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Optimized pyrogallol based superoxide scavenging assays for testing antioxidants

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Abstract: Four *in vitro* methods have been optimized for detection of superoxide generated from autoxidation of pyrogallol in alkaline solution. The methods have been linear over 0.1 through 1.0 µmole pyrogallol with pyrogallol autooxidation assay (PAA), pyrogallol autoxidation assay in presence of iron (II) (PAAI) and iodometric pyrogallol autoxidation assay (IPAA), and over 0.04 through 0.20 µmole pyrogallol with pyrogallol-mediated MTT reduction assay (PMTTRA).The relative sensitivity order of the assays based on their regression estimates (the mean values indicated within parenthesis) has been: PMTTRA (2.464) >> PAAI (0.596) ≥ PAA (0.568)>IPAA (0.283). Flavonoids, phenolics, thio compounds, ascorbic acid and anionic salts have differently responded to these assays. The assays provide inexpensive tools to employ these as a part of any primary screening program for testing antioxidants for any scavenging activity against superoxide radicals.
Key words: Pyrogallol, Autoxidation, MTT reduction, Iron (II), Superoxide scavenging activity.

Introduction

Evaluation of antioxidants for any superoxide scavenging potential is a priority owing to the implications of free radicals in health and disease[1]. Pyrogallol is an inexpensive substrate to generate superoxide anion when compared to other expensive non-enzymatic[2-6] and enzymatic substrates[5, 7, 8]. Pyrogallol has long been known to undergo autoxidation rapidly particularly at alkaline pH, and the color change in the medium remains persistent for a few hours[9]. The end-product of pyrogallol oxidation is accepted to be the hydroxylated benztropolone called purpurogallin showing characteristic absorption maxima (270–275; 310–320; 425 mµ) [10]. The original method has employed its monitoring at 420 nm, was developed specifically for determination of SOD[9], and now constitutes part of a battery of tests for antioxidants[5] and widely used for measuring superoxide-scavenging potential of other antioxidants[5, 11-14]. Recently, a simple and reliable alternative method, monitoring pyrogallol autoxidation at 325 nm, has been advanced for assaying all antioxidants[14]. A microtiter plate assay, based on pyrogallol superoxide mediated reduction of MTT, has been developed for SOD[7]. These assays[7, 14] have yet to find any place in screening antioxidants. The primary focus of present study has been to optimize few inexpensive methods for screening antioxidants while using pyrogallol as a substrate for generating superoxide anion. The rationale for the work has been based on the following: (i) pyogallol is an inexpensive and established substrate to generate superoxide anion on alkaline autoxidation; (ii) pyrogallol as a phenolic has strong affinity for iron[15]; (iii) autoxidation of pyrogallol in presence of KI solution was found to release iodine quantitatively enabling its assay by iodometry (unpublished data); and (iv) superoxide from pyrogallol is reportedly a moderately strong reducing agent to reduce MTT to formazan[7]. The experiments were accordingly designed to optimize four assays based on autoxidation of pyrogallol at alkaline pH: (i) autoxidation of pyrogallol with monitoring absorbance at a convenient time than within 5 minutes as is otherwise a common practice[7, 9, 12, 14], (ii) autoxidation in presence of iron (II); (iii) iodometric method for

monitoring autoxidation reaction by its ability to release iodine from KI solution; and (iv) reduction of tetrazolium dye, MTT, to formazan by the reducing action of superoxide anion.. The assays have been standardized, and verified for their linearity, sensitivity and capability to detect scavenging activity in test antioxidants.

Experimental

The experiments were carried out at an ambient temperature of 16.6±1.4 ^{0}C. The chemicals used were of standard purity and quality obtained from reputed sources in India. The reaction samples were centrifuged at 8000 rpm for 5 minutes when required for obtaining transparent solutions. Spectrophotometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan).

Reagents and test agents

Rutin trihydrate (Rut), quercetin dehydrate (Quer) and morin hydrate (Mor):

The requisite dilutions were made from their respective stock solutions made in methanol providing respectively as 2.0, 5.0 and 2.0 µmole of the flavonoid mL^{-1} methanol.

