We have described a modified method for evaluating inhibitor of peroxyl radicals, a well-recognized and -documented radical involved in cancer initiation and promotion as well as diseases related to oxidative stress and ageing. We are reporting hydrophilic and lipophilic as well as natural and synthetic forms of antioxidants revealing a diversified behaviour to peroxyl radical in a dose-dependent manner (1 nM–10 μM). A simple kinetic model for the competitive oxidation of an indicator molecule (ABTS) and a various antioxidant by a radical (ROO∙) is described. The influences of both the concentration of antioxidant and duration of reaction (70 min) on the inhibition of the radical cation absorption are taken into account while determining the activity. The induction time of the reaction was also proposed as a parameter enabling determination of antioxidant content by optimizing and introducing other kinetic parameters in 96-well plate assays. The test evidently improves the original PRTC (peroxyl radical trapping capacity) assay in terms of the amount of chemical used, simultaneous tracking, that is, the generation of the radical taking place continually and the kinetic reduction technique (area under curve, peak value, slope, and \( V_{\text{max}} \)).

1. Introduction

Peroxyl radicals (ROO∙), the chain carrying analogs of perhydroxyl radical (HOO∙) where the H atom is replaced by an organic group (R), are formed due to the oxidation of proteins and lipids [1–4]. Activation of neutrophil during oxidative stress related inflammation also produces peroxyl radicals [5, 6]. They are stable (reduction potential \([+][-] 0.77–[+][-] 1.44 \text{ V}\) and do not dissociate into oxygen because of the stable C–O bond. Its formation depends on the concentration of oxygen and other reactants as well as the hydrophobic/hydrophilic environment in which the reaction occurs [7–9]. These radicals not only occur in a cell but also transpire in aquatic systems (lakes, rivers, streams, and oceans) and in atmosphere (water droplets). Such successive reactions involving free radicals in biological systems lead to many physiological and pathological progressions [10–18].

In study allied to human disease, lipid peroxidation is correlated with peroxyl radical arbitrated reaction [7, 19, 20], induction of DNA damage by superoxide is additively boosted by peroxyl radicals which is implicated in modification of protein, lipid, and DNA cleavage, nevertheless arsenic-mediated ROS generation produces dimethylarsinic peroxyl radicals [7, 21]. The peroxyl radical, besides playing an outstanding character in radicals dependent shabbiness of membranes and proteins, is also fretful in the pathogenesis of a number of human diseases and disorders involving autoxidation of lipids, inactivation of certain enzymes, cleavage of phosphodiester bond resulting in single and double strand breaks, oxidizing thymidine resulting in mutagenic 5-methyl oxidation products, and causing transversion at deoxyguanosine [22–26]. Involvement of peroxyl radicals in carcinogenesis relevant to tumor initiation and promotion is well reported. Investigation related to cancer and redox
2. Materials and Methods

2.1. Chemicals. 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) also known as 2,2′-azobis (2-amidopropane) (ABAP), 2,2′azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammoniumsalt (ABTS) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), hydrogen peroxide (H₂O₂), L-ascorbic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemical Company, USA, while butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), tert-butyl hydroperoxide (tBHP), butylated hydroxyanisole (BHA), tocopherol, n-propyl gallate, quercetin, and β-carotene were purchased from Merck India Ltd. All the chemicals, solvents and reagents used were either of analytical grade or higher.

2.2. Experimental. Stock solution of 0.1 M sodium phosphate buffer (pH 7.0) was made by mixing solutions of Na₂HPO₄ (0.1 M) and NaH₂PO₄ (0.1 M) while stock solution of ABTS (5 mM) and AAPH (200 mM) was made in deionized water. 10 mM stock solution of tBHP, BHT, L-ascorbic acid, trolox, nicotinic acid, and hydrogen peroxide was made in deionized water, tocopherol, BHA and n-propyl gallate was made in ethanol, TBHQ in methanol, quercetin in DMSO, and β-carotene in acetone. Dilution was done in 0.1 M sodium phosphate buffer. The concentrations used for the analysis were 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM, and 0.001 μM.

Colorimetric peroxyl radical averting assay (PRAA) was done following method described by Bartosz et al. (1998) [43] with modification including increase in the time period from 10 min to 70 min and reduction in the volume of the reaction cocktail by 20 times. The additional feature of the modified method comprises kinetic reduction technique (area under curve, peak value, slope, time of half maximum, mean, and Vₘₐₓ). Total reaction volume (150 μL) of the modified method in 96-well plate contains 15 μL of samples (10x), 15 μL AAPH (200 mM), 4.5 μL ABTS (5 mM), and rest phosphate buffer. The phosphate buffer was preheated to 37°C for 30 min followed by the addition of other components including AAPH (source of peroxyl radical) added at the last and the absorbance was taken at 414 nm in a kinetic mode by presetting the spectrophotometer at 37°C for 0–70 min. From the kinetic curve, time-dependent increase in the absorbance was read for each compound along with control. Appropriate solvent blank was run in each assay.

