Methods for the determination of antioxidant activity of plant extracts *in vitro*

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Abstract

Plants are rich sources of secondary metabolites that exhibit diverse biological and pharmacological effects. Some plant ingredients, primarily phenolics, have significant *in vitro* antioxidant activity, which implies their contribution to the maintenance of redox balance in the body. These potential antioxidant agents are structurally very diverse, having different mechanisms of antioxidant activity. Since there is a growing necessity to detect, develop and understand effective antioxidant compounds, interest in the identification and the measurement of antioxidants in various plant isolates is persistently growing and many methods are being established. Most of the available *in vitro* tests are affordable and easy to perform, but due to the complex composition of plant extracts, different kinetics, mechanisms and specificity of the chemical reactions underlying these tests, there is no universal parameter for the assessment of antioxidant activity. In this paper, some of the currently most used *in vitro* methods for investigating and evaluating antioxidant activity of plant extracts are presented, emphasizing their advantages and weaknesses.

Key words: antioxidants, plant extracts, *in vitro* tests

Introduction

Oxygen is used in aerobic organisms for respiration and energy production, synthesis of some structural molecules and host defence. Moreover, the reactive species of oxygen (ROS) are involved in gene expression and signalling transduction. On the other hand, ROS and other reactive species (e.g., reactive species of nitrogen, RNS) cause damage to various biomolecules in the body, but the body's antioxidant network maintains normal redox balance and is able to counteract and reduce harmful effects of oxidants. In some cases, redox homeostasis could be substantially imbalanced (1). This disruption in redox signalling and control is called "oxidative stress" and is also defined as "the imbalance in the redox status of the cell between physiological antioxidants and oxidants/prooxidants in favour of the latter". (2) Oxidative stress is considered to be the main cause of oxidative damage of biomolecules and consequent development of certain diseases (e.g., cardiovascular, neurodegenerative, diabetes, cancer) and ageing (3). In that sense, it was postulated that exogenous intake of antioxidants, through food and supplements, can contribute to the antioxidant protection of the body and maintenance of good health and longevity. Research on antioxidants from plants is continuously growing in order to detect, develop and understand effective compounds that could serve as a shield against oxidative stress-related conditions (4). Due to the undesirable effects of some synthetic substances, antioxidants of natural origin have attracted enormous attention, especially secondary plant metabolites such as different phenolics (tannins, flavonoids, anthocyanins and phenolic acids). (5)

Considering the structural and chemical diversity of antioxidant molecules and their possible application in various fields, many different methods have been developed and used for their evaluation. However, some aspects of these tests, such as reliability, accuracy, validity, and overall utility, have always been a subject of debate. The significance of these tests is also questionable considering the low correlation between the results of *in vitro* and *in vivo* performed assays (4, 6).

Antioxidants

According to some authors, an antioxidant could be defined as "any substance that, when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate" (7). The nature of substrates varies broadly. A substrate could be a food, and in that case antioxidants inhibit, slow down or retard oxidation and subsequently protect food components, especially lipids, from deterioration (5). In industry, antioxidants are used in the production of rubber, plastics, and paints to control polymerization, to protect oils from deterioration, and to protect clear plastics against ultraviolet light (7). In living organisms, antioxidants prevent damage of biological macromolecules (lipid, protein, DNA, etc.) *via* different reactive species (RS) (3). Therefore, antioxidants are considered to be significant contributors to the quality and safety of food and other products, as well as health protection (4).

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Antioxidants are represented with a diverse group of substances (Table I), both of synthetic and natural origin, with the latter being more favourable due to some hazardous effect of the synthetic ones (4). In the group of natural antioxidants, compounds of plant origin are of particular interest, since they are common in various herbal products and food, and thus available in everyday life.

Table I Classes of antioxidants
Tabela I Klase antioksidanasa

Synthetic		Butylated hydroxyanisole (BHA) Butylated hydroxytoluene (BHT) Ethylenediaminetetraacetic acid (EDTA) Tertiary butylhydroquinone (TBHQ) TROLOX® Propylene glycol (PG)
Natural	**Enzymes**	Superoxide dismutase (SOD) Catalase (CAT) Glutathione peroxidase (GSH-Px) Glutathione reductase (GSHR) Glutathione S-transferase (GSH-ST) Peroxidase (Px)
	Compounds	**Endogenous**
		Lipoic acid, uric acid, glutathione, bilirubin, melatonin, polyamine, ubiquinon
		Plant/dietary origin
		Vitamins C, E, A Se, Cu, Fe, Mn, Zn (enzyme cofactors) Plant phenolics (flavonoids, anthocyanins, tannins hydroxycinnamic acids, coumarins), alkaloids, amino acids, carotenoids etc.

