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Review

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Iwaoka's Assay Applied to Natural Products: Principles, Methodological Developments, and Future Perspectives

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How To Cite: Collevecchio, C.; Fiorito, S.; Epifano, F.; et al. Iwaoka's Assay Applied to Natural Products: Principles, Methodological Developments, and Future Perspectives. *Natural Products Analysis* **2026**, *2*(1), 100004. https://doi.org/10.53941/npa.2026.100004

Received: 2 February 2026
Revised: 8 April 2026
Accepted: 10 April 2026
Published: 17 April 2026

Abstract: Glutathione peroxidases (GPxs) are essential antioxidant enzymes that catalyze the reduction of hydrogen peroxide and organic hydroperoxides, thereby maintaining cellular redox homeostasis. The search for small-molecule GPx mimetics has stimulated the development of biomimetic chemical assays capable of evaluating peroxide-reducing catalytic activity under controlled conditions. Among these, Iwaoka's assay has emerged as a robust and mechanistically informative method. The assay monitors the catalyst-mediated oxidation of 1,4-dithiothreitol (DTT^{red}) to its disulfide form (DTT^{ox}) in the presence of hydrogen peroxide, typically using ^{1}H NMR, UV-Vis spectroscopy, or more recently hyphenated chromatographic techniques such as HPLC-DAD and GC-MS. Although originally designed for synthetic organoselenium compounds, Iwaoka's assay has increasingly been applied to natural products, including phenylpropanoids and terpenophenolic cannabinoids. These studies reveal that certain natural scaffolds can promote peroxide reduction through redox cycling mechanisms. Importantly, the assay distinguishes true catalytic turnover from simple stoichiometric radical scavenging, offering mechanistic insight beyond conventional antioxidant tests. This review critically examines the principles, analytical evolution, and application of Iwaoka's assay to natural products, highlighting methodological challenges, structure-activity relationships, and future directions aimed at improving standardization, biological relevance, and integration with cellular oxidative stress models.

Keywords: catalytic antioxidants; glutathione peroxidase mimetics; Iwaoka's assay; natural phenolic compounds; redox cycling mechanisms

1. Introduction

1.1. General Aims and Scope of Iwaoka's Assay

Oxidative stress represents a fundamental biochemical imbalance between the production of reactive oxygen species (ROS) and the capacity of biological systems to detoxify these species [1]. Among ROS-detoxifying enzymes, glutathione peroxidases (GPx) occupy a central role, catalyzing the reduction of hydrogen peroxide and organic hydroperoxides using glutathione (GSH) as the reducing substrate (Figure 1)

The catalytic efficiency of GPx derives largely from its active-site selenocysteine residue, which cycles between reduced and oxidized states during peroxide reduction [2]. Because selenium-dependent enzymes are not always easily modulated pharmacologically, the development of small-molecule GPx mimetics has attracted considerable attention. Iwaoka's assay emerged as a rational chemical tool to evaluate the GPx-like catalytic behavior of synthetic molecules under controlled conditions. Rather than measuring enzymatic activity in cell lysates or tissues, the assay provides a biomimetic chemical model that reproduces the essential redox

transformation: thiol oxidation coupled to peroxide reduction. The central reaction involves the oxidation of 1,4-dithiothreitol (DTT^{red}) to its disulfide form (DTT^{ox}) in the presence of H_2O_2 and a candidate catalyst. In the absence of a catalyst, DTT oxidation proceeds slowly. In the presence of a catalytically competent redox mediator, the reaction is accelerated. The assay thus serves to identify compounds capable of catalytic peroxide detoxification, rather than simple stoichiometric antioxidant action [3,4]. The primary aims of Iwaoka's assay are:

(i) to provide a standardized chemical model for assessing peroxide-reducing catalytic activity;
(ii) to enable comparative evaluation of structurally diverse small molecules;
(iii) to facilitate structure–activity relationship (SAR) investigations, especially in organoselenium chemistry;
(iv) to distinguish true catalytic behavior from simple stoichiometric antioxidant effects.

While initially applied to organoselenium chemistry, Iwaoka's assay has increasingly been employed to explore the catalytic redox potential of natural products, especially phenolic and terpenophenolic metabolites. This expansion reflects a conceptual shift from viewing natural antioxidants solely as radical scavengers to evaluating their capacity for redox cycling and catalytic turnover.

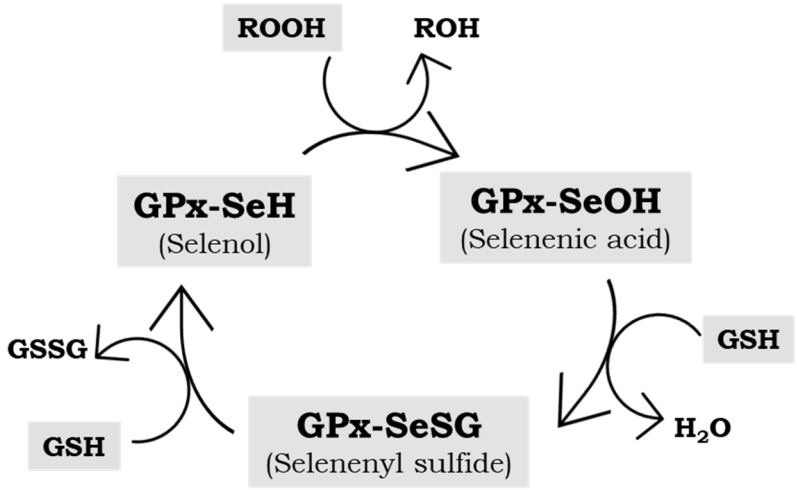


Figure 1. Catalytic cycle of glutathione peroxidase.

