Original research paper

Extraction, Partial Purification and Characterization of Peroxidase from *Calotropis procera* leaves

ABSTRACT

Aim: This study was aimed at the isolation, partial purification and characterization of peroxidase from low cost material, *Calotropis procera* leaves.

Materials and Methods: Partial purification of crude enzyme extract was done by ammonium sulfate precipitation followed by dialysis against Tris-HCl buffer. Peroxidase activity was measured spectrophotometrically.

Results: It was observed that after partial purification, the enzyme specific activity was increased as compared to crude enzyme extract. Peroxidase from *Calotropis procera* leaves was purified up to 2.04 fold with specific activity of 2.68 U/mg after dialysis. The partially purified peroxidase displayed optimum activity at temperature 50^{o}C and pH 6.0. The kinetic data shows that guaiacol is a better substrate than ABTS. All the tested metal ions (Fe^{3+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Zn^{2+}) and EDTA exhibited strong inhibitory effects on the *Calotropis procera* leaves peroxidase.

Conclusion: It is more evident that *Calotropis procera* leaves is a good source of peroxidase. It is therefore, concluded that further purification and full biochemical characterization of this enzyme may serve as a promising option to be explored for industrial purposes.

Key words: *Calotropis procera*, peroxidase, purification, characterization

1. INTRODUCTION

Peroxidases (E.C. 1.11.1.7) are ubiquitous enzymes, widely distributed in plants, micro-organisms and animals [1, 2]. They are versatile biocatalyst capable of oxidizing a broad variety of organic compounds including phenols, aromatic amines, indoles, and sulfonates using hydrogen peroxide as the oxidant [3, 4, 5]. In plants, they serve many functions, including lignification, suberization/wound healing, protection against pathogen attack, and the scavenging of damaging hydrogen peroxide from the cell [6]. The applications of peroxidases span the bioscience and biotechnology spectra, ranging from bioremediation and biocatalysis through to diagnostics and biosensors to recombinant protein expression, bioinformatics, and even to therapeutics [3, 7]. Peroxidase have numerous industrial applications which include decolourization of waste [8], and remediation of waste water containing phenolic compounds [9, 10]. Presently, Horseradish (*Amoracia rusticana*) roots is used as the major commercial source of peroxidases for industrial applications such diagnostic, biosensing, and biotechnological applications because of its high stability in aqueous solution [11]. Thus, there is need for a continuous research in search of novel peroxidase for various industrial and biotechnological applications. Therefore, numerous studies have been carried out in search for alternative source of peroxidase with low cost, higher stability, availability, degree of purification and substrate specificity. Peroxidase has been purified and characterized from different plant sources in search of an

alternative novel enzyme, *e.g. Moringa oleifera* leaves [12], oil palm leaf [13], apple [14], vanilla bean [15], etc.

Calotropis procera belongs to the family Asclepidaceae and it is distributed in tropical and subtropical region of Asia and Africa. It is a well-known plant for its multifarious medicinal properties [16]. Different parts of leaves, roots, flowers and latex from this plant are used in several medicinal preparations [17]. Furthermore, the root bark and leaves of *Calotropis procera* were used by various tribes of central India as a curative agent for jaundice [18]. The chloroform extract of the root has protective activity against carbon tetrachloride induced liver damage [19]. The milky white latex of this plant exhibit potent antiinflammatory, analgesic and weak antipyretic activity in various experimental models [20, 21]. It was also reported to possess antioxidant and anti-hyperglycemic property [22] and there is an empirical relationship between antioxidant property and residual peroxidase activity. The leaf of *Calotropis procera* is a natural coagulant used traditionally in waste water treatment and it has also been reported that *Calotropis procera* leaf is effective in removal of environmental pollutant, polyphenolic crystal violet dye from aqueous solution of textile effluent [23], which is suspected to be attributed to the presence of peroxidase in *Calotropis procera* leaf which oxidized phenols to phenoxy radicals. Therefore, this study seek to isolate, partially purify and characterize peroxidise as antioxidant enzyme from *Calotropis procera* leaf which may offer a low cost peroxidase biocatalyst for industrial applications such as treatment of industrial waste water containing phenols and aromatic amines and organic synthesis.

2. MATERIALS AND METHODS

2.1 Materials/Reagents

The fresh leaves of *Calotropis procera* was collected from Toro L.G.A of Bauchi State. Ammonium sulphate, Ciocalteu reagent, bovine serum albumin, guaiacol/ 2, 2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) [ABTS], and hydrogen peroxide were obtained from Sigma-Aldrich. All other chemicals used in this study were of research grade and obtained from commercial sources.

2.2 Methods

2.2.1 Crude extraction

Peroxidase was extracted from *Calotropis procera* by the method of [24] with slight modifications. 50g of healthy leaves of *Calotropis procera* were thoroughly washed with distilled water. The leaves were homogenized with 200 ml of 0.1 M Tris-HCl buffer, pH 7.5 in an Akia homogenizer for 5 to 10 minutes. The homogenate was filtered using a cheese cloth arranged in three folds to remove suspended particles. The filtrate was centrifuged at 10,000 g for 15 minutes at 4°C using refrigerated centrifuge to remove cell debris. The supernatant will be removed carefully from the sediments and filtered through Whatman No 1 filter paper to get clearer crude soluble *Calotropis procera* leaves enzyme extract.

