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Iodimetric assay for evaluating potential of antioxidants by iodine reducing activity

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Abstract: A photometric iodimetric assay has been optimized to enable evaluation of antioxidants on the basis of their iodine reducing activity (IRA). Iodine mass is monitorable at a wide range of wavelengths: 365, 400, 430 and 450 nm with regression estimate decreasing with increase in choice of wavelength (r= - 0.99). Monitoring for the present work has been done at 430 nm, which provided more stable values, with least interference from flavonoids and cover a wider range of iodine concentrations. IRA has been expressed in unit, µmole iodine reduced µmole^{-1} test agent. The presence of acid (1% HCl) has affected IRA varyingly; commencing from no effect (ascorbic acid, hydroquinone) to increase in activity (TGA), to marked decrease in activity (flavonoids, resorcinol, pyrogallol, thiourea) to complete blockade of action (catechol, phenol, guaiacol, gallic acid). The assay has enabled to provide notable structure activity relationship features in test flavonoids and phenolics.The assay is anticipated to be used as a part of battery of tests for screening antioxidants for their further investigation by advanced techniques.

Key-words: Iodimetric assay, Photometric assay, Antioxidants, Iodine reducing activity.

Introduction

A large number of techniques are available for screening antioxidants[1,2]. Simple and cost-effective methods are superior to those requiring expensive equipments and specialized expertise in particular for laboratories having financial constraints to continue work in areas of medical interest. Redox iodimetry has been one of the oldest and reliable methods to monitor iodine concentrations. The method involves transfer of electrons accompanied with change in color of solution due to change of iodine to iodide. The technique has been employed for standardization of thiosulfates, hydrogen sulfide, sulfurous acid, tin (II) chloride, arsenic (III), antimony (III) and hydrogen peroxide[3]. Limited efforts have been made to use the procedure for evaluation of antioxidants. Redox iodimetry has been employed for determining ascorbic acid in natural samples or in pharmaceuticals[4-8], for determination of thiols in pharmaceuticals [9] and has been used to assess and compare antioxidant potentials of extracts from fruits and vegetables in student demonstrations[10]. The assay has been employed as a reference standard for testing advanced methods developed for assaying ascorbic acid in pharmaceuticals[11]. The redox titrimetry involving iodine has been considered one of the standard reliable methods for determining total antioxidant capacity of human serum[12]. Iodometric methods by titrimetry and spectrophotometry at 350 nm have been recommended as simple and reliable methods for monitoring lipid peroxidation[13]. Based upon these observations, the work was mooted and experiments designed with the objective of testing chemically diverse antioxidants for their iodine reducing activity by photometry with antioxidant potentials expressed as µmole iodine reduced µmole^{-1} test agent. In view of known effects of acidity

and alkalinity of solution on iodine color[3,9,14], the responses to antioxidants were also studied in absence and presence of dilute acid.

Experimental

The experiments were carried out at an ambient temperature of 15.5 ± 0.5 ^{0}C. The drugs and chemicals used were of standard purity and quality obtained from reputed sources in India. Spectrophtometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan).

Reagents

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 taking 25 mL of aliquot until the color made palish-yellow; then added 0.5 mL starch solution (0.5 % w/v in boiled and cooled water) and titration continued till blue color is just discharged . Working solutions as 1 and 5 µmole iodine mL^{-1} water were made freshly at the time of use.

Dilute HCl solution: 10 %(v/v) HCl in water.

Dilute acetic acid solution: 0.6 % (v/v) glacial acetic acid in water.

Rutin trihydrate (Rut), quercetin dihydrate (Que) and morin hydrate (Mor) solutions: The solutions was prepared as 0.2 % (w/v) (Rut) and 0.1 % (w/v) (Que and Mor) in methanol with consideration to their respective minimum labeled purity as 90% (Rut), 98 % (Que) and 95% (Mor). The requisite dilutions were made in methanol to provide 0.2 and 0.5 µmole flavonoid mL^{-1} methanol.

