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In vitro photometric assays for screening antioxidants using methylene blue, potassium dichromate, ferric and copper reduction assays

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Abstract: Four simple and inexpensive photometric assays have been developed for testing antioxidants *in vitro*. These include two new introductions based on reduction of methylene blue (MBRA) and potassium dichromate(DRA), and two improvised methods based on reduction of iron (III) (IRA) and copper (II) (ICRA). The assays have shown perfect linearity (r±S.E, 0.999±0.001) with relative order of regression coefficients in units of absorbance versus µmole test oxidant (the mean values within parenthesis) as: MBRA (13) > IRA (1.9) > DRA (0.10)>ICRA (0.085). The methods have shown distinct differentiating responses to the test antioxidants including amongst others flavonoids, phenolics, ascorbic acid, mannitol and thio compounds. The improvised iron (III) assay has detected thio compounds that remain otherwise unresponsive in existing iron (III) reduction assays. The results suggest that the methods would constitute a part of battery of tests required for any primary screening of test agents for their further evaluation as antioxidants. Using a standard parameter for activity evaluation, nmoles of oxidant reduced µmole^{-1} test agent, has enabled relative comparisons between antioxidants within or across different assays.
Key-words: Dichromate reduction, Methylene Blue reduction, Iron reduction, Copper reduction, Antioxidants.

Introduction

An increasing interest to search antioxidants for human use has necessitated requirement for a variety of antioxidant methods to accomplish the objective. Of large numbers of such methods, those based on iron reduction have been very popular[1-5]. Lately, copper based reduction assays have become popular for their simplicity and better performance[6-12] especially their ability to detect thio compounds where iron assays fail[4, 6, 7, 12]. Methylene blue and dichromate ions in acidic medium are easily reducible. There have been very limited efforts to utilize these for antioxidant assays[13-16]. Earlier efforts from this laboratory to improvise iron reduction assays by using either ferric salicylate method[3] or ferric thocyanate method[5] for monitoring iron reduction have been encouraging. Ferric thiocyanate assay has shown applicability to assay antioxidant activity of beta-carotene and alpha-tocopherol[5]. The ability of cupric salts to oxidize soluble iodides to release iodine has improved the prospects of designing the assay for antioxidant studies based on copper-based iodometry. Similarly, assessment of dichromate reduction as an antioxidant assay has deserved attention.

The objectives for the present work were set to improvise these methods for making simple *in vitro screens* for antioxidants. Experiments were accordingly designed: (i) to optimize inexpensive assay systems including iron reduction assay based on thiocyanate reactivity[5]; copper reduction monitored by iodomtery; dichromate and methylene blue reduction assays for photometric evaluation; (ii) to determine linear ranges of detection and regression estimates for each assay; and (ii) to evaluate different antioxidants for their relative

potentials in these assay systems using a standard unit of antioxidant potential as nmoles of oxidant reduced per µmole of test agent to simplify intra-agents and intra-assay comparisons.

Experimental

The experiments were carried out at an ambient temperature of 17.8 ± 0.7^0C. The chemicals used were of standard purity and quality obtained from reputed sources in India. The samples developing turbidity or discoloration during the assay were centrifuged at 8000-9000 rpm for 5 minutes. Spectrophotometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan).

Reagents and test agents

Rutin trihydrate, quercetin dihydrate and morin hydrate:

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

Diosmin and daflon:

Laboratory standards for synthetic diosmin and for daflon were prepared from the pharmaceuticals: Venex-500 (Elder Pharma.Ltd., Mumbai) (labeled mass 500 mg synthetic diosmin per tablet) and Daflon (Serdia Pharma. Ltd., Mumbai) (labeled contents 90 % diosmin and 10 % hesperidin) following standard procedure[17] to the strength of 0.25 % (w/v) equivalent to 4.1µmole flavonoid mL^{-1} 0.1 M NaOH. The requisite dilutions were always made in 0.1 M NaOH from these stock solutions.

Catechol, resorcinol, guaiacol, hydroquinone, phenol, pyrogallol, gallic acid, thioglycollic acid, thiourea, ascorbic acid solutions:

Each working solution was made by dilution in water from working stock solutions of 25 µmole mL^1 water each.

Mannitol, citric acid, tartaric acid, oxalic acid:

Each was appropriately made to provide 100 µmole mL^1 water.

