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The assessment of rosehip (*Rosa Canina* L.) pseudo-fruit antioxidant capacity: electrochemical vs. spectrophotometric methods pre- and post- *in vitro* gastrointestinal digestion

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Abstract

Extracts of Rosa canina L. *have been traditionally utilized in nutrition due to their high concentration of vitamin C and other bioactive compounds, including polyphenols. The total antioxidant capacity (TAC) of these bioactive components serves as a critical parameter for evaluating the biological value of the extract. Conventional spectrophotometric methods have been widely employed for TAC quantification. However, electrochemical techniques present distinct advantages, including rapid analysis, lower costs, and minimal sample preparation. This study aims to compare electrochemical and spectrophotometric methods for TAC determination in Rosa canina* L. *pseudo-fruit extract before and after in vitro gastrointestinal digestion. TAC was assessed in dried pseudo-fruit extract, the liquid phase post-digestion, and the solid phase post-digestion using cyclic voltammetry (CV), differential pulse voltammetry (DPV), and the ABTS and Folin-Chiocalte spectrophotometric assay. The results indicate that the TAC of the undigested pseudo-fruit extract was significantly higher than that of the digested samples, with the liquid phase exhibiting greater TAC than the solid phase. The electrochemical methods (CV and DPV) produced highly consistent results, whereas the ABTS and Folin-Chiocalte assay yielded slightly lower TAC values post-digestion. The electrochemical approaches demonstrated a substantial reduction in analysis time, as TAC measurements were obtained within seconds without the need for extensive sample preparation, unlike spectrophotometric methods. These results underscore the potential of electrochemical techniques, especially differential pulse voltammetry, as reliable, rapid alternatives to traditional spectrophotometric assays for routine TAC determination in plant extracts. Moreover, electrochemical methods may overcome the limitations of spectrophotometric analysis, such as lower sensitivity and prolonged analytical procedures, thereby enhancing the efficiency and applicability of antioxidant capacity assessments in nutritional and pharmaceutical research.*

Keywords: *Rosa canina* L.; *total antioxidant capacity; ABTS; TPC; cyclic voltammetry; differential pulse voltammetry.*

INTRODUCTION

Rosa canina L., known as rosehip, is a perennial shrub of the family *Rosaceae*. *Rosa canina* L. produces a pseudo-fruit, which is widely used for its beneficial effects on human health. Dried pseudo-fruit extracts are highly valued in nutrition due to their high content of vitamin C and polyphenols, significantly contributing to their total antioxidant capacity (TAC). TAC serves as a key indicator of the extract's overall biological activity, reflecting its potential to neutralize free radicals and support physiological functions [1-6].

Antioxidants are molecules that help protect cells from oxidative damage caused by free radicals or reactive oxygen species (ROS). Oxidative stress refers to a state of disrupted redox balance within cells, resulting from excessive production and/or inadequate removal of reactive species, free radicals, or ROS. It has not yet been fully elucidated whether the damage caused by reactive species is due to genetic disorders, cellular homeostasis disturbances, metabolic incidents, normal physiological processes, or perhaps a combination of these factors. Oxidative damage to biomolecules leads

to changes such as altered cell membrane permeability, signaling pathways, gene expression, transcription of regulatory gene factors, protein replication, and inhibition of proteases. Overall, oxidative stress is responsible for a complex pathophysiological response in the organism [7]. Antioxidants neutralize free radicals by donating electrons, and their redox properties are crucial for determining the capacity of a compound to neutralize ROS and free radicals by undergoing oxidation itself [8]. Antioxidants play a crucial role in maintaining cellular health, preventing oxidative stress, and supporting overall well-being. Dietary sources rich in antioxidants, such as *Rosa canina* L. extracts, can enhance the body's defense mechanisms against oxidative damage [2,6,9].

