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A new Fenton assay for Hydroxyl radical scavengers by monitoring Catechol oxidation

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Abstract: A new, simple assay has been optimized to generate and detect hydroxyl radical by Fenton reaction. The assay is based on oxidation of catechol, monitored at 360 nm following incubation at room temperature for ca. 30 minutes, with linearity over 0.25 through 2.5 µmole H_2O_2 using Fe (II) (0.5 µmole) and acidified catechol (0.5 mg). The Fenton reaction has been characterized by registering: (i) about 13 folds increase in response to H_2O_2 in presence of iron (II), (ii) inhibition by agents chelating iron and/or neutralizing H_2O_2 and, (iii) antagonism by established hydroxyl radical scavengers. Hydroxyl radical scavenging potential of the test agents, expressed as µmole scavenged µmole^{-1} test agent, has been found in the order: quercetin > curcumin> morin > rutin> daflon> diosmin > ascorbic acid > thiourea > DMSO > mannitol > benzoic acid. Citric acid, oxalic acid, tartaric acid, acetic acid and their salts, disodium EDTA, sodium metabislphite, ethanol, methanol and acetone have been evaluated for their influence on the assay when present during or following color development. A comparable Fenton reaction has been also demonstrated while using 1 mg catechol and 10 µmole Cu (II) with linearity over 0.25 through 5.0 µmole H_2O_2.
Key-words: Fenton reaction, Hydroxyl radical scavengers, Inhibitors of Fenton reaction, Dihydroxybenzenes.

Introduction

Fenton reaction is a convenient generator of the hydroxyl radical with known implications in health and disease [1-4]. The reaction traditionally involves reduction of H_2O_2 with Fe (II), but can also occur in presence of copper [5]. In view of a very brief half- life of the radical (10^{-9} to 10^{-10} sec), the methods for its detection are limited and indirect, and include electro chemical detection[3], electron spin resonance[6] and fluorimetry[7,8]. A couple of spectrophotometric methods have been developed based on either decolorization of dyes [9-12] or degradation of deoxy-D-ribose caused by the radical[9,10, 13, 14]. These techniques have been employed for screening anti-oxidants [7,9, 14, 15] or for understanding the reaction mechanisms[1,11,16]. Most of the methods require expensive infrastructure and expertise so remain largely beyond the reach of common laboratories. A need to develop a simple protocol for the purpose was mooted on the following rationale: (i) Fenton reaction involving a transition metal such as iron or copper generates hydroxyl radical as a powerful oxidizing species [5]; (ii) dihydroxybenzenes including catechol have accelerated Fenton reaction[12, 17, 18] and (iii) catechol is oxidized to brown-colored compounds [19] due to formation of semi-quinone and quinine[20]. Therefore, the experiments were designed to optimize the

Fenton reaction, and validate the assay by testing its response to inhibitors of Fenton reaction and to scavengers of hydroxyl radical using catechol oxidation model.

Experimental

The experiments were carried out at an ambient temperature of 23.5 ± 0.6 ^{0}C. The chemicals and drugs used were of standard purity and quality obtained from reputed sources in India. Spectrophotometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan). The samples when required were analyzed for pH by a pocket pH meter (pH Scan 3, Eutech Instruments, Malaysia). Centrifugation of test samples, whenever required, was done at 8000 rpm for 5 minutes.

Reagents and drugs

Phenolics: Stock solutions of resorcinol, hydroquinone and catechol were each made as 1 % (w/v) in 0.1 % HCl. Acidified and non-acidified catechol solutions were respectively prepared as 0.05 % (w/v) in 0.005 % HCl and water.

Hydrogen peroxide solution: The stock solution was titrated against standard $KMnO_4$ which has been standardized against 0.05 M oxalic acid. Working solution, made freshly before use, provided 5 µmole H_2O_2 mL^{-1} water.

Dilute HCl solutions: The solutions were prepared in three dilutions as 10 %, 1 % and 0.5 % (v/v) concentrated HCl in water.

