Optimized In Vitro Methods For Generating Superoxide Anion From Hydroxylamine, And Their Response To Flavonoids, Curcumin And Ascorbic Acid

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Abstract: Six different methods have been optimized for *in vitro* generation of super-oxide radical from auto-oxidation of hydroxylamine in alkaline medium. The methods are listed as follows with decreasing order of sensitivity, based on linear range (within parenthesis) of responses induced by the hydroxylamine in alkaline solution such as nitrite formation (0.01-0.15 µmoles HA), MTT reduction (0.02 – 0.2 µmoles HA in water, 0.1 - 0.4 µmoles in acid-ethanol), ferricyanide reduction (0.5– 2.0 µmoles HA), sodium aquoferrocyanide formation from reduction of SNP (2-20 µmoles HA), copper oxide formation as detected by phosphomolybedate reagent (2-4 µmoles HA) and copper amine reduction (10-40 µmoles HA). The assay systems have been evaluated with respect to different flavonoids (i.e. diosmin, rutin, and morin), curcumin and ascorbic acid. The present study provides an opportunity to generate super oxide anion from hydroxylamine under different test conditions for screening test agents for scavenging super oxide anion.
Key words: Super-oxide anion, Hydroxylamine, Flavonoids, Curcumin, Ascorbic acid.

INTRODUCTION

Hydroxylamine is an unstable compound that undergoes auto-oxidation in alkaline medium to generate highly toxic super oxide anion. Hydroxylamine tends to show both reducing and oxidizing properties as it is capable of reacting in its hydroxyl form, H_2NOH (I), or its amine oxide form, H_3NO (II)[1]. Super oxide in aqueous solution is poorly reactive, acting as a moderately strong reducing agent[2]. Generation of a super oxide anion from hydroxylamine serves as a useful technique to screen anti-oxidants capable of scavenging super oxide[3, 4]. The common assay system employs reduction of nitro blue tetrazolium (NBT) by hydroxylamine[5], and test agents are identified by their ability to reduce reduction potential of super oxide from hydroxylamine. However, reduction of another tetrazolium dye, MTT, has been employed as a basis to screen anti-oxidants capable of scavenging super oxide generated from auto-oxidation of pyragallol[2]. Nitrous oxide and subsequent nitrite are important reaction products of autoxidation of hydroxylamine in an alkaline medium[6]. Nitrite is measurable by diazotization method using Griess reaction[7.] The objective of the present investigation has been to optimize different in *vitro* methods to generate super oxide anion from hydroxylamine, and to test their efficiency against selected test agents with known anti-oxidant potential including flavonoids, curcumin and ascorbic acid[8-11]

MATERIALS AND METHODS

The experiments were carried out at a room temperature of $19.6 \pm 0.7 \ ^0C$ (n = 100). The chemicals and the drugs used were, respectively, as reagent and pharmaceuticals grade obtained from reputed sources of India. Unless otherwise indicated, the working solutions of test reagents were made in double distilled water.

Reagents

Hydroxylamine HCl stock solution: The solution was prepared as 0.7 % (w/v) hydroxylamine hydrochloride (HA) in water providing 100 µmoles per mL water.

Nitrite stock solution: The solution was made from sodium nitrite as 1 µmole per mL water.

Sulfanilamide solution: Sulfanilamide was prepared as 0.025 % (w/v) in 1 % HCl in water

NEDA solution: The solution was prepared by dissolving N-naphthyl-ethylene-diamine dihydrochloride (NEDA) in 1 % HCl in water to provide 0.05% (w/v).

HCl solution: A 50 % (v/v) concentrated HCl in water.

Standard NaOH solutions: 4% (w/v) solution of sodium hydroxide in water (1 M).

MTT solution: MTT dissolved in methanol as 0.1 % (w/v).

Ferricyanide solution: 0.33 % (w/v) potassium ferricyanide in water (10 µmoles^{-1}ml).

Sodium carbonate solution: 0.4 M in water.

SNP solution: 0.3 % sodium nitroprusside (w/v) in water equivalent to 10 µmoles per mL.

Copper acetate solution: 2.0 % copper acetate monohydrate (w/v) in water equivalent to 100 µmoles copper per mL water.

Ammonia solution: 5 % (v/v) ammonia in water (stock 25% diluted 5-folds).

Oxalic acid solution: 0.1 M in water.

