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Original Article

Overview Of In Vitro – Antioxidant Models

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ABSTRACT
Laboratory-based antioxidant tests have turned into essential tools for evaluating the antioxidant characteristics of various substances and extracts. These in vitro evaluations serve as a rapid and efficient method for measuring antioxidant capabilities. This overview presents an in-depth examination of the most commonly used in vitro antioxidant assays, including DPPH, ABTS, FRAP, among others. The fundamental concepts, advantages, and limitations of each method are discussed, along with their applications in pharmaceuticals, food science, and cosmetics. The importance of standardization, validation, and high-throughput screening is highlighted, indicating future pathways for in vitro antioxidant research.
Keywords: Antioxidant, antioxidant potential, cost-effective

INTRODUCTION

Antioxidant: Free Radicals: In the body, unstable molecules referred to as free radicals arise from external influences, whereas antioxidants are substances that can lessen or avert the damage inflicted on cells by these free radicals.

Functions:

1. Antioxidants have the ability to neutralize free radicals and prevent further harm by supplying them with electrons.
2. Antioxidants help to alleviate oxidative stress by targeting free radicals and reducing inflammation.
3. While free radical damage contributes to aging and various illnesses, antioxidants can protect cells from this kind of harm.[1]

In investigations centered on the essential mechanisms driving various illnesses such as cancer, mental disorders, and heart-related diseases, reactive oxygen species (ROS) are acknowledged as vital components. Antioxidants are seen as promising methods for warding off or controlling these health issues, thanks to their capacity to neutralize or eliminate ROS. There has been a notable rise in the quest to discover and evaluate natural antioxidants derived from fruits, vegetables, and plants.Laboratory tests aimed at antioxidants have become vital instruments for assessing the antioxidant properties of diverse substances and extracts. These evaluations offer a quick and economical approach to measuring antioxidant effectiveness, enabling researchers to pinpoint candidates for deeper exploration. However, the complex characteristics of antioxidant actions, along with the multitude of reactive oxygen species (ROS), can make the task of choosing appropriate evaluations and interpreting their findings more challenging. This article intends to outline the key concepts, benefits, and drawbacks of the most frequently used in vitro antioxidant tests. We will also explore how these evaluations are applied in various sectors such as food technology, cosmetics, and medical care. Furthermore, we will discuss the difficulties and future opportunities within the field of in vitro antioxidant testing.[2]

Advantages:

1. Rapid verification
2. Diverse range of applications
3. Regulated setting
4. Evaluation before experimentation
5. Minimized use of animals.[3]

Disadvantages:

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1. Limited predictive ability
2. Lack of biological relevance
3. Interference from testing materials.[4]

Antioxidant In-vitro Models:

A scientific examination referred to as the in-vitro antioxidant assessment is utilized to assess the antioxidant properties of a particular material, extract, or combination.

An in vitro antioxidant assessment is crucial.

1. Origin of natural substances
2. Creation of novel antioxidants
3. Consistency of botanical extracts
4. Analysis of dietary antioxidants
5. Assessment of therapeutic efficacy.[5]

Mechanism of action of antioxidants:

Mechanism through various methods:

Free Radical Neutralization

1. Electron Contribution: Antioxidants provide electrons to free radicals, which aids in their stabilization and prevents further damage.
2. Hydrogen Contribution: Antioxidants deliver hydrogen atoms to free radicals, which deactivates them.

Inhibition of Oxidative Enzymes

1. Enzyme Disruption: Antioxidants interfere with enzymes that cause oxidative stress, such as xanthine oxidase and cyclooxygenase.
2. Management of Redox Enzymes: Antioxidants regulate redox-sensitive enzymes to maintain cellular equilibrium.

