

A Sensitive *In vitro* Spectrophotometric Hydrogen Peroxide Scavenging Assay using 1,10-Phenanthroline

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ABSTRACT

Introduction: Hydrogen peroxide (H₂O₂) is a biologically important, non-radical reactive oxygen species (ROS) that can influence several cellular processes. Ability of anti-oxidants to scavenge H₂O₂ can be measured by several methods but all the methods are suffering from several lacunae due to poor reproducibility, lack of specificity, inaccuracy, high cost etc.

Objective: The present study aimed towards development of a rapid, low cost, reproducible, specific as well as sensitive and accurate method to detect H₂O₂ scavenging activity of anti-oxidants. **Methods:** We have used 1,10-phenanthroline and ferrous ammonium sulphate to detect H₂O₂ scavenging activity of anti-oxidants. **Results:** We revealed that our assay is able to detect the *in vitro* H₂O₂ scavenging activity of both phenolic and non-phenolic anti-oxidants. Furthermore, we found that this assay is highly specific, as scavengers of other types of ROS were unable to show any detectable effect on H₂O₂ through this assay. Finally, we tested commercially available non-steroidal anti-inflammatory drugs that are known to possess H₂O₂ scavenging activity and in such drugs also we were able to detect their H₂O₂ scavenging activity.

Conclusion: In conclusion, the proposed spectrophotometric method was rapid, cost effective, reproducible and highly specific to detect *in vitro* H₂O₂ scavenging activity of diverse compounds.

Key words: Anti-oxidant, H₂O₂, Phenolics, Reactive oxygen species, ROS.

INTRODUCTION

Reactive oxygen species (ROS) is a collective term used directly for free radicals or molecular species capable of generating free radicals. Most of the reactive oxygen species including superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂) and hydroxyl radical (•OH) are inevitably produced as byproducts of normal aerobic metabolisms and are increased during infections, exercise, stress conditions, radiations etc.¹ Amongst the ROS, H₂O₂ is an important molecule as although it is not toxic by itself, but can be

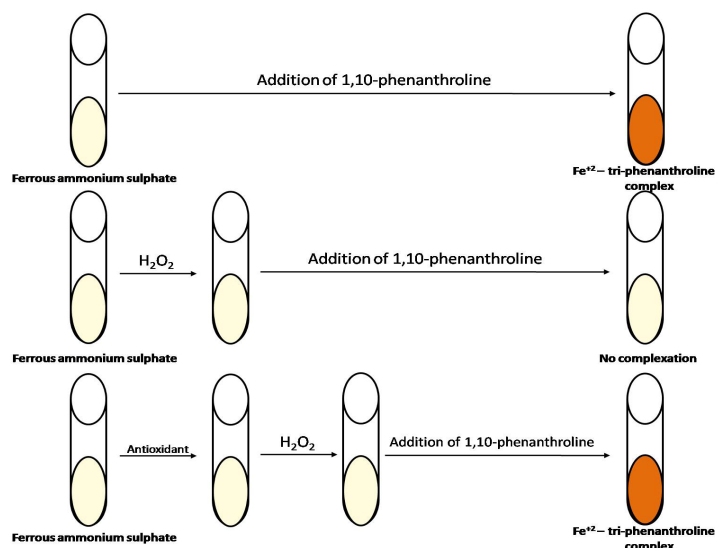
converted to other even more toxic radicals such as •OH by fenton reaction or hypochlorous acid by the enzyme myeloperoxidase (MPO, EC 1.11.2.2).² The generation of H₂O₂ by activated phagocytes is known to play an important role as bactericidal and antifungal since it also acts as mediators of inflammation by activation of signal transduction pathways.³

Uncontrolled generation of ROS is known to cause redox imbalance and oxidative stress which is harmful and responsible for various diseases including cancer, neurodegenerative disorders, autoimmunity etc. Conversely, live organisms can develop complex system of enzymatic and non-enzymatic anti-oxidant defences which can counteract the harmful effects of free radicals. Amongst the enzymatic system, superoxide dismutase (SOD, EC

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Graphical Abstract

1.15.1.1), glutathione peroxidase (GPx, EC 1.11.1.9), ascorbate peroxidase (EC 1.11.1.11), catalase (CAT, EC 1.11.1.6) are important, however, in non-enzymatic branch albumin, glutathione, ascorbic acid, α -tocopherol, β -carotene, uric acid, bilirubin and some flavonoids have been widely investigated.^{4,5}

Different anti-oxidants have various scavenging activities against $O_2^{\cdot-}$, H_2O_2 , $\cdot OH$ or peroxy radicals and many methods for measuring these properties have been established.⁶ Systems available for measuring H_2O_2 scavenging activity are based on direct UV absorption measurement (A_{230}) of H_2O_2 with/without incubation with scavenger, with the help of Fox reagent (Xylenol orange based), horseradish peroxidase (HRP, EC 1.11.1.7), spectrofluorimetric assay using homovanilic acid (HVA) and by replacement titration method.⁷⁻¹⁰ However, none of the methods are free from limitations like high background absorbance, non-specificity or reliability.⁸⁻¹⁰ These facts clearly necessitate for a robust H_2O_2 scavenging assay that could be applicable to diverse array of compounds.

