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## BIOCHEMICAL CHARACTERIZATION OF PEROXIDASE FROM *CALOTROPIS PROCERA* LATEX

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### Abstract:-

Peroxidase from *Calotropis procera latex* was purified by benzene treatment, DEAE-Sepharose and Sephacryl S-200 column. The purity was determined from the high specific activity (2200 units/mg protein), purification fold (7.0), and a single band in native PAGE, SDS-PAGE. Peroxidase had molecular mass of 45 kDa. Phenylenediamine had the highest peroxidase activity (140%), o-dianisidine had moderate activity (83%). p-Aminoantipyrine (42%) and pyrogallol (33%) had low affinity towards enzyme. The apparent  $K_m$  value of peroxidase from *C. procera latex* for  $H_2O_2$  and guaiacol were 6.8 and 11.4 mM/ml, respectively. The enzyme showed a pH optimum at 5.5, whereas the optimal temperature was 40°C. The enzyme activity was remained stable up to 40°C. Both  $Cu^{2+}$  and  $Fe^{2+}$  caused the highest activation at 2 and 5 mM (260, 115 and 290, 230%, respectively).  $Hg^{2+}$  caused the highest inhibitory effect. In conclusion, *Calotropis procera latex* could be a new and potential source for peroxidase enzyme.

**Keywords:** *Calotropis procera*, Latex, Peroxidase, Purification, Characterization.

### INTRODUCTION

Peroxidases are widely distributed in plants, microbes, fungi and vertebrates. These enzymes act as an oxidoreductase that catalyzes a reaction, in which  $H_2O_2$  acts as the acceptor and another compound act as the donor of hydrogen atoms (Ikehata *et al.*, 2005). Peroxidases could be broadly classified into three major classes. Plant ascorbate peroxidase, cytochrome c peroxidase and bacterial peroxidase belong to same evolutionary branch and are designated as class I, whereas secretory fungal enzymes, ligninolytic peroxidase (Moreira *et al.*, 2005), and manganese (Mn) peroxidase from fungi (Moreira *et al.*, 2006) are classified as class II peroxidases. Secretory plant peroxidases and vacuolar peroxidases such as horseradish peroxidases that usually contain a ferriprotoporphyrin IX prosthetic group linked to His residue are designated as class III peroxidases (Onsae *et al.*, 2004). Class III peroxidases have been reported to play physiological roles such as catabolizing phenolic compounds for biosynthetic and catabolic functions (Hamid and Khalil-ur-Rehman, 2009), cross linking of cell wall polysaccharide, cell elongation regulation, wound healing, abiotic stress, ethylene biosynthesis, scavenging of peroxides, oxidation of toxic compounds, defense mechanism towards pathogens, biodegradation reactions, metabolism of plant hormone, indole acetic acid (IAA) oxidation, lignification of cell wall (Manu and Rao, 2009) and anthocyanin degradation (Zhang *et al.*, 2005).

Peroxidases were involved in production of alkaloid, biosensor construction, air pollution damage control (Lee, 2002), food processing, food storage, biotransformation of organic

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compounds, treatment of industrial waste water containing phenols and aromatic amines (Rudrappa *et al.*, 2007), production of oxidants, lignin degradation in fuels, bio-bleaching process, site directed mutagenesis (Dubey *et al.*, 2007), production of secondary antibodies for research and medical diagnosis, production of dimeric alkaloids, preventing deterioration of many frozen fruits Quality, biotransformation of organic compounds, preventing deterioration of prepared foods (Dicko *et al.*, 2006) and the combination of peroxidase with IAA was introduced as a novel cancer therapy (Kim *et al.*, 2004).

*Calotropis procera* was known to possess multifarious medicinal properties. The root of the plant was used as a carminative in the treatment of dyspepsia (Kumar and Arya, 2006). Furthermore, the root bark and leaves of *Calotropis procera* were used by various tribes of central India as a curative agent for jaundice (Samvatsar and Diwanji, 2000). The chloroform extract of the root was shown to exhibit protective activity against carbon tetrachloride induced liver damage (Basu *et al.*, 1992). The milky white latex of this plant was reported to exhibit potent anti-inflammatory, analgesic and weak antipyretic activity in various experimental models (Kumar and Arya, 2006; Kumar and Basu, 1994; Dewan *et al.*, 2000a,b), besides, it was also demonstrated to possess antioxidant and anti-hyperglycemic property (Roy *et al.*, 2005). The aim of present work is to study the biochemical properties of peroxidase as antioxidant enzyme from latex of *Calotropis procera* in view of its applications in biotechnology and food industries.