2.2.2 Thermal treatment

To selectively inactivate the contaminating traces of catalase moieties, crude enzyme extract was heated at 65°C for 3 min in a water bath and cooled promptly by placing it in ice bucket for 30 min [25]. After thermal inactivation, the final extract was preserved at 4°C until further use.

2.2.3 Protein and enzyme assay

Total protein concentration was determined by the method of [26] using bovine serum albumin as the standard.

Peroxidase activity was measured spectrophotometrically with guaiacol/ 2, 2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)[ABTS]as substrates according to the method of [27] with slight modifications. To 2.5 ml of 0.1 M Tris-HCl buffer solution (pH 7.5) 100 µl of crude enzyme extract and 200 µl of substrate (guaiacol) were added. The reaction was initiated by addition of 200 µl 30% of hydrogen peroxide and the absorbance was read at (470 nm for guaicol and 315 for ABTS) every 30 second interval up to 3 minutes. Peroxidase activity was determined at each stage of purification.

2.2.4 Ammonium sulphate precipitation and dialysis

Ammonium sulphate was added to the crude enzyme extract until it was 85% saturated and kept for 4 hours at 4°C for complete precipitation. The resulting precipitate was collected by centrifugation at 10,000 g for 15 min at 4°C. After centrifugation, the supernatant was removed and sediment was dissolved in small amount of buffer in which the enzyme was originally extracted. The solution was kept in a dialysis bag after sealing securely, and dialyzed against 0.1 M Tris-HCl buffer solution (pH 7.5) for 8 hours with four regular change of the buffer after every 2 hours interval. The dialyzed enzyme was used as partially purified peroxidase to carry out further analysis.

2.2.5 Kinetic constants/substrate specificity

In order to determine the kinetic parameters (K_m and V_{max}) of the peroxidase, activity was measured at varying substrates concentrations (0.5-3 mM) of two well-known peroxidase substrates: (guaiacol/ABTS) with a suitable amount of purified enzyme, and H_2O_2. From the data, the kinetic parameters of the partially purified peroxidase using the two substrates was calculated from the equation of straight line (Lineweaver-Burk).

2.2.6 Determination of optimum pH

The optimum pH value for the peroxidase activity was determined by assaying for enzyme activity at varying pH values, using the following buffers: 0.1M glycine-HCl buffer (pH 4.0 to 5.0), 0.1M phosphate buffer (pH 6 to 7) and 0.1M Tris-HCl (pH 8.0 to 10).

2.2.7 Determination of optimum temperature

The optimum temperature of the peroxidase activity was determined by assaying enzyme activity at different temperatures from 10 to 100^{o}C at pH 6.0. Temperature was controlled by using water bath

2.2.8 Determination of effect of chemicals and metal ions on peroxidise activity

The effect of metal ions/chemicals on peroxidase activity was determined by pre-incubating the enzyme with varying concentrations (1, 2, 5, 10 and 20 mM) of individual divalent metal ion (Mg^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}) or chemical, ethylene diamine tetra acetic acid (EDTA) for 30 minutes at 37°C prior to starting the reaction. The peroxidase activity in the absence of metal ion and chemical was taken as a control.

2.3 Statistical Analysis

Data was expressed as mean values of three replicates. All calculation and data analysis was done using Microsoft excel.

3. RESULTS AND DISCUSSION

The results of peroxidise purified from *Calotropis procera* leaves is summarized in Table 1. The ammonium sulfate fraction obtained at 85% showed maximum activity. This primary purification step resulted in 1.613-fold purification of peroxidase from the crude extract. Following ammonium sulfate precipitation, the enzyme was dialyzed with the dialyzed fraction having 2.04 purification folds.

Table 1: Summary of Purification of Peroxidase from *Calotropis procera* Leaves

Purification Steps	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	1002.3	764	1.31	1	100
$(NH_4)_2SO_4$ ppt	881.3	416.5	2.12	1.61	87.93
Dialysis	697.14	260	2.68	2.04	69.55

In order to determine the substrate specificity, kinetic studies were carried out under standard conditions using guaiacol and ABTS as substrates. Figures 1 and 2 showed the Linweaver-Burk plot using guaicol and ABTS respectively. Apparent Km and V_{max} values were 3.567mM and 432.78mM/min respectively for guaiacol as a substrate as shown in Table 2. Whereas, the Km and V_{max} of peroxidase were 3.84mM and 400.00mM/min respectively for ABTS as a substrate. The turnover rate of guaiacol by *Calotropis procera* leaves peroxidase was higher than that of ABTS as a substrate and the affinity of the partially purified enzyme towards guaiacol was higher than ABTS. However, the Km values are higher than the ones reported by [28] for spring cabbage peroxidase and [12] for *Moringa oleifera* leaves peroxidase.