The objective of this study is to develop a rapid, cost effective, robust method using commonly available instruments like spectrophotometer/colorimeter for H_2O_2 scavenging activity determination.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were of analytical grade. 1,10-phenanthroline monohydrate, ammonium iron (II) sulphate hexahydrate, sulphuric acid 98%, sodium azide, ascorbic acid and

hydrogen peroxide 30% were purchased from Merck, Bangalore, India. Mannitol, gallic acid, sodium pyruvate and uric acid were from Loba Chemie, Mumbai, India. Di methyl sulphoxide (DMSO) was from SRL, Mumbai, India. Indomethacin and Etodolac were purchased from Jagsonpal Pharmaceuticals Ltd, Uttarakhand, India and Ipcal Laboratories, Dehradun, India respectively.

Apparatus

Absorption spectra and intensities were measured at 25°C with an Evolution 201 uv-visible spectrophotometer from Thermo Fisher scientific using 1 cm x 1 cm quartz cells.

Principle

This assay is based on the reaction of ferrous ion (Fe^{+2}) with 1,10-phenanthroline (Figure 1a). Ferrous ion specifically forms red-orange tri-phenanthroline complex which absorbs maximally at 508-510 nm (A_{510}) and this assay has been used for a long time for quantitative measurement of iron in various samples.¹¹ It is known that if hydrogen peroxide is added to the tube before addition of 1,10-phenanthroline, then H_2O_2 will oxidize all the ferrous ion to ferric ion which is incapable of forming red-orange complex with 1,10-phenanthroline (Figure 1b) and a sharp reduction in A_{510} can be seen. This concept has been exploited for determination of H_2O_2 in the samples.^{12,13} Herein, we took advantage of this assay further in a way that after adding ferrous ion, the scavenger is added and followed by known amount of H_2O_2 for few minutes. If the scavenger is capable enough to scavenge the H_2O_2 added in the sample, no ferrous to ferric conversion would occur and detected by addition of 1,10-phenanthroline which

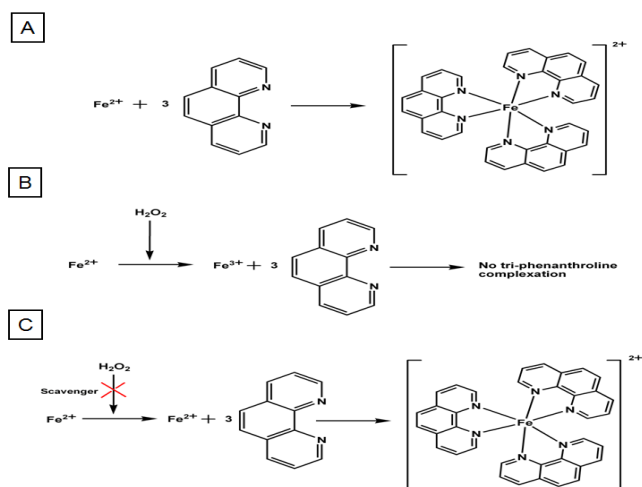


Figure 1: Schematic illustration of the reaction pathway leading to detection of hydrogen peroxide scavenging ability by anti-oxidants

(A) Reaction between Ferrous iron and 1,10-phenanthroline and formation of orange colored tri-phenanthroline complex. (B) Oxidation of ferrous ion to ferric by H_2O_2 leading to non-complexation of 1,10-phenanthroline (C) Addition of scavenger causes minimization of the effect of H_2O_2 resulting in formation of orange colored tri-phenanthroline complex.

yields a red-orange complex (Figure 1c). Conversely, if the scavenger is unable to scavenge H_2O_2 , then H_2O_2 converts all the ferrous ion to ferric which is unable to form coloured complex with 1,10-phenanthroline. Hence, the generation of red-orange ferrous-triphenanthroline complex will be directly proportional with the ability and concentration of the scavenger. Accordingly, the calculation of the ability of compounds having hydrogen peroxide scavenging activity was calculated using following formula:

$$\% \text{H}_2\text{O}_2 \text{ scavenging activity} = \frac{A_{\text{test}}}{A_{\text{blank}}} \times 100$$

where A_{blank} is the absorbance of solution containing only ferrous ammonium sulphate and 1,10-phenanthroline and A_{test} is the absorbance of the solution containing ferrous ammonium sulphate, hydrogen peroxide along with test compound having expected hydrogen peroxide scavenging activity and 1,10-phenanthroline. All the assays were done in triplicate and the data were showed in the graph as mean \pm SD.