Diosmin: Laboratory standard for synthetic diosmin was prepared from Venex-500 mg tablets (Elder Pharmaceuticals Ltd., Mumbai) as per standard procedure already outlined[15]. The concentration of diosmin was adjusted to 0.25 % (w/v) labeled diosmin in 0.1 M NaOH (equivalent to 4.1 µmole diosmin mL^{-1}). The requisite dilutions were made in 0.1 M NaOH to provide 0.5 µmole diosmin mL^{-1}.

Daflon: The flavonoid mixture contained in Daflon tablets was extracted from the powder in the manner applied to Diosmin tablet powder[15]. The final strength was adjusted to 0.25 % (w/v) flavonoid mixture (90 % diosmin and 10 % hesperidin as per label) in 0.1 M NaOH (equivalent to 4.1 µmole bioflavonoid mixture mL^{-1}). The requisite dilutions were made in 0.1 M NaOH to provide 0.5 µmole daflon mL^{-1} 0.1 M NaOH.

Ascorbic acid (AA), thiourea (TU) and thioglycollic acid (TGA) solutions: Working solutions were made by dilution in water as 0.2 µmole (TU), 0.4 µmole (AA) and 1.0 µmole (TGA) mL^{-1} from respective stock solutions of 10 µmole each mL^{-1} water.

Hydrogen peroxide solution: Working solution of hydrogen peroxide contained 10 µmole hydrogen peroxide mL^{-1} water. The stock solution, 42 µmole H_2O_2 mL^{-1} water, was standardized against potassium permanganate solution.

DMSO solution: The working solution contained DMSO 10 µmoles mL^{-1} water.

Phenol (Phe), guaiacol (Gua), resorcinol (Res), hydroquinone (HQ), catechol (Cat), gallic acid (Ga), pyrogallol (Pyr) solutions: The solutions were appropriately made in water and diluted to provide working solutions of 0.1 µmole (Ga), 0.5 µmole (Pyr), 1 µmole (Res, Cat, HQ), 10 µmole (Gua) and 25 µmole (Phe) mL^{-1} water.

Glucose (Glu), sucrose (Suc), mannitol (Man), citric acid (Ca), tartaric acid (Ta) and oxalic acid (Ta) solutions: Each compound was appropriately dissolved in water to provide 25 µmole (CA, OA, Ta) and 100 µmole (Glu, Suc, Man) mL^{-1} water.

Analytical technique

For assaying test agents in absence of acid, test agents were taken in three to four concentrations with or without standard mass of iodine, 2 µmole, in 5.0 mL volume of water matched for appropriate solvent, allowed standing at room temperature for 30 to 40 minutes and monitored for absorbance at 430 nm. Unless otherwise indicated each concentration was taken in triplicate. For assays involving comparison of effect of test agent in absence and presence of acid, single concentration of test agent within the linear range was employed. The acid group contained 0.5 mL 10 % HCl in 5 mL volume providing overall 1 % HCl. Each assay included simultaneously run standard at 3 to 4 concentrations of iodine (0, 0.5 through 2 µmole) as a check for observing any change in sensitivity of the assay. Based on previous experience (unpublished data), estimations were done with respect to single standard employed with test agent for convenience of calculations. Diosmin and daflon contained in alkaline solution, i.e.1 mL 0.1 M NaOH, were assayed following their neutralization with 1 mL of dilute acetic acid. Quercetin, morin and rutin were added in 1 mL methanol. All other test agents were added in water. The samples were appropriately matched for volume and type of solvent used.

Optimization studies

Optimization studies were focused to find linear ranges for iodine masses at various wavelengths viz., 365, 400, 430 and 450 nm to select the appropriate wavelength that would cover wider range of iodine concentrations and showing minimal interferences with test agents. Iodine for this purpose was taken in varying masses from 0.2 through 5 µmole. The effect of varying masses of dilute HCl, 0.5 through 2 mL, on absorbance of standard mass of iodine was also monitored to find *pe se* if any effect of acid on iodine absorbance. Since methanol was employed as a solvent for quercetin, rutin and morin, it was found desirable to check influence of methanol on response to standard iodine and IRA of these flavonoids. Comparable effects of ethanol and acetone on iodine were also recorded as these are commonly employed for extraction of phytochemicals.