In addition to their chemical structure, an important feature of antioxidant molecules concerns their hydro/lyposolubility and mechanism of action. Antioxidants show great diversity in the mechanism of action (Table II), with some antioxidants having multiple and overlapping mechanisms.

Table II Mechanisms of antioxidant action

Tabela II Mehanizmi antioksidativnog delovanja

Inhibition of ROS/RNS* production
Scavenging of ROS/RNS
Chelating of transition metal ions
Inhibition of lipid peroxidation (LP)
Redox activity
Inhibition of ROS generating enzymes
Effect on gene expression – induction of antioxidant enzymes and compounds
Effect on redox signalling pathways – adaptive mechanisms

*ROS-Reactive Oxygen species; RNS-Reactive Nitrogen Species

Antioxidants *in vivo* do not act alone and isolated, but are part of a tremendous network of interconnected molecules; e.g., glutathione can reduce dehydroascorbate to regenerate ascorbate, which in turn can reduce an α-tocopheroxyl radical to α-tocopherol (3).

Antioxidants are usually classified as chain-breaking (primary) and preventative (secondary) antioxidants (8, 9). Preventive antioxidants (enzyme catalase, chelators of transition metal ions) interfere with the initiation and impede the early formation of radical species (R•), while chain-breaking antioxidants (i.e. flavonoids) react with already formed peroxyl radicals (ROO•) more rapidly than the oxidizable substrate to form species that do not propagate the oxidation chain (Figure 1) and slow down (or block) autoxidation (9).
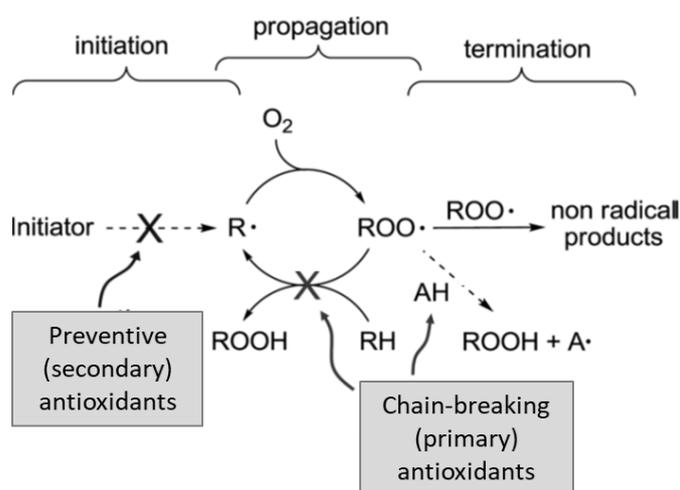

Figure 1. **Simplified scheme of autoxidation and antioxidative effect of preventive and chain-breaking antioxidants (according to ref. 9)**

Slika 1. **Pojednostavljena shema autooksidacije i antioksidativnog efekta preventivnih i „chain-breaking" antioksidanasa (prema ref. 9)**

Because of that, chain-breaking antioxidants are regarded as the most important type of antioxidants. The efficiency of these compounds is primarily related to the kinetics of reaction with peroxyl radicals. A certain compound able to react with "some radical species" could be considered an antioxidant if the following criteria are met:

- the free radical produced is truly involved in the redox chain-reaction,
- the reaction of antioxidant and radical (AH + ROO$^{\bullet}$) is much faster than the reaction of the radical with the substrate to protect, and
- the products of the chain reaction (AH$^{\bullet}$) are not able to multiply further and propagate the cycle.

Typical chain-breaking antioxidants are represented by various phenolic compounds (9).

Antioxidant assays

There are numerous assays for evaluating antioxidant activity. They were developed mainly depending on the purpose and type of the antioxidant, i.e., the product in which it was necessary to prevent oxidative changes (plastics, edible oils, food, cosmetic products) (10).

Significant progress in understanding oxidative processes and underlying mechanisms also resulted in a huge growth of knowledge on different antioxidants, their mechanisms of action and their significance both in *in vitro* and *in vivo* systems. That led to substantial revisions of the interpretations of results obtained in the past decades, as well as of the importance given to some antioxidants. Critical revision also included some widely and universally accepted assays for determining antioxidant activity and some excellent papers on this matter were presented by several research groups (3, 6, 11, 12). For example, The United States Department of Agriculture (USDA) removed the oxygen radical absorbance capacity (ORAC) database (13) from the web "due to mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health" (3).

In antioxidant assays, it is important to clearly identify and explain the initiators, possible targets, interaction between antioxidants or oxidant species, reaction rates, and reaction conditions such as pH, temperature and solvents. With such a versatility of factors involved, it is still hard to compare and correlate results obtained in antioxidant assays, even within one laboratory, and especially among different ones (3). If the protective action of antioxidants toward a biologically relevant substrate is measured, it is very important to know which biomolecule (e.g., lipid, protein, DNA, nucleic acid, etc.) is protected from which reactive species (14). The composition of plant isolates (i.e., extracts, essential oils) is complex and comprises numerous compounds with different chemical structures and different antioxidant properties. It is thus very difficult to detect and isolate individual antioxidants in such complex matrices and determine their true antioxidant activity (8).

