

 <p>ISSN NO. 2320-5407</p>	<p>Journal Homepage: -www.journalijar.com</p> <h2 style="text-align: center;">INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</h2> <p style="text-align: center;">Article DOI:10.21474/IJAR01/9524 DOI URL: http://dx.doi.org/10.21474/IJAR01/9524</p>	 <p>INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR) ISSN 2320-5407 Journal Homepage: http://www.journalijar.com Article DOI:10.21474/IJAR01/9524</p>
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RESEARCH ARTICLE

RESPONSES TO CADMIUM- INDUCED OXIDATIVE STRESS IN MARINE MACROALGA *ULVA LINZA*.

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Manuscript Info

Manuscript History

Received: 06 June 2019

Final Accepted: 08 July 2019

Published: August 2019

Key words:-

Ulva linza, Cd, Antioxidant enzyme, Antioxidant activity, Gene expression.

Abstract

The activities of antioxidant enzymes and gene expression of antioxidant enzymes were determined in *Ulva linza* under different Cd concentrations (0, 50, 100, 200, 300, 400 μM CdCl₂) for 3 days. The results clearly demonstrated that Cd induced stimulation of antioxidant activity. The Cd accumulation in thalli increased linearly as Cd concentration increased. The decrease in growth rate, photosynthetic efficiency and protein contents with increasing of H₂O₂ and malondialdehyde (MAD) contents in response to elevated Cd concentrations indicates the induction of the oxidative stress. As in response to the increased oxidative stress, Cd treatments induced the activities and transcripts of superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6) and glutathione reductase (GR; EC 1.6.4.2). In general the ascorbate pool increased by treatments with different Cd concentrations. A steady slight decrease in glutathione content with increasing Cd concentration was indicated. The oxidized glutathione (GSSG) content and the glutathione (GSH) /GSSG ratio were not significantly influenced by the tested Cd concentrations, except the concentration of 300 μM , which caused a 18% decline in GSSG content and the concentration of 400 μM which decreased GSH/GSSG ratio by 23%. The different Cd concentrations induced a conspicuous increase in proline content of the investigated alga.

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Introduction:-

The bio-concentration factor is a useful parameter to evaluate the potential of plants for accumulating metals and this value was calculated on a dry weight basis (Raskin et al., 1994). It is known that marine macroalgae can accumulate heavy metals to a large extent (Baumann et al., 2009 ; Jiang et al., 2013). It is known that algal cell walls are often porous and allow the free passage of molecules and ions in aqueous solutions (Hope and Walker, 1975). In addition, the constituents of the cell wall provide an array of ligands with a mosaic of functional groups capable of binding various metallic ions. Indeed, it has been shown that many metal binding mechanisms are involved in the biosorption process; these include ion exchange, complexation, coordination, and microprecipitation (Chen et al., 2002 ; Flores-Garnica et al., 2013). Cd is a non- essential element for growth, the plants may actively exclude or sequester such to minimize the metal toxic impact (Xia et al., 2004 ; Williams and Salt, 2009).

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Metal toxicity has high effect and importance to plants and other autotrophs and since these organisms are primary producers, it will consequently affect the whole ecosystem. Heavy metal toxicity emerges through non specifically bonding of metals with coordination sites of important biological molecules such as enzymes and other proteins, thereby altering normal metabolic functions and affect various physiological and biochemical processes of the cell (Sunda, 1989 ; Arunakumara and Xuecheng, 2008). Toxicity can also be deeply related to the production of reactive oxygen species (ROS) and the resulting unbalanced cellular redox status (Sharma and Dietz, 2009; Panda et al., 2016). Cadmium with no reported biological function (non-essential) for most living organisms except one occasion as a cofactor for carbonic anhydrase in marine diatom which catalyzes the reversible hydration of carbon dioxide (Park et al., 2008 ; Hurd et al., 2014). It seems to be more or less toxic to plants and micro-organisms (Benavides et al., 2005; Gratão et al., 2005). Performed studies in different plant species have showed that Cd can intervene with several metabolic processes such as photosynthesis, respiration or nutritional status (Sandalo et al., 2001 ; Dourado et al., 2014), although the mechanisms involved in its toxicity has not been well established (Romero-Puertas et al., 2007 ; Tchounwou et al., 2014). Heavy metals such as copper, zinc, mercury, lead, cadmium, chromium, aluminium and arsenic motivate oxidative stress in plants and thereby the accumulation of oxidized macromolecules (Valko et al., 2005; Sharma and Dietz, 2009). The oxidative stress condition in plants is alleviated by increasing of antioxidant enzymes activities such as superoxide dismutase (SOD) which detoxify superoxide anions ($O_2^{\bullet -}$), catalase (CAT), ascorbate peroxidase (APX), dehydro ascorbate reductase (DHAR) and glutathione reductase (GR), which detoxify hydrogen peroxide (H_2O_2) (Sharma and Dietz, 2009; Gill and Tuteja, 2010 ; Caverzan et al., 2016).

Furthermore, oxidative stress can be buffered by the production of non enzymatic water soluble antioxidant compounds such as ascorbate (AsA) and glutathione (GSH) which directly reduce reactive oxygen species (Foyer and Noctor, 2011; Takami et al., 2012 ; Caverzan et al., 2016) and/or act as substrates for antioxidant enzymes such as APX, dehydro-ascorbate reductase (DHAR) or GR. In addition, proline can be added to a group list of non-enzymatic antioxidants that is required by microbes, animals, and plants to confront the inhibitory effects of ROS (Chen and Dickman, 2005; Liang et al., 2013). Linking physiological responses to stress