Diosmin (Dios) and daflon (Daf):

Laboratory standards for synthetic diosmin and for daflon were prepared from their respective pharmaceutical tablets: Venex-500 (Elder Pharmaceuticals Ltd., Mumbai) (labeled mass 500 mg synthetic diosmin per tablet) and Daflon (Serdia Pharmaceuticals Ltd., Mumbai) (labeled contents 90 % diosmin and 10 % hesperidin) as per standard procedure[16] to the strength of 0.25 % (w/v) equivalent to 4.1µmole flavonoid mL^{-1} 0.1 M NaOH. The requisite dilutions were made in 0.1 M NaOH from these stock solutions.

Ascorbic acid (AA), thioglycollic acid (TGA), thiourea (TU), dimethyl sullfoxide

(DMSO), phenol (Phe), resorcinol (Res), hydroquinone (HQ), guaiacol(Gua), catechol (Cat) and gallic acid (Ga) solutions:

The requisite dilutions were made in water from respective stock solutions made in water as 100 (TU, DMSO), 20 (TGA) and 25 µmole mL^{-1} (others).

Citrate (Cit), oxalate (Oxa) and tartrate (Tart) solutions:

Each solution was prepared in water from the respective salts viz., tri-sodium citrate, potassium oxalate and potassium sodium tartrate provide 50 µmole of anion mL^{-1} water.

Standard iodine solution:

It was prepared approximately as a 100 mL solution containing 1.4 g resublimed iodine crystals, 3.6 g of KI with a drop of dilute HCl. The solution was standardized against standard sodium thiosulfate solution (0.1 M) by titrimetry. The working solutions were made by dilution in water as per need.

Pyrogallol stock solution:

Stock solution was prepared by dissolving 100 mg pyrogallol (purity 98 %) in 15.8 mL water containing 0.2 mL 1 % HCl to provide 50 µmole mL^{-1}. Working solutions were made by dilution in water as per need.

MTT reagent:

0.1 % (w/v) [(3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) in ethanol was used as a working solution.

Dilute HCl solutions:

1 % or 10 % (v/v) concentrated HCl in water.

Iron reagents:

Working solutions were made by dilution in water from stock solution of ferrous ammonium sulfate (FAS-II) and ferric ammonium sulfate (FAS-III) prepared in 0.001 M HCl as 25 µmole mL^{-1}.

KI solution:

5 %(w/v) KI in 0.001M NaOH.

Sodium hydroxide solution:

0.1 M NaOH in water.

Sodium bicarbonate solution:

2.1 % (w/v) sodium bicarbonate in water, ca. 0.25 M.

Alkaline mixture:

The solution was prepared by mixing 10 mL of 0.25 M bicarbonate solution with 10 mL water and 5 mL of 0.1 M sodium hydroxide solution. Each aliquot of 0.5 mL provided 50 µmole of bicarbonate and 10 µmole of sodium hydroxide.

Analytical methods

1. Pyrogallol autoxidation assay (PAA)

For linearity setting, 3.5 mL aliquots of aqueous solution containing pyrogallol as 0, 0.1 through 1 µmole were added 0.5 mL alkaline mixture, allowed standing at room temperature for 40 to 50 minutes and read at 415 nm. For antioxidant studies, the appropriate mass of test material was incubated with or without standard mass of pyrogallol 0.8 µmole. The calibration standard was run using pyrogallol 0.2, 0.4 and 0.8 µmole for linearity checking at the time of the assay.

2. Pyrogallol autoxidation assay in presence of iron (PAAI)

For linearity testing, 2.6 mL aqueous samples containing 0, 0.1 through 1 µmole pyrogallol were added 2 µmole of FAS (II) in 0.4 mL water, added each 0.5 mL alkaline mixture, and allowed standing for 5 minutes. The reaction was stopped by addition of 0.5 mL dilute HCl, and the samples were monitored at 430 nm at 30 to 40 minutes following alkalinization. Test antioxidants were assayed by incubating known mass in absence and presence of standard mass of pyrogallol (1 µmole).

3. Iodometric pyrogallol-based autoxidation assay (IPAA)

For linearity setting, 3 mL aqueous samples containing pyrogallol 0, 0.1 through 1.0 µmole were added 1 mL KI solution followed by 0.5 mL of alkaline mixture.The samples were allowed standing at room temperature for 10 minutes, the reaction was stopped by addition of 0.5 mL 10 % HCl, and absorbance monitored for iodine at 430 nm at about one hour. Control and blank samples contained all reagents including pyrogallol with addition of acid before adding pyrogallol to prevent super oxide generation. Test antioxidants were assayed by incubating known mass in absence and presence of standard mass of pyrogallol (0.5 µmole).