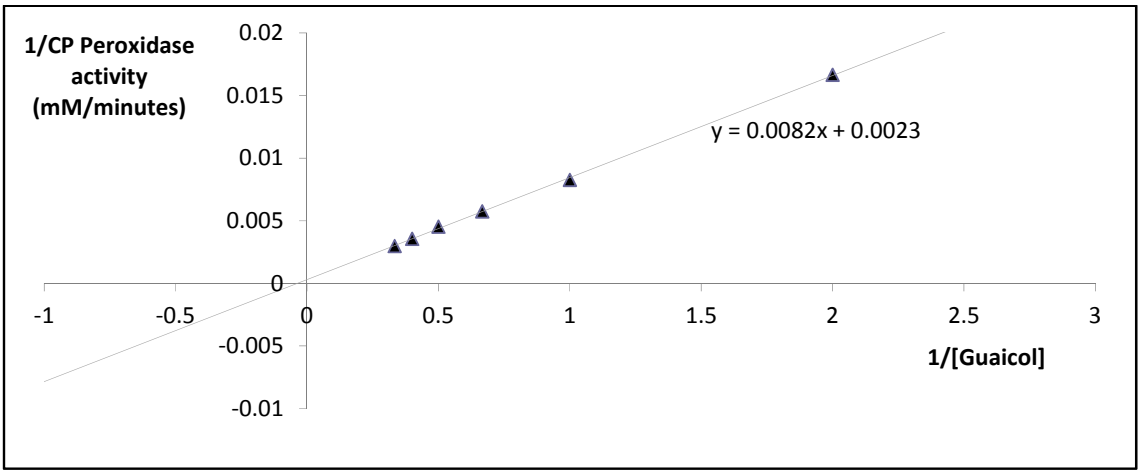

Figure 1: Linweaver-Burk plot for *Calotropis procera* leaves peroxidase using guaicol as substrate

*Linweaver-Burk equation 1/V=Km/Vmax * 1/[S] + 1/Vmax*

*Y= m * x + c*

Therefore, m=Km/Vmax, c=1/Vmax. Hence Km and Vmax can be obtained from the equation of straight line

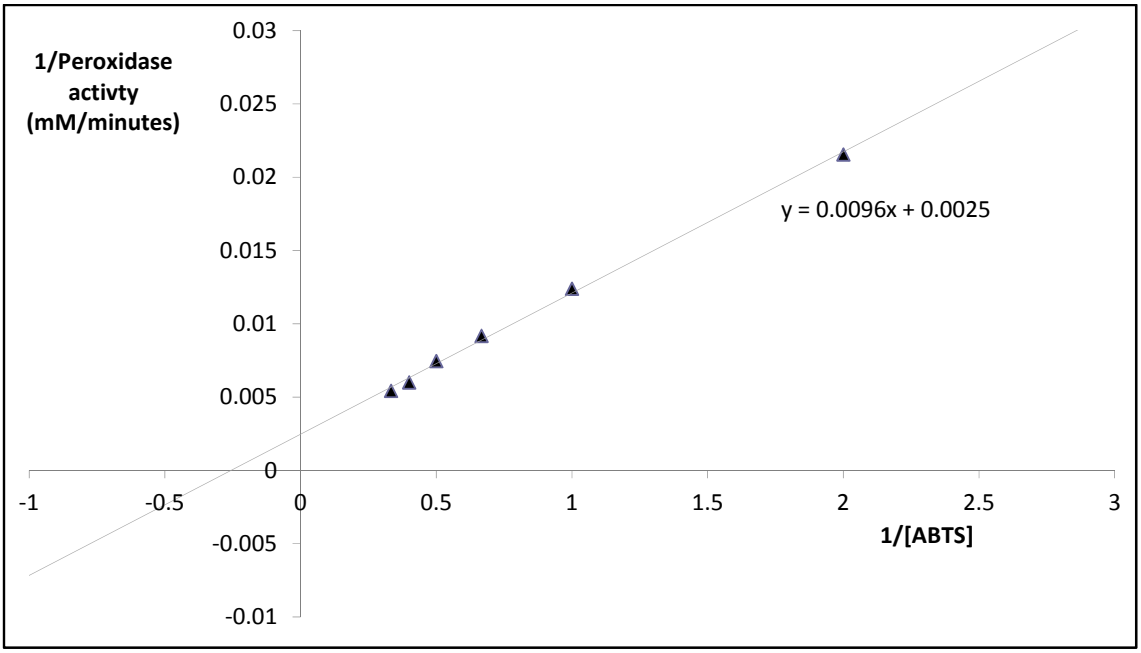

Figure 2: Linweaver-Burk plot for *Calotropis procera* leaves peroxidase using ABTS as substrate

*Linweaver-Burk equation 1/V=Km/Vmax * 1/[S] + 1/Vmax*

*Y= m * x + c*

Therefore, m=Km/Vmax, c=1/Vmax. Hence Km and Vmax can be obtained from the equation of straight line

Table 2: Kinetic parameters of *Calotropis procera* leaves peroxidase

Substrate	Kinetic Parameters	
	Km (mM)	Vmax (mM/min)
Guaiacol	434.78	3.57
ABTS	400.00	3.84

Calotropis procera leaves peroxidase maintained above 50% over a temperature range of 20-70 with optimum temperature 50^{o}C (Figure 3). The optimum temperature is in agreement with the earlier reports of [12] for *Moringa oleifera* leaves peroxidase. Optimum activity was also reported at 55^{o}C by [29] from the soft stem of *Leucaena leucocephala* peroxidases The enzyme activity increased sharply and reaches peak at temperature of 50^{o}C and declined gradually with near or total loss of activity at temperature of 90-100^{o}C which indicates loss in conformational structure of the enzyme.