1.2. Chemical Principles and Mechanistic Foundations

The assay is designed to reproduce, in a chemically simplified and controllable *in vitro* system, the essential features of the catalytic cycle of GPx. In biological systems, GPx protects cells from oxidative damage by reducing hydrogen peroxide (H_2O_2) to H_2O through a well-defined selenol–selenenic acid redox cycle (Figure 1) [1–4]. The active site of the enzyme contains a selenocysteine residue that exists in the reduced selenol form (SeH). Upon reaction with H_2O_2, this selenol is oxidized to a selenenic acid (SeOH) intermediate. The oxidized enzyme is subsequently regenerated by two equivalents of glutathione (GSH), which reduce the selenenic intermediate back to the active selenol state, forming oxidized glutathione (GSSG) in the process. This continuous regeneration enables true catalytic turnover and efficient peroxide detoxification under physiological conditions. In Iwaoka's small-molecule model, this enzymatic process is translated into a minimal chemical system that retains the core redox logic while avoiding the complexity of proteins and cellular components. Specifically: (a) DTT replaces glutathione as a low-molecular-weight dithiol reductant; (b) H_2O_2 serves as the oxidizing substrate; and (c) the test compound under evaluation functions as a putative redox mediator or catalyst. Thus, the assay monitors the oxidation of DTT^{red} to its corresponding disulfide form DTT^{ox} in the presence of H_2O_2 and a candidate catalytic compound (Figure 2). The extent and rate of DTT oxidation provide a quantitative measure of the compound's ability to mediate peroxide reduction through a catalytic cycle.

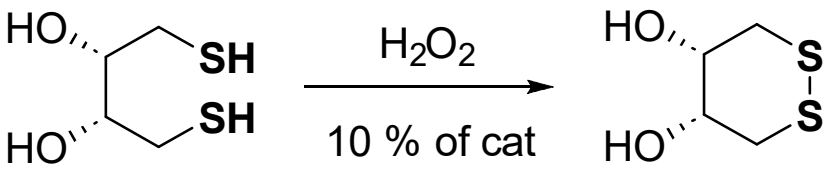


Figure 2. The reaction of the Iwaoka's assay.

Under methanolic conditions and in the absence of any catalyst, the direct reaction between DTT and H_2O_2 proceeds slowly. This low background rate is essential, as it allows clear discrimination between non-catalyzed oxidation and catalyst-accelerated turnover. When a catalytic species capable of activating H_2O_2 and undergoing reversible redox cycling is present, the rate of DTT oxidation increases significantly. The enhanced reaction rate reflects the ability of the test compound to shuttle oxidative equivalents from H_2O_2 to the thiol substrate in a cyclic manner.

Mechanistically, compounds that display GPx-like activity typically follow three fundamental steps:

(i) oxidation by H_2O_2—the reduced form of the catalyst reacts with H_2O_2 to generate an oxidized intermediate;
(ii) thiol oxidation—the oxidized intermediate transfers the oxidative equivalent to DTT, promoting formation of the disulfide (DTTox);
(iii) catalyst regeneration—the catalyst is reduced back to its original active form, completing the catalytic cycle and enabling further turnovers.

For organoselenium compounds, this mechanism closely parallels that of native GPx and generally involves interconversion between selenol and selenenic acid intermediates, sometimes proceeding through selenenyl sulfide species during thiol exchange. These systems often show high catalytic efficiency because selenium readily undergoes rapid and reversible two-electron redox transformations [5,6]. In contrast, for phenolic natural products, the redox cycle may proceed through the formation of quinone, semiquinone, or phenoxyl radical intermediates. Although such species can mediate peroxide-dependent thiol oxidation, their redox cycling is typically less efficient and may compete with side reactions such as irreversible oxidation or radical coupling. Consequently, catalytic turnover numbers are often lower than those observed with selenium-based mimetics.

Importantly, this assay does not simply measure radical-scavenging or direct antioxidant capacity. Instead, it evaluates the ability of small molecules to mediate catalytic peroxide reduction via repeated redox cycling. The distinction is critical: a true GPx mimic must function catalytically-being regenerated during the reaction, rather than being consumed stoichiometrically as a sacrificial antioxidant. This review aims to provide a comprehensive and critical overview of Iwaoka's assay as a biomimetic chemical platform for evaluating glutathione peroxidase (GPx)-like catalytic activity, with particular emphasis on its application to natural products. It focuses on the underlying chemical principles, methodological developments, and analytical implementations of the assay, as well as its capacity to distinguish true catalytic peroxide reduction from simple stoichiometric antioxidant behavior. Additionally, the review explores structure–activity relationships across different classes of natural compounds and discusses current limitations, standardization challenges, and future perspectives for improving the assay's biological relevance and translational potential. The literature data were collected through a systematic search of major online scientific databases (e.g., Scopus, PubMed, Google Scholar), including peer-reviewed articles relevant to Iwaoka's assay, GPx mimetics, and catalytic antioxidant activity of natural products