Since numerous antioxidant methods have been developed and utilized, different classifications have been used. Depending on their application, these methods can be *in vitro* and *in vivo* (15), with *in vitro* assays further classified as chemical- and cell-based assays (4, 16). Chemical *in vitro* tests use artificial substances whose spectral features (absorbance, fluorescence) are changed upon reactions involving antioxidants. Novel chemical tests include the usage of nanoparticles (NPs): antioxidant capacity of an extract is estimated from the generation and growth of nanoparticles from a suitable cation solution (i.e., gold NPs from an Au^{3+} solution) (16).

In the cellular antioxidant activity (CAA) assay, different cultured cells (i.e., human hepatocarcinoma HepG2 cells, including Caco-2 matured differentiated intestinal cells, human macrophage cell line U937, vascular endothelial cells EA.hy926, erythrocytes) loaded with dihydrodichlorofluorescein ($DCFH_2$) are used. Under induced cellular oxidative stress (using $ROO^{\bullet}$ radicals or H_2O_2), $DCFH_2$ is easily oxidised to fluorescent dichlorofluorescein (DCF). In the presence of an antioxidant, the fluorescence is decreased (16). Other cellular tests evaluate the effects of antioxidants on the inhibition of cell membrane lipids peroxidation or activation of endogenous antioxidant systems (enzyme activity, modulation of gene expression) (4, 16).

Different instrumental methods and techniques (spectrophotometric, electrochemical, and chromatographic) are applied in order to achieve better overall characteristics of antioxidant assays. Among them, spectrophotometric methods prevail (Table III).

According to their mechanism of action, antioxidant assays may be categorized as hydrogen atom transfer (HAT)- and electron transfer (ET)-based assays (8).
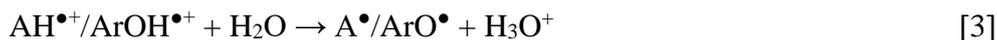
Hydrogen atom transfer (HAT)-based assays measure the capability of an antioxidant (AH/ArOH) to quench free radicals (usually $ROO^{\bullet}$) by H-atom donation:

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet} \qquad [1]$$

As it was previously mentioned, an effective antioxidant should react with free radicals faster than the substrate it protects. Reactions in HAT-based assays are performed in a competitive manner, meaning that both the probe (in most cases fluorescent compound) and antioxidant react with free radicals. The antioxidant activity is determined by measuring the fluorescence decay curve of the probe in the presence and absence of antioxidants, using the area under these curves (AUC) calculation (8). Leading HAT-based assays are ORAC (oxygen radical absorbance capacity), TRAP (total radical-trapping antioxidant parameter), crocin and β-carotene bleaching assays. Each assay has its specificities in terms of initiators, fluorescent probes and free radicals generated (3). The "lag-phase" approach to quantification is marked as the main flaw, because not all antioxidants have an apparent lag-phase, and thus the antioxidant activity of samples following the lag-phase is ignored. Endpoint observations can also be ambiguously interpreted (17). Still, their advantage is mainly

the HAT mechanism, which is the main mechanism underlying free radical reactions *in vivo*, and a certain resemblance of reaction conditions with the physiological environment.

In electron transfer (ET)-based assays, the reactions of antioxidants and free radicals are accomplished by electron transfer coupled with proton transfer:

$$ROO^{\bullet} + AH/ArOH \rightarrow ROO^{-} + AH^{\bullet+}/ArOH^{\bullet+} \qquad [2]$$
$$AH^{\bullet+}/ArOH^{\bullet+} + H_2O \rightarrow A^{\bullet}/ArO^{\bullet} + H_3O^{+} \qquad [3]$$

In ET-based assays, antioxidants react with different oxidizing probes whose initial form is spectrally distinguishable from the chemically reduced one. During reaction, the probe is converted to a colored (or fluorescent or chemiluminescent) species, or the initial absorbance (or fluorescence) is reduced. Assays can be performed as competitive or non-competitive. The broadly used ET-based assays include FC (Folin-Ciocalteau), FRAP (ferric reducing antioxidant power), CUPRAC (cupric reducing antioxidant capacity), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid)/TEAC (Trolox equivalent antioxidant capacity) and DPPH (1,1-diphenyl-2-picryl-hydrazyl) assay (3). Compared to HAT assays, these are relatively slower, and also solvent and pH dependent. The fact that the artificial chemical probes used in these tests are compounds that do not occur in physiological or pathophysiological processes considerably reduces the significance of these tests for *in vivo* systems (17).

