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Spectrophotometric Assay based on Horseradish Peroxidase- Catalysed Hydrogen Peroxide using Amino Antipyrine and Resorcinol as Chromogenic Reagents for Sensitive Detection of Peroxidase in Plant Extracts

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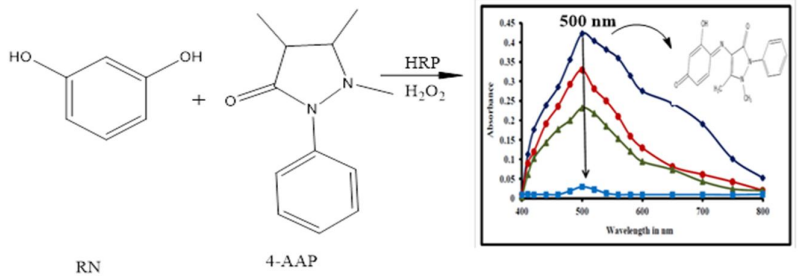
***Abstract*— We reported a unique enzyme- catalyzed hydrogen peroxide system for the detection of Horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). The catalytic system involving 4-Aminoantipyrine (4-AAP) and resorcinol (RN) as chromogens. In this study, the red-colored quinone-imine radical generated by the oxidation of RN with peroxidase catalyzed H_2O_2 oxidation, coupling with the aminyl radical of 4-AAP that has absorption peak at 500 nm. The absorption intensity of the quinone-imine radical scaled linearly with H_2O_2 concentration in the ranges of 1.1522µM-294.98 µM and 0.5761- µM- 82.96 µM with a detection limit of 0.12 µM and quantification limit of 0.225 µM and peroxidase concentration 0.0425-5.8 nM and 0.02-2.99 nM for kinetic and fixed time methods could be achieved. The kinetic parameters K_m and V_{max} were found to be 172.41 µM and 0.1136 EU min^{-1}. Moreover, compared with standard guaiacol system, HRP/ H_2O_2/4-AAP/RN system had wide linear ranges, low detection limit, Km and Vmax value. Noteworthy, the present study was successful applied to monitor peroxidase concentration in different plants extracts with satisfied results. Overall, the proposed system can be adopted as a candidate system for the determination of H_2O_2.**



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***Index Terms*— Resorcinol, Horseradish peroxidase, Michaelis -Menten constant, guaiacol, Hydrogen peroxide.**

I. INTRODUCTION

Enzymes, which are biocatalysts that have catalytic activity that catalyze various bio-chemical processes in organisms. These catalysts are key to increase and regulate the chemical reactions in cell media. According to the reaction that they catalyze, they are widely divided into six major classes. Among enzymes, Peroxidases (E.C.1. 11..1.X) enzyme found abundance in bacteria and eukaryotes, belongs to oxidoreductases class [1]. Peroxidases are distinguished into two forms, heme and nonheme peroxidases based on the heme (iron protoporphyrin IX) group [2]. Horseradish peroxidase (EC.1.11.1.7) enzyme is a class III member of heme peroxidase family, has a heme protein consisting of iron (III) metal ion with two calcium atoms. composed of different isoenzymes, and the commercial peroxidase is commonly isolated from the root of horseradish (*Armoracia rusticana*) plant which belongs to the Brassicaceae family [3]. Nevertheless, innumerable plants can serve as sources of peroxidases enzyme for diverse applications. Therefore, depending on the source, peroxidases with different properties are found such as differ in their amino acid composition, pH, thermal stability and substrate specificity [4]. among peroxidases, HRP has been widely studied and used enzyme due to its high selectivity and specificity and it plays an important factor in widespread applications in various fields such as pharmaceuticals, food industry, biotechnology, biology, agriculture and environmental applications but it is highly applicable in biological sector due to its catalytic action it destroys phenolic compounds during an oxidation process [5]. Nevertheless, an important limitation of this biocatalysts are low activity and stability in some conditions when compared to chemical catalysts. Peroxidase is extensively used as an index of blanching due to its heat thermal stability [6] Peroxidases are involved in the conversion of toxic phenolic compounds into quinones hence it plays a major role in the plant defense system. It is also used in the removal of amines and phenols from industrial wastewater and the detection of nucleic acid [7]. Peroxidases play an important role in the diagnostic assay, polymer synthesis, biosensors, and various organic syntheses [8-10]. The peroxidase reaction has widespread applications in the field of biochemistry due to the formation of H_2O_2 by the oxidase reaction can be used in the determination of clinically important biomarkers such as cholesterol, glucose, and uric acid [11-13]. In plants, peroxidase is responsible for building cell walls and growth regulation due to the metabolism of the auxin hormone [14]. Peroxidase is widely employed for the manufacture of enzyme-conjugated antibodies for use in diagnostic kits [15]. Because of its significance many chromogenic reagents and many analytical methods are designed for the quantification of peroxidase activity, namely amperometry [16], HPLC [17], spectrofluorimetric [18], electrochemical [19], flow injection analysis [20], cyclic voltammetry [21], radiometer biosensor [22], Raman scattering [23], luminescence [24], microtiter plate reader [25], and potentiometric assay [26]. However, they have some demerits, too expensive, less versatile, poor sensitivity, multi-step, high incubation period, and need of expensive biocatalyst. Some of the reagents used for the quantification of peroxidase are 2,4-DMA [27], Paraphenylenediamine/mequinol [28], Para-acetylaminophenol [29], iminodibenzyl/ paraphenylenediamine dihydrochloride [30], N, N-diethyl-p-phenylenediamine sulphate/3-Aminophenol [31], para phenylene diamine/3-Dimethylaminobenzoic acid [32], 2,5-DMA [33], 8-hydroxyquinoline/ para-phenylenediamine dihydrochloride [34]. Some of these reagents used are low sensitivity, toxic, carcinogenic, poor solubility, and Mutagenicity.