Preparation of reagents

To prepare the ferrous salt, we used ferrous ammonium sulphate in 1 mM concentration. For 100 ml of 1 mM of the ferrous salt, we dissolve 40 mg of ferrous ammonium sulphate in 95 ml of double distilled water and 5 ml of 0.01N sulphuric acid. Hydrogen peroxide was prepared fresh everyday because it is photo labile. All the reagents

were dissolved in double distilled water except etodolac and indomethacin which were dissolved in absolute ethanol.

Determination of reaction time point between Fe^{+2} and 1,10-phenanthroline

To determine the end point of the reaction i.e. time required for the formation of the Fe^{+2} -tri-phenanthroline complex, we incubated 0.25 mL of 1 mM Fe^{+2} salt with 1.5 mL, 1mM 1,10-phenanthroline which was found to be sufficient to form red-orange Fe^{+2} -tri-phenanthroline complex. We incubated the tubes for 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 45 minutes and finally for 60 minutes at room temperature (25°C). After each incubation period absorbance was taken at 510 nm against a blank solution that contained only 1,10-phenanthroline solution.

Standardization of the volume of 1,10-phenanthroline

Although it has been shown earlier that the Fe^{+2} : 1,10-phenanthroline ratio should be at least 1:6 or more for optimum complexation,^[11] but in a recent paper it is proved that even 1:4 ratio between Fe^{+2} and 1,10-phenanthroline could be sufficient enough to form complex which can give strong absorbance at 510 nm.¹² To resolve the issue, we took a range of Fe^{+2} : 1,10-phenanthroline from 16:1 to 2:1 by volume (using 1 mM each of Fe^{+2} salt and 1,10-phenanthroline) and checked for the ratio where optimum complexation i.e. maximum absorbance could be observed.

Optimization of H_2O_2 incubation time, concentration and volume

Initially we incubated ferrous ammonium sulphate with 0.5 mL of 10 mM hydrogen peroxide for 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes and 30 minutes. Thereafter 1.5 mL of 1,10-phenanthroline was added and incubated for 10 minutes at room temperature. Finally absorbance was measured at 510 nm to determine the minimum absorbance i.e. maximum activity of H_2O_2 .

After determining the time, concentration of hydrogen peroxide was optimized by addition of different concentrations of hydrogen peroxide ranging from 10 mM-0.078 mM and incubated for 5 minutes. Thereafter absorbance was measured after addition of 1,10-phenanthroline followed by 10 minutes of incubation at 510 nm in room temperature.

Finally we standardized the volume of hydrogen peroxide by incubating 0-500 μL 5 mM hydrogen peroxide for 5 minutes with ferrous ammonium sulphate (0.25 mL, 1

Table 1: Hydrogen peroxide scavenging action of various anti-oxidant compounds found by the 1,10-phenanthroline method

Name of the compound	Mode of action	H ₂ O ₂ scavenging activity (IC ₅₀ value)
Gallic acid	General anti-oxidant	53.68 ± 2.91 µg/mL
Pyrogallol	General anti-oxidant	47.35 ± 3.01 µg/mL
Ascorbic acid	General anti-oxidant	426.80 ± 24.82 µg/mL
Sodium pyruvate	H ₂ O ₂ scavenger	>1000 µg/mL
Mannitol	OH. Scavenger	No effect
Dimethyl Sulphoxide (DMSO)	OH. Scavenger	No effect
Sodium azide	Singlet oxygen scavenger	No effect
Uric acid	Peroxynitrite scavenger	No effect
Etodolac	Anti-inflammatory drug	2699 ± 479.50 mM
Indomethacin	Anti-inflammatory drug	>5000 mM

*Values were Mean ± S.D, all assays were done in triplicate.

mM). Absorbance at 510 nm was measured after addition of 1.5 mL of 1,10-phenanthroline for 10 minutes in room temperature.

Optimization of incubation time with different concentrations of a known hydrogen peroxide scavenger

After standardization of all parameters as mentioned above, our next aim was to optimize the appropriate incubation time for the scavenger molecule, so that it could effectively scavenge the hydrogen peroxide and restore the ferrous ion which then subsequently forms a red-orange Fe⁺²-tri-phenanthroline complex with 1,10-Phenanthroline. We have used pyrogallol, a gallic acid derivative and known scavenger of hydrogen peroxide for our experiments in the range of 0-125 µg/mL.¹⁴ We have also tried higher concentrations including 1 mg/mL, 500 µg/mL, 250 µg/mL but at these concentrations, pyrogallol forms a red/brown complex with hydrogen peroxide which strongly absorbs 510 nm light, so we decided not to include those concentrations. During the experiment, we have tested a diverse incubation time period, ranging from 5 minutes to 60 minutes to determine and confirm the optimum incubation time for the assay.