Iron reagents: Working solutions of ferrous ammonium sulfate (FAS-II) and ferric ammonium sulfate (FAS-III), 1 µmole mL^{-1}, were made by dilution in water at the time of use from their respective stock solutions, 25µmole mL^{-1} in 0.005 M HCl.

Copper acetate: Working solution of 10 µmole copper mL^{-1} 0.01 M acetic acid was prepared freshly from stock solution of 100 µmole mL^{-1} 0.01 M acetic acid.

Iron-catechol reagent: The reagent was prepared freshly on the day of use by mixing stock solutions of catechol and FAS-II, respectively as 2.5 and 1 mL in 50 mL water.

Salicylate reagent: 3.2 % (w/v) sodium salicylate in water provided 0.2 M reagent.

Test solutions: Test solutions prepared were prepared in water as 0.005 M (ascorbic acid), 0.05 M (citric acid, oxalic acid, tri-sodium citrate, potassium oxalate), and 0.1 M (acetic acid, tartaric acid, disodium EDTA, sodium metabisulphite, potassium sodium tartrate, D-mannitol, DMSO, thiourea). Curcumin and benzoic acid solutions were prepared in methanol as 0.001 M and 1 M, respectively.

Flavonoids: Working solutions of quercetin, rutin and morin were prepared each as 1 µmole mL^{-1} methanol. Diosmin and daflon (bioflavonoid mixture with labeled composition: 90% disomin and 10 % hesperidin) were extracted in 0.1 M NaOH as 0.25 % (w/v) solutions (equivalent to 4.1 µmole mL^{-1} with molar mass of each taken as 608) from pharmaceuticals, respectively, Venex-500 mg tablets (Elder Pharmaceuticals Ltd., Mumbai) and Daflon-500 (Serdia Pharmaceuticals Ltd., Mumbai)) as per standard procedure[21]. The pH of the test solutions was adjusted few minutes before use with 1 % HCl and 0.1 M NaOH to provide approximately 1 µmole flavonoid mL^{-1} water with pH in the range 3.4 to 3.8.

Standard assay protocol

One milliliter catechol iron reagent was added 1 mL water or appropriate solvent with or without test agent followed by addition of 1 mL water containing varying masses of H_2O_2 (0, 0.25 through 2.5 µmole). The samples were allowed standing at room temperature for 20 minutes to complete oxidation, and then each sample was added 1 mL water, and the absorbance was monitored at 360 nm within next 10 minutes.

Optimization Studies

Unless otherwise indicated, the reactants were used in standard masses as 0.5 mg

catechol, 0.5 µmole FAS-II and 2 µmole H_2O_2 in 4 mL volume. The assay was optimized with respect to variables: (i) FAS-II over 0.1 through 0.5 µmole, (ii) catechol over 0.1 through 1 mg, (iii) incubation time from 10 through 240 minutes, (iv) response of catechol with FAS-II, FAS-III and hydrogen peroxide in standard masses individually and in combination, (v) order of addition of hydrogen peroxide as first or the last reagent, (vi) addition of iron and catechol separately or in combination, and (vii) varying incubation volume as 3 mL versus 4 mL. Besides, structural isomers of catechol (1, 2–dihydroxy benzene) namely resorcinol (1, 3 – dihydroxy benzene) and hydroquinone (1, 4-dihydroxy benzene) were also compared for their responses using standard procedure. Spectral analysis of the reactants was performed to find appropriate absorbance maxima for routine use. The randomized data were also evaluated for absorbance at 360 nm and 400 nm.

Fenton reaction as affected by the chelants of iron and organic solvents Standard protocol was conducted in absence and presence of 25 µmole of each of the test agents including citric acid, tri-sodium citrate, oxalic acid, potassium oxalate, tartaric acid and potassium sodium tartrate, acetic acid, acetate buffer, disodium EDTA, potassium dihydrogen phosphate, sodium metabisulphite and in presence of 1 mL each of organic solvents ethanol, methanol and acetone. Test agents in 3 mL reaction volume were kept incubated for about 15 minutes, and then each sample was added 1 mL water before taking absorbance at 360 nm at about 30 minutes following addition of hydrogen peroxide. In a separate set of experiments, test agents baring salts of organic acids were added in 1 mL volume in appropriate solvent at 20^{th} minute following color development.