## MATERIAL AND METHODS

### Plant materials:

The latex of *Calotropis procera* was collected by breaking the stem.

### Purification of peroxidase from *Calotropis procera* latex:

The latex was separated from rubber content by adding 0.5 ml of benzene to each 1.0 ml of latex followed by centrifugation at 16500 Xg for 15 min at room temperature, and two layers was obtained. The upper benzene layer designated as crude extract. The crude extract was loaded on a DEAE-Sepharose CL-6B column (10 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.2. The enzyme was eluted with a stepwise gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Protein fractions exhibiting peroxidase activity were pooled in one peak. DEAE-Sepharose peroxidase fraction was concentrated through dialysis against solid sucrose and loaded on Sephacryl S-200 column (90 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.2 and developed at a flow rate of 30 ml/h and 3 ml fractions were collected.

### Molecular weight determination:

Enzyme molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90 x 1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and  $\alpha$ -amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume ( $V_0$ ). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and  $\alpha$ -lactalbumin (14,200) were used for the calibration curve.

### Protein determination:

Protein was determined either by the method of Bradford (1976) using bovine serum albumin as a standard.

### Peroxidase assay:

Peroxidase activity was carried out according to Miranda *et al.* (1995). The reaction mixture contained in one ml, 8 mM  $H_2O_2$ , 40 mM guaiacol, 50 mM sodium acetate buffer, pH 6.5 and least

amount of enzyme preparation. The change of absorbance at 470 nm due to guaiacol oxidation was followed at three minutes intervals. One unit of peroxidase activity is defined as the amount of enzyme that increases the O.D. 1.0 per min under standard assay conditions.

**Characterization of peroxidase**

**Substrate specificity:**

The substrate specificity of the enzyme was determined using a number of potential natural electron donors are used under the standard assay conditions.

**Kinetic constant:**

Kinetic parameters of the peroxidase for hydrogen peroxide and guaiacol as substrates were determined at pH 5.5. The values of Michael's constants (Km) were determined from Lineweaver Burk plot.

**Optimum pH and temperature:**

The optimum pH for the peroxidase activity was determined by assaying the activity at different pH values, using the following buffers: 50 mM sodium acetate buffer (pH 4.5 to 6.5) and 50 mM Tris-HCl buffer (pH 7.0 to 8.5). The optimum temperature for peroxidase activity was determined by assaying the enzyme at temperatures from 30 to 80°C at pH 5.5.

**Thermal stability:**

Heat stability was measured by incubating the enzyme at 30 to 80°C for 15 min in 0.05 mM sodium acetate buffer, pH 5.5. After heat treatment, the enzyme solution was cooled and the residual activity was assayed under standard assay conditions.

**Metal ion effect:**

The effects of various metal ions on enzyme activity were determined by pre-incubating the enzyme with 2 and 5 mM metal ions for 15 min before assaying the enzyme activity. The activity in absence of metal ions was considered as 100%.

