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Development and validation of a radical scavenging antioxidant assay using potassium permanganate

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Abstract

The main objective of the study was to develop and validate a novel radical scavenging antioxidant assay using KMnO4. In this method tannic acid at concentration 6.25 µg/ml – 200 µg/ml was used as the standard antioxidant. KMnO4 solution (80.00 µg/L) in phosphate buffer (pH= 9) was developed as the prooxidant. Absorbances of $KMnO_4$ both in the presence and absence of the antioxidant were taken spectrophotometrically at a wavelength of 526 nm after 30 minutes incubation period. The results obtained were then compared to results from reference method which employs 40.00 µg/L DPPH in methanol prepared and subjected to similar experimental conditions. The results showed that $KMnO_4$ solution prepared in a phosphate buffer (pH= 9), was most stable and had the highest molar absorptivity. The method developed was precise, accurate and specific. The method developed passed all the validation parameters according to the ICH guidelines. This offers a new antioxidant method that is relatively cheap and safe to use.

Keywords: Potassium permanganate, DPPH, Validation, Radical scavenging method, Tannic acid

INTRODUCTION

During aerobic metabolism and other cellular processes, there is the production of reactive oxygen species (ROS) [1-3]. These ROS exert deleterious effects on biological macromolecules, giving rise to protein, lipid, and DNA damage, cell aging, oxidative stress-originate diseases (e.g., cardiovascular and neurodegenerative diseases), and cancer [3,4]. Antioxidants scavenge or quench ROS and reactive nitrogen species (RNS) produced by cellular metabolism and environmental pollution [3]. Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease, rheumatoid arthritis, cataracts and altitude sickness [5].

Due to the recent interest of the importance of antioxidants and the fact that several of the fruits, vegetables and other food stuffs beneficial to man are due to their presence, a number of antioxidant, a number of antioxidant assay methods, exploring different mechanisms of action, have evolved [6-10]. Some of these assays include Oxygen Radical Absorbance Capacity method (ORAC), DPPH radical scavenging assay and Ferric Reducing Power method (FRAP) [9, 10].

The use of DPPH as an antioxidant assay method is one of many methods used in the assay of antioxidants, due to its merits of rapidity, simplicity and the use of only a UV spectrophotometer. However, DPPH is an expensive chemical (especially for under resourced laboratories) and potentially toxic, with a high risk of inhalation due to its powdery nature [11]. $KMnO_4$ is an oxidant like DPPH and can be reduced in the presence of antioxidants. It is relatively non-toxic and its reduction results in a colour change which can be measured spectrophotometrically [12]. Therefore in the present studies, $KMnO_4$, a relatively cheap and readily available oxidant was explored for its possible use as a free radical in a novel antioxidant assay method development.

MATERIALS AND METHODS

Materials

All chemicals used (comprising tannic acid, DPPH, sodium hydroxide and potassium hydroxide pellets, sodium dihydrogen orthophosphate, potassium dihydrogen orthophosphate, methanol, etc.) were

analytical grade BDH chemicals and/or Hopkins & Williams (H&W) laboratory chemicals. Bells "Duke Brand" of Potassium permanganate was purchased from a local community pharmacy.

Equipment

A double beam UV-Vis Spectrophotometer ($T90^+$ UV Spectrophotometer) with a cell of 1cm width was used for measuring absorbance. A PW254 Laboratory Balance (Adams Equipment) was used for weighing samples and a pH 2700 Benchtop Meter for checking pH.

