



Quercetin as an effective antioxidant against superoxide radical

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ABSTRACT

Background: Quercetin is considered one of the most studied flavonols widely found in fruits and vegetables. Food preparation and storage affect the level of quercetin in food, as these processes can cause a partial or complete reduction in flavonol levels. Quercetin, as a bioactive compound, has beneficial effects on the human body and with various diseases due to its potent antioxidant properties. Quercetin is a scavenger of free radicals and exerts its effects in various body fluids (e.g., saliva, synovial fluid, blood). Because of this, it is essential to know the effect of pH on its antioxidant properties.

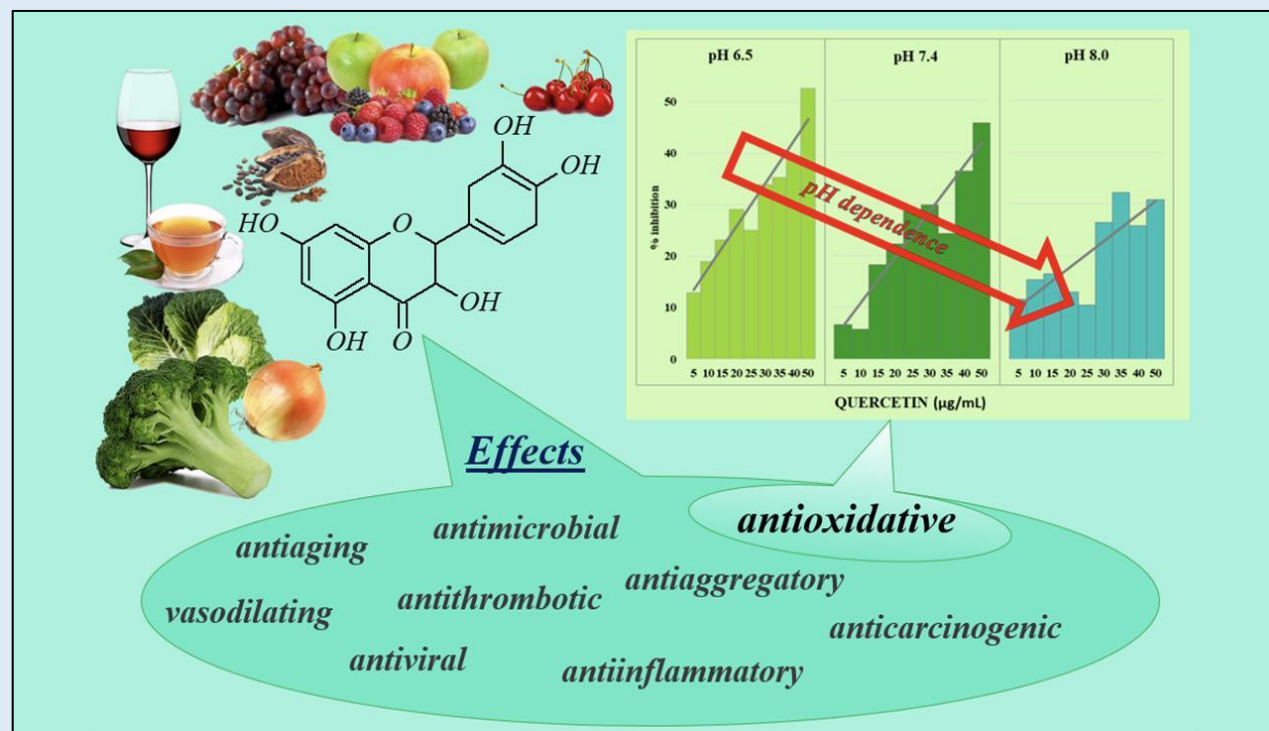
Objective: This study aims to determine the antioxidant capacity of quercetin against superoxide radicals depending on pH using a spectroscopic method.

Methods: The antioxidant properties of quercetin in the concentration range from 5 to 50 µg/ml against superoxide radicals were measured at pH values of 6.5 to 8. Superoxide radicals were generated in the photooxidation reaction of methionine with riboflavin. Inhibition of superoxide radicals by quercetin was detected spectrophotometrically in reaction with NTB.

Results: Quercetin is an effective superoxide radical scavenger with the highest antioxidant capacity observed at a slightly acidic pH of 6.5. The antioxidant activity of quercetin increased with increasing concentration; effective antioxidant capacity of quercetin was in the experiment at a concentration above 30 µg/ml.