In some cases, certain thermodynamic parameters of antioxidants in a given reaction surrounding could induce the so-called sequential proton-loss electron transfer (SPLET) mechanism. This so-called "mix-mode" mechanism seems to be dominant in DPPH and ABTS assays (6, 17).

In addition to these methods, various methods have been developed in order to investigate the effect of different antioxidants against specific ROS/RNS formed *in vivo* (e.g. $OH^{\bullet}$, $O_2^{\bullet-}$, $NO^{\bullet}$). Some of the widely used methods include TRAP (total radical-trapping antioxidant parameter), TOSC (total oxyradical scavenging capacity), 2-deoxyribose test and others (11, 16, 18).

Methods based on lipid peroxidation have also found their place in a diverse palette of antioxidant assays (4, 11, 18).

Table III Methods and techniques for *in vitro* antioxidant assays

Table III Metode i tehnike u *in vitro* antioksidantnim testovima

What is measured	Methods and techiques for end-product detection	Assay
Reducing ability of antioxidants	Spectrophotometry	FC assay
	Cyclic voltametry (CV)	
Metal ion reducing antioxidant potential	Spectrophotometry	FRAP, CUPRAC
DPPH radical scavenging activity	Spectrophotometry, HPLC-DAD/LC-DAD-MS, GC-FID/GS-MS, Electron spin resonance (ESR)	
ROO$^{\bullet}$ scavenging activity	Fluorimetry	ORAC, luminol-CL* assay, CAA assay
	GC-FID (ethylene)	TOSC
O$_2^{\bullet-}$ scavenging activity	Fluorimetry	PCL**
	Spectrophotometry	
	GC-FID (ethylene)	
	ESR	
OH$^{\bullet}$ scavenging activity	Spectrophotometry	2-Deoxyribose assay
	Fluorimetry	
	HPLC-ED	
Inhibition of oxygen uptake	Polarography, fluorimetry	TRAP
Lipid peroxidation	Spectrophotometry	
	GC-MS/MS, LC-MS/MS	
	GC-FID (4-hydroxynonenal, 4-HNE)	

*CL – chemiluminescence; **PLC - photochemiluminescence assay

*CL – hemiluminiscencija; **PLC – fotohemiluminiscentni test

The assays reviewed in this paper were selected based on their common and almost universal application in research on antioxidants in plant extracts. These methods are quite simple, easily available and affordable for most investigators, requiring no particular equipment. They are mostly used as screening tests, revealing the activity of samples in different reaction conditions and enabling further investigations through other more specific methods, in order to assess the real potential of antioxidant agents.

Folin-Ciocalteu (FC) assay

The method was originally used for the analysis of proteins in urine, but now many other matrices are also investigated. It is based on the reaction of phenolics with a molybdotungstophosphate heteropolyanion reagent (FC reagent) in alkaline solution, forming a blue coloured product with an absorbance maximum between 750 and 765 nm (Figure 2):
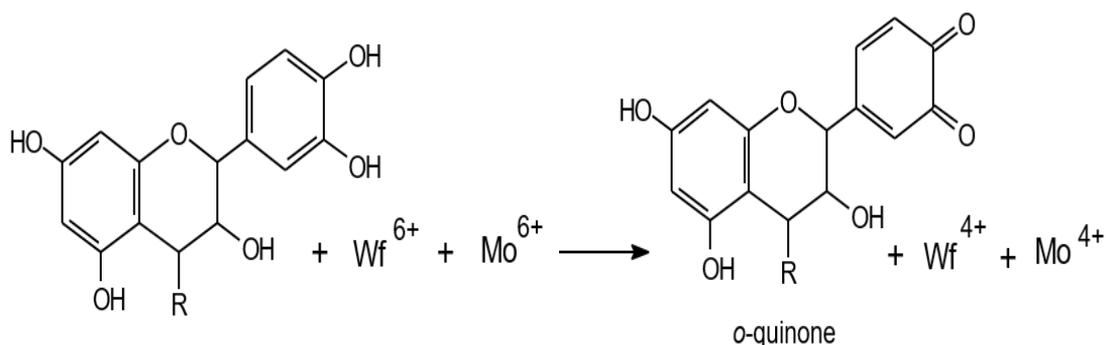

Figure 2.   Reaction of a phenolic antioxidant compound and a Folin-Ciocalteu reagent
Slika 2.   Reakcija fenolnog antioksidansa i Folin-Ciocalteu reagensa

This method is simple, fast, robust, and does not require specialized equipment. Another advantage is minimal interference with the matrix due to the long wavelength of measurement (17). The assay is routinely used to determine polyphenol content in various plant isolates, foods and beverages (19), and it has been adopted as the official procedure for measuring total phenolic levels in wines (20). The official method of the European Pharmacopoiea (Ph. Eur.) also uses FC reagent for the determination of tannin content in herbal drugs (21). The FC reagent is a strong oxidizing agent, so it oxidizes many non-phenolic reducing compounds, including weak reductants (e.g., aromatic amines, sulfites, ascorbic acid, Cu(I), Fe(II), etc.); the possible interference with some reducing agents can thus lead to overestimation of the phenolic compounds content. Other disadvantages are the unrealistically high pH reaction conditions and applicability of the conventional FC reagent only for water-soluble antioxidants (6, 11). Still, some modifications and standardization of the method have been made in order to enable measurement in both lipophilic and hydrophilic samples (22).