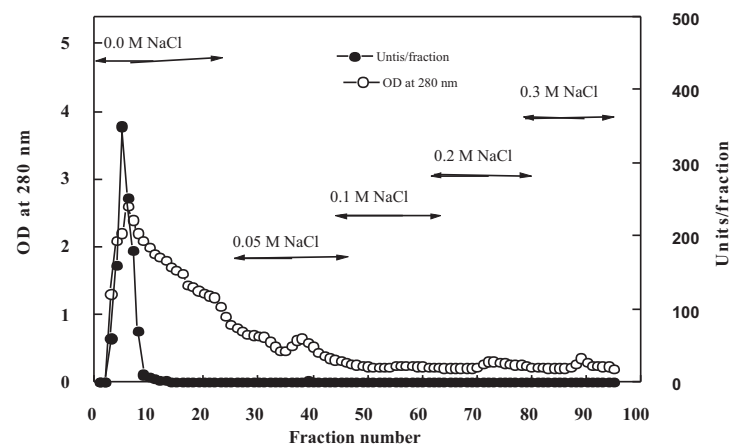
**RESULTS AND DISCUSSION**

The purification procedure of peroxidase from *Calotropisprocera* latex included chromatography on DEAE-Sephacryl column followed by Sephacryl S-200 column. The purification was summarized in Table (1). The elution profile of the chromatography on DEAE-Sephacryl column showed that peroxidase activity was detected in one peak eluted by 0.0 M NaCl. The specific activity of peroxidase was 632 units/mg protein that represented 2.0 fold purification over the crude extract with 80 % recovery (Fig. 1). A Sephacryl S-200 column was used to obtain peroxidase with the highest possible specific activity of 2200 units/mg protein that represented 7.0 fold purification over the crude extract with 56 % recovery (Fig. 2). The *C. procera* latex peroxidase is proved to be pure after Sephacryl S-200 column as judged by SDS-polyacrylamide gel electrophoresis (Fig. 3) The molecular weight of peroxidase from *C. procera* latex was calculated from a Sephacryl S-200 calibration curve and estimated to be 45kDa. This was confirmed by SDS-PAGE (Fig. 3), and estimated to be 44 kDa, as single subunit.

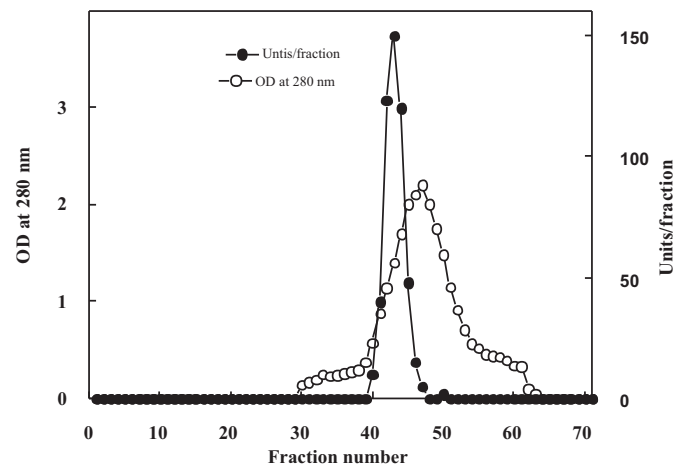
**Table 1: Purification scheme of peroxidase from *C. procera* latex.**

Step	Total protein (mg)	Total activity (units*)	S.A. (units/mg protein)	Fold purification	Recovery %
Crude extract	3.1	980	316	1	100
Chromatography on DEAE- Sepharose 0.0 M NaCl	1.25	790	632	2.0	80
Gel filtration on Sephacryl S-200 Peroxidase	0.25	550	2200	7.0	56

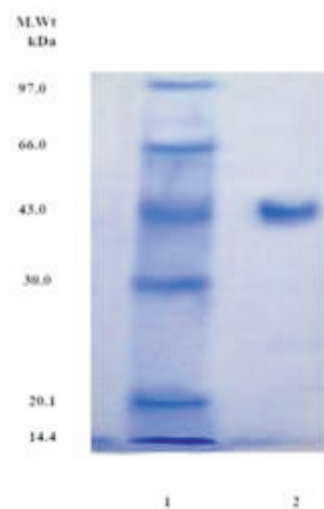
\*One unit of peroxidase activity is defined as the amount of enzyme that increases the O.D. 1.0 per min under standard assay conditions.



**Fig. 1:** A typical elution profile for the chromatography of peroxidase from *C. procera* latex on DEAE-Sephacryl column (10 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.



**Fig. 2:** Gel filtration of peroxidase from *C. procera* latex from DEAE-Sephacryl fraction on Sephacryl S-200 column (90 x 1.6 cm i.d.). The column was equilibrated with 20 mM Tris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions.