Hence, the purpose of this work was to (I) construct an innovative catalytic spectrophotometric system for the determination of peroxidase and H_2O_2 on the basis of enzyme-catalyzed oxidation of RN, (II) Investigate the kinetic parameters (such as reaction rate, effect of co-substrates, pH, temperature, substrates, and buffer, (III) Investigate the stability of 4-AAP/RN system, (IV) Applying the 4-AAP/RN system to analyse peroxidase concentration in plants crude extracts.

The proposed catalytic system is based on the enzymatic consumption of H_2O_2 using 4-AAP/RN as a chromogenic co-substrate that had a strong absorption of red-colored species at λ_{max} 500nm. To validate the proposed system, the results were compared with the results obtained from the reference guaiacol method [35].

II. PROCEDURE

A. Instrumentation

The absorbance measurements were recorded on a UV-Vis spectrophotometer (UVIDEC-610, Jasco model) using 1 cm quartz cuvettes. The pH measurements were measured on a digital pH meter (Version- EQ-614,

Equip- Tonics model, Mumbai, India). A water bath shaker (Version-206-88950-93, Japan) with temperature controller was used for the color development of working solutions.

B. Chemicals

All reagents used in the PPD/AN system were of pure grade chemicals. All required reagent solutions were made by deionized water. Peroxidase (E.C. 1.11.1.7, 100 units/mg) from Horseradish, 4-Aminoantipyrine (4-AAP), resorcinol (RN) was purchased from Sigma-Aldrich and used as received. Potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) was supplied by Hi media laboratories, Mumbai, India and used without further purification.

C. Crude Extract Sample Preparation

Disease-free flowers portion of *Leucas aspera, Catharanthus roseus, Nerium oleander, ixora coccinea, Taberna Montana divaricate*, were collected from the local agricultural field as a source of peroxidase and transported at 4 ºC to the laboratory, and the sample was stored at -20 ºC till used. 5g of the sample was washed using double distilled water and blended using 100 mL of 100 mM phosphate buffer at pH 7.8 using a blender. The extract was filtered using cheesecloth and centrifuged the filtrate at 12000g for 15mins and supernatant was collected as a crude extract.