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FRAP (ferric reducing antioxidant power) assay

The FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+} by antioxidants in the presence of 2,4,6-tripyridyl-*s*-triazine (TPTZ) ligand. The complex of TPTZ with Fe^{2+} is yellow coloured and can be spectrophotometrically measured at 593 nm (Figure 3) (17):
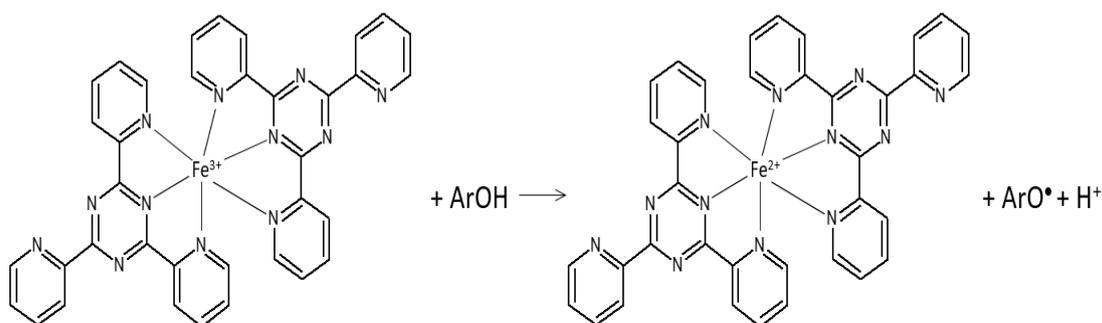

Figure 3. Reduction of Fe^{3+} ions by an antioxidant (ArOH) in the presence of a TPTZ ligand

Slika 3. Redukcija Fe^{3+} antioksidansom (ArOH) u prisustvu TPTZ liganda

The FRAP values are usually calculated by measuring the increase in absorbance and compared to a Fe^{2+} standard solution (e.g., $FeSO_4$) (11). The main advantages of the FRAP assay are its simplicity, practicality and low cost. In many cases, additivity of antioxidant capacity is observed in mixtures. Reaction conditions allow selective oxidation of most antioxidants, but not citric acid, simple sugars, thiols and carotenoids (17). In certain cases, lower than actual antioxidant capacity results can be expected: polyphenols with slow kinetics (i.e., caffeic acid, ferulic acid) are not fully oxidised within the FRAP assay protocol time (usually up to 5 minutes). Since the reaction pH is quite high, phenolic antioxidants are not fully dissociated (6, 11, 17), while precipitation of proteins in samples may occur (11). Another disadvantage of the FRAP assay, as well as of other Fe^{3+} reduction ET-based assays, is the fact that an excessive amount of Fe^{2+} is generated, which can lead to an increase in reactive species formation (such as $OH^\bullet$) through Fenton-like reaction with H_2O_2, therefore causing "redox-cycling" of phenolics and producing incorrect results (17).

CUPRAC (cupric reducing antioxidant capacity) assay

One of the recently established ET-based assays considers the reduction of Cu^{2+} to Cu^{1+} by an antioxidant in the presence of neocuproine (2,9-dimethyl-1,10-phenanthroline). Blue coloured bis(neocuproine)copper(II) chelate reacts with antioxidants, being reduced to orange coloured chelate (Figure 4) (23, 24).
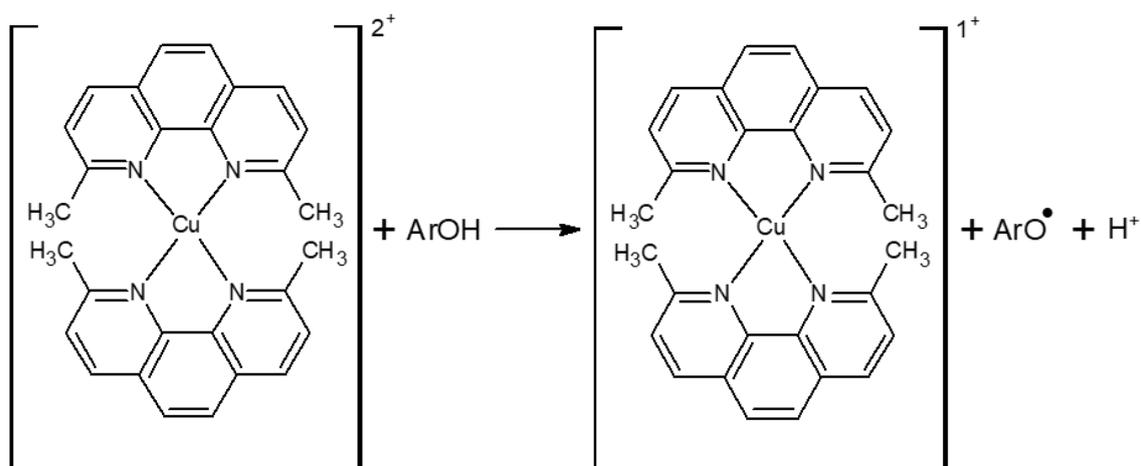
Figure 4. Mechanism of a CUPRAC reaction
Slika 4. Mehanizam CUPRAC reakcije