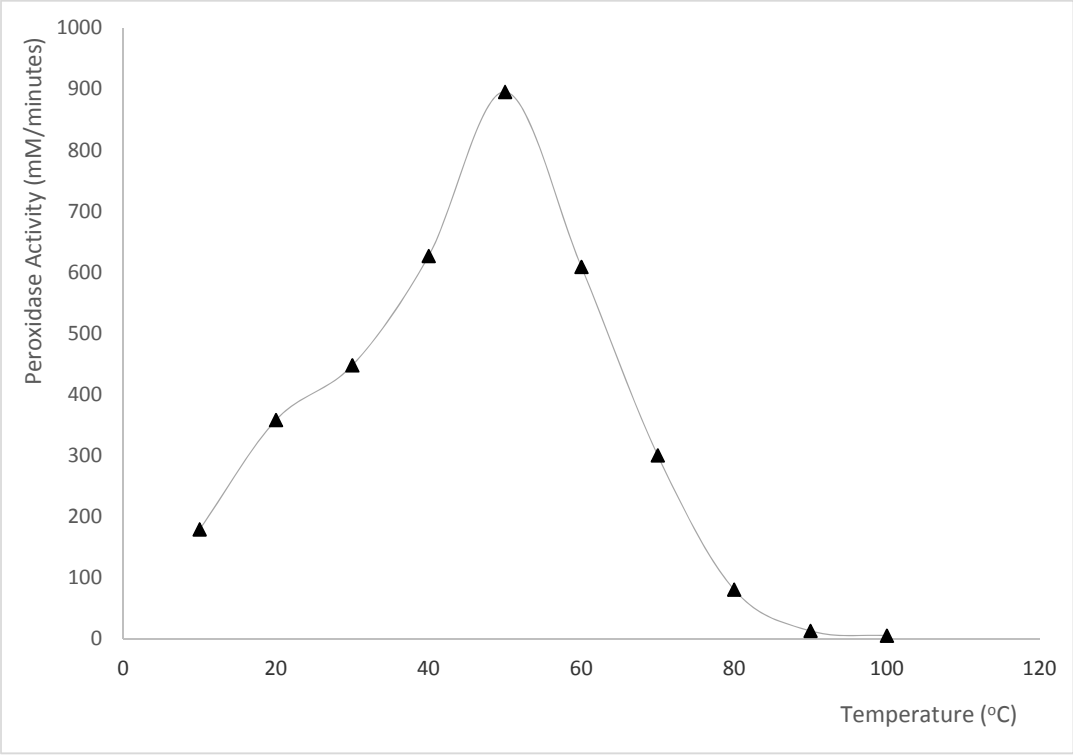

Figure 3: Effect of temperature on the activity of *Calotropis procera* leaves peroxidase

Calotropis procera leaves peroxidase has an optimum activity at pH 6.0 as showed in Figure 4. A rapid decrease in activity was observed on either neutral or extreme acidic side of the pH. There was near or total loss of activity at the basic side of the pH. This is in agreement with the previous reports of [12] for *Moringa oleifera* leaves peroxidase. Also, similar optimum was observed for peroxidase from *capaifera longsdorffii* leaves [30].

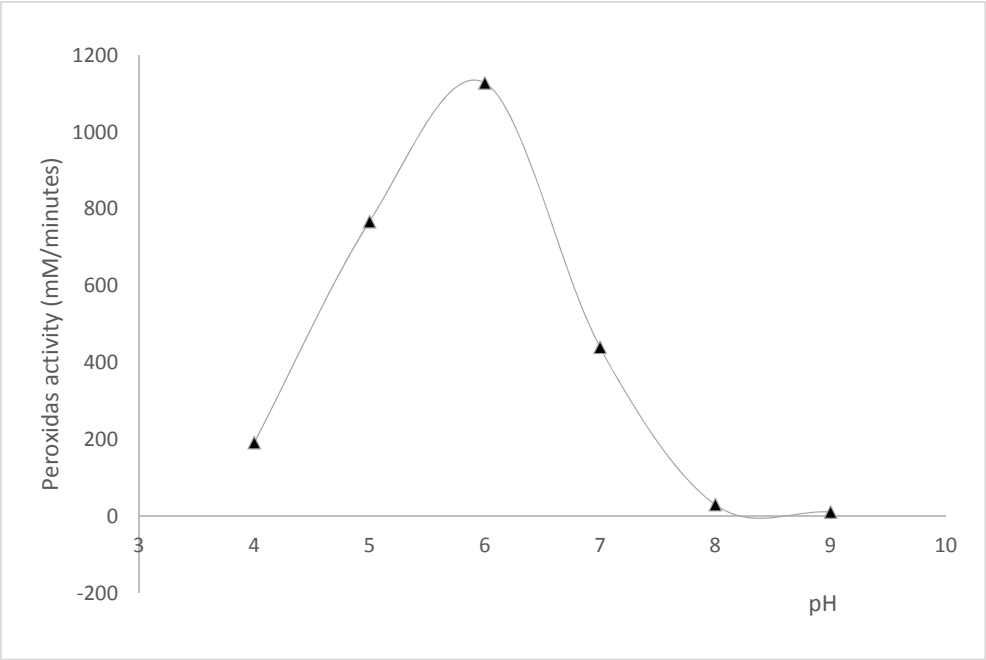

Figure 4: Effect of pH on the activity of *Calotropis procera* leaves peroxidase

Figures 5, 6, 7,8, 9 and 10 shows the effects of metal ion, Fe^{3+}, Co^{2+}, Ni^{2+}, Mg^{2+}, $Zn^{2+}$and chemical (EDTA) respectively on the partially purified peroxidase. The results suggest that all the tested metal ions and EDTA exerted a strong inhibitory effect. [12], reported similar inhibitory trend for *Moringa oleifera* leaves peroxidase.