Various assays, such as DPPH, ABTS, FRAP, and Folin-Ciocalteu assay, rely on redox reactions to quantify antioxidant potential [10,11]. The ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay is a widely used spectrophotometric method for evaluating the TAC of samples, based on the ability of antioxidants to neutralize the $ABTS^{\cdot+}$ radical cation. The assay provides a reliable measure of the radical scavenging activity of bioactive compounds, particularly polyphenols and vitamin C, present in fruit extracts, like *Rosa canina* L. extracts. The Folin-Ciocalteu reagent assay can be used to determine the total polyphenol content in plants and food products. It is based on the electron transfer from polyphenols to the Folin-Ciocalteu reagent in an alkaline medium, resulting in blue complexes that are independent of the structure of phenolic compounds, eliminating the possibility of coordination complex formation between metals and phenolic compounds [12-15]. Spectrophotometric methods are commonly used for TAC quantification; however, electrochemical techniques offer advantages in speed, cost, and sample preparation. Unlike spectrophotometry, electrochemistry can differentiate antioxidants based on their redox potentials, providing more detailed information about the sample's composition. Electrochemical techniques are also less susceptible to interference from turbid samples, making them suitable for crude extracts. Furthermore, they often exhibit higher sensitivity, enabling the detection of low concentrations of antioxidants. Certain electrochemical approaches require fewer or no reagents, reducing costs and simplifying the analysis process [16,17].

In addition to *in vitro* evaluation of fruit extract effects, it is essential to examine their properties following digestion to better understand their bioavailability and potential health impact. Studies have shown that the concentration of total phenols and antioxidant capacity in fruit juices can significantly change after simulated gastrointestinal digestion [18]. These findings highlight the importance of investigating the stability and activity of antioxidants post-digestion to provide relevant insights into their potential biological effects. The methods utilizing static digestion models are based on simplifying the gastrointestinal system into a basic chemical bioreactor. Despite its limitations, the *in vitro* static digestion model allows for the assessment of bioavailability, and the obtained results can correlate with findings from *in vivo* studies conducted on human and animal models. Static models are the most commonly used systems for simulating digestion, where the oral phase is typically simulated by the addition of salivary alpha-amylase, and the gastric phase is mimicked by adding pepsin and a fixed temperature for a defined period. Subsequently, the intestinal phase can be simulated within the same bioreactor by adding pancreatic enzymes, with or without bile. The method developed by Aura *et al.* 1999 [19] is an *in vitro* static digestion method that includes bile during the intestinal phase of digestion. This method involves the sequential simulation of oral, gastric, and intestinal digestion, while parameters such as electrolytes, enzymes, bile, dilution, pH, and digestion time are based on available physiological data. The method can be employed to evaluate the outcomes of food digestion by analyzing the digestion products, functional properties of digests, or assessing the bioaccessibility of bioactive compounds from the food matrix [20-22].

This study aims to compare electrochemical and spectrophotometric methods for assessing the TAC of *Rosa canina* L. pseudo-fruit extract before and after simulated *in vitro* gastrointestinal (GI) digestion. Additionally, it seeks to evaluate the changes in antioxidant capacity of commercially available lyophilized *Rosa canina* L. pseudo-fruit pre- and post-digestion to explore its potential health benefits.

MATERIAL AND METHODS

Sample preparation

In this study, a lyophilized, commercially available pseudo-fruit of Rosehip (*Rosa canina* L.), with seeds removed, was taken for the sample (ELBI DOO Valjevo).

In vitro gastrointestinal digestion of Rosa canina L. *pseudo-fruit extract*

An *in vitro* GI digestion protocol described by Batinić *et al.* 2024 [20] was implemented, with its schematic representation provided in **Figure 1**. For this purpose, 1.5 g lyophilized sample of *Rosa canina* L. pseudo-fruit was combined with 15 mL of deionized water, 10 mL of 0.85% sodium chloride (NaCl), and 100 µL of a salivary α-amylase solution (50 IU activity) prepared in phosphate buffer to simulate the physiological conditions of the oral cavity. The mixture was incubated in a water bath, maintained at 37 °C, and subjected to continuous shaking (120 rpm) for 5 minutes, ensuring a stable pH of 6.9. Following the oral phase, the gastric phase was simulated by adding 4.5 mL of 0.15 M HCl and 1 mL of pepsin (Sigma P-7012, 0.6–2.0 mg) dissolved in 20