Conclusion: Quercetin is a widely occurring flavonoid with many beneficial effects on the human body related to its antioxidant properties. Quercetin is an effective scavenger of superoxide radicals. Its correct dosage, processing, and

storage, which significantly affect the antioxidant properties of quercetin, have a considerable impact on the effects of quercetin in the prevention and treatment of diseases. Since pH significantly affects biochemical processes in living organisms, our results could contribute to expanding information on the antioxidant properties of quercetin as a rich natural source of medicinal substances, depending on pH.



Keywords: quercetin, antioxidant, superoxide radical, spectrophotometry

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INTRODUCTION

Flavonoids (Fig 1A) represent an important group of plant metabolites, widely found in vegetables, fruits, grains, bark, roots, coffee, tea, and wine. Their structure is composed of three rings (C6-C3-C6) labeled as ring A, ring B, and ring C [1-2]. Flavonoids are subdivided into subgroups based on structure: flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins, and chalcones. This large group of plant metabolites is widely known due to their positive effects on the human body in health and diseases. They show anti-oxidative,

anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties associated with their capacity to scavenge reactive nitrogen and oxygen species and alter the function of key cellular enzymes (cyclooxygenase, lipooxygenase, xanthine oxidase, CYP1A2, CYP2A6 and CYP2C8) [2-3]. The largest and most common subgroup of flavonoids is flavonols. (Fig 1B). Quercetin (3,3',4',5,7-pentahydroxyflavone, Fig 1C) is considered one of the most studied flavonols.

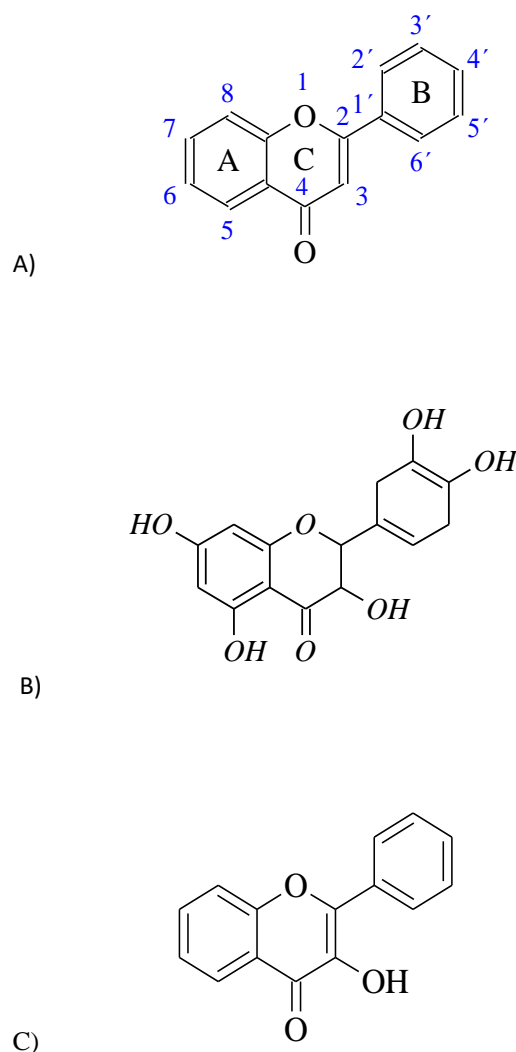


Figure 1. Structure of A) flavonoid, B) flavonol and C) quercetin.

Fruits and vegetables, such as apples, cherries, berries, kale, broccoli, and onion, are primary sources of quercetin in the diet. Its presence was also found in cocoa, red wine, and tea [4]. One crop containing the highest amount of quercetin is onion, which has about 300 mg of quercetin per 1 kg of fresh weight, compared to kale (100 mg/kg), blackcurrants (40 mg/kg), and broccoli or apples (30 mg/kg both) [5-7].