2. Analytical Implementations: Methodological Evolution and Critical Appraisal

2.1. ^{1}H NMR-Based Monitoring

The earliest implementations of the assay relied on ^{1}H NMR spectroscopy to track the progressive disappearance of signals corresponding to DTT^{red} alongside the concomitant appearance of resonances attributable to its oxidized form DTT^{ox} [7,8]. This strategy offers a high degree of structural specificity, enabling unambiguous differentiation between redox states without the need for secondary probes or chromogenic reporters. Importantly, it allows the direct observation not only of substrate conversion but also of transient catalyst-derived intermediates, which can provide valuable insight into the redox cycling process. Such capability makes NMR particularly advantageous for mechanistic investigations, as it eliminates reliance on indirect spectroscopic proxies and minimizes the risk of signal misinterpretation arising from coupled reactions or side processes. Despite these strengths, NMR-based implementations suffer from several practical limitations. First, relatively high analyte concentrations are typically required to achieve adequate signal-to-noise ratios, which may not reflect physiologically or environmentally relevant conditions. Second, temporal resolution is inherently constrained by acquisition times, making it difficult to capture rapid kinetic events or short-lived intermediates. Third, the technique is intrinsically low-throughput compared with optical or electrochemical alternatives, limiting its applicability in screening workflows. These constraints are further amplified when studying natural products. Complex phytochemical mixtures often display extensive signal overlap in the proton spectrum, complicating spectral assignment and quantitative analysis. In addition, many bioactive metabolites are present in their native matrices at very low concentrations, frequently below the sensitivity threshold of conventional NMR methods without extensive pre-concentration or purification steps. Nevertheless, NMR remains indispensable for mechanistic validation, particularly in distinguishing true catalytic turnover from simple stoichiometric oxidation processes. Its ability to provide molecular-level confirmation of redox cycling continues to make it a gold standard

in assay development and validation. Future methodological advances are expected to enhance its utility, especially through the integration of quantitative NMR (qNMR) and real-time kinetic monitoring approaches. These developments could enable more precise determination of rate constants, turnover numbers, and catalytic efficiencies, thereby strengthening the mechanistic interpretation of redox-active natural products and related systems.

2.2. UV-Vis Spectrophotometric Monitoring

Subsequent adaptations of the assay transitioned toward UV-visible spectroscopic monitoring, most commonly tracking absorbance changes around ~310 nm associated with the formation of DTT^{ox} [9]. This shift significantly improved analytical practicality, as UV detection allows rapid data acquisition, straightforward instrumentation, and the possibility of parallel measurements in multi-well plate formats. As a result, kinetic analyses can be performed with greater temporal resolution compared to NMR-based approaches, enabling more accurate monitoring of reaction progress over short time scales. In addition, the reduced sample requirements and compatibility with automated workflows make UV-based methods particularly attractive for screening studies and comparative assessments of redox-active compounds. However, this convenience comes at the cost of reduced molecular specificity. UV absorbance is inherently less selective than spectroscopic techniques that provide structural information, and signals at ~310 nm may overlap with those arising from endogenous chromophores present in complex mixtures. This limitation is especially relevant when working with natural products, many of which contain conjugated systems, such as phenolics, flavonoids, quinones, and other aromatic constituents, that exhibit intrinsic UV absorption in the same spectral region. Such overlap can introduce background contributions or false-positive signals, complicating quantitative interpretation. Matrix effects may further exacerbate this issue, particularly when crude extracts are analyzed without prior purification. In these cases, co-extracted pigments or degradation products may either enhance or mask the apparent formation of DTT^{ox}. Consequently, careful baseline correction, appropriate controls, and, where feasible, complementary analytical validation are necessary to ensure that observed absorbance changes genuinely reflect thiol oxidation rather than unrelated UV-active species. Despite these challenges, UV-based monitoring remains a widely adopted compromise between analytical speed and mechanistic rigor, particularly when supported by orthogonal methods to confirm redox cycling activity.

2.3. Chromatographic Methods

More recent methodological refinements have incorporated chromatographic detection, most notably through HPLC coupled with diode array detection (HPLC-DAD) or gas chromatography–mass spectrometry (GC–MS) [10,11]. These platforms markedly enhance both sensitivity and selectivity compared to direct spectroscopic approaches, making them especially suitable for evaluating complex natural compounds that either exhibit intrinsic UV absorption or generate secondary products capable of interfering with simpler optical readouts. By introducing a separation step prior to detection, chromatographic methods resolve DTT^{red} and DTT^{ox} forms into distinct peaks, thereby minimizing spectral overlap and eliminating ambiguity arising from coexisting matrix components. This separation provides several key advantages. First, it allows accurate and independent quantification of DTT^{red} depletion and DTT^{ox} formation, improving mass balance assessment within the assay. Second, it facilitates discrimination between true catalytic turnover and competing side reactions, such as irreversible thiol modification or degradation processes that may otherwise be misinterpreted as redox cycling. Third, it ensures analytical compatibility with challenging classes of natural products, including highly colored extracts, polyphenols, and quinonoid compounds, that often compromise direct UV-based monitoring. In addition, the use of DAD or MS detection enables confirmation of peak identity through spectral matching or mass fragmentation patterns, further strengthening analytical reliability. These refinements have been instrumental in broadening the applicability of the assay beyond simple model systems, allowing its extension to structurally diverse natural molecules with complex redox behavior. As a result, chromatographic approaches now represent a critical evolution in assay design, bridging the gap between mechanistic rigor and practical applicability in natural product research.