 Phosphomolybedate reagent: The reagent was prepared by dissolving 7 g molybdic acid, 1 g sodium tungstate and 4 g sodium hydroxide pellets in about 80 mL of water. The mixture is boiled for 20-30 minutes, cooled to room temperature, and added 27 mL of o-phosphoric acid (85%), and volume made to 100 mL. The solution was stored in a dark colored plastic container.

Acid-ethanol: Solvent for rutin prepared as 55 % (v/v) ethanol in 5 % (v/v) glacial acetic acid in water. Working solution is prepared as 10 % in water.

Acetic acid: 1 % (v/v) glacial acetic acid in water.

Dugs

Diosmin: Extracted from Venex-500 Tablets (Elder, India) using 0.1 M NaOH as extractant. Each tablet contained 500 mg of synthetic diosmin. The extract was approximated to contain 0.01% of labeled synthetic diosmin (0.01 %) in 0.1 M NaOH.

Rutin: Prepared as stock solution in acid-ethanol as 0.09 % (w/v). Working solution was made as 90 µg mL^{-1} in 10 % acid-ethanol.

Morin: Prepared as 0.1 % (w/v) in methanol.

Curcumin: Prepared as stock solution of 0.05 % (w/v) in methanol. Working solution was prepared as 50 µg mL^{-1} in 50% methanol.

Ascorbic acid: Prepared as stock solution of 20 µmoles per mL water. Working solutions was made in water by dilution.

Nitrite formation reduction method

Two milliliter water samples containing 0, 0.1 through 1.5 ml of hydroxylamine solution (0.1 µmole^{-1} mL water) were each added 1 mL of 0.1 M NaOH solution and allowed to stand 1 hour at room temperature to generate nitrite. Each sample is added 0.4 mL sulfanilamide solution followed by 1 mL HCl solution, allowed to

diazotize for 1-2 min, and then added 0.4 mL of NEDA solution and volume made 5.0 mL with water. The color is monitored at 540 nm after 40-50 minutes standing. Mass of nitrite formed per unit μmole of hydroxylamine is calculated.

Test samples are evaluated by incubating each sample with standard mass of hydroxylamine (1 mL of 0.1 μmole) in 2 mL water and 1 mL sodium hydroxide solution. The control sample contains matched volume of test substance in 3 mL volume containing 1 mL of alkali solution. Simultaneously run standard hydroxylamine solutions are matched with appropriate volume of the diluent used in making test substance. Thereafter, the procedure remains as above.

The mass of nitrite formed is estimated in terms of simultaneously run calibration curve for nitrite while using 0.2, 0.4 and 0.8 mL of 0.1 μmole nitrite per mL water with appropriate matched volume of diluent in 2 mL volume. The remainder procedure remains exactly as above. The mass of hydroxylamine antagonized per unit mass of test material is estimated.

MTT reduction method

The procedure has been run in two versions. In absence of acid-ethanol (Method-1), MTT solution 0.1 mL is added 3.4 mL water containing 0, 0.02 through 0.2 μmole hydroxylamine followed by addition of 0.5 mL of 0.1 M NaOH solution. The samples are read at 60 minutes at 535 nm. The test is applicable for agents soluble in water. In another version necessitating presence of acid-ethanol as diluent (Method-2), MTT solution 0.1 mL is added 0.4 mL 10% acid-ethanol followed by addition of 3 mL water containing 0, 0.1 through 0.4 μmole of hydroxylamine. Each sample is added 1.5 mL 0.1 M NaOH solution and color monitored at 535 nm at 60 minute. Range of concentrations of hydroxylamine has been chosen on the basis of observed linearity between the concentrations of hydroxylamine vis-à-vis increase in absorbance at 535 nm due to formation of formazan.

Sample tests are carried out while using MTT 0.1 mL and 0.12 or 0.20 μmole hydroxylamine in absence and presence of standard mass of test material. The reaction is initiated by addition of alkali, and color monitored as usual. Control samples contain MTT with matched volume of test material in absence of hydroxylamine.

Potassium ferricyanide reduction method

To prepare standard curve for potassium ferricyanide, 3.5 mL water samples containing 0,and 0.25 through 2.5 μmoles of potassium ferricyanide solution were added each 0.5 Ml 0.1 M NaOH solution. The samples are read at 410 nm after 1 hour incubation at room temperature. Linear response for hydroxylamine has been obtained by taking 1 mL of standard concentration of potassium ferricyanide (2.5 μmoles) with 0 and 0.5 through 2.0 μmoles of hydroxylamine in 2.5 mL water. Each sample is added 0.5 mL 0.1 M NaOH solution, and color intensity monitored at 410 nm following 1 hour standing at room temperature.