Evaluating hydroxyl radical scavengers

The standard protocol was conducted in presence of test hydroxyl radical scavengers in appropriate solvent with simultaneously run H_2O_2 at masses of 0.5, 1 and 2 µmole as standard matched for the solvent. Test agents have included ascorbic acid (1 µmole), thiourea (25 µmole), DMSO and D-mannitol (80 µmole each) in water; curcumin, quercetin, morin, rutin (0.1 µmole each) and benzoic acid (200 µmole) in methanol; and diosmin and daflon (0.4 µmole each) in 0.1 M NaOH.

Estimating conversion of iron (II) to iron (III) during Fenton reaction

Mass of iron (III), generated from Fenton reaction was estimated by running simulating test employing ferric-salicylate method [22]. Each milliliter of water containing 0.5 µmole FAS-II was added 0.5 mL of sodium salicylate reagent followed by addition of 1.5 mL water containing 0, 0.05, 0.10, 0.15 and 0.2 µmole H_2O_2 The samples were allowed standing at room temperature for 30 minutes and then monitored for absorbance at 480 nm. The assay included simultaneously run standard using 0, 0.1 through 0.5 µmole FAS-III in 3 mL volume excluding addition of H_2O_2.

Catechol oxidation based on copper Fenton reaction

One milliliter solution containing 10 µmole copper (II) in 0.01 M acetic acid was added 1 mL water with 1 mg catechol and 1 mL water with H_2O_2 0.25 through 5 µmole. The samples were incubated at room temperature for 20 minutes, and then added 1 mL 0.5 % HCl, and monitored for absorbance at 360 nm. The response of the reactants in standard concentrations of copper (II) 10 µmole, catechol 1 mg and H_2O_2 2 µmole were monitored in different combinations to validate the reaction generated hydroxyl radical.

Calculations

The data were subjected to routine statistical analysis. The scavenging or inhibiting activity, expressed as µmole hydroxyl radical scavenged or reduced per µmole of test agent, was calculated as follows.

Scavenging activity = (C - [(A* C)/B])/M

Where C = Concentration of standard H_2O_2 in µmole used for the assay; A= Mean absorbance with test agent in presence of H_2O_2; B= Mean absorbance with standard mass of H_2O_2; M = Mass of test substance used in µmole. Where the value was less than unity, the result was multiplied by 1000 to express the activity in nmoles.

An alternative formula employs parameters obtained from regression analysis of simultaneously run standard, such as y-intercept (c) and regression coefficient (b), to make the calculations: Scavenging activity = (C - [(A-c)/b])/M.

Results and Discussion

Standard linear curve for optimized Fenton assay

As evident from **Table 1,** the assay is linear over 0.25 through 2.5 µmole H_2O_2 under standard conditions suggesting a linear increase in generation of hydroxyl radical with linear oxidation of catechol with peak absorbance at about 360 nm (r ± S.E = 0.9994 ±0.0005, b ± S.E = 0.181 ± 0.003). The absorbance peak at 360 nm has been confirmed from spectral analysis.

The pooled data from five randomized assays covering 0.5, 1 and 2 µmole hydrogen peroxide have shown mean absorbance values correspondingly as 0.106 ± 0.001, 0.196 ± 0.002 and 0.374 ± 0.002 (r±S.E. = 0.9999±0.0001, b ± S.E. = 0.1786 ± 0.0007, n=15 each) with over all mean RSD 2.7 ± 0.3 (range 2.1 through 3.2). The assay conditions have been based on optimization studies.