4. Pyrogallol based MTT reduction assay (PMTTRA)

One milliliter aqueous samples containing 0, 0.04 through 0.2 µmole pyrogallol were each added 0.1 mL MTT solution followed by 0.9 mL water. Each sample was added 1 mL of appropriate solvent followed by addition of 0.5 mL alkaline solution. The reaction was allowed at room temperature for 20 minutes, added 0.5 mL DMSO solution (50 µmole) and 1 mL water. The samples were monitored at 560 nm for formazan formation about 10 to 15 minutes following addition of DMSO. Test materials were used in appropriate mass with or without standard pyrogallol mass 0.08 µmole.

Each assay has been optimized for optimal conditions required for linearity setting and conduct of the assay. While conducting assay of test antioxidants, the samples were matched for appropriate solvent and its volume in standard, blank and control samples.

Calculations

The data were analyzed by routine statistical methods for regression and variance studies. The activity in the test sample in presence of standard was estimated by the formula:

Activity, PEA or PSA (nmoles µmole^{-1} test agent) = [(C-A-B)/B)]* Factor Positive value indicates potentiating activity (pyrogallol-equivalent activity, PEA) and negative value indicates antagonistic activity (i.e. pyrogallol scavenging activity, PSA). A, B and C are absorbanace values due, respectively, to test agent with known mass (T, in µmole) in absence of pyrogallol (as control value), standard mass of pyrogallol (S, in nmoles) (as standard value), and test agent in presence of pyrogallol (as treated value). When treated samples have been measured setting control to zero, the value of A is taken as zero. The factor is given by the quotient of S and T.

The value given by [(A/B)* Factor] reveals inherent standard equivalent activity present in the test agent in absence of the standard.

Results and Discussion

The test agents have included known antioxidants: flavonoids, phenolics, ascorbic acid and thio compounds. Citrate, oxalate and tartrate salts were included because major antioxidants are known to possess carboxylic acid moeity[14]. Pyrogallol *per se* is known to be a potent reducing agent[15] and its autoxidation product is known to be moderately strong reductant[7]. As evident (Tables 4 and 5) the test agents have not responded uniformly to the assays. Both potentiation as well as reduction in pyrogallol-mediated response has been recorded. Besides, some agents have expressed their own reducing potential by reducing MTT in absence of pyrogallol (Table 5). The spectral analysis with PAA, PAAI and IPOA has revealed that absorbance maxima were much higher at 400 nm than at 430 nm. However, in view of higher interference by flavonoids at 400 nm than at 430 nm, the preferred monitoring for AOA was kept 415 nm ($p>0.1$ with respect to 400 nm) and 430 nm for other two assays. The flavonoid interference was still high with PAA because flavonoids impart yellowish coloration to the solution at alkaline pH. Consequently, treated samples were always read against corresponding controls set as zero. The interference got largely eliminated in PAAI and IPOA where 0.5 mL dilute HCl was used to terminate the reaction. Scavenging activity has been found to be related to pH. This was quite evident while using anionic salts in PAA and MTTRA. Citric acid, oxalic acid and tartaric acid at 50 µmole each and 80 nmoles pyrogallol and 0.5 mL alkaline mixture in 4 mL volume failed to evoke any response. Upon addition of 1 mL 0.1 M NaOH, the absorbance appeared with linear relation to observed pH showing increase in absorbance from 0.01 through 0.39 with increase in pH from 6.1 through 9.7 ($r=0.96$; $b=0.10$). With MTTRA, addition of 0.5 mL 10 % HCl was found to reduce mean absorbance by the salts from 71 to 76 %. Similar observations have been observed with flavonoids in PAA Incorporation of acid in PAAI and IPOA has enabled using pyrogallol even in blanks by adding first acid followed by additions of pyrogallol and alkaline mixture. This prevented autoxidation of pyrogallol. It has been normal practice to estimate and compare relative potentials of antioxidants by using per cent inhibition criterion[2, 3, 11, 13]. The present study has employed standardized criterion, nmole substrate (scavenged or equivalent) per µmole test agent. This has obvious advantages of enabling comparisons across the assays.

1. Autoxidation assay in absence of iron

1.1 Optimization and linearity studies

As apparent, the assay has been linear over 0.1 through 1.0 µmole pyrogallol (Table 1) with improvement over 0.1 through 0.8 µmole (0.999±0.001; 0.597±0.012) and steepest regression over 0.1 through 0.4 µmole (0.999±0.001; 0.630±0.016).