Evaluation of test samples was done by running simultaneously three tests. Test-1 included standard potassium ferricyanide (0, 0.5, 1.5 and 2.5 μmoles of potassium ferricyanide in 3.5 mL water). Test-2 included standard hydroxylamine amine (0 .5, 1.0 and 1.5 μmoles hydroxylamine) with standard concentration of potassium ferricyanide (2.5 μmoles) in 3.5 mL water. Test-3 included running standard concentrations of potassium ferricyanide in presence of standard concentration of test substance in absence and presence of standard concentration of hydroxylamine (1 or 1.5 μmoles of hydroxylamine). The samples were matched for any appropriate volume of diluent. The diluents included 10 % acid-ethanol (5.5 % ethanol in 0.5 % acetic acid) in case of rutin and 0.2 mL methanol in case of morin and curcumin. In case of diosmin prepared in 0.1M NaOH, each sample was initially added 0.2 mL of 1 % acetic acid and then matched volume of 0.1 M NaOH.

The reaction in each sample was initiated with addition of 0.5 mL 0.1 M NaOH solution, and the color monitored at 410 nm after 1 hour.

Effects of test substances in altering color of potassium ferricyanide directly or countering the reduction in color intensity by hydroxylamine were monitored and compared for any statistical significance.

SNP reactivity reduction method

Each sample of 2 mL volume in water containing 0 and 2 through 20 μmoles of hydroxylamine was added 0.5 mL of SNP solution (10 μmoles^{-1} mL) followed by 0.5 mL 0.4 M Na_2CO_3 solution. The color was monitored at 410 nm at 5 through 60 minute. The peak effect is observed at 5th minute and thereafter decreases @ about 1 % per minute but linearity remains throughout with b ranging from 0.039 at 5th minute to 0.014 at 60th minute.

Test material is taken in 1.5 mL water containing 5 µmoles of SNP and 0.2 mL 1% acetic acid in case the test material is other than rutin and quercetin. The samples are added 0 or 20 µmoles of hydroxylamine in 1 mL water. Standard contains 5, 10 and 20 µmoles of hydroxylamine with 5 µmoles of SNP and any diluent, if required, in 2.5 mL volume without any test material. Each sample is added 0.5 mL of carbonate solution, and color monitored at 410 nm following 15 or 30 minute incubation at room temperature.

Monitoring was done by recording increase in absorbance with increase in hydroxylamine, and alteration in absorbance in presence of test substance.

Copper oxide formation reduction method

Standard copper solution 10 µmoles in 0.5 mL water is added 1.2 mL water containing 0, 2 through 4 µmoles of hydroxylamine, and 0.3 mL of 1 M NaOH solution. The samples are well mixed and allowed standing about 2 minutes. Each sample is added 0.2 mL phosphomolybedate reagent, mixed up and allowed to stand 2 minute followed thereafter by addition of 0.5 mL of oxalic acid solution, and volume made 5 mL with water. The color is monitored at 540 nm at 20-40 minutes.

Linear response for copper was taken under given conditions using copper concentration range 2 through 6 µmoles of copper with standard mass of hydroxylamine (4 µmoles). Test agents were evaluated using standard mass of copper (10 µmoles) in absence and presence of standard mass of hydroxylamine (2 µmoles). The samples were matched for appropriate volumes of the diluents. Monitoring was done by recording increase in absorbance with increase in hydroxylamine, and alteration in absorbance in presence of test substance.

Copper amine reduction method

The reaction mixture contains 1 mL water providing 0, 10, 20, 30, 40 and 50 µmoles of hydroxylamine, 0.5 mL of standard copper solution (50 µmoles), 1 mL ammonia solution and 1.5 mL water, added in that order. The color developed is monitored at 30 to 60 minutes at 605 nm.

Standard for copper is simultaneously run by using 0, 5, 10, 20, 30 and 50 µmoles in 3 mL water and added 1 mL ammonia solution. The remaining protocol remains same.

Test is carried out by using standard mass of copper (50 µmoles) with known mass of test agent with and without standard mass of hydroxylamine (30 or 40 µmoles) containing matched volume of any diluent wherever required. The sample is made 3 mL each with water and then added 1 mL ammonia solution, and the color monitored as indicated.

