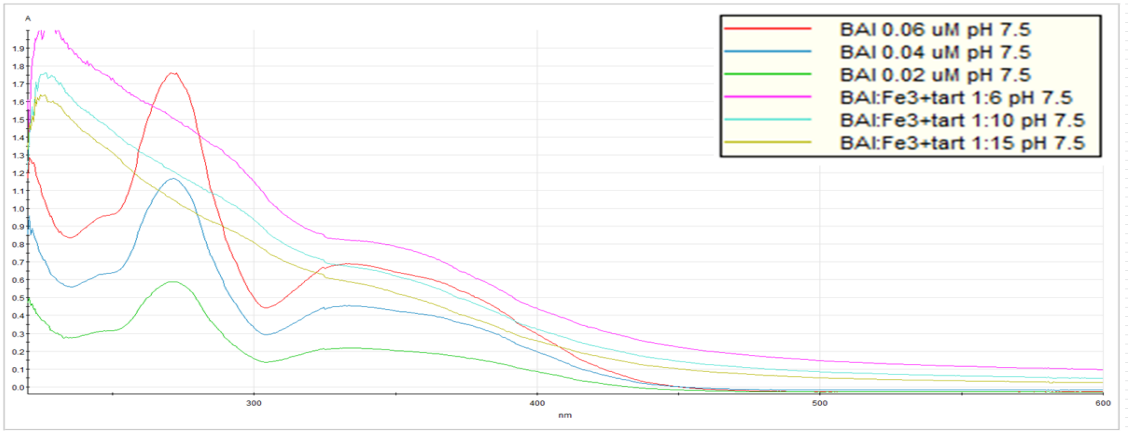


Figure 21: Absorption spectra of baicalein (BAI) and its complex with Fe(III) at pH 7.5

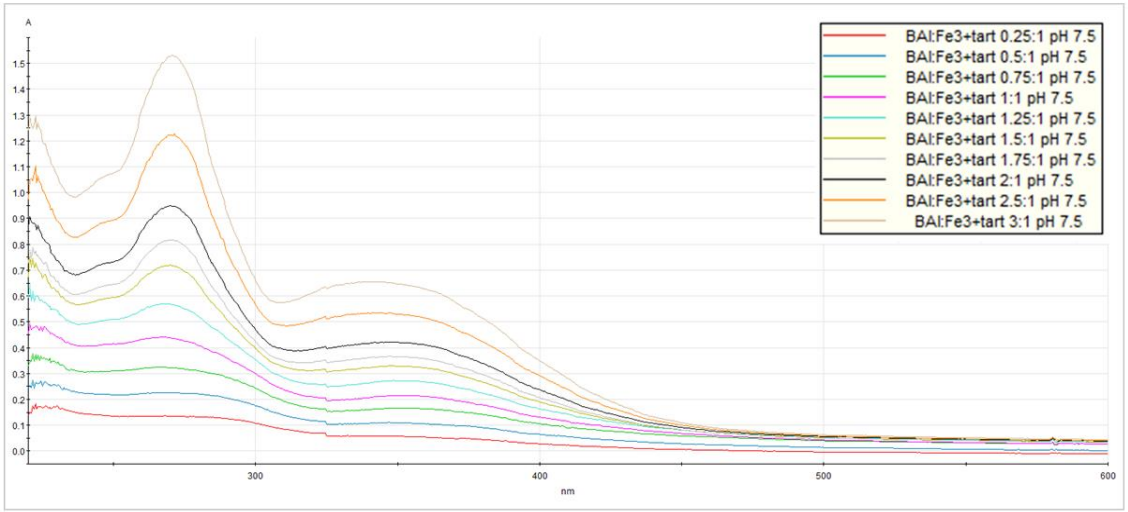


Figure 22: UV – VIS spectra - complementary approach for baicalein (BAI) with Fe(II) at pH 7.5

4.1.2 Experimental results of baicalin

Similar experiments as with baicalein were performed with baicalin (Figs. 23, 25, 27, 29, 31, 33, 35, 37).

Fe (II), pH 4.5

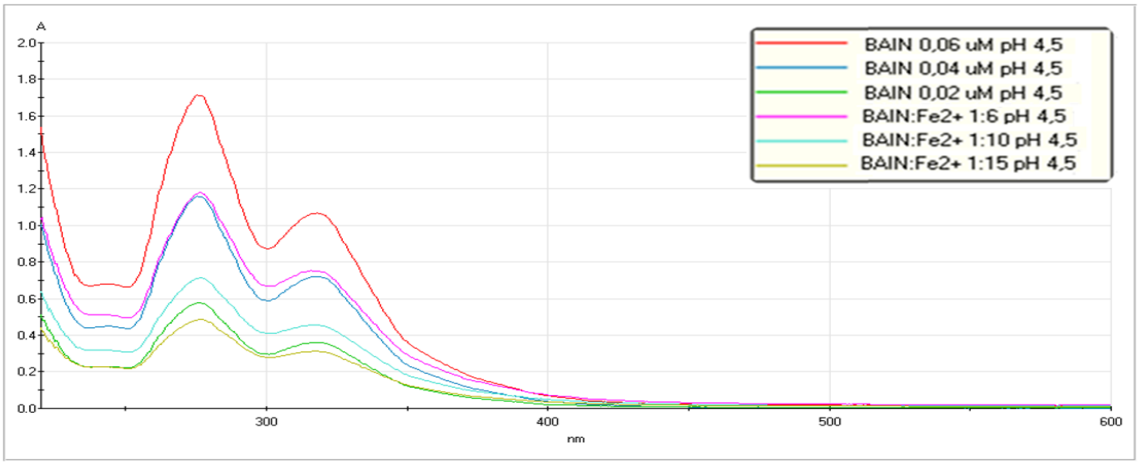


Figure 23: Absorption spectra of baicalin (BAIN) and its complex with Fe(II) at pH 4.5

