Heavy Metals Effect on the Activity and Kinetics of Peroxidase Enzyme in Crude Extracts of *Rosmarinus officinalis* and *Eruca Sativa*

Abstract

The catalytic activities and kinetic parameters of the enzyme peroxidase (POX), extracted from two plant species namely *Rosmarinus officinalis* and *Eruca Sativa*, were investigated in the presence and absence of various heavy metals. The activation effects of heavy metals Co^{2+}, Fe^{3+} and Pb^{2+} have performed a noncompetitive inhibition on the enzyme activity in the crude extract of *Eruca Sativa*. In contrast, Cd^{2+}, Ni^{+} and Cu^{2+} have an uncompetitive inhibition for the two selected plants. Furthermore, Fe^{3+}, Al^{3+}, Pb^{2+} and Mo^{2+} were found to be acted as noncompetitive inhibitors on the enzyme activity in the crude extract of *Rosmarinus officinalis*. Presence of heavy metals altered the enzyme activity by acting as uncompetitive or noncompetitive inhibitors depending on the type of heavy metals.

Key words: **Relative activities, Uncompetitive inhibition, Noncompetitive inhibition**.

1.Introduction

Oxygen plays a major role in energy metabolism as the final acceptor for electrons in the electron transport chain. However, when cells use oxygen to produce energy, free radicals are created as a consequence of ATP realization by the mitochondria. These by-products are mostly reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) generated from various cellular redox processes (Halliwell & Gutteridge, 2007). These reactive species play a dual role as both toxic and beneficial compounds *(*Young and Woodside, 2001*;* Valko *et al.*, 2005).

In general, free radicals can be categorized as endogenous and exogenous free radicals. Endogenous free radicals are those generated as a consequence of immune cell activation, inflammation, mental stress, immoderate exercise, ischemia, infection, cancer and aging (Valko *et al.*, 2005). While, exogenous ROS and RNS are those sourced from polluted air and water, smoking, alcohol, heavy or transition metals, certain drugs, pesticides and industrial solvents, cooking (smoked meat, used oil, fat), radiation (increased exposure to sunlight), etc. (Young and Woodside, 2001*;* Chatterjee *et al.*, 2007). A number of heavy metals such as cobalt, iron, manganese, molybdenum, nickel, zinc and copper are essential micronutrients for normal biochemical and physiological functions such as redox reactions, electron transfer and other important metabolic processes in plants(Paul *et al.*, 2012). Other heavy metals which are nonessential (Pb^{+2}, Cd^{+2}, Cr^{+2}, Hg^{+2} etc.) are potentially highly toxic for plants (Sebastianil *et al.*, 2004). Recently, the increasing accumulation of these toxic heavy metals in large areas of lands resulting from urban activities was stressed. Their impacts on the plantation and the quality of the produced crops were also studied (Khan *et al.*, 2000*;* Clemens, 2001). Plants under stress exert some defense mechanisms to protect themselves from the harmful effect of oxidative stress. ROS scavenging is one among the common defense reaction against a biotic stresses (Vranova *et al.*, 2002; Jaleel *et al.*, 2007a). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatic reduced molecules (antioxidants) and enzymatic antioxidants such as polyphenol oxidase and peroxidase (Jaleel *et al.*, 2007b). Peroxidases are group of enzymes found in animal and plant tissues as well as in microorganisms that catalyze the oxido-reduction reaction between H_2O_2 and various reductants. Besides their main function as a catalysts in the oxido-reduction reaction between H_2O_2 and various reductants, peroxidase

(POX) in plants also participates in many other functions such as hormone regulation, defense mechanisms, indolacetic degradation and lignin biosynthesis (Serrano-Martínez *et al*., 2008). Rocket (*Eruca sativa*) is a dark green annual plant, about 20 to 50 cm in height, with a spicy-pungent taste (Morales and Janick, 2002). Rocket extract significantly scavenged several ROS and RNS. It has an effective antioxidant and renal protective action and stop oxidative damage inflicted to the kidney (Gurpreet-Singh *et al*., 2007). Rosemary (*Rosmarinus officinalis*) extracts formulations are the only ones commercially available for use as antioxidants in the European Union and the United States, and they are marketed in an oil-soluble form, as a dry powder, and in water-dispersible or water-miscible formulations (Aguilar, *et al*., 2008, Bozˇin *et al*., 2007). Rosemary scavenges superoxide radicals, repress lipid oxidation and chelate metals. The phenolic content of rosemary is approximately 150 mg/g (Peschel *et al*., 2007). In the present study, the kinetic parameters of POX in crude leaves extract of rocket and rosemary in presence and absence of various heavy metals were investigated to illustrate the potential activities of peroxidase enzyme to be used in different industrial applications.