Determination of specificity of the assay

Our final aim was to assess the specificity of the assay i.e. to show whether this assay is capable of detecting hydrogen peroxide only, not any other ROS such as hydroxyl radical which might be generated during assay by fenton reaction. To achieve this goal, we have used several scavengers specific for each type of ROS (Table 1) and both phenolic and non-phenolic scavengers of hydrogen peroxide (Table 1).

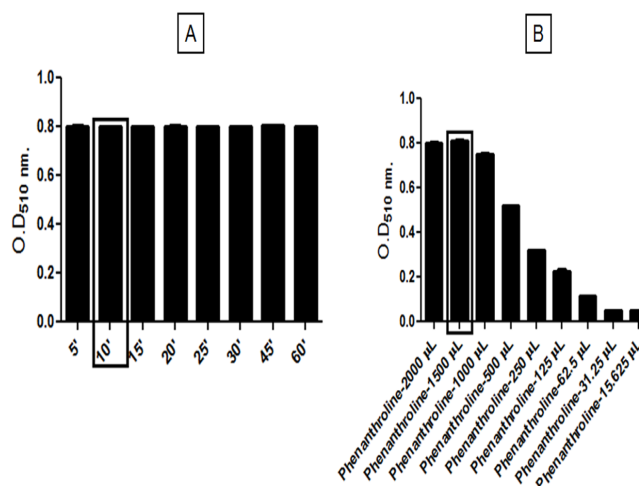


Figure 2: Determination of optimum time point of the reaction and accurate ratio between ferrous ammonium sulphate and 1,10-phenanthroline

(A) The absorbance of tri-phenanthroline complex formation started from 5 minutes and remains stable up to 60 minutes shows that the complex is stable. (B) The ratio of ferrous ammonium sulphate and 1,10-phenanthroline from 16:1 (right most) to 1:8 (left most), shows that the ratio of 1:6 gives the best result.

H₂O₂ scavenging activity of non-steroidal anti-inflammatory drugs (NSAIDs)

To determine the ability of H₂O₂ scavenging activity of NSAIDs we first measured the average dry weight of the indomethacin capsules and etodolac tablets and then measured the average content of the respective drugs. Finally, after crushing the capsular content of indomethacin and etodolac tablets to a fine powder with the help of mortar and pestle, we dissolved the drugs in alcohol at a concentration of 5 mM from which respective dilutions of 2.5, 1.25, 0.625 and 0.3125 mM were made. The assay was performed as described previously except 0.2 mL of 0.5 M H₂SO₄ was added before addition of the drugs or alcohols to maintain the acidic pH of the solution required for the

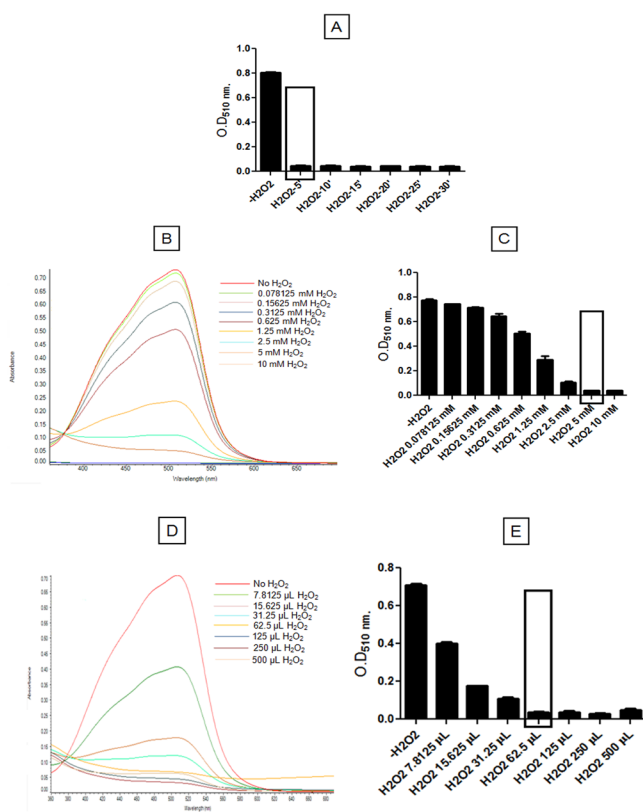


Figure 3: Standardization of time point, concentration and volume of H_2O_2 for oxidation of ferrous to ferric iron

(A) Incubation with H_2O_2 for variable time point showed that 5 minutes was sufficient for conversion of ferrous to ferric as evident in sharp decrease in absorbance at 510 nm. (B) Absorption spectra of solutions containing variable concentrations of H_2O_2 . (C) Bar diagram showed that with increasing concentrations of H_2O_2 absorbance was decreased, the optimum concentration being 5 mM. (D) Absorption spectra of solutions containing variable volumes of H_2O_2 at 5 mM concentration. (E) Bar diagram showed that with 62.5 μL of H_2O_2 was sufficient to convert ferrous to ferric iron.

stable complexation between ferrous ammonium sulphate and 1,10-phenanthroline.