Methods

Preparation of Stock Solutions

About 32.9226 g of Potassium hydroxide (KOH) was weighed and dissolved in 100 mL of distilled water and made up to 500 mL with distilled water to produce a 1M KOH solution [13, 14]. Eight thousand seven hundred milligram of Potassium dihydrogen orthophosphate (KH_2PO_4) was accurately weighed and dissolved in 400 mL of distilled water and the pH adjusted with 1M KOH [13]. The solution was then made up to 500 mL with distilled water. Twenty milligram of DPPH was weighed, dissolved and made up to 100 mL using methanol to obtain 200 mg/L solution. Ten milliliters of the methanolic solution was pipetted into a 50 mL volumetric flask and made up to volume using methanol to obtain 40 mg/L. 0.0200 g of $KMnO_4$ was also weighed, dissolved and made up to 100 mL using phosphate buffer (Ph= 9), to obtain 200 mg/L. 20 mL of the $KMnO_4$ solution was pipetted into a 50 mL volumetric flask and made up to mark using phosphate buffer to obtain 80 mg/L. 0.1000g of tannic acid was weighed and dissolved in water to produce 100ml (1000 µg/ml). Serially diluted standard solutions of concentrations 6.25 µg/ml – 200 µg/ml, were prepared from the stock [13, 14].

Determination of the absorption maxima of $KMnO_4$

Similar quantities of $KMnO_4$ crystals were separately dissolved in distilled water and phosphate (KH_2PO_4) buffers of pH 7, 8, 9 and 10 to finally produce 200 mg/L solution for each. Using distilled water and the corresponding phosphate buffer solutions as blanks, replicate absorbances for $KMnO_4$ was taken in the Vis range 400 - 800 nm to determine the absorption maxima [15].

Scavenging effect of Tannic acid on $KMnO_4$

The scavenging effect of tannic acid on $KMnO_4$ was determined using the DPPH method described by Sharma and Bhat [8], Villano *et al* [16] and Gulcino *et al* [17] with few modifications. 1ml each of standard tannic acid solution (200 - 6.25 µg/ml) was placed in a test tube with 3ml of 80.00 µg/L $KMnO_4$ solution (pH = 9). The resulting solutions were incubated at 25 ºC for 30 mins and the absorbance of the residual $KMnO_4$ determined at wavelength 525 nm using the $T90^+$ UV-VIS Spectrophotometer with a solution of $KMnO_4$ in phosphate buffer solution only incubated at 25 ºC for 30 mins as a control. Results were expressed as percentages of blank (percentage scavenging) [9, 10]. The concentration required to cause a 50% decrease in the absorbance (EC_{50}) was calculated [9, 16]. Each test was carried out in triplicate. The % $KMnO_4$ scavenging effect of the antioxidant was calculated as follows:

$$\% \ KMnO_4 \text{ scavenging effects} = \frac{(Ac - At)}{At} \times 100\% ,$$

Where Ac = absorbance of the control, At = Absorbance of test sample (tannic acid solution).

Method development and Validation

The *in-vitro* method for scavenging effect of tannic acid on $KMnO_4$ was developed based on the comparative results with tannic acid against DPPH as the radical scavenger. $KMnO_4$ is reactive in the presence of light [12]. The method was thus, developed in the absence of light. The medium for dissolution of the crystals was optimized to increase absorptive properties of $KMnO_4$ (Figure 1). $KMnO_4$ was observed to be a pro-oxidant in the alkaline medium (Scheme 1). It also undergo a colour change as a result of reduction (gain of electrons and addition of hydrogen) [10]. Hence, its effect could be measured spectrophotometrically. The developed method was then validated against parameters as outlined in the International Conference on Harmonization (ICH) guidelines [Q_2 (R1)] [18].

$$\underset{\text{oxidised form}}{MnO_4^-} + \underset{\text{electron from antioxidant}}{e^-} + OH^- \longrightarrow \underset{\text{reduced form}}{MnO_4^{2-}} + H_2O + \underset{\text{generated oxygen}}{O_2}$$

Scheme 1 – Equation showing the reduction of Potassium permanganate in alkaline medium

Specificity

The specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components [18]. The specificity was performed by determining the effect of the presence of Tannic acid on absorbance of $KMnO_4$. $KMnO_4$ generates oxygen radicals, recording high absorbance. In the presence of different concentrations of the antioxidant agent (tannic acid), the absorbance decrease, as the radicals get scavenged. The solvent, methanol was expected not to have any effect on the absorbance.

