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Assaying antioxidants for nitrous acid scavenging activity with four assay systems: a comparative analysis

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Abstract: Four different assay systems for determination of nitrous acid have been optimized to evaluate a group of potential antioxidants for their nitrous acid scavenging activity (NASA). The methods show linear ranges for detection within 100 nmole nitrite with relative sensitivity order based on their regression estimates (mean values within parenthesis): iron (II) oxidation (38.1) > diazotization (8.9) > iodometric (6.5) > ferrocyanide (5.55). Relative response order of the assays to test antioxidants, based on per cent test agents responding to the given assay, has been (approximate values within parenthesis): ferrocyanide (95) >diazotization (68) > iron (II) oxidation (53)> iodometric (42). Highly active agents with potency calculated as mean NASA (nmoles nitrite scavenged per µmole test agent) by different methods (the mean values within parenthesis) and their relative order has been found as: TGA (413) > ascorbic acid (56.4) > quercetin (39.3)> gallic acid (31.9)> resorcinol (26)> morin (24.8)> curcumin (23.7)> daflon (22.6)> diosmin (21.4). Weak to moderately active agents included hydroquinone (14.2) ≥ thiourea (14) > rutin (13.7) > catechol (5.8) > guaiacol (3.3)>phenol (1.8)>oxalic acid (0.6). DMSO (0.11), citric (0.09) and tartaric acids (0.15) have shown negligible activity those too detectable with only ferrocyanide method while other methods failed to detect using 50 µmole mass each. Solvents viz. ethanol, methanol, acetone and dilute sodium hydroxide have shown characteristic affects in one or more test assay systems. The study provides opportunity to screen test antioxidants for their NASA using a variety of assay protocols.

Key words: Nitrous acid scavengers, Assays, Flavonoids, Phenolics, Ascorbic acid, Thiourea, TGA, Curcumin.

Introduction

Nitric oxide (NO) is an established bio-active molecule with implications in health and disease[1]. The half-life of NO is very brief, 1 to 30 seconds, necessitating requirement for sophisticated assay systems for its direct measurement. These facilities are beyond the scope of common laboratories. Therefore, NO is measured indirectly by measuring formation of nitrous acid (NA) from its stable metabolite nitrite. Monitoring of NA is mostly done by diazotization method[2]. This method has been extensively used to screen plant extracts and standard chemical agents for their NO scavenging activity[3-13]. The standard scavengers of NA have included curcumin[3, 4], gallic acid[8] and ascorbic acid[9-11]. The NASA in plant extracts and test agents has been most often linked to flavonoids including notably quercetin, rutin, morin and catechin [6-8, 10, 12, 14-16], phenolics[14, 15, 7], curcumin[3, 11, 12], ascorbic acid[12, 14], triterpinoids and essential oils[10], alkaloids and saponins[8], xanthones [13] and organic acids[4]. Comparative studies have often been limited in choice of test agents, and many known antioxidants remain un-evaluated for NASA. Besides, the activities have been expressed in non-standard terms

as per cent inhibition by test agent of standard response. There exists opportunity to look for development of additional methods, besides diazotization, to enhance confidence and scope for evaluation of the test agents for NASA. Three new photometric assay protocols for nitrous acid monitoring were mooted:(1) iodometric assay based on oxidation of acidified KI solution by nitrous acid to release iodine[17-18]; (2) ferric estimation by thiocyanate based on oxidation of acidified iron (II) to iron (III) by nitrous acid [17-18]; and (3) nitro-ferrocyanide method based on direct interaction of nitrous acid with acidified potassium ferrocyanide[19]. Diazotization method is an established method for monitoring nitrous acid concentrations. Ferrocyanide method has been advocated as an alternative method to diazotization for micro-determination of nitrite in water[19]. Other two methods based on oxidizing property of nitrous acid were required to be shown their suitability for the purpose. The present work was accordingly designed with the objectives to: (i) optimize new assay systems for suitability of determination of NASA in comparison to the standard diazotization method[12]; (ii) compare relative sensitivity of the test assays with respect to the ranges of nitrous acid determination and regression estimates; (iii) evaluate potential antioxidants belonging to different chemical categories including flavonoids, phenols, organic acids, thiourea, thioglycollic acid, DMSO and curcumin for NASA activity if any with different assay systems; and (iv) express NASA in standard terms, nmoles nitrous acid reduced per µmole of test agent.