Hydrogen peroxide solution:

It was made freshly by dilution in water from standardized stock containing 40 µmole of hydrogen peroxide mL^{-1} water.

Nitrite solution:

Working solutions 50 through 200 nmoles mL^{-1} were made freshly by dilution with water as per need from 0.1 M stock solution of sodium nitrite in water (containing 0.1 % chloroform as preservative).

Copper acetate solution:

The required dilutions were made in water at the time of use from stock solution of 0.1 M copper acetate (0.2 % w/v in water).

Dilute HCl and H_2SO_4 solutions:

10 %(v/v) concentrated acid in water.

Potassium iodide solution:

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

Sodium bicarbonate solution:

The working solution was made by dilution in water as 0.1 M sodium bicarbonate from 0.25 M stock (2.1 % (w/v) in water).

Methylene Blue (MB) solution:

Working solution of 100 nmoles dye mL^{-1} water was prepared from stock solution 0.1 % (w/v) of methylene blue made in water.

Dichromate solution:

Working solution of potassium dichromate 1 and 10 µmoles mL^{-1} water was prepared from stock solution 0.2 N potassium dichromate in water.

Ferric ammonium sulfate (FAS-III):

The stock solution (25 µmole iron (III) mL^{-1} 0.005 N HCl in water) was appropriately diluted in water freshly as per need.

Standard iodine solution:

The solution was approximately made 0.1 M by dissolving 1.4 g resublimed iodine crystals and 3.6 g potassium iodide with a drop of 10% HCl in water to make 100 mL solution. The solution was standardized against standard 0.1N sodium thiosulfate by titrimetry using starch as indicator.

Potassium thiocyanate solution:

10 % (w/v) KSCN in water.

Analytical technique

Methylene blue reduction assay (MBRA)

MB solution as 0, 5 through 60 nmoles was taken in 4.5 mL water, acidified with 0.5 mL dilute HCl, allowed standing at room temperature for about 30 minutes and monitored at 650 nm. Test substances in appropriate solvents were taken with standard mass of MB, 40 nmoles. For calibration standard, MB was used at three masses as 20, 40 and 60 nmoles, matched with appropriate solvent in 5 mL volume.

Dichromate reduction assay (DRA)

Potassium dichromate solution as 0, 0.5 through 8 µnmoles was taken in 3.5 mL water, acidified with 0.5 mL dilute sulfuric acid, allowed standing at room temperature for about 30 to 40 minutes and monitored at 430 nm. Test substances were taken in appropriate solvent with standard mass of dichromate, 3 µmoles. For calibration assays, potassium dichromate was taken in three masses as 1, 3 and 5 µmoles in 4 mL volume with appropriate matching volume of solvent.

Iron (III) reduction assay (IRA)

The standard curve was run using FAS-III over 0.05 through 0.5 µmole range. The samples in 3 mL water were acidified with 0.5 mL dilute HCl, allowed standing at room temperature for about 20 minutes, and added 0.5 mL thiocyanate solution, and absorbance measured at 480 nm following further about 20 minutes. For assay, test samples in appropriate solvent were used with and without 0.5 µmole FAS-III, and subjected to identical conditions. The standard calibration assay employed 0, 0.2, 0.3 and 0.5 µmole FAS-III with matching volume of the solvent.

Iodometric copper (II) reduction assay (ICRA)

Copper acetate samples as 0, 1 though 30 µmole in 2 mL water were added 0.2 mL bicarbonate solution followed by 1.3 mL water and 0.5 mL 10 % HCl in that order. Each sample was added 1 mL KI solution, allowed standing for about 30 minutes at room temperature, and subjected to centrifugation to remove any insoluble matter. The supernatant was read at 430 nm at about 30 minutes following addition of KI solution. Test agents were taken with and without standard Cu (II) 5 µmole, and the calibration standards were run using as 1, 3 and 5 µmole Cu (II), each matched for solvent.

Calculations

The data were subjected to routine statistical analysis. Test agents were evaluated for their antioxidant activity by their ability to reduce the mass of the reactant in nmoles (viz., MB, dichromate, ferric or copper ion) per µmole of test agent. Initially estimations were done both directly (A) by using single matching standard as well as indirectly (B) by employing regression analysis parameters (y-intercept and regression coefficient) obtained from simultaneously run calibration assay. The following formulae were used to estimate antioxidant activity of test agents:

Reducing activity, nmoles reactant reduced µmole^{-1} test agent

= [C*(1 - (T/S))] / M (A)
= [C - ((T-c)/b)*Factor] / M (B)
Where
T is absorbance due to standard mass of the reactant in presence of test substance
S is absorbance due to standard mass of the reactant in absence of test substance
C is mass of reactant used in nmoles; M is mass of test substance used in µmole(s);
c is y-intercept value from regression analysis data; b is regression coefficient from regression analysis data; Factor = 1000, to convert µmoles into nmoles.

