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REVIEW

Protocols for Antioxidant Testing: A Mini Review of Common Assays and Approaches

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ABSTRACT

Antioxidants play a pivotal role in mitigating damage caused by oxidative stress, which is implicated in aging, inflammation, neurodegeneration, and various chronic diseases. With the growing interest in natural and synthetic antioxidants across nutraceutical, pharmaceutical, and food industries, the need for reliable, reproducible, and standardized testing methods has become increasingly urgent. This mini-review provides a structured overview of the most widely used antioxidant testing protocols, categorized into in vitro chemical-based assays (e.g., DPPH, ABTS, FRAP, metal chelation), cell-based assays (e.g., DCFH-DA for intracellular ROS detection), and in vivo biomarker-based models. Each method's principle, experimental protocol, advantages, and limitations are critically discussed. The review further highlights the importance of assay selection based on mechanistic relevance, distinguishing between primary radical scavenging and secondary antioxidant mechanisms. Finally, it addresses major challenges in assay variability, poor in vitro–in vivo correlation, and calls for methodological standardization and integration of emerging biosensing and high-throughput approaches for future advancements.

Keywords: Antioxidant assays, Free radical scavenging, DPPH/ ABTS/ FRAP, Oxidative Stress biomarkers, In vitro and in vivo protocols

1. Introduction

Antioxidants play a pivotal role in mitigating damage caused by oxidative stress, which is implicated in aging, inflammation, neurodegeneration, and various chronic diseases [1, 2]. This imbalance has profound biological implications, serving as a central pathological mechanism underlying various diseases and conditions, including aging [3], cancer [4], inflammatory disorders [5], and neurodegeneration [6, 7]. The accumulation of oxidative damage to cellular components such as DNA, proteins, and lipids contributes significantly to disease progression and cellular dysfunction [8]. The growing recognition of the therapeutic potential of antioxidants has created an urgent need for reliable, standardized, and reproducible antioxidant assays for compound screening, especially in food science, pharmaceutical development, and nutraceutical research[9,10]. The complexity of antioxidant mechanisms and the diversity of available testing methods have led to significant variability in results and interpretation challenges across different laboratories and research groups [11, 12]. This comprehensive review aims to address these challenges by covering widely used in vitro and biological assays with a particular focus on their underlying methodologies and comparative applicability. The scope encompasses both chemical-based and cell-based approaches, providing researchers with detailed protocols and critical

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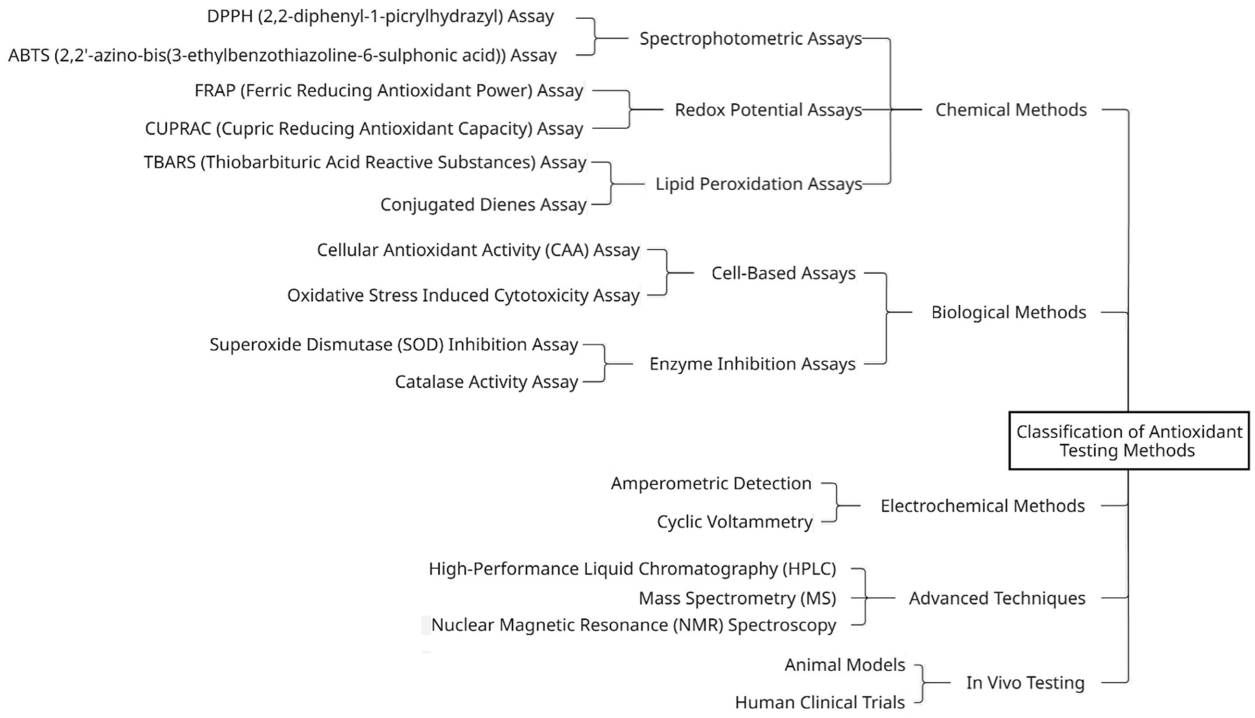