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 assay samples developing turbidity or discoloration during the assay were centrifuged at 8000-9000 rpm for 5 minutes. This happened usually while using diosmin and daflon (all four assays), quercetin (all baring ferrocyanide assay) and curcumin (diazotization, ferrocyanide assays). 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 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 and daflon: 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[20] 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.

Curcumin: Working solution contained 1.0 µmole curcumin mL^{-1} methanol.

Ascorbic acid, phenol, resorcinol, hydroquinone, guaiacol, catechol and gallic acid solutions: The requisite dilutions were made in water from respective stock solutions made in water as 25 µmole mL^{-1}.

Citric acid, tartaric acid and oxalic acid solutions: Each chemical grade acid was dissolved in water appropriately to provide 50 µmole organic acid mL^1water.

Thioglycollic acid solution: Working solutions were made by dilution in water from stock solution containing 20 µmole thioglycollic acid mL^{-1}water.

DMSO & thiourea solutions: The working solutions were made appropriately by dilution from respective stocks as 100 µmoles mL^{-1} water.

Nitrite solution: Stock solution was prepared as 6.9 % (w/v) $NaNO_2$ in water (containing 0.1 % chloroform as preservative) providing 100 µmole nitrite mL^{-1}. Working solutions of 50, 100 & 200 nmoles mL^{-1} water were made freshly by dilution with water as per need.

Sulfanilamide solution: The solution contained sulfanilamide 250 µg mL^{-1} 1% (v/v) HCl in water.

NEDA solution: The solution contained N-1-(naphthyl) ethylenediamine dihydrochloride (NEDA) as 500 µg mL^{-1} 1% (v/v) HCl in water.

HCl solutions: Dilute and strong solutions were made respectively as 10 and 50 % (v/v) HCl in water.

Potassium iodide solution: The solution contained 5% (w/v) KI in 0.001M NaOH in water.

Sodium bicarbonate solution: The solution contained 2.1 % (w/v) sodium bicarbonate in water, providing 0.25 M bicarbonate. The working solution was made by dilution in water as 0.1 M sodium bicarbonate.

Iron solutions (II & III): Working solutions of ferrous ammonium sulfate (FAS-II) and ferric ammonium sulfate (FAS-III) were made by dilution in water from their respective stock solutions of 25 µmole iron mL^{-1} 0.001 M HCl.

Thiocyanate solution: 10 % (w/v) KSCN in water.

Potassium ferrocyanide solution: 1.5 % (w/v) potassium ferrocyanide was prepared in 1% (w/v) sodium carbonate solution in water. Working solution was made by dilution with water 1:1 at the time of use.

Standard iodine solution: It was prepared approximately by adding 1.4 g resublimed iodine crystals to 50 mL water containing 3.6 g of KI with a drop of dilute HCl solution. The mixture was diluted to 100 mL with water. The iodine solution was standardized against standard sodium thiosulfate solution (0.1 M) by titrimetry. The working solutions were made by dilution in water as 1 and 5 µmole iodine mL^{-1} solution.