Time optimization revealed that in delay in measurement by 40 to 50 minutes reduced regression estimate by 8 % ($p<0.01$, $n=5$ each). Alkaline mixture was better than either sodium hydroxide or sodium bicarbonate providing better absorbance ($p<0.01$, $n= 5$ each) with mean absorbance in order: NaOH + $NaHCO_3$> NaOH > $NaHCO_3$.

1.2 Response of test agents

Per cent response to test agents was 94 ; 11 and 5 agents have shown, respectively, scavenging and potentiating activity while resorcinol has failed to modify pyrogallol action Pyrogallol action has been reduced

by 11 to 61 % with scavengers. Ga (100) proved most potent scavenger followed by (per cent of Ga activity indicated within parenthesis): AA (98), Mor (64), Dios (59), TGA (54), Rut (36), Daf (26) and HQ (12). Weakly active scavengers include Cit (0.6), Tart (0.5) and Oxa (0.4). Potentiators have enhanced absorbance by 3 to 47 % with most potent Quer (100) followed by TU (10), Cat (4), Gua (0.7) and Phe (0.7). Acidification of samples with 0.5 mL 10 % HCl (or 0.5 mL 0.6 % acetic acid) reduced color by over 90 %. However, this downgraded PSA of Dios to107 ± 2 and of Daf to 64±1, and even standard pyrogallol suffered reduction in absorbance by over 70 %. Similar behavior was seen with other flavonoids. Ethanol, methanol and acetone have potentiated activity of pyrogallol, respectively, by 49, 33 and 51 % showing PEA per mL basis respectively as 395 ±5, 266±6 and 816 ±3. Sodium hydroxide failed to modify standard pyrogallol response ($p>0.1$). None has shown any activity in absence of pyrogallol.

2. Autoxidation assay in presence of iron (II)

2.1. Optimization and linearity studies

Table 1: Pyrogallol dependent autoxidation assays in absence and presence of iron (II)

Pyrogallol mass, µmole	Absorbance in absence of iron (II)[a]	Absorbance in presence of iron (II)[b]
0.1	0.078±0.001	0.045±0.002
0.2	0.141±0.001	0.101±0.002
0.4	0.267±0.003	0.244±0.007
0.6	0.381±0.003	0.366±0.010
0.8	0.496±0.003	0.485±0.021
1.0	0.582±0.004	0.567±0.009
Statistical analysis		
r±S.E.	0.998±0.002	0.997±0.002
b±S.E.	0.568±0.015	0.596±0.019

[a] Pyrogallol auto-oxidation in absence of iron and acid
[b] Pyrogallol auto-oxidation in presence of acid and iron
The values are mean ± S.E. of 5 observations each.

In presence of 2 µmole iron (II), the assay has been linear over 0.1 through 1.0 µmole pyrogallol (Table 1) showing improved regression estimate over 0.1 through 0.8 µmole (0.999±0.001; 0.636±0.013); steepest over 0.1 through 0.4 µmole (0.998±0.002; 0.671±0.024). The absorbance with acid addition over 5 to 15 minutes has been significantly better (peak 10^{th} minute) than at 3^{rd} minute following alkalinization ($p<0.01$, n=5 each). This suggests initial alkalinization is critical for autoxidation to proceed. The reaction was better when iron was added before alkalinization than when added just before or following acidification ($p<0.01$, n=5 each).This suggests iron is presumably interacting with the oxidation product of pyrogallol at an earlier stage. The mean absorbance has increased linearly with increase in mass of iron (II) over 1, 1.5 and 2 µmole (0.264 ±0.001; 0.999±0.001) with 1 µmole pyrogallol. The regression estimate with 2 µmole iron (II) was 2.2 times more than with 1.5 µmole iron (II). The preliminary studies revealed that either form of iron (II) or (III) can be used for the assay. However, with iron (III), reactivity to thiocyanate showed linear decline from control with increase in pyrogallol while with iron (II), the reactivity was enhanced at least 25 times at lower mass of pyrogallol (0.1 to 0.2 µmole) and thereafter fell linearly. The analysis implied iron (II) maintained ferrous/ferric ratio >1.0 while iron (III) favored ratio <1.0. Consequently, reducing form of iron was preferred for its less likely chances to mask reducing activity of moderately strong reducing super oxide anion from pyrogallol.[7].