Monitoring was done by recording decrease in absorbance with increase in hydroxylamine, and alteration in absorbance in presence of test substance.

Calculations

The mass of nitrite generated over linear range of hydroxylamine has been determined in terms of standard nitrite using calibration curve for nitrite run simultaneously:

Nitrite, nmoles = $(A_{st} - C)/ b$

Where A_{st} is absorbance of standard hydroxylamine sample and C and b are, respectively, the values of Y-intercept and regression estimate of linear curve for standard sodium nitrite over 10 through 80 nmoles.

In tests where the test substance produced effect comparable to the effect of hydroxylamine on test reagent, hydroxylamine-like activity in test substance (HLATS, µmoles HA equivalent per mg test substance) was determined by using the above formula wherein C & b parameters referred to the parameters of linear curve of hydroxylamine with respect to the test reagent.

Anti-oxidant potential of any test substance (AOATS) was measured as µmoles of hydroxylamine antagonized by each unit mass of test substance taken in mg. The values were determined by using the formula:

AOATS = $(A_{st} - A_{st+ts}) (C_{st}/M_{ts})$

wherein AOATS is anti-oxidant activity of test substance with units µmoles hydroxylamine antagonized^{-1}mg test substance; C_{st} is concentration of standard hydroxylamine used in µmoles; M_{ts} is mass of test substance used in mg; A_{st} and A_{st+ts} are respectively the absorbance values of standard hydroxylamine sample in absence and presence of test substance.

RESULTS AND DISCUSSION

Nitrite formation reduction method

Hydroxylamine is auto-oxidized to nitrite under test conditions using standard mass of alkali upon incubation at room temperature for about an hour. The conversion is linear over hydroxylamine concentration range of 10 through 150 nmoles with each µmole hydroxylamine forming an average of 668 nmoles of nitrite (Table 1). The observed regression estimate is nearly 67 % of the theoretical stoichiometric value wherein one µmole of nitrite is generated from one µmole of hydroxylamine:

$NH_2OH.HCl + 2\ NaOH + O_2 \quad NaNO_2 + 3H_2O + NaCl$
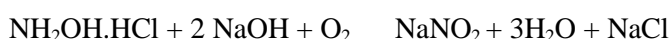
The mean pH with addition of standard alkali remained 11. 83 ± 0.01 and 11.90 ± 0.03 respectively in presence and absence of 0.4 mL 10 % acid-ethanol (P>0.1, n= 4 each). Nitrite over test range 10 through 80 nmoles showed perfect linearity (r= 0.9999, b= 0.0082, c= 0.0034). The various test substances have been screened for their ability to reduce formation of nitrite from standard mass of hydroxylamine. Control samples remained colorless in presence of test agents indicating no inherent nitrite like activity. Per cent reduction in mean absorbance values with test materials rutin, morin and curcumin (Table 2) have been, respectively estimated as 21.3, 8.2 and 19.9 (P<0.05, n= 5 each) compared to the value obtained in absence of test material while diosmin exhibited no effect. Ascorbic acid 1 µmole prevented formation of nitrite from hydroxylamine by 62 ± 4 per cent (Table 2; P <0.01) showing HA antagonizing activity equivalent to 175 ±4 nmoles per mg. At higher concentration, 5 µmoles, ascorbic acid completely prevented nitrite formation from 0.05 µmoles hydroxylamine. Acid-ethanol (0.4 mL 10 %) caused 82.2 ±0.2 % reduction in nitrite formation (P<0.01). Ethanol and methanol in matched volumes did not affect nitrite formation (P>0.1, n=5 each) while acetic acid 0.2 mL 1 % matching mass in acid-ethanol reduced nitrite estimate by 19.4 % (P<0.01). This implied action of acid-ethanol is attributed to the acetic acid component in the solvent.

Table 1 : Relation between mass of hydroxylamine and nitrite formation

Hydroxylamine mass, µmoles	Mean ± S.E., Absorbance values	Mean ± S.E , nitrite formed, nmoles
0.01	0.045 ± 0.002	5.1 ± 0.2
0.03	0.172 ± 0.002	20.6 ± 0.2
0.05	0.306 ± 0.001	36.9 ± 0.1
0.10	0.585 ± 0.009	70.9 ± 1.0
0.15	0.811 ± 0.017	98.5 ± 2.0
Statistical analysis		
r ± s.e.	0.9974 ± 0.0023	
b± s.e..	5.4789 ± 0.0002	667.9 ± 21.6

The values are mean ± s.e. of five observations each.