mM HCl. The gastric phase was simulated for 2 hours with continuous mixing (120 rpm) at 37 °C, at a pH of 2.5. The subsequent duodenal and jejunal phases were simulated by adding 4 mL of bile acids (Sigma B-8381, 0.4–0.6 g) dissolved in 0.15 M sodium bicarbonate ($NaHCO_3$), 4 mL of pancreatin (Sigma P-1750, 10–75 mg, 163.5 U/mg) dissolved in 0.15 M $NaHCO_3$, and 1 mL of mucin (Sigma M-2378, 75 mg) dissolved in water. These phases proceeded for 3 hours under continuous shaking (120 rpm) at 37 °C, with pH maintained within the range of 6.5–7.5. The ileal phase was simulated by adding 5.5 mL of water, followed by centrifugation for 20 minutes at 4000 rpm. Upon completion of *in vitro* GI digestion, the soluble fraction was separated from the insoluble fraction, frozen, and lyophilized (INOFD-10S, AMTAST, US). After GI digestion, the soluble and insoluble fractions were separated, frozen, and lyophilized. For the control sample, 1.5 mL of distilled water was used, while all other steps and reagents were replicated as in the main trial.

tracted in ethanol). For the lyophilized insoluble fraction and raw sample, 1 gram of the sample was mixed with 10 mL of 80% ethanol. The mixture was then placed on a shaker for 2 hours, followed by centrifugation at 4000g for 5 minutes. The obtained insoluble fraction was subsequently used for the analysis of antioxidant capacity. The total antioxidant capacity of the dry pseudo-fruit extract, liquid phase post-digestion, and solid phase post-digestion was determined using spectrophotometric and electrochemical methods.

The total antioxidant capacity was evaluated using the ABTS assay, following the method described by

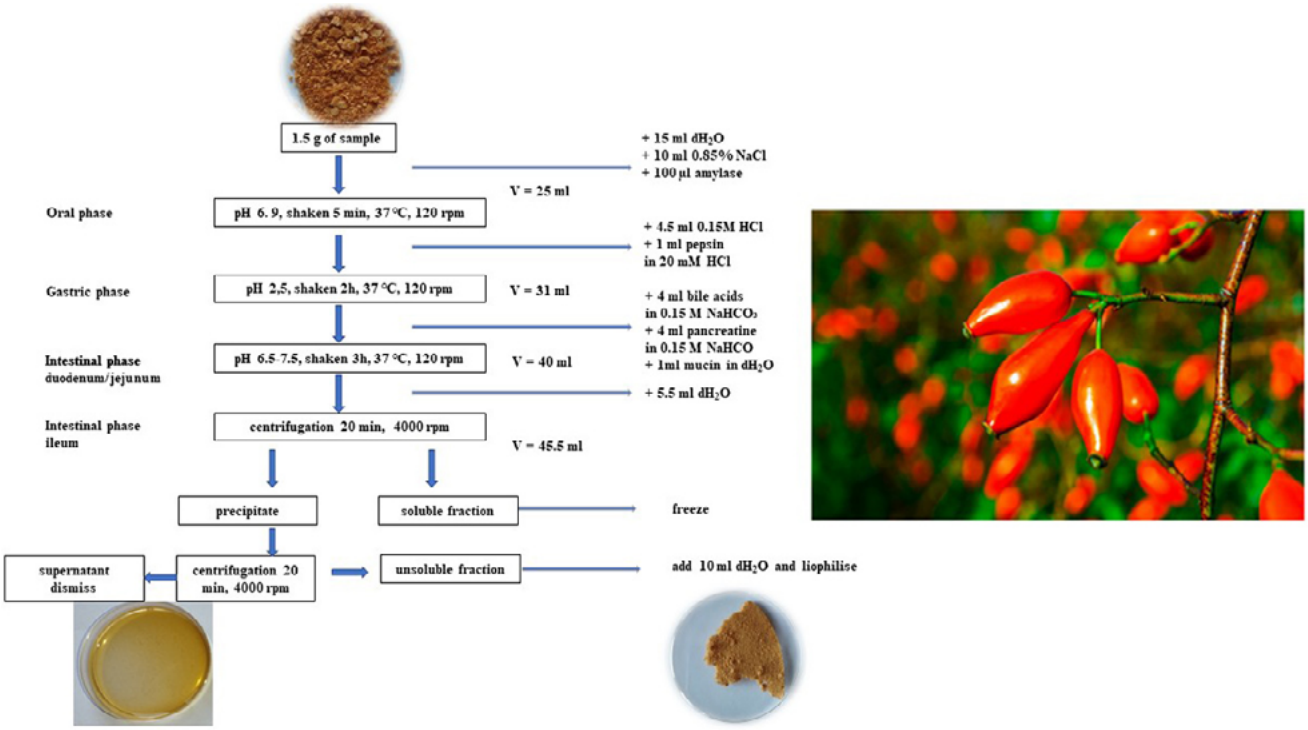


Figure 1. Structured scheme of *in vitro* GI digestion protocol for *Rosa canina* L. pseudo-fruit extract.