Fig. 1. Classification of antioxidant assays.

insights into the selection and implementation of appropriate antioxidant testing strategies for their specific research objectives [13, 14].

2. Classification of antioxidant testing approaches

Antioxidant testing methodologies can be systematically classified into several distinct categories based on their experimental design and biological relevance. In vitro assays represent the most commonly employed initial screening approach, encompassing both chemical-based methods such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, as well as cell-based systems utilizing fluorescent probes like 2',7'-dichlorofluorescin diacetate (DCFH-DA) [15, 16]. In vivo assays provide more physiologically relevant information through biomarker analysis in animal models, typically involving rodent studies where oxidative stress markers and antioxidant enzyme activities are measured in various tissues and biological fluids [17, 18]. These approaches offer valuable insights into the bioavailability, metabolism, and actual protective effects of antioxidant compounds under physiological conditions [19]. A mechanism-based categorization further distinguishes between primary antioxidants, which function as direct radical scavengers and can be effectively measured using assays like DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [20, 21], and secondary antioxidants, which operate through indirect mechanisms such as metal chelation, enzyme induction, or prevention of radical formation [22, 23]. This classification is particularly important as it influences the selection of appropriate testing methods and the interpretation of results. Fig. 1 represents a comprehensive hierarchical classification of antioxidant testing techniques, organized into five major categories: Chemical Methods, Biological Methods (including Cell-Based and Enzyme Inhibition Assays), Electrochemical Methods, Advanced Analytical Techniques, and In Vivo Testing. Each branch includes representative assays or tools such as DPPH, FRAP, CAA, SOD inhibition, HPLC, and animal models, reflecting the diversity of approaches used to assess antioxidant activity across in vitro, ex vivo, and in vivo systems.

The distinction between direct and indirect antioxidant capacity evaluation is fundamental to understanding the limitations and applicability of different assays [24]. Direct methods measure the immediate radical scavenging capacity of compounds, while indirect methods assess the compound's ability to enhance cellular antioxidant systems or prevent oxidative damage through various protective mechanisms [25, 26].

Table 1. Summary of common in vitro antioxidant assays.

Assay	Target Radical/Mechanism	Detection Method	Wavelength (nm)	Reference Standard	Advantages	Limitations
DPPH	DPPH•scavenging	UV-Vis spectrophotometry	517	Trolox, Ascorbic acid	Simple, Fast	hydrophilic samples
ABTS	$ABTS^{\bullet+}$ scavenging	UV-Vis spectrophotometry	734	Trolox	Broad pH range	Requires radical-generation step
FRAP	Reducing power ($Fe^{3+} \rightarrow Fe^{2+}$)	UV-Vis spectrophotometry	593	$FeSO_4$ (Fe^{2+})	Reproducible	Does not reflect radical-scavenging activity
Metal chelation	Fe^{2+} chelation	UV-Vis spectrophotometry	562	EDTA	Measures secondary antioxidant activity	Not ROS-specific

3. Free radical scavenging assays: Protocols and principles

Table 1 summarizes widely used in vitro antioxidant assays based on their target mechanisms (e.g., radical scavenging, reducing power, metal chelation), detection methods, optimal wavelengths, reference standards, and key advantages and limitations. The comparison highlights methodological distinctions and supports the appropriate selection of assays depending on compound properties and experimental goals.