2.3. Assay Validation, Data Scrutiny, and Statistical Analysis. Data represented are mean/average ± standard deviation of three independent experiments in duplicate. IC₅₀ values were calculated from dose-responsive curve by using Table Curve 2D Windows version 4.07 (SPSS Inc., Chicago, IL, USA). Using the advanced kinetic reduction technique available in SoftMax Pro Microplate Data Acquisition and Analysis Software Version 5.3 (Molecular Devices Corporation, Sunnyvale CA, USA) various kinetic parameters were calculated:
The decomposition of AAPH/ABAP and formation of peroxyl radicals:

\[
\begin{align*}
\text{AAPH} & \xrightarrow{\Delta} \text{Thermal lysis at 37°C} \\
\text{H}_2\text{N} & + \text{H}_2\text{N} + \text{N}_2 \\
\text{Dissolved oxygen in buffer at 37°C} & \\
\text{H}_2\text{N} & + \text{H}_2\text{N} + \text{N}_2 \\
\text{AAPH peroxy radical} & \\
\text{ABTS} & \\
\text{AN} = \text{NA} & \rightarrow 2\text{A}' + \text{nitrogen} \\
2\text{A}' + \text{O}_2 & \rightarrow 2\text{AOO}' \\
2\text{AOO}' + 2\text{LH} & \rightarrow 2\text{AOOH} + 2\text{L}' \\
2\text{L}' + \text{O}_2 & \rightarrow 2\text{LOO}' \\
2\text{LOO}' + 2\text{LH} & \rightarrow 2\text{LOOH} + 2\text{L}' \\
\text{LOO}' + \text{LOO}' & \rightarrow \text{LOO}' + \text{AOO}' \\
\text{AOO}' + \text{AOO}' & \rightarrow 2\text{L}' + 2\text{L}' \\
\text{Chain breaking antioxidant} & \\
\text{Green color} & \\
\text{Bleaching/prevention of green color formation} & \\
\end{align*}
\]

**Figure 1:** Decomposition of AAPH/ABAP and formation of Peroxyl radicals.

**Table 2:** Properties of the compound studied.

| Name               | Pubchem ID | Molecular formula | Molecular weight (g/mol) | XLogP3 | H-bond donor | H-bond acceptor |
|--------------------|------------|-------------------|--------------------------|--------|--------------|-----------------|
| Trolox             | 4063       | C₁₅H₁₆O₄          | 250.29032                |        |              |                 |
| α-Tocopherol       | 14985      | C₂₀H₄₀O₂          | 430.7061                 |        |              |                 |
| Nicotinic acid     | 982        | C₆H₅NO₂           | 123.11                   |        |              |                 |
| β Carotene         | 5280489    | C₁₀H₁₆O₂          | 353.87264                |        |              |                 |
| Ascorbic acid      | 54670067   | C₈H₈O₆            | 302.24                   | −1.6   | 4            | 6               |
| Quercetin          | 5280343    | C₁₀H₁₆O₇          | 176.12412                |        |              |                 |
| n-Propyl gallate   | 4947       | C₁₀H₁₄O₃          | 212.19928                |        | 3            | 5               |
| BHA                | 8456       | C₁₀H₁₄O₂          | 180.24354                |        | 1            | 2               |
| BHT                | 31404      | C₁₀H₁₄O₂          | 220.35046                | AA: 5.3| 1            | 1               |
| TBHQ               | 16043      | C₁₀H₁₄O₂          | 166.21696                |        |              |                 |
| tBHP               | 6410       | C₁₀H₁₄O₂          | 90.121                   | AA: 0.6| 1            | 2               |
| Hydrogen peroxide  | 784        | H₂O₂              | 34.01468                 | −0.9   | 2            | 2               |
percent values for \( V_{\max} \) (milli OD/min), peak, slope, and time of half maximum, mean, and area under curve using the formula: ((Control – Unknown)/Control*100). Pearson and RSQ values were also calculated for different parameters at higher tested concentration.

3. Results and Discussion

In the present work, a competitive kinetic method using ABTS as an indicator molecule was employed to estimate the reactivity of antioxidants towards AAPH/ABAP-derived peroxyl radical formation. The results obtained indicate that the relative protection afforded by a given compound strongly depends upon the experimental conditions employed and emphasize the role of secondary reactions of the phenol-derived radicals initially formed. Herein we are reporting hydrophilic and lipophilic as well as natural and synthetic forms of antioxidants revealing a diversified behaviour to peroxyl radical in a dose-dependent manner (1nM–10 \( \mu \)M). Hydrogen peroxide and tert-butyl hydroperoxide were used as checks. The influence of concentration of the antioxidants, duration of reaction system, and inhibition of the radical (cation or structure of antioxidant) absorption were taken into account while determining the scavenging potential.

Figures 2(a)–2(l) were plotted with control which showed a linear gradient in absorbance (optical density) from 0.06 to 0.6 within 70 min at 414 nm which gradually reaches maximum (0.8–1.0) in 24 h. The time period of 70 min (10 time increase in OD) was chosen in order to get a clear picture of the compound which was not apparent in 10 min incubation time. For IC\(_{50}\) calculation, the percent scavenging value of \( V_{\max} \) (milli absorbance/min) was chosen over the lag time period earlier reported to determine the scavenging capacity. It is evident from our plotted curves that not only lag time period but other features should also be considered while evaluating the ability of a particular compound as an effective scavenger of peroxyl radicals. The behaviour of the compounds varies which can be manifested by the graph not following the pattern of the control and the echelon of the offtrack hints the individual ability of both concentration and time-dependent.