1. Material and Methods

2.1 Plant samples

Fresh Plant samples of *Eruca sativa* have been obtained from local market, while *Rosmarinus officinalis* was collected from local garden (Alkarak region) in interval between June-November, 2013.

2.2 Chemicals:

Cobaltus chloride, Lead IV acetate 3- hydrate (AVONDALE Laboratories, England); Di Sodium Hydrogen phosphate, Cupric sulfate anhydrous (FULKA, Spain); Aluminium sulfate 2-hydrate (F.E.R.O.S.A, England); Ammonium molybdate, Cadmium nitrate 4-hydrate, Phenol reagent (folin and ciocalteu), Sulphuric acid 98%, Sodium dehydrogenate (Laboratory rasayan, India); Iron IV sulfate hydrogen (BDH Technical laboratory supplies, Englan); Nickel chloride (SIGMA, USA).

2.3 Preparation of crude plant extracts

Leaves of the selected plants were homogenized in 50 mM sodium phosphate buffer (pH 6.8) in the ratio 1:1 (w/v) in a blender for 3 min. The homogenate was filtered using cloth sheet and then was centrifuged at 13,000 rpm (Sigma112, Germany) for 20 min at room temperature. The supernatant was collected as crude enzyme solution and was kept at 4°C until use (Hammerschmidt *et al.* 1982).

2.4 Protein estimation

Total protein concentration was determined in triplicate by Lowry *et al*. (1951) method using bovine serum albumin (BSA) as a standard. The amount of the soluble protein was calculated from the standard curve as mg of protein per ml of test sample.

2.5 Enzyme assay

Peroxidase activity was measured using the method described by Kumar and Khan (1982). The assay mixture of POX contained 2 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H_2O_2 and 0.5 mL of the enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 mL of 2.5 M H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance

at 420 nm against a blank prepared by adding the extract after the addition of 2.5 M H_2SO_4 at zero time.
The activity was expressed in unit mg^{-1} protein. One U is defined as the change in the absorbance per one min per mg protein (Kumar and Khan, 1982).

2.6 Kinetic Determination

POX activity in crude extract was measured with catechol as a substrate at varying concentrations (1-20 mM) at 495 nm. Lineweaver-Burk double reciprocal plots were used to calculate the enzyme kinetic parameters (V_{max}, K_m) in the absence or presence of selected heavy metals (Cd^{2+}, Co^{2+}, Al^{3+}, Cu^{2+}, Fe^{3+}, Mo^{2+}, Ni^{2+} and Pb^{2+}) at 400µM concentration (Lineweaver, H. & Burk, 1934).

2.7 Heavy Metals solutions

Fresh stock solutions of different heavy metals (Cd^{2+}, Co^{2+}, Al^{3+}, Cu^{2+}, Fe^{3+}, Mo^{2+}, Ni^{2+} and Pb^{2+}) of 1M were prepared (Gao *et al.*,2003). Different concentrations were used (200, 400, 800 µM). Maximum effects were seen at concentration 400µM. Therefore, for all experiments, 400µM concentration of heavy metal was used.

2.8 Specific activity (Unit mg^{-1}):

The specific activity was calculated by dividing V_{max} value by concentration of protein for each selected plant.

2.9 Relative activity (%)

The relative activity (%) of POX enzyme in crude plant extract was calculated by dividing V_{max} value of the treated sample (with heavy metal) by V_{max} value of control sample (not treated) multiplied by 100% (Gulzar and Amin, 2012).

2.10 Statistical analysis:

For all experiments, samples of the selected plants were analyzed and all the assays were carried out in triplicate. The results were expressed as mean ± standard deviation using Microsoft excel 2007**.**

3 Results and Discussion

Metalloenzymes comprise approximately one-third of the known enzymes and require stoichiometric quantities of transition metal ions for their catalytic activities. Peroxidases are important detoxifying enzymes serving to eliminate cells of excess H_2O_2 under normal and stress conditions (Ellis, 2006). Peroxidases in plants are mainly haem-containing enzymes where the prosthetic group is protoporphyrin IX (Laloue *et al*., 1997). So, it is considered as a metallo enzyme contains a tightly bounds metals which are needed to maintain the structural integrity of enzyme and participate in electrophilic catalysis (Ellis, 2006). It was reported that peroxidases remain active in the presence of a number of metal ions, but recent reports have indicated their inhibition by certain metal ions (Keyhani *et al*., 2003).

