

Antioxidant assays by reducing potential and 2,2-diphenyl-1-picrylhydrazyl radical scavenging techniques as affected by pH and ion concentrations

Babatunde J. Oso^{1,*} and Clement O. Ogidi²

¹Department of Biochemistry. McPherson University. Seriki-Sotayo. Ogun State. Nigeria. Email: basjoe08@gmail.com.

²Department of Biological Sciences. Kings University. Odeomu. Osun State. Nigeria.

Abstract. The aim of the study is to investigate the effect of varying pH and different metal ion concentrations on the analyses of antioxidants by reducing potential (RP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging techniques. The investigation was conducted by examining the effects of various pH values (4, 5, 6, 7, 8 and 9) and potassium chloride concentrations (50, 100, 150, 200, 250 and 300 µg/mL) on the optical densities of reducing potential and DPPH radical scavenging potential of aqueous infusions of *Cassia alata* (L.) Roxb. The determinations were also conducted on the extraction media with the intention of identifying the probable source of variation in the investigation. The antioxidant potentials for both the aqueous infusion and media were most efficient at the least pH 4.0. Moreover, the antioxidant potentials decrease as the ion concentrations increase. The study revealed that the colorimetric methods for the determinations of DPPH radical scavenging and RP could be liable to errors arising from slight changes in acidity and concentrations of the metal ions thus affecting the performance characteristics in terms of repeatability and reproducibility of reports and meaningful comparisons of antioxidant capacity of dietary products among different authors.

Keywords: 2,2-diphenyl-1-picrylhydrazyl; *Cassia alata*; Ionic strength; pH; Reducing power.

Introduction

There have been increasing interest in recent years on the assessment of antioxidant capacities of dietary products ever since diets which are rich in antioxidants had been known to provide health benefits through the reduction of accumulative elevation of

free radicals in the system along with their essential role in delaying oxidative rancidity of natural products. Consequently, various techniques with exclusive principles of reactions have been developed in order to determine the antioxidant capacity in various dietary products. Moreover, the antioxidant activity of a given sample

Received
January 10, 2019

Accepted
April 23, 2019

Released
April 30, 2019



Full Text Article



ORCID

0000-0001-8667-7108
Babatunde J. Oso

0000-0002-4154-6750
Clement O. Ogidi

cannot be concluded based on the report from a single test model; this is because there are different sources of antioxidants in the biological ranging from proteins and peptides to small molecules such as vitamins and phytochemicals with different antioxidant principles and besides there are numerous free radical sources (Apak et al., 2007).

Generally, the assessment of antioxidant capacity through chemical reactions in *in vitro* studies are broadly divided into hydrogen atom transfer and single electron transfer reactions based assays and these assays are either based on the utilization of radicals or metal ions as the oxidizing agents (Apak et al., 2016). The ability of an antioxidant compound to donate hydrogen atom and quench free radicals formed the basis of the hydrogen atom transfer reaction-based assays while electron transfer based reaction depends on redox reaction (Prior et al., 2005).

Reducing potential (RP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays are methods commonly employed in the evaluation of the antioxidant capacity of dietary products. They are redox-coupled methods that utilize the antioxidants as reducing agents. Redox properties of natural products which allow them to act as reducing agents, hydrogen donors and radical scavengers define their antioxidant potentials. Major factors that may influence the reaction mechanism and the efficacy of the redox potential of antioxidant compounds include ionization potential and bond dissociation energy (Wright et al., 2001). These factors, in turn, are influenced by substances which alter the acidity of the reaction medium such as acids, alkalis, and buffers. Moreover, plant extracts from different species of botanicals have been shown to exhibit different hydrogen potentials. For instance, methanol extract of peanut hulls showed higher DPPH radical scavenging activity at neutral and acid pH, extracts from cocoa had higher

radical scavenging potential at alkaline pH while the antioxidant properties of Thai hot curry paste was higher at slightly acidic pH 6 and the methanolic leaf extract of *Ocimum sanctum* showed a maximum phenolic content at pH 7.2 (Yen and Duh, 1983; Azizah et al., 1999; Silas et al., 2012; Padmaja and Srinivasulu, 2016). Moreover, extraction of antioxidant compounds such as phenolics and flavonoids and the antioxidant activities had been shown to be influenced by pH of the extracting medium (Settharaksa et al., 2012).