Calculations

The data were subjected to routine statistical analysis. Iodine reduction to iodide is a reversible redox reaction involving participation of electrons and consequently potential antioxidants cause quantitative reduction of iodine, and hence decolorization of iodine solution. Therefore test substances were evaluated photometriclly for antioxidant activity using standard parameter iodine reducing activity (IRA) expressed as µmole iodine reduced per µmole test agent. The following formula was used to calculate IRA of test substances:

$$IRA = [C*(1 - (T/S))] / M$$

where, T is absorbance due to standard mass of iodine in presence of test substance; S is absorbance due to standard mass of iodine in absence of test substance; C and M are respectively masses of iodine and test antioxidant used in µmole(s). If the value of IRA was found to be less than unity, the result was multiplied by 1000 to express the activity in nmoles.

Results and Discussion

Choice of wavelength

Iodimetry has been conducted at 350 nm in environmental and biological samples for determination of iodine species[16] or for spectrophometric monitoring of lipid peroxidation[13]. Monitoring iodine at 350 nm or 365 nm has been optimal depending upon nature of solvent[17]. As evident from Table 1, iodine shows linear relation with each of test wavelengths from 365 nm to 450 nm over varying ranges of iodine mass. The maximum absorbance and regression estimate are shown at 365 nm compared to other wavelengths. Increase in wavelength from 365 nm though 450 nm has linearly decreased regression estimate from 0.873 through 0.142 (r =-0.99; b= -0.008778). However, monitoring of iodine at 430 nm has been followed during this work for following reasons: (i) flavonoids absorbed excessively at 365 nm than in visible range. For example, diosmin and daflon controls exhibited absorbance > 1.0 at 365 nm, > 0.1 at 400 nm and <0.04 at 430 nm; (ii) monitoring at 430 nm has given opportunity to cover wider range of iodine under linear region (1 to 5 µmole) compared to narrower range (0.2 to 1 µmole) provided by monitoring at 365 nm; and (iii) the absorbance values showed

least fluctuation and greater stability at 430 nm than at 365 nm. Consequently, it was desirable to make all measurements at 430 nm.

Table1. Linear relation absorbance and mass of iodine at various wavelengths

Wavelength, nm	Iodine mass µmole	Mean absorbance	r±S.E.	b±S.E.
365	0.2 0.3 0.5 1.0	0.043± 0.001 0.094±0.002 0.227±0.002 0.727±0.002	0.994 ±0.006	0.873±0.048
400	1.0 1.5 2.0 3.0	0.190±0.003 0.366±0.002 0.636±0.006 1.165±0.007	0.997±0.003	0.497±0.019
430	1.0 1.5 2.0 3.0 4.0 5.0	0.109±0.001 0.191±0.001 0.311±0.002 0.528±0.005 0.763±0.007 1.026±0.009	0.999±0.001	0.230±0.004
450	1.0 1.5 2.0 3.0 4.0 5.0	0.089±0.001 0.144±0.001 0.224±0.001 0.357±0.002 0.499±0.004 0.658±0.007	0.999±0.001	0.142±0.003

The values are Mean ± S.E. of 3 observations each

Experimental variations

Centrifugation: The control samples of quercetin developed opalescence on standing in absence of iodine. The samples were, therefore, centrifuged at 8000 rpm for 5 minutes to obtain colorless centrifugate. Rutin which is chemically quercetin-3-rutinoside remained transparent and did not require any centrifugation. This suggests the importance of the sugar link in rutin favoring solubility of the flavonoid.