Assay systems for NASA

The first common step in each of the test assay has been to incubate standard sodium nitrite solution with optimal dilute HCl to generate nitrous acid. For a given assay, linear range for detection of nitrite was established with respect to standardized conditions. For conducting assays, standard mass of nitrite was incubated with or without known mass of test agent. Unless otherwise indicated the test agent was always added before addition of acid to nitrite solution. The samples were matched for volume of any diluent used for test agent. Calibration curve assays for standard within linear response range of concentrations of nitrite were taken simultaneously under matching conditions. The mass of nitrous acid generated and hence reduced by presence of test agent was calculated. Each test agent was assessed for its nitrous acid reduction ability by the four test procedures: (i) diazotization of sulfanilamide; (ii) iodine releasing potential of nitrous acid from potassium iodide; (iii) conversion of ferrous ions into ferric ions, and (iv) scavenging of nitrous acid by ferrocyanide. The solvents used for assaying test agents have included 1 mL 0.1 M NaOH for diosmin and daflon, 1 mL methanol for quercetin, rutin, morin and curcumin or water for rest test agents.

Method 1: Nitrous acid assay by diazotization method

The assay procedure[12] has been used with modification to match the experimental conditions. For linearity testing, 1 mL water containing nitrite 0, 10 through 100 nmoles was added 1 mL solvent and 0.5 mL dilute HCl. The mixture was allowed standing for 5 minutes to generate nitrous acid. The samples were then added 0.4 mL sulfanilamide solution, allowing diazotization for 1-2 minutes. Thereafter, the samples were added 0.4 mL NEDA solution as coupling agent and volume made 5 mL each with 1.7 mL water. The samples were read for color development at 540 nm at about 1 hour following acidification. For assaying a test agent, an appropriate mass of test agent was incubated with or without standard mass of nitrite (60 nmoles) followed by acidification. The calibration standard was run using 20, 40 and 60 nmoles nitrite with matching volume of solvent.

Method 2: Nitrous acid assay by ferrous oxidation method

A new optimized assay protocol was developed for this purpose. For linearity studies 1 mL water containing 0, 5 through 40 nmoles nitrite was added 1 mL FAS-II solution (1 µmole) and 1.5 mL water. The samples were added 0.5 mL of dilute HCl and incubated at room temperature for 10 minutes, and then added 1 mL KSCN solution. The color developed was monitored at about 60 minutes at 480 nm. The assay was optimized for time of incubation over 30 through 120 minutes for color development following acidification. A linearity setting for iron (III) was conducted simultaneously using FAS-III over the range 0, 0.1 through 0.5

µmole under matched conditions. This enabled to estimate oxidizing potential of nitrous acid in converting iron (II) into iron (III) under matching conditions. Test agent was added to standard mass of nitrite (30 nmoles) before acidification, and calibration standard was run using 10, 20 and 30 nmoles nitrite with matching volume of solvent. The order of addition of reagents was slightly modified for assaying flavonoids prepared in dilute hydroxide. Standard nitrite was added following acidification: test sample or diluent→ acid → nitrite. This was necessitated because alkali otherwise increased oxidation potential of nitrous acid over 6-folds.

Method 3: Nitrous acid assay by iodometry

A new optimized assay protocol was developed for this purpose. For linearity setting, 2 mL water containing nitrite as 0, 20 through 120 nmoles was added 0.5 mL sodium bicarbonate solution, 1 mL of KI solution, and 1 mL water. The samples were mixed and added 0.5 mL dilute HCl solution; allowed standing at room temperature and read at 430 nm about 1 hour following acidification. The assay was optimized for acid requirements using 0.5 through 2 mL dilute acid and time for incubation period over 0.5 through 4 hour using appropriate mass of nitrite. A linearity testing with standard iodine solution was made over the range 0, 1 through 5 µmoles iodine under the test conditions. This enabled estimation of oxidizing potential of nitrous acid for converting iodide into iodine. For assaying test agent, appropriate mass of test agent, added before acidification, was incubated with standard mass of nitrite (100 nmoles) and matched for solvent. The calibration for nitrite run simultaneously used nitrite as 30, 50 and 100 nmoles nitrite with matching volume of solvent.