3.1 Protein content

The protein content in the crude leaves extracts of rosemary and rocket were measured by Lowry method using BSA as standard protein. The result showed that the crude leaves extract of rosemary has 0.786 mg mL^{-1} protein concentration, while crude leaves extract of rocket has 0.22 mg mL^{-1} as listed in table (1).

Table (1): Protein content (mg mL^{-1}) in the crude extracts of leaves of rosemary, and rocket. Mean ±SD, (n=3)

Selected Plants	Protein amount (mg mL^{-1})
Rosmarinus officinalis	0.786 ±0.247
Eruca stavia	0.22 ±0.6

3.5 Relative activities :

The relative activities (%) of the enzyme POX in crude extracts of the studied plants in the presence of heavy metals showed a reduction in the enzyme activity compared to control (Figures 1, 2).

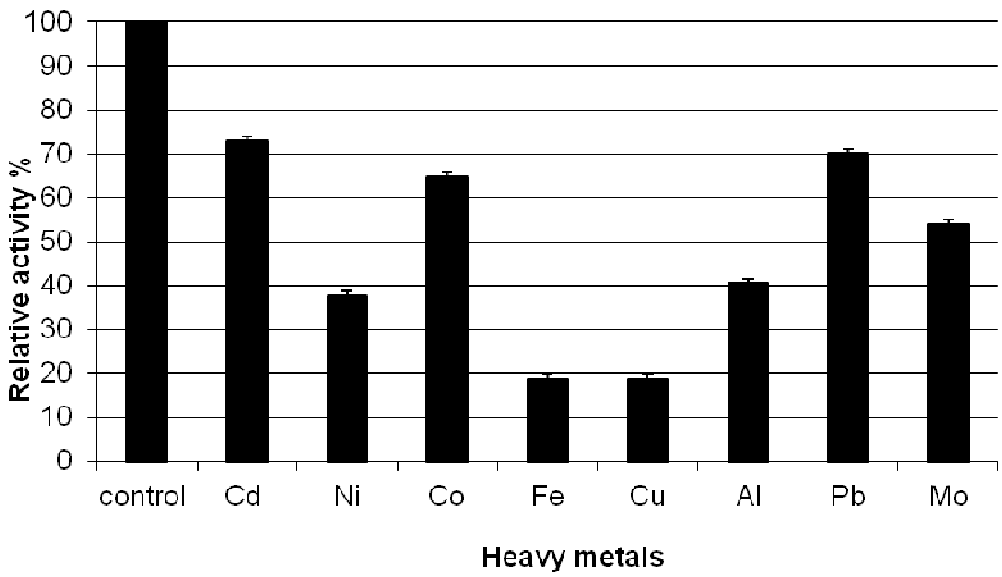

Figure (1): Relative activity (%) of POX enzyme in the crude extracts of *Rosmarinus officinalis* in control and in presence of different heavy metals. Mean ±SD (n=3).

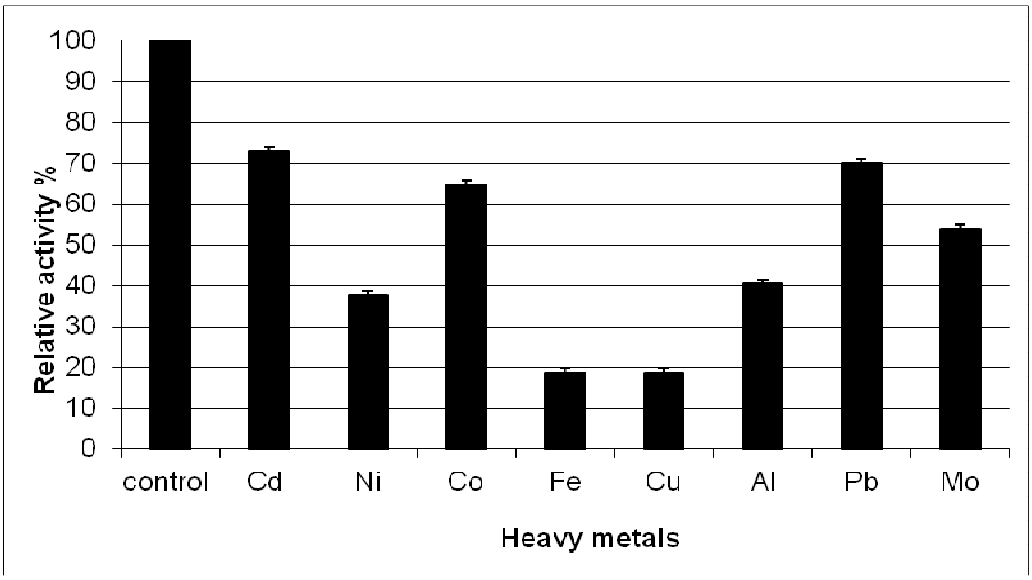

Figure (2): Relative activity (%) of POX enzyme in the crude extracts of *Eruca sativa* in control and in presence of different heavy metals. Mean ±SD (n=3).