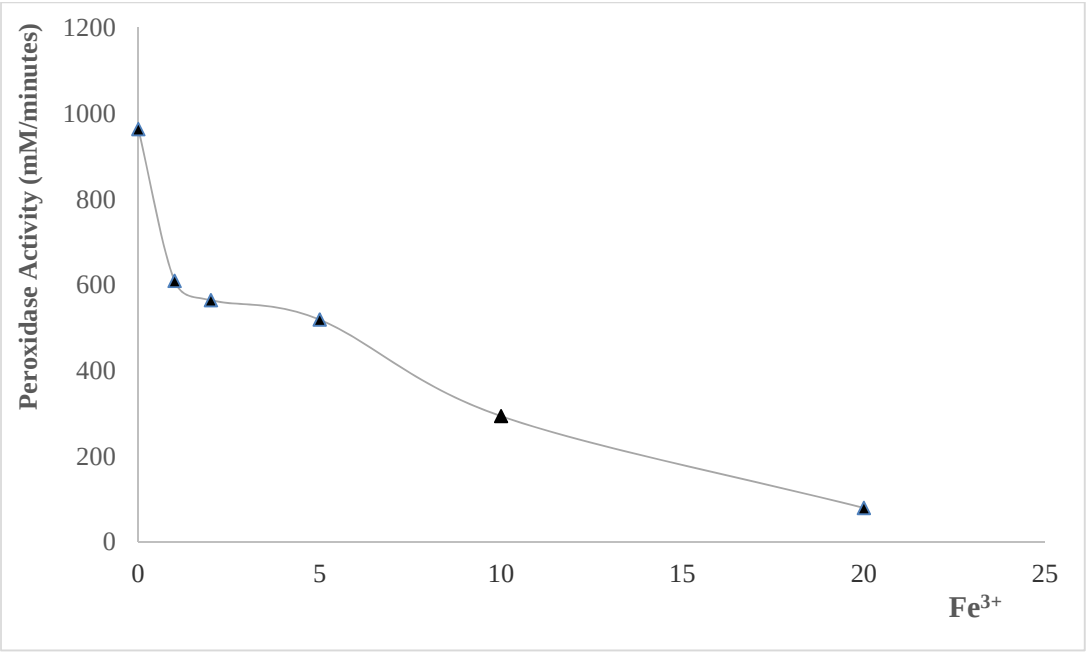

Figure 5: Effect of Fe^{3+} on the activity of *Calotropis procera* leaves peroxidase

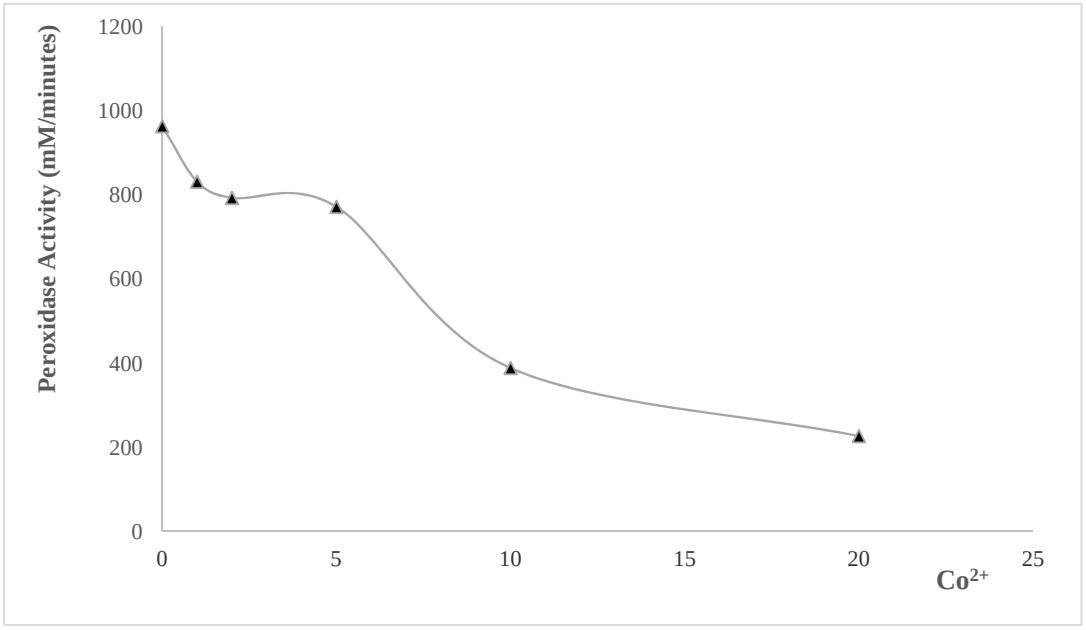

Figure 6: Effect of Co^{2+} on the activity of *Calotropis procera* leaves peroxidase