Precision (Repeatability and Intermediate Precision)

Repeatability

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. It is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements [18].

The method precision was determined by analysing replicate absorbances produced from the developed method at different concentrations of tannic acid. The relative standard deviations (RSD) were determined for each concentration term [19]. The RSDs were expected to be less than 2% [18].

Intermediate Precision (Ruggedness)

The ruggedness of an analytical method is the degree of reproducibility of test results (absorbances) obtained from the analysis of the same samples under variety of conditions, such as analysts, instruments or days. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from analyst to analyst [18].

The ruggedness of the method was determined by analysing absorbances obtained from the developed method on different times of the day (intra-day) as well as absorbances on different days of analysis (inter-day) [18]. The results were also subjected to One-Way ANOVA analysis at 95% confidence interval from GraphPad Prism (version 5).

The RSDs calculated [19] should not be more than 2.0%. There should also not be significant difference between results obtained in different analyses.

Linearity and range

The linearity of the method is its ability to elicit results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [18].

Linearity was measured by analysing standard concentrations of $KMnO_4$ solution across a range (20 - 200 µg/ml) and plotting the absorbance against the concentration [18]. Linearity was also established for the concentration range (6.25 - 200 µg/ml) employed for the antioxidant assay. The linearity of the plot is expressed as the Regression Coefficient [19]. The data was further supported with the y-intercept, slope of the regression line and residual sum of squares [18]. The regression coefficient was expected not to be less than 0.9950 [18].

Accuracy

The accuracy for the developed method was determined by comparing the results (percentage scavenging) obtained from the developed method with that of an established method, that is, with the DPPH radical scavenging assay [18]. In determining accuracy, concentrations within the range of 6.25µg/ml - 200.00µg/ml for tannic acid were tested against 80.00 µg/L $KMnO_4$ in phosphate buffer of pH 9 in the developed method whereas a range of 6.25 µg/ml – 100.00 µg/ml were tested against 40.00 µg/L DPPH in the reference method [18]. The results obtained for each day were compared statistically using Graph Pad Prism (version 5) at a confidence level of 95%. Statistically, it was expected that there will be no significant difference in the results from both methods.

Robustness

Robustness is a measure to establish that small but deliberate variations in the parameters of the method do not influence the result. It is an indicator of the reliability of a method in normal routine use [18]. In this test, the composition of the buffer was changed, from KH_2PO_4 to NaH_2PO_4 and the results were analysed statistically. There should be no significant difference in the results from the two methods.

RESULTS AND DISCUSSION

Absorption maxima of $KMnO_4$

In the development of the method, the absorptive property of $KMnO_4$ was evaluated in media of different pH. The absorbance and λmax (s) were recorded (Table 1, Fig. 1) and their corresponding specific absorptivities [A (1%, 1cm)] calculated from the Beer-Lambert's law [20]. The higher the specific absorptivity, the more sensitive the substance is to absorb electromagnetic radiation. $KMnO_4$ showed two λmax in each media. $KMnO_4$ in phosphate buffer (pH= 9) at wavelength 525 nm (Fig. 2), gave the highest absorptivity and thus was selected as the best workable wavelength of maximum absorption for $KMnO_4$ (Table 1, Fig. 1). $KMnO_4$ is relatively stable at a basic pH [12].