Method 4: Nitrous acid assay by ferrocyanide method

The assay developed for nitrite determination[19] has been adopted for this work. For linearity setting, 1 mL water containing 0, 20 through 100 nmoles nitrite was added 2 mL water. Each sample was added 0.8 mL potassium ferrocyanide solution and 1.2 mL strong acid solution. The samples were allowed standing at room temperature for 60 to 90 minutes and read at 430 nm. For assaying test agents, the agent in appropriate mass was added to standard mass of nitrite (100 nmoles) before acidification, and calibration standard was run using 40, 60 and 100 nmoles nitrite with matching volume of solvent.

Test agents

Test agents have included flavonoids (diosmin, daflon, quercetin, rutin and morin), phenols (resorcinol, catechol, guaiacol, hydroquinone, phenol), organic acids (oxalic acid, gallic acid, ascorbic acid, citric acid, tartaric acid), sulfur containing agents (thiourea, TGA), DMSO and curcumin. Appropriate masse of a test agent for a given assay was chosen from trial experiments to get absorbance values within the linear range of the assay. Effects of solvents viz., ethanol, methanol, acetone and sodium hydroxide on the assays were also conducted to find their interference if any with the assay.

Calculations

The data were subjected to routine statistical analysis. As a rule, potential test agents were analyzed for their nitrous acid scavenging activity (NASA) employing a standard unit, nmoles nitrous acid scavenged per µmole of test agent, that would help comparisons between different test agents assayed by different assays. The parameter was determined by using the following formula:

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

Where, T is absorbance due to standard mass of nitrite in presence of test substance; S is absorbance due to standard mass of nitrite in absence of test substance; C is mass of nitrite used in nmoles, and M is mass of test substance used in µmole(s)

Results and Discussion

Nitrous acid monitoring systems

Two steps are required for evaluation of test agents for NASA under test conditions: (i) to generate nitrous acid (NA) and (ii) to monitor the mass of generated NA. The main source of NA is nitrite treated with dilute HCl or using a NO- donor such as SNP[5, 6, 8, 9, 11] which forms stable nitrite on standing. NA is most

commonly monitored by photometry employing most commonly diazotization and coupling reaction based on Griess reaction[2-13]. This constituted a standard assay system for the present study. The choice for taking additional assay systems have been based on known oxidizing and coupling properties of NA with reagents like KI, iron (II)[17,18] and ferrocyanide[19]. This necessitated optimizing and evaluating the three new assay systems in relation to standard diazotization method[12]. The diazotization technique involves generation of nitrous acid to affect diazotization of an aromatic amine such as sulfanilic acid[5,7,13] or most often sulfanilamide[2,8-12], and coupling of diazotized aromatic amine with another aromatic amine called coupling agent such as naphthaylamine[7] or most often NEDA[2,8-12] to form an azo-dye monitored by photometry. Iodometric and ferrous oxidation methods are based on oxidation property of nitrous acid: KI in acidic solution is oxidized to form iodine which is detected directly by photometry, and ferrous ions are oxidized to ferric ions which are detected selectively by thiocyanate reagent for photometric determination. Ferrocyanide ions appear to interact with nitrous acid directly rather than affecting its conversion to ferricyanide[19].