Metal Ion Binding

1.Attachment to Metal Ions: Antioxidants connect with metal ions like iron and copper, preventing them from initiating oxidative reactions.[6]

Categories:

1. DPPH scavenging activity
2. Hydrogen peroxide (H2O2) neutralization test
3. Evaluation of Ferric Ion Reduction Capacity (FRAP)
4. Nitric oxide inhibition test
5. Overall radical-trapping antioxidant measurement (TRAP) technique
6. Trolox equivalent antioxidant ability (TEAC) approach/ABTS radical cation decolorization test
7. Activity of scavenging superoxide radicals (SOD)
8. ABTS (2, 2'-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid) diamonium salt) technique
9. Method utilizing xanthine oxidase
10. Overall reducing capacity

1. DPPH scavenging activity:

The placement of the extra electron within the molecule prevents dimer formation, setting it apart from numerous other free radicals. Therefore, the compound known as 1, 1-diphenyl-2-picrylhydrazyl, frequently referred to as α,α-diphenyl-β-picrylhydrazyl or simply DPPH, is recognized as a stable free radical. When it is dissolved in ethanol, DPPH exhibits a rich violet hue, with an absorption peak located at approximately 517 nm. This violet hue fades in response to interaction with a substance (AH) that can provide a hydrogen atom, leading to a more stable configuration. To evaluate the antioxidant potential, we examine how different substances can neutralize free radicals by observing alterations in the optical density of the DPPH radicals. Manzocco et al. (1998) state that the extract sample should first be diluted with 0.2 mL of methanol prior to its combination with 2 mL of a 0.5 mM DPPH solution. After a waiting period of thirty minutes, the absorbance is measured at 517 nm. The percentage of DPPH radical scavenging is calculated using the formula:

DPPH scavenging effect (%)/% Inhibition = (A0 – A1) / A0 × 100

In this equation, A0 represents the absorbance of the control sample, while A1 indicates the absorbance of the experimental sample.[7]

2.Hydrogen peroxide (H2O2) neutralization test:

Humans typically encounter hydrogen peroxide through agricultural products, ingesting about 0.28 mg per kg daily. The absorption of hydrogen peroxide occurs not just through inhaling mists or sprays but also via direct exposure to skin or eyes. When it degrades swiftly, hydrogen peroxide turns into water

and oxygen, resulting in the production of hydroxyl radicals, known as OH radicals. These substances have the potential to harm DNA and trigger lipid peroxidation within the body. In 1989, Ruch and colleagues developed a method to determine the ability of plant extracts to lessen the impact of hydrogen peroxide. They create a phosphate buffer solution containing 50 mM hydrogen peroxide, yielding a final concentration of 40 mM at a pH of 7.4. To quantify the level of hydrogen peroxide, absorbance readings are taken at 230 nm using a spectrophotometer. A solution of plant extract is prepared with concentrations ranging from 20 to 60 µg/mL, mixed with distilled water, and introduced to the hydrogen peroxide solution. After a ten-minute waiting period, the absorbance at 230 nm is assessed and compared with a control solution prepared solely from the phosphate buffer without hydrogen peroxide. Subsequently, the extent of hydrogen peroxide degradation is calculated using the formula:

H2O2 (%) Scavenged: [(A0-A1)/A0] × 100.

In this equation, A0 denotes the absorbance of the control solution, while A1 indicates the absorbance of the treated sample.

3.Evaluation of Ferric Ion Reduction Capacity (FRAP):

To evaluate the potential for reduction, mix 1.0 ml of the extract with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide solution (30 mM), then incubate this combination at a temperature of 50°C for 20 minutes. Following this incubation, add 2.5 ml of trichloroacetic acid (600 mM) to the mixture and centrifuge at a speed of 3000 rpm for 10 minutes. After centrifugation, collect the upper liquid layer (2.5 ml) and combine it with 2.5 ml of distilled water and 0.5 ml of FeCl3 (6 mM), then assess the absorbance at a wavelength of 700 nm (Arthukorala et al., 2006). For the positive controls, one may use ascorbic acid, butylated hydroxyanisole (BHA), α-tocopherol, and trolox (Oyaizu et al., 1986), along with butylated hydroxytoluene (BHT) (Jayaprakasha et al., 2001).[8]

4. Nitric oxide inhibition test:

1. To create a solution containing sodium nitroprusside, combine 2 mL of a 10 mM solution with 0.5 mL of phosphate-buffered saline set at a pH level of 7.4.
2. Next, add 0.5 mL of the sample extract and maintain a constant temperature of 25°C.
3. After allowing the mixture to incubate for 150 minutes, add 0.5 mL of Griess reagent, which is made up of 1% sulfanilamide, 2% H3PO4, and 0.1% naphthylethylenediamine dihydrochloride.
4. Let the reaction mixture remain at room temperature for another 30 minutes.
5. The subsequent step is to measure the absorbance at a wavelength of 546 nm.
6. Using this information, calculate the percentage of inhibition.