2.2 Response of test agents

Per cent response to test agents was 94 (16 out of 17). TGA has failed to change absorbance by standard ($p>0.1$, n= 5 each); while 10 and 6 agents have, respectively, shown scavenging and potentiating activity. Scavengers caused reduction in absorbance by 5 to 79 %. AA (100) ranked as most potent scavenger followed by TU (57), Dios (50), Daf (40), Oxa (17) and HQ (12). Res (1.4), Phe (0.9), Tart (0.4) and Cit (0.11) have shown weak but significant scavenging activity ($p<0.01$, n=5 each). Potentiators have increased absorbance by 5 through 34 % with most potent potentiator GA (100) followed by Mor (60), Rut (20), Quer (16), Gua (2.2) and Cat (1).

Ethanol, methanol and acetone have enhanced pyrogallol action by 7 to 10 % with PEA on mL basis respectively as 159 ± 9, 102 ±7 and 70 ±7. Sodium hydroxide 1 mL 0.1 M reduced pyrogallol action by 6 % showing PSA as 57 ±2 per mL.

3. Iodometric assay

3.1. Optimization and linearity studies

Table 2: Pyrogallol superoxide anion dependent iodometric assay

Pyrogallol mass, µmole	Absorbance
0.1	0.051±0.001
0.2	0.089±0.001
0.3	0.128±0.001
0.4	0.159±0.002
0.5	0.183±0.002
0.6	0.199±0.006
1.0	0.314±0.005
Statistical analysis	
r±S.E.	0.996±0.003
b±S.E.	0.283±0.010

The values are mean ± S.E. of 5 observations each.

The iodometric assay has been linear over 0.1 through 1.0 µmole pyrogallol (Table 2)showing improved regression over 0.1 through 0.5 µmole (0.334±0.013; 0.996 ± 0.004); steepest over 0.1 through 0.4 µmole (0.363±0.008; 0.999±0.001). The alkaline mixture increased absorbance by over 175 % and 24 % as compared to those found respectively with sodium bicarbonate and sodium hydroxide, ($p<0.01$, n= 5 each).

3.2 Response of test agents

Per cent response to test agents was 88 (14 out of 16) with 9 and 5 showing, respectively as, scavenging and potentiating activity. The scavengers reduced standard absorbance by 3 through 92 %. AA (100) has ranked most potent followed by TGA (80), Res (29), TU (28), Daf (25), Dios (17) and Mor (10). Cit (0.44) and Oxa (0.09) have been weak to least active scavengers ($p<0.01$). Potentiating agents increased absorbance by 4 through 31 % with most active GA (100) followed by HQ (53), Rut (7), Quer (7) and Phe (3).

Ethanol and methanol imparted faint tinge in control samples (absorbance <0.04) while acetone and NaOH had no effect. Methanol enhanced absorbance of standard by 45 % (PEA, 221±13 per mL) while acetone, ethanol and sodium hydroxide reduced absorbance by 9 to 37 % showing PSA, respectively, as 185±8, 44±3 and 99±2.

Oxidizing potential of alkaline pyrogallol has been estimated in terms of simultaneously run standard iodine (1 through 5 µmole: b= 0.23, c= -0.1445 and r =0.999) under identical conditions in 5 mL volume containing 0.5 mL 10 % HCl releasing iodine equivalent to 1.58 ±0.04 µmole per µmole pyrogallol (r±S.E., 0.999±0.001) .

4. MTT reduction assay

PMTTRA actually measures reducing activity of alkaline pyrogallol superoxide[7].The reducing potential is indicated by formazan formation in absence of pyrogallol. While in presence of pyrogallol, both reducing as well as scavenging potentials get expressed. Antioxidants *per se* are reducing agents so capable of reducing MTT. In presence of pyogallol, the magnitude of observed absorbance would reflect whether reducing or scavenging potential is dominant. This is done by subtracting sum of absorbance values obtained with standard and test agent individually from the value obtained when used together. If the net absorbance is positive in sign it reflects reducing potential of standard has been potentiated by the test agent. If the sign of the value is negative, it reflects antagonism, or scavenging activity of the test agent.

4.1 Optimization and linearity studies

Table 3: Pyrogallol superoxide anion dependent MTT reduction assay

Pyrogallol mass, µmole	Absorbance
0.04	0.098±0.001
	0.204±0.003
0.10	0.259±0.004
0.12	0.309±0.001
0.16	0.412±0.002
0.20	0.488±0.002
Statistical analysis	
r±S.E.	0.998±0.002
b±S.E.	2.464±0.064

The values are mean ± S.E.of 5 observations each.