Table 2 : Effect of test agents on HA-mediated nitrite formation

Test material, µg used	Hydroxylamine antagonizing activity, µmoles HA reduced per mg test material
Diosmin, 20	Nil
Rutin, 18	1.58 ± 0.16
Morin, 20	0.43 ± 0.01
Curcumin, 20	1.05 ± 0.13
Ascorbic acid, 176	0.175 ± 0.004

The values are mean ± s.e. of five observations each.

MTT reduction method

Under test conditions, hydroxylamine in alkaline solution causes quantitative reduction of MTT dye to formazan. The dye is monitored at 535 nm, and test agents could be evaluated by their ability to reduce reduction potential of hydroxylamine.

The mean pH with addition of standard alkali has been 11. 83 ± 0.01 and 11.85 ± 0.01 respectively in presence and absence of 0.4 mL 10 % acid-ethanol (P>0.1, n= 4 each).

Method-1, conducted in presence of water, is linear over 0.02 through 0.2 µmole of hydroxylamine (r± S.E. = 0.9941 ± 0.0053, b ± S.E. = 3.82 ± 0.19) while Method-2, conducted in presence of the matched volume of acid-ethanol, is linear over 0.1 through 0.4 µmole hydroxylamine (r± S.E. = 0.9916 ± 0.0096, b ± S.E. = 1.636 ± 0.123) monitored at about one hour. Acid-ethanol 0.4 mL (10%) caused 22.5 % decrease in HA-induced effect (0.4 µmole; P<0.01) which is accountable to acetic acid that produced 23.5 % decrease in effect (P<0.01) while matched mass of ethanol failed to modify action of standard hydroxylamine (P>0.1) with mean absorbance values in water, acid-ethanol, acetic acid and ethanol being, respectively as, 0.761± 0.006, 0.590 ± 0.010, 0.582 ± 0.011 and 0.754 ± 0.031. Methanol 0.2 mL did not affect MTT nor modified effect of HA (P>0.1). Acid-ethanol 0.4 mL caused 49 ± 1 per cent reduction in HA activity (P<0.01) showing 388 ± 11 nmoles HA antagonized per mL. Diosmin 20 µg neither produced any direct affect on MTT nor modified effect of hydroxylamine on MTT. Rutin, morin and curcumin varyingly reduced MTT directly like hydroxylamine (morin > >curcumin > rutin) and baring morin, rutin and curcumin potentiated HA-effect on MTT (rutin>curcumin). Ascorbic acid was nearly 1/3rd as active as curcumin in causing MTT reduction while it showed 1/20th as antagonistic potential against hydroxylamine induced effect (Table 3).

Table 3: Effect of test agents on MTT reduction and HA-mediated MTT reduction

Test material, µg used	MTT reducing activity equivalent to HA µmoles per mg test material	Activity in presence of Hydroxylamine
Diosmin, 20	Nil	Nil
Rutin, 9	3.5 ±0.2	36.5 ± 1.6[a]
Morin, 5	71.0 ± 0.6	Nil
Curcumin, 5	25.8 ± 0.9	106.8 ± 1.4 [a]
Ascorbic acid, 35.2	7.62 ± 0.13	4.91± 0.07[b]

[a] Potentiation activity, as µmoles HA equivalent mg^{-1} test agent
[b] Hydroxylamine antagonizing activity as µmoles HA antagonized mg^{-1} test agent
The values are mean ± s.e. of five observations each.

Potassium ferricyanide reduction method

Hydroxylamine causes quantitative reduction of potassium ferricyanide leading to concentration related decrease in optical density measured at 410 nm. Test agents are evaluated by their potential to cause decrease in reduction in color intensity caused by standard concentration of hydroxylamine in presence of standard concentration of potassium ferricyanide. The mean pH at the point of addition of standard alkali has been 11. 15 ± 0.01 and 11.46 ± 0.01 in presence and absence of 0.4 mL 10 % acid-ethanol (P<0.01, n= 4 each).

Standard curve potassium ferricyanide

The method is linear over 0.25 through 2.5 µmoles of potassium ferricyanide with mean ± S.E. (n=5 each) at 0.25, 0.50, 1.50 and 2.50 µmoles, respectively as, 0.062 ±0.001, 0.118 ± 0.001, 0.345 ±0.002 and 0.595 ±0.002, with r ± S.E. = 0.9997±0.0003 and b ± S.E. = 0.236 ± 0.003 and c = -0.0006).