Determination of Total Antioxidant Capacity

For the analysis of the TAC, samples before and after GI digestion were used. The first sample (1) represented dry pseudo-fruit extract (undigested lyophilized pseudo-fruit of rosehip extracted in 80% ethanol), the second sample (2) the liquid phase post-digestion (soluble fraction after *in vitro* GI digestion), and the third sample (3) the solid phase post-digestion (lyophilized insoluble fraction and undigested rosehip sample ex-

Milinčić *et al.* 2022 [23]. The stock ABTS solution was prepared by dissolving 0.03845 g of ABTS in 8 mL of water, followed by the addition of 0.0662 g of potassium persulfate. The final volume was adjusted to 10 mL with distilled water. After incubation in the dark at room temperature for 16 hours, 1 mL of the working solution was mixed with 30 mL of ethanol. The absorbance was adjusted to a range of 0.7 to 1 at a wavelength of 734 nm. This prepared solution also served as the control used in further analysis. A volume of 50 µL of the sample or Trolox standard was pipetted into a microplate, followed by the addition of 1 mL of the ABTS working solution. Absorbance was measured at 734 nm after a 7-minute incubation in the dark using a microplate UV/VIS spectrophotometer (FlexA 200, Amtast, USA Manufacturing Inc., FL, USA). The percentage of ABTS inhibition was calculated based on the obtained values using the following formula:

% inhibition = [(Ab – As)/As] x 100
Ab – the absorbance of the blank; As – the absorbance of the sample extract

The results were expressed as milligrams of Trolox equivalents per gram of dry weight of the sample (mmol Trolox/100 g). The linear range of the standard curve was 50-250 µg/mL ($R^2 = 0.99$).

The total antioxidant capacity expressed as total polyphenolic content (TPC) was determined using the Folin-Ciocalteu method developed by Singleton *et al.* 1998 [24], with minor modifications by Pešić *et al.* 2019 [12]. An aliquot of the sample (250 µL) was mixed with 1250 µL of Folin-Ciocalteu reagent and incubated for 5 minutes at room temperature. Subsequently, 1000 µL of 7.5% Na_2CO_3 was added. The reaction mixture was left to incubate for 1.5 hours at room temperature in the dark. The samples were then centrifuged, and the absorbance of supernatants was measured at 765 nm using a UV/VIS spectrophotometer. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight of the sample (mg GAE/ g DW). The linear range of the standard curve was 10-300 µg/mL ($R^2 = 0.99$).

Electrochemical determination of redox properties was conducted using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements with a CH1760B electrochemical instrument (CH Instruments, Austin, USA). All electrochemical experiments were performed in a three-electrode configuration within a quartz cuvette (5 mL total volume). A glassy carbon electrode (GCE, Model CHI 104) was used as the working electrode, while a platinum electrode (Model CHI 221) and an Ag/AgCl reference electrode (Model CHI 111) functioned as the auxiliary and reference electrodes, respectively. To ensure an inert environment, nitrogen was continuously introduced into the electrochemical cell, maintaining anaerobic conditions throughout the measurements.

The CV experiments were conducted over a potential range of 0 to 1000 mV at a scan rate of 100 mV/s, while DPV measurements were performed within the same potential window, but with a scan rate of 1 mV/s. Prior to each electrochemical measurement, the GCE was polished sequentially with 1.0 µm and 0.5 µm alumina powder (Buehler, IL, USA), followed by thorough rinsing with distilled water to remove residual particulates. All measurements were carried out at room temperature (20 °C). Test samples were prepared by dissolution in deionized water before analysis to ensure consistency and homogeneity. Electrochemical responses were analyzed based on two distinct oxidation peaks observed at approximately 350 mV and 650 mV, expressed as mol vitamin C/g. Calibration curves were constructed using peak area (Ah) from CV and DPV measurements, specifically focusing on the peak at 350 mV for quantitative analysis.

The UV absorption spectra of the samples were recorded in the wavelength range of 200–400 nm, with a recommended absorbance range of 0.0–4.0 A. The measurements were conducted using a scan pitch of 0.5 nm to ensure high-resolution spectral acquisition.