Besides, there could be considerable variations in these factors during analyses of biological materials based on practical modifications by different laboratories. Plant-based natural products may be exposed to processes that could alter their neutral pH and have profound effects on their nutritional and functional properties. The biological relevance of the effects of pH on the activities of antioxidant products could be substantiated as pH range of different human fluid and tissues vary widely (Muzolf-Panek et al., 2012).

Thus, the aim of the present study is to assess the impact of varying pH and ionic strength on the analyses of antioxidants by reducing potential and DPPH radical scavenging techniques.

Materials and methods

Sample preparation

Phosphate buffer (0.1 M) was prepared and adjusted to different pH of 4, 5, 6, 7, 8 and 9 in separate beakers. Also, solutions of different concentrations of potassium chloride (50, 100, 150, 200, 250 and 300 µg/mL) were prepared in separate beakers. Exactly 5.0 g of the dried leaf of *C. alata* (L.) Roxb were extracted separately with 50 mL from each of the prepared solutions with varying pH and ionic strength. The extracts were filtered through the Whatman filter paper and

the filtrates were kept for the subsequent analyses.

Reducing power

The assay was carried out as described by Oyaizu (1986). Exactly 2.0 mL of each of the samples (media with an aqueous infusion of *C. alata* (L.) Robx and the media without extract), 2.0 mL of 0.1 M sodium phosphate buffer (pH 6.6) and 2.0 mL of 1% potassium ferricyanide prepared in distilled water were added into a test tube and mixed. The mixture was incubated at 50 °C for 20 min. Afterward, 2.0 mL of 5% trichloroacetic acid prepared in distilled water was added. The reaction mixture was centrifuged at $650 \times g$ for 10 min and 2.0 mL of the supernatant was taken and dispensed into a test tube containing 2.0 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. The contents were gently mixed and the optical density was measured at 700 nm.

DPPH radical scavenging potential

The DPPH radical scavenging technique was carried out as described by Shirwaikar et al. (2006). The solution of the radical was prepared by dissolving 60 mg of DPPH in 300 mL of methanol. In

a test tube, 3 mL of DPPH solution was mixed with 100 μ L of either of the media with an aqueous infusion of *C. alata* (L.) Robx or 500 μ L of either of the media without extract. The optical density was read at 517 nm after the test tubes had been kept in the dark for 30 minutes.

Results

The results of the experiments that investigated the influence of pH and ionic strength on the optical densities of FR assays were presented in Figures 1 and 2. The results presented in Figure 1 reveal a decrease in the measurements of the optical densities as the pH increases for the experiments with the extract of *C. alata* (L.) Robx and without extract. Similarly, there was a decrease in the optical density as the ionic strength increases from 50 to 150 μ g/mL; however, there was a slight surge at 200 μ g/mL and further decrease. The resulting optical densities of the influence of pH and ionic strength on DPPH scavenging potential are depicted in Figures 3 to 6. The coloration increased parallel to the increases in pH and KCl concentration, with the sharpest increase occurring at pH 9 and KCl concentration of 200 μ g/mL.