3.1. DPPH assay protocol

The DPPH assay represents one of the most widely utilized methods for evaluating antioxidant activity due to its simplicity, reproducibility, and cost-effectiveness [27, 28]. The principle underlying this assay involves the use of $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), a stable violet-colored free radical that exhibits strong absorption at 517 nm [29]. When antioxidants are present, they donate hydrogen atoms or electrons to $DPPH^{\bullet}$, reducing it to the yellow-colored DPPH-H (2,2-diphenyl-1-picrylhydrazine), resulting in a measurable decrease in absorbance [30].

Detailed Protocol:

1. Prepare a 0.1 mM DPPH solution in methanol and store in darkness to prevent photodegradation
2. Prepare serial dilutions of test compounds in appropriate solvents (typically methanol or ethanol)
3. Mix 1 mL of DPPH solution with 1 mL of test compound at various concentrations in test tubes
4. Prepare control samples by mixing 1 mL DPPH solution with 1 mL of pure solvent
5. Incubate all samples in darkness for 30 minutes at room temperature (20–25 °C)
6. Measure absorbance at 517 nm using a spectrophotometer
7. Calculate percentage scavenging activity using the formula (1) [31]:

$$\%\ Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

The EC50 value, representing the concentration of antioxidant required to scavenge 50% of DPPH radicals, serves as a standard parameter for comparing antioxidant potencies [32, 33].

3.2. ABTS assay protocol

The ABTS radical cation decolorization assay offers several advantages over the DPPH method, including its applicability to both hydrophilic and lipophilic antioxidants and its ability to function across a wide pH range [34, 35]. The principle involves the generation of the blue-green $ABTS^{\bullet+}$ radical cation (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), which exhibits characteristic absorption at 734 nm and is readily decolorized by antioxidants [21, 36].

Detailed Protocol:

1. Generate $ABTS^{\bullet+}$ radical stock solution by mixing 7 mM ABTS with 2.45 mM potassium persulfate in equal volumes
2. Allow the mixture to stand in darkness for 8–16 hours at room temperature to ensure complete radical formation
3. Dilute the $ABTS^{\bullet+}$ stock solution with phosphate buffer (pH 7.4) or ethanol to achieve an absorbance of approximately 0.7 ± 0.02 at 734 nm
4. Add 100 μL of test sample to 1 mL of diluted $ABTS^{\bullet+}$ solution
5. Mix thoroughly and incubate for exactly 6 minutes at room temperature

6. Measure absorbance reduction at 734 nm against an appropriate blank
7. Calculate antioxidant activity as Trolox equivalent antioxidant capacity (TEAC) values

3.3. Superoxide scavenging assay (NBT assay)

The superoxide anion radical scavenging assay utilizing nitroblue tetrazolium (NBT) provides specific information about a compound's ability to neutralize one of the most biologically relevant ROS [37, 38]. The principle relies on the non-enzymatic generation of superoxide radicals through the phenazine methosulfate (PMS)/NADH system, which subsequently reduces NBT to form a blue formazan product with maximum absorption at 560 nm [39, 40].

Detailed Protocol:

1. Prepare reaction mixture containing 50 μL of NBT (0.1 mM in phosphate buffer, pH 7.4)
2. Add 50 μL of NADH (0.1 mM in phosphate buffer)
3. Include 50 μL of PMS (0.1 mM in phosphate buffer, prepared fresh)
4. Add varying concentrations of test compounds (50 μL)
5. Adjust total volume to 300 μL with phosphate buffer
6. Incubate for exactly 5 minutes at room temperature under normal lighting conditions
7. Measure absorbance at 560 nm immediately after incubation
8. Calculate percentage inhibition using the standard formula (1)

3.4. Hydroxyl radical scavenging assay (Deoxyribose assay)

The deoxyribose degradation assay represents the gold standard for evaluating hydroxyl radical scavenging capacity, as hydroxyl radicals are among the most reactive and damaging ROS encountered in biological systems [41, 42]. This assay is based on the principle that hydroxyl radicals, generated through the Fenton reaction, attack deoxyribose to produce thiobarbituric acid-reactive substances (TBARS) that form a pink-colored complex with thiobarbituric acid (TBA) [43, 44].