Comparative sensitivity range of test assays

The diazotization assay in the present set up has shown linearity over 10 through 100 nmoles of nitrite (b=8.906) with 10 through 60 nmoles of nitrite exhibiting steepest regression (b=10.0) (Table 1). The iron (II) oxidation method was found to be most sensitive, ca. 4.4 folds more than that of diazotization method, with linear range 5 through 40 nmoles nitrite (b = 38.1) with steepest regression (b=43.7) over 20 through 30 nmoles (Table 2). Time optimization study using 5, 10 and 30 nmoles nitrite has shown a definite increase in oxidation of iron (II) providing regression estimates at 30, 60 and 120 minutes, respectively, as 25.3 ± 1.1, 31.3±1.1 and 36.9 ±1.0. About an hour was considered optimal for the conduct of present study. The iodometric (Table 3) and ferrocyanide methods (Table 4) have been somewhat less sensitive as compared to the diazotization method. The regression value with iodometric assay (b=6.54) has remained nearly 73 % of diazotization method over 20 through 120 nmoles nitrite with steepest regression (b=6.97) over 40 through 100 nmoles nitrite. Acid optimization using 0.5 through 2 mL 10 % HCl with 100 nmoles nitrite did not reveal any significant difference, the mean absorbance values have ranged from 0.589 ± 0.004 to 0.592±0.008 ($p>0.1$, n=6 each). Time optimization studies while using 10 through 200 nmole nitrite with monitoring over 30 through 240 minutes have provided estimated regression coefficients at 30, 60, 120 and 240 minutes of incubation (r=0.999 each) correspondingly as 4.71 ± 1.05, 5.54 ±0.12, 5.78 ±0.13 and 5.76 ±0.13 showing no difference in estimations over the 4 hour observation period ($p>0.1$). Monitoring at about an hour was accordingly considered optimal for the present study. The assay based on ferrocyanide reagent has shown least sensitivity. Its regression estimate (b=5.55) remaining about 62 % of that obtained with diazotization method with linearity registered over 20 through 100 nmoles of nitrite. The assay could be monitored over 30 through 90 minutes with peak absorbance at 90 minutes[19]. Based on regression estimate comparisons, the mean relative order of sensitivity of test methods has been found approximately as: iron (II) oxidation method (7.4 X) > diazotization method (1.7 X) > iodometric method (1.2 X)> ferrocyanide method (1X) including mean values of regression estimate over steepest linear ranges of nitrite.

Table 1: Linear relation between nitrous acid and diazotization of sulfanilamide

Nitrite mass, µmole	Absorbance values
0.01	0.106 ±0.002
0.02	0.209 ±0.002
0.04	0.385 ±0.002
0.06	0.613±0.009
0.08	0.748 ±0.010
0.10	0.898 ±0.002
Statistical analysis	
r ±S.E.	0.997 ±0.002
b ±S.E.	8.9 ±0.3

The values are mean ± S.E. of five observations each; the linearity is perfect with peak regression over 10 through 60 µmoles nitrite (r ±S.E. = 0.998 ±0.002; b ±S.E. = 10.0 ± 0.3)

Table 2: Linear relation between nitrous acid and iron (II) oxidation

Nitrite mass, µmole	Absorbance values
0.005	0.048 ±0.002
0.01	0.139 ±0.006
0.02	0.462 ±0.010
0.025	0.674±0.012
0.03	0.899 ±0.010
0.04	1.374 ±0.049
Statistical analysis	
r ±S.E.	0.991 ±0.007
b ±S.E.	38.1 ±2.1

The values are mean ± S.E. of five observations each; the linearity is perfect with peak regression over 20 through 30 µmoles nitrite (r ±S.E. = 0.999 ±0.001; b ±S.E. = 43.7± 0.4)

Table 3: Linear relation between nitrous acid and iodine release from potassium iodide

Nitrite mass, µmole	Absorbance values
0.02	0.065±0.003
0.04	0.163 ±0.001
0.06	0.299 ±0.002
0.08	0.453±0.003
0.10	0.576 ±0.009
0.12	0.720 ±0.004
Statistical analysis	
r ±S.E.	0.999 ±0.001
b ±S.E.	6.5 ±0.1

The values are mean ± S.E. of five observations each; the linearity is perfect with peak regression over 40 through 100 µmoles nitrite (r ±S.E. = 0.999 ±0.001; b ±S.E. = 7.0 ± 0.1)

Table 4: Linear relation between nitrous acid and its interaction with potassium ferrocyanide

Nitrite mass, µmole	Absorbance values
0.02	0.071±0.002
0.04	0.189 ±0.002
0.06	0.299 ±0.004
0.08	0.401±0.002
0.10	0.520 ±0.008
Statistical analysis	
r ±S.E.	0.999 ±0.001
b ±S.E.	5.55 ±0.01