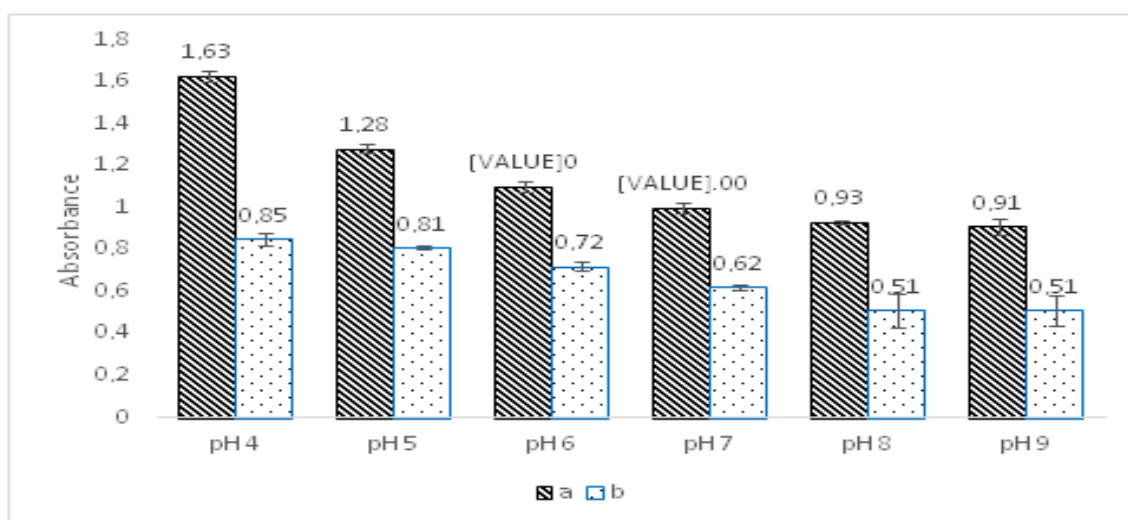


Figure 1. The influence of varying pH on the ferric reducing potential of (a) aqueous extract of *Cassia alata* (L.) Roxb and (b) extraction medium.

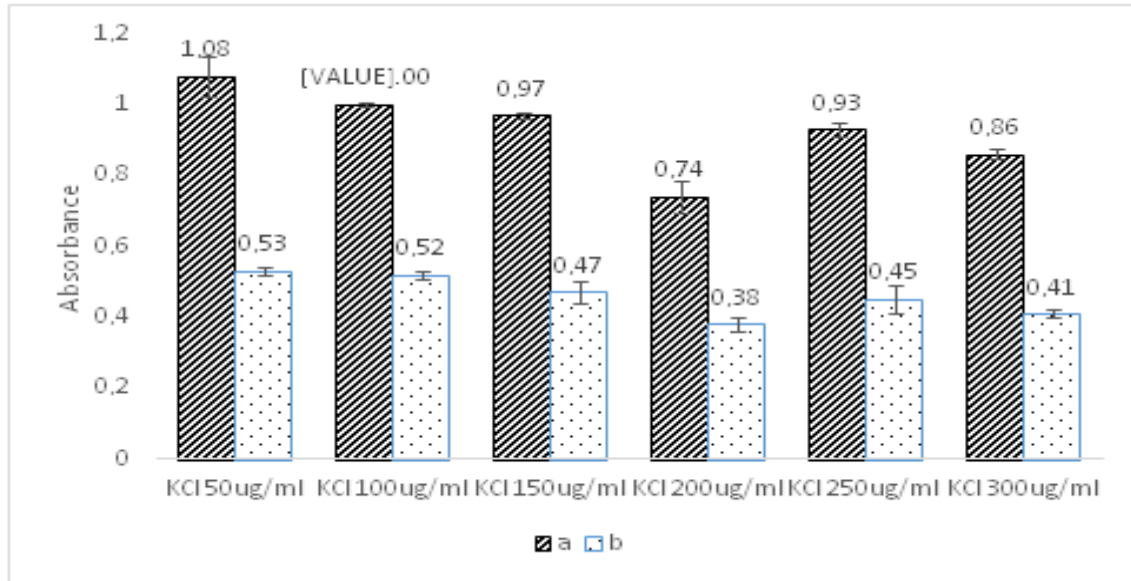


Figure 2. The influence of varying ion concentrations on the ferric reducing potential of (a) aqueous extract of *Cassia alata* (L.) Roxb and (b) extraction medium.