3. Application to Natural Products

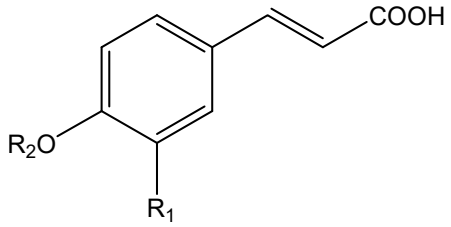
3.1. Rationale for Studying Natural Products: Moving beyond Radical Scavenging Paradigms

Natural products represent one of the richest sources of chemically diverse redox-active molecules, many of which have been historically associated with antioxidant effects [12]. These effects are typically assessed using conventional radical-scavenging assays such as DPPH and ABTS, which quantify the ability of a compound to donate an electron or hydrogen atom to neutralize a stable radical species [13]. While these methods are rapid and widely accepted, they fundamentally measure stoichiometric reactivity, that is, the capacity of a molecule to be

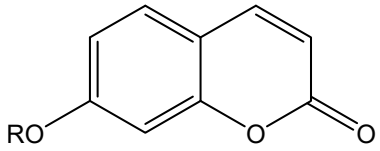
consumed in the process of reducing an oxidant. This paradigm implicitly treats antioxidants as sacrificial agents, meaning their biological role is interpreted as being depleted during oxidative stress. However, this model does not adequately reflect the behavior of endogenous antioxidant systems in living organisms. Biological redox homeostasis is largely maintained by catalytic systems, such as peroxidases and thiol-based enzymes, which repeatedly reduce reactive oxygen species without being consumed. Herein lies the conceptual limitation of traditional antioxidant screening: radical-scavenging capacity does not necessarily translate into biologically relevant redox function [14]. A molecule that performs well in DPPH or ABTS assays may simply act as a one-time electron donor, lacking the ability to participate in sustained redox cycling under physiological conditions. Applying Iwaoka's peroxide-reduction assay to natural products offers a powerful strategy to overcome this limitation. Rather than measuring single-turnover radical quenching, this approach evaluates whether a compound can: (a) facilitate catalytic reduction of peroxides; (b) undergo reversible redox transformations; (c) operate in the presence of biological reductants; and finally (d) mimic enzyme-like antioxidant behavior. In doing so, it allows researchers to address a critical mechanistic question: do natural products function as true redox catalysts, or are they merely consumed as sacrificial antioxidants? This distinction has profound pharmacological implications. Catalytic antioxidants could: (a) provide sustained protection against oxidative damage; (b) act at lower concentrations; (c) reduce the need for continuous replenishment; and (d) more closely resemble endogenous defense mechanisms. Conversely, stoichiometric antioxidants may offer only transient benefits and may even generate secondary oxidation products upon depletion. Therefore, moving beyond radical-scavenging assays toward catalytic peroxide-reduction models enables a more biologically meaningful evaluation of natural products. It shifts the focus from simple chemical reactivity to functional redox behavior, an essential step in understanding their true therapeutic potential in oxidative stress-related pathologies.

3.2. Phenylpropanoids and Simple Phenolics

Studies on compounds such as ferulic acid **1** and *p*-coumaric acid **4** (Figure 3) reveal modest glutathione peroxidase (GPx)-like activity when evaluated under peroxide-reducing conditions.



1 $R^1 = OCH_3$, $R^2 = H$, **2** $R^1 = OCH_3$, R^2 = 3,3-dimethylallyl, **3** $R^1 = OCH_3$, R^2 = geranyl, **4** $R^1 = R^2 = H$, **5** $R^1 = H$, R^2 = 3,3-dimethylallyl, **6** $R^1 = H$, R^2 = geranyl



7 R = H, **8**, R = 3,3-dimethylallyl, **9** R = geranyl, **10** R = farnesyl

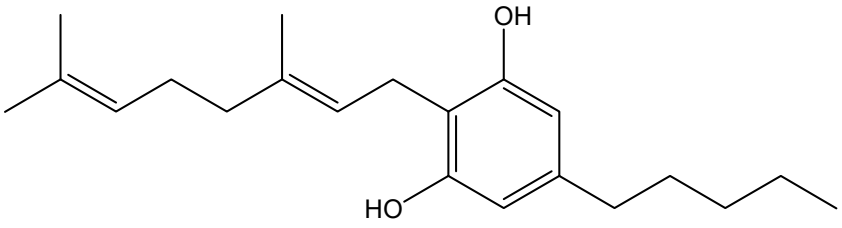
Figure 3. Structure of phenylpropanoids and their oxyprenylated counterparts.