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

It was considered appropriate to evaluate nitrous acid for its oxidizing potential in two test methods: iron (II) and KI oxidations. Estimation of ferric iron in iron (II) oxidation method was made in terms of simultaneously run ferric standard over its linear range. Iron (III) over 0.1 through 0.5 µmoles showed regression estimate of 1.540 ± 0.0006 with perfect linearity (0.999 ± 0.001). The regression data revealed an estimated 25.4 ± 1.5 µmole iron (II) was oxidized per µmole of nitrous acid (0.99 ± 0.01, n = 5). A similar level of potential was noticed with respect to iodide oxidation. Each µmole of nitrous acid produced 22.4 ± 1.3 µmole of iodine (0.992 ± 0.004, n=5 each). Iodine over 1 through 5 µmoles showed regression estimate of 0.2300 ± 0.0042 with perfect linearity (0.999 ± 0.001). The two mean values exhibiting oxidizing potential of nitrous

acid in the two assay systems did not differ significantly (P>0.1, n=5 each) confirming consistency of response. As regards ferrocyanide assay, it has been already evidenced that the interaction between nitrous acid and ferrocyanide is direct one and unlikely to be an oxidation process [19].

Nitrous acid scavenging potential of test agents

The results obtained with four standardized methods are tabulated (Table 5). As evident, ferrocyanide method has responded to nearly all test agents (per cent response within parenthesis) (ca. 94) followed by diazotization method (ca.88), iron (II) oxidation method (ca.69) and iodometric method (ca. 56). Agents capable of exhibiting NASA in all test systems include diosmin, daflon, curcumin, thioglycollic acid, ascorbic acid and resorcinol. Mean NASA, calculated as mean of means obtained with responding assays reveals their relative potency order (within parenthesis) as TGA, 413 (17.4 X), ascorbic acid, 56.4 (2.4X), curcumin 23.7, (1X), daflon, 22.6(0.95 X), diosmin , 21.4(0.9 X) and resorcinol, 26 (1.1 X). Each assay as a rule exhibited a different value for a given test agent indicating sensitivity of test agents are varying with type of assay. NASA was shown in three assay protocols with respect to quercetin, 39.3 (1.7 X); gallic acid, 31.9 (1.3 X); oxalic acid,0.56 (0.024X); catechol, 5.8 (0.245 X). NASA for morin, 24.8 (1.05X); hydroquinone, 14.2 (0.6X); guaiachol,3.3 (0.14 X); thiourea and 14.0 (0.6X) have been seen in only two assay systems.

Table 5: Nitrous acid scavenging potential of some select anti-oxidants with four different assay systems