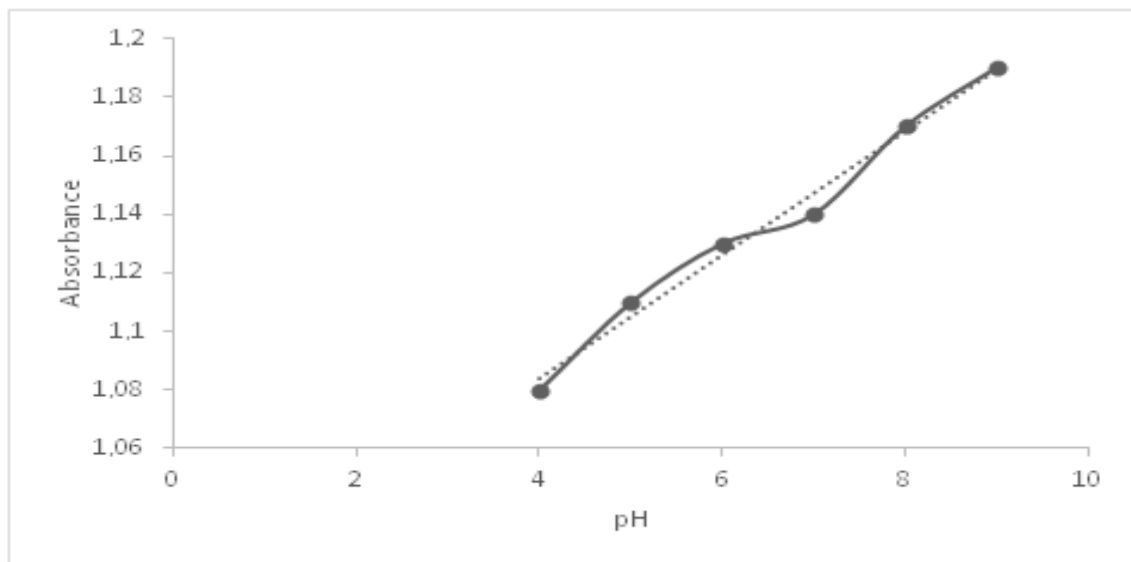


Figure 3. The influence of varying pH on DPPH scavenging potential of aqueous extract of *Cassia alata* (L.) Roxb.

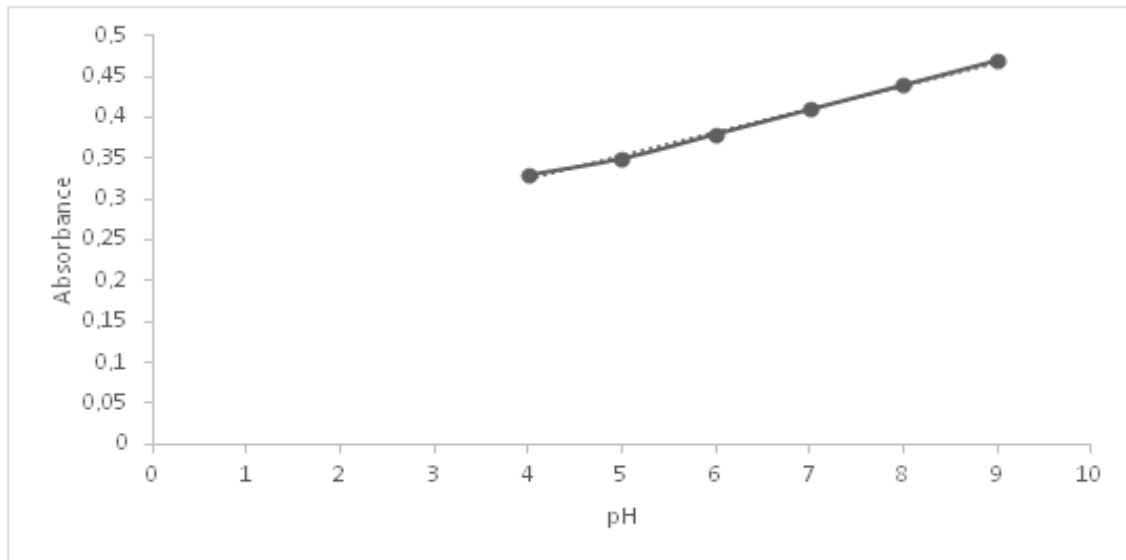


Figure 4. The influence of varying pH on DPPH scavenging potential.