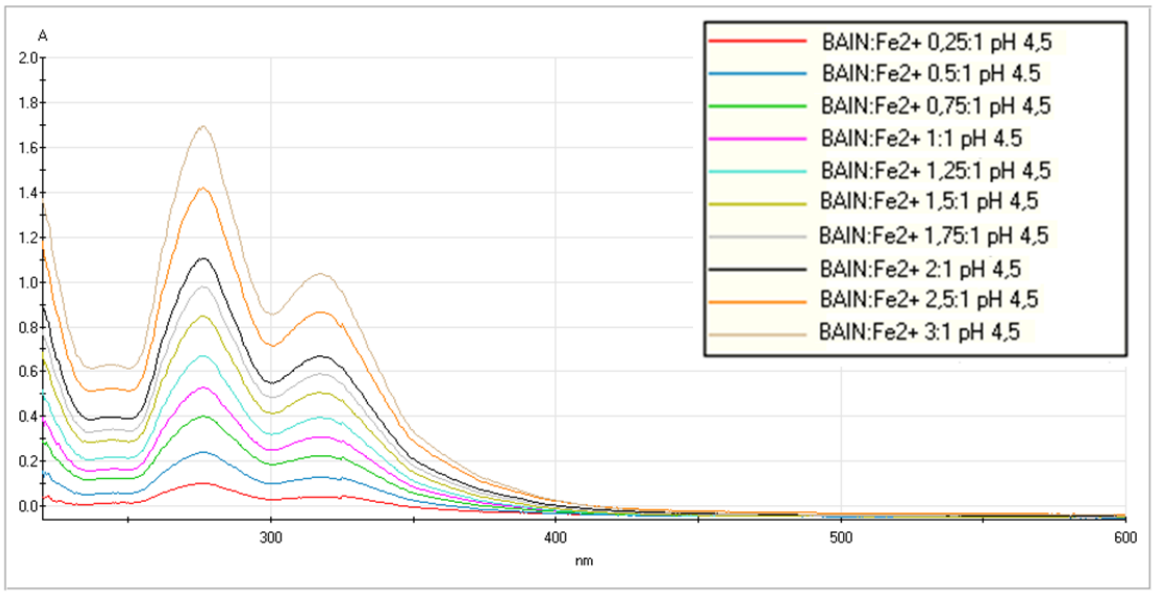


Figure 24: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(II) at pH 4.5

Fe(II), pH 5.5

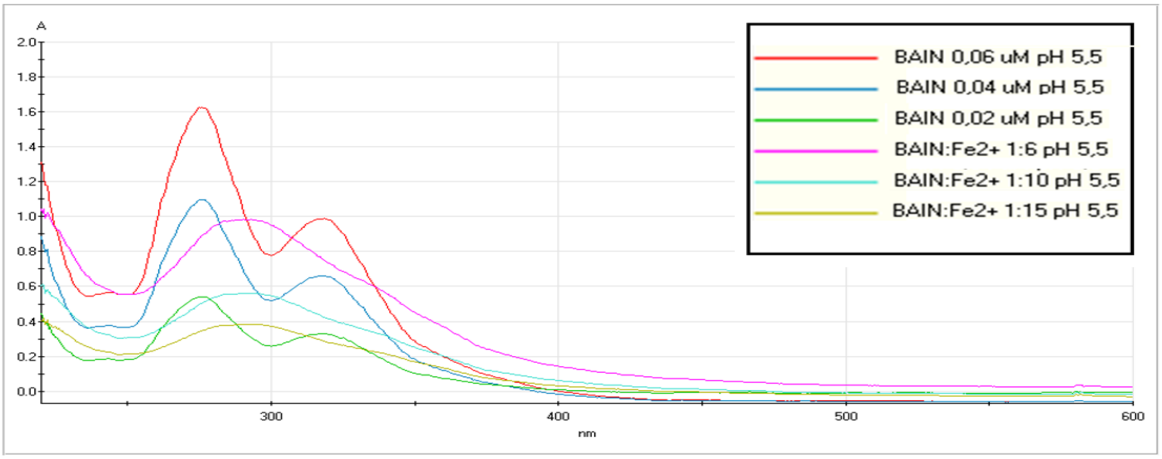


Figure 25: Absorption spectra of baicalin (BAIN) and its complex with Fe(II) at pH 5.5

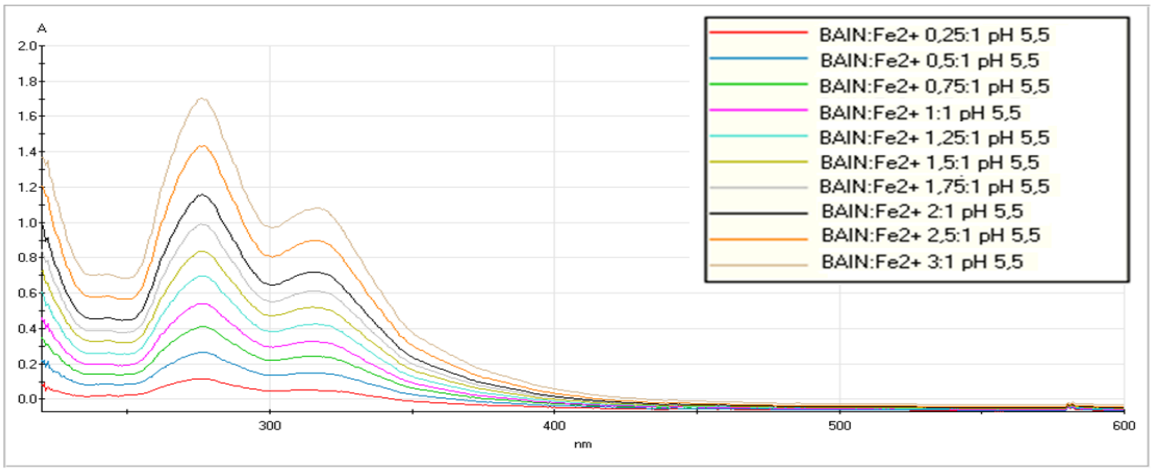


Figure 26: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(II) at pH 5.5

Fe(II), pH 6.8

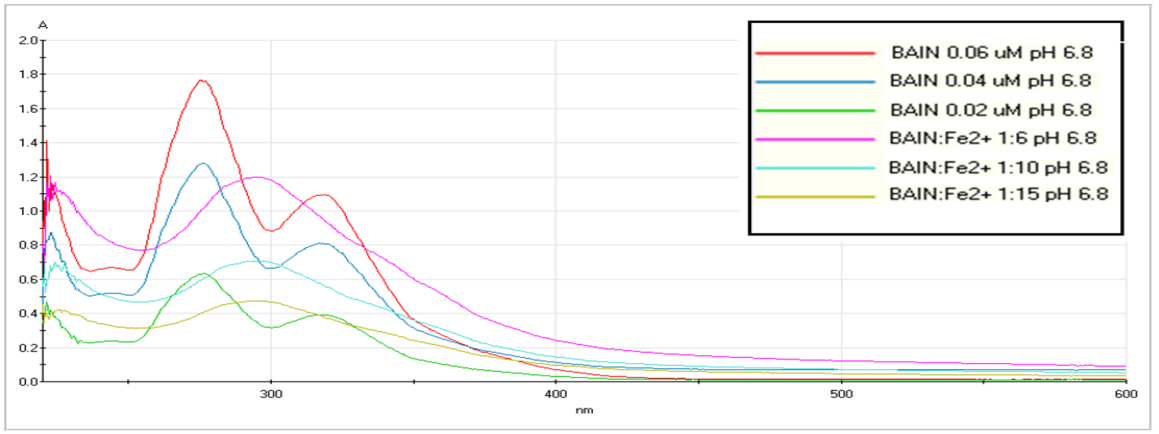


Figure 27: Absorption spectra of baicalin (BAIN) and its complex with Fe(II) at pH 6.8