Table 1: Wavelength, absorbance and specific absorptivity of KMnO4 in different media

Condition	λ max$_1$ (nm)	Absorbance$_1$	Specific absorptivity	λ max$_2$ (nm)	Absorbance$_2$	Specific absorptivity
Distilled Water	546	0.288	144.0	526	0.298	149.0
pH 7	546	0.279	139.5	526	0.291	145.5
pH 8	545	0.326	163.0	525	0.339	169.5
pH 9	545	0.371	185.5	525	0.383	191.5
pH 10	546	0.362	181.0	526	0.372	186.0

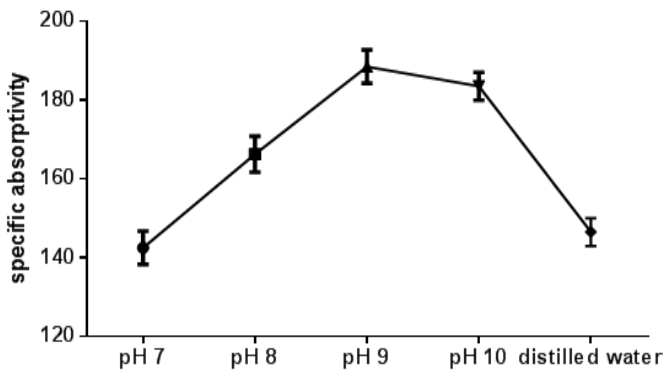

Figure 1: Specific absorptivities of $KMnO_4$ in different pH media

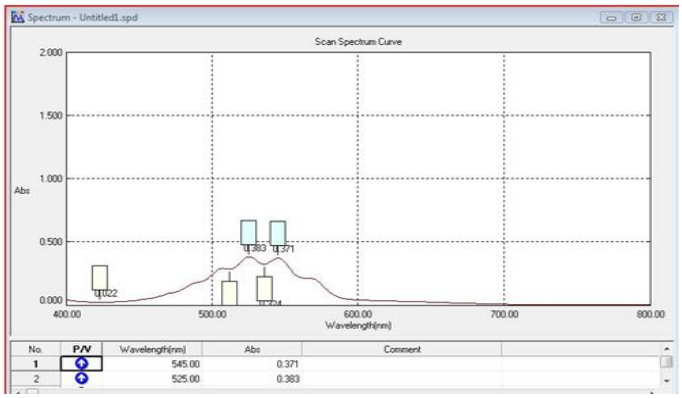

Figure 2: UV spectrum of KMnO4 in phosphate buffer (pH= 9)

Scavenging effect of tannic acid on $KMnO_4$

The use of $KMnO_4$ in place of DPPH is informed by the fact that, it is an oxidizing agent and can therefore mimic the ROS (Reactive Oxygen Species) present in the body. Tannic acid, an antioxidant (with reducing effects), causes the reduction of the MnO_4^- to MnO_4^{2-} and mops up ROS [10]. The reduction process resulted in change of colour from purple to light yellow as a result of gain of electrons with consequent bathochromic shift [15, 21], depending on the concentration of the antioxidant used.

The scavenging effect of tannic acid on 80.00 µg/L $KMnO_4$ solution in phosphate buffer (pH= 9), was evaluated over a concentration range of 6.25 - 200 µg/ml. It was observed that the percentage scavenging was concentration dependent, with the highest concentration mopping up most of the ROS leading to decreased absorbance and high percentage scavenging (Fig. 3). The EC_{50} for the method was determined to be 50.67 µg/ml.

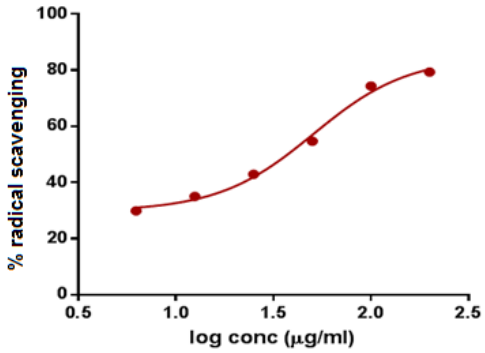

Figure 3: Scavenging effect of tannic acid on $KMnO_4$

Validation

Validation of an assay method is done to ensure the consistency, reliability and accuracy of data from the method [18]. In validating the developed scavenging method, the parameters considered included specificity, precision, linearity and range, accuracy and robustness [18, 19].