A comprehensible concentration-dependent (1nM–10 \( \mu \)M) diminishing interaction with peroxyl radical was observed in trolox (Figure 2(a)). Maximum kinetic inhibition was detected at 10 \( \mu \)M and percent inhibition (in terms of \( V_{\max} \), milli absorbance/min) was found to be in the range of –8.2 to 67.2 proving it as an effective scavenger of peroxyl radical formation in a dose-dependent manner. Trolox, a cell-permeable, water soluble imitative of vitamin E with compelling antioxidant assets [44, 45], is frequently used as a standard or positive control in antioxidant assays. It is also used to gauge the job of oxidative injury in cell death and ageing [46, 47] and as an effective therapy in the treatment of certain cancers [48]. Noteworthy effect was monitored in \( \alpha \)-tocopherol (Figure 2(b)) at 10 \( \mu \)M and 1 \( \mu \)M concentration. Percent inhibition was found to be in the range of –8.0 to 81.5 compared to control. \( \alpha \)-Tocopherol is the most imperative (90%) among eight natural tocopherol, as peroxyl radicals scavenger/repressor of lipid peroxidation. Mechanistic study reveals that hydrogen atom is abstracted from the OH group in \( \alpha \)-tocopherol by a lipid peroxyl radical [\( AOO^-\)] producing fairly inert tocopheroxyl radical [TocO\(^+\)], which may then react with a second radical [\( AOO^-\)] to yield a nonradical product, AOO-Toc, thus destroying two radicals and terminating the radical chain reactions thereby contributing two electrons as a chain breaking antioxidant [49]. Inspite of scavenging peroxyl radicals, they are unable to act as a potent scavenger of hydroxyl, alkoxyl, nitrogen dioxide, and thyl radicals in vivo [50]. Behaviour of nicotinic acid (NA) was uncanny till 40 min and afterwards trifling scavenging started with time (Figure 2(c)). Percent inhibition was found to be in the range of –6.1 to 37.4 as the concentration increases from 1nM to 10 \( \mu \)M. NA, a colorless, water soluble derivative of pyridine with a carboxyl group at the 3-position, is a cofactor for NAD and NADP acting as coenzymes for more than thousand hydrogenases involved in almost every aspect of cell metabolism [51, 52]. Additionally, it also reduces LDL, VLDL-C, and triglycerides but effectively increases HDL [53] and affects vascular endothelial oxidative and inflammatory events [54]. Much litigious \( \beta \)-carotene showed good scavenging effect at 10 \( \mu \)M while at other concentrations the effect was equivalent to basal values (Figure 2(d)). Percent inhibition was found to be in the range of –4.4 to 40.5. Belonging to carotenoid family, \( \beta \)-carotene also known as provitamin A has an unsaturated and long aliphatic hydrocarbon chain ultimately splitting into two molecules of vitamin A. Unlike phenolic antioxidants, they do not have reactive hydrogen to contribute to radicals, which make it difficult to use conventional probes for the assessment of their radical scavenging capacity [55]. Earlier investigators [56, 57] observed that it inhibits peroxyl radical-initiated autoxidation of both tetralin and methyl linoleate and it is more effective antioxidant at 15 torr oxygen concentration than at 150 torr. Others have noticed a reticent increase in its action against liposomes at 15 torr [56, 58] and at about 4 torr in microsomes [56, 59]. \( \beta \)-Carotene eventually form a resonance-stabilized, carbon-centered radical adduct \([\text{AOO-} \beta \text{-C}^*\]) . Interaction of second peroxyl radical to the adduct produces a nonradical product and results in an overall trapping of two peroxyl radicals per \( \beta \)-carotene consumed [56]. L-Ascorbic acid was found as an excellent scavenger at 10 \( \mu \)M and 1 \( \mu \)M, an effect which decreases at lower concentrations (Figure 2(e)). Percent inhibition was found to be in the range of –2.5 to 97.7. Also known as vitamin C, ascorbic acid is an important water soluble antioxidant in extracellular fluid present in its deprotonated state under most physiologic conditions [60–62]. It is effective scavenger of superoxide anion radical, hydrogen peroxide, hypochlorite, the hydroxyl radical, and peroxyl radicals [60, 63–69]. Comparatively, it is more effective in inhibiting lipid peroxidation initiated by a peroxyl radical than other human plasma components, such as protein thiol, urate, bilirubin, and \( \alpha \)-tocopherol [60, 70]. Ascorbic acid can also protect membranes against peroxidation by enhancing the action of tocopherol and thereby reestablishing the radical scavenging activity [60, 71–74]. Quercetin showed a preeminent scavenging of peroxyl radical formation both at
Figure 2: Continued.
10 μM and 1 μM concentration (Figure 2(f)) and the percent scavenging effect was found to be in the range of 11.48 to 98.3 in a concentration-dependent manner. It is a flavonoid phytochemical naturally occurring in the rind and bark of numerous plants. Chemically it is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one where B-ring is the active center for scavenging and stabilizing the free radicals [75–77]. Best effect of averting action of peroxyl radical by n-propyl gallate was detected at 10 μM with marginal to no scavenging was found at 0.1 μM, 1 μM, 10 nM, and 1 nM concentration (Figure 2(g)). Percent averting action was found to be in the range of 15.7 to 79.57. Propyl gallate, obtained from natural gallic acid (3,4,5-trihydroxybenzoic acid, C₆H₆(OH)₃COOH), is one of the most effective antioxidant-based antimicrobials for the food industry. It has two functional groups hydroxy and carboxyl and its two analogues were more effective than trolox in preventing cell lysis of human erythrocytes induced by peroxyl radical initiator [78]. Butylated hydroxyanisole (BHA) and butyl hydroxytoluene (BHT) are synthetic antioxidants exhibiting diverse effect. Significant inhibition was observed at 10 μM and 1 μM which decreases at lower dose (Figures 2(h) and 2(i)). Percent inhibition was found to be in the range of 23.75 to 96.6 and –10.9 to 96.2 for BHA and BHT, respectively. BHA consists of a mixture of two isomeric organic compounds, 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole. It is a waxy solid used as a food additive (E320), as an antioxidant and preservative in food, food packaging, animal feed, cosmetics, rubber, and petroleum products to prevent from rancidity and developing objectionable odors. It is also used in medicines, such as isotretinoin, lovastatin, and simvastatin [79]. BHT is a lipophilic organic compound which behaves as a synthetic analogue of vitamin E, primarily acting as a terminating agent that suppresses autoxidation by converting peroxyl radicals to hydroperoxides through donating a hydrogen atom [80]. EC₅₀ value for BHA and BHT was found to be 18.94 ± 0.38 and 182.69 ± 13.7 μM, respectively, in an ABAP generated peroxyl radicals, measured by inhibition of dichlorofluorescein oxidation [33]. Like BHT, the conjugated aromatic ring of BHA is able to stabilize free radicals through sequestration. Tertiary butylhydroquinone (TBHQ) flaunted good forestalling action at 10 μM while fairly negligible effects were seen at other concentration (Figure 2(j)). Percent inhibition was found to be in the range of 19.97 to 61.37 in a concentration-dependent manner. TBHQ, a derivative of hydroquinone, substituted with tert-butyl group reacts with peroxyl radicals to form a semiquinone resonance hybrid which undergo different reactions to form more stable products reacting with one another to form dimers, dismutate, and regenerate as semiquinones before finally counteracting with another peroxyl radical [81]. Interaction with peroxyl radicals exhibited no effect with tert-butyl hydroperoxide (Figure 2(k)) and hydrogen peroxide (Figure 2(l)). Both tert-butyl hydroperoxide (tBuOOH/tBHP) and hydrogen peroxide (H₂O₂) were used as check compound for seeing the effect in peroxyl radical formation. tBuOOH/tBHP used in a variety of oxidation processes depletes GSH, induces lipid peroxidation, and tempts ROS formation, involved in PLA(2) activation in hepatocyte injury [82], responsible for K⁺ leakage [83]. Hydrogen peroxide (H₂O₂) is a potent oxidant and is even more toxic to cells than superoxide radicals removed by enzymes, such as catalase, glutathione peroxidases, and cysteinyl peroxidase [49].