Neocuproine is a selective chelating agent for copper cations, stable and easily accessible. The copper(II)neocuproine reagent can react with many types of biologically important antioxidants (vitamins C, A and E, reduced glutathione (GSH), uric acid, bilirubin). It also reacts with different plant phenolics, such as flavonoids and phenolic acids, regardless of the chemical type or hydrophilicity (24). The usage of an acetate buffer enables the reaction conditions at a physiological pH, giving the CUPRAC test precedence over the FC or FRAP assay. The method can be applied to hydrophylic and lipophylic antioxidants. Several modifications of the original method have been applied with great success to antioxidants in food, plant isolates and human plasma. In that way, the measurement of total antioxidant capacity and scavenging activity of antioxidants to various reactive oxygen species (i.e., $OH^{\bullet}$, $O_2^{\bullet-}$) could be performed simultaneously (17, 24).

DPPH assay

The stable, purple coloured 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$) radical with a maximum of absorbance at 515 nm has been used for almost half a century to investigate different antioxidants (25). Due to its simplicity and accessibility, this assay is broadly used as a standard method for evaluating antioxidant activity by many research groups, and numerous variations in methods have been developed (17, 26). Though usually classified as an ET-based method, the reaction of $DPPH^{\bullet}$ with an antioxidant in fact follows the HAT mechanism (Figure 5):
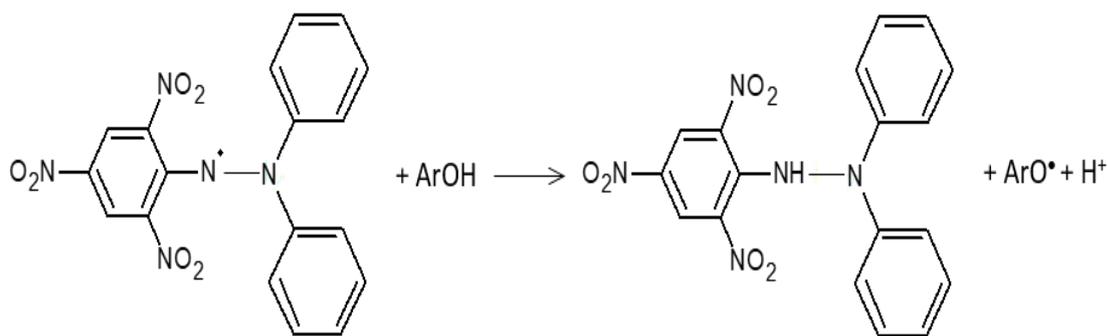
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Figure 5. HAT-based mechanism of a DPPH reaction
Slika 5. „ HAT" mehanizam DPPH reakcije

Since the reaction is highly dependent on the pH and solvent used, in most cases, especially in phenol-ionizing solvents such as methanol, the ET-mechanism predominates (6).

$$DPPH^\bullet + ArOH \rightarrow DPPH + ArO^\bullet + H^+ \qquad [4]$$

For that reason, many authors classify this assay as a SPLET or "mix-mode" assay (17). Apart from the pH and type of solvent (DPPH is hydrophobic), DPPH reactions are also affected by the presence of oxygen and exposure to light. DPPH is sterically hindered and its radical centre is strongly protected, which enables small molecules with fast initial rates (i.e., pyrogallol) to react at low concentration and show a linear increase in activity. On the other hand, in complex mixtures such as plant extracts, large molecules spatially interfere with each other, so that at low concentrations access to DPPH is hindered and at high concentrations the reaction is blocked. That is why the phenolic composition and content of the investigated extract has to be known, and the activity of the extract should be investigated in a range of concentrations (6). Although activity against DPPH is usually expressed as a SC_{50} value (concentration that causes 50% of radical quenching), allowing easier comparison between different measurements, many modifications of the test protocol by different researchers have made these results almost incomparable. Certain recommendations toward overcoming these obstacles have been proposed (26). DPPH test was successfully applied as a dot-blot technique (27), allowing the identification of individual antioxidant compounds from mixtures. The hyphenated techniques were also successfully applied to the DPPH assay (3), such as HPLC-MS (28), online extraction–DPPH–HPLC–DAD–QTOF-MS (29) and GC-MS-DPPH (30), allowing rapid and simultaneous on-line activity detection and structure elucidation of the individual antioxidant in various plant isolates (28).