Table 1 Fenton reaction mediated linear catechol oxidation

Hydrogen peroxide, µmole	Absorbance
0.25	0.063 ±0.001
0.5	0.114 ±0.001
0.75	0.161 ±0.001
1.5	0.301 ±0.002
2.0	0.390 ±0.001
2.5	0.468 ±0.002
Statistical analysis	
r ± S.E	0.9994 ±0.0005
b ± S.E	0.1809 ±0.0026
c	0.02333

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

Time optimization studies

The reaction is faster in onset with effect discernible within a few minutes following addition of the reactants. The mean absorbance values remained almost similar during first thirty minutes, from 0.355 ± 0.003 at 10^{th} minute to 0.352 ± 0.002 at 30^{th} minute of incubation ($p>0.1$, n=5 each). Thereafter, the values decreased by about 4 % at 40^{th} minute, 0.342 ± 0.003, and by about 6 % by 1 hr, 0.335 ± 0.003, ($p<0.01$). Following one hour, the values remained stable for up to 4 hours with mean absorbance at 4^{th} hour, 0.335 ± 0.002 ($p>0.1$). Effects of generated hydroxyl radical from Fenton reaction are largely complete within first 10 minutes of reaction [23, 24]. The remaining time in the present study is presumably required to complete the oxidation of the catechol. Thus, it has been reasonable to monitor the reaction from 20 to 30 minutes following addition of reactants. However, if monitoring is delayed then readings can be safely taken up to 4 hour observation period anticipating a decrease in absorbance by about 6 %.

Table 2 Demonstration of Fenton reaction as catechol oxidation

Treatment	Absorbance values[a]
Catechol+ H_2O_2	0.030 ±0.003
Catechol+ FAS-II	0.008 ±0.002
Catechol+ FAS-III	0.083 ±0.002
Catechol+ FAS-II + H_2O_2	0.399 ±0.003
Catechol+ FAS-III + H_2O_2	0.458 ±0.003

The values are mean ± S.E. of 11 observations each. The concentrations of catechol, H_2O_2, FAS-(II or III), have been correspondingly as 0.5 mg, 2 µmole and 0.5 µmole.

Reactant optimization studies

With standard masses of H_2O_2 and catechol, varying mass of FAS-II has induced concentration related increase in absorbance with mean values at 0.1, 0.2, 0.3 and 0.5 µmole, respectively as, 0.260 ± 0.002, 0.311 ± 0.002, 0.322 ± 0.004 and 0.347 ± 0.006 with perfect linear regression over 0.2 through 0.5 µmole FAS-II (r ± S.E. = 0.9995 ± 0.0006; b ± S.E. = 0.121 ± 0.002). This has enabled using mass of iron (II) as 0.5 µmole for the assay throughout the study. This mass induced negligible background absorbance, 0.008 (Table 1), and on complete oxidation would generate maximally 0.5 µmole iron (III) capable of producing background absorbance maximally 0.083 (Table 2). Mass of catechol as 0.5 mg was considered choice for the assay on the basis of observation that there was linear increase in absorbance with catechol over 0.1 through 0.5 mg (r ± S.E. = 0.98 ±0.02; b ± S.E. = 0.45 ± 0.05), and the response tapered at 1 mg catechol. The mean values at 0.1, 0.3, 0.5 and 1.0 mg catechol were found to be, respectively, as 0.168 ± 0.001, 0.287 ± 0.001, 0.348 ± 0.002 and 0.391 ± 0.003 (n = 5 each).

Reaction volume and manner of adding reactants

The addition of acidified catechol and iron as a single reagent vis-à-vis when added separately have yielded comparable absorbance values, respectively as, 0.366 ± 0.002 and 0.363 ± 0.003 in 4 mL volume and correspondingly as 0.392 ± 0.002 and 0.387 ± 0.002 in 3 mL volume ($p>0.1$, n= 6 each). As apparent, the mean absorbance values in 3 mL reaction volume are significantly increased by about 7 % ($p<0.01$, n= 6 each). The absorbance values have increased by 5.1 % when H_2O_2 was added as the last reagent compared to its addition as first reagent, and the mean values were respectively as 0.349 ±0.001 and 0.332 ± 0.001 ($p<0.01$, n = 6 each).