Antioxidants deactivate free radicals either by reduction via electron or by hydrogen atom. The end point result is the same regardless of the mechanism but the kinetics differ. For kinetic analysis we have used software in which kinetic data reduction options were present. We tried different
options present to get a vivid picture of our experimental observations. All the kinetic parameters were calculated with respect to control as percent inhibition. The advantage of calculating using percent inhibition brought all kinetic parameters on the same platform in order to judge the kinetic behaviour characteristics independently. Percent inhibition of \( V_{\text{max}} \) (milli OD/min) of the kinetic curve was plotted for all twelve compounds (Figure 3) and IC\(_{50}\) values were calculated from table curve as given in Table 3. \( V_{\text{max}} \) is the maximum slope of the kinetic display of mOD/min, calculated by measuring the slopes of a number of straight lines, where \( V_{\text{max}} \) points determine the number of contiguous points over which each straight line is defined. This is an alternative method for analyzing nonlinear kinetic reactions that reports the elapsed time until the maximum reaction rate is reached, rather than reporting the maximum rate itself. \( V_{\text{max}} \) rate is reported as signal/min (milli-OD/min) for a kinetic read. It is calculated using a linear curve fit, \( y = Ax + B \). A creeping iteration is performed using \( V_{\text{max}} \) points and the slope of the steepest line segment is reported as \( V_{\text{max}} \) rate. The first slope is calculated for a line drawn beginning at the first reading as defined by lag time and ending at a total number of readings equal to the \( V_{\text{max}} \) points setting. The second and any subsequent slopes are calculated beginning at the second time point and ending at a total number of readings. The steepest positive or negative slope is reported as \( V_{\text{max}} \). Decreasing order for the highest concentration used (10 \( \mu \)M) is quercetin > L-ascorbic acid > BHA > BHT > \( \alpha \)-tocopherol > trolox > TBHQ > \( \beta \)-carotene > n-propyl gallate > nicotinic acid > tBHP > \( H_2O_2 \).

Percent inhibition of area under curve is represented in Figure 4. Defined by the data within the reduction limits, plots are treated as a series of trapezoids with vertices at successive data points and at the \( x \)-axis coordinates of the data points. The areas defined by each of the trapezoids are then computed and summed. Order was found to be trolox > quercetin > n-propyl gallate > \( \alpha \)-tocopherol > L-ascorbic acid > \( \beta \) carotene > BHA > TBHQ > nicotinic acid > \( H_2O_2 \). 

Implication of the percent values with respect to control was also calculated on the basis of time to half maximum of the kinetic curve (Figure 6). It denotes half of maximum OD to the time that falls within the reduction limits. The software determines the kinetic point that has maximum OD, divided by 2 thus getting 1/2 maximum values; further it finds the time at this 1/2 maximum value. Order of the percent values was recorded as BHT > quercetin > trolox > TBHQ > L-ascorbic acid > nicotinic acid > \( \alpha \)-tocopherol > tBHP > BHA > \( H_2O_2 \). 