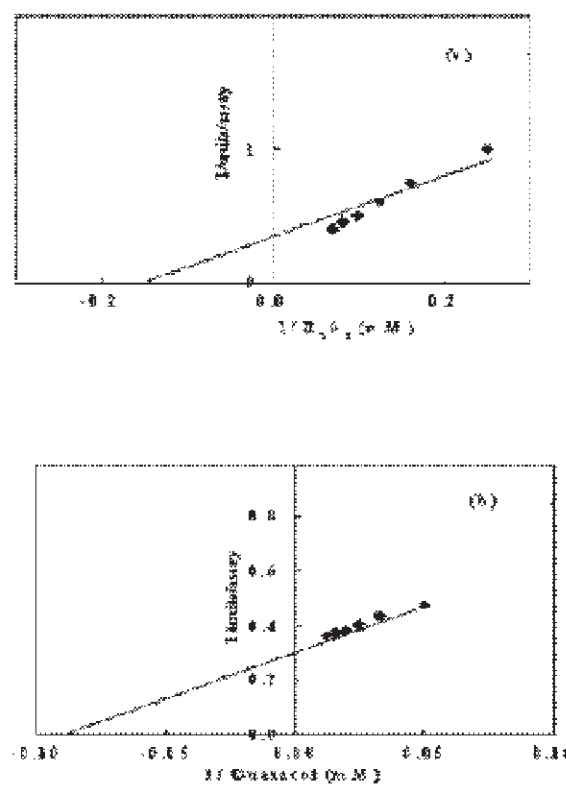
**Fig. 3: SDS-PAGE for homogeneity and molecular weight determination of peroxidase from *C. proceralate* .1- Protein markers; 2- Sphacryl S-200 peroxidase.**

This molecular weight was similar to that reported for peroxidases from *Ficus sycomorus latex* (43 kDa) (Mohamed et al., 2011a), *Raphanus sativus* (44 kDa) (Kim et al., 2005) and *Withania somnifera* (34-48 kDa) (Johri et al., 2005) Most of the peroxidases reported to date are monomers. Some exceptions were reported for peroxidases from coconut (tetramer subunit molecular weight of 55 kDa) (Mujer et al., 1983), oil palm leaf (native and tetramer subunit molecular weights of 200 and 48 kDa, respectively) (Deepa and Arumughan 2002) and vanilla bean (native and tetramer subunit molecular weights of 186 and 46.5 kDa, respectively) (Marqueza et al., 2008).

The substrate specificity of peroxidase from *C. proceralate* latex was examined using a number of potential natural electron donor substrates (Table 2). The activity with the guaiacol is regarded as 100% activity. o-Phenylenediamine had the highest peroxidase activity (140%), while o-dianisidine had moderate activity (83%). p-Aminoantipyrine (42%) and pyrogallol (33%) had lower affinity towards the enzyme. Similarly, o-phenylenediamine was found to be the best substrate for peroxidase from chewing stick miswak followed by guaiacol, o-dianisidine, pyrogallol and p-aminoantipyrine (Mohamed et al., 2012). Horseradish peroxidase exhibited high activity toward o-phenylenediamine horseradish peroxidase (Mohamed et al., 2011b). Substrates commonly used in immunodiagnostic kits as 2,2-azino-bis [3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS), 4-chloro-1-naphthol (4C-1N), o-phenylenediamine and 3,3',5,5'-tetramethylbenzidine (TMB) were found to be the best substrates for peroxidase from *Ficus sycomorus latex* (Mohamed et al., 2011a). *Withania somnifera* peroxidases readily catalyzed the oxidation of phenolic substrates like guaiacol and o-dianisidine but did not catalyze ascorbic acid (Johri et al., 2005). Kim et al. (2005) reported that *Raphanus sativus* peroxidase had higher affinity towards o-dianisidine than guaiacol and o-phenylenediamine. o-Dianisidine was also found to be the best substrate for black gram husk peroxidase followed by guaiacol and pyrogallol (Ajila and Rao, 2009).

**Table 2: Relative activities of peroxidase from *C. proceralate* toward different substrates.**

Substrate	Relative activity (%)
Guaiacol	100
<i>o</i> -Phenylenediamine	140
<i>o</i> -Dianisidine	83
<i>p</i> -Aminoantipyrine	42
Pyrogallol	33



**Fig. 4: Lineweaver-Burk plot relating *o*peroxidase from *C. procera* latex reaction velocities to HQ(a) and guaiacol (b) as substrate concentration.**