III. Proposed Experiment Protocol

A. Quantification of Hydrogen peroxide

The H_2O_2 concentration was examined in a 3ml of reaction mixture containing 164 µM 4 AAP, 1210.8 µM RN, 5.8 nm peroxidase in 100 mM KH_2PO_4/NaOH buffer at pH 7.3. The H_2O_2 concentration used in the range of 0.5761-294.98 µM. The change in the absorbance was continuously recorded at 500nm by adding 100 µl of different concentrations of H_2O_2 at a 1min time interval. The λ_{max} linearity range of the H_2O_2 concentration lies between 1.1522µM-294.98 µM and 0.5761- µM- 82.96 µM from the kinetic and fixed time method. The calibration graph for the effect of H_2O_2 on the rate of reaction is shown in Fig. 1 and 2.

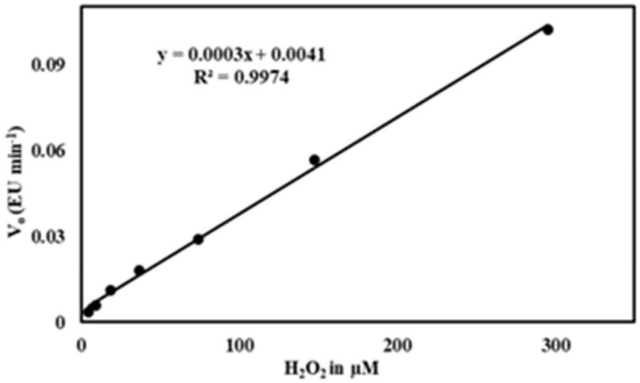

Figure 1. Calibration graph for the quantification of H_2O_2 from the rate method

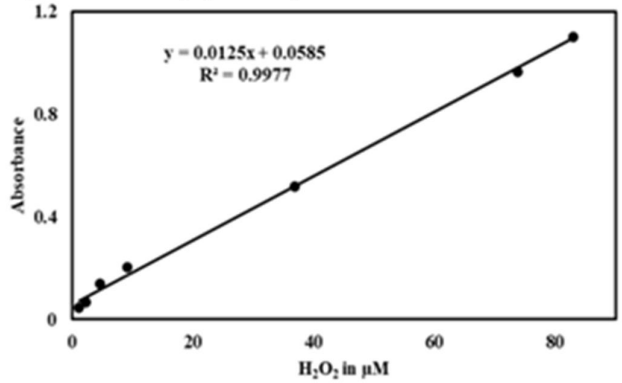

Figure 2. Calibration graph for the quantification of H_2O_2 from the fixed time method

B. Peroxidase Assay

The quantification of peroxidase was measured with change in absorbance concerning time for 5min at 1min interval, a 3ml reaction mixture containing 164 µM 4 AAP, 1210.8 µM RN, KH_2PO_4/NaOH buffer at pH 7.3, 294.8 µM H_2O_2, and 100 µl of different concentrations of peroxidase varying in the range from 0.0425-5.8 nM. The linearity range of the peroxidase by the fixed time method was evaluated by incubating the 3ml reaction mixture for 10min at optimized temperature (30 ^{0}C) and recording the absorbance of the red-colored solution at λ_{max} 500nm is shown in Fig. 3 and 4.

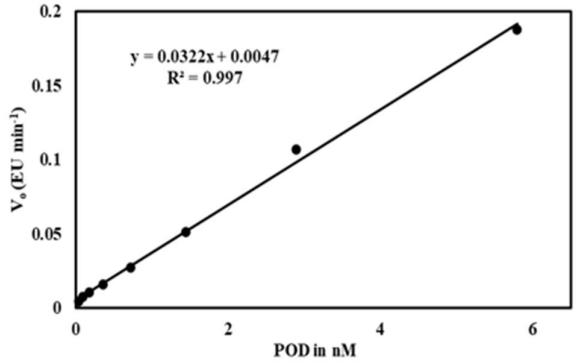

Figure 3. Calibration graph for the quantification of peroxidase from the rate method.

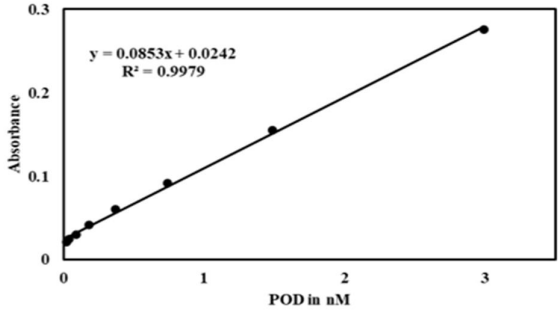

Figure 4. Calibration graph for the quantification of peroxidase from fixed time method.