The bioavailability of quercetin is more significant when it is consumed as an integral food component. The degree of glycosylation rate increases the bioavailability

of quercetin as well as the properties of various glycosides conjugated to quercetin [8]. Food preparation and storage also influence the concentration of the bioavailable quercetin in food. General food processing, but also trimming, peeling, pitting, as well as picking certain leaves, could cause a partial or complete reduction in flavonol levels [9]. Different content of flavonols was observed when using different manufacturing procedures and techniques on food, especially in the processing of juices and vinification. It is known that fried and boiled food contains less quercetin than raw sources, while baking and the subsequent loss of water can cause an increase in quercetin in the ready meal. Boiling causes the most significant reduction in quercetin content; the reason is thermal degradation and boiling water leaching [10]. Different quercetin concentrations were observed when different methods of onion preparation were used. After baking and sautéing, quercetin concentrations in onion increased by 7–25%, and after boiling, decreased by 18% [11]. The content of quercetin in the final product also varies depending on the cultivar, as demonstrated by Kahle et al. [12]. They determined the content of quercetin and its derivatives in the concentration of 0.4–27 mg/l in freshly produced juices from different cultivars of dessert and cider apples [12]. When considering storage conditions, onions can lose 25-33% of quercetin content in the first twelve days of storage, but after that time, the losses are minimal [13]. The amount of quercetin in strawberries increased by approximately about 32% when stored at -20°C for nine months. The plant's growing process influences its flavonoid content [10].

Recently, there has been a growing interest in utilizing plant-based products to prevent diseases through proper nutrition within a new discipline of the science of nutrition, the Functional Food Science. The Functional Food Center (FFC) has agreed on the current definition for functional foods as: "Natural or processed foods that contain biologically-active compounds, which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms" [14]. The FFC with the Academic Society of Functional Foods and Bioactive Compounds (ASFFBC) in collaboration with the FDA and other governmental agencies, decided to bring a definition and classification of Functional Foods [15]. Recently, the FFC described and proposed a 16-step path, according to which foods could be classified as functional. [14]. The bioactive compounds include chemicals in plant, yeast, insect, single-cell, and animal-based food products, usually in small quantities. These compounds support the optimal health of the human body. It is essential to know their effective and safe doses, at which these compounds may be able to induce a positive response in individuals supporting their health. Bioactive compounds influence the body due to their acting on cell functions and metabolism [16].

There is a lot of evidence about the beneficial effect of quercetin on the human body; quercetin showed multiple beneficial effects in various diseases; there were reported antioxidative, neuroprotective anticarcinogenic, anti-inflammatory, antithrombotic, antiaggregatory, vasodilating, antimicrobial, antiviral, antiobese

and antiaging effects [4,17-22]. It is precisely its antioxidant properties that come to the forefront, thanks to which quercetin is widely used in traditional Chinese medicine and botanical medicine.

Quercetin shows strong antioxidant potency, which is associated with its effect on glutathione (GSH), signal transduction pathways, enzymatic activity, and reactive oxygen and nitrogen species (ROS, RNS) [4]. ROS and RNS represent highly reactive molecules derived from oxygen and nitrogen, superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), singlet oxygen (1O_2), peroxy radical (ROO^{\bullet}), ozone (O_3), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite anion ($ONOO^-$), and hypochlorous or hypobromous acid (HOCl, HOBr) [23]. At low physiological levels, at small (nanomolar) quantities, the ROS and RNS are beneficial for the human body, act as signaling molecules, strengthen the immune defense, and contribute to the natural aging process via induction of cell differentiation and apoptosis. Elevated levels of free radicals led to the formation of oxidative stress, which is associated with damage and chemical transformations of biomolecules, including, for instance, peroxidation of lipids, denaturation of proteins, also oxidation of DNA. Oxidative stress is associated with the development of various diseases (e.g., vascular, autoimmune, neurodegenerative, respiratory diseases) [24-26].

Some studies have pointed out eventual toxicity problems of high brain concentrations of some antioxidants, including quercetin. In this sense, quercetin dosage is critical due to its possible pro-oxidative properties, especially at high doses [27-28].

MATERIALS

All chemicals used in the experiments were of the highest quality available. Quercetin, L-methionine, nitrotetrazolium blue (NTB), and riboflavin were purchased from MERCK (Merk Schuchardt OHG, Hohenbrunn, Germany). The EDTA, K_2HPO_4 , and KH_2PO_4 were purchased from ITES (ITES, Vranov nad Topľou, Slovakia). Stock solutions of the reagents were prepared as follows: 50 mM potassium phosphate buffer with the following pH values: 6.5 - 7.0 - 7.4 - 7.6 - 8.0, 0.2 mM riboflavin, 5 mM NTB, and 5 to 50 $\mu\text{g/ml}$ quercetin.