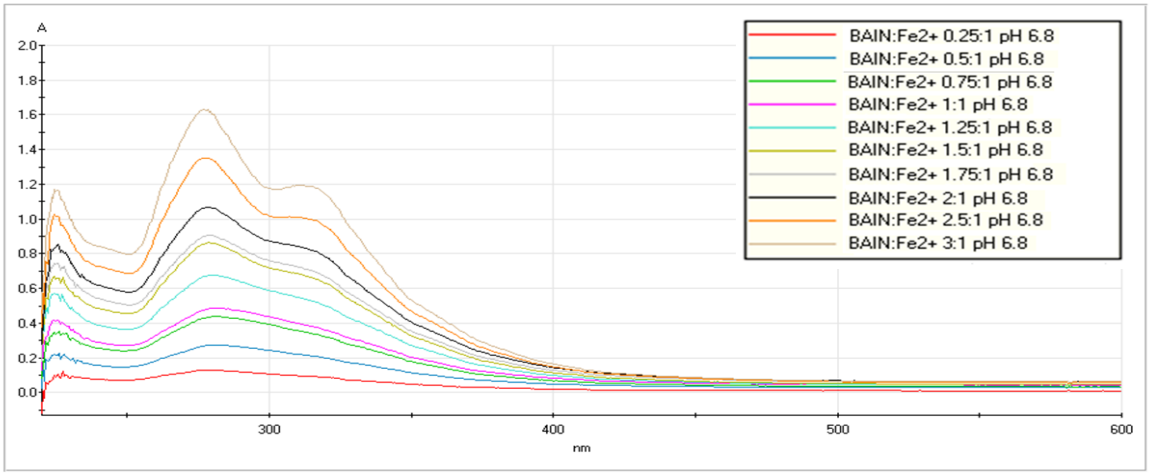


Figure 28: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(II) at pH 6.8

Fe(II), pH 7.5

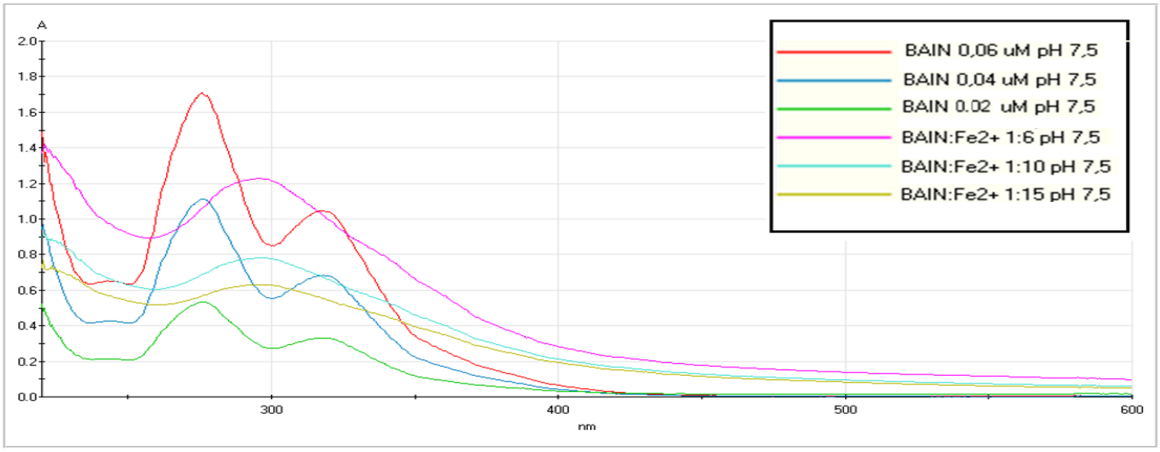


Figure 29: Absorption spectra of baicalin (BAIN) and its complex with Fe(II) at pH 7.5

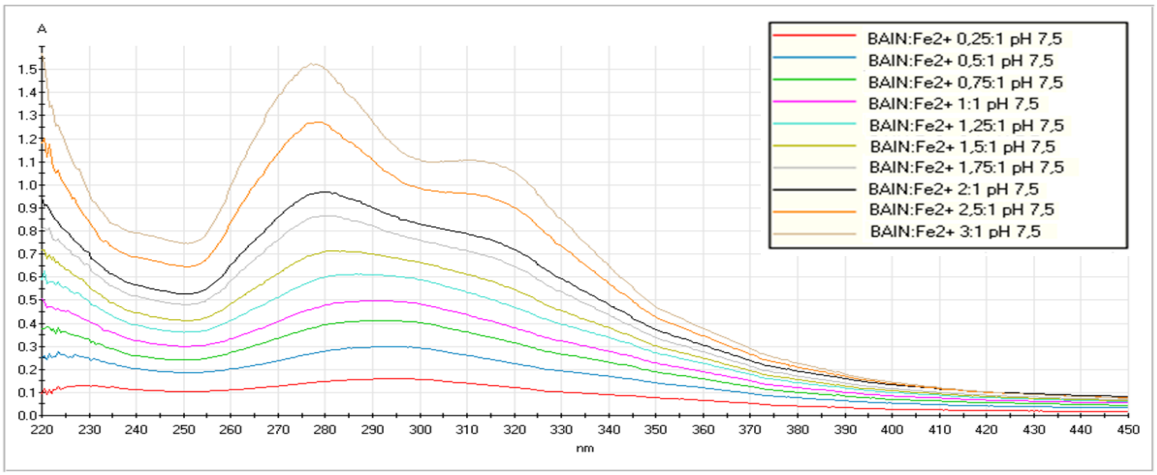


Figure 30: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(II) at pH 7.5

Fe(III), pH 4.5

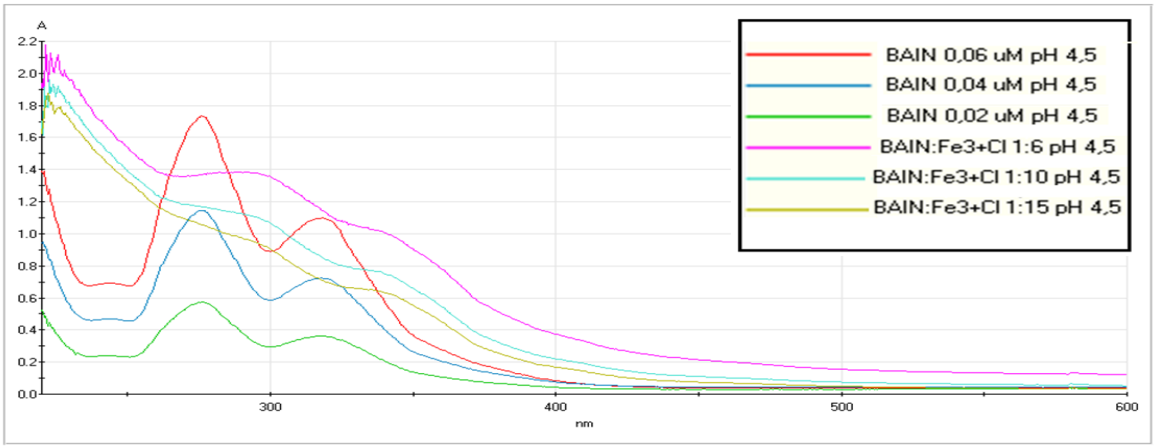


Figure 31: Absorption spectra of baicalin (BAIN) and its complex with Fe(III) at pH 4.5