These simple phenylpropanoids provide a useful structural framework for understanding how naturally occurring redox-active molecules may participate in catalytic antioxidant processes rather than acting solely as sacrificial scavengers. Preliminary evidence suggests that several structural features influence their activity. First, the presence of electron-donating substituents (such as methoxy or hydroxyl groups) enhances the electron density of the aromatic ring, facilitating oxidation and promoting interaction with peroxide substrates. Second, extended conjugation across the phenylpropanoid scaffold appears to support more efficient charge delocalization during redox cycling, lowering the energetic barrier for intermediate formation. Third, and perhaps most critically, the ability to stabilize quinone or semiquinone intermediates plays a central role in sustaining catalytic turnover. Molecules capable of forming relatively persistent oxidized states are more likely to re-enter the catalytic cycle rather than undergoing irreversible degradation. Despite these favorable features, the overall catalytic efficiency of these phenolic acids remains lower than that of the selenium-based GPx mimetic Ebselen, used as reference,

which benefit from inherently superior redox flexibility and faster peroxide reduction kinetics (48.4% for ferulic acid **1** and 46.7% for p-coumaric acid **4**, respectively) [10]. In phenolic systems, the catalytic cycle is often limited by competing side reactions, including overoxidation or polymerization of reactive intermediates. This limitation is reflected experimentally by the observation that the initial acceleration of DTT oxidation frequently declines over time. Such behavior suggests that the active species undergoes gradual deactivation, possibly through irreversible oxidation to stable quinones, radical coupling, or structural fragmentation. As a result, these compounds may display transient catalytic behavior rather than sustained turnover. When the OH function is etherified with emiterpenyl, terpenyl or sesquiterpenyl side chians the observed activity largely dimished to values comprised in the range 25.6–10.3% respect to Ebselen. A similar pattern has been observed also when umbelliferone **7** and its terpenylated derivatives **8**–**10** were tested. To advance the field, future studies should aim to establish a systematic relationship between intrinsic redox properties and catalytic performance. In particular, measuring oxidation and reduction potentials via cyclic voltammetry could provide valuable insight into the thermodynamic feasibility of redox cycling. Correlating these electrochemical parameters with turnover efficiency in Iwaoka's assay may enable the identification of predictive descriptors for GPx-like activity in phenolic natural products. Such an approach would support the rational selection or design of natural-product-inspired scaffolds with improved catalytic antioxidant function

3.3. Cannabinoids and Terpenophenolic Structures

Cannabidiol (CBD) **11** and cannabigerol (CBG) **12** (Figure 4) represent structurally distinctive natural-product scaffolds in which phenolic functionality is embedded within a terpenoid framework.



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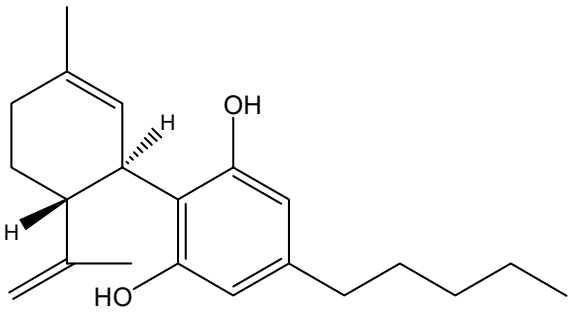

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Figure 4. Structures of cannabigerol **11** and cannabidiol **12**.

This hybrid architecture differentiates them from simpler polyphenols and may influence both their redox behavior and interaction with peroxide substrates. Their good activity in Iwaoka's assay (84.5% and 79.8% activity respect to reference Ebselen) indicates that the phenolic moieties present in these molecules are capable of participating in peroxide reduction, suggesting a potential role as redox mediators rather than merely passive radical scavengers [11]. At a mechanistic level, their behavior raises several important questions. One key issue is whether these compounds operate through the formation of phenoxyl radical intermediates, as commonly proposed for many phenolic antioxidants. The stabilization of such intermediates by the conjugated aromatic system and the steric environment provided by the terpenoid portion could enable reversible redox cycling, an essential requirement for catalytic activity. Another unresolved aspect concerns the sustainability of catalytic turnover. While initial activity may be detectable, it remains unclear whether CBD and CBG can undergo multiple redox cycles without structural degradation. Phenolic oxidation can lead to quinone formation or radical coupling reactions, both of which may terminate catalytic function and render the process self-limiting. Additionally, the

pronounced lipophilicity of these cannabinoids introduces an important variable not typically present in simpler phenolic systems. Their affinity for hydrophobic environments may influence substrate accessibility, local redox microenvironments, and the diffusion of reactive intermediates. In biological or membrane-mimicking systems, this property could either enhance or hinder effective redox cycling depending on the spatial distribution of peroxide species. To elucidate these mechanistic uncertainties, further investigation is required. Integrating kinetic modeling with electron paramagnetic resonance (EPR) [12] spectroscopy would allow direct detection of transient radical intermediates and help determine whether phenoxyl species are formed during catalysis. Such studies could clarify whether the observed activity reflects genuine catalytic behavior or arises from limited, self-consuming redox events. Ultimately, this mechanistic insight would inform the broader evaluation of cannabinoid-derived scaffolds as potential peroxide-reducing agents.