Equipment: Mercury-vapour (Hg) UV lamp, Shimadzu MultiSpec-1501 UV/Vis spectrophotometer with wavelengths set to 450 and 560 nm.

METHODS

Test principle: The antioxidant properties of quercetin were measured by the test reaction according to Beauchamp and Fridovich [29], in which riboflavin-mediated photo-oxidation of methionine led to the superoxide radical anion formation, which was spectrophotometrically detected in reaction with NTB. The initially yellow-colored NTB, with maximum absorption at 560 nm, is oxidized in the presence of superoxide radical anion to the blue-colored NTB-formazan product at maximum absorption of 450 nm. In the presence of quercetin, superoxide radical anions are scavenged, which leads to the inhibition of the photochemical oxidation of NTB. EDTA added in excess removed interfering trace amounts of metal ions (Fig 2).

Test procedure: The reaction mixture contained 50 mM phosphate buffer of different pH to yield pH 6.5, 7.0, 7.4, 7.6, and 8.0 with 13 mM L-methionine and 0.1 mM EDTA in a total volume of 8.7 ml and 30 μl of 5 mM NTB. A blank solution was prepared by adding distilled water to the solution. Quercetin was added at concentrations ranging from 5 to 50 $\mu\text{g/ml}$. The last component added was 0.2 mM riboflavin in the amount of 300 μl . Measurements at each concentration at each pH were repeated three times. The reaction was initiated by placing the reaction mixture under a Hg lamp to perform UV illumination, which lasted 20 min. The percentage of inhibition of the NTB-formazan production expressed the antioxidant properties of quercetin. It was calculated from the absorbances at both wavelengths 450 and 560 nm using the following formulas:

$$\% \text{ of inhibition} = \frac{((A_{c0,t20} - A_{c0,t0}) - (A_{cx,t20} - A_{cx,t0}))}{(A_{c0,t20} - A_{c0,t0})} \times 100$$

- $A_{c0,t0}$ is the absorbance of the reaction mixture with 0 $\mu\text{g/ml}$ quercetin in time 0 min.
- $A_{c0,t20}$ is the absorbance of the reaction mixture with 0 $\mu\text{g/ml}$ quercetin in time 20 min.
- $A_{cx,t0}$ is the absorbance of the reaction mixture with $x = 5$ to 50 $\mu\text{g/ml}$ quercetin in time 0 min.
- $A_{cx,t20}$ is the absorbance of the reaction mixture with $x = 5$ to 50 $\mu\text{g/ml}$ quercetin in time 20 min.
- A similar formula was used to calculate the % of inhibition after 10 minutes, with data corresponding to the 10th minute of the experiment.