The regression data has been based on three concentrations of the standard reactant (in µmoles) as x-axis vis-à-vis their corresponding absorbance values as y-axis.

On the basis of the inferences drawn from the comparative analysis of the two methods, direct method was employed throughout for ease of convenience in calculations.

Classifying anti-oxidants as per potencies

Taking highest recorded activity (nmoles oxidant reduced µmole^{-1} test agent) in a given assay system by a test agent as 100 per cent, the categorization of the agents was made as follows for comparative evaluation of their relative potencies :

Category	**Activity (% of maximum recorded)**
Highly active	≥ 10
Moderately active	1 to <10
Weakly active	0.1 to < 1
Least active	<0.1
Inactive	No activity for given mass

The same trend was followed while comparing overall mean activities for the test agents obtained with different assay systems to make comparisons across the assays.

Results and Discussion

Linearity studies and effect of solvents

The solvents for the test agents have included methanol for quercetin, rutin and morin, 0.1 M NaOH for diosmin and daflon, and water for the rest. The assays were standardized in aqueous medium for linearity and regression studies. However, calibration assays and assays for test agents were conducted using matching volumes of particular solvent depending upon the assay requirements. In aqueous medium, the linear ranges for the reactant species (the values within parenthesis) have been in the order: MBRA (0.005 – 0.06 µmole) < IRA (0.05 – 0.5 µmole) < DRA (0.5 – 8 µmole) < ICRA (1 – 30 µmole) (Tables 1 through 4). The regression coefficients for the assays have been (mean values within parenthesis): MBRA (12.91) steepest over 0.01 - 0.04 µmole (13.41) followed by IRA (1.9004) followed by DRA (0.1014) which has been somewhat equal to that obtained with ICRA (0.0854) with steepest value over 3 – 10 µmole range (0.1044). Thus MBRA and IRA have been, respectively, about 128 and 19 times as sensitive as either DRA or ICRA. A perusal of regression estimates in calibration assays has revealed that the values increased with methanol (3 to 20 %) and decreased with NaOH (9 to 30 %) throughout except in DRA where the alkali increased regression coefficient by about 8 %. Similar effects were observed while comparing effects of ethanol and methanol added in 1 mL volume

increasing absorbance, respectively, by 15 and 18 % (IRA), 24 and 16 % (MBRA) and 53 and 51 % (ICRA). In DRA, the two solvents, ethanol and methanol, have decreased absorbance by 81 and 27, respectively, showing thereby *per se* reduction of 2430 ± 20 and 817 ± 24 nmole dichromate mL^{-1}solvent. Baring ICRA, addition of acetone 1 mL has increased absorbance by 6 % (DRA), 23% (IRA) and 30 % (MBRA). The response of ICRA to acetone has been noteworthy. It caused almost complete decolorization of the standard (97.5% reduction) at 1 mL. Lower masses, 0.01 through 0.05 ml acetone, have caused linear reduction of copper (II) to the extent of 70.1 ± 1.8 µmoles mL^{-1} with perfect linearity (0.999±0.001). The reducing effects of acetone were probably due to presence of reducing impurities in the solvent as the label indicated the presence of permanganate reducing impurities to the extent of 0.002%. This would further suggest the applicability of the technique in testing reducing impurities in test solutions.

Table 1.Linear relation: absorbance with mass of methylene blue

Methylene Blue, µmole	Absorbance
0.005	0.096±0.002
0.01	0.160±0.002
0.02	0.292±0.001
0.03	0.430±0.001
0.04	0.561±0.003
0.05	0.687 ±0.003
0.06	0.796 ±0.004
Statistical analysis	
r ±S.E	0.9995±0.0004
b ±S.E[a].	12.91 ±0.15

[a] Unit expressed as absorbance per nmole dye
The values are mean ± S.E. of 5 observations each.
The curve is steepest over 10 through 40 nmoles
(r ±S.E =0.9999±0.0001; b ±S.E =13.41±0.09)