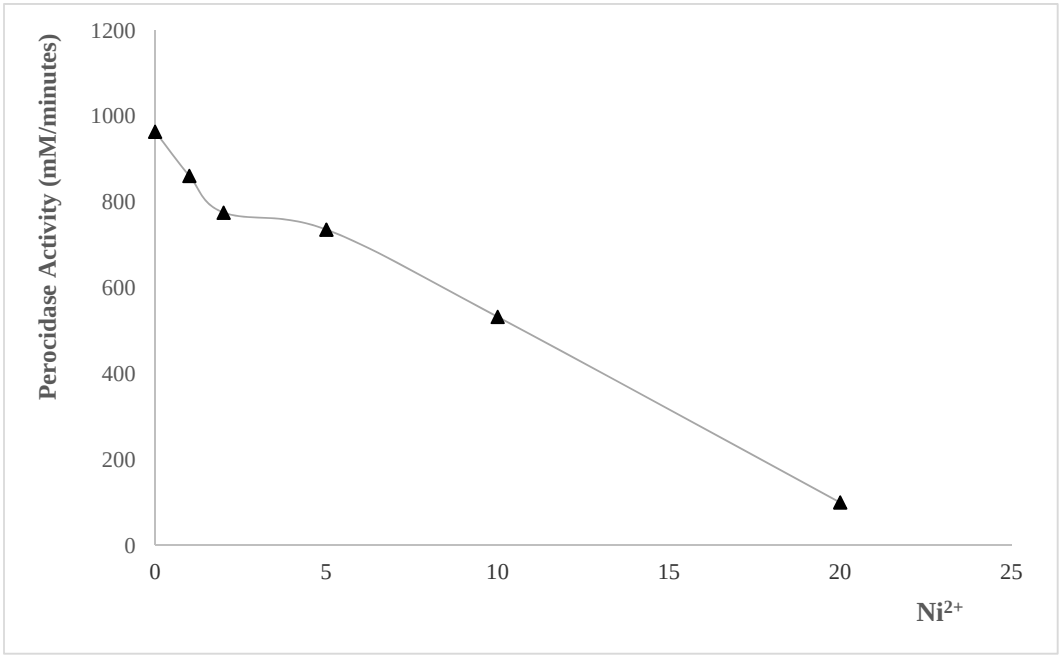

Figure 7: Effect of Ni^{2+} on the activity of *Calotropis procera* leaves peroxidase

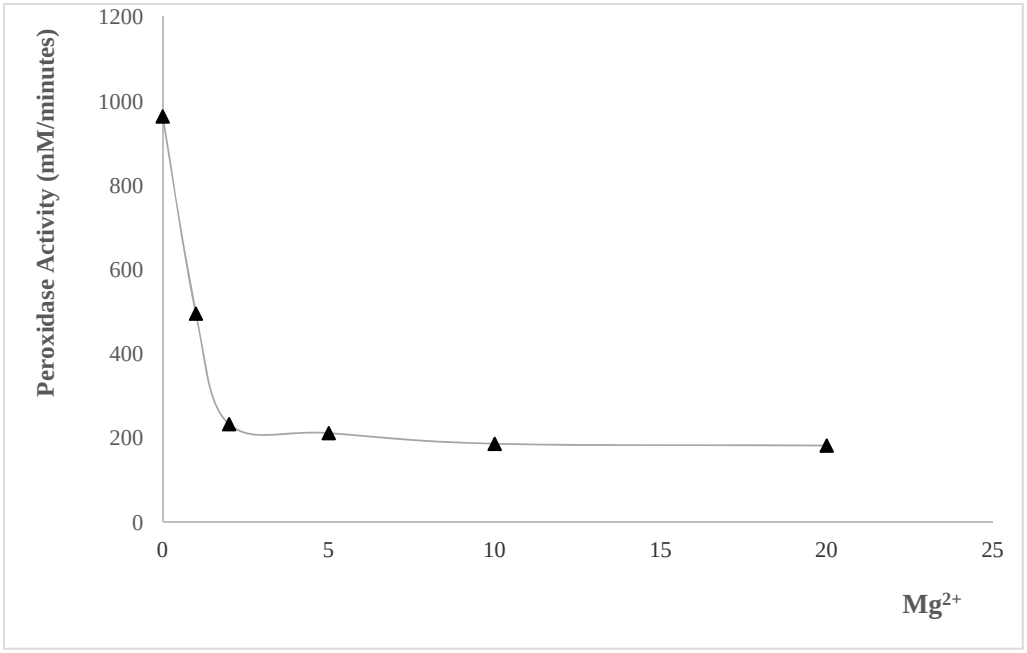

Figure 8: Effect of Mg^{2+} on the activity of *Calotropis procera* leaves peroxidase