All chemicals used for the preparation of buffers and solutions were of analytical grade. Standard reference compounds, including gallic acid, were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). The Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Milli-Q water was utilized for the preparation of standard solutions and sample dilutions to ensure high purity and reproducibility in all analytical procedures.

Statistical analysis

All tests were performed in triplicate, and the results are expressed as mean values ± SD.

RESULTS AND DISCUSSION

The results of TAC for *Rosa canina* L. pseudo-fruit extract before and after *in vitro* GI digestion using spectrophotometric and electrochemical methods are shown in **Figure 2**. The obtained results indicate that *in vitro* GI digestion of lyophilized *Rosa canina* L. pseudo-fruit extract significantly influences their TAC following digestion. This suggests potential modifications in the bioavailability and stability of antioxidant compounds during the digestive process, which may affect the extract's overall biological activity.

The results demonstrate that TAC of the undigested *Rosa canina* L. pseudo-fruit extract was significantly higher compared to the digested sample (**Figure 2**). Among the digested fractions, the liquid phase exhibited greater TAC than the solid phase. The ABTS antioxidant activity of dried fruit extract was 144.73 ± 1.51 mg Trolox/g dry weight, liquid phase after *in vitro* GI digestion 77.63 mg Trolox/g DW, and solid phase was 14.76 mg Trolox/g DW (**Figure 2A**).

The TPC antioxidant test (**Figure 2B**) indicates that the raw sample had 59.53 ± 3.35 mg GAE/g, soluble fraction after *in vitro* GI digestion had 50.73 ± 1.21 mg GAE/g, and insoluble fraction had 5.26 ± 0.14 mg GAE/g content of compounds with polyphenolic antioxidant properties in the analyzed samples. Compared to the raw sample (100% TAC), the total antioxidant capacity in the soluble fraction decreased to 85.23% of its original activity, while in the insoluble fraction, it was reduced to 8.84% of the initial activity. These findings indicate a substantial redistribution of antioxidant compounds between the soluble and insoluble phases during digestion, highlighting the impact of the digestive process on the extract's bioactive properties.