Standard curve hydroxylamine

At 2.5 µmoles potassium ferricyanide, hydroxylamine 0.5 through 2 µmoles causes concentration related decrease in color with mean µmoles of potassium ferricyanide reduced at 0.5, 1.0, 1.5 and 2.0 µmoles of hydroxylamine, respectively as, 0.74±0.04, 1.26±0.01, 1.75±0.01 and 2.26±0.16 (r=0.9999±0.0001, b= 1.01±0.01, c = 0.24). As evident, each µmole of hydroxylamine caused reduction of 1.01 µmole of potassium ferricyanide.

Standard NaOH (0.1 M) was significantly better than standard $Na_2 CO_3$ (0.4M) with 2 µmoles hydroxylamine showing reduction in absorbance from standard values of 0.833 ± 0.002 to 0.049 ± 0.002 (94 % reduction) with

sodium hydroxide compared to reduction from 0.799 ± 0.002 to 0.151 ± 0.004 (81 % reduction) with sodium carbonate.

The assay for evaluating test substances employed 2.5 µmoles of potassium ferricyanide and 1 or 1.5 µmoles of hydroxylamine. Methanol and acid-ethanol in matched volumes did not affect potassium ferricyanide ($P>0.1$). Methanol potentiated effect of HA by 29 ± 1 per cent ($P<0.01$) showing HA-equivalent activity as 1.29 ± 0.06 µmole per mL. Acid-ethanol did not affect HA activity ($P>0.1$). Test agents baring diosmin exhibited ferricyanide reducing potential: ascorbic acid >rutin > morin >curcumin ($P<0.05$) while the effect of hydroxylamine was significantly reduced in the order: rutin > diosmin > curcumin >morin >> ascorbic acid (($P<0.05$)) (Table 4).

Table 4: Effect of test materials on ferricyanide reduction and HA -induced reduction of ferricyanide

Test material, mass µg	Ferricyanide reducing potential, µmoles mg^{-1}	Hydroxylamine antagonizing potential, µmoles mg^{-1}
Diosmin, 20	Nil	75.4 ±0.8
Rutin, 18	16.1 ± 0.8	78.2 ±0.9
Morin,20	13.9 ±1.0	67.4 ±0.6
Curcumin,20	5.8 ±0.3	70.8 ±0.6
Ascorbic acid , 52.8	19.3 ±0.6	12.7 ±0.3

The values are mean ± s.e. of five observations each
The effects are significant ($P<0.05$) except by those indicated nil ($P>0.1$)

SNP reactivity reduction method

The assay is based on the reaction of reduction of sodium nitroprusside by hydroxylamine in alkaline solution forming an intense yellow compound (sodium aquoferrocyanide, $Na_3 Fe (CN)_5 .H_2O$) [12]. The color exhibited maximum absorbance at 410 nm. Preliminary tests were conducted to optimize linear range for using hydroxylamine with respect to the reagents with color change monitored over 5 through 60 minutes. The optimized reaction was used to evaluate test agents for their ability to reduce the reaction potential of standard hydroxylamine. Therefore, decrease in absorbance by HA in presence of test material compared to that in absence of test material is indicative of anti-oxidant potential of the test material.

The mean pH at the point of addition of standard alkali has been 10.33 ± 0.01 and 10.48 ± 0.01 in presence and absence of 0.4 mL 10 % acid-ethanol ($P<0.01$, n= 4 each). SNP 5 µmoles react quantitatively with 2 through 25 µmoles HA with peak absorbance at 410 nm. The method is linear over 5 through 60 minutes of observation period with peak response at 5th minute. At 5, 15, 30 and 60 minutes, r ± S.E and b ± S.E., have remained respectively and correspondingly as 0.9924 ± 0.0068 and 0.0394 ± 0.0022; 0.9964 ± 0.0032 and 0.0304 ± 0.0012; 0.9965 ± 0.0031 and 0.0228 ± 0.0009; and 0.9973 ± 0.0024 and 0.0142 ± 0.0005. As apparent the regression coefficient shows regular decrease over 60 minute observation period @ ca. 1 % decrease per minute. During the present study measurements of absorbance have been made at 15 through 30 minutes. Using 40 µmoles HA, the method enables detection of 25 through 1000 nmoles of SNP in a 3 mL reaction volume (r ± S.E = 0.9997 ± 0.0002 and b ± S.E = 0.00085 ± 0.00001). Acid-ethanol did not affect SNP and hydroxylamine reactivity ($P>0.1$) while ascorbic acid (1 µmole) caused 26 ± 4 per cent reduction in HA activity equivalent to 400 ± 25 µmoles HA-antagonized per mg ($P<0.05$). Methanol reduced HA activity by 10.5 ± 1.5 per cent showing 8.7 ± 0.9 µmoles HA-antagonized per mL. Test agents significantly antagonized hydroxylamine induced reduction of sodium nitroprusside with order of potency as morin > diosmin > ascorbic acid> rutin = curcumin ($P <0.05$) (Table 5). This would indicate that the method is a useful technique for screening anti-oxidant potential of these test agents.