The kinetic parameters of peroxidase from *C. procera* latex for HQ and guaiacol were obtained (Fig. 4). The apparent  $K_m$  value of peroxidase from *C. procera* latex for HQ and guaiacol were 6.8 and 11.4mM/ml, respectively. Different  $K_m$  values of HQ were reported for peroxidases from *C. jambhiri* (0.5 mM) (Mohamed *et al.*, 2008), *Ficus sycomorus* latex (1.2 mM) (Mohamed *et al.*, 2011a), chewing stick miswak (0.9 mM) (Mohamed *et al.*, 2012), turnip (0.85 mM) (Duarte-Vazquez *et al.*, 2001), marula fruit (1.77 mM) (Mdluli, 2005), *Raphanus sativus* (1.27 mM) (Kim *et al.*, 2005). The apparent  $K_m$  value of peroxidase from *C. procera* latex for guaiacol was 11.2 mM/ml. Different  $K_m$  values of guaiacol were reported for peroxidases from buckwheat seeds (0.202-0.288 mM guaiacol) (Suzuki *et al.*, 2006), *Pleargonium graveolense* (7.3 mM guaiacol) (Seoket *et al.*, 2001), *Brassica napus* (3.7 mM guaiacol) (Duarte-Vazquez *et al.*, 2001), *chewing stick miswak* (17.33 mM guaiacol) (Mohamed *et al.*, 2012) and *C. jambhiri* (5 mM guaiacol) (Mohamed *et al.*, 2008).



The pH profile for the purified peroxidase from *C. procera* latex was carried out. Peroxidase activity exhibited a pH optimum at 5.5 (Fig. 5). pH optimum of 5.5 was detected for peroxidases from horseradish (Mohamed *et al.*, 2011b), chewing stick miswak (Mohamed *et al.*, 2012), black gram husk (Ajila and Rao, 2009), date palm leaves (Al-Senaidy and Ismael, 2011) and Citrus jambhiri cv. Adalia (Mohamed *et al.*, 2008).

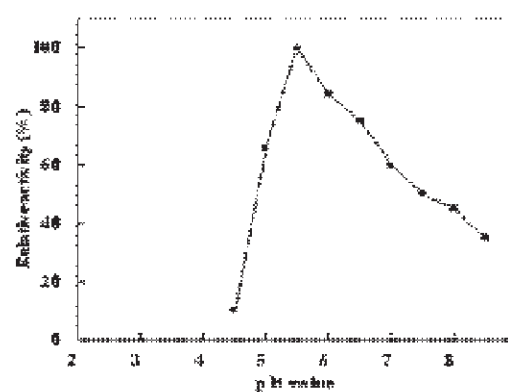


Fig. 5: pH Optimum of peroxidase from *C. procera* latex.

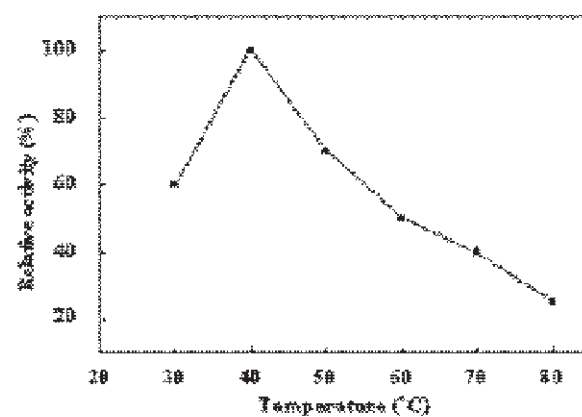


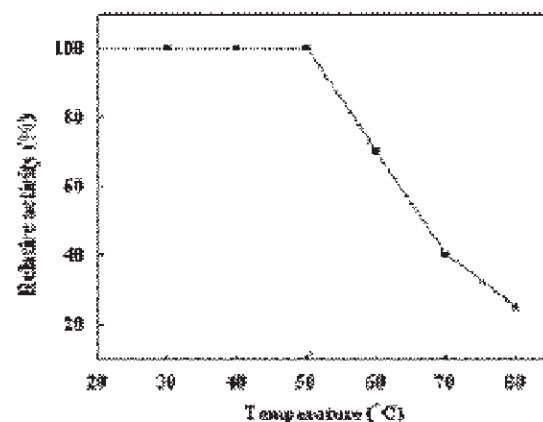
Fig. 6: Optimum temperature of peroxidase from *C. procera* latex.