RESULTS

Optimization of reaction time point as well as the ratio between ferrous ammonium sulphate and 1,10-phenanthroline

We have used a broad incubation time range to assess the time of reaction between ferrous ammonium sulphate and 1,10 phenanthroline and observed that after 5 minutes the reaction is completed and a stable red-orange complex was formed which was stable upto 60 minutes (Figure 2a). Additionally, we have determined the optimum ratio between ferrous ammonium sulphate and 1,10-phenanthroline as there is conflicting data regarding the ratio between this two reagents.^{11,12} Our result showed that the optimum ratio between ferrous ammonium sulphate and 1,10-phenanthroline was 1:6 (Figure 2b).

Hydrogen peroxide time, concentration and volume standardization

After initial standardization of reaction time and volume of ferrous ammonium sulphate and 1,10-phenanthroline, our next aim was to optimize the incubation time after the addition of hydrogen peroxide (H_2O_2) for complete oxidation of ferrous to ferric along with its concentration and volume. We initially took a high concentration of H_2O_2 (10 mM) and incubated with the ferrous salt for 5 minutes to 30 minutes in different tubes with a 5 minute interval. Our result revealed that within 5 minutes H_2O_2 was able to oxidize all the ferrous salt to ferric for which the complex coloured tri-phenanthroline complex was not formed and the absorbance was definitely low (Figure 3a).

After the time point calibration we aimed to standardize the concentration of H_2O_2 which was initially used at a high concentration (10 mM). We took a range of H_2O_2 concentration from 10 mM-0.078 mM and incubated with ferrous ammonium sulphate for previously optimized 5 minutes. We observed that the lowest concentration of H_2O_2 that was able to convert maximum ferrous to ferric in 5 minutes was 5 mM (Figure 3b and 3c).

After calibration of time point and concentration, our final aim was to fix the volume of the H_2O_2 . In all the previous assays (Figure 3a, b and c) we used 0.1 mL of H_2O_2 . Now, we took a full volume range from 500 μL to 7.81 μL . We observed that at the volume of 62.5 μL for incubation time of 5 minutes, 5 mM H_2O_2 was able to convert all ferrous ions to ferric ions (Figure 3d and 3e).

Determination of hydrogen peroxide scavenging potential of a known anti-oxidant

For this particular experiment, we have used pyrogallol (a polyphenolic compound, derivative of gallic acid) which is known for its H_2O_2 scavenging ability.¹⁴ Here we used different concentrations of pyrogallol (125-1.95 $\mu g/mL$) to measure its potency. We took a range of incubation time with 1,10-phenanthroline from 5-60 minutes to finalize the reaction time point with the scavenger molecule and found that after 10 minutes the difference of scavenging activity by different concentrations of pyrogallol revealed best result (Figure 4). Moreover, our result showed that with increasing concentrations of pyrogallol the effect of H_2O_2 on ferrous ammonium sulphate was minimized and tri-phenanthroline complexation was found to be increased as evident by increase in absorbance at 510 nm (Figure 4).

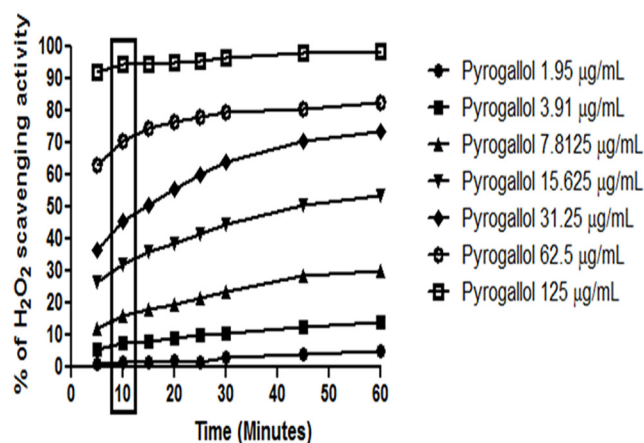


Figure 4: % of H₂O₂ scavenging activity by pyrogallol (µg/mL) vs. incubation time (minutes) curves

Result showed that as minimum as 10 minutes was able to detect the differences of H₂O₂ scavenging activity by different concentrations of pyrogallol.