Table 5: Effect of test materials on HA-mediated formation of colored complex with sodium nitrusside

Test material, mass µg	Hydroxylamine antagonizing potential, µmoles mg^{-1}
Diosmin, 20	512 ±8
Rutin, 18	334 ±7
Morin,20	678± 29
Curcumin,20	321 ± 14
Ascorbic acid , 52.8	400 ± 25

 The values are mean ± s.e. of five observations each.

Reduction of copper oxide formation method

Hydroxylamine causes quantitative reduction of copper (II) to copper oxide (I) at alkaline pH , the copper oxide reacts quantitative to phosphomolybedate reagent to form a deep blue color with peak absorbance at 540 nm. Test agents capable of scavenging super oxide anion from hydroxylamine would reduce reduction potential of hydroxylamine and raise absorbance values. The test is linear over 2 through 4 µmoles of hydroxylamine using standard mass of copper as 10 µmoles.

The mean pH at the point of addition of standard alkali has been 12.22 ± 0.01 and 12.22 ± 0.04 in presence and absence of 0.4 mL 10 % acid-ethanol (P>0.1, n= 4 each).

The method is linear over 2 through 6 µmole of copper using constant mass of HA as 4 µmoles per sample (r ±S.E. = 0.9979 ± 0.0021, b ± S.E. = 0.1518 ±0.0049). The mean absorbance values at 2, 3, 4 and 6 µmole of copper are, respectively, as 0.119 ± 0.005, 0.258 ± 0.003, 0.448 ± 0.008 and 0.719 ± 0.013 (n= 5 each). Using 10 µmoles copper acetate, hydroxylamine over 2 through 4 µmoles produces linear reduction of copper (II) to copper (I) with mean absorbance values at 2, 2.5, 3, 3.5 and 4 µmoles of HA, respectively, as 0.087 ± 0.002, 0.194 ± 0.013, 0.380 ± 0.026, 0.526 ± 0.020 and 0.660 ± 0.019 (n=5 each, r ± S.E. = 0.9974 ±0.0951, b ± S.E. = 0.2956 ± 0.1087).

Acid-ethanol has no effect on copper reduction but showed 1.44 ±0.31 µmole HA-antagonizing activity per mL. Test flavonoids and curcumin failed to reduce copper *per se* while ascorbic acid showed 7.0 ± 0.1 µmole HA-like activity on copper and potentiated effect of HA on copper reduction. The effect in combination, 13.3 ± 0.3 µmoles per mg is significantly more than sum of individual effects by HA (2 µmoles) and that produced by ascorbic acid (as 7 µmoles per mg). The absorbance values with HA, ascorbic acid and HA + Ascorbic acid are, respectively as, 0.163 ± 0.021 (n= 3), 0.504 ±0.004 and 0.957 ±0.021 (n=5 each) showing an increase by about 44% in combination (P<0.01). Methanol did not affect copper reduction nor modified effect of HA on copper (P>0.1). Rutin effectively antagonized reducing potential of hydroxylamine on copper to the extent of 21.2 ± 1.7 µmoles HA mg $^{-1}$ test agent (P<0.01) (Table 6).

Table 6: Effect of test materials on HA-mediated reduction of copper (II) to Copper (I) oxide as determined by phosphomolybedate action.