C. Absorption Spectrum with Colored Product

The absorption spectra of the red-colored product obtained at different concentration of hydrogen peroxide were measured by the proposed assay in the wavelength range 400-800 nm. The working solution was incubated at 30 ^{0}C for 5 mins against the corresponding blank reagent after the spectra were recorded at a 2 nm/sec scan rate on a spectrophotometer. Fig. 5 depicts the absorption spectra of the red-colored product. The maximum absorbance of the proposed assay was found to be at 500 nm.

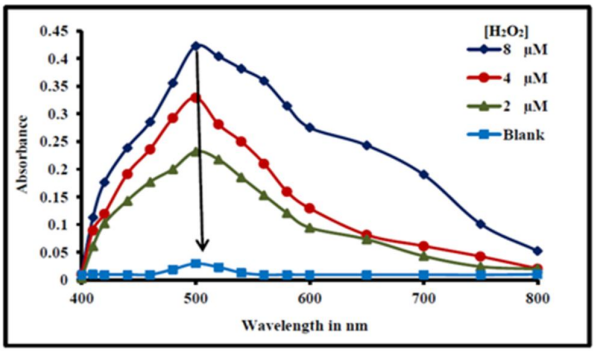

Figure 5. Absorption spectra of the red-colored product.

D. Optimization of experimental conditions

Optimizations of experimental variables such as the effect of co-substrates, different buffer concentrations, the effect of substrates, temperature, and incubation period, these experimental conditions affect enzyme assay have been studied.

E. Effect of co-substrates on enzyme activity

The influence of co-substrates, 4-AAP, and RN on the rate of reaction was studied,

The effect of different concentrations of 4-AAP varying in the range of 41 µM to 620 µM and resorcinol varying in the range of 37.8375 µM to 1513.5 µM. The results showed that the rate of reaction increased by increasing the concentration of 4-AAP up to 164 µM and RN up to 1210.8 µM. Farther on there is no considerable increase in the rate of reaction. Hence 164 µM 4-AAP and 1210.8 µM RN were chosen as optimized concentrations for further assays. The linearity graph of co-substrates is shown in Fig. 6 and 7.

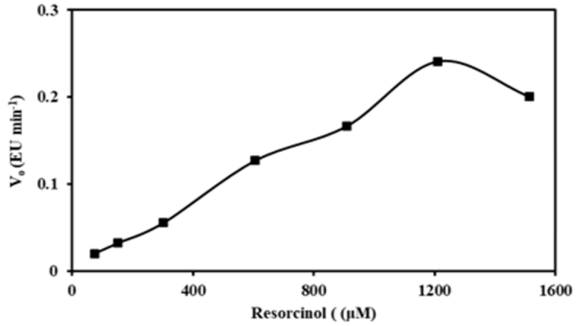

Figure 6. Effect of RN on the reaction rate

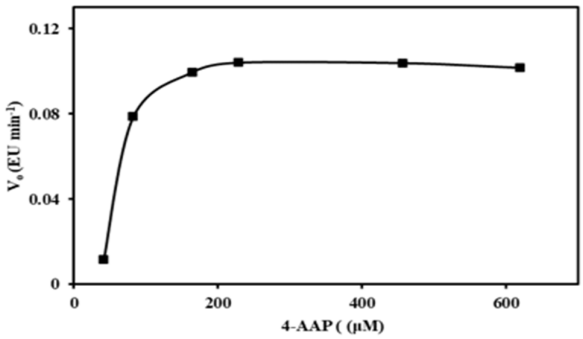

Figure 7. Effect of RN on the reaction rate

F. Effect of Temperature on enzyme activity

The temperature effect on the enzyme activity was determined by 3ml of the reaction mixtures containing 1210.8 µM RN, 164 µM 4 AAP, 294.98 µM H_2O_2, 5.8 nM peroxidase and 100 mM KH_2PO_4/NaOH buffer of pH 7.3 and incubate the reaction mixtures at a temperature in the range of 0-80 ^{0}C for 10 mins. The enzyme activity increased up to 30^{o}C and thereafter decreased. Hence 30 ^{0}C temperature was selected for further assays as depicted in Fig. 8.