Table 3: IC\(_{50}\) values and lag time of the compounds in peroxyl radical averting assay.

| Name                  | IC\(_{50}\) (\( \mu \)M) | Lag time (min) | Concentration |
|-----------------------|--------------------------|----------------|---------------|
| Trolox                | 0.119                    | 30             | 10 \( \mu \)M |
| \( \alpha \)-Tocopherol| 0.289                    | 35             | 1 \( \mu \)M  |
| Nicotinic acid        | ND                       | ND             | ND            |
| \( \beta \)-Carotene  | 91.95                    | 15             | 10 \( \mu \)M |
| Ascorbic acid         | 1.38                     | 15             | 1 \( \mu \)M  |
| Quercetin             | 0.239                    | 20             | 0.1 \( \mu \)M|
| n-Propyl gallate      | 5.8                      | 45             | 10 \( \mu \)M |
| BHA                   | 0.21                     | 35             | 0.1 \( \mu \)M|
| BHT                   | 0.885                    | 40             | 1 \( \mu \)M  |
| TBHQ                  | 8.93                     | 15             | 10 \( \mu \)M |
| tBHP                  | ND                       | ND             | ND            |
| Hydrogen peroxide     | ND                       | ND             | ND            |

ND: not detected. Each data is mean average values of three independent experiments.

![Figure 3: Percent inhibition calculated on the basis of \( V_{\text{max}} \) (milli OD/min) of the kinetic curve.](image-url)
Order was found to be BHT > trolox > L-ascorbic acid > quercetin > BHA > TBHQ > n-propyl gallate > α-tocopherol > β-carotene > nicotinic acid > H₂O₂ > tBHP. Figure 8 represents percent inhibition calculated from peak values of the kinetic data with respect to control, representing maximum absorbance of the compound at 414 nm. Order was found to be BHT > trolox > quercetin > L-ascorbic acid > BHA > TBHQ > n-propyl gallate > α-tocopherol > nicotinic acid > β-carotene > H₂O₂ > tBHP. Pearson “r” denotes the Pearson product moment correlation coefficient (Table 4). RSQ “r²” returns the square of the Pearson product moment correlation coefficient through the given data points. “r²” value is interpreted as the proportion of the variance in y attributable to the variance in x, where x and y represent different parameters in sequence. “r²” is the square of this correlation coefficient presented in Table 5.

4. Conclusion

Redox biology, an inescapable field known for its beneficial/detrimental property is being studied extensively. Radicals can wreak devastation on macromolecules/metabolites and may cause short/long term effects on cell signalling. Lipid peroxidation has been the issue of far-reaching scrutiny of mechanistic cell signalling and its involvement in human diseases/disorders. The development of a high-throughput absorbance assay for monitoring kinetics of peroxyl radical reactions in vitro is described in this paper where the evolution of the increase in absorbance values over time provides a rapid, facile method to conduct competitive kinetic studies in the presence of different antioxidants. A quantitative treatment formulated for the temporal evolution of the kinetic interpretation in terms of different parameters is presented. Combined, competitive kinetic assay and the data analysis provides a new method to obtain, in a rapid, parallel format, relative antioxidant capacity to retard the
Table 4: Pearson values calculated for 10 μM of various kinetic parameters.

|            | $V_{max}$ | Peak | Slope | Mean | $T$: (1/2) max | Area |
|------------|-----------|------|-------|------|----------------|------|
| $V_{max}$  | 1.00      | 0.95 | 0.67  | 0.97 | 0.45           | 0.54 |
| Peak       | 0.95      | 1.00 | 0.63  | 0.97 | 0.36           | 0.54 |
| Slope      | 0.67      | 0.63 | 1.00  | 0.63 | $-0.01$        | 0.81 |
| Mean       | 0.97      | 0.97 | 0.63  | 1.00 | 0.39           | 0.54 |
| $T$: (1/2) max | 0.45    | 0.36 | $-0.01$ | 0.39 | 1.00           | $-0.01$ |
| Area       | 0.54      | 0.54 | 0.81  | 0.54 | $-0.01$        | 1.00 |

Table 5: RSQ values calculated for 10 μM of various kinetic parameters.

|            | $V_{max}$ | Peak | Slope | Mean | $T$: (1/2) max | Area |
|------------|-----------|------|-------|------|----------------|------|
| $V_{max}$  | 1.00      | 0.89 | 0.45  | 0.94 | 0.20           | 0.30 |
| Peak       | 0.89      | 1.00 | 0.40  | 0.94 | 0.13           | 0.30 |
| Slope      | 0.45      | 0.40 | 1.00  | 0.40 | 0.00           | 0.65 |
| Mean       | 0.94      | 0.94 | 0.40  | 1.00 | 0.15           | 0.29 |
| $T$: (1/2) max | 0.20    | 0.13 | 0.00  | 0.15 | 1.00           | 0.00 |
| Area       | 0.30      | 0.30 | 0.65  | 0.29 | 0.00           | 1.00 |

Figure 7: Percent mean values with respect to control. Mean values represent the average values (OD) generated during the time specified.

formation of peroxyl radicals. These data underpin the key role which the lipid environment plays in modulating the rate of reaction of antioxidants characterized by different inherent chemical reactivity/membrane mobility. The accuracy of these measurements depends mainly on the pH of buffer, solvent form, temperature, AAPH/ABAP and ABTS solution preparation. Amalgamation of AAPH/ABAP and ABTS is a highly accurate combination as ABTS solution does not react with the compounds in absence of AAPH/ABAP, is not light sensitive, and does not require sophisticated techniques. On the whole, this method with kinetic analysis part is a simple way of analyzing and interpreting character and behaviour of the molecule. Altogether, a novel, facile method of study, new insights, and a quantitative understanding of the critical role in modulating peroxyl radical formation by antioxidants are reported.

Conflict of Interests

The authors declare no conflict of interests.