ABTS/TEAC assay

In this assay, an intensely green coloured radical is produced from 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) by an oxidising agent (Figure 6). The formed ABTS$^{\bullet+}$ cation radical is then used as a colorimetric probe reacting with an antioxidant compound.
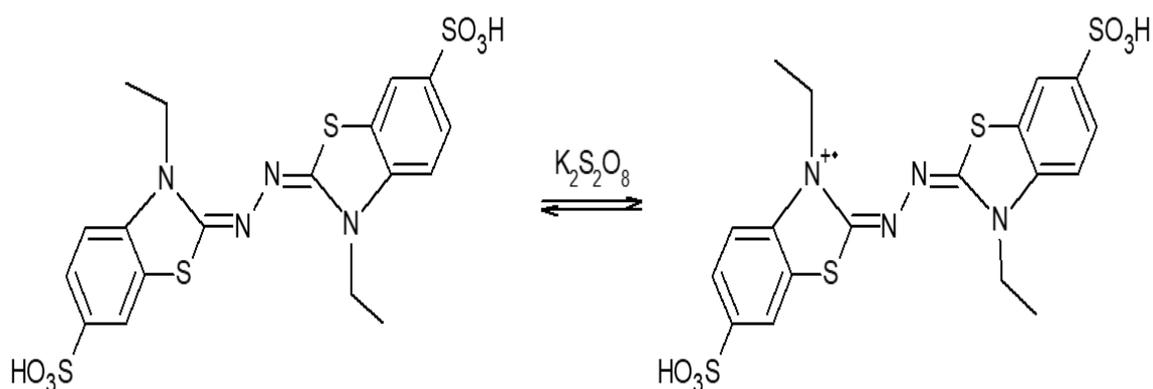

Figure 6. **ABTS oxidation and formation of an ABTS$^{+\bullet}$ radical**
Slika 6. **Oksidacija ABTS i nastanak ABTS$^{+\bullet}$ radikala**

Initially, the ability of an antioxidant to intercept initial oxidation and prevent ABTS$^{\bullet+}$ radical formation was measured. In order to avoid possible double reaction and improve reaction conditions, a modification was introduced where ABTS$^{\bullet+}$ is produced in excessive amounts using potassium sulfate as oxidising agent. In the modified method, a stabilized ABTS$^{\bullet+}$ radical reacts with the antioxidant, causing an absorbance decrease (6):

$$ABTS^{\bullet+} + ArOH \rightarrow ABTS + ArO^{\bullet} + H^{+} \qquad [5]$$

The activity of the sample is estimated by the amount of ABTS$^{\bullet+}$ quenched after certain period (usually 5 minutes) and is usually compared to the activity of Trolox (for this reason the assay is also known as the Trolox Equivalent Antioxidant Capacity (TEAC) assay) (12). As in the case of DPPH assay, ABTS also follows "mix-mode" mechanisms. At the same time, reaction rate and kinetics are ignored, because absorbance drop is provided only by reaction stoichiometry (6, 12). Although various compounds can be used to generate ABTS$^{\bullet+}$ (usually potassium permanganate or potassium persulfate), great importance should be attached to the choice of oxidant, because the results obtained for the same compound (e.g., reduced glutathione, GSH) can vary greatly (17).

ORAC test

The Oxygen Radical Absorbance Capacity (ORAC) assay is one of the most used methods for evaluating the peroxyl radical (ROO•) quenching capacity of different samples (11). Peroxyl radicals are produced by the thermal degradation of 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH) in buffer solution: as it decomposes, nitrogen is eliminated, and two carbon-centered radicals (R•) are left behind. They almost instantly react with oxygen and produce reactive peroxyl radicals (ROO•) (Equation 6), which can either attack fluorescent target/probe (equation 7) or react with the antioxidant (equations 8 and 9) (6):

$$\text{R-N=N-R} \rightarrow N_2 + 2R^\bullet \rightarrow 2\ ROO^\bullet \qquad [6]$$

$$ROO^\bullet + \text{target/probe} \rightarrow ROOH + \text{oxidised product (no fluorescence)} \qquad [7]$$