G. Effect of pH and Concentrations of Buffers on Enzyme Activity

The influence of pH on the enzyme activity was quantified by using the concentrations ranging from 0.1 M to 2.0 M of different buffers such as sodium acetate/ acetic acid buffer with pH in the range of 3.6-5.6, KH_2PO_4/NaOH buffer pH 6.0–8.0, citric acid/potassium citrate buffer of pH ranging from 3.6–5.6, KH_2PO_4/K_2HPO_4 buffer 5.93-7.5, Tris- HCl buffer of pH 9.8, The experimental result (Fig. 9) depicts that the enzyme activity was maximum at KH_2PO_4/NaOH buffer at pH 7.3. Hence KH_2PO_4/NaOH buffer of pH 7.3 was selected as optimized pH. The volume of the buffer added (0.1–1ml) also influences the activity of the enzyme, in a 0.1 ml buffer the reaction shows maximum activity hence 100 µL was chosen as optimized pH concentration.

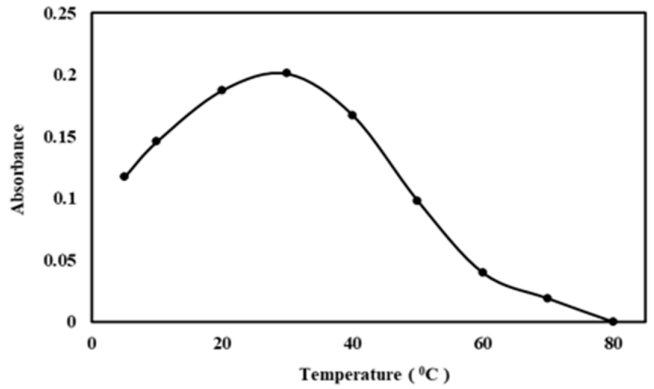

Figure 8. Effect of the temperature on the reaction

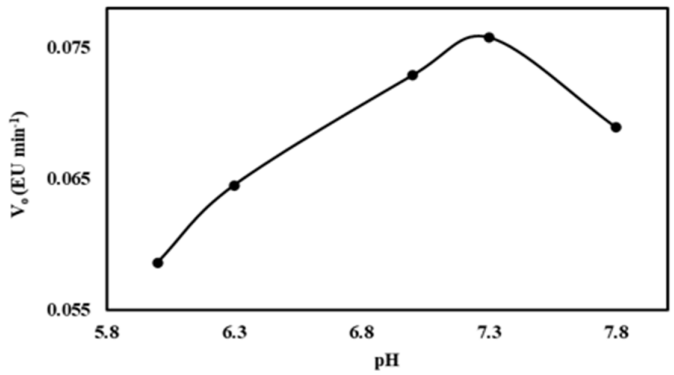

Figure 9. Effect of pH on the rate of reaction.

H. Order of Reagent Addition

The enzyme activity is also affected by the order of reagent addition hence the order of reagent addition is also quantified. The experimental results depict that the reaction was optimum when the reagents were added in the following order – 4-Aminoantipyrine, resorcinol, buffer, H_2O_2, water, and Peroxidase. So, this order of addition of reagents was selected for subsequent assays.

IV. Assessment of Analytical Parameters on Enzyme Activity

The kinetic factors of the proposed assay protocol were analyzed under optimized conditions. **Fig. 10** depicts the Line weaver- Burk plot (L-B plot) used to calculate Michaelis -Menten constant (K_m) for the projected method with the linear regression equation ($1/V_0$=1496.2(1/C H_2O_2) +8.7966) with a correlation coefficient of 0.9973. The Km value for PPDA- AN coupling assay was witnessed to be 172.41 µM, the Km value shows the affection between the enzyme active site and substrate. The K_m value of the proposed method is low as compared to earlier reported methods [27,28,32,33,36] the lower value of the K_m indicates more affinity between enzyme and substrate of the proposed assay. The maximal velocity (V_{max}) of the projected peroxidase catalyzed reaction is found to be 0.1136 EU min^{-1}, the V_{max} value signifies how rapidly the enzyme peroxidase catalyzed the reaction, obtained by the L-B plot.