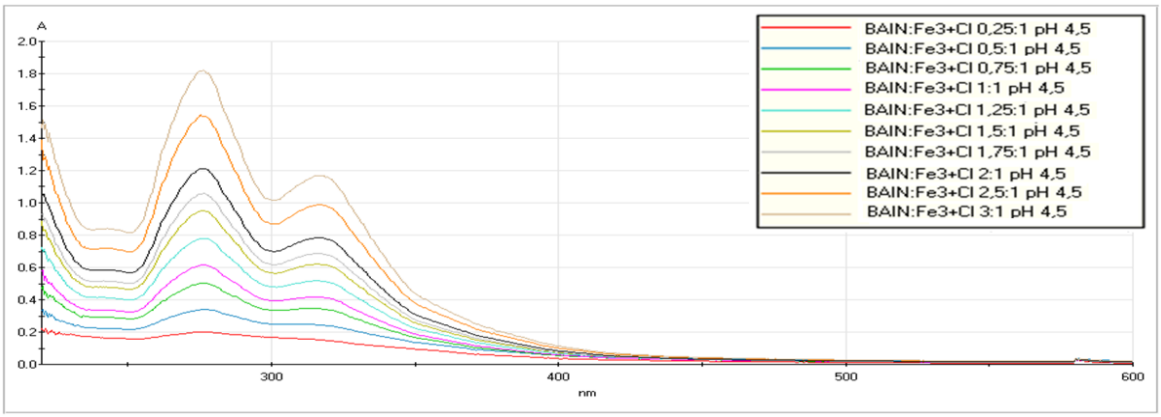


Figure 32: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(III) at pH 4.5

Fe(III), pH 5.5

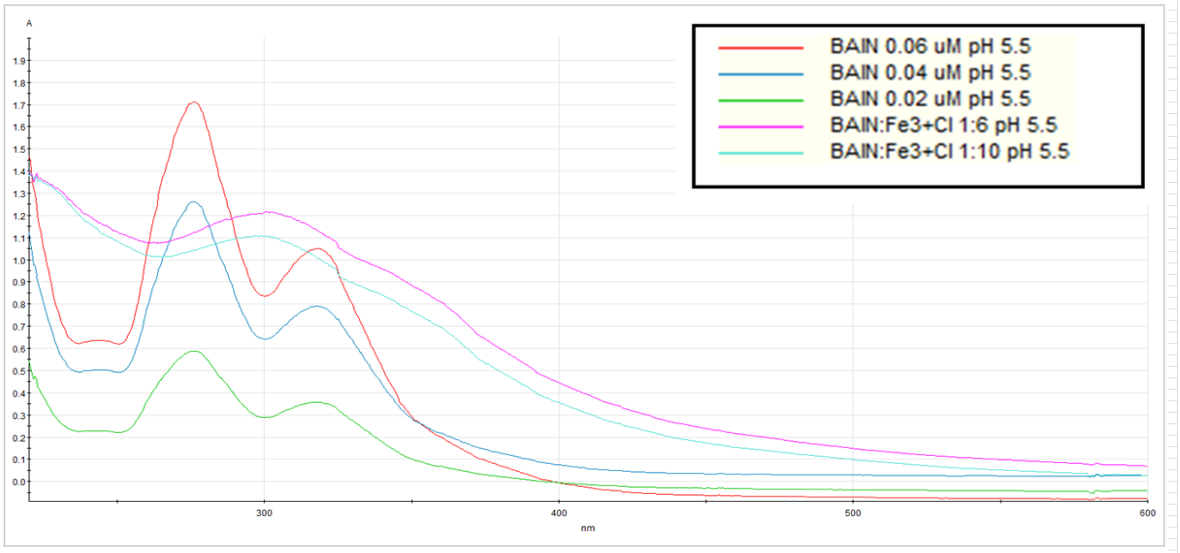


Figure 33: Absorption spectra of baicalin (BAIN) and its complex with Fe(III) at pH 5.5

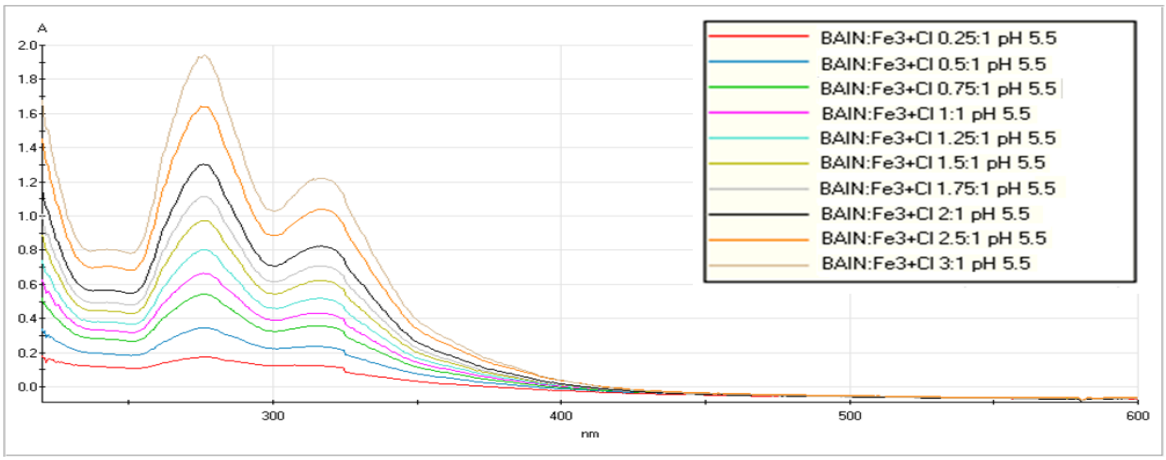


Figure 34: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(III) at pH 5.5

Fe(III), pH 6.8

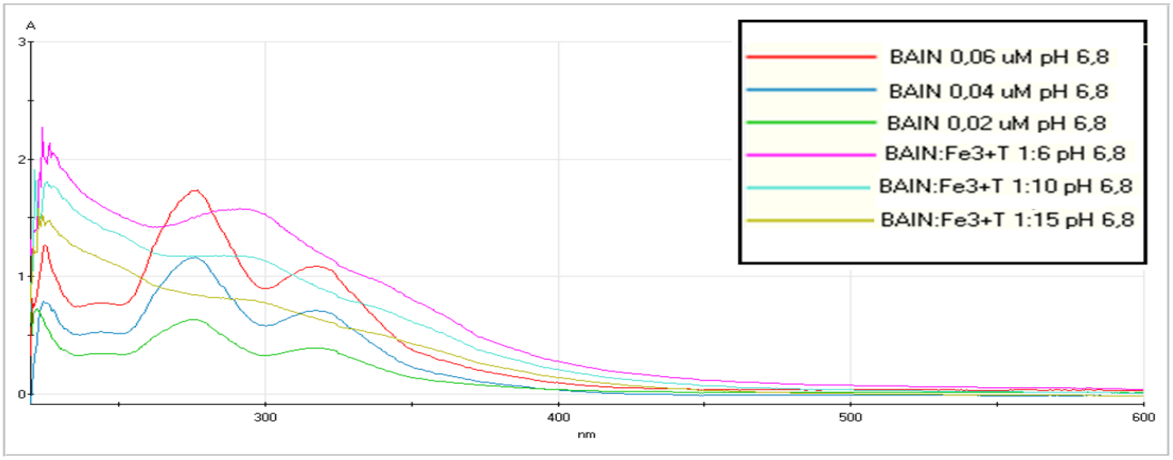


Figure 35: Absorption spectra of baicalin (BAIN) and its complex with Fe(III) at pH 6.8