Demonstration of Fenton nature of the reaction

The Fenton reaction has been characterized by: (i) a linear increase in oxidation of catechol as a function of mass of H_2O_2 (Table 1), (ii) H_2O_2 action was 10.5 and 4 folds more in presence of FAS-II and FAS-III, respectively, than sum of their individual responses ($p<0.01$, n= 11 each) and estimated response as net absorbance value due to H_2O_2 has increased 13 folds by FAS-II and 12.5 folds by FAS-III (Table 2), (iii) Fenton reaction has been inhibited from 20 to 100 % in presence of agents chelating/precipitating iron (organic acids, their salts, EDTA, phosphate) or neutralizing H_2O_2 (sodium metabisulphite) or affecting both (pH exceeding 5.0) (Table 3), and (vi) ability of known hydroxyl scavengers to inhibit the oxidation of catechol as per their relative potential (Table 4).

Catechol oxidation as affected by the inhibitors and organic solvents

Fenton reaction has been inhibited by iron chelants such as phosphate, EDTA, citric/oxalic acid, formaldehyde[25] with metal chelating potential in the order: EDTA>citric acid>tartaric acid[26].Sodium metabisulphite neutralizes hydrogen peroxide[25]. Our observations are consistent with these findings (Table 3).

Based on extent of inhibition at equimolar masses, the inhibitors of Fenton reaction may be conveniently listed into three categories: (i) most active inhibitors with inhibition exceeding 75 %: tri-sodium citrate (>100) ≥ sodium metabisulphite (>100) > disodium EDTA (97) > potassium dihydrogen phosphate (81); (ii) moderately active inhibitors with inhibition in the range 25 through 75 %: oxalic acid (72) > potassium sodium tartrate (69) > potassium oxalate (62) > citric acid (43), and (iii) least active inhibitors with inhibition < 25 %: tartaric acid

(17.5) > acetate buffer (10.4) > acetic acid (8.7). Ethanol and acetone have improved catechol oxidation, respectively, by 22 and 41 per cent ($p<0.01$ each) while methanol has reduced absorbance by ca. 7 % ($p<0.01$).The latter response has been responsible for lower absorbance values in control samples wherever solvent was matched with 0.1 mL methanol (Table 4). The effect of methanol is presumably due to its oxidation to formaldehyde and formic acid [27] inhibiting Fenton reaction [1, 25].

As apparent from Table 3, the agents have been poorly active when added following color development with per cent inhibition (within parenthesis) in the order as: sodium metabisulphite (20)> EDTA (10.4)> citric acid (9.8)> tartaric acid (9.5) ($p<0.01$ each). Least inhibitory agents include: phosphate (5.4, $p<0.01$)> oxalic acid (2.5, $p<0.01$)> acetic acid (1.4, $p>0.1$). Mean absorbance has increased by 5 to 8 % with test organic: acetone (7.8)>ethanol (5.3) >methanol (4.7) ($p<0.01$). The foregoing observations reveal that the Fenton reaction is quite sensitive to presence of agents interfering with action of iron and /or H_2O_2, and the response is relatively tolerant to their presence once oxidation is complete.