Scavenging assay using 1,10-phenanthroline is highly specific for H₂O₂

We used different types of ROS scavengers to determine the specificity of this H₂O₂ scavenging assay as mentioned in Table 1. Furthermore, we confirmed that the assay was able to detect activity of both polyphenolic (pyrogallol and gallic acid) and non-polyphenolic (ascorbic acid and sodium pyruvate) H₂O₂ scavengers (Figure 5a-d). Our assay also proved to be sensitive enough as it was able to detect the H₂O₂ scavenging activity of polyphenolic compounds in as low as 1.95 µg/mL and 7.81 µg/mL for non-polyphenolic compounds (Figure 5a-d). In terms of specificity, we proved that this assay is highly specific for detection of hydrogen peroxide scavengers, as scavengers of hydroxyl radicals (mannitol, DMSO), singlet oxygen (sodium azide), peroxyxynitrite (uric acid) were unable to form any detectable colour and thus showed no scavenging activity (Figure 5e-5g).

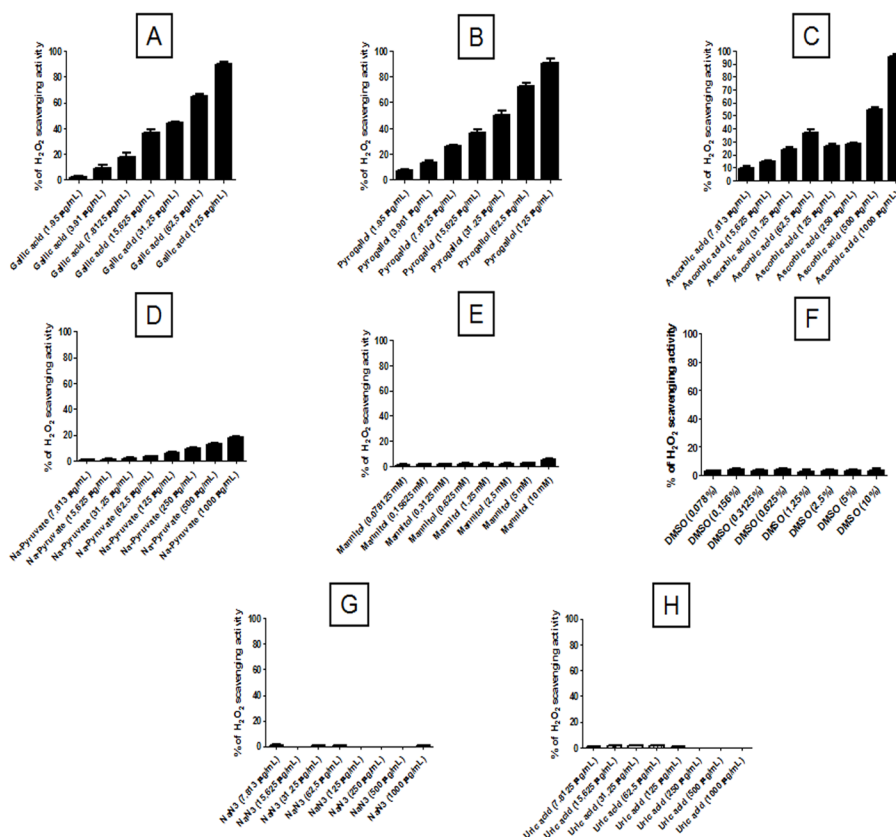


Figure 5: Determination of specificity of the assay for H₂O₂

(A) Bar diagram showing the % of H₂O₂ scavenging activity of gallic acid in different concentration (1.95 to 125 µg/mL). (B) Bar diagram showing the % of H₂O₂ scavenging activity of pyrogallol in different concentration (1.95 to 125 µg/mL). (C) Bar diagram showing the % of H₂O₂ scavenging activity of ascorbic acid in different concentration (7.813 to 1000 µg/mL). (D) Bar diagram showing the % of H₂O₂ scavenging activity of sodium pyruvate in different concentration (7.813 to 1000 µg/mL). (E) Bar diagram showing that mannitol has no H₂O₂ scavenging activity in the concentration range of 0.078125 to 10 mM. (F) Bar diagram showing that DMSO has no H₂O₂ scavenging activity in the concentration range of 0.078 % to 10 % v/v solution. (G) Bar diagram showing that sodium azide has no H₂O₂ scavenging activity in the concentration range of 7.8125 to 1000 µg/mL. (H) Bar diagram showing that uric acid has no H₂O₂ scavenging activity in the concentration range of 7.8125 to 1000 µg/mL.