NO Inhibition = [(A546 Control – A546 Sample) / A546 Control] × 100. [9]

5. Overall radical-trapping antioxidant measurement (TRAP) technique:

The fundamental concept of this method revolves around how antioxidants enhance the degradation of R-ficolitrin (R-PE) fluorescence when subjected to oxidative heating. ABAP hydrochloride acts as a free radical generator, which aids in diminishing the fluorescence observed in R-ficolitrin. The presence of antioxidants leads to a significant quenching effect. This decrease in color strength is used to assess the antioxidant potential. A mixture is prepared containing 120 µl combined with 2.4 ml of a phosphate buffer, adjusted to a pH of 7.4, along with 375 µl of distilled water and 30 µl of a diluted R-PE solution. The kinetic reactions are monitored over a span of 45 minutes at a temperature of 38°C utilizing a luminescence spectrometer. Following this interval, 75 µl of ABAP is added (Giselli et al., 1995). To determine the TRAP value, the duration of the lag phase in the sample is contrasted with that of the reference standard.

6. Trolox equivalent antioxidant ability (TEAC) approach/ABTS radical cation decolorization test:

This technique utilizes a diode array spectrophotometer to assess the color change caused by the reaction between an antioxidant and the blue-green chromophore ABTS•+ (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). The presence of an antioxidant leads to a color transformation, converting ABTS•+ into its stable form, ABTS. The

persistent radical, ABTS•+, does not occur naturally in the human body. The method introduced by Seeram and colleagues in 2006 enables the assessment of the efficacy of antioxidants. Specifically, an amount of 80 mg of manganese dioxide is incorporated into a 20 mL solution containing 5 mM of ABTS, using a sodium/potassium buffer at pH 7 with a concentration of 75 mM to form the ABTS radical cation. Trolox, which is a water-soluble derivative of vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), serves as the standard antioxidant. The concentrations of Trolox evaluated include: 0, 50, 100, 200, 300, and 350 µM. The antioxidant's activity influences the changes applied to the samples within the sodium/potassium buffer maintained at pH 7. A total of 200 µl of the ABTS radical cation solution is mixed with the diluted samples in a 96-well plate, and absorbance is measured at 750 nm after five minutes using a microplate reader. A standard curve for Trolox is prepared to calculate TEAC values, reported in terms of Trolox equivalents (in mM).

7. Activity of scavenging superoxide radicals (SOD):

Although it is considered a weak oxidizing agent, the superoxide anion plays a crucial role in producing singlet oxygen and highly reactive, dangerous hydroxyl radicals, which lead to elevated levels of oxidative stress (Meyer and Isaksen, 1995). The method described by Roebuck and Gryglewski (1988) provides a way to evaluate the ability to eliminate superoxide anions. The solution will contain 3.0 ml of Tris-HCl buffer (16 mM, pH 8.0), in addition to 0.5 ml of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 ml of NADH solution (0.936 mM), 1.0 ml of the extract, and another 0.5 ml of Tris-HCl buffer (16 mM, pH 8.0) to aid in producing superoxide anion radicals. To kick off the reaction, add 0.5 ml of phenazine methosulfate (PMS) solution (0.12 mM) to the mixture. Next, let the solution sit at 25 °C for five minutes prior to measuring the absorbance at 560 nm with a blank control. [10]

8. ABTS (2, 2'-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid) diamonium salt) technique:

In terms of the quantity of antioxidants present, this serves as a measure of their potency. It may encompass specific antioxidants that do not exert considerable physiological impacts. Additionally, it offers a way to assess the efficiency of various antioxidant combinations, aiding in distinguishing between additive and synergistic effects. This approach was utilized to assess the antioxidant capabilities of various wines. The assessment is based on the interaction between the antioxidant and the radical cation ABTS+, which shows distinctive color shifts at the primary wavelengths of 645, 734, and 815 nm.[11]