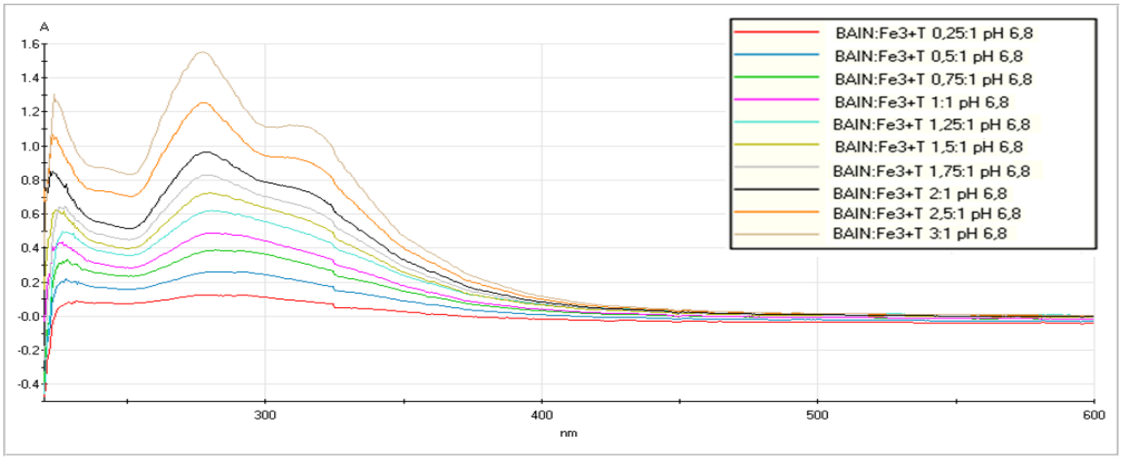


Figure 36: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(III) at pH 6.8

Fe(III), pH 7.5

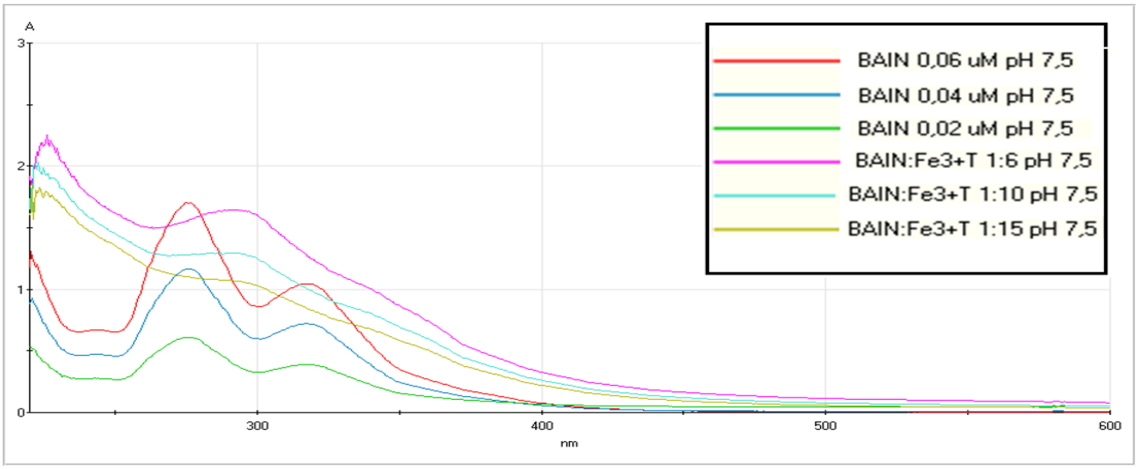


Figure 37: Absorption spectra of baicalin (BAIN) and its complex with Fe(III) at pH 7.5

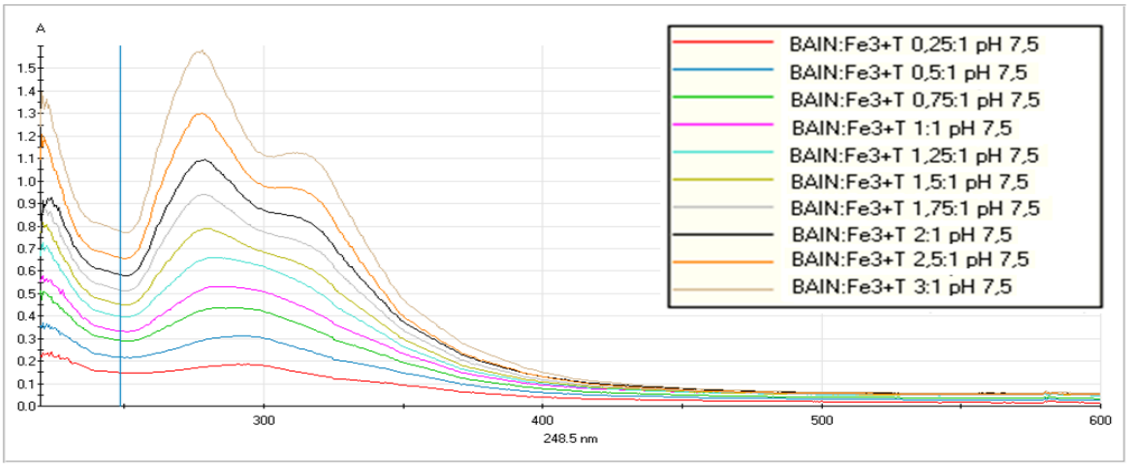


Figure 38: UV – VIS spectra - complementary approach for baicalin (BAIN) with Fe(III) at pH 7.5

Having mixed BAI or BAIN with the excess of ferrous or ferric ions, a significant absorption spectra change was detected at all tested pH levels (Figs. 7, 9, 11, 13, 15, 17, 19, 21, 25, 27, 29, 31, 33, 35, 37). This implies the formation of the substance-iron complex. The only exception in the overall complex formation process was BAIN at pH 4.5 with ferrous ions as seen in the Figure 23, where apparently no complex formation was observed.

In contrast to BAIN, BAI formed a complex at this pH with ferrous ions (Fig. 7) but from the absorption spectra, it is difficult to predict the stoichiometrical ratio (Fig. 8). At other pH values, it is easier to predict the chelation ratio of the BAI and iron from the absorption spectra by using the complementary approach (Figs. 10, 12, 14). It seems most likely that

the chelation ratio is 1:1 (pink line) in all measurements. The shape of this particular line is reminiscent of the absorption spectra of the pure substance; and if we compare it to the next line (light blue, chelation ratio 1.25:1), whose shape differs from the absorption spectra of the substance, it seems safe to assume that the chelation ratio is 1:1. From the light blue line and all the other lines, it is obvious that all iron has been consumed in the complex formation process, because with higher chelation ratios the absorption spectra become more similar to the absorption spectra of the tested substance, indicating the surplus of the substance.

When BAI was mixed with ferric ions, from the absorption spectra at pH 4.5 and 5.5 (Figs. 16, 18) it is difficult to predict the precise stoichiometrical ratio, as there seems to have been no significant spectra change after the addition of the extra BAI. It is possible that the formation process was insufficient or that the method used provides unreliable ratio predictions. The absorption lines are quite similar to the absorption spectrum of the substance, indicating that little or no complex was formed during the procedure. In contrast to the measurements at pH 4.5 and pH 5.5, it is easier to predict the formation of the complex from the absorption spectra using the complementary approach (Figures 20, 22). It seems that the chelation ratio is 1:1 (pink line), as the absorption spectra in subsequent lines become more similar to the absorption spectra of the pure substance. It seems safe to conclude that after this particular ratio all iron has been used for the complex formation and that there is a surplus of the substance.

As mentioned, when BAIN was mixed with ferrous ions, from the substance-complex absorption spectra (Fig. 23), it can be ascertained that at pH 4.5 BAIN failed to produce a complex with Fe(II). Only the absorption spectrum of the pure substance was observed. The measurement was performed twice, so it is safe to conclude that, in these conditions, no chelation with Fe(II) occurred. The absorption spectrum obtained by means of the complementary approach (Fig. 24) confirms that only the pure substance was measured (as indicated by lines that are similar to the substance absorption spectra) and that no complex was formed. There is only one peak in the measurement of complex formations at other pH values, which indicates that the amount of the complex formed is actually quite small. Very low chelation value is also seen in the absorption spectra obtained by the complementary approach at pH 5.5 and 7.5 (Figs. 26, 30). It is difficult to predict the

chelation ratio, because there seems to have been no significant spectra change with addition of the extra BAIN. Thus, the most probable explanation is that no or very small amount of complex was formed. The absorption spectrum of the complementary approach at pH 6.8 (Fig. 28) indicates that the most probable chelation ratio is 1:1 (pink line), because the next line (light blue, chleation ratio 1.25:1) is positioned significantly higher and approaches the shape of the absorption spectrum of the pure substance, indicating the surplus of the substance.