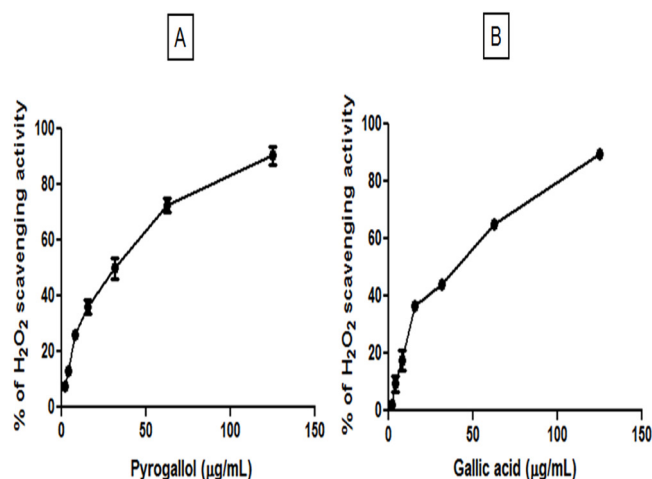


Figure 6: Efficiency of H₂O₂ scavenging activity of pyrogallol and gallic acid

(A) Effect of concentration on the H₂O₂ scavenging activity of pyrogallol (1.95 to 125 µg/mL) using the 1,10-phenanthroline method. (B). Effect of concentration on the H₂O₂ scavenging activity of gallic acid (1.95 to 125 µg/mL) using the 1,10-phenanthroline method.

Final optimized procedure

To a series of test tubes, 0.25 mL of ferrous ammonium sulphate (1 mM) were added. Then 1.5 mL of different concentrations of pyrogallol or gallic acid were added and mixed. Hydrogen peroxide at a concentration of 5 mM, 62.5 µL was added thereafter incubated at room temperature in dark (because hydrogen peroxide can be photo bleached) for 5 minutes. After incubation, 1.5 mL of 1 mM 1,10-phenanthroline was added to each tube, mixed well and incubated for 10 minutes at room temperature. Finally absorbance was taken at 510 nm through a spectrophotometer (Figure 6a and 6b). The blank solution contained only ferrous ammonium sulphate (0.25 mL, 1mM), water (1.562 mL) and 1,10-phenanthroline (1.5 mL, 1 mM). The blank tube showed maximum absorbance. An additional reagent blank was also prepared which contained 1,10-phenanthroline only and the absorbance of this tube was subtracted from all tube.

Etodolac is better scavenger of H₂O₂ than Indomethacin

Finally our method of assay showed that both etodolac and indomethacin were able to scavenge H₂O₂ in a dose dependent manner starting from 312.5 mM (Figure 7). Etodolac was shown to be a much better scavenger than indomethacin as it possessed almost a 4 fold higher scavenging ability (Figure 7).

DISCUSSION

Measurement of anti-oxidant activity of medicinally important plant extracts, anti-inflammatory drugs has

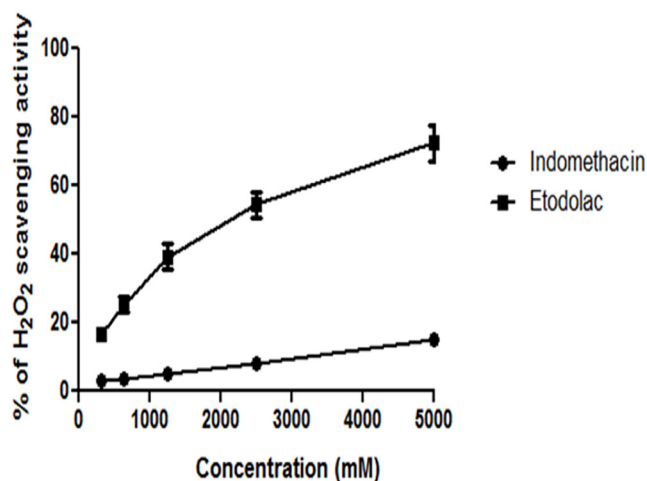


Figure 7: H₂O₂ scavenging action of commercial non-steroidal anti-inflammatory drugs

Hydrogen peroxide scavenging action of etodolac and indomethacin in the concentration range of 312.5 to 5000 mM.

been widely reported for a long time because of the wider applications of anti-oxidants in food-nutrition and cosmetics industries. This is because ROS is proved and proposed to be one of the most important factors in the pathogenesis of diverse diseases including autoimmune diseases like rheumatoid arthritis, diabetes, cardiovascular diseases, neurodegenerative disorders like Alzheimer's, Parkinson's as well as majority of the cancer types.¹⁵⁻¹⁷ Hydrogen peroxide (H₂O₂) is regarded as less reactive because of its weak oxidizing ability and relatively less toxicity but as it is stable under physiological pH and temperature with the capacity to penetrate membrane as well as diffuse to long distances, it plays a key role in pathogenesis of many diseases.¹⁵ Furthermore, in presence of transition metal, it is converted to more toxic hydroxyl radical that can mediate cellular damages. It can also generate singlet oxygen by reacting with superoxide anion or with hypochlorous acid (HOCl) or chloramines in living systems.⁸ Thus, measurement of H₂O₂ scavenging activity is an important part for assessment of the anti-oxidant activity.