Peroxidase from *C. procera* latex was found to have temperature optimum at 40°C (Fig. 6). The same optimum temperature was detected for peroxidases from horseradish (Mohamed *et al.*, 2011b) and *Citrus jambhiri* (Mohamed *et al.*, 2008). The optimum temperature for *Ficus sycomorus* latex peroxidase was 35 to 40°C (Mohamed *et al.*, 2011a). The lowest temperature optima were detected for peroxidases from miswak (30°C) (Mohamed *et al.*, 2012), buckwheat seed (10-30°C) (Suzuki *et al.*, 2006) and vanilla bean (16°C) (Marqueza *et al.*, 2008). The effect of temperature on the thermal stability of peroxidase from *C. procera* latex was obtained (Fig. 7). The enzyme activity remained stable up to 40°C. The enzyme restored 70 and 50% of its activity at 50 and 60°C, respectively. Similarly, the thermal stability of peroxidases from horseradish (Mohamed *et al.*, 2011b), Citrus jambhiri (Mohamed *et al.*, 2008), apple (Valderrama and Clemente, 2004), orange (Clemente, 1998), mango (Khan and Robinson, 1993), *Withania somnifera* (Johri *et al.*, 2005) and

marula fruit (Mdluli, 2005) was reported to remain stable up to 40°C. Peroxidases from buckwheat seeds exhibited low thermal stability (20-30°C) (Suzuki *et al.*, 2006).

**Table 3: Effect of metal ions on activity of peroxidase from *C. procera* latex.**

Metallon	Relative activity (%)	
	2 mM	5 mM
Cu <sup>2+</sup>	260	290
Fe <sup>2+</sup>	115	230
Co <sup>2+</sup>	105	130
Zn <sup>2+</sup>	95	60
Ca <sup>2+</sup>	84	73
Ni <sup>2+</sup>	86	57
Fe <sup>3+</sup>	81	48
Pb <sup>2+</sup>	80	43
Hg <sup>2+</sup>	54	33



The effect of different metal ions on peroxidase from *C. procera* latex was examined (Table 3). Cu<sup>2+</sup>, Fe<sup>2+</sup> and Co<sup>2+</sup> were found to enhanced activity of peroxidase, while both Cu<sup>2+</sup> and Fe<sup>2+</sup> caused the highest activation at 2 and 5 mM (260, 115 and 290, 230%, respectively). The other metals had slight inhibitory effect at 2 mM, while at 5 mM moderate inhibitory effect on enzyme activity was detected. Hg<sup>2+</sup> caused the highest inhibitory effect compared to the other tested metals. The same result was detected for peroxidase from horseradish except Hg<sup>2+</sup> had strong inhibitory effect (Mohamed *et al.*, 2011b). Mohamed *et al.* (2008) reported that most of examined metal ions (Ba<sup>2+</sup>, Co<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Cu<sup>2+</sup> and Ni<sup>2+</sup>) had very little effect on Citrus jambhiriperoxidase. The moderate inhibitory effect on enzyme was found for Li and Zn, while Hg<sup>2+</sup> had strong inhibitory effect. It was reported that Zn<sup>2+</sup>, Li<sup>+</sup>, Mg<sup>2+</sup>, Ba<sup>2+</sup>, Ca<sup>2+</sup> and Fe<sup>3+</sup> stimulated the activity of black gram husk peroxidase at 5 mM, whereas Mn<sup>2+</sup>, Cd<sup>2+</sup>, Al<sup>3+</sup>, Na<sup>+</sup> and K<sup>+</sup> moderately inhibited the enzyme at the same concentration (Ajila and Rao, 2009). Most of the metal ions tested had partially inhibitory effects on peroxidase from chewing stick miswak, while Co<sup>2+</sup>, Fe<sup>3+</sup> and Ca<sup>2+</sup> were greatly able to enhance the activity (Mohamed *et al.*, 2012). The activities of *Metroxylon sagu* peroxidases were highly enhanced by Al<sup>3+</sup>, Ca<sup>2+</sup> and Fe<sup>3+</sup> but they were moderately inhibited by Zn<sup>2+</sup> (Onsaet *et al.*, 2004). However, vanilla bean peroxidase was strongly inhibited by Fe<sup>2+</sup> and Hg<sup>2+</sup> (Marquezaet *et al.* 2008). In conclusion, *Calotropis procera latex* could be a new and potential source for peroxidase which

might be used in several applications.

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