The low chelation value can be observed in the absorption spectra obtained by the complementary approach with BAIN and ferric ions (Figs. 32, 34, 36). No significant spectra change was observed after the addition of the extra BAIN, making any predictions about the chelation ratio difficult. The absorption spectrum of the complementary approach at pH 7.5 (Fig. 38) indicates that the most probable chelation ratio is 1:1 (pink line), because the next line (light blue, chleation ratio 1.25:1) is higher and takes on the shape of the absorption spectrum of the pure substance, indicating the surplus of the tested substance.

Mathematical analysis of data

For adequate mathematical analysis of data and calculation of stoichiometric ratio we needed the data of absorption maxima and molar absorption coefficient of pure compound and corresponding complex. The absorption maxima of BAI, BAIN and their complex with ferrous and ferric ions are summarized in Table IV and Table V and their molar absorption coefficients are summarized in Table VI and VII.

Table IV: Absorption maxima of baicalein and its iron complexes (values are the average of 2 or 3 performed measurements)

pH	λ_{max} baicalein (nm)	λ_{max} baicalein + Fe(II) (nm)	λ_{max} baicalein (nm)	λ_{max} baicalein + Fe(III) (nm)
pH 4.5	274.0 324.0	274.0*	274.5 324.0	276.0 343.5
pH 5.5	273.5 324.0	274.0 346.5	274.5 324.0	267.0 346.0
pH 6.8	273.0 324.5	271.5 345.5	273.5 324.5	375.5 343.5
pH 7.5	271.5 329.5	272.5 345.5	271.5 333.5	274.0 341.0

***only one measurement due to the low chelation**

Table V: Absorption maxima of baicalin and its iron complexes (values are the average of 2 or 3 performed measurements)

pH	λ_{max} baicalin (nm)	λ_{max} baicalin + Fe(II) (nm)	λ_{max} baicalin (nm)	λ_{max} baicalin + Fe(III) (nm)
pH 4.5	275.5 318.0	X	276.0 317.0	292.5*
pH 5.5	275.5 317.5	293.0*	276.0 317.0	300.5*
pH 6.8	276.0 318.0	294.5*	276.5 317.5	291.0*
pH 7.5	276.0 318.0	297.0*	275.5 317.0	291.5*

X – no complex formation was observed, *only one measurement due to the low chelation

Table VI: Molar absorption coefficients of baicalein and its complex with iron

pH	ε baicalein ($mol^{-1}cm^{-1}l$)	ε baicalein + Fe(II) ($mol^{-1}cm^{-1}l$)	ε baicalein ($mol^{-1}cm^{-1}l$)	ε baicalein + Fe(III) ($mol^{-1}cm^{-1}l$)
pH 4.5	26033.3 9092.5	/	27204.2 17875.0	16847.1 11524.3
pH 5.5	22350.0 13475.0	7714.3 6420.0	25875.0 16175.0	17871.4 11528.6
pH 6.8	22750.0 12500.0	11640.0 8280.0	26787.5 14650.0	19251.4 10384.3
pH 7.5	26675.0 11800.0	14160.0 8400.0	29250.0 11825.0	17678.6 9587.1

Table VII: Molar absorption coefficients of baicalin and its complex with iron

pH	ε baicalin ($mol^{-1}cm^{-1}l$)	ε baicalin + Fe(II) ($mol^{-1}cm^{-1}l$)	ε baicalin ($mol^{-1}cm^{-1}l$)	ε baicalin + Fe(III) ($mol^{-1}cm^{-1}l$)
pH 4,5	28550.0 17700.0	/	27262.5 17562.5	15784.3
pH 5,5	27625.0 16987.5	24188.6	28100.0 17275.0	11160.0
pH 6,8	28375.0 17600.0	29074.3	27450.0 17400.0	29708.6
pH 7,5	29375.0 17875.0	26760.0	27250.0 16400.0	22594.3

For accurate calculations of stoichiometrical ratios we also needed the following parameters – absorbencies at absorption maxima (Tables VIII - XI), slopes of calibration curve of the tested substance at absorption maximum, slopes of calibration curve of the tested substance with iron complex at absorption maximum and intercepts of calibration curve of substance. Tables VIII-XI are summarized values of measured absorbencies at two different absorption maxima (λ_{max}) at particular substance and its iron complexes (Tables IV and V).

Table VIII: Absorbencies of baicalein and its Fe(II) complexes (values are the average of 2 or 3 performed measurements)

	absorbance of substance		**absorbance of complex**	
pH	first peak	second peak	first peak	second peak
4.5	/*	/	/	/
5.5	0.596	0.342	0.596	0.281
6.8	0.577	0.300	0.578	0.278
7.5	0.682	0.336	0.681	0.338

*- there was no peak

Table IX: Absorbencies of baicalein and its Fe(III) complexes (values are the average of 2 or 3 performed measurements)

	absorbance of substance		**absorbance of complex**	
pH	first peak	second peak	first peak	second peak
4.5	0.823	0.538	0.820	0.378
5.5	0.709	0.438	0.651	0.293
6.8	0.692	0.348	0.685	0.312
7.5	0.719	0.323	0.709	0.329

Table X: Absorbencies of baicalin and its Fe(II) complexes (values are the average of 2 or 3 performed measurements)

	absorbance of substance		**absorbance of complex**	
pH	first peak	second peak	first peak	second peak
4.5	0.817	0.502	/*	/
5.5	0.811	0.507	0.523	/
6.8	0.771	0.577	0.660	/
7.5	0.704	0.550	0.637	/

*-there was no peak

Table XI: Absorbencies of baicalein and its Fe(III) complexes (values are the average of 2 or 3 performed measurements)

	absorbance of substance		**absorbance of complex**	
pH	first peak	second peak	first peak	second peak
4.5	0.902	0.590	0.610	/*
5.5	0.936	0.601	0.527	/
6.8	0.692	0.513	0.628	/
7.5	0.771	0.590	0.698	/

*-there was no peak

Interestingly, because the molar absorption coefficient for the complex was generally lower than that of the pure compound (Table VI and VII), some calculations may not lead the reasonable results. This is true for the pH 4.5 and ferrous ions, where a clear shift of spectra was observed in the case of iron excess (Fig. 6), but in comparable concentration of iron to BAI, the spectra of the complex had similar shape as the pure compound (Fig. 6 vs. Fig. 7). This means that the complex is formed but likely in low quantity. No complex stoichiometry could be calculated for this particular pH. Summarized results of the data obtained by mathematical analysis for BAI, BAIN and their complexes with iron are shown in Tables XII, XIII and XIV.