Test material, mass µg	Response of copper acetate	Response of Hydroxylamine mediated action
Diosmin, 20	Nil	Nil
Rutin, 18	Nil	21.2 ± 1.7 [a]
Morin, 20	Nil	Nil
Curcumin,20	Nil	Nil
Ascorbic acid , 52.8	7.0 ± 0.1[b]	13.3 ± 0.3 [b]

[a] HA-antagonism equivalent to µmoles HA mg $^{-1}$ test agent (P<0.01)
[b] HA-like reduction activity equivalent to µmoles HA mg $^{-1}$ test agent (P<0.01)
 The values are mean ± s.e. of five observations each.

Copper amine reduction method

Copper acetate in presence of ammonia forms copper amine with peak absorbance at 605 (600-610 nm) and hydroxylamine was found to cause quantitative reduction in copper amine formation and thus of absorbance

values. Test agents capable of scavenging super oxide anion from hydroxylamine would prevent copper amine reducing potential of hydroxylamine. This provided basis for screening the test agents.

The mean pH at the point of addition of standard ammonia has been 10.95 ± 0.01 and 11.00 ± 0.02 in presence and absence of 0.4 mL 10 % acid-ethanol (P>0.1, n= 4 each).

Copper amine method over 0 and 10 through 40 µmoles of HA with standard mass of copper as 50 µmoles shows mean absorbance values at 0, 10, 20, 30 and 40 µmoles of HA, respectively, as 0.625 ± 0.001, 0.615 ± 0.005, 0.539 ± 0.009, 0.433 ± 0.012 and 0.311 ± 0.016 . The decrease in absorbance shows linearity over 10 to 40 µmoles of HA (r± S.E. = 0.9948 ± 0.0052, b ± S.E. = 0.8362 ± 0.0005) with perfect linearity over 20 through 40 µmoles (r± S.E. = 0.9992 ± 0.0009, b ± S.E. = 0.9365 ± 0.0003).

Copper is measurable linearly over 5 through 50 µmoles showing linear increase in absorbance at 605 nm as copper amine (r± S.E. = 0.9999 ± 0.0001, b ± S.E. = 0.0122 ± 0.0001). Ascorbic acid 0.3 and 5 µmoles did not affect copper amine (P>0.1). Lower mass did not modify action of HA on copper amine (P>0.1) while higher mass antagonized the effect of HA to the extent of 14.5 ± 2.1 µmoles HA antagonized per mg ascorbic acid.

Acid-ethanol 0.4 mL and methanol 0.2 mL did not modify action of HA but acid-ethanol caused increase in color intensity of copper amine by 3.3 ± 0.2 % (P<0.01). Diosmin and ascorbic acid *per se* failed to affect copper directly while rutin, morin and curcumin at caused significant reduction in copper at higher masses: morin > curcumin = rutin while lover masses were ineffective. Only ascorbic acid showed significant antagonizing activity against hydroxylamine mediated reduction in copper ions, and others were ineffective and curcumin significantly potentiated effect of hydroxylamine on copper reduction (Table 7). The method overall showed poor response to the test agents but has potential as a screening method.

Table 7: Effect of test materials on copper amine and HA-mediated reduction of copper amine

Test material, mass µg		Response of copper amine	Response of Hydroxylamine mediated action
Diosmin	20	Nil	Nil
	40	Nil	Nil
Rutin	18	Nil	Nil
	36	2.4 ± 0.2 [a]	Nil
Morin	20	Nil	Nil
	40	10.6 ± 0.2 [a]	Nil
Curcumin	20	Nil	Nil
	40	3.0 ± 0.2 [a]	351 ± 16 [b]
Ascorbic acid	52.8	Nil	Nil
	880	Nil	14.5 ± 2.1 [c]

[a] Per cent increase in absorbance in copper amine compared to that of the standard
 copper amine concentration of 50 µmoles (P<0.01)
[b] Reducing potential of hydroxylamine potentiated in units of µmoles HA equivalent
 mg^{-1} test agent
[c] Hydroxylamine antagonizing activity as µmoles HA equivalent mg^{-1} test agent
 (P<0.01)
 The values are mean ± s.e. of five observations each.

CONCLUSIONS

Currently, reduction of tetrazolium dye is the only potential method used for detecting super oxide anion from autoxidation of hydroxylamine in alkaline solution, and the potential test agents are evaluated by employing this model. Present study has revealed that a battery of optimized tests can be employed for generating super oxide anion from auto-oxidation of hydroxylamine in alkaline solution to enable screening of diverse anti-oxidants for their scavenging activity including flavonoids, curcumin and ascorbic acid. These procedures can be routinely used for screening potential anti-oxidant agents acting through scavenging of super oxide anion radicals.

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