For specificity, results obtained showed that both buffer and methanol did not contribute significantly to the absorbance recorded (Table 2). The absorbance obtained for both phosphate buffer and methanol read 0.00 at 525 nm because they do not absorb within the chromophore (visible) range [15]. This also confirms the specificity of the tannic acid for the $KMnO_4$ since the absorbance of $KMnO_4$ only reduced when the tannic acid was added. Tannic acid mopped up the permanganate radicals hence a lower absorbance was recorded for the $KMnO_4$ after the incubation period. Thus, the method could be said to be specific to the presence of the radical scavenger, the tannic acid. The higher the concentration of the tannic acid, the lower the absorbance of $KMnO_4$ recorded.

In testing for precision (repeatability), the RSDs determined were less than 2.0% (Table 3). This showed that the method was precise for results generated within any testing period. An unpaired Student *t-test* analysis showed that there was no significant difference at 95% confidence interval. The range of RSDs was observed to be 0.00% - 0.93% (Table 3). The smaller the RSDs, the better the precision [19].

For intermediate precision, the RSDs determined for triplicate tests within each day were less than 2.0% (Table 4). This showed that the method was precise within day determinations. An unpaired Student *t-test* analysis for intra-day determinations showed that there were no significant differences (p = 0.9753, t = 0.03178, df = 10 for day 1; p = 0.9012, t = 0.1273, df = 10 for day 2; p = 0.9040, t = 0.1237, df = 10 for day 3) at 95% confidence interval. If a method is precise, it means the degree of variation obtained within different experiments done on the same day is small. Hence the experiment can be repeated on the same day at different times and similar results would be obtained provided the necessary experimental conditions are met.

Table 2: Specificity determination for tannic acid on $KMnO_4$

	DAY 1	DAY 2	DAY 3
KMnO4 + Phosphate buffer	1.360	1.370	1.400
Phosphate buffer only	0.001	0.000	0.001
KMnO4 + Phosphate buffer + Tannic acid (20 µg/ml) + Methanol	0.285	0.283	0.289
Methanol only	0.000	0.000	0.000

Table 3: Repeatability of developed method

CONCENTRATION (µg/ml)	DAY 1			DAY 2			DAY 3		
	Mean	SEM	RSD	Mean	SEM	RSD	Mean	SEM	RSD
200	0.2857	0.0012	**0.73%**	0.2707	0.0015	**0.93%**	0.2893	0.0003	**0.20%**
100	0.3490	0.0006	**0.29%**	0.4123	0.0012	**0.50%**	0.4137	0.0003	**0.14%**
50	0.6160	0.0029	**0.81%**	0.6283	0.0023	**0.64%**	0.6853	0.0009	**0.22%**
25	0.7763	0.0003	**0.07%**	0.8333	0.0018	**0.37%**	0.8450	0.0	**0.00%**
12.5	0.8833	0.0035	**0.69%**	0.9590	0.0030	**0.54%**	0.9393	0.0003	**0.00%**
6.25	0.9537	0.0027	**0.50%**	1.052	0.0015	**0.25%**	0.9993	0.0003	**0.06%**

Table 4: Intermediate precision

Concentration (µg/ml)	Day 1			Day 2			Day 3		
	Mean	SEM	**RSD**	Mean	SEM	**RSD**	Mean	SEM	**RSD**
200	0.2858	0.0008	**0.68%**	0.2863	0.0013	**1.07%**	0.2892	0.0003	**0.26%**
100	0.3467	0.0011	**0.81%**	0.4217	0.0014	**0.83%**	0.4213	0.0013	**0.73%**
50	0.6238	0.0013	**0.51%**	0.6108	0.0014	**0.54%**	0.6817	0.0017	**0.61%**
25	0.7798	0.0018	**0.55%**	0.8143	0.0013	**0.38%**	0.8195	0.0027	**0.82%**
12.5	0.8778	0.0029	**0.82%**	0.9423	0.0016	**0.41%**	0.9425	0.0015	**0.38%**
6.25	0.9585	0.0025	**0.64%**	0.9685	0.0024	**0.60%**	0.9865	0.0057	**1.43%**

Inter-day (ruggedness) results were also analysed statistically and was observed not to be significantly different from each other, further proving the consistency of the method (Table 5).