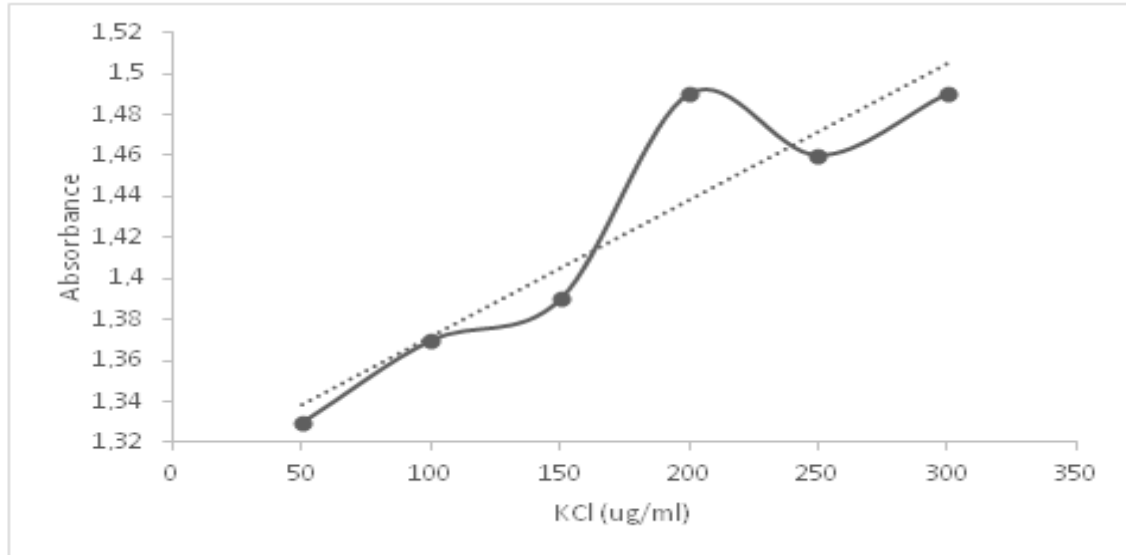


Figure 5. The influence of varying ion concentrations on DPPH scavenging potential of aqueous extract of *Cassia alata* (L.) Roxb.

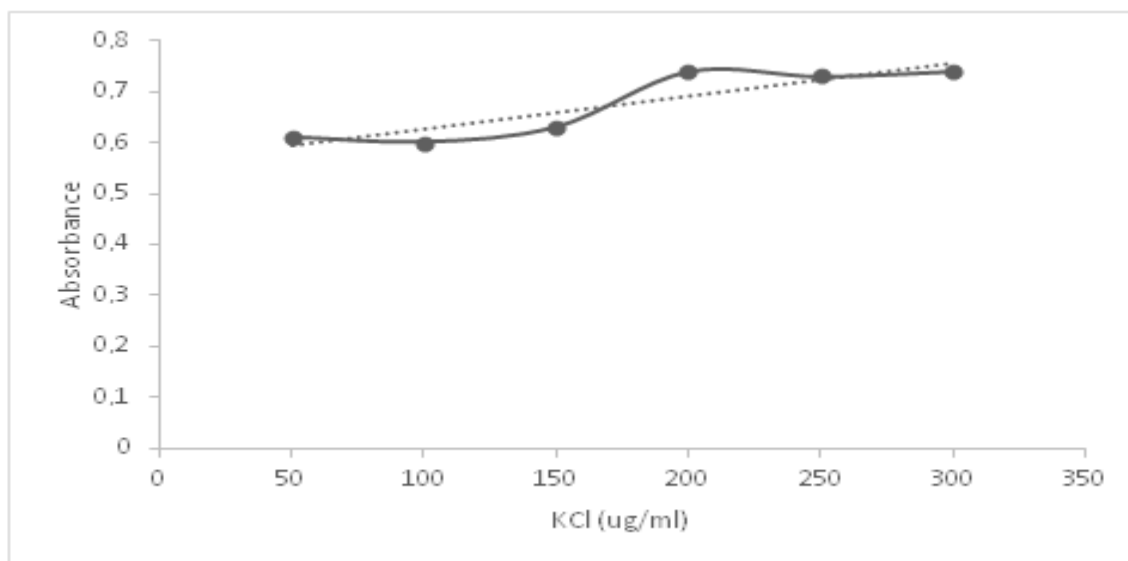


Figure 6. The influence of varying ion concentrations on DPPH scavenging potential.