As evident (Table 3), the assay has been linear over 0.04 through 0.2 µmole pyrogallol. The regression is better over 0.04 through 0.16 µmole (0.999 ± 0.001; 2.618±0.052) with steepest regression over 0.04 through 0.10 µmole (0.999 ± 0.001; 2.679±0.069). Addition of DMSO has been recommended to terminate the reaction and to solubilize formazan[6]. DMSO addition at 20th minute of incubation has provided mean absorbance as good as that of standard without DMSO ($p>0.1$, n=5 each).The alkaline mixture provided mean absorbance value which was about 2 times more than those provided by hydroxide and bicarbonate individually ($p<0.01$, n=5 each). Besides, the mean absorbance remained more stable with alkaline mixture than with either of the bases used separately when monitored over a 2 hour observation period ($p<0.01$, n=5 each).

4.2 Response of test agents

As evident (Table 5), in presence of pyrogallol, there has been 100 per cent response by test agents while in absence of pyrogallol, 7 out of 16 have failed to reduce MTT ($p>0.1$, n=5 each) including notably Cit, Oxa, Tart, Gua, TU, Dios and Daf. Nine have reduced MTT just like standard pyrogallol ($p<0.01$, n= 5 each). The most active reducing agent was Quer (100) followed by GA (62), Mor (34), AA (21), HQ (0.05), Rut (0.02), TGA (0.015), Cat (0.013) and Res (0.005). In presence of pyrogallol, 10 and 6 test agents have, respectively, shown potentiating and scavenging activity. The potentiators have enhanced absorbance of standard by 17 through 79 % with most potentiating agent Mor (100) followed by Quer (94), TGA (47), Rut (44), Dios (34), TU (13), Daf (11), Gua (3), Res (2) and Cat (1). The scavengers have reduced mean absorbance of standard by 7 through 60 % with most active GA (100) followed by AA (96) , HQ (13), Tart (0.6), Oxa (0.3) and Cit (0.2). As evident, salts have shown significant but weaker activity, about 30 to 90 times lesser than that of catechol. In absence of pyrogallol, acetone and NaOH showed no action on MTT while ethanol and methanol increased reduction showing PEA per mL basis, respectively as, 20±2 and 26±1. In presence of pyrogallol, acetone did not modify response ($p>0.1$) while ethanol enhanced MTT reduction by 17 % showing PEA 28±4 per mL, and NaOH and methanol reduced absorbance of standard by 19 and 15 % showing PSA respectively as 30 ±2 and 23 ± 2.

Table 4. Response of test agents to pyrogallol superoxide anion: autoxidation versus iodometric assay systems

S.No	Test agent	Autoxidation	Autoxidation with FAS (II)	Iodometric
1	Diosmin	-295 ± 31 (1)	-394±32 (0.25)	-49±6 (1)
2	Daflon	-133± 14 (1)	-314±43 (0.25)	-71± 3 (1)
3	Quercetin	+750 ± 40 (1)	+105±40 (0.5)	+ 20 ± 2 (1)
4	Rutin	-179± 2 (1)	+133 ±49 (0.5)	+22 ± 3 (1)
5	Morin	-319± 5 (1)	+407 ± 26 (0.5)	-28 ± 4 (1)
6	Resorcinol	NAD (5)	-11 ±2 (5)	-83 ± 3 (1)
7	Guaiacol	+5 ±1 (5)	+15 ± 2 (5)	NAD (1)
8	Catechol	+28±1 (5)	+7 ±2 (5)	NAD (1)
9	Phenol	+5±1 (5)	-7 ± 0.7 (10)	+9± 0.5 (10)

10	HQ	-61 ± 2 (5)	-94 ±1 (5)	+156± 4 (1)
11	GA	-502±3 (0.5)	+676 ± 13 (0.5)	+296 ±15 (0.2)
12	Ascorbic acid	-491 ± 8 (1)	-794±11 (1)	-287 ± 20 (0.5)
13	TGA	-269 ±7 (0.5)	NAD (0.5)	-231 ±2 (2)
14	TU	+76 ±9 (1)	-450 ±2 (0.5)	-79 ± 2 (1)
15	Citrate	-2.8 ±0.1 (50)	-0.9 ±0.1 (50)	-0.41 ±0.07 (50)
16	Oxalate	-2.0 ±0.1 (50)	-137±1 (5)	-0.25 ±0.07 (50)
17	Tartrate	-2.4 ±0.1 (50)	-3.4 ±0.1 (50)	Not conducted

NAD, no activity detected (p>0.1); test masses are given within parenthesis as µmole; + prefix for potentiation, pyrogallol-equivalent activity; PEA; - prefix for antagonism, pyrogallol scavenging activity, PSA; the values are mean ± S.E. of 5 observations each.