Effect of acidity: The acidity *per se* has no effect on absorbance of iodine. Using standard iodine mass as 2.0 µmole without and with dilute HCl as 0.2 through 1 mL in 5 mL total volume, the mean absorbance values have remained in the range 0.217 ±0.002 through 0.220±0.002 in absence and presence of varying acid ($p>0.1$, $n=5$ each). While testing diosmin and daflon for IRA, hydroxide ions in addition to intensifying flavonoid color have affected decolorization of iodine[3, 9]. Trial experiments revealed that addition of minimal mass of acid was necessary to neutralize hydroxide and to keep acidity as minimal as possible; increasing acidity, pH less than 2.5, has largely reduced the response to these flavonoids. Each mL of 0.1 M NaOH required 0.3 mL of 4% HCl or 1 mL of 0.6 % acetic acid to eliminate alkalinity and retain acidity within 3 to 5 pH units. This also minimized control absorbance which remained 0.009 through 0.017 at 0.1 through 0.4 µmole of diosmin and daflon. No significant difference was observed between the acetic acid and HCl in indicated masses on mean absorbance of diosmin ($p>0.1$, $n= 6$ each); the mean absorbance with two acids remained respectively as 0.196 ±0.003 and 0.204 ±0.002 and per cent decrease with respect to control value, 0.241 ±0.002, remained ca. 19 and 15 ($p<0.01$, $n=6$ each). Acetic acid was preferred for use as it maintained higher limit of pH ca. 4.6 than HCl which kept pH around 3.

Control samples of morin have shown excessive coloration in methanol. The samples were rendered almost colorless by addition of 0.5 mL of 0.6 % acetic acid, and absorbance in controls remained within 0.005 through 0.011 with 0.1 through 0.3 µmole of morin. None other samples needed any special treatment. Quercetin and rutin control samples showed a faint tinge with absorbance within 0.02 unit.

Iodine reducing activity of antioxidants

As evident (Tables 2 and 3), flavonoids and phenolics as a class have shown high IRA. Test agents may be grouped on the basis of their relative IRA values while assigning 100% to most potent agent:

Highly potent, those with activity ≥ 50 % of most active agent; Moderately potent, those with activity in the range 30 to 50 %; Less potent, those with activity in the range of 10 to 30 %; and Least potent, those with activity less than 1 %

Table 2: Iodine reducing potential of test flavonoids and phenolics

Test agent	Mass, µmole	IRP, µmole	r ± S.E.	b[a] ± S.E.
Quercetin	0.04 0.08 0.12 0.16	0.180±0.044 0.474±0.048 0.792±0.010 1.002±0.010	0.997±0.003	6.96 ± 0.27
Morin[b]	0.10 0.20 0.30	0.660±0.014 1.198±0.007 1.626±0.008	0.998±0.002	4.83 ± 0.18
Rutin	0.12 0.16 0.20 0.30	0.107±0.035 0.237±0.021 0.331±0.009 0.574±0. 053	0.998±0.002	2.55 ± 0.08
Diosmin[b]	0.20 0.30 0.40	0.487±0.022 0.657±0.009 0.820±0.004	0.999±0.001	1.67 ± 0.01
Daflon[b]	0.20 0.40 0.50	0.425±0.008 0.729±0.002 0.865±0.003	0.999±0.001	1.47 ± 0.02
Gallic acid	0.04 0.06 0.08 0.10	0.131±0.007 0.262±0.017 0.361±0.009 0.480±0.004	0.999±0.001	5.73 ± 0.13
Pyrogallol	0.05 0.10 0.20	0.162±0.005 0.365±0.009 0.744±0.012	0.999±0.001	3.87 ± 0.03
Resorcinol	0.10 0.20 0.40	0.181±0.011 0.452±0.032 1.067±0.045	0.999±0.001	2.97 ± 0.08
Hydroquinone	0.10 0.20 0.40 0.60	0.209±0.008 0.452±0.006 0.891±0.003 1.405±0.005	0.999±0.001	2.37 ± 0.05
Catechol	0.10 0.20 0.50	0.116±0.013 0.242±0.017 0.812±0.022	0.997±0.003	1.78± 0.08
Guaiacol	3.0 5.0 10.0	0.071±0.025 0.171±0.039 0.408±0.035	0.999±0.001	48.0 ± 0.4[c]
Phenol	5.0 10.0 20.0	0.210±0.003 0.302±0.007 0.569±0.018	0.996±0.005	24.3 ± 1.2[c]