Table 3 Comparative evaluation of test agents affecting Fenton reaction

Test agent, 25 µmole	**Added during incubation**		**Added following incubation**	**Mean pH**
	Change[a]	**Potential[b]**	**Change[a]**	
Citric acid	42.9 ↓	34.3 ± 0.3	9.8 ↓	2.88
Tri-sodium Citrate	>100 ↓	> 80.0	NA	7.87
Oxalic acid	72.1 ↓	57.6 ± 0.5	2.5 ↓	2.63
Potassium oxalate	62.5 ↓	50.0 ± 0.2	NA	7.08
Tartaric acid	17.5 ↓	13.9 ± 0.3	9.5 ↓	3.05
Potassium sodium tartrate	68.6 ↓	54.8 ± 0.1	NA	5.41
Acetic acid	8.7 ↓	34.3 ± 0.3	1.4 ↓	3.35
Acetate buffer	10.4 ↓	9.5 ± 0.4	NA	4.45
$KH_2(PO_4)_3$	81.2 ↓	64.9 ± 0.4	5.4 ↓	4.02
EDTA disodium	96.6 ↓	77.3 ± 0.2	10.4 ↓	4.42
Sodium metabisulphite	> 100↓	> 80.0	20.1↓	3.81
Ethanol[c]	21.5 ↑	424 ± 3↑	5.3 ↑	3.37
Methanol[c]	6.8 ↓	157 ± 4 ↓	4.7 ↓	3.56
Acetone[c]	40.7 ↑	810 ± 20↑	7.8 ↑	3.74

The values are mean ± S.E. of 5 observations each. [a] Per cent of untreated standard (absorbance range from 0.358 ± 0.001 to 0.415 ± 0.003); [b]nmoles hydroxyl radical µmole^{-1} test agent or mL^{-1} in case of organic solvent;[c] 1 mL each.; NA, addition of salts of organic acids not attempted post-incubation

Comparative evaluation of hydroxyl radical scavengers

The scavenging potential of the test agents, expressed as µmole hydroxyl radical scavenged µmole^{-1} test agent, has been found in the order: quercetin (10.6 X)> curcumin (5.9X)> morin (5.2 X) >rutin (2.9 X) >daflon (1.2 X) >diosmin (1X)> ascorbic acid (0.4 X) >thiourea (0.03 X) > DMSO (0.01 X)> mannitol (0.007 X> benzoic acid (0.0009 X) (Table 4). Hydroxyl radical scavenging activity has been demonstrated for ascorbic acid[14,15,28], mannitol[1, 29], sodium benzoate [1], thiourea[1,30], and flavonoids[28,31- 33]. Curcumin has favored generation of hydroxyl radical[10,30] as well shown it's scavenging[10]. The relative potency in different combinations has been found varyingly in the order as: quercetin>rutin > ascorbic acid[28]; quercetin > morin > diosmetin [33], and thiourea > mannitol > benzoate[1]. Flavonoids are generally considered powerful anti-oxidants due to their free hydroxyl

groups which chelate transition metals and scavenge free radicals. Rutin (quercetin-3-rutinoside) is relatively less potent than its aglycone quercetin [31] due to blockade of one hydroxyl function by rutinose [32].

Table 4 Comparative evaluation of hydroxyl radical scavengers by the catechol oxidation assay

Test agent, µmole used	**Absorbance**	**Absorbance, per cent of control**	**Inhibitory potential[a]**	**Mean pH**
Quercetin, 0.1	**C** 0.221 ± 0.001 **T** 0.033 ± 0.004	85.0 ↓	17.0 ± 0.3	3.62
Rutin, 0.1	**C** 0.224 ± 0.002 **T** 0.171 ± 0.002	18.7 ↓	4.7 ± 0.2	3.38
Morin,0.1	**C** 0.224 ± 0.002 **T** 0.132 ± 0.001	41.0 ↓	8.3 ± 0.1	3.41
Diosmin, 0.4	**C** 0.335 ± 0.001 **T** 0.256 ± 0.001	23.6 ↓	1.6 ± 0.1	4.30
Daflon, 0.4	**C** 0.335 ± 0.001 **T** 0.247 ± 0.004	26.3 ↓	1.9 ± 0.1	4.25
Curcumin, 0.1	**C** 0.230 ± 0.002 **T** 0.158 ± 0.001	31.3 ↓	9.5 ± 0.1	3.46
Ascorbic acid, 1	**C** 0.375 ± 0.004 **T** 0.276 ± 0.002	26.4 ↓	576 ±9[b]	3.43
Thiourea, 25	**C** 0.367 ± 0.002 **T** 0.161 ± 0.001	56.1 ↓	44.9± 0.1[b]	3.42
DMSO, 80	**C** 0.384 ± 0.004 **T** 0.139 ± 0.002	63.8 ↓	15.7 ± 0.1[b]	3.60
Mannitol, 80	**C** 0.384 ± 0.004 **T** 0.202 ± 0.001	47.4 ↓	11.6 ± 0.1[b]	4.84
Benzoic acid, 200	**C** 0.221 ± 0.003 **T** 0.191 ± 0.002	13.6 ↓	1.4± 0.1[b]	2.95