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References

[1] L. J. Marnett, “Peroxy free radicals: potential mediators of tumor initiation and promotion,” Carcinogenesis, vol. 8, no. 10, pp. 1365–1373, 1987.

[2] M. Kappler, A. B. Gerry, E. Brown, L. Reid, D. S. Leake, and S. P. Gieseg, “Aqueous peroxy radical exposure to THP-1 cells causes glutathione loss followed by protein oxidation and cell death without increased caspase-3 activity,” Biochimica et Biophysica Acta, vol. 1773, no. 6, pp. 945–953, 2007.

[3] M. J. Davies, S. Fu, and R. T. Dean, “Protein hydroperoxides can give rise to reactive free radicals,” Biochemical Journal, vol. 305, no. 2, pp. 643–649, 1995.

[4] U. P. Steinbrecher, S. Parthasarathy, D. S. Leake, J. L. Wittzum, and D. Steinberg, “Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids,” Proceedings of the National Academy of Sciences of the United States of America, vol. 81, no. 12, pp. 3883–3887, 1984.

[5] C. N. Oliver, “Inactivation of enzymes and oxidative modification of proteins by stimulated neutrophils,” Archives of Biochemistry and Biophysics, vol. 253, no. 1, pp. 62–72, 1987.

[6] A. J. Kettle and C. C. Winterbourn, “Myeloperoxidase: a key regulator of neutrophil oxidant product,” Redox Report, vol. 3, no. 1, pp. 3–15, 1997.

[7] M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, and M. Mazur, “Free radicals, metals and antioxidants in oxidative stress-induced cancer,” Chemico-Biological Interactions, vol. 160, no. 1, pp. 1–40, 2006.

[8] P. C. Burcham, “Genotoxic lipid peroxidation products: their DNA damaging properties and role in formation of endogenous DNA adducts,” Mutagenesis, vol. 13, no. 3, pp. 287–305, 1998.

[9] N. A. Porter, S. E. Caldwell, and K. A. Mills, “Mechanisms of free radical oxidation of unsaturated lipids,” Lipids, vol. 30, no. 4, pp. 277–290, 1995.

[10] J. M. Gebicki, J. Collins, C. Gay, S. Duggan, and S. Gieseg, “The dissection of oxidative changes in human blood serum and U937 cells exposed to free radicals,” Redox Report, vol. 5, no. 1, pp. 55–56, 2000.

[11] S. Gieseg, S. Duggan, and J. M. Gebicki, “Peroxidation of proteins before lipids in U937 cells exposed to peroxy radicals,” Biochemical Journal, vol. 350, no. 1, pp. 215–218, 2000.

[12] J. Du and J. M. Gebicki, “Proteins are major initial cell targets of hydroxyl free radicals,” International Journal of Biochemistry and Cell Biology, vol. 36, no. 11, pp. 2334–2343, 2004.

[13] R. T. Dean, S. Fu, R. Stocker, and M. J. Davies, “Biochemistry and pathology of radical-mediated protein oxidation,” Biochemical Journal, vol. 324, no. 1, pp. 1–18, 1997.

[14] J. A. Simpson, S. Narita, S. Gieseg, S. Gebicki, J. M. Gebicki, and R. T. Dean, “Long-lived reactive species on free-radical-damaged proteins,” Biochemical Journal, vol. 282, no. 3, pp. 621–624, 1992.

[15] S. Gebicki and J. M. Gebicki, “Crosslinking of DNA and proteins induced by protein hydroperoxides,” Biochemical Journal, vol. 338, no. 3, pp. 629–636, 1999.

[16] M. B. Hampton, P. E. Morgan, and M. J. Davies, “Inactivation of cellular caspases by peptide-derived tryptophan and tyrosine peroxides,” FEBS Letters, vol. 527, no. 1–3, pp. 289–292, 2002.

[17] N. A. Porter, S. E. Caldwell, and K. A. Mills, “Mechanisms of free radical oxidation of unsaturated lipids,” Lipids, vol. 30, no. 4, pp. 277–290, 1995.

[18] E. Niki, Y. Yoshida, Y. Saito, and N. Noguchi, “Lipid peroxidation: mechanisms, inhibition, and biological effects,” Biochemical and Biophysical Research Communications, vol. 338, no. 1, pp. 668–676, 2005.

[19] J. M. C. Gutteridge, “Lipid peroxidation and antioxidants as biomarkers of tissue damage,” Clinical Chemistry, vol. 41, no. 12, pp. 1819–1828, 1995.

[20] E. Cadenas and H. Sies, “The lag phase,” Free Radical Research, vol. 28, no. 6, pp. 601–609, 1998.

[21] K. Yamanaka, F. Takabayashi, M. Mizoi, Y. An, A. Hasegawa, and S. Okada, “Oral exposure of dimethylarsinic acid, a main metabolite of inorganic arsenics, in mice leads to an increase in 8-oxo-2′-deoxyguanosine level, specifically in the target organs for arsenic carcinogenesis,” Biochemical and Biophysical Research Communications, vol. 287, no. 1, pp. 66–70, 2001.

[22] K. U. Ingold, “Peroxy radicals,” Accounts of Chemical Research, vol. 2, no. 1, pp. 1–9, 1969.

[23] L. J. Marnett, “Policyclic aromatic hydrocarbon oxidation during prostaglandin biosynthesis,” Life Sciences, vol. 29, no. 6, pp. 531–546, 1981.

[24] C. A. Gee, K. J. Kittridge, and R. L. Willson, “Peroxy free radicals, enzymes and radiation damage: sensitisation by oxygen and protection by superoxide dismutase and antioxidants,” British Journal of Radiology, vol. 58, no. 687, pp. 251–256, 1985.