Table 5: Summary of inter - day analysis

	DAY 1 – DAY 3
p-value	0.9999
$F_{(5,30)}$	0.01566
Summary	ns

ns: not significant

The linearity plot from the method was expressed as the regression coefficient [19]. The graph obtained (Fig. 4A) showed that there was a linear relationship between $KMnO_4$ and its absorbance at the concentrations employed. Fig. 4B is a residual plot of figure 3A and it further confirmed the linear relationship that existed between $KMnO_4$ and its absorbance. Fig. 4C is a graph of $KMnO_4$ plus different concentrations of tannic acid against their absorbance. The higher the concentration of the tannic acid, the lower the absorbance of $KMnO_4$ recorded. The data was supported with the y-intercept, slope of the regression line and residual sum of squares [18]. For a relation to be linear, the regression coefficient (R^2) should not be less than 0.9950 [18]. From the results obtained (Table 6), the relation can be said to be linear over the concentration range 6.25 – 200 µg/ml.

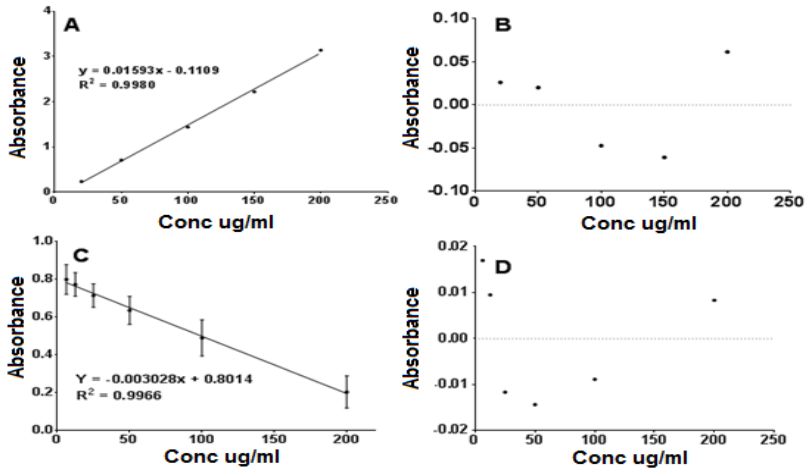

Figure 4: Linearity plot of developed method method
[A] – Line of regression for absorbance from different concentrations of $KMnO_4$. [B]- Residual plot signifying correlation between KMnO4 with their corresponding absorbance. [C] – Regression line for absorbance recorded from the assay. [D] – Residual plot from assay results

Table 6: Statistical data for establishing Linearity

Parameter	Standard $KMnO_4$	Assay
Slope	0.01593 ± 0.0004115	-0.003028 ± 8.887e-005
y-intercept	-0.1109 ± 0.05053	0.8014 ± 0.008377
R^2	0.9980	0.9966
Sy.x	0.06008	0.01473
F	1499	1161
	Confidence interval	
Slope	0.01462 to 0.01724	-0.003275 to -0.002781
y-intercept	-0.2717 to 0.04991	0.7782 to 0.8247

In establishing accuracy of the method, the results obtained from $KMnO_4$ were compared with that from DPPH (Fig. 5A, B & C). Testing the percentage scavenging from the two methods, using a two-tailed unpaired Mann Whitney test from GraphPad Prism (version 5) at 95% confidence interval showed that there was no significant difference (p = 0.0823) (Figure 5D). This signified that $KMnO_4$ and DPPH possessed comparable effects in the presence of tannic acid at the concentrations employed. Hence $KMnO_4$ could be used in place of DPPH in a radical scavenging antioxidant assay.