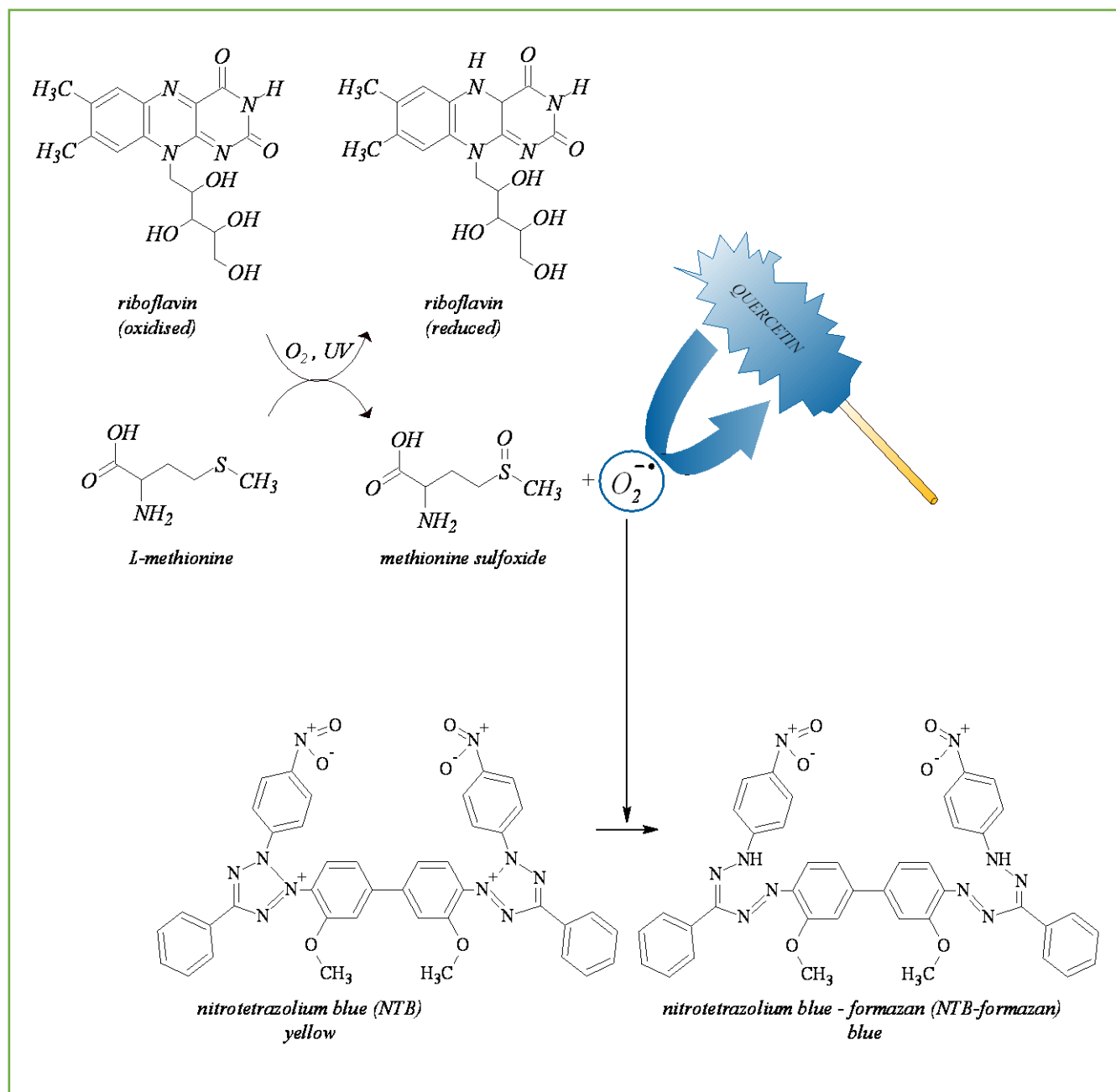


Figure 2: Representation of used method in formulas.

RESULTS

The antioxidant capacity of quercetin (5 to 50 $\mu\text{g/ml}$) was tested in reaction with superoxide radicals generated by the methionine-riboflavin generator at different pH levels (pH 6.5 - 8). The spectroscopic method determined the antioxidant properties of quercetin in different pH

conditions. The absorbance of the reagent solution was measured at 450 nm and 560 nm after 0, 10, and 20 minutes of illumination (Fig 3).