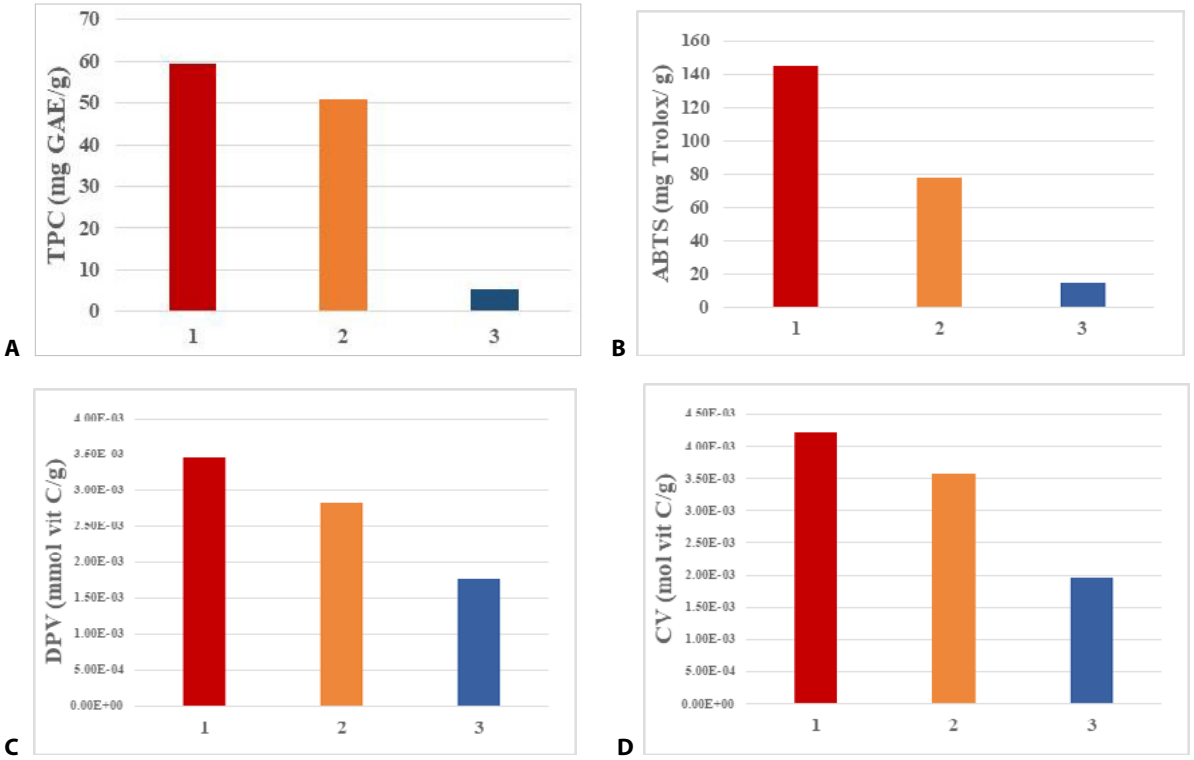


Figure 2. Comparison of spectrophotometric (A-ABTS and B-TPC) and electrochemical (C-CV and D-DPV) methods for determination of TAC of *Rosa canina* L. pseudo-fruit extract - dried fruit extract (1), liquid phase of digest after *in vitro* GI digestion (2), and solid phase of digest after *in vitro* GI digestion (3).

The characterization of *Rosa canina* L. pseudo-fruit extract was performed using both electrochemical and spectrophotometric techniques to assess its antioxidant properties and redox behavior. **Figure 3A** shows CV voltammograms of different samples expressed as mol Vitamin C. The results follow the same trend as those obtained from the TPC and ABTS tests, with the highest antioxidant activity 4.22×10^{-3} mol vit C/g (100%) observed in the undigested raw sample (1), followed by the soluble fraction 3.57×10^{-3} mol vit C/g (2), and the lowest activity 1.97×10^{-3} mol vit C/g in the insoluble fraction (3) (**Figure 2C**).

Different components contribute to the overall antioxidant activity of the extract, as seen in **Figure 3B** at 0.2 V, 0.4 V, and 0.6 V for the water and solid phase before and after GI digestion. The DPV values for samples 1, 2, and 3 were 0.00345, 0.00289, and 0.00177 mol Vit C/g, corresponding to 100%, 83.76%, and 51.30 %, respectively, following the same trend as observed in the CV results (100%, 84.59% and 46.68%).

The UV absorption spectra (**Figure 3C**) were recorded within a range that captures the characteristic absorbance bands of key bioactive compounds such as polyphenols and vitamin C, which typically exhibit strong absorbance in the UV region. These spectra provide valuable insights into the electronic transitions and structural characteristics of the analyzed compounds.

Results obtained using the electrochemical methods, specifically differential pulse voltammetry, demonstrate the same trend as those observed using spectrophotometric techniques (TPC, ABTS). The highest TAC was detected in the undigested raw sample (1), followed by the soluble fraction post-digestion (2), while the lowest antioxidant activity was observed in the insoluble fraction after GI digestion (3). Although the TPC method reflected a similar trend for samples 1 and 2 (100% and 85.23%, respectively), sample 3 showed significantly lower activity compared to the raw sample (8%). The ABTS assay also indicated lower antioxidant activity for both the soluble (2) and insoluble (3) fractions post *in vitro* GI digestion.

All tests confirmed a high level of the total antioxidant capacity in the soluble fraction after *in vitro* GI digestion. This fraction contains compounds likely to be bioavailable during the intestinal phase of digestion and could be absorbed by intestinal epithelial cells. Although the insoluble fraction exhibited lower antioxidant activity, it may still have biological significance. Compounds in this fraction could contribute to a positive antioxidant environment in the colon, benefiting the colonic microbiota and being absorbed by the epithelial cells of the colon. The remainder of this fraction, which is not absorbed, is eliminated from the body.

The decrease in TAC observed after *in vitro* GI digestion suggests that antioxidant-reducing compounds interact with other components of the rosehip pseudo-fruit matrix and/or digestive enzymes, forming complexes that exhibit reduced antioxidant properties. It is also possible that during GI digestion, compounds