3.4. Complex Polyphenols and Extracts

Extending Iwaoka's assay from isolated compounds to complex plant extracts opens an important avenue for evaluating redox catalysis under more realistic chemical conditions. Unlike single-molecule systems, plant matrices contain intricate mixtures of polyphenols, terpenoids, alkaloids, and other secondary metabolites that may interact dynamically. These interactions introduce the possibility of synergistic redox behavior, where individual constituents, ineffective on their own, may collectively support sustained peroxide reduction through complementary electron-transfer pathways. For example, one component may facilitate peroxide activation while another regenerates the active reduced form of the catalyst, effectively mimicking the cooperative mechanisms seen in biological antioxidant networks. Such emergent behavior could enhance catalytic turnover beyond what would be predicted from the activity of isolated constituents. However, this complexity also presents significant analytical challenges. Overlapping redox processes, competing side reactions, and spectroscopic interference from strongly absorbing polyphenols can obscure kinetic readouts. Additionally, some extract components may act as stoichiometric reductants rather than true catalysts, complicating the distinction between transient antioxidant effects and sustained catalytic activity. To address these challenges and harness the potential of plant matrices, future research should prioritize several strategic directions:

(i) *Fractionation-guided screening*: systematic separation of extracts into chemically defined fractions would enable localization of catalytic activity and reduce matrix interference. Comparing activity across fractions may reveal whether catalysis arises from single dominant constituents or cooperative mixtures.
(ii) *Integration with metabolomics*: coupling activity data with untargeted metabolomic profiling could facilitate correlation between chemical composition and catalytic performance;
(iii) multivariate analysis may help identify patterns linking specific metabolite classes to enhanced peroxide-reducing behavior;
(iv) *identification of catalytic "hot spots"*: within complex polyphenolic networks, certain structural motifs, such as catechol, hydroquinone, or conjugated phenolic systems, may disproportionately contribute to redox cycling. Mapping these functional clusters could guide the rational enrichment or reconstruction of catalytically active mixtures;
(v) by combining chemical fractionation, systems-level analysis, and functional screening, it may become possible to move beyond single-compound evaluation and uncover cooperative catalytic mechanisms inherent to plant-derived redox networks.

4. Methodological Limitations and Standardization Needs

A recurring issue in applying Iwaoka's assay to natural products is the lack of methodological standardization, which complicates meaningful comparison across studies. Experimental outcomes are highly sensitive to several variables, including catalyst concentration, the stoichiometric ratio of H_2O_2, reaction time, and solvent composition. Even minor adjustments in these parameters can shift the balance between catalytic turnover and simple stoichiometric oxidation, potentially leading to misinterpretation of a compound's true redox behavior. For instance, excessive peroxide equivalents may accelerate catalyst degradation rather than reflect enhanced activity, while insufficient oxidant may mask catalytic potential. Similarly, solvent polarity can influence both substrate solubility and the stability of redox intermediates, thereby affecting observed kinetics. Reaction time is another critical factor: short assays may capture only initial activity, whereas longer durations can reveal whether turnover is sustained or self-limiting. To improve reproducibility and comparability, future studies should adopt more rigorous reporting standards. In particular, it is essential to include:

(i) turnover numbers (TON) and turnover frequencies (TOF) to distinguish catalytic processes from single-turnover antioxidant reactions;

(ii) catalyst stability profiles over time, providing insight into whether activity is maintained or declines due to degradation;
(iii) control experiments confirming regeneration cycles, such as monitoring catalyst integrity or demonstrating repeated peroxide reduction under continuous reductant supply.

Moreover, most current implementations rely heavily on organic solvents to ensure solubility of hydrophobic natural products. While practical, these conditions diverge from biologically relevant environments. Transitioning toward partially aqueous systems or mixed solvent models that better mimic physiological conditions would enhance the translational relevance of the assay. Such adaptations may also reveal differences in redox behavior that are masked under purely organic conditions, ultimately providing a more accurate assessment of catalytic antioxidant potential in natural-product scaffolds.

5. Comparison with Alternative Biomimetic Assays

Alternative methods for evaluating peroxide-reducing activity include coupled NADPH-dependent systems and enzyme-based GPx assays, both of which are designed to more closely mimic physiological redox pathways [15,16]. In these systems, peroxide reduction is typically linked to NADPH consumption through enzymatic cascades involving glutathione, glutathione reductase, or peroxidases. Such approaches provide valuable insight into how candidate molecules may interact with endogenous antioxidant networks and support redox homeostasis under biologically relevant conditions. In contrast, Iwaoka's assay offers notable advantages in terms of experimental simplicity and mechanistic clarity. By relying on a defined chemical reductant and monitoring peroxide reduction directly, it enables precise evaluation of catalytic behavior without the confounding influence of multiple enzymatic steps. This makes it particularly useful for distinguishing between true catalytic redox cycling and single-turnover antioxidant reactions. However, this simplicity also represents a limitation. The assay lacks the biological complexity inherent to cellular systems, including compartmentalization, competing metabolic pathways, and the presence of regulatory redox partners. As a result, activity observed *in vitro* may not directly translate into functional antioxidant effects in living systems. To bridge this gap, an integrated evaluation strategy is warranted. Initial screening using Iwaoka's assay can identify compounds capable of catalytic peroxide reduction under controlled conditions. These candidates could then be advanced to cellular reactive oxygen species (ROS) assays, where their impact on intracellular oxidative stress can be assessed [17]. Such a tiered approach would combine mechanistic insight with biological relevance, enabling a more comprehensive assessment of natural products and helping to prioritize scaffolds with genuine therapeutic potential