Table 2: Linear relation: absorbance with mass of potassium dichromate

Potassium dichromate, µmole	Absorbance
0.5	0.052±0.001
1.0	0.103±00.002
2.0	0.203±0.002
3.0	0.294±0.003
4.0	0.397 ±0.003
5.0	0.512 ±0.007
6.0	0.607 ±0.003
8.0	0.814 ±0.004
Statistical analysis	
r ±S.E	0.9998 ±0.0002
b ±S.E.	0.1014 ±0.0001

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

Table 3: Linear relation: absorbance with mass of ferric ammonium sulfate

Ferric ammonium sulfate, µmole	Absorbance
0.05	0.085±0.001
0.1	0.170±00.003
0.2	0.363±0.005
0.3	0.549±0.006
0.4	0.743 ±0.014
0.5	0.938 ±0.002
Statistical analysis	
r ±S.E	0.9999 ±0.0001
b ±S.E.	1.9004 ±0.0002

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

Table 4 .Linear relation: absorbance with mass of copper acetate

Copper acetate, µmole	Absorbance
1	0.132±0.003
3	0.281±0.007
5	0.485±0.006
10	1.055±0.004
30	2.602±0.005
Statistical analysis	
r ±S.E	0.997±0.003
b ±S.E[a].	0.0854 ±0.0030

The values are mean ± S.E. of 5 observations each.
[a] Regression is steeper over 1 through 10 µmoles (0.1044 ± 0.0040) and steepest over 3 through 10 µmoles (0.1112 ±0.0029; 0.999±0.001)

Direct versus calibrated estimations

The data for this study covered 40 separate estimates made with two different techniques (MBRA and DRA) involving different antioxidants. The comparisons of the estimates by direct and indirect methods have revealed that the estimated mean from direct method, 6.19 ± 1.55, deviated hardly by about 2 % from the value, 6.30 ±1.59 obtained from calibration curve ($p>0.1$, $n= 40$).Consequently, only direct method was employed throughout for estimating antioxidant activity of test agents.

Evaluation of test agents by different techniques

The test agents for the study have included flavonoids (5), phenolics (7), organic acids (4), thio-compounds (2) and mannitol, all known for their reducing potentials (Table 5). Besides, nitrite and hydrogen peroxide were also included in some studies for their possession of both oxidizing and reducing abilities[16], and to test if their reducing abilities are expressed in these assays.

Methylene blue reduction assay

The response ranking of the assay has been 57 %. As evident(Table 5), quercetin has ranked top amongst highly active agents with its mean activity 41.6 nmole MB reduced $\mu mole^{-1}$(100 %) followed in that order (with per cent of quercetin activity shown within parenthesis) by ascorbic acid (74); rutin (25), diosmin (20), daflon (20) and morin (13).Thioglycollic acid (1.3) and oxalic acid (1.2) were moderately active. Pyrogallol (0.7), thiourea (0.5) and nitrite (0.4) were weakly active. Ascorbic acid has been most active from amongst non-flavonoid anti-oxidants while tartaric acid (0.04) least active in this assay. Compared to hydrogen peroxide, nitrite at 5 µmole has demonstrated weak activity. Both were inactive at 1 µmole mass. Phenolics as a

class, mannitol, citric acid, and hydrogen peroxide have failed to reduce MB. On the other hand, gallic acid significantly increased absorbance of standard by 2.5 % ($p<0.05$, n=5).

Concentration related MB decolorization was recorded with flavonoids (0.3 thorugh 1 µmole), ascorbic acid (0.1 through 1µmole; linear over 0.1 through 0.5 µmole), thiourea (20 through 100 µmoles) and thioglycollic acid (10 through 30 µmole). Failure to get activity with phenolics and high activity with flavonoids would suggest that these residues are not contributing to antioxidant potential of flavonoids for this activity in this assay. Besides, 4 times increased activity with quercetin compared to its rutinose derivative, rutin implies free hydroxyl function at C-3 in quercetin is very essential for maximal activity expression.