Detailed Protocol:

1. Prepare reaction mixture containing 0.1 mL of 2.8 mM deoxyribose in phosphate buffer (pH 7.4)
2. Add 0.1 mL of 0.1 mM $FeSO_4 \cdot 7H_2O$
3. Include 0.1 mL of 0.1 mM EDTA
4. Add 0.1 mL of 0.1 mM ascorbic acid
5. Include 0.1 mL of 1 mM H_2O_2
6. Add test sample (0.1 mL at various concentrations)
7. Adjust total volume to 1.0 mL with phosphate buffer
8. Incubate at 37 °C for 1 hour with gentle shaking
9. Add 1.0 mL of 2.8% trichloroacetic acid (TCA)
10. Add 1.0 mL of 1% thiobarbituric acid in 0.05 M NaOH
11. Heat the mixture in a boiling water bath for 15 minutes
12. Cool rapidly in an ice bath
13. Measure absorbance at 532 nm against an appropriate blank
14. Calculate the hydroxyl radical scavenging activity as a percentage inhibition of deoxyribose degradation

4. Reducing power & metal chelation assays

The reducing power and metal chelation capacity of compounds represent fundamental mechanisms of antioxidant action that complement free radical scavenging activities. These assays provide critical insights into the electron-donating capacity and metal-binding properties of potential antioxidants, which are essential for understanding their protective mechanisms against oxidative damage [45, 46].

4.1. FRAP (ferric reducing antioxidant power) protocol

The FRAP assay represents one of the most widely adopted methods for measuring the reducing power of antioxidants, based on the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in acidic conditions [47, 48]. The principle underlying this assay involves the reduction of the colorless ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to the intensely blue ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex at low pH, with maximum absorption at 593 nm [49, 50]. A concise summary of the key assay conditions is presented in Table 2.

Detailed Protocol:

1. Prepare FRAP reagent fresh daily by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ solution
2. Warm FRAP reagent to 37 °C before use
3. Add 100 μL of test sample to 3.0 mL of FRAP reagent
4. Mix thoroughly and incubate at 37 °C for exactly 4 minutes

Table 2. Protocol overview for each key assay.

Assay	Key Reagents	Incubation Time	Temperature	Quantification Formula	Instrument
DPPH	DPPH in methanol	30 minutes	Room temperature (20–25 ˚C)	Eq. (1)	UV-Vis
ABTS	ABTS + $K_2S_2O_8$	6 minutes	Room temperature	Trolox equivalents	UV-Vis
Superoxide	NADH, PMS, NBT	5 minutes	Room temperature	% Inhibition of formazan	UV-Vis
FRAP	TPTZ, $FeCl_3$	4 minutes	37 ˚C	μmol Fe^{2+} equivalents	UV-Vis
DCFH-DA	DCFH-DA, H_2O_2	30–60 minutes	37 ˚C	Fluorescence Intensity	Microplate reader

5. Measure absorbance at 593 nm against a reagent blank
6. Prepare standard curve using $FeSO_4{\cdot}7H_2O$ solutions (100–1000 μM)
7. Express results as μmol Fe^{2+} equivalents per gram or milliliter of sample

The FRAP assay offers several advantages including simplicity, reproducibility, and the ability to measure both water-soluble and fat-soluble antioxidants when appropriate solvents are used [51, 52]. However, the assay's acidic conditions may not reflect physiological pH, and it measures only electron-donating capacity rather than hydrogen atom transfer mechanisms [53].