S.No.	Test agent	Nitrous acid scavenging activity, nmoles per µmole agent			
		Iron (II) oxidation assay	Diazotization assay	Iodometric assay	Ferrocyanide assay
1	Diosmin	11.6±1.0 (1)	14.7±0.6 (1)	41.3±1.3(1)	18.0±0.3 (1)
2	Daflon	11.0±0.3 (1)	11.2±0.5 (1)	45.3±2.0(1)	23.0±0.9 (1)
3	Quercetin	NRD (1)	18.1±1.6 (1)	19.8±1.1(1)	80.0±1.3 (1)
4	Rutin	NRD (1)	13.7±0.2(1)	NRD (1)	NRD (1)
5	Morin	7.6±0.3 (1)	NRD (1)	NRD (1)	42.0±2.0 (1)
6	Resorcinol	10.6±0.1 (2.5)	61.7±1.7 (0.5)	1.5±0.1(5)	30.3±0.5 (1)
7	Catechol	1.3±0.1 (2.5)	3.6±0.1 (5)	NRD (5)	12.6±0.2 (5)
8	Guaiacol	NRD (5)	3.1±0.2(5)	NRD (5)	3.4±0.3 (5)
9	Hydroquinone	NRD (5)	10.4±0.3 (2.5)	NRD (5)	18.0±0.1 (5)
10	Phenol	NRD (10)	NRD (10)	NRD (10)	1.8 ±0.2 (10)
11	Gallic acid	2.7±0.4(1)	31.8±0.6 (0.5)	NRD (1)	61.3±0.1 (1)
12	Oxalic acid	0.5±0.01 (50)	0.91±0.01 (50)	NRD (50)	0.27±0.05 (20)
13	Ascorbic acid	17.9±0.6(1)	96.4±0.8 (0.5)	62.3±0.2(1)	48.9±1.0 (1)
14	Citric acid	NRD (50)	NRD (50)	NRD (50)	0.09 ± 0.01 (50)
15	Tartaric acid	NRD (50)	NRD (50)	NRD (50)	0. 15 ± 0.01 (50)
16	DMSO	NRD (50)	NRD (50)	NRD (50)	0.15 ± 0.01 (50)
17	Thioglycollic acid	275±1.0 (0.1)	924±12 (0.04)	37.9±0.7(1)	416±1 (0.2)
18	Thiourea	NRD (2)	NRD (2)	15.1±0.7(1)	12.8±0.5 (2)
19	Curcumin	19.7±0.3 (1)	13.7±0.7 (1)	21.2±0.9(1)	40.1±2.0 (1)

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

However, NASA for rutin, 13.7 (0.58X); and phenol, 1.8 (0.08 X) were demonstrated only in one assay systems. NASA values for DMSO, citric and tartaric acids have been very low, demonstrated only by ferrocyanide assay with mean activity respectively as 0.11 ± 0.01, 0.09± 0.01 and 0.15 ±0.01 using very high mass 50 µmole each.

As apparent, TGA has ranked most active agent followed by ascorbic acid, quercetin and gallic acid in that order. Activity of curcumin has been somewhat comparable to those of morin, diosmin and daflon. Category wise, quercetin appears most active amongst flavonoids while activity of its rutinose derivative rutin

has been least, about 1/3rd of quercetin. Diosmin, daflon (mixture of 90 % diosmin and 10 % hesperidin) and morin have shown almost equipotent comparable activity. Resorcinol has been most active amongst phenols with gallic acid, a phenolic acid, showing better response than due to resorcinol. Hydroquinone, catechol, guaiacol and phenol have been comparatively weaker in that order. The potential of HQ catechol, guaiacol and phenol have been about 50, 22, 13 and 7 per cent of resorcinol activity. These observations may indicate that resorcinol residue present in flavonoids may be contributing significantly to the overall NASA of flavonoids. Poor response of rutin compared to its aglycone flavonoid quercetin would suggest importance of 3-OH function in the flavonoid as important for nitrous acid scavenging potential.

There have been limited reports of comparative evaluation of test agents for NASA. The activity is more often expressed in non-standard units as per cent inhibition by test agent as compared to the effect of standard. Strong activity in plant extracts has often being linked to their flavonoid and phenolic contents[7,10,14-16] with activity presumably due to their phenolic and phenolic hydroxyl groups[15] including those in curcuminoids[3]. Ascorbic acid has been most often employed as standard, and is known to be a potent scavenger of nitroso-compounds[13]. Rutin, curcumin, morin and ascorbic acid have been found to be active scavengers of super oxide anion from hydroxylamine, decreasing generation of nitrite with relative potency order: rutin>curcumin>morin> ascorbic acid and diosmin failing to show any such activity[12].