[25] M. Martini and J. Termini, “Peroxy radical oxidation of thymidine,” Chemical Research in Toxicology, vol. 10, no. 2, pp. 234–241, 1997.

[26] M. R. Valentine, H. Rodriguez, and J. Termini, “Mutagenesis by peroxy radical is dominated by transversions at deoxyguanosine: evidence for the lack of involvement of 8-oxo-dG1 and/or abasic site formation,” Biochemistry, vol. 37, no. 19, pp. 7030–7038, 1998.

[27] L. Zennaro, M. Rossetto, P. Vanzani et al., “A method to evaluate capacity and efficiency of water soluble antioxidants as peroxy radical scavengers,” Archives of Biochemistry and Biophysics, vol. 462, no. 1, pp. 38–46, 2007.

[28] S. Lussignoli, M. Fraccaroli, G. Andrioli, G. Brocco, and P. Bellavite, “A microplate-based colorimetric assay of the total peroxy radical trapping capability of human plasma,” Analytical Biochemistry, vol. 269, no. 1, pp. 38–44, 1999.

[29] Y. M. A. Naguib, “A fluorometric method for measurement of peroxy radical scavenging activities of lipophilic antioxidants,” Analytical Biochemistry, vol. 265, no. 2, pp. 290–298, 1998.

[30] F. A. Kuypers, J. J. M. van den Berg, C. Schalkwijk, B. Roelofs, and J. A. E. O. D. Kamp, “Parinaric acid as a sensitive fluorescent probe for the determination of lipid peroxidation,” Biochimica et Biophysica Acta, vol. 921, no. 2, pp. 266–274, 1987.

[31] R. J. DeLange and A. N. Glazer, “Phycoerythrin fluorescence-based assay for peroxy radicals: a screen for biologically relevant protective agents,” Analytical Biochemistry, vol. 177, no. 2, pp. 300–306, 1989.
P. Cos, N. Hermans, M. Calomme et al., “Comparative study of eight well-known polyphenolic antioxidants,” Journal of Pharmacy and Pharmacology, vol. 55, no. 9, pp. 1291–1297, 2003.

B. Tadolini, C. Juliano, L. Piu, F. Franconi, and L. Cabrini, “Resveratrol inhibition of lipid peroxidation,” Free Radical Research, vol. 33, no. 1, pp. 105–114, 2000.

H. J. Jang, S. Hwang, K. Y. Cho, D. K. Kim, K.-O. Chay, and J.-K. Kim, “Taxol induces oxidative neuronal cell death by enhancing the activity of NADPH oxidase in mouse cortical cultures,” Neuroscience Letters, vol. 443, no. 1, pp. 17–22, 2008.

M. G. Benedetti, A. L. Foster, M. C. Vantipalli et al., “Compounds that confer thermal stress resistance and extended lifespan,” Experimental Gerontology, vol. 43, no. 10, pp. 882–891, 2008.

Z. Diaz, A. Laurenzana, K. K. Mann, T. A. Bismar, H. M. Schipper, and W. H. Miller Jr., “Trolox enhances the anti-lymphoma effects of arsenic trioxide, while protecting against liver toxicity,” Leukemia, vol. 21, no. 10, pp. 2117–2127, 2007.

R. Wolf, D. Wolf, and V. Ruocco, “Vitamin E: the radical protector,” Journal of the European Academy of Dermatology and Venereology, vol. 10, no. 2, pp. 103–117, 1998.

E. Niki, “Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence,” Free Radical Biology Medicine, vol. 8, no. 66, pp. 3–12, 2014.

P. Wan, S. Moat, and A. Anstey, “Pellagra: a review with emphasis on photosensitivity,” British Journal of Dermatology, vol. 164, no. 6, pp. 1188–1200, 2011.

N. Ishii and Y. Nishihara, “Pellagra among chronic alcoholics: clinical and pathological study of 20 necropsy cases,” Journal of Neurology Neurosurgery and Psychiatry, vol. 44, no. 3, pp. 209–215, 1981.

T. C. Villines, A. S. Kim, R. S. Gore, and A. J. Taylor, “Nicotinamide: the evidence, clinical use, and future directions,” Current Atherosclerosis Reports, vol. 14, no. 1, pp. 49–59, 2012.

V. S. Kamanna, S. H. Ganji, and M. L. Kashyap, “Recent advances in niacin and lipid metabolism,” Current Opinion in Lipidology, vol. 24, no. 3, pp. 239–245, 2013.

M. Takashima, M. Shichiri, Y. Hagihara, Y. Yoshida, and E. Niki, “Capacity of peroxyl radical scavenging and inhibition of lipid peroxidation by β-carotene, lycopene, and commercial tomato juice,” Food Function, vol. 3, pp. 1153–1160, 2012.

T. A. Kennedy and D. C. Liebler, “Peroxyl radical scavenging by β-carotene in lipid bilayers. Effect of oxygen partial pressure,” The Journal of Biological Chemistry, vol. 267, no. 7, pp. 4658–4663, 1992.

R. W. Burton and K. U. Ingold, “β-Carotene: an unusual type of lipid antioxidant,” Science, vol. 224, no. 4649, pp. 569–573, 1984.

R. Stocker, Y. Yamamoto, and A. F. McDonagh, “Bilirubin is an antioxidant of possible physiological importance,” Science, vol. 235, no. 4792, pp. 1043–1046, 1987.