The specificity constant (k_{sp}) of the assay was found to be 0.0001 $\mu m^{-1} min^{-}1$, it conducts how efficiently enzyme convert substrates into products, the k_{sp} measured by the equation,

$$specificity\ constant = \frac{k_{cat}}{K_m}$$

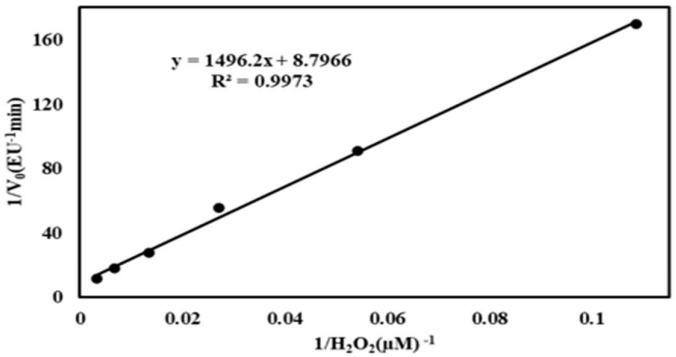

Figure 10. Lineweaver- Burk plot for POD.

The Catalytic constant (k_{cat}) of the projected method was found to be 0.0195 min^{-1}, it measures enzymes' maximum rate, the more the k_{cat} greater the number of substrates get turned into product per second.

$$catalytic\ constant = \frac{V_{max}}{[E]_0}$$

where $[E]_0$= enzyme concentration

The catalytic power (K_{pow}) was found to be, 0.0006 EU $\mu m^{-1}min^{-1}$. the more value of K_{pow} shows the substrate used for enzymatic reactions is best, catalytic power is deliberated by

$$catalytic\ power = \frac{V_{max}}{K_m}$$

The catalytic efficiency(k_{cat}) was found to be 0.0001 $\mu m^{-1}min^{-1}$, the value of k_{cat} shows that how efficiently an enzyme catalyzes a reaction.

$$catalytic\ efficiency = \frac{1}{slope} \times \frac{1}{[E]_0}$$

V. METHOD VALIDATIONS

A. Analytical Performance

The quantification of hydrogen peroxide was carried out under experimental optimized conditions, the proposed assay reveal that the reaction rate increases with increasing the H_2O_2 concentration up to 294.98 µM. the excellent linearity range of H_2O_2 concentration lies between 1.1522µM-294.98 µM from the rate method and 0.5761 µM- 82.96 µM by the fixed time method from the calibration graph. the limit of detection (LOD) and limit of quantification (LOQ) analyzed by fixed time method were found to be 0.12 µM and 0.225 µM, respectively.

The peroxidase assay reveals that the enzymatic reaction rate increased up to 2.99 nm concentration, the linearity range of peroxidase lies between 0.0425-5.8 nm and 0.02-2.99 nm by rate and fixed time, respectively.

B. Study of Interferences

The effect of interfering species in the determination of peroxidase was examined by using various ions and some commonly accompanying amino acids under the optimized experimental conditions containing 128 µM H_2O_2. The results are calculated as tolerance ratio which corresponds to the concentration of foreign species with respect to the concentration of peroxide used. The result showed that apart from Cu^{2+}, ascorbic acid, Fe^{3+}, L-Tryptophan, L-Tyrosinase, L- cysteine, L- Cystine, Fe^{2+}, Zn (II), citric acid, uric acid and L-Serine none of the other interfering species investigated interfered in the reaction. The results are shown in Table I.

C. Accuracy and Precision Studies

The accuracy and precision of the proposed assay were quantified by studying various solutions with the known concentrations of hydrogen peroxide. the experimental outcome shows that intra precision was 0.59-2.3% (n=10) and inter precision was 1.95-2.98% (n=10), respectively. The accuracy range for hydrogen peroxide concentrations of 16 and 32 µmol/L was 90-94% and 94-98%, and for 64 µmol/L of hydrogen peroxide, the accuracy range was 95-103%, respectively. results are exposed in Table 2.