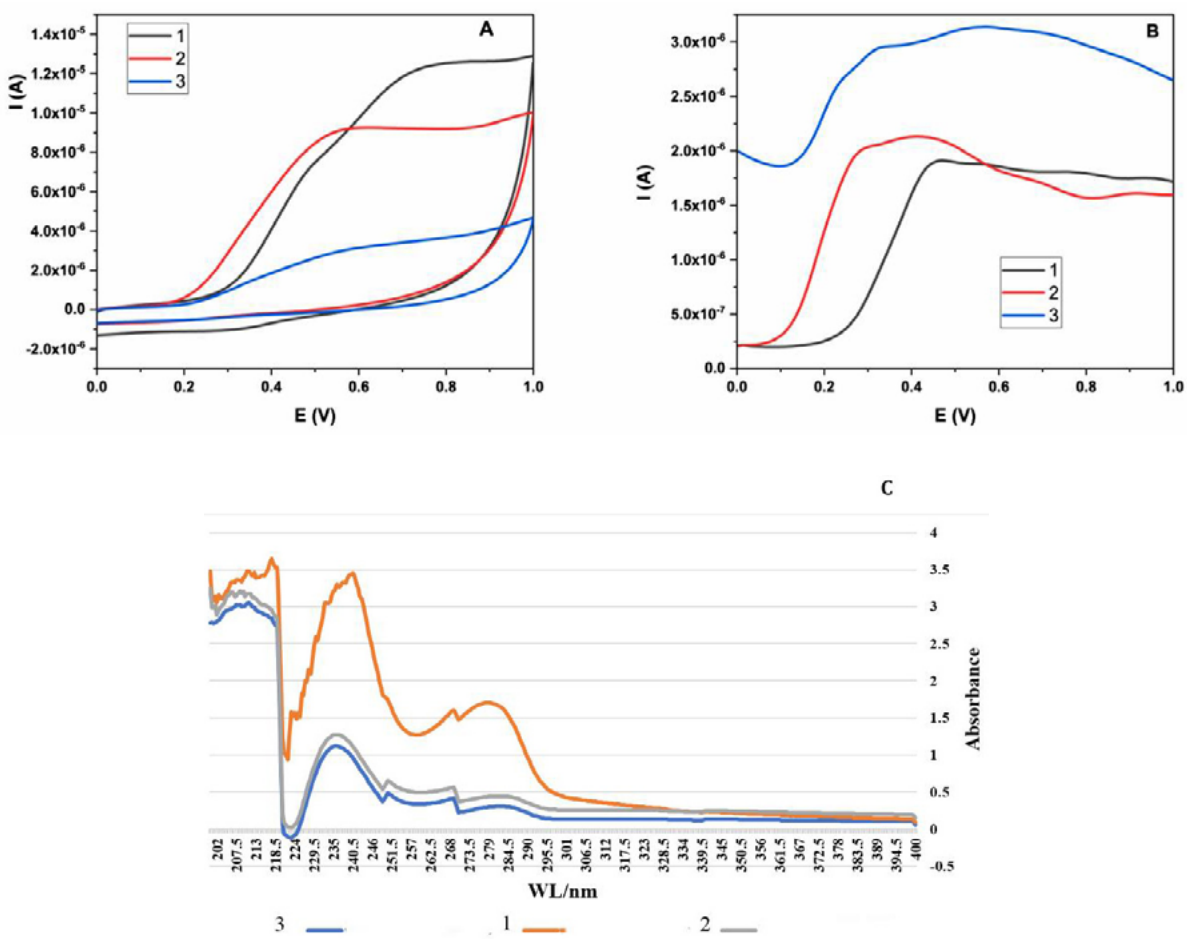


Figure 3. Characterization of spectra using electrochemical and spectrophotometric methods for detection of different samples of *Rosa canina* L. pseudo-fruit: A) CV voltammogram, B) DPV voltammogram of samples, C) UV spectrum (1 – raw water extract; 2 –extract after *in vitro* GI digestion, water phase; 3 – solid phase after *in vitro* GI digestion).

with high initial antioxidant activity are transformed into less potent forms [23]. Other researchers have also observed a reduction in the antioxidant properties of digests rich in phenolic compounds after *in vitro* GI digestion due to low recovery of phenolic compounds [12,23,25].

The antioxidant activity observed using the TPC method is likely attributed to flavonoids, phenolic acids, vitamin C, tocopherols, and carotenoids present in the rosehip pseudo-fruit matrix. Additionally, other reducing compounds, such as reducing sugars, divalent iron, and organic acids, may also contribute to the observed TPC results [14]. Further studies are required to determine the specific distribution of these compounds between the soluble and insoluble fractions of the digesta. The results obtained in this study align with those reported by Koczka et *al.* 2018 [26] for lyophilized rosehip extracts. The soluble fraction, rich in bioactive compounds, is potentially bioavailable for gastrointestinal absorption and systemic distribution, where it may exert antioxidant effects. On the other hand, the insoluble fraction contains compounds that could either be utilized by the gut microbiota, absorbed by intestinal epithelial cells, or eliminated from the body. These compounds were not released during *in vitro* GI digestion.