Evaluation of H₂O₂ scavenging activity has been described by various authors with diverse technologies like UV absorption method,¹⁸ fluorimetry using homovanilic acid (HVA),¹⁹ colorimetry,^{9,14,20} chemiluminescence,²¹ by Raman spectroscopy etc.²² Among these methods, UV absorption at 230 nm by H₂O₂ has been used most extensively but this method has serious shortcomings in that,⁸ most of the plant derived compounds or polyphenols have a strong intrinsic absorption at that region which limits the precision, accuracy and thus applicability of this method. (data not shown)^{14,23} When considering other methods

used for the determination of H_2O_2 scavenging activity, such as fluorescence spectroscopic method based on HVA and peroxidase are most common.¹⁹ Being a fluorescence method it is highly selective and sensitive, but fluorescence generated can be lost either by quenching, resonance energy transfer and inner filter effect in the presence of various endogenous phytochemicals. Furthermore, fluorescence spectrometry is a costly method.²⁰ H_2O_2 scavenging activity of plant extracts can be measured by replacement titration method.¹⁰ This method is based on iodide oxidation by hydrogen peroxide and is a time consuming macro method where a titration is involved and its sensitivity is very low (data not shown).²⁴ Other methods of colorimetric detection using tetra-methyl benzidine (TMB) along with Horse radish peroxidase (HRP) are although sensitive but are still a costly method. The chemiluminescent methods based on HRP along with chemiluminogenic probes like luminol and peroxyoxalates also have some disadvantages. Several compounds like ascorbate, thiols like glutathione are substrates for HRP and therefore can compete with the probe during detection resulting in erroneous measurement of scavenging activity. Moreover, these methods are expensive and require precision skill due to stringent experimental conditions.²⁵

Our assay utilized the reaction between ferrous ammonium sulphate and 1,10-phenanthroline which formed a red-orange coloured Fe^{+2} -tri-phenanthroline complex that absorbed at 510 nm. This method has been utilized routinely for measurement of iron in many types of samples and is considered as very sensitive for the same.²⁶ We in this paper, improvise this technique to measure H_2O_2 scavenging activity and showed that this method is highly sensitive as scavenging action can be detected in very low concentration of scavenger (Figure 4), specific (Figure 5), reproducible and most importantly able to detect H_2O_2 scavenging property of both phenolic as well as non phenolic anti-oxidants (Figure 5a-d). Moreover, this method is rapid (total time taken is within 20 minutes), requires simple UV-visible spectrophotometer which is present in most laboratories, cost effective as all the chemicals required for the assay is

inexpensive than the ingredients of other available methods and requires less skill. In terms of chemistry, the principle is easy to understand (Figure 1), it is compatible with common solvents such as water and alcohol. We also observed that although di methyl sulphoxide (DMSO) itself has no H_2O_2 scavenging activity up to 10% (v/v in water) concentration (Figure 5f) but when it was used as the solvent, formation of tri-phenanthroline complex was prevented to some extent (data not shown). We measured the H_2O_2 scavenging activity of two non steroidal anti-inflammatory drugs (NSAIDs) namely indomethacin and etodolac which are already proved to have anti-oxidant activity.²¹ Our assay showed that etodolac is a better scavenger of H_2O_2 than indomethacin (Figure 7). This proves that the assay can be readily used for detection of H_2O_2 scavenging potential of commercially available compounds.

CONCLUSION

The present spectrophotometric method can be considered as a precise and accurate alternative approach for quantification of H_2O_2 scavenging activity. This method will hopefully be convenient for the evaluation of the anti-oxidant activity of phenolic and non-phenolic as well as natural and synthetic anti-oxidants in various biological or commercial samples.

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CONFLICTS OF INTEREST

Authors have no conflict of interest. Funders have no role in writing the manuscript or in data analysis.

Highlights of the Paper

- Measurement of hydrogen peroxide scavenging ability of anti-oxidants was measured using a common ferrous iron probe 1,10-phenanthroline.
- The assay was proved to be very rapid (as it took only 20 minutes), cheap method.
- The assay showed remarkable specificity and sensitivity.
- Both polyphenolic and non phenolic anti-oxidants showed good results.
- This method is found to be suitable for commercial products also.

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