4.2. Metal chelation assay (Ferrozine method)

The metal chelation assay using ferrozine provides specific information about a compound's ability to bind metal ions, particularly iron, which plays a crucial role in catalyzing oxidative reactions through Fenton chemistry [54, 55]. The principle is based on the formation of a stable, intensely colored complex between Fe^{2+} and ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid), which exhibits maximum absorption at 562 nm [56, 57].

Detailed Protocol:

1. Prepare 0.1 mM $FeSO_4{\cdot}7H_2O$ solution in distilled water
2. Prepare 0.25 mM ferrozine solution in distilled water
3. Mix 1.0 mL of $FeSO_4$ solution with 1.0 mL of test sample at various concentrations
4. Allow the mixture to react for 5 minutes at room temperature
5. Add 1.0 mL of ferrozine solution to initiate complex formation
6. Mix thoroughly and incubate at room temperature for 10 minutes
7. Measure absorbance at 562 nm against an appropriate blank
8. Calculate metal chelation activity regarding Eq. (1)
9. Determine IC_{50} values representing the concentration required for 50% chelation

EDTA is typically used as a positive control due to its well-known metal chelating properties [58, 59]. The assay can be adapted for other metal ions by using different chromogenic reagents and appropriate metal solutions [60].

5. Cell-based antioxidant assays

Cell-based antioxidant assays represent a significant advancement in antioxidant testing, providing more physiologically relevant information compared to chemical assays by incorporating cellular uptake, metabolism, and bioavailability factors [56, 61]. These assays evaluate antioxidant activity within living cellular systems, offering insights into the actual protective effects against oxidative stress under biological conditions [10, 18].

5.1. DCFH-DA assay (intracellular ROS detection)

The 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay is the most widely used method for detecting intracellular ROS production and evaluating antioxidant protection in living cells [64, 65]. The principle involves the cellular uptake of the non-fluorescent DCFH-DA probe, which is subsequently deacetylated by intracellular esterases to form DCFH, and then oxidized by various ROS to produce the highly fluorescent dichlorofluorescein (DCF) [66, 67].

Detailed Protocol:

1. Seed cells (1×10^4 cells/well) in 96-well black plates and incubate overnight at 37˚C
2. Remove growth medium and wash cells twice with phosphate-buffered saline (PBS)
3. Load cells with 10 μM DCFH-DA in serum-free medium for 30 minutes at 37 ˚C in darkness
4. Wash cells three times with PBS to remove excess probe
5. Pre-treat cells with test compounds at various concentrations for 1 hour
6. Induce oxidative stress with appropriate ROS generators (e.g., 500 μM H_2O_2, 100 μM tert-butyl hydroperoxide, or 50 μM menadione)

7. Measure fluorescence immediately and at regular intervals using a microplate reader (excitation: 485 nm, emission: 535 nm)
8. Calculate antioxidant activity regarding Eq. (2):

$$\% \text{ Protection} = \frac{F_{\text{oxidant}} - F_{sample}}{F_{\text{oxidant}} - F_{control}} \times 100 \quad (2)$$

The DCFH-DA assay can be effectively coupled with viability assays such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or resazurin to evaluate antioxidant-induced cytoprotection. This combination provides comprehensive information about both ROS scavenging capacity and cellular protection against oxidative damage [68].

5.2. MTT coupling protocol

1. After completing the DCFH-DA measurement, remove medium and add 100 μL of MTT solution (0.5 mg/mL)
2. Incubate for 4 hours at 37 °C
3. Remove MTT solution and add 100 μL of DMSO to dissolve formazan crystals
4. Measure absorbance at 570 nm with 630 nm as the reference wavelength
5. Calculate cell viability regarding Eq. (3)

$$\% \text{ Viability} = \frac{A_{sample}}{A_{control}} \times 100 \quad (3)$$

6. In vivo antioxidant testing and biomarker assessment

In vivo antioxidant testing represents the gold standard for evaluating the actual biological efficacy of antioxidant compounds under physiologically relevant conditions. These studies typically employ rodent models subjected to controlled oxidative stress conditions, followed by comprehensive biomarker analysis to assess the protective effects of test compounds [69].