This study also found that the TAC of the dry pseudo-fruit extract was significantly higher than that of the digested samples, with the liquid phase showing a higher TAC compared to the solid phase. The results from cyclic voltammetry and differential pulse voltammetry were in good agreement, whereas the ABTS method yielded slightly lower values for post-digestion samples. Notably, the time required to obtain TAC measurements using voltammetry is much shorter, as these analyses take only a few seconds and do not require the additional sample treatments necessary for spectrophotometric methods. These results indicate that electrochemical methods offer several advantages over spectrophotometric methods for determining the total antioxidant capacity of plant extracts. These methods are generally faster, enabling high-throughput analysis.

CONCLUSIONS

Electrochemical methods can be used for determining the total antioxidant capacity before and after *in vitro* GI digestion of *Rosa canina* L. pseudo-fruit samples. Our results provide valuable data for further research on electrochemical methods for rapid, routine determination of TAC in plant extracts before and after GI digestion. Electrochemical techniques can exceed the limits of spectrophotometric analysis, such as lower sensitivity and slower response, and can be successfully applied as a replacement for long-running spectrophotometric methods.

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Procena antioksidativnog kapaciteta lažnog ploda šipurka (*Rosa Canina* L.): elektrohemijske i spektrofotometrijske metode pre i posle *in vitro* gastrointestinalne digestije

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Kratak sadržaj

Ekstrakti Rosa canina L.*, tradicionalno se koriste u ishrani zbog visokog sadržaja vitamina C i drugih bioaktivnih jedinjenja, uključujući polifenole. Ukupni antioksidativni kapacitet (TAC) ovih bioaktivnih komponenti predstavlja ključni parametar za procenu biološke vrednosti ekstrakta. Konvencionalne spektrofotometrijske metode široko su primenjivane za kvantifikaciju TAC-a. Međutim, elektrohemijske tehnike nude određene prednosti, uključujući bržu analizu, niže troškove i minimalnu pripremu uzoraka. Ova studija ima za cilj da uporedi elektrohemijske i spektrofotometrijske metode za određivanje TAC-a u ekstraktima lažnog ploda Rosa canina* L. *pre i nakon in vitro digestije. Ukupni antioksidativni kapacitet je procenjen u ekstraktu sušenog ploda pre digestije, tečnoj fazi nakon digestije i čvrstoj fazi nakon digestije primenom spektrofotometrijskih metoda ABTS i TPC, kao i elektrohemijskih metoda cikličke voltametrije (CV) i diferencijalne pulsne voltametrije (DPV). Rezultati odredjivanja ukupnog antioksidativnog kapaciteta ukazuju da je kod ekstrakta sušenog ploda značajno viši u odnosu na digestovane uzorke, pri čemu je aktivnija tečna faza nakon digestije u poređenju sa čvrstom fazom. Elektrohemijske metode (CV i DPV) dale su visoko konzistentne rezultate, dok su ABTS i TPC metode pokazale nešto niže vrednosti nakon digestije. Elektrohemijski pristupi omogućili su značajno smanjenje vremena analize, jer su rezultati merenja dobijeni u roku od nekoliko sekundi, bez potrebe za opsežnom pripremom uzoraka, za razliku od primenjenih spektrofotometrijskih metoda. Ovi rezultati naglašavaju potencijal elektrohemijskih tehnika, naročito diferencijalne pulsne voltametrije, kao pouzdanih i brzih alternativa tradicionalnim spektrofotometrijskim metodama za rutinsko određivanje TAC-a u biljnim ekstraktima. Elektrohemijske metode mogu prevazići ograničenja spektrofotometrijske analize, poput niže osetljivosti i produženih analitičkih procedura, čime se poboljšava efikasnost i primenljivost procene antioksidativnog kapaciteta u nutricionističkim i farmaceutskim istraživanjima.*

Ključne reči: *šipurak; ukupni antioksidativni kapacitet; ABTS; TPC; ciklična voltametrija; diferencijalna pulsna voltametrija.*