In testing for robustness, the composition of the buffer was altered but the pH was maintained. Instead of KH_2PO_4, NaH_2PO_4 was employed for the scavenging test. The result was shown to be comparable. Further testing statistically showed that there was no significant difference (p = 0.9587; t =0.05315, df = 10) (Figure 6).

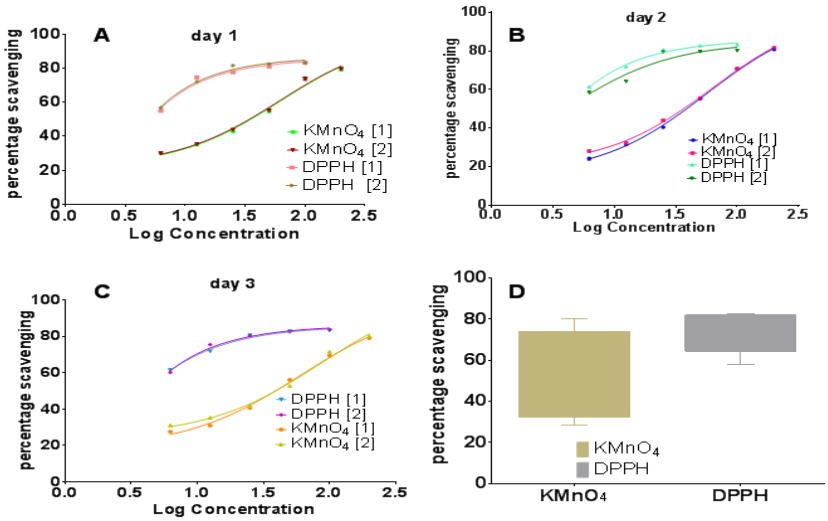

Figure 5: Determination of the accuracy of the method
[A] – [C] – Comparing the percentage scavenging effects of using $KMnO_4$ and DPPH as sources of radicals for the scavenging test. [D] – Comparing the Mean ± SEM of percentage scavenging for the two methods.

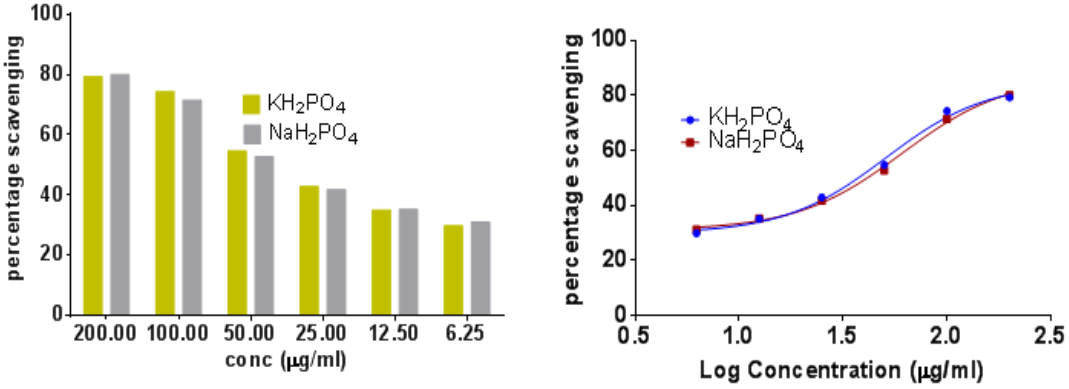

Figure 6: Establishing robustness of method by altering buffer agent
(A)Comparing % scavenging produced from the use of different buffer agents maintained at the same pH (B)Sigmoidal graph establishing similarity in response from the two different buffer agents

CONCLUSION

In the present study a radical scavenging antioxidant method using potassium permanganate was developed and validated in accordance with the ICH parameters. The method was validated and found to be simple, accurate, and precise. $KMnO_4$ radical scavenging assay could be used for routine screening for antioxidants.

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