These effects of heavy metals on POX activity in the crude leaves extracts of rosemary and rocket extracts will be analyzed according to their type of inhibition: Uncompetitive inhibition or noncompetitive. All results are summarized in tables 2 and 3.

Uncompetitive inhibitor (UCI) binds to the enzyme-substrate complex (EIS complex) and it effectively reduces the concentration of that complex by converting some of it into the ternary EIS complex. The effect of inhibitor realized on increasing the amount of substrate which binds to the enzyme, giving an apparent increase in enzyme-substrate affinity and a decrease in K_m values. As the inhibitor is not competed out by large amounts of substrate, quite the opposite as it needs substrate to bind to the enzyme first, it is effective at high substrate concentrations and therefore decreases V_{max} values. Once the inhibitor has bound to enzyme, it will prevent it from turning the substrate into product by direct interaction, or due to a change in conformation of the active site (Voet & Voet, 2003). Therefore, the ratio of V_{max}/K_m of the POX enzyme in the crude extract will not be affected. The V_{max}/K_m ratio is called the "catalytic power" (Rocha *et al.*, 1998) and is a good parameter for finding the most effective or ineffective heavy metal (Baritaux *et al.*, 1991). It was found that Cd^{2+}, Ni^{2+} and Cu^{2+} have an uncompetitive inhibition on the POX activities in the crude extract of the selected plants by decreasing both K_m and V_{max} values. Similar effects were seen in the POX activity of the crude extract of rosemary by Co^{2+} (Table 2). In contrast, Mo^{2+}, Al^{3+} and Pb^{2+} had the same effects on the crude extract of rocket (Tables 3).

According to heavy metals, it may inhibit or promote the activities of antioxidant enzymes involved in the oxidative defense system (Ahmad *et al.*, 2011). It was reported by Radeva *et al*. (2010) that antioxidant enzymes including peroxidase, polyphenyl oxidase and catalase activities in pea seedlings were gradually increased by increasing the concentrations of Cd^{2+}.

Noncompetitive inhibitors (NCI) bind to an inhibitor site which is remote from the active site. This means that the inhibitor is not competed out by high substrate concentrations and works equally well at low and high concentrations of the substrate. A classical noncompetitive

inhibitor (pure noncompetitive) has no effect on substrate binding, so the enzyme-substrate affinity not changed. A mixed inhibitor allows the substrate to bind, but reduces its affinity, so the K_m value is increased. At high substrate concentrations, noncompetitive inhibitors of both classical and mixed types reduce V_{max} value. Moreover, they will lower the catalytic power at low substrate concentrations and as a result they will decrease the ratio of V_{max}/K_m (Shengwen *et al.,* 2013 ; Amine *et al.*, 2006).

As shown in tables (2,3), the V_{max} value of POX decreased and its K_m value may increase or unchanged which illustrate that no effect on the enzyme-substrate affinity under effect of these heavy metals. It was found that Fe^{3+}, Al^{3+} and Pb^{2+} were acted as a mixed NCI on the POX activity of crude extract of rosemary by decreasing V_{max} value from 0.11μmol min^{-1} (control) to 0.02, 0.07 and 0.035 μmol min^{-1}, and increasing K_m value from 4 mM (control) to 4.99, 7.76 and 5 mM, respectively. Moreover, Fe^{3+}, $Pb^{2}$and Co^{2+} have the same effects on the POX activity of the crude extract of rocket.

In contrast, Mo^{2+} was the only heavy metals that exhibit the crude extract of rosemary as a pure NCI (K_m value unchanged, V_{max} value decreased). The ratio of V_{max}/K_m which reflects the specificity of enzyme to substrate, it was found to be 0.0275 and 0.038 in the crude extracts of rosemary and rocket, respectively. Moreover, catalytic power was decreased under noncompetitive inhibitors, and it reached the lowest value in the crude extracts of rosemary and rocket with Fe^{3+} (0.004 , 0.003), respectively.