The values are mean ± S.E. of 3 observations each; [a] b reflects iodine reducing activity (IRA), unit, µmole iodine reduced µmole^{-1}, [b] Added 1 mL 0.6 % acetic acid to control back-ground color (morin) or neutralize alkali solvent (diosmin and daflon); [c] nmoles iodine reduced µmole^{-1} test agent

Table 3: Iodine reducing potential of thiourea, thioglycolic acid and ascorbic acid

Test agent	Mass, µmole	IRP, µmole	r ± S.E.	b[a] ± S.E.
Thiourea	0.02 0.06 0.10 0.20	0.096±0.010 0.223±0.002 0.353±0.008 0.658±0.005	0.999±0.001	3.12 ± 0.02
Thioglycollic acid	0.20 0.40 0.60 0.80	0.117±0.007 0.343±0.006 0.560±0.005 0.734±0.014	0.998±0.002	1.03 ± 0.03
Ascorbic acid	0.08 0.16 0.24 0.32	0.080±0.025 0.225±0.014 0.387±0.004 0.562±0.004	0.999±0.001	2.01 ± 0.05

[a] b is an estimate of iodine reducing activity, µmole iodine reduced µmole^{-1} test agent; the values are mean ± S.E. of 3 observations each.

As per this protocol, quercetin is most active of all (100) followed by gallic acid (82), morin (69), and pyrogallol (56). Moderately potent antioxidants include thiourea (45) followed by resorcinol (43), rutin (37), and hydroquinone (34). Ascorbic acid (29), catechol (26), diosmin (24), daflon (21) and thioglycollic acid (15) appear less potent.

Phenol and guaiacol have shown least IRA, respectively, as 0.35 and 0.69 % of quercetin. Ascorbic acid, an important antioxidant, is most frequently standardized by its ability to cause quantitative decolorization of iodine solutions. The present work demonstrated it to be comparatively less potent compared to flavonoid and phenolics in general. The IRA value of 2.01 is equivalent to its theoretical stoichiometric ratio whereby each micromole of ascorbic acid is equivalent to 2 micromoles of iodine as per standard redox titrimetric assay[8].

Ability of iodimetric assay to respond to sulfur-containing antioxidants is a special feature. This is advantageous compared to failure of one of the most commonly employed antioxidant assays such as FRAP to respond to thio compounds[18-20]. In the present assay, thiourea has shown 3 times more IRA than TGA (Table 3).

Structure activity relationship in flavonoids and phenolics: It is interesting to note that quercetin is about 3 times more potent than its rutinoside analog rutin. This suggests importance of free –OH at carbon-3 of heterocyclic ring-C of the flavonoid[21] which is bound by rutinose in rutin. Quercetin has been generally observed to be more potent than rutin as antioxidant. High activity of quercetin and morin, both aglycones, compared to diosmin, daflon (mixture of 90 % diosmin and 10% hespeidin) and rutin, all being glycosides, may suggest sugar rutinose is interfering with IRA. Correlating number of free hydroxyl functions in test flavonoids (diosmin and daflon, 3 each; rutin, 4 and morin and quercetin, 5 each) with their individual IRA values has revealed good correlation (r = 0.92, b= 2.16), and the correlation was still better when number of free hydroxyl groups were matched to their mean IRA values (r=0.95, b=2.16). The correlation analysis between number of free hydroxyl functions in phenolics including monohydroxy benzenes (phenol, guaiacol), dihydroxy benzenes (catechol, resorcinol and hydroquinone) and trihydroxy benzenes (gallic acid and pyrogallol) with their mean IRA respectively as 0.036, 2.37 and 4.8 µmole iodine reduced µmole^{-1} has revealed perfect correlation (r =0.9999, b= 2.382). Extra methoxy function in guaiacol than in phenol is presumably responsible for its increased activity which is nearly twice that of phenol. Gallic acid has extra –COOH than pyrogallol and shows about 1.5 times more IRA. A comparison of three dihydroxy benzene phenols (catechol, resorcinol and hydroquinone) with potency order resorcinol (1.7X) > hydroquinone (1.3 X) >catechol (X) suggests orientation and neighborhood of hydroxyl functions on aromatic ring is contributing to IRA of test agents. Chemically the flavonoids are 2-phenylbenzopyrans composed of two aromatic rings A and B with interposed heterocyclic ring C as bezopyran[21]. Structurally phenols are residues of test flavonoids: resorcinol (as ring A) is a common residue with all, with additional residue as ring B includes resorcinol (morin), catechol (quercetin and rutin) and guaiacol (diosmin and hesperidin). The contribution of these phenolic residues in particular resorcinol and catechol in their IRA is not ruled out. Interestingly resorcinol is more active than diosmin and daflon, presumably because in these flavonoids one hydroxyl function in resorcinol moiety (ring A) at C-7 is blocked