Table 6: Effect[a] of water soluble solvents on nitrous acid activity

Assay system	Ethanol	Methanol	Acetone	NaOH, 0.1 M
Iodometric	37.4 % ↑	27.4 % ↑	22 through 72 % ↓[b] 1275 ± 47[c]	10.6 % ↑
Iron (II) oxidation assay	12.5 % ↓ 3.8 ± 0.8 [c]	12.0 % ↓ 3.7 ± 0.7 [c]	3.5 % ↑	59.3 % ↑[d]
Ferrocyanide assay	21.5 % ↓ 22.5 ± 2.1 [c]	21.0 % ↓ 21.1 ± 0.7 [c]	31.6 % ↓ 31.7 ± 1.9 [c]	NSC
Diazotization assay	43.3 % ↓ 26.0 ± 0.4 [c]	50.4% ↓ 30.2 ± 0.4 [c]	26.8 % ↓ 16.6 ± 0.8 [c]	NSC

The values are mean ± S.E. of 5 observations each; [a] The effect as per cent increase or decrease in absorbance with 1 mL solvent using standard mass of nitrite as per assay; [b] 10, 20 and 50 µL acetone caused per cent reduction in absorbance and nmole reduction in nitrite as 22.1, 32.0 & 72.3 (0.998 ± 0.002; 1275 ±47 nmole nitrite scavenged per mL acetone);[c] Activity expressed as nmole nitrite scavenged per mL test solvent; [d] NaOH 20 through 100 µmole caused linear enhancement in iron (II) oxidation (r=0.996) with 20 µmole causing over 6-folds increase in absorbance by standard nitrite. NSC, no significant change observed with test solvent.

Solvents have affected the absorbance values characteristically (Table 6). Sodium hydroxide 100 µmole did not affect the mean absorbance by standard nitrite with either diazotization method or ferrocyanide method ($p>0.1$) while it caused increase in absorbance by 10.6 % in iodometric assay ($p<0.01$) and by 59.3 % in iron (II) oxidation method. As little as 20 µmole sodium hydroxide was found to increase iron (II) oxidation over 6-folds ($p<0.01$). The absorbance levels over 20 through 150 µmole of NaOH increased oxidation of iron(II) by nitrite (6.24 ± 0.04) folds higher than in absence of NaOH (0.269 ±0.005). Organic solvents behaved differently with test assay systems. Ethanol enhanced absorbance with iodometric assay by 37.4 % ($p<0.01$) while it decreased absorbance values in other assays, showing NASA in units nmoles scavenged per mL solvent, as 26 ±0.4 (diazotization method), 22.5 ±2.1 (ferrocyanide method) and 3.8 ±0.8 (iron (II) oxidation method). Methanol has shown activity pattern matching that of ethanol. There was 27.4 % increase in mean absorbance with iodometric assay ($p<0.01$), and reduction in absorbance by 50.4, 21 and 12 per cent correspondingly with diazotization, ferrocyanide and iron (II) oxidation methods with NASA values

comparable to those obtained with ethanol (Table 6). Compared to ethanol and methanol, acetone has shown a slight increase in absorbance, 3.5 % with iron (II) oxidation method and reduction in all assays. Reduction has been highest with iodometric assay exhibiting a linear reduction to the extent of 1275 ± 47 nmole NASA per mL. Comparatively lower NASA values for acetone were observed with ferrocyanide and diazotization assays respectively as 32 ± 2 and 17±1. No attempt has been made to use distilled acetone. The label indicated presence of permanganate reducing agents in acetone to the extent of 0.002 %. These observations indicate that solvent must be matched while conducting assays.

Conclusions

The work has introduced new optimized assay systems to monitor nitrous acid scavenging potential of test agents. The work offers opportunity to employ choice method(s) as alternative to existing one for a given setting in case of any observed incompatibility with the test agents and reagents. The comparative data in the study has been based on standard parameter giving actual NASA in standard units, nmoles nitrite scavenged per micromole of test agent. This obviates use of non-standard evaluation parameters based on per cent inhibition of standard activity by the test agent. The study has revealed that the given test assays are capable of demonstrating nitrous acid scavenging potential of various potential agents including flavonoids, phenolics, organic acids (ascorbic acid, oxalic acid), sulfur-containing compounds (thiourea, thioglycollic acid) and curcumin. The work has been found quite handy in screening extracts from some local medicinal plants for NASA (to be published).

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