The values are mean ± S.E. of 5 observations each; C and T are absorbance values, correspondingly, in absence and presence of test agent in the common solvent. Morin, quercetin, rutin, curcumin and benzoic acid were added in methanol as 0.1 mL, diosmin and daflon with 25 µmole acetate buffer, benzoate in pH adjusted solution and others in water. Activity unit µmoles[a] or nmoles[b] hydroxyl radical scavenged per µmole test agent.

Catechol oxidation in relation to pH

Fenton reaction is critically determined by ambient pH with optimal range within 3 to 6 units[25]. Maximum efficiency has been observed at pH 2.5 with hydroquinone and at pH 3.0 in presence of catechol and resorcinol [12]. Acidity provided by 0.005% HCl for catechol reagent was considered most appropriate during the present study based on trial experiments. This has provided an overall mean pH with pooled data over all assays as 3.52 ± 0.13 (n=42) with mean values during the standard curve as 3.27 ± 0.03 (n=9). Increased pH over 5.0 has been partly responsible for decreased response observed with tri-sodium citrate, potassium oxalate and potassium sodium tartrate (Table 3). Fenton reaction has reacted poorly at pH <3.0 and failed to occur at pH > 5.0 with sample discoloration including blanks. The Fenton reaction is not favored by pH lower than 3 or higher than 6. In fact, an increased pH leads to decomposition of hydrogen peroxide or iron precipitation[34]. In simulated experiments with standard masses, no activity was observed at pH 6 and 9, and the solutions remained colorless at pH 4.76. The color changed with increase in pH from light blue (pH 5.5) to deep blue (pH 8.4) to reddish (pH 9.1 to 10.4). The color alterations were apparently due to action of pH on catechol and iron.

Fenton reaction mediated generation of iron (III) from iron (II)

The simulated experiment demonstrated that iron (II) was quantitatively getting oxidized to iron (III) during the assay confirming its Fenton nature. H_2O_2 0.05 through 0.20 µmole in presence of 0.5 µmole iron (II) has lead to a linear increase in formation of iron (III) with regression estimate expressed in units as µmole iron (III) generated per each µmole of hydrogen peroxide (b ± S.E. = 1.04 ± 0.06, r ±S.E. = 0.993 ± 0.007, n=4). The assay was linear over 0.1 through 0.5 µmole iron (III) (r ± S.E = 0.9999 ± 0.0001, b ± S.E. = 0.959 ± 0.007).

Comparative response of dihydroxybenzenes to Fenton reaction

Fenton reaction is basically reduction of hydrogen peroxide by iron (II) to form hydroxyl radical and iron (III), the reaction continues owing to regeneration of iron (II) from iron (III) by reducing intermediates/agents in the medium. The reaction occurs even in presence of iron (III) or copper (II) wherein the transition metals are first reduced by hydrogen peroxide or other reducing agents to reduced form[5,16,23]. Dihydroxybenzenes including catechol and its structural isomers have accelerated Fenton reaction[12,17,18] by regenerating reduced iron with potency order as hydroquinone >catechol >resorcinol [12]. Catechol forms stable complexes with iron and copper and is easily oxidized to semiquinone and then to o-benzoquinone (colored brownish products) in presence of oxidizing agents, and participates in the oxidation-reduction cycle[20]. Our findings are perfectly in tune with these reported observations. At equivalent mass of 0.5 mg (ca. 4.5 µmole phenolic), three test phenolics reacted in a comparable manner with mean absorbance values for resorcinol, hydroquinone and catechol respectively as 0.341 ± 0.002, 0.348±0.001 and 0.372 ±0.003 (n=6 each). Spectral analysis revealed with catechol has revealed single peak at 359 nm while hydroquinone revealed a single peak at 341 nm. In contrast, resorcinol did not reveal any peak but displayed a smooth rising curve around 340 nm. Therefore, oxidized product(s) of catechol and its structural isomers are appropriately monitorable, respectively, at 360 and 340 nm. Consequently, the magnitude of mean absorbance values at about 1.25 hours at 360 nm was found in the order: catechol (0.354 ±0.001)> hydroquinone (0.341 ±0.001)> resorcinol (0.318 ±0.004) while at 340 nm, the order was found to be as hydroquinone (0.375 ± 0.001) > resorcinol (0.355 ± 0.001) >catechol (0.329 ±0.001).