by rutinose such that both diosmin and hesperidin are 7-rutinoside forms of, respectively, diosmetin and hesperitin aglycones.

Effect of dilute acid on effect of test agents

Table 4: Iodine reducing potential as affected by presence of acidification

Activity profile category	Test agent, µnole	Iodine reducing activity[a]		Per cent change from non-acid values
		Acid +	Acid -	
Activity unaffected by acid[b]	Hydroquinone, 0.2	2.26± 0.05	2.30±0.02	1.7 ↓
	Ascorbic acid,0.2	1.28±0.06	1.16±0.05	10.3 ↑
Activity fails to occur in acid[c]	Catechol, 0.2	No response	1.54±0.04	100 ↓
	Phenol, 10	No response	60.3±1.2[d]	100 ↓
	Guaiacol, 5	No response	37. 8±5.5[d]	100 ↓
	Gallic acid. 0.1	No response	5.15±0.46	100 ↓
Activity increased in acid	Thioglycollic acid, 0.5	1.96 ±0.02	1.28±0.01	53 ↑
Activity in acid is markedly reduced	Rutin,0.2	0.50 ±0.02	1.81 ±0.23	72.4↓
	Morin,0.2	2.12 ±0.11	5.96 ±0.0.06	64.4↓
	Diosmin,0.3	0.38 ±0.02	1.04 ±0.05	63.5 ↓
	Daflon , 0.3	0.44 ±0.02	0.93 ±0.05	52.7 ↓
	Quercetin,0.1	3.21 ±0.12	5.72 ±0.24	43.9↓
	Thiourea,0.1	2.79 ±0.29	4.75 ±0.14	41.3↓
	Resorcinol, 0.2	2.49±0.11	4.19±0.10	40.6↓
	Pyrogallol, 0.1	2.77 ±0.07	4.26 ±0.10	35↓

The values are mean ± S.E. of 5 observations each; [a]Activity, µmole or nmole[d] iodine reduced µmole^{-1} test agent; [b] the mean values in acid and non-acid are not different ($p>0.1$, n=5 each); [c] mean absorbance in acid is similar to untreated standard ($p>0.1$, n=5 each)

Iodimetry is known to be affected by pH of the solution; alkaline hydroxide favors conversion of iodine into iodide and acid enhances potential of some reducing agents and, with respect to iodide favors its rapid oxidation to iodine[3]. In view of these observations, each test agent that responded positively in absence of acid was tested simultaneously in absence and presence of overall 1 % HCl for its IRA. As evident (Table 4), four types of responses have been recorded. The acid has failed to modify response to hydroquinone and ascorbic acid; the mean absorbance in absence of acid was decreased to the same extent as in presence of acid, about 23 and 12 per cent for respective antioxidants ($p>0.1$, n=5 each) and their IRA remained unaffected (Table 4). On the other hand, the acid has completely blocked response to catechol, phenol, guaiacol and gallic acid; the mean absorbance in presence of acid deviated from standard by 0 to 1.8 % ($p>0.1$, n=5 each) whereas in absence of acid, per cent decrease in mean absorbance with respect to the corresponding standard was recorded as 10 (guaiacol), 15 (catechol), 26 (gallic acid) and 30 (phenol) ($p<0.01$, n= 5 each) reflected by their corresponding IRA values (Table 4). IRA for thioglycollic acid has shown marked increase in presence of acid; the mean absorbance in acid decreased by 49 % from standard while in absence of acid, it decreased by only 32 % ($p<0.01$, n=5 each) showing over 50% increase in IRA in acid (Table 4). Acid has markedly reduced IRA of flavonoids, thiourea, pyrogallol and resorcinol. The decrease in mean absorbance in acid was in the range of about 5 to 25 per cent compared to ca. 15 to 60 % observed in absence of acid ($p<0.01$, n=5 each). As evident (Table 4), the order of influence in decreasing IRA amongst test agents, from greater to lesser, has been as under:

rutin>morin ≥ diosmin>daflon >quercetin> thiourea ≥ resorcinol > pyrogallol

Effect of water soluble organic solvents: Methanol has enhanced absorbance of iodine; an increase in methanol from 0 to 20, and 90 per cent in 5 mL volume has affected linear increase in iodine absorbance (r=0.99) using iodine mass as 1 µmole(b ±S.E., 0.0038± 0.0003) or 1.5 µmole (b ±S.E., 0.0058± 0.0005). The regression estimate of iodine over 0.5 through 2.0 µmole iodine in 100 % methanol has been, 0.47 ± 0.02 (r ±S.E., 0.997± 0.003), nearly 3 times more than the value obtained in water over the same range at 430 nm (b ±S.E., 0.16± 0.01; r ±S.E., 0.995± 0.004).

The regression estimates at 2 mL methanol with morin, quercetin and rutin were respectively found to be (test ranges within parenthesis) as 5.10±0.37 (0.1 to 0.3 µmole), 7.65 ±0.11 (0.04 to 0.10 µmole) and 1.26 ±0.06 (0.12 to 0.30 µmole) compared to regression estimates obtained with 1 mL methanol (Table 2.). This showed increase in methanol from 1 to 2 mL has increased regression estimate of morin and quercetin by about 6 and 10 per cent while decreased that of rutin by about 51%. Compared to methanol, ethanol and acetone have been found to enhance absorbance of iodine to greater extents in that order. At 1 mL each with 2 µmole iodine; the solvents have increased absorbance respectively by 15.4, 25.7 and 49.8 per cent compared to mean absorbance in water as 0.253 ±0.003 showing correspondingly mean absorbance values as , 0.292±0.001 , 0.318±0.003 and 0.379±0.004 ($p<0.01$, n=3 each).

Sugars, mannitol and carboxylic acids: Glucose, sucrose and mannitol (50 and 100 µmole each) and organic acids containing carboxylic acid moieties such as citric acid, oxalic acid and tartaric acid (10 and 25 µmole each) have failed to affect absorbance of 2 µmole iodine in absence or presence of 0.5 mL of 10 % HCl ($p>0.1$, n=3 each).

Hydrogen peroxide and DMSO: The mean absorbance with DMSO (10 µmole with 2 µmole of iodine) has remained unaffected in absence or presence of acid ($p>0.1$, n=5 each). With hydrogen peroxide at 10 µmole using 2 µmole of iodine, the absorbance values have increased by about 13 and 41 % in absence and presence of acid ($p<0.01$, n=5 each). DMSO has been considered a good solvent to solubilize iodine as tri-iodide for estimating amylose[17] and hydrogen peroxide as oxidizing agent tends to reconvert any iodide to iodine[16].

Conclusions

An iodimetric assay has been standardized to evaluate antioxidants for their relative antioxidant potency based on their iodine reducing activity (IRA). The assay has shown ability to demonstrate activity in flavonoids, phenolics, ascorbic acid and sulfur containing reducing agents. A standard parameter has been employed to compare relative potencies. The parameter is obtained as a regression coefficient while employing linear concentration range of test antioxidants with unit, µmole iodine reduced $\mu mole^{-1}$ test agent. The presence of acid has varyingly affected the response of test agents. The assay may be employed as a general assay as a part of battery of tests for screening potential antioxidants for their further evaluations using more advanced antioxidant assay systems,

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