Dichromate reduction assay

Overall the response ranking of the technique has been 52%. Gallic acid has ranked top amongst highly active category (100) followed by ascorbic acid (95), catechol (64), hydroquinone (54), thioglycollic acid (49), hydrogen peroxide (47) and thiourea (34). Nitrite (6) and mannitol (1) have been moderately active while oxalic acid (0.2) and tartaric acid (0.7) have been weakly active. The assay shows somewhat contrasting behavior towards the test agents as otherwise seen with MBRA such that flavonoids and some phenolics (resorcinol, pyrogallol, guaiacol and phenol) failed to produce reduction. In fact the absorbance values have increased by about 60, 24, 24, 20 and 16 %, respectively, with rutin, morin, diosmin, quercetin and daflon (Table 5) ($p<0.01$, 5 each). This necessitated to take lower masses for evaluation else photometric readings could not be taken. Similarly, guaiacol and resorcinol (0.5 µmole each) increased mean absorbance by ca. 47 and 8 % respectively ($p<0.01$, 5 each). Response to citric acid was comparable to those observed in MBRA and ICRA, all showing it to be inactive.

Iron (III) reduction assay

The assay has ranked second top in its response to test agents showing per cent response of 67 (Table 5). Ascorbic acid has proven most potent amongst highly active agents (100) followed by much lesser active in this category shown by pyrogallol (20), gallic acid (13), quercetin (12) and morin (10). Diosmin and daflon have characteristically failed to demonstrate any activity. In fact, they increased absorbance, respectively, by about 1.1 % ($p>0.1$, n=5) and 3.4 % ($p<0.05$). Mannitol, nitrite and hydrogen peroxide failed to evoke any response. Organic acids have either failed (oxalic acid, tartaric acid) or evoked a very weak response (citric acid, 0.006). Moderately active response has been observed with hydroquinone (6), thioglycollic acid (4.3), thiourea (4), catechol (3), resorcinol (1.6), guaiacol (1.5) and rutin (1.4). As seen with MBRA, quercetin has shown much higher potency (about 9 times more) than that observed with rutin in this assay. Phenol (0.4) has been weakly active while citric acid (0.006) has shown least potency. Demonstration of activity by thio compounds is remarkable as existing iron (III) reduction assays suffer from the limitation of not being able to remain responsive to thio compounds[4,6,7,12] .Consistent with known ability of iron assay to detect citric acid where copper assay fails[12], citric acid has remained responsive to IRA than to ICRA (Table 5).

The assay in general has shown its suitability in detecting phenolics, thio compounds, ascorbic acid and some flavonoids like quercetin, rutin and morin, and not suitable for diosmin, daflon, mannitol, nitrite and hydrogen peroxide, and carboxylic acids.

Iodometric copper (II) reduction assay

ICRA has ranked highest in its response to test agents showing 72 % response as 13 out of 18 tested compounds responded to the assay (Table 5). The order of reagent addition has not affected absorbance values nor presence or absence of bicarbonate ($p>0.1$, n=5). Bicarbonate solution was added to release CO_2 to remove any residual oxygen from the samples to obviate its possible interference with the assay.

Interestingly, the flavonoid morin (100) has demonstrated highest activity from amongst all assays. Most test agents (10 out of 13) have shown high activity. These included quercetin (50), ascorbic acid (38), rutin (35), diosmin (33), gallic acid (25), hydroquinone (25), thioglycollic acid (18), daflon (17), and thiourea (13). Resorcinol (5.7) and oxalic acid (1.6) have shown moderate activity while catechol (0.8) has been weakly active. The assay has failed to register any activity in mannitol, citric acid, tartaric acid, phenol and guaiacol. The aforelisted activities for flavonoids have been observed in absence of bicarbonate. The presence of bicarbonate has interfered with the assay of flavonoids. For instance, in presence of bicarbonate the activity of daflon (33) was higher than that of diosmin (22) and other flavonoids failed to demonstrate any activity, showing an enhanced absorbance with quercetin (0.606 ± 0.006), rutin (0.635 ±0.004) and morin (0.636

±0.005) with 1 µmole of each and 5 µmole of copper as compared to standard copper (0.600 ±0.004). This necessitated conducting test in absence of bicarbonate and adding acid at towards end. Then absorbance values then were respectively recorded as 0.371 ± 0.008, 0.533±0.002 and 0.190 ±0.005 as compared to that with standard copper 5 µmole (0.586±0.002). The phenolic control samples have developed color especially with hydroquinone, so required centrifugation, and the treated samples were read against corresponding sample controls.

Simultaneously run standard iodine (0, 1 through 5 µmole iodine) under matching conditions indicated that the mean oxidizing potential of Cu(II) to oxidize KI to I_2 ranged from 0.371± 0.013 (0.999± 0.001) to 0.484 ± 0.013 (0.999± 0.001) µmole µmole^{-1} Cu(II), respectively, over general and steepest portions of the curve (Table 4).