Table 5: Response of MTT to test agents in absence and presence of pyrogallol[a]

S. No.	Test agent	Mass used, µmole	Activity in absence of pyrogallol	Activity in presence of pyrogallol
1	Diosmin	0.5	NAD	+ 90 ± 7
2	Daflon	0.5	NAD	+29 ± 3
3	Quercetin	0.2	+1006 ± 14	+250 ± 15
4	Rutin	0.2	+24 ± 2	+117 ± 5
5	Morin	0.2	+347 ± 7	+266 ± 15
6	Resorcinol	5	+4.9 ± 0.2	+5.8 ± 0.2
7	Guaiacol	5	NAD	+7.8 ±0.5
8	Catechol	5	+13.1 ± 0.5	+2.8 ±0.4
9	HQ	5	+52.7 ± 0.8	-9.5 ±0.3
10	GA	0.2	+625 ± 6	-71 ± 6
11	Ascorbic acid	0.5	+213 ±3	-68 ± 3
12	TGA	0.5	+15.4 ± 1.0	+126 ± 5
13	TU	0.5	NAD	+35 ± 2
14	Citrate	50	NAD	-0.14 ± 0.02
15	Oxalate	50	NAD	-0.21 ± 0.02
16	Tartrate	50	NAD	-0.46 ± 0.02

NAD, no activity detected (p>0.1) at test mass; + prefix for potentiation, pyrogallol-equivalent activity; PEA; - prefix for antagonism, pyrogallol scavenging activity, PSA; the values are mean ± S.E. of 5 observations each.

[a]Pyrogallol standard mass 80 nmoles per test

An overview of the performance of the assays has revealed that out of 17 compounds, 14 have responded positively to scavenging activity. Quercetin, guaiacol and catechol have exhibited only potentiating effects. Quercetin has been used as a positive control while using PMS+NADH assay to generate superoxide to reduce NBT[6]. Ascorbic acid has proven as most potent scavenging agent, responding in all assays. Carboxylic acid salts have also responded to all assays with weaker but significant scavenging activity. Ascorbic acid has been employed as a positive control in PMS+NADH+NBT assay[1-3], and carboxylic acid moiety is considered a characteristic feature of antioxidants for scavenging activity[13]. Diosmin appears as most potent flavonoid followed by almost equipotent rutin, morin and daflon. Rutin has been employed as a positive control[13]. The scavenging potency order of flavonoids in PMS assay: rutin>quercetin>morin, has been shown to be unrelated to their antioxidant activity[17]. Gallic acid with mean scavenging ranking second to ascorbic acid has been most potent amongst phenolics followed by dihydroxy benzenes such as hydroquinone and then resorcinol. This lends credence to the observation that dihydroxy benezene rings including HQ are useful in the development of potent antioxidants[17]. Scavenging activity of flavonoids, and phenolics has been attributed to their hydroxyl functions[12]. Of thio compounds, mean scavenging activity is slightly better for TU than TGA while mean potentiating activity is over twice with TGA than with TU (Tables 4 and 5). The scavenging or reducing potential is obviously due to –SH group: GSH has been found to be more potent than ascorbic acid in pyrogallol autoxidation assay[10].

Conclusions

The work has provided a comparative evaluation of test antioxidants for their response in four optimized assays employing inexpensive pyrogallol as a substrate for generating superoxide anion. MTT reduction assay has been most sensitive method and with a detection range, about one-eighth to one-fourth of other assays, demonstrating both reducing and scavenging potential of test antioxidants. Autoxidation in absence and presence of iron (II) have been equally sensitive with iron based assay showing better regression than plain autoxidation assay. Iodometric assay has been least sensitive. Different test agents have shown differential pattern of responses in the assay systems. These assays provide simple tools as a part of test battery for any evaluation scheme for testing antioxidants for superoxide scavenging activity..

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