Table XII: Summarized results of stoichiometrical ratios of baicalein and iron, according to the complementary approach

	Fe(II)				Fe(III)			
method/pH	**4.5**	**5.5**	**6.8**	**7.5**	**4.5**	**5.5**	**6.8**	**7.5**
Method I	x	*1:1**	x	x	x	x	x	x
Method II	x	x	*1:1*	*1:1*	x	x	1:1	x
Method III	x	x	x	x	x	x	x	x
Method IV	x	x	x	x	x	x	1:1	x
Method V	x	1:1	1:1	*1:1*	*1:1*	1:1	1:1	1:1
Method VI	x	*1:1*	1:1	1:1	1:1	1:1	1:1	1:1

x - after mathematical calculation adequate ratio were not clear

*chelation ratio is the most probable, mathematical calculation did not give accurate results

Table XIII: Summarized results of stoichiometrical ratios of baicalin and iron, according to the complementary approach

	Fe(II)				Fe(III)			
method/pH	**4.5**	**5.5**	**6.8**	**7.5**	**4.5**	**5.5**	**6.8**	**7.5**
Method I	x	x	x	x	x	x	x	x
Method II	x	x	x	*1:1**	x	x	x	*1:1*
Method III	x	x	x	x	x	x	x	x
Method IV	x	x	x	1:1	x	x	x	x
Method V	x	x	1:1	1:1	x	x	*1:1*	x
Method VI	x	x	1:1	1:1	x	x	1:1	1:1

x - after mathematical calculation adequate ratio were not clear

*chelation ratio is the most probable, mathematical calculation did not give accurate results

Table XIV: Summarized results of stoichiometrical ratio obtained by mathematical analysis

Substance	Iron	pH	Complementary approach
Baicalein	Fe(II)	4.5	↓*
		5.5	1:1
		6.8	1:1
		7.5	1:1
	Fe(III)	4.5	1:1
		5.5	1:1
		6.8	1:1
		7.5	1:1
Baicalin	Fe(II)	4.5	0*
		5.5	↓
		6.8	1:1
		7.5	1:1
	Fe(III)	4.5	↓
		5.5	↓
		6.8	1:1
		7.5	1:1

***↓ - low chelation, 0 - no chelation at all**

5. DISCUSSION

BAI and BAIN are two bioactive flavonoid substances that originate from the medicinal herb Baikal skullcap (*Scullateria baicalensis* Georgi) and are recognized under the name of »Ogon« in Japan and »Huang qin« in China. For centuries, both substances have been used in the treatment of various symptoms, most commonly fever, insomnia, and hyperhidrosis. Because of their promising positive effects on human health, the consumption of BAI and BAIN is increasing throughout the United States and Europe. In recent years, BAI and its glucuronide BAIN have been the main focus of numerous studies whose findings suggest that they might offer a wide spectrum of health benefits, the most promising of which seems to be their antioxidant activity (34).

“Free” iron in the LIP can participate in the Fenton reaction and hence it can react with endogenous H_2O_2; the end products of this reaction are hydroxyl radicals, which are known for their high reactivity and short half-lives. Hydroxyl radicals have a deleterious effect on the human organism, as they can oxidize proteins, nucleic acids, or cell membranes. Flavonoids are ubiquitous plant secondary metabolites found in almost all parts of plants; structurally, they fall into the category of diphenylpropanes (C6-C3-C6). Their general chemical structure is of a 15-carbon skeleton with 2 aromatic rings (A and B), connected with heterocyclic ring (C) (11, 34).

The aim of our experiment was to determine the effect of the 6,7-dihydroxy group in flavones on iron chelation. The substance under study was BAI (5,6,7-trihydroxyflavone) and its corresponding glycoside BAIN (baicalein 7-O-β-D-glucuronide). Because both substances have »iron-binding motifs«, it is expected that they will bind iron. The stoichimetrical ratio of iron:flavonoid complex differs in relation to pH levels and structural differences. Iron binding is one of the possible mechanisms of the antioxidant activity of flavonoids; the SARs for scavenging free radicals and iron binding differ significantly (11, 15, 34).

The iron chelation capacities of the two substances were measured with the UV-VIS spectrophotometry using the complementary approach. What is typical of the complementary approach is that the concentration of the iron was constant throughout the

measurement (0.02 mM), while the concentration of the tested substance was increased progressively. The substance-iron molar ratio ranged from 0.25:1 to 3:1. Capacities of iron binding in the conjugated ring system of tested substance over iron are probably associated with spectroscopic changes, which can be attributed to delocalization of π electrons. The acquired data were analysed with mathematical calculations designed to determine the chelation stoichiometry (28, 31).

5.1 Impact of pH

It is well known that catechol, gallol, and certain other functionalized derivatives are recognized as effective metal chelators. For efficient metal binding it is vital that they are present in the deprotonated form, which is why both functional groups are referred to as gallate group and catecholate group. Both iron ions (Fe(II) and Fe(III)) have the preference for octahedral geometry and are therefore able to coordinate up to 3 catecholate or gallate groups. Since polyphenols form a structurally diverse group of plant metabolites, the formation of the complexes is pH-dependent and frequently exhibits variable coordination modes. In the presence of iron, polyphenol compounds are effortlessly deprotonated at or below physiological pH, despite that pK_a values for the most acidic phenolic hydrogen are in the range between 7 and 9. pH value plays a very important role in the process of complex formation. Flavonoids act as weak polybasic acids and thus remain undissociated at the lower pH values (pH < 3), which is unfavourable for complex formation. The optimal pH in this regard is about 6, but the precise value can vary according to the specific metal ion. At higher pH values flavonoids are deprotonated and consequently the ratio of formed complexes is higher (12, 22). Our substances were tested in 4 media with different pH values – 4.5, 5.5, 6.8 and 7.5. Lower pH values facilitate iron absorption in the proximity of the gastro-duodendal junction. In addition, the most acidic pH used in our experiment is also present in lysosomes, cellular organelles that are important in iron metabolism. Lower pH values are also documented in tumours. Other two acidic pH levels (5.5, 6.8) mimic the pathophysiological conditions of moderate to severe ischemia, where the role of iron in tissue damage is well-documented (31).

5.2 Baicalein

According to theory, the degree of chelation at lower pH values should be low or maybe non-existent because of the undissociated form, but our results from the absorption spectra showed BAI to form complexes with Fe(II) at all tested pH levels. The same results were observed with Fe(III). It should be noted, however, that the molar absorption coefficient of the complex was generally lower than that of the pure compound and thus the results may not be fully reliable. Obtained UV-Vis spectra were analysed by complementary approach for adequately determination of the accurate chelation values. The analysis of the spectra with Fe(II) at pH 4.5 was unable to provide an accurate prediction of the stoichiometric ratio of the complex, because the spectra closely resemble the shape of the pure compound. For this reason, i.e. because so little of the complex was formed under the specified conditions, no mathematical calculations were performed in this particular example. Having performed the appropriate mathematical calculations on all other samples, we were able to determine the exact chelation ratio of the tested compound with iron. The results showed that at other pH values the chelation ratio is 1:1 (Table XIV). With Fe(III) the complementary spectra of the tested substance mixed with iron at pH 4.5 and 5.5 were not as clear, but it was observed that some complex formation did occur. The results of mathematical calculations revealed that, at all measured pH levels, the chelation ratio was 1:1 (Table XII). The experimental results thus suggest that BAI is a highly effective iron chelator, especially because of its pronounced chelation capacities at different pH values. For this reason, BAI might have high-ranging therapeutic potential *in vitro*.

5.3 Baicalin

BAIN is BAI with glucuronic acid attached to the position 7 of the A-ring. In comparison to BAI, the results showed a lower degree of chelation with both ferric and ferrous ions. In addition, the mathematical analysis revealed that in the complementary approach the rate of complex formation was lower in BAIN, and that BAIN had lower affinity for iron in comparison to BAI (this holds true for both sources of iron). The only instance where no complex was formed, was the mixture of BAIN and Fe(II) at pH 4.5; the shape of the absorption curve turned out to be the same as the shape of the pure compound. Measurements were performed 3 times, giving strong support to the claim that at this particular pH level no complex formation occurred. At pH 5.5, the formation of the

complex did occur, but only in very small quantity. For this reason, it was difficult to mathematically determine the level of chelation. According to absorption spectra at pH 6.8 and 7.5, it was clear that the chelation ratio is 1:1. The accurate chelation ratio was also confirmed with mathematical analysis. Similar results were observed with Fe(III). Complex formation was observed at all pH levels, but at pH 4.5 and 5.5, the complex was formed in small quantity, and it was therefore impossible to determine the stoichiometry accurately. At pH 6.8 and 7.5, the chelation ratio turned out to be 1:1.