Discussion

The results showed that pH 4 exhibited a relatively high antioxidant potential. The antioxidant potential decreased as the pH and ion concentration increased suggesting poor activities of the antioxidant compounds in the extract at higher pH. This is in agreement to the report from literature data that acid pH could increase the antioxidant potential of the antioxidant compounds (Friedman and Jurgens, 2000; Ruenroengklin et al., 2008; Muzolf-Panek et al., 2012). Though, a contrary result was reported by Gosh et al. (2015) which stated that phenolic concentration and antioxidant activity of palm wine and palm vinegar increased as pH increased. Studies had however indicated that phenolic compounds tend to demonstrate greater stability at low pH partly due to the prevention of oxidation of the phenolic compounds (Li et al., 2007). For instance, anthocyanins in red onion extract had been demonstrated to be more stable at low pH values (1.0) and decreased significantly at pH > 4.5 (Oancea and Drăghici, 2013). Moreover, the antioxidant potential of phenolics depends on the position and number of

hydroxyl and methoxy groups (Cai et al., 2006).

Changes in pKa values of reaction system have been shown to be related to the degree of ionization and deprotonation of functional groups of the compounds and deprotonation of phenolic compounds could alter the thermodynamics of the hydrogen atom transfer (Lemańska et al., 2001; Amorati et al., 2006; Leopoldini et al., 2006). The oxidation potential of antioxidant compounds is inversely related to their antioxidant activities and could be altered by pH of the reaction system. The report of the present study showed that increasing the pH of the system could increase the oxidation potentials of the compounds. Therefore, the pH-dependent behavior of these antioxidant compounds results in an altered antioxidant potential of the antioxidant compounds. Furthermore, pH could induce a change in the steric structure of the antioxidant compounds and this could also influence the antioxidant potential of the compounds (Muzolf-Panek et al., 2012). The remark from this study could have been responsible for the claims that antioxidant potentials of natural products could be favored by

extracting at acid pH (Ruenroengklin et al., 2008).

Similarly, there was an inverse relationship between the metal ion concentration and antioxidant potential of the extract, although the least antioxidant capacity was observed at 200 µg/mL KCl. This is in agreement with the report of Pękal and Pyrzynska (2015) that desalting process using cation-exchange resin decreased the content of metal ions in tea infusion and subsequently increased the DPPH scavenging potential of the infusion. The influence of metal ions on any reaction could vary as it depends on the type and concentration of the metal ion initiating a shift in the main reaction mechanism. Elevated metal ion concentration in this study could correspondingly be responsible for observed decreased in antioxidant activity in the alkaline region. Metal ions which form complexes which are difficult to be reduced could also interfere with the colorimetric determinations of DPPH radical scavenging and the RP. Moreover, the pH effect on deprotonation could alter the redox potential of metal ions (Stupka et al., 2005).

It is noteworthy to observe that while this present study agreed with previous reports on the possible stabilities of antioxidant compounds at low pH, further investigation on the influence of varying pH values and metal ion strength on the stability and biophysical states of the reagents indicated that pH and ionic strength of the reaction medium without an antioxidant compounds alter the redox status of the reagents. The observed reduction in the color intensity of the DPPH radical dye and a concomitant increase in the coloration and absorbance of the FR indicate that pH and/or metal ion strength of the reaction media for antioxidant assay could confer a false antioxidant capacity on the analytes. Thus, analysis conducted at acid pH or with medium with relatively higher metal ion concentration could

present a false positive antioxidant potential which may not be compared with other reports.

Conclusion

The study clearly showed that the reported antioxidant potential of a specific natural product largely depends on the chemical properties of the extracting solution. Elevated pH and ion concentration tend to lower the antioxidant capacity of the plant material. Moreover, changes in pH and ionic strength of the reaction media without an antioxidant compound could alter the redox potential of the reagents thus conferring a false antioxidant capacity. Thus, this study suggests that variations in the pH and ionic strength could lead to a shift in the central reaction mechanism and resulting in inconsistent and incomparable results. The study, therefore, recommends the consideration of pH and ionic strength in establishing standard methods in order to control variations in reports and obtain meaningful comparisons of antioxidant capacities of natural products.

Conflicts of Interest

The authors declare no conflict of interest relevant to this article.

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