Table I. Influence of interfering substances in the real sample analysis

Interfering species	Tolerance ratio[a]
Cu (II)	0.088
Ascorbic acid	0.1316
Fe (III), L-Tryptophan	0.987
L-Tyrosinase, L-Cysteine, L-cystine	1.25
Fe (II)	1.82
Zn (II), Citric acid, Uric acid and L-Serine	2.76
DL-Methionine, Oxalic acid and F-	7.60
D-Asparagine, Isoleucine and Nitrate	11.23
L-Histidine	18.25
DL-Threonine	54.78
Ammonium	56.21
Potassium and Chloride	78.45
Urea	88.16
Sodium	102.55
Glycine	123.52
Lactose and sulfate	202.14
D-galactose and Fructose	343.09
Sucrose	779.35
Glucose	1180.23

* [a]Tolerance ratio corresponds to the ratio of the limit of $\frac{[Interfering\ species]}{[Hydrogen\ peroxide]}$.

Table 2. Inter-day and Intra-day precision of the assay.

Inter- day precision			Accuracy range %	Intra- day pre :ision			Accuracy range %
H_2O_2 (µM)	SD (n=10)	CV	-	H_2O_2 (µM)	SD (n=10)	CV	-
16	0.000192	2.3	90.42-93.45	16	0.000143	2.98	91.65-94.16
32	0.000236	1.76	94.84-97.75	32	0.000561	2.47	90.00-97.38
64	0.000558	0.59	95.21-102.97	64	0.00191	1.95	95.32-100.86

D. Evaluation of peroxidase activity in plant extracts

The application of the proposed assay was investigated by using medicinal plants as a source of peroxidase such as *Leucas aspera, Catharanthus roseus, Nerium oleander, ixora coccinea, and Taberna Montana divaricates*. The evaluation of peroxidase activity in crude plant extracts was analyzed by using different buffers with varying pH ranges and 5:1 to 15;1mL/g buffer to tissue ratio. The enzyme activity was observed maximum in KH_2PO_4/NaOH buffer of pH 7.3 with a 10:1 mL/g ratio. hence this was chosen as the optimum pH and tissue ratio for further assays. Of these, *Centella Catharanthus roseus* and *ixora coccinea* crude extract was found to give maximum peroxidase activity by the proposed and reference guaiacol method whereas *Nerium oleander* crude extract was found to be less peroxidase activity and the results obtained by 4-AAP/RN system and standard guaiacol system are tabulated in Table 3. The relative half saturation points of the 4-AAP/RN with reference to the guaiacol system is <1, indicating higher interaction between the enzyme active site and chromogenic substrates. The catalytic parameters like Km, Vmax, Kpow and relative catalytic power for the plant extracts determined by proposed and standard assay method are tabulated in Table 4.

E. Reaction pathway for the POD-catalyzed reaction of PPD with AN

The probable reaction pathway for the HRP/H_2O_2 – catalyzed reaction of 4-AAP with RN is presented in Scheme 1. The mechanism involved oxidation of RN loses 2 e- and 2 H^+ in the presence of H_2O_2 and POD forming a quinone intermediate which by coupling with 4-AAP derived aminyl or imine radical results in the formation of red-colored quinone-imine intermediate with absorption at 500 nm. The proposed enzymatic mechanism is similar to that suggested by Ngo and Lenhoff for enzyme catalyzed oxidative coupling of MBTH/amines forming indamine dyes [37-39].

Table 3. Quantification of peroxidase activity in plant crude extracts as per the 4-AAP/RN and guaiacol system.

Sample	Activity(units)		$\frac{K_m^{AR}}{K_m^G}$
	PPDA-AN	Guaiacol	
Leucas aspera	22.18	17.31	0.9142
Catharanthus roseus	62.01	56.71	0.0762
Nerium oleander	16.22	19.5	0.7198
ixora coccinea	44.21	40.34	0.2319
Taberna Montana divaricates	36.85	31.27	0.1440

Table 4 Kinetic parameters for plant crude extracts from the 4-AAP/RN and guaiacol system.