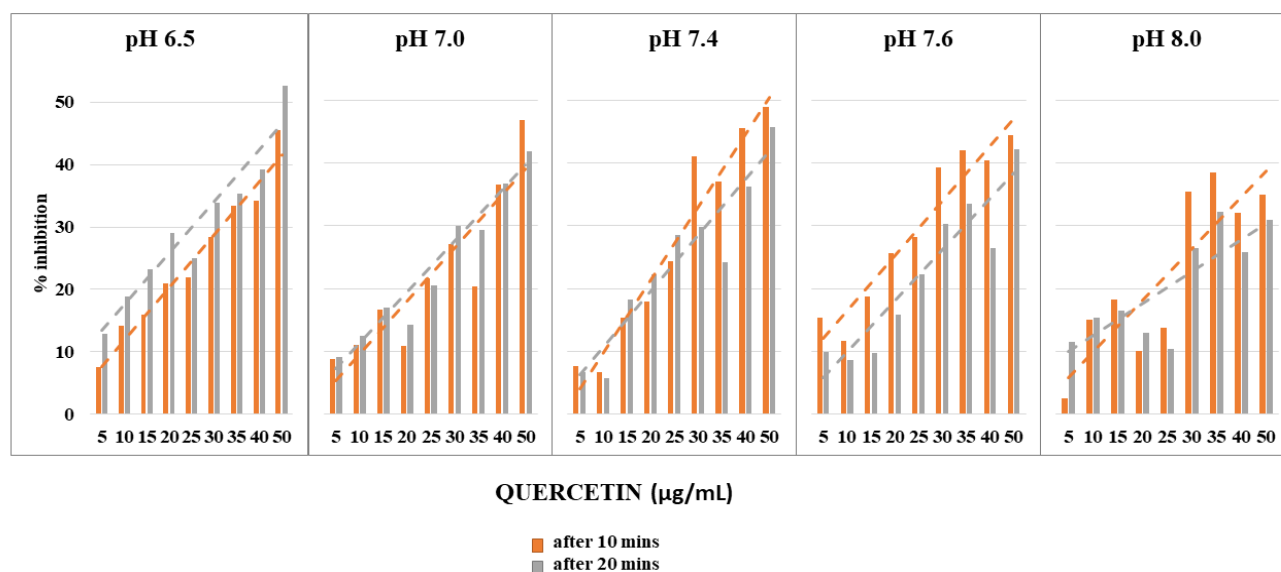


Figure 3. Antioxidant properties of quercetin against superoxide radical at different pH values.

The range of tested pH is the most common range within the human body. Experiments express that quercetin is an effective superoxide radical scavenger in all tested pH values with slight differences, dependent mainly on its concentration. The best antioxidant capacity is in a slightly acidic environment at pH 6.5 with decreasing % of inhibition with increasing pH value. At a pH 8, the better antioxidant capacity is achieved at concentrations above 30 µg/mL. Experiments also show that the antioxidant properties of quercetin are steadily increasing with a concentration throughout time while only in an acidic environment (at pH 6.5). In an alkaline environment, the antioxidant capacity in time is not stable, and even decreases. This effect is even more pronounced at higher concentrations.

DISCUSSION

In general, flavonoids are known as effective exogenous antioxidants. Their antioxidant activity is due to their ability to scavenge free radicals and reduce free radical formation; however, the human body's metabolism and absorption must be considered. Despite significant advances in the field of flavonoid metabolism and

absorption, it is still an underexplored area. Due to the limited information and knowledge about their uptake in humans, the antioxidant efficacy of flavonoids *in vivo* needs to be better documented. Most of the flavonoids taken in food are broken down into numerous phenolic acids, some of which still have the ability to scavenge free radicals [30]. A relationship between the structure of polyphenolic compounds and their antioxidant properties in terms of their ability to scavenge free radicals has been observed in many studies. The antioxidant activity of phenolic compounds strongly depends on the number and position of hydroxyl and carboxyl groups attached to the benzene ring [31-33].

The presence of methoxyl (-OCH₃) and phenolic hydroxyl (-OH) groups increases the antioxidant activity of phenolic acids compared to the presence of a carboxylic group (-COOH) [34-36]. Jovanovic [35] pointed out in his work that antioxidant capacity is enhanced due to the presence of more hydroxyl groups, especially on the B-ring [34]. Quercetin, the flavonol with its specific chemical structure, consists of three rings and five hydroxyl groups. There is evidence about the antioxidant capacity of quercetin in reducing reactive

oxygen species (ROS) due to the presence of the free hydroxyl group on the third carbon [37]. Our results confirmed this statement: quercetin is an effective superoxide radical scavenger. During the reaction of quercetin with a free radical, quercetin donates a proton and becomes a radical. The resulting unpaired electron is delocalized by resonance in the flavonoid molecule. The reactivity of quercetin radical is relatively low due to its energetic stability. The formation of hydrogen bonds between the hydroxyl groups of the B ring and the electronegative atoms of the remaining rings conditions this stability. Of five hydroxyl groups in the quercetin structure, the one in position three on the C ring is the most essential for the antioxidant properties of this molecule. The reactivity of quercetin radical is relatively low due to its energetic stability. In addition to the natural resonance structures resulting from the aromatic character of the benzene nucleus, mesomeric effects of other functional groups also contribute to other resonance structures [34,38]. These functional groups contribute to quercetin's ability to maintain stability and support antioxidant action in reactions with free radicals: the B ring *o*-dihydroxyl groups, the 4-oxo group in conjugation with the 2,3-alkene, and the 3- and 5-hydroxyl groups [39-40].