6. Future Research Directions: Mechanistic and Application Perspectives

Several promising avenues of investigation could significantly advance both the mechanistic understanding and practical application of peroxide-reducing activity in natural products. One important direction involves electrochemical correlation. Integrating cyclic voltammetry with Iwaoka's kinetic assay would allow researchers to directly relate catalytic performance to intrinsic redox properties such as oxidation potential, reversibility, and electron-transfer kinetics. Establishing quantitative relationships between electrochemical descriptors and turnover behavior could help identify the electronic features that enable sustained catalytic activity, moving the field toward predictive structure–activity frameworks. A second priority is the development of aqueous or water-compatible assay variants. Most current implementations rely on organic solvent systems to maintain solubility of hydrophobic natural products. Adapting the assay to function in water-rich or mixed media would improve physiological relevance and facilitate a smoother transition from chemical screening to biologically meaningful environments. Such modifications could also reveal whether catalytic redox cycling persists under conditions that more closely resemble intracellular oxidative stress. Another promising avenue is the creation of high-throughput screening platforms. Miniaturizing Iwaoka's assay into microplate-based formats, coupled with LC or LC-MS detection, would enable rapid evaluation of large natural product libraries derived from plants, fungi, or marine organisms. This scalability would be particularly valuable for fractionated extracts, where identifying active components currently remains time-intensive. Finally, deeper mechanistic studies are essential to clarify the nature of the catalytic cycles involved. Techniques such as EPR spectroscopy [18] or transient absorption spectroscopy [18,19] could provide direct evidence for short-lived intermediates formed during peroxide reduction.

7. Bridging towards Biological Relevance

An essential challenge about the Iwaoka's assay lies in translating this chemical activity into meaningful biological outcomes. Demonstrating catalytic turnover *in vitro* does not automatically imply that a compound will

exert protective effects under physiological conditions. Natural phenolic compounds, for instance, may display measurable peroxide-reducing activity in simplified assay systems yet encounter significant barriers *in vivo* [20]. These include limited bioavailability, rapid metabolic transformation, and restricted cellular uptake, all of which can diminish their effective concentration at sites of oxidative stress. Additionally, conjugation reactions such as glucuronidation [21] or sulfation [22] may alter redox properties, potentially suppressing catalytic function altogether. Beyond pharmacokinetic constraints, the intracellular environment itself presents complexities absent from chemical assays. Compartmentalization, competing redox pathways, and fluctuating oxidant levels can all influence whether a compound participates in sustained catalytic cycles or becomes inactivated after a single oxidative event [23]. For these reasons, it is essential to move toward integrated evaluation strategies that connect chemical reactivity with biological performance.

8. Conclusions

Iwaoka's assay has emerged as a valuable and versatile chemical platform for probing glutathione peroxidase (GPx)-like catalytic behavior in small molecules, including structurally diverse natural products [10,11]. In this review article we have demonstrated how the value of Iwaoka's assay lies not only in benchmarking activity but also in fostering a more nuanced understanding of antioxidant function. Looking ahead, continued progress will depend on methodological refinement, including improved standardization and adaptation to biologically relevant conditions. Parallel mechanistic studies aimed at elucidating intermediate species and reaction pathways will further clarify how natural compounds participate in redox cycles. Ultimately, integrating chemical screening with cellular and physiological models will be critical for determining whether natural products can meaningfully contribute to the development of catalytic antioxidants with real-world biomedical potential. Together, these efforts will help define the role of natural-product-derived molecules within the broader landscape of catalytic redox research.

Author Contributions

C.C., S.F., F.E. and S.G.: conceptualization, methodology, software data curation, writing—original draft preparation; visualization, investigation; supervision; software, validation; F.F.: writing, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest. Given the role as Editorial Board Member, Francesco Epifano had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

References

1. Sies, H. Oxidative Stress: A Concept in Redox Biology and Medicine. *Redox Biol.* **2015**, *4*, 180–183. https://doi.org/10.1016/j.redox.2015.01.002.