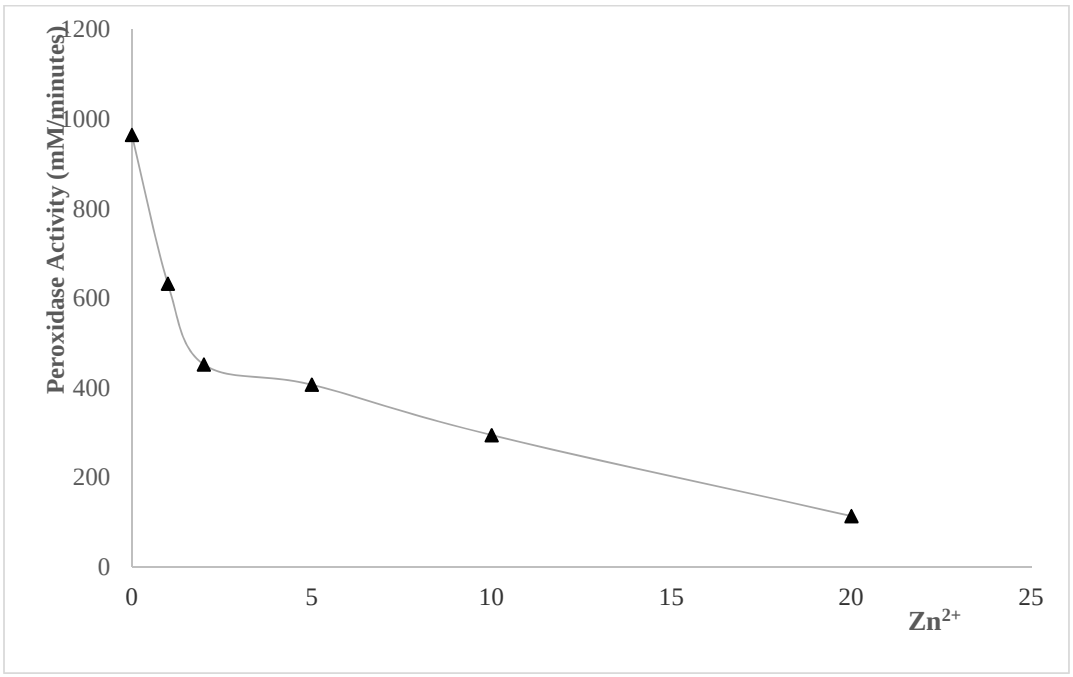

Figure 9: Effect of Zn^{2+} on the activity of *Calotropis procera* leaves peroxidase

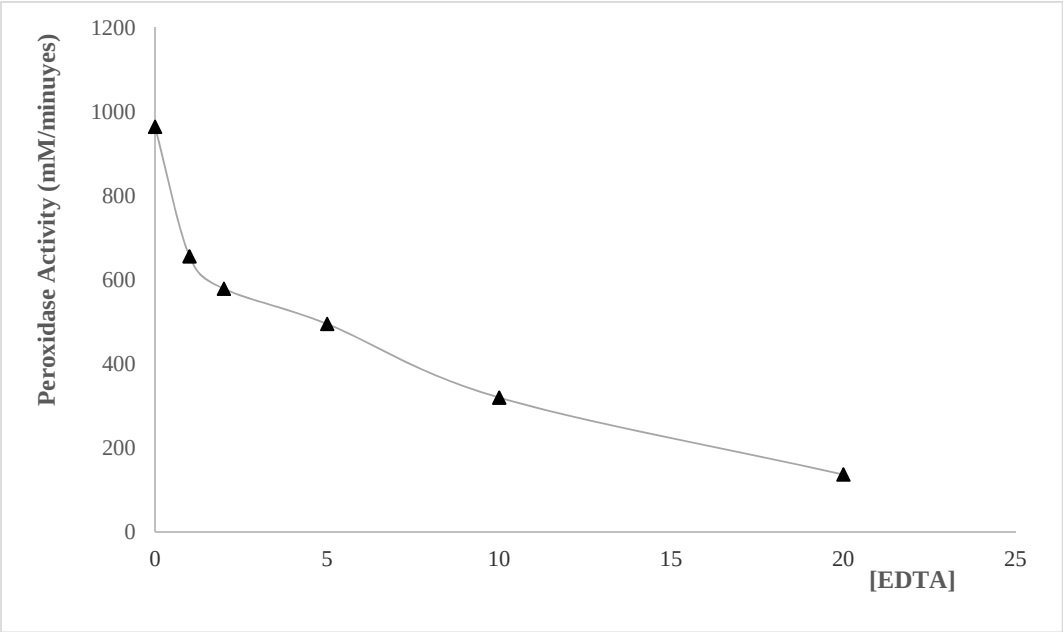

Figure 10: Effect of EDTA on the activity of *Calotropis procera* leaves peroxidase

4. CONCLUSION

Due to the abundance and availability of fresh leaves of *Calotropis procera* plant in Nigerian habitat and with availability of advance purification method, this plant may provide a very cheap source of peroxidase for bioremediation of phenolic pollutants in industrial waste such as oil spill in the Niger Delta of Nigeria. It is therefore, recommended that full industrial purification and stability studies of *Calotropis procera* leaves peroxidise be carried out as *Calotropis procera* may provide potential alternative peroxidase that can compete with commercially available peroxidases for industrial and biotechnological applications.

REFERENCES

[1] Brill AS. Peroxidase and Catalase. In: Florkin and Stotz, Editors, (3rd ed.). Comp. Biochem. 1971; 14: 447-479.

[2] Zia MA, Kousar M, Ahmed I, Iqbal HMN, Abbas RZ.Comparative study of peroxidase purification from apple and orange seeds. African J. of Biotechnol. 2011; 10(33): 6300-6303.

[3] Veitch NC, Smith AT. Horseradish peroxidase. Adv. Inorg. Chem. 2001; 51:107–162.

[4] Veitch NC. Horseradish peroxidase: a modern view of a classic enzyme. Phytochemistry, 2004; 65: 249-259.

[5] Adam W, Lazarus M, Saha-Moller CR, Weichold O, Hoch U, Haring D, Schreier P. Biotransformations with peroxidases. AdV. Biochem. Eng. Biotechnol.1999; *63*:73-108.