Our results correlate with the study of Fazilatun et al. [41], who proved the antioxidant activity of quercetin. In addition, the authors confirmed the higher antioxidant activity of quercetin compared to other flavonoids. They concluded that flavonoids with a free hydroxyl group are more active than their methylated derivatives [41].

Quercetin represents a substantial phytochemical present in *Cissus quadrangularis* (L). Dhanasekaran et al. [42] investigated free radical scavenging and anticancer efficacy of quercetin in an extract from the aerial parts of *C. quadrangularis*. They confirmed that quercetin shows an ability to scavenge superoxide anion radicals in a dose-dependent manner. Our results are consistent with their

observation that the antioxidant properties of quercetin increase with concentration, mostly over 30 µg/ml. Some studies confirm its effect as a valid component of functional foods in cancer prevention, enhancing the antioxidant activity with a cytoprotective role against oxidative stress. The dosage of quercetin is crucial due to its possible pro-oxidative properties, especially at high doses, even though some studies point to the benefits of quercetin as a pro-oxidant, for example, in the treatment of cancer by inhibiting tumorigenesis [42]. Pro-oxidant properties of quercetin tested in concentrations 5 to 50 µg/ml were not observed in our study.

Recent research has proven that the material of the microcapsule itself significantly affects the properties of the transferred bioactive material regarding its intake, absorption, metabolism, and antioxidant activity. The efficient antioxidant activity of quercetin against reactive oxygen species (ROS) was observed in the study of Noelia et al. [43]. They demonstrated that chitosan and its glucosamine derivative are suitable biopolymers for quercetin microencapsulation, controlled release, and increased bioavailability [43].

Polyphenols seem to be sensitive to environmental conditions, including pH, temperatures, and light [44-45]. Therefore, it is also important to study the effects of these factors on the antioxidant activity of polyphenolic compounds under precise experimental conditions. It is well known that polyphenols contain many hydroxyl groups capable of dissociation. The pH environment influences the hydroxyl groups' dissociation rates and therefore affects polyphenol compounds' free radical scavenging activity [46]. Not only the pH of the mixture from which the polyphenols are isolated but also the isolation procedures, the processes of incorporating polyphenols into functional foods, the pH of the environment of the human digestive tract and the tissues into which the polyphenols are absorbed, and in which

they are further metabolized and exert their effects, have a significant impact on their functioning. Quercetin is known to be most stable in a pH environment from 1 to 6, and above these pH values, it degrades, most significantly above pH 9 [47]. Low water solubility, poor bioavailability, and gastrointestinal instability account for the low availability of orally administered quercetin [48]. The knowledge about the higher superoxide radical scavenging ability of quercetin from our experiment limited to slightly acidic conditions could have a substantial impact on tissue compartment-specific or pathological conditions-specific activities. The application of appropriate doses of quercetin may be of particular importance in conditions related to acidification of the internal environment in many pathological processes (e.g., conditions related to hypoxia, diabetes mellitus, etc.) as protection against oxidative stress, including some types of cancer. Many solid tumors and leukemias are well known to be associated with excessive production of acids due to the Warburg effect and, at the same time, the ability to excrete these acids outside the tumor cell. Such acidification of the extracellular matrix then promotes the spread of tumors, metastasis, and chemotherapy resistance, being directly involved in the mechanism of aggressiveness of the tumor. The most aggressive tumors create a micro-environment with pH 6.8, and less aggressive tumors create a slightly less acidic microenvironment but still below pH 7.0 [49].

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CONCLUSION

Quercetin is a widely occurring flavonoid, representative of the flavonol subclass, with much evidence of its beneficial effects on the human body in different body fluids (e.g., blood, saliva, synovial fluid). Since pH significantly influences biochemical processes in living organisms, our results could contribute to expanding the information on the correct pH adjustment of functional foods containing quercetin as a rich natural source of medicinal substances with antioxidant properties, as well as potential supplementary therapy during many pathological states related to acidification.

List of Abbreviations: GSH: glutathione, ROS: reactive oxygen species, RNS: reactive nitrogen species, NTB: nitrotetrazolium blue

Conflict of interest: The authors have no conflicts of interest to declare.

Author's contributions: *Beata Cizmarova performed the experimental research, **Anna Birkova and ***Beata Hubkova performed analytical calculation and contributed to interpretation of results. All authors contributed to the analysis, provide critical feedback, discussed the results and contributed to the final manuscript.

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