The chelation ratio in BAIN was expected to be lower than in BAI. In most cases, the chelation of flavonoids with sugar moiety is lower than their corresponding aglycones. This was also confirmed by some other studies (12, 22, 34). Our experimental results confirm these previous findings, with differences being particularly pronounced at lower pH values. Nevertheless, BAIN did show some iron chelation potential, and might therefore prove useful as iron chelator in therapeutic contexts.

5.4 SAR

Numerous studies have been performed with the aim of determining the structure activity relationships for flavonoids and their antioxidant activities. As mentioned before, SARs for different antioxidant mechanisms differ significantly. Our points of interest are functional groups of flavonoids that are important for iron chelation. Results and implications for the SAR in question are different, and sometimes inconsistent. Flavonoids containing metal-complex domains play the key role in the iron chelation processes. These domains, located on the B-ring, are following: (i) between the 4-carbonyl and the 5-hydroxyl group, (ii) between the 4-carbonyl and the 3-hydroxyl group, and (iii) between the 3', 4'-dihydroxyl group. pH value is of crucial importance, since not all positions are equally preferable for iron chelation under different pH levels. The chelation area between the 3', 4'-dihydroxyl group seems to be preferable, but is, in contrast to the first two groups, not readily available under more acidic conditions (15). In one study (34), the NMR spectra change indicates assumption that molecular site on the A ring, where iron is binding, is near the H_8 proton, therefore at the O_6 and O_7. Figure 38 shows the proposed structure for the BAI-Fe(II) (34). This study also suggests that the process of iron chelation plays a crucial role in the ability of BAI to inhibit the damage triggered by the Fenton reactions. Thus, the antioxidant capacities of BAI seem to be

associated primarily with the chelation of Fe(II) and not with the scavenging of free radicals although the latter cannot be excluded.

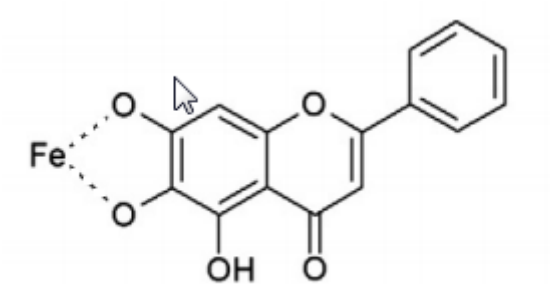


Figure 39: Proposed iron binding with baicalein (1:1) (31)

The importance of the 6,7-dihydroxy group for the iron chelation processes was also confirmed by a study (31), in which 26 different flavonoids were tested in 4 media with different pH values. The main purpose of the study in question was to compare the selected flavonoids with deferoxamine, a clinically used iron chelator. Results showed that the efficiency of BAI was similar to that of deferoxamine (molar ratio 1:1), which is in stark contrast to other flavonols, whose efficacy was considerably lower (molar ratio 10:1). The results of study showed that the 6,7-dihydroxy structure had the most promising results in iron binding. The importance of the 7-OH group in the A-ring was also confirmed by another study (22), whose goal was to determine the acidity of polyphenols. In polyphenols, metal chelation often occurs through the deprotonated hydroxyl. The most favoured deprotonation site for flavonoid compounds was the 4'-position in the B-ring and the second favoured one was the 7-OH group in the A-ring.

In contrast to these findings, another study (35) observed that the function of phenol at the C-6 position is to donate the labile hydrogen. In the experiment preformed, compounds with OH group at the position 7 of the A-ring were inactive. A series of tests on various substances revealed that for the effective ROS scavenging activity, the pyrogallol moiety of the B-ring and/or -OH group in position 6 in the A-ring is favoured. Another study (36) suggests that -OH groups attached to the A-ring perform no significant antioxidant activity. The comparison of 4 different flavonoids revealed that BAI was not successful in suppressing the Fenton reaction. The conclusion was that hydroxylation at the B-ring have

a significant impact on the antioxidant activity, and that the 5,6- and 6,7-hydroxy groups of the A-ring do not have a positive effect on the iron chelation process. Similar conclusions were reached in another study (9), where it was observed that phenol substituents at the-B ring have higher antioxidant potential than those at the-A ring. These findings indicate the important contribution of a catechol functional group at the B-ring to antioxidant activity and iron binding. Another confirmation of this hypothesis was provided by the study (12), which measured the complex formation of BAI. The results of the study imply that there is no detectable complexation in acidic conditions. The provided explanation was that these results stem from the lack of the chelation capacity or the instability of the complex at pH levels below 6. It was observed in 6,7-dihydroxyflavone that the hydroxylation at the position 7 of the A-ring is unfavourable for antioxidant activity (16). It seems probable that not only does the 7-hydroxyl group fail to provide a marked increase in the scavenging activity, but that methylation or glycoslyation might actually help to decrease it (15). Another research (37) suggests that, for the efficient free-radical scavenging to take place, ortho-di or tri-phenolic groups need to be present in the flavones.

Studies suggest that the importance of the antioxidant activity of hydroxylation on the A-ring is still a matter of some debate. The experimental results of our study indicate that BAI might function as a highly effective iron chelator, capable of iron-binding at different pH values. Considering the chemical structure of BAI and the lack of hydroxylation on the B-ring, it seems safe to conclude that the 6,7-dihydroxy group is of significant importance in the iron chelation process. Some studies suggest that there was no detectable chelation at the more acidic pH levels, whereas the results of our study imply that BAI functions as an effective iron chelator at the acidic pH levels as well. Our experimental results with BAIN provided additional confirmation of the key impact attributed to the 7-OH group in the A-ring. Sugar moiety is at the mentioned position, and after the OH group is removed from it, the chelation capacity of flavonoids becomes significantly reduced.

6. CONCLUSION

BAI and BAIN possess iron-binding motifs that are crucial for effective iron chelation. Our main point of interest was the effect of 6,7-dihydroxygroup in the A-ring on flavone skeleton on the iron chelating capacity. After the addition of excess iron, the complex formation occurred in both, BAI and BAIN; the only exception was the mixture of Fe(II) and BAIN at pH 4.5. In a complementary approach molar absorption coefficient was often lower than that of the pure compound. It is possible that in these cases the complex was formed in very low quantities, which made further mathematical analysis impossible. BAIN, a glucuronide of BAI, was supposed to have a lower degree of iron chelation and the results of our study confirm this assumption. In comparison to BAI there were fewer cases at different pH values where stoichiometric ratio could be determined accurately with selected mathematical analysis. With BAI there was only one condition (with Fe(II) at pH 4.5) where precise ratio could not be determined. From our experiments we can conclude that for both tested substances the predominant stoichiometric ratio is 1:1. Particularly clear absorption spectra and consequent accurate determination of stoichiometric ratio with mathematical analysis was for both substances at pH 6.5 and 7.5. Therefore, both BAIN and BAI are potent iron chelators *in vitro*. For further confirmation of these tentative results, additional measurement methods and *in vivo* studies are needed.

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