$$ROO^\bullet + AH \rightarrow ROOH + A^\bullet \qquad [8]$$

$$ROO^\bullet + A^\bullet \rightarrow ROO\text{-}A \qquad [9]$$

The fluorescent target/probe is usually fluorescein, whose fluorescence is lost when attacked by free radicals. When an antioxidant is present, it quenches the formed ROO• radicals by hydrogen atom transfer (Equation 8) or addition of a radical (Equation 9), and fluorescence loss is slowed. The reaction is monitored for a fixed time period (30 minutes and more). The oxidation during this time is measured and quantified based on the area under the fluorescence decay curve (AUC). The protective effect of antioxidants is estimated from the difference in AUC of the sample with and without the investigated antioxidant, and the results are usually compared to Trolox. As in some other tests using AUC calculations, the ORAC assay can be applied for antioxidants that exhibit distinct lag-phases and to those having no lag-phases, making it useful for plant extracts and food samples with multiple constituents and having complex reaction kinetics. The inability to investigate lipophilic samples, one of the main disadvantages of this assay, was dealt with by using lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) (18). Despite its complexity, the ORAC assay is still superior to the DPPH and ABTS assays due to using ROO• radicals, and thus representing a better model of antioxidant reactions that occur *in vivo* and in complex plant matrices. ORAC considers an entirely HAT-based mechanism and provides continuous flux of free radicals on a realistic time scale, further mimicking physiological conditions. Another advantage of ORAC is the possibility to test reactivity against a variety of free radicals by changing reaction initiators (6). The popularity of this assay resulted in forming an antioxidant database of ORAC values of selected foods in combination with the total phenolics content. This database was intended to "provide reliable values for epidemiological studies to assess the relationship between the dietary intake of bioactive compounds and health status" (13), but as previously mentioned, the database was removed due to a lack of evidence that certain bioactive compounds, such as phenolics, have beneficial effect on human health and the fact that ORAC values were largely misrepresented (3, 6).

The principles of the ORAC assay were modified to determine the activity toward other ROS, and a broad array of *in vitro* tests was developed in order to expand the initial ORAC and overcome the detected flaws and disadvantages (elaborated in detail in refs. 6 and 11).

Conclusion

Since there is a growing necessity to detect, develop and understand effective antioxidant compounds, interest in the identification and measurement of antioxidants in various plant isolates is persistently growing and many methods are being established. Most of the available *in vitro* tests are affordable and easy to perform, but due to the complex composition of plant extracts, different kinetics, mechanisms and specificity of the chemical reactions of these tests, there is no universal parameter for the assessment of antioxidant activity. Results obtained from *in vitro* tests cannot be directly extrapolated to *in vivo* systems, which further reduces their practical significance.

Nevertheless, studies of antioxidant activity by *in vitro* methods are important, because they allow a better understanding of the antioxidant mechanisms and the identification of individual bioactive molecules. It is of crucial importance to select and apply the most suitable method for a specific application. Because antioxidants can exert their effects through complex and often overlapping mechanisms, it is important to clarify which feature of the antioxidant molecule is being measured, which in turn determines the antioxidant assay to be applied. Therefore, a combination of different assays should be conducted in order to comprehensively investigate the antioxidant properties of selected samples (17).

A rational and critical assessment of all the advantages and limitations of currently available *in vitro* methods is essential, because it significantly affects their improvement and standardization, and the development of new analytical procedures. Currently available and future *in vitro* methods for determining the antioxidant activity of plant isolates should contribute to a better assessment of their real effectiveness as antioxidants and to the optimization of their use.

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Metode određivanja antioksidativne aktivnosti biljnih ekstrakata *in vitro*

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Kratak sadržaj

Biljke su bogati izvori sekundarnih metabolita koji ispoljavaju raznovrsne biološke i farmakološke efekte. Neki biljni sastojci, u prvom redu polifenolna jedinjenja, ispoljavaju značajnu *in vitro* antioksidativnu aktivnost, zbog čega se smatra da mogu doprineti održanju redoks ravnoteže u organizmu. Ovi potencijalni antioksidativni agensi strukturno su veoma raznovrsni i deluju različitim antioksidantnim mehanizmima. S obzirom na rastuću potrebu za iznalaženjem, razvojem i razumevanjem efikasnih antioksidanasa, interesovanje za ispitivanje antioksidanasa u različitim biljnim izolatima kontinuirano raste, pa su shodno tome i razvijeni mnogi testovi. Većina dostupnih *in vitro* testova je pristupačna i jednostavna za izvođenje, ali zbog složenog sastava biljnih ekstrakata, različite kinetike, mehanizama i specifičnosti hemijskih reakcija na kojima se ovi testovi zasnivaju, još uvek ne postoji univerzalni parametar za procenu antioksidativne aktivnosti. U ovom radu su prikazane neke od trenutno najkorišćenijih *in vitro* metoda za ispitivanje i procenu antioksidativne aktivnosti biljnih ekstrakata, s naglaskom na njihove prednosti i nedostatke.

Ključne reči: antioksidansi, biljni ekstrakti, *in vitro* testovi