G. F. Vile and C. C. Winterbourn, “Inhibition of Adriamycin-promoted microsomal lipid peroxidation by β-carotene, α-tocopherol and retinol at high and low oxygen partial pressures,” FEBS Letters, vol. 238, no. 2, pp. 353–356, 1988.

H. Sies and W. Stahl, “Vitamins E and C, β-carotene, and other carotenoids as antioxidants,” The American Journal of Clinical Nutrition, vol. 62, no. 6, pp. 1315S–1321S, 1995.

U. Moser and A. Bendich, “Vitamin C” in Handbook of Vitamins, U. Machlin, Ed., pp. 195–232, Marcel Dekker, New York, NY, USA, 1991.

R. Stocke and B. Frei, “Endogenous antioxidant defense in human blood plasma,” in Oxidative Stress: Oxidants and Antioxidants, H. Sies, Ed., pp. 213–243, Academic Press, London, UK, 1991.

M. Nishikimi, “Oxidation of ascorbic acid with superoxide anion generated by the xanthine xanthine oxidase system,” Biochemical and Biophysical Research Communications, vol. 63, no. 2, pp. 463–468, 1975.

R. S. Bodannes and P. C. Chan, “Ascorbic acid as a scavenger of singlet oxygen,” FEBS Letters, vol. 105, no. 2, pp. 195–196, 1979.

D. E. Cabelli and B. H. J. Bielski, “Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO2/O2- radicals. A pulse radiolysis and stopped-flow photolysis study,” The Journal of Physical Chemistry, vol. 87, no. 10, pp. 1809–1812, 1983.
B. Halliwell, M. Wasil, and M. Grootveld, “Biologically significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by ascorbic acid. Implications for antioxidant protection in the inflamed rheumatoid joint,” *FEBS Letters*, vol. 213, no. 1, pp. 15–17, 1987.

A. Dwenger, M. Funck, B. Lueken, G. Schweitzer, and U. Lehmann, “Effect of ascorbic acid on neutrophil functions and Hypoxanthine/Xanthine Oxidase-Generated, oxygen-derived radicals,” *European Journal of Clinical Chemistry and Clinical Biochemistry*, vol. 30, no. 4, pp. 187–191, 1992.

B.-M. Kwon and C. S. Foote, “Chemistry of singlet oxygen. 50. Hydroperoxide intermediates in the photooxygenation of ascorbic acid,” *Journal of the American Chemical Society*, vol. 110, no. 19, pp. 6582–6583, 1988.

B. Frei, L. England, and B. N. Ames, “Ascorbate is an outstanding antioxidant in human blood plasma,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 16, pp. 6377–6381, 1989.

E. Niki, A. Kawakami, Y. Yamamoto, and Y. Kamiya, “Oxidation of lipids. VIII. Synergistic inhibition of oxidation of phosphatidylethanolamine liposome in aqueous dispersion by vitamin E and vitamin C,” *Bulletin of the Chemical Society of Japan*, vol. 58, no. 7, pp. 1971–1975, 1985.

D. D. M. Wayne, G. W. Burton, K. U. Ingold, L. R. C. Barclay, and S. J. Locke, “The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma,” *Biochimica et Biophysica Acta*, vol. 924, no. 3, pp. 408–419, 1987.

T. Dob, G. W. Burton, and K. U. Ingold, “Antioxidant and co-antioxidant activity of vitamin C. The effect of vitamin C, either alone or in the presence of vitamin E or a water-soluble vitamin E analogue, upon the peroxidation of aqueous multilamellar phospholipid liposomes,” *Biochimica et Biophysica Acta*, vol. 835, no. 2, pp. 298–303, 1985.

P. Lambelet, F. Saucy, and J. Loliger, “Chemical evidence for interactions between vitamins E and C,” *Experientia*, vol. 41, no. 11, pp. 1384–1388, 1985.

H.-Y. Zhang, L.-F. Wang, and Y.-M. Sun, “Why B-ring is the active center for genistein to scavenge peroxyl radical: a DFT study,” *Bioorganic and Medicinal Chemistry Letters*, vol. 13, no. 5, pp. 909–911, 2003.

W. Bors, W. Heller, C. Michel, and M. Saran, “Flavonoids as antioxidants: determination of radical-scavenging efficiencies,” *Methods in Enzymology*, vol. 186, pp. 343–355, 1990.

P. J. O’Malley, “The reaction profile for hydrogen atom transfer from phenol to peroxyl free radicals,” *Chemical Physics Letters*, vol. 364, no. 3–4, pp. 318–322, 2002.

J. Wu, H. Sugiyama, L.-H. Zeng, D. Mickle, and T.-W. Wu, “Evidence of Trolox and some gallates as synergistic protectors of erythrocytes against peroxyl radicals,” *Biochemistry and Cell Biology*, vol. 76, no. 4, pp. 661–664, 1998.

L. K. T. Lam, R. P. Pai, and L. W. Wattenberg, “Synthesis and chemical carcinogen inhibitory activity of 2-tert-butyl-4-hydroxyanisole,” *Journal of Medicinal Chemistry*, vol. 22, no. 5, pp. 569–571, 1979.

G. W. Burton and K. U. Ingold, “Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro,” *Journal of the American Chemical Society*, vol. 103, no. 21, pp. 6472–6477, 1981.

P. K. J. P.D. Wanasundara and F. Shahidi, “Antioxidants: science, technology, and applications,” in *Bailey’s Industrial Oil and Fat Products*, F. Shahidi, Ed., pp. 431–489, John Wiley & Sons, Hoboken, NJ, USA, 2005.