Spectral analysis

Spectral analysis of the reactants in isolation and in combination have revealed that at chosen standard concentrations in 4 mL volume, hydrogen peroxide, FAS-II and catechol absorbed poorly within 300 to 700 nm range, the values 537 to 539 did not exceed 0.05. Catechol and hydrogen peroxide absorbed maximally at 357 nm (absorbance 0.398) with FAS-II, and at 363 nm (0.452) with FAS-III. This enabled choice of 360 nm as appropriate for the assay. A comparison of data observed at 360 and 400 nm indicated that the values at 360 nm were 1.79 ± 0.03 times (n= 20) more than the corresponding values at 400 nm. This observation would enable use of technique in laboratories where ordinary spectrophotometric measurements are possible within visible range only.

Copper Fenton reaction

Copper (II) has been found produce comparable catechol oxidation as seen with FAS-II. The assay has been linear over 0.25 through 5 µmole H_2O_2 with 1.0 mg catechol and 10 µmole of copper (II) with mean absorbance values at 0.25, 0.5, 1, 2, 3, 4 and 5 µmole of H_2O_2 respectively as 0.045 ± 0.001, 0.090 ± 0.002, 0.173 ± 0.003, 0.321 ± 0.004, 0.460 ±0.004, 0.591 ± 0.002 and 0.707± 0.003 (r ± S.E.= 0.999 ± 0.001, b ± S.E.=0.140 ± 0.003, n = 5 each) with steepest response over 0.25 through 2 µmole of H_2O_2 (r ± S.E. = 0.999 ± 0.001, b ± S.E. =0.157 ± 0.003). The mean pH has remained 2.70 ± 0.01 during the assay. Catechol (1 mg) *per se* failed to react with copper (10 µmole) or H_2O_2 (2 µmole) nor copper reacted with H_2O_2, mean absorbance remained either zero or less than zero. Catechol oxidation was evident only when the three reactants were present together with mean absorbance 0.345 ± 0.001. This validated that copper (II) has acted as catalyst to generate hydroxyl radical like iron [5, 32].

Conclusions

The work has validated the criteria necessary to suggest that the hydroxyl radical was produced by a Fenton-type reaction: (i) oxidation of catechol and its structural isomers by H_2O_2 in presence of iron (II), (ii)

enhancement of hydroxyl radical production by iron (II), (iii) inhibition of oxidation by inhibitors of Fenton reaction viz., agents chelating iron and/or neutralizing H_2O_2, and (vi) antagonism of oxidation by hydroxyl scavenging agents[3]. The work is further supported by comparable response shown by copper based Fenton reaction. The optimized assay protocol is simple and inexpensive, within the reach of common laboratories, to compare and screen agents capable of favoring or inhibiting or scavenging hydroxyl radical from Fenton reaction. The procedure has been used by the authors for evaluating extracts from indigenous medicinal plants for their hydroxyl scavenging activity (to be published separately). Further studies are required to develop and validate the assay with copper as transition metal. However, copper based Fenton reaction may not be compatible to use with flavonoids [35].

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