Comparative evaluation of techniques

Ascorbic acid, thiourea and TGA have been responsive to all test assays while morin, quercetin, rutin, catechol, hydroquinone, gallic and oxalic acids have responded each to the three assays varyingly (Table 5). Citric acid, guaiacol, phenol, mannitol and H_2O_2 have been assayable only by one of the assays and not in others. Tartaric acid responded effectively to only DRA with a milder response in BMRA (Table 5).

Table 5: Comparative potential of test antioxidants using reduction assays: methylene blue, dichromate, ferric and copper (II) ions

S No.	Test agent	Reduction activity, nmoles per µmole test agent			
		Methylene Blue	Dichromate	Ferric ions	Cupric ions
1	Diosmin	8.4 ±0.1 (1)	NRD (0.4)	NRD (1)	1208±38[a] (1)
2	Daflon	8.7 ±0.4 (1)	NRD (0.4)	NRD (1)	633±57[a] (1)
3	Quercetin	41.6±5.1 (0.3)	NRD (0.3)	256±5 (0.5)	1837 ± 72[b] (1)
4	Rutin	10.6±0.4 (1)	NRD (0.3)	28.0±1.8 (0.5)	1292 ± 17[b] (1)
5	Morin	5.6 ±0.6 (1)	NRD (0.5)	216.8 ±4.3 (0.5)	3682±33[b] (1)
6	Resorcinol	NRD (10)	NRD (0.5)	33.9±0.4 (2)	210±15 (5)
7	Catechol	NRD (10)	347±29 (0.5)	63.1±0.7 (2)	31±9 (5)
8	Guaiacol	NRD (10)	NRD (0.5)	31.8±1.3 (2)	NRD (5)
9	Hydroquinone	NRD (10)	297±8 (2)	125.0±0.7 (2)	938±12 (5)
10	Phenol	NRD (20)	NRD (20)	8.1±0.2 (10)	NRD (10)
11	Pyrogallol	0.29±0.01 (5)	NRD (1)	409 ± 6 (1)	Not conducted
12	Gallic acid	NRD (10)	546±6 (1.25)	277±4 (0.5)	904±8 (2)
13	Oxalic acid	0.50±0.01 (50)	0.91±0.01 (50)	NRD (50)	60 ±1 (20)
14	Ascorbic acid	30.6±1.0 (0.5)	518±11 (2.5)	2066±5 (0.2)	1402 ±19 (1)
15	Citric acid	NRD (100)	NRD (100)	0.13±0.02 (100)	NRD (50)
16	Tartaric acid	0.017± 0.001 (100)	3.9±0.5 (100)	NRD (100)	NRD (50)
17	Thiourea	0.21±0.01 (40)	184±3 (5)	84.3±3.0 (1)	465±66 (1)
18	Thioglycollic acid	0.54±0.02 (20)	265±3 (5)	89.3 ± 4.7 (1)	650±36 (1)
19	Mannitol	NRD (100)	5.8 ± 0.1 (100)	NRD (100)	NRD[c] (50)

The values are mean ± S.E. of 5 observations each; NRD, no reduction detected at test concentration (within parenthesis as µmole);

[a] In absence of bicarbonate while in presence of the bicarbonate, the values for diosmin and daflon were 797±88 and 1200 ± 91, respectively;

[b] In absence of bicarbonate, the effect was masked in presence of bicarbonate; [c] Absorbance increased by 11%

Table 6: Relative anti-oxidant activity profile of test agents with different assay systems