	4-AAP -RN method			Guaiacol method			Relative catalytic power
Sample	K_m	V_{max}	K_{pow}	K_m	V_{max}	K_{pow}	$\frac{K_{pow}^{AR}}{K_{pow}^G}$
	µM	EU min^{-1}	10^{-3} min^{-1}	µM	EU min^{-1}	10^{-3} min^{-1}	
Leucas aspera	298.4	0.346	1.159	329.4	0.021	0.0063	18.39
Catharanthus roseus	30.4	0.052	1.71	398.9	0.030	0.0752	15.41
Nerium oleander	167.5	0.463	2.764	232.7	0.317	1.3622	49.28
ixora coccinea	44.1	0.075	1.70	190.1	0.055	0.2893	17.01
Taberna Montana	56.8	0.139	2.447	394.2	0.44	1.1161	45.61

VI. Conclusions

The substantial literature analysis verified that no work promulgated by the coupling of 4-AAP and RN for the assay of peroxidase. the reagents are inexpensive, versatile, steady, and have no technical risks. The higher catalytic efficiency, molar absorptivity, and the lower limit of detection and relative standard deviation values and the red-colored reaction product absorbs at a longer wavelength region claim the superiority of the proposed assay. the lower value of K_m (172.41 µM) and V_{max} (0.1136 EU min^{-1}) indicate the specificity and selectivity of the proposed system compared to the reported assay systems. The broad linearity range of H_2O_2 concentration between 1.1522 -294.98 µM and 0.5761 - 82.96 µM range by rate and fixed time method and peroxidase linearity in the range of 0.04-5.8 nm and 0.02-2.99 by rate and fixed time methods, the linearity shows the superiority of the assay. The interference studies show that the reaction interferes with very few foreign materials and the evidence from the kinetic parameters shows 4-AAP-RN/HRP system can be a better substitute for the reference guaiacol system [35].

Acknowledgement

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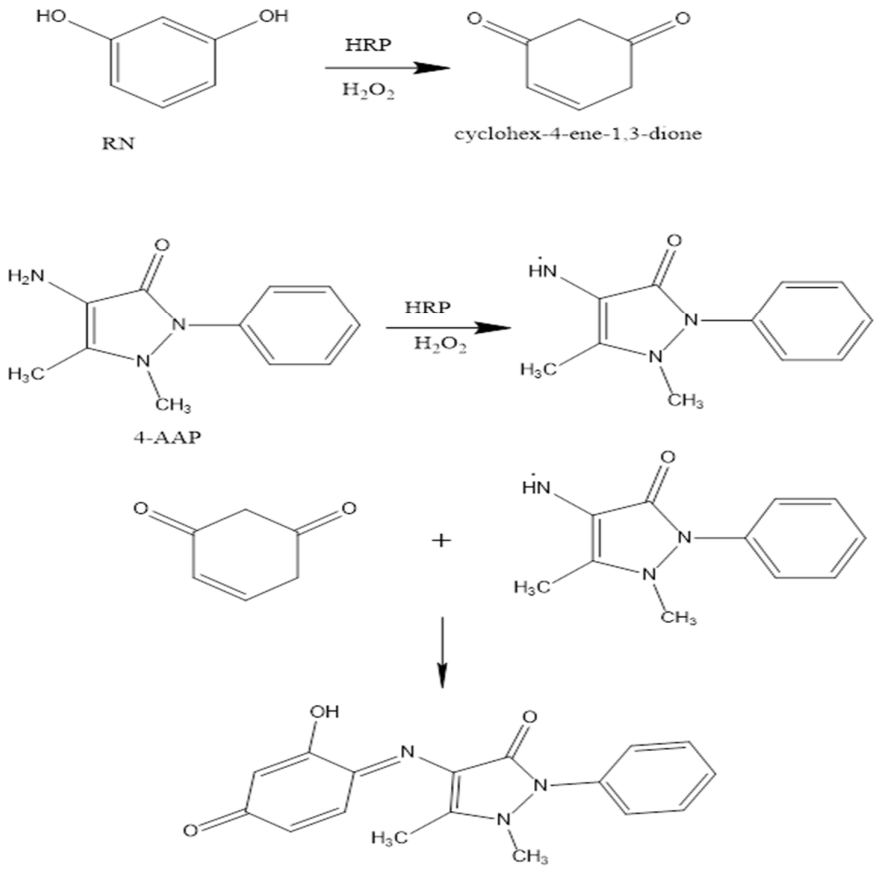

(*E*)-4-((2-hydroxy-4-oxocyclohexa-2,5-dien-1-ylidene)amino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one

Scheme 1: Proposed reaction mechanism for the formation of red-colored product

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