[6] Hiraga S, Sasaki K, ItoH, Ohashi Y, Matsui H. A large family of class III plant peroxidases. *Plant Cell Physiol.* 2001; *42*:462-468.

[7] Ryan O, Smyth MR, O´'Fa´ga´in C. Horseradish peroxidase: the analyst's friend. In: Ballou, D.K. (Ed.), Essays in Biochemistry, Vol. 28. Portland Press, London, pp. 129-146.2011.

[8] Jedhav UU, Dawkar VV, Telke AA, Govindwar SP. Decolorization of Direct Blue GLL with enhanced lignin peroxidase enzyme production in *Comamonas sp* UVS. J. Chemical Technol & Biotechnol. 2009; 84(1):126-132.

[9] Lai YC, Lin SC. Application of immobilized horseradish peroxidase for the removal of p-chloorophenol from aqueous solution. *Process Biochem*. 2005; 40:1167-1174.

[10] Dalal S, Gupta MN. Treatment of phenolic waste water by Horseradish peroxidase immobilized by bioaffinity layering. Chemosphere. 2007; 67: 741-747.

[11] Alpeeva IS, Sakharov IY. Soybean peroxidase-catalyzed oxidation of luminol by hydrogen peroxide. J. Agric. and Food Chem. 2005;*53*: 5784-5788.

[12] Khatun S, Ashraduzzaman M, Karim MR, Pervin F, Absar N, Rosma A. Purification and characterization of peroxidase from *Moringa oleifera* L. leaves. Bioresources. 2012; 7: 3237-3251.

[13] Deepea SS, Arumughan C. Purification and characterization of soluble peroxidase from oil palm (Elaeis quineensis Jacq) leaf. Phytochem. 2002; 61:503-511.

[14] Dudey A, Diwakar SK, Rawat KK, Kumar P. Characterization of ofionically bound peroxidase from apple (*Mallus pumilus*) fruits. Prep. Biochem. Biotechnol. 2007; 37: 47-58.

[15] Marquez O, Waliszewski KN, Oliart RM, Pardio VT. Purification and characterization of cell wall-bound peroxidase from vanilla bean. *LWT*. 2008; 41: 1373-1379.

[16] Verma R, Satsangi GP, Shrivastava JN. Ethno-medicinal profile of different plant parts of *Calotropis procera* (Ait.) R. Br. Ethnobotanical Leaflets. 2010; 14: 721–742.

[17] Murti Y, Yogi B, Pathak D. Pharmacognostic standardization of leaves of *Calotropis procera* (Ait.) R. Br. (Asclepiadaceae). Int. J. Ayurveda Res. 2010; 1: 14–17.

[18] Samvatsar S, Diwanji VB. Plant sources for the treatment of jaundice in the tribals of Western Madhya Pradesh of India. J. Ethnopharmacol. 2000; 73: 313 – 316.

[19] Basu A, Sen T, Ray RN, Chaudhuri, A. Hepatoprotective effects of *Calotropis procera* root extract on experimental liver damage in animals. Fitoterapia. 1992; 63: 507-514.

[20] Kumar L, Basu N. Antiinflammatory activity of the latex of *Calotropis procera*. Journal of Ethnopharmacology.1994; 44: 123 – 125.

[21] Kumar VL, Arya S. Medicinal uses and pharmacological properties of *Calotropis procera*. In: Recent Progress in Medicinal Plants, 11. (Govil E. D., ed.). Studium Press, Houston, TX, USA, pp. 373 – 388. 2006.

[22] Roy S, Sehgal R, Padhy BM, Kumar VL. Antioxidant and protective effect of latex of *Calotropis procera* against alloxan-induced diabetes in rats. J. Ethnopharmacol. 2005; 102: 470-473.

[23] Ali H, Muhammad SK. Biosorption of crystal violet from water on leafe biomass of *Calotropis procera*. J. Environmental Sc. Technol. 2008; 1(3): 143-150.

[24] Geng Z, Rao J, Bassi AS, Gijzen M, Krishnamoorthy N. Investigation of biocatalytic properties of soybean seed hull peroxidise. Catal. Today. 2001; 64: 233.

[25] Wang MH, Lee SY, Rhee HI. Properties of anionic peroxidases purified from Chinese cabbage roots. Plant Physiol. Biochem. 1999; 37: 459.

[26] Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin-Ciocalteu's reagent. J. Biol. Chem. 1951; 193: 265-275.

[27] Rad AM, Ghourchian H, Moosavi-Movahedi AA, Hong J, Nazari K. Spectrophotometric assay for horseradish peroxidase activitybased on pyrocatechol–aniline coupling hydrogen donor. Anal. Biochem. 2007; 362: 38-43.

[28] Belcarz A, Ginalski G, Kowalewska B, Pawel K. Spring cabbage peroxidase-potential tool in biocatalysis and bioelectrocatalysis. Phytochem. 2008; 69. 627-636.

[29] Pandey VP, Dwivedi UN. Purification and characterization of *Leucaena leucocephala*, a tree legume. J. molecular catal. B: Enzymatic. 2011; 68: 168-173.

[30] Maciel HPF, Gouvea CMCP, Toyama M, Smolka M, Marangoni S, Pastore GM. Extraction, Purification and biochemical characterization of a peroxidase from *Copaifera langsdorffii* leaves. Quim. Nova. 2007; 30: 1067-1071.