9. Method utilizing xanthine oxidase:

Two separate methods can be employed to evaluate the effectiveness of scavenging superoxide anion: the first method applies a non-enzymatic approach using nitroblue tetrazolium (NBT), while the second utilizes an enzymatic method with cytochrome C, as noted by McCord and Fridovich in their research from 1969. In 1990, Lu and Zhang discovered that superoxide anions could be generated through the cytochrome C pathway along with xanthine and the mechanism involving xanthine oxidase. The assessment of oxidase activity depends on the substrate that is used. This is carried out by capturing absorbance readings with a spectrophotometer, based on methods established in 1983. A solution was prepared by combining 0.1 mg/ml and 100 µg/ml concentrations of allopineol (dissolved in methanol) with 1.3 ml of a phosphate buffer solution (0.05 M, pH 7.5) and 0.2 ml of xanthine oxidase solution. After this, 1.5 ml of the xanthine substrate was added, and the mixture was kept at a stable temperature of 25°C for 10 minutes while absorbance was measured at 293 nm (with a sample volume of 0.5 ml) using the spectrophotometer. Subsequently, the mixture was left to incubate for another 10 minutes at room temperature (25°C), and absorbance was again checked at 293 nm (also utilizing 0.5 ml). A spectrophotometric analysis of 0.5 ml was performed for evaluation. It's vital to keep the mixture at room temperature (25°C) for a duration of 30 minutes. The solution was made by combining 0.2 ml of xanthine oxidase with 1.3 ml of phosphate buffer in methanol. The control was set up by mixing 0.5 ml of methanol, 1.3 ml of phosphate buffer, 0.2 ml of xanthine oxidase, and 1.5 ml of the xanthine substrate. To determine the percentage of inhibition, the formula used was: $[1 – (As / Ac)] \times 100$. In this equation, As and Ac refer to the absorbance values of the experimental group and the control group,

respectively. Positive controls, such as BHT (as mentioned by Chang et al. In 1996) or catechin (as reported by Schmeda-Hirschmann et al. In 1996), can also be utilized.[12]

10. Overall reducing capacity:

The technique developed by Yen and Duh in 1993 was employed to assess the capacity for reduction. An equal volume of 500 µl of a phosphate buffer solution (20 mM, pH 6.6) was combined with 500 µl of a 1% potassium ferricyanide solution, and various extracts were added, ranging from 0.1 to 0.9 mg/ml. Following this, the mixture was incubated at 50 degrees Celsius for 20 minutes, then subjected to centrifugation at 2500 rpm for 10 minutes. Afterward, 300 µl of 0.1% ferric chloride and 1.5 ml of distilled water were mixed into the supernatant, and the absorbance was measured at 700 nm. This whole procedure was carried out in triplicate. An increase in the absorbance of the analyzed solution indicated an improvement in reduction capacity.[13]

CONCLUSION:

Research focused on antioxidants has undergone significant transformation due to lab-based antioxidant systems, which provide a swift, affordable, and effective approach to assessing antioxidant capabilities.

Key Insights:

1. Variety of Evaluations: Tests such as DPPH, ABTS, FRAP, and ORAC are among the various assessments utilized in laboratory antioxidant evaluations.
2. Fast and Simple: These evaluations are quite quick and uncomplicated, promoting timely analysis of a large number of samples.
3. Cost-Effective: The cost-efficient aspect of laboratory antioxidant systems eliminates the need for costly and prolonged experiments involving living subjects.
4. Wide Applications: These systems are applicable in multiple fields, including food, health care, and cosmetic products.

Future Perspectives:

1. Consistency: It is crucial to standardize in vitro antioxidant tests to guarantee consistency and the feasibility of result comparisons.
2. Verification: In order to evaluate the predictive accuracy of in vitro antioxidant frameworks, it is important to perform comparisons with in vivo research.
3. Accelerated Screening: Developments in high-throughput screening techniques will enhance the speed of identifying novel antioxidants.

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