2. Pei, J.; Pan, X.; Wei, G.; et al. Research Progress of Glutathione Peroxidase Family (GPX) in Redoxidation. *Front. Pharmacol.* **2023**, *14*, 1147414. https://doi.org/10.3389/fphar.2023.1147414.
3. Barbosa, N.V.; Nogueira, C.W.; Nogara, P.A.; et al. Organoselenium Compounds as Mimics of Selenoproteins and Thiol Modifier Agents. *Metallomics* **2017**, *9*, 1703–1734. https://doi.org/10.1039/c7mt000873a.
4. Orian, L.; Toppo, S. Organochalcogen Peroxidase Mimetics as Potential Drugs: A Long Story of a Promise Still Unfulfilled. *Free Radic. Biol. Med.* **2014**, *66*, 65–74. https://doi.org/10.1016/j.freeradbiomed.2013.03.006.
5. Kadian, R.; Singh, V.P. Therapeutic Potential of Se-N Heterocycles: Advances in Biological and Antioxidant Applications. *Chem. Asian J.* **2025**, *20*, e00845. https://doi.org/10.1002/asia.202500845.
6. Chillar, B.; Brito, J.M.; Sodhi, N.; et al. Aminic Organoselenium Compounds: Promising Antioxidant Agents. *ACS Omega* **2025**, *10*, 43311–43326. https://doi.org/10.1021/acsomega.5c04072.
7. Kumakura, F.; Mishra, B.; Priyadarsini, K.I.; et al. A Water-Soluble Cyclic Selenide with Enhanced Glutathione Peroxidase-Like Catalytic Activities. *Eur. J. Org. Chem.* **2010**, *2010*, 440–445. https://doi.org/10.1002/ejoc.200901114.
8. Arai, K.; Tashiro, A.; Osaka, Y.; et al. Glutathione Peroxidase-Like Activity of Amino-Substituted Water-Soluble Cyclic Selenides: A Shift of the Major Catalytic Cycle in Methanol. *Molecules* **2017**, *22*, 354. https://doi.org/10.3390/molecules22030354.
9. Cindric, M.; Cepo Kusalic, T.; Marinc, S.; et al. Determination of Dithiothreitol in Complex Protein Mixtures by HPLC-MS. *J. Sep. Sci.* **2008**, *31*, 3489–3496. https://doi.org/10.1002/jssc.200800207.
10. Fiorito, S.; Epifano, F.; Palumbo, L.; et al. A Revised Version of the Iwaoka's Assay: Application of Hyphenated Techniques. *J. Pharm. Biomed. Anal.* **2022**, *212*, 114652. https://doi.org/10.1016/j.jpba.2022.114652.
11. Collevecchio, C.; Fiorito, S.; Epifano, F.; et al. Glutathione Peroxidase-Like Activity of Natural and Semisynthetic Phenylpropanoids and Cannabinoids: An Analytical Investigation by a GC-MS- and HPLC-DAD-Based Iwaoka's Assay. *J. Pharm. Biomed. Anal.* **2026**, *272*, 117381. https://doi.org/10.1016/j.jpba.2026.117381.
12. Sahu, I.D.; Lorigan, G.A. Advances in EPR Approaches for Studying Structural Properties of Membrane Proteins. *Magn. Reson. Chem.* **2026**, *64*, 456–476. https://doi.org/10.1002/mrc.70085.
13. Gulcin, I. Antioxidants: A Comprehensive Review. *Arch. Toxicol.* **2025**, *99*, 1893–1997. https://doi.org/10.1007/s00204-025-03997-2.
14. Munteanu, I.G.; Apetrei, C. Analytical Methods Used in Determining Antioxidant Activity: A Review. *Int. J. Mol. Sci.* **2021**, *22*, 3380. https://doi.org/10.3390/ijms22073380.
15. Foti, M.C. Use and Abuse of the DPPH• Radical. *J. Agric. Food Chem.* **2015**, *63*, 8765–8776. https://doi.org/10.1021/acs.jafc.5b03839.
16. Gaber, A.; Tamoi, M.; Takeda, T.; et al. NADPH-Dependent Glutathione Peroxidase-Like Proteins (Gpx-1, Gpx-2) Reduce Unsaturated Fatty Acid Hydroperoxides in Synechocystis PCC 6803. *FEBS Lett.* **2001**, *499*, 32–36. https://doi.org/10.1016/s0014-5793(01)02517-0.
17. Figueroa, D.; Asaduzzaman, M.; Young, F. Real Time Monitoring and Quantification of Reactive Oxygen Species in Breast Cancer Cell Line MCF-7 by 2′,7′-Dichlorofluorescin Diacetate (DCFDA) Assay. *J. Pharmacol. Toxicol. Methods* **2018**, *94*, 26–33. https://doi.org/10.1016/j.vascn.2018.03.007.
18. Gertsenshteyn, I.; Giurcanu, M.; Vaupel, P.; et al. Biological Validation of Electron Paramagnetic Resonance (EPR) Image Oxygen Thresholds in Tissue. *J. Physiol.* **2021**, *599*, 1759–1767. https://doi.org/10.1113/JP278816.
19. Chen, E.; Chance, M.R. Nanosecond Transient Absorption Spectroscopy. *Methods Enzymol.* **1993**, *226*, 119–147. https://doi.org/10.1016/0076-6879(93)26008-w.
20. Verma, A.R.; Vijayakumar, M.; Mathela, C.S.; et al. *In Vitro* and *in Vivo* Antioxidant Properties of Different Fractions of *Moringa oleifera* Leaves. *Food Chem. Toxicol.* **2009**, *47*, 2196–2201. https://doi.org/10.1016/j.fct.2009.06.005.
21. Stachulski, A.V.; Yates, E.A.; Teriosina, A.; et al. Dietary Substances and Their Glucuronides: Structures, Occurrence and Biological Activity. *Nat. Prod. Rep.* **2025**, *42*, 1924–1935. https://doi.org/10.1039/d5np00002e.
22. Pei, S.; Dou, Y.; Zhang, W.; et al. O-Sulfation Disposition of Curcumin and Quercetin in SULT1A3 Overexpressing HEK293 Cells: The Role of Arylsulfatase B in Cellular O-Sulfation Regulated by Transporters. *Food Funct.* **2022**, *13*, 10558–10573. https://doi.org/10.1039/d2fo01436j.
23. Nwachukwu, I.D.; Sarteshnizi, R.A.; Udenigwe, C.C.; et al. A Concise Review of Current *in Vitro* Chemical and Cell-Based Antioxidant Assay Methods. *Molecules* **2021**, *26*, 4865. https://doi.org/10.3390/molecules26164865.