Comparing specific activities of the enzyme (POX) in the crude leaves extracts (control) in the absence of heavy metals (0.140) and (0.227 unit mg^{-1}) for rosemary and rocket with the specific activity of the enzyme (POX) in the crude extracts of treated with heavy metals revealed that it was decreased in all types of inhibition (Tables 2, 3).

K_i defined as an equilibrium constant for the inhibitor binding to the enzyme, which reflects the strength of the interaction between the enzyme and the inhibitor (Voet & Voet, 2011). So, in the absence of inhibitor K_i=0, low K_i value mean tight binding between enzyme and inhibitor and high K_i value mean weak binding. It was clearly noted that low K_i value in the uncompetitive inhibition reflects strong binding between the heavy metals and the enzyme. Tables (2 and 3) showed that K_i value in mixed non competitive inhibition is high relatively (compared to uncompetitive inhibition) that reflects the loose binding between the enzyme and the inhibitor.

One of the characteristics of noncompetitive inhibitors that they can work at high and low substrate concentrations, which decrease the ratio of V_{max}/K_m (Shengwen *et al.,* 2013). It was reported that, both redox-active metals (Fe^{3+} and Cu^{2+}) and redox-inactive metals (Pb^{+2} and Cd^{+2}) may rise the production of ROS species including hydroxyl radical (HO.), superoxide radical ($O.^{2-}$) or hydrogen peroxide (H_2O_2) (Ercal *et al.,* 2001) and eventually, such rise in ROS species inactivates antioxidant enzymes including peroxidases, catalases, superoxide dismutases that responsible for free radical detoxification (Sahw *et al.,* 2004).

Table (2): Kinetic parameters of POX enzyme in the crude leaves extracts of *Rosmarinus officinalis* in control and in presence of different heavy metals. Mean ±SD (n=3).

Kinetic parameters						
Heavy metals	**K_m(mM)**	**V_{max}(µmol min^{-1})**	**K_i (µM)**	**V_{max}/ K_m**	**Specific activity (unit mg^{-1})**	**Effects**
Control	4.0	0.11	0	0.0275	0.140	Normal
Cd	3.0	0.067	-0.0016	0.022	0.087	Un competitive
Ni	1.7	0.01	- 0.00047	0.0058	0.012	Un competitive
Co	0.57	0.023	- 0.00046	0.040	0.030	Un competitive
Fe	4.99	0.02	0.0016	0.004	0.025	Mixed non competitive
Cu	0.5	0.0035	- 0.00045	0.007	0.0045	Un competitive
Al	7.76	0.07	0.004	0.009	0.090	Mixed non competitive
Pb	5	0.035	0.0016	0.007	0.044	Mixed non competitive
Mo	3.8	0.036	-0.001	0.009	0.045	Pure non competitive

Table (3): Kinetic parameters of POX enzyme in the crude leaves extracts of *Eruca sativa* in control and in presence of different heavy metals. Mean ±SD (n=3).

Kinetic parameters						
Heavy metals	**K_m(mM)**	**V_{max}(µmol min^{-1})**	**K_i (µM)**	**V_{max}/ K_m**	**Specific activity (unit mg^{-1})**	**Effect**
Control	1.3	0.05	0	0.038	0.227	Normal
Cd	0.8	0.005	- 0.001	0.006	0.0027	Un competitive
Ni	0.48	0.009	-0.0006	0.01875	0.040	Un competitive
Co	1.67	0.012	0.0013	0.007	0.050	Mixed non competitive
Fe	13.4	0.04	0.00004	0.003	0.180	Mixed non competitive
Cu	0.7	0.023	- 0.0008	0.033	0.100	Un competitive
Al	0.38	0.003	- 0.0005	0.0035	0.004	Un competitive
Pb	2.5	0.036	0.0004	0.014	0.160	Mixed non competitive
Mo	0.53	0.02	-0.0006	0.037	0.090	Un competitive

4. Conclusions

The crude extracts of plant green leaves of *Rosmarinus officinalis and Eruca sativa* were analyzed for POX activities. Results demonstrated that POX enzyme in the crude leaves extracts of the selected plants have potential activities according to the K_m and V_{max} values. Presence of heavy metals altered these activities by acting as uncompetitive or noncompetitive inhibitors depending on the type of heavy metals. For example, Fe^{3+}, Al^{3+}, Pb^{2+} and Mo^{2+} were found to act as noncompetitive inhibitors on POX enzyme in the crude extract of rosemary, while Co^{2+}, Fe^{3+} and Pb^{2+} acted as noncompetitive inhibitors on POX enzyme in the crude leaves extract of rosemary. In contrast, Cd, Ni and Cu have uncompetitive inhibition on POX activities in both crude extracts.

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