Assay system	Highly active	Moderately active	Weakly active	Least active	Inactive
Iodometric copper reduction assay	Mor(100), Quer(50), AA(38), Rut(35), Dios(22,33)[a]; Daf(17,33)[a]; HQ,GA (25), TGA (18), TU(13)	Res (5.7) OA(1.6)	Cat (8)	-	Gua Phe CA TA Man
Ferric ion reduction assay	AA(100), Pyr(20), GA(13), Quer(12), Mor (10)	HQ(6) TGA(4.3) TU(4) Cat(3) Res(1.6) Gua(1.5)	Phe(0.4)	CA (0.006)	Dios Daf OA TA Man Nit HP
Dichromate reduction assay	GA (100), AA(95), Cat(64),HQ(54), TGA(49),H2O2(47),TU(34)	Nit(6) Man(1)	TA(0.7) OA(0.2)	-	Dios, Daf, Quer, Rut, Mor, Res, Gua,Phen, Pyr, CA
Methylene blue reduction assay	Quer(100), AA(74), Rut(25), Dios(20), Daf(20), Mor(13)	TGA(1.3) OA (1.2)	Pyr(0.7) TU(0.5) Nit(0.4)	TA (0.04)	Res, Cat, Gua, HQ, Phen, GA, CA, Man, HP
Overall	Mor (100),AA (77), Quer (55), Dios (31, 47), Daf (25,46), Ga(44),HQ(35), Rut (34), HP(20), TGA(19), Pyr(16), TU(14), Cat (11)	Res (9) Gua (2.4) Oxa (1.6) Nit(1.4)	Phe(0.6) Man(0.4 Ta (0.2)	CA (0.009)	All assayable varyingly

AA, ascorbic acid; CA, citric acid; Cat, catechol; Daf, daflon; Dios, diosmin; GA, gallic acid; Gua, guaiacol; HP, hydrogen peroxide; HQ, hydroquinone; Man, mannitol; Mor, morin; Nit, nitrite; OA, oxalic acid; Phe, phenol; Pyr, pyrogallol; Quer, quercetin; Res, resorcinol; Rut,Rutin; TA, tartaric acid; TGA, thioglycollic acid;TU, thiourea; [a] Differing activity noticed in presence and absence of bicarbonate; the values within parenthesis are per cent of most active agent in the category

Application of test criterion (according 100 % potency to most active agent) (Table 5) has enabled ranking of the test agents for comparative assessment of their relative potency orders within and across the assay systems (Table 6).

As evident ascorbic acid, gallic acid and TGA are correspondingly most active agents from non-flavonoids, phenolics and thio compounds with *inter se* relative potency in that order. Rutin has been least potent amongst flavonoids, much less potent than its aglycone quercetin suggesting blockade of hydroxyl function by rutinose in rutin has been cause for its decreased potency.

A comparative evaluation of techniques reveals notable features. MBRA has been least sensitive with failure to respond to phenolics except pyrogallol. ICRA has been better than others including IRA for testing flavonoids, thio compounds and gallic acid. IRA has been most sensitive of all for ascorbic acid. Copper based assays are considered better than iron assays for detecting flavonoids, polyphenols, ascorbic acid and in particular thio compounds[6, 7, 11] and have also shown better response to thiourea than to mannitol[11]. The iron assay as used in present study is an improvement over existing iron assays which fail to detect thio compounds[4,

[6, 7, 12]. DRA has failed to respond to flavonoids and some phenols (resorcinol, guaiacol, phenol and pyrogallol) but has shown better response than IRA for testing thio compounds and gallic acid. Whereas dichromate assay failed to respond to flavonoids and responded to favorably to phenols like catechol, hydroquinone and gallic acid; MB assay reacted favorably to flavonoids but failed to respond to these (Table 5). MB assay showed generally poor response to carboxylic acids compared to dichromate and thio compounds showed better response with dichromate than MB assay. Iron and copper assays show comparable response though magnitude of responses to copper assay were much higher with striking differences especially diosmin and daflon failing to respond in iron assay compared to copper assay, and response to other flavonoids, thio compounds, gallic acid, HQ, resorcinol were several folds higher with copper assay than those obtained with iron assay.

The foregoing observations suggest that the four techniques are to be taken as a battery for routine test screening to avoid any mistake in missing the potential antioxidant.

Conclusions

The study has provided four simple and inexpensive techniques for primary screening of test agents, including flavonoids, phenolics, ascorbic acid, thio compounds and mannitol, for any antioxidant activities. Since the methods respond varyingly to different antioxidants so would demand to be used together as a battery to increase chances of detecting potential antioxidants. The methods include new introductions based on reduction of methylene blue and of dichromate, and inexpensive, simple versions, for existing copper (II) and iron (III) reduction based assays. Improvised iron assay has responded to thio compounds that remain otherwise unresponsive to existing versions of the technique. A standard protocol has been outlined to compare and grade anti-oxidant potentials uniformly employing the activity, as nmole reduction per unit micromole of test agent, as test criterion. This obviates making any arbitrary classification of antioxidants based on non-standard parameters using per cent inhibition response with respect to some standard.

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