Experimental Design: Animal models commonly utilize chemical inducers of oxidative stress, such as carbon tetrachloride (CCl_4), hydrogen peroxide, or doxorubicin, to create reproducible oxidative damage. Following treatment with test antioxidants and oxidative stress induction, various tissues (liver, kidney, brain, heart) and biological fluids (plasma, urine) are analyzed for oxidative stress biomarkers [69].

Key Biomarker Categories:

Lipid Peroxidation Assessment: The thiobarbituric acid reactive substances (TBARS) assay remains the most widely used method for quantifying lipid peroxidation products, primarily malondialdehyde (MDA) [70].

TBARS Protocol:

1. Prepare tissue homogenates (10% w/v) in ice-cold phosphate buffer (pH 7.4)
2. Mix 0.5 mL of tissue homogenate with 0.5 mL of 30% trichloroacetic acid (TCA)
3. Add 0.5 mL of 0.8% thiobarbituric acid (TBA) solution
4. Heat the mixture in a boiling water bath for 30 minutes
5. Cool rapidly in an ice bath and centrifuge at 3000 rpm for 10 minutes
6. Measure absorbance of the supernatant at 532 nm
7. Calculate MDA concentration using a standard curve prepared with 1,1,3,3-tetramethoxypropane

Antioxidant Enzyme Activity Assays: Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) activities are routinely measured to assess the enzymatic antioxidant defense system [17].

SOD Activity Protocol:

1. Prepare tissue homogenate in 50 mM phosphate buffer containing 0.1 mM EDTA
2. Mix reaction components: 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, and 75 μM NBT
3. Add tissue sample and expose to fluorescent light for 15 minutes
4. Measure absorbance at 560 nm
5. Calculate SOD activity as the amount of enzyme causing 50% inhibition of NBT reduction

Non-enzymatic Antioxidant Markers: Glutathione (GSH) levels serve as a critical indicator of cellular antioxidant status and are typically measured using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB) [17].

GSH Measurement Protocol:

1. Deproteinize tissue samples with 10% TCA and centrifuge
2. Mix supernatant with 0.4 M Tris-HCl buffer (pH 8.9)
3. Add DTNB reagent and measure absorbance at 412 nm
4. Calculate GSH concentration using a standard curve

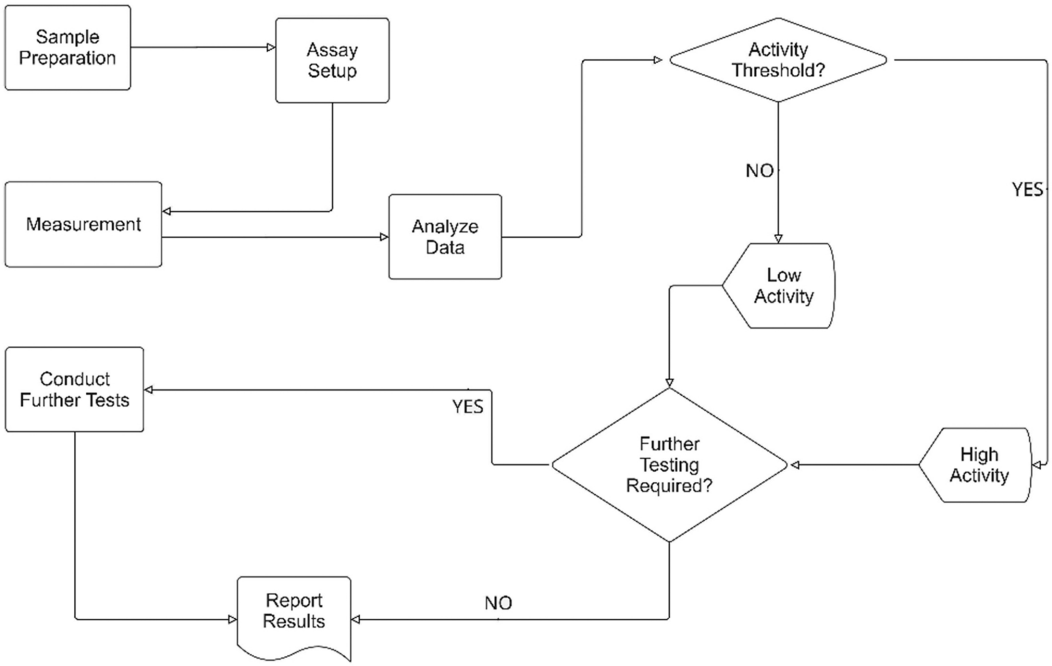

Fig. 2. Workflow diagram of a typical antioxidant screening pipeline.

7. Challenges, standardization, and future directions

The field of antioxidant testing faces numerous methodological and interpretive challenges that significantly impact the reliability and comparability of research findings across different laboratories and applications. Fig. 2 illustrates the stepwise process of antioxidant testing, beginning with sample preparation and assay setup, followed by measurement and data analysis. Based on the results, samples are classified according to activity thresholds (low or high). Decisions on further testing are guided by activity outcomes, ensuring that promising compounds undergo additional validation before final reporting. This workflow emphasizes iterative testing for reliability and reproducibility in antioxidant evaluation.

7.1. Major limitations

Methodological Variability: Significant variations exist in protocols, solvent systems, pH conditions, temperature, and incubation times across different laboratories, leading to poor reproducibility and comparability of results.

Poor In Vitro-In Vivo Correlation: Chemical antioxidant assays often fail to predict biological efficacy due to their inability to account for factors such as bioavailability, cellular uptake, metabolism, and tissue distribution. Many compounds showing strong antioxidant activity in vitro demonstrate limited or no protective effects in biological systems.

Assay Specificity and Mechanism Limitations: Individual assays typically measure only specific aspects of antioxidant activity, such as radical scavenging or reducing power, while ignoring other important mechanisms like enzyme induction, metal chelation, or membrane stabilization. This narrow focus can lead to incomplete characterization of antioxidant potential.

7.2. Critical needs for advancement

Protocol Standardization: The development of internationally accepted standard operating procedures (SOPs) for major antioxidant assays is essential for ensuring reproducibility and enabling meaningful comparison of results across different research groups. This includes standardization of reagent preparation, reaction conditions, and data reporting formats.

Multiplexed Approaches: Future antioxidant testing should integrate multiple complementary assays that combine chemical reactivity measurements with biological relevance assessments. This approach would provide more comprehensive antioxidant profiles and better predictive value for biological efficacy.

Integration of Advanced Technologies: The incorporation of omics-based biomarkers, including genomics, proteomics, and metabolomics approaches, offers unprecedented opportunities to understand the molecular mechanisms of antioxidant action and identify novel biomarkers of oxidative stress. High-

throughput screening platforms enable rapid evaluation of large compound libraries, while advanced biosensor technologies provide real-time monitoring of antioxidant activity.

Cross-Disciplinary Harmonization: Future directions must focus on harmonizing antioxidant testing approaches across diverse fields including food science, pharmaceutical development, nutraceutical research, and environmental toxicology. This harmonization should consider field-specific requirements while maintaining scientific rigor and biological relevance.

The development of predictive models incorporating physicochemical properties, structural features, and biological activity data through machine learning and artificial intelligence approaches represents a promising avenue for improving antioxidant discovery and development. Additionally, the integration of systems biology approaches will enhance our understanding of antioxidant networks and their interactions within complex biological systems.

8. Conclusion

The accurate evaluation of antioxidant activity is essential for the development and validation of bioactive compounds targeting oxidative stress-related diseases. This mini-review summarizes the most common and validated protocols for testing antioxidant potential, ranging from rapid in vitro screening assays to more biologically relevant cell-based and in vivo approaches. While assays such as DPPH, ABTS, and FRAP remain foundational, cell-based systems like DCFH-DA and animal models offer insights into physiological relevance. Persistent challenges—including methodological variability, lack of standardized protocols, and limited concordance between experimental platforms—continue to hinder cross-study comparisons and translational accuracy. Future progress will depend on the adoption of harmonized operating procedures and the integration of multiplexed platforms, biosensors, and omics-driven readouts to improve precision, reproducibility, and predictive value across antioxidant research.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Not applicable.

Data availability

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Author contributions

AH: Conceptualization, methodology, writing—original draft. SA: Writing—review & editing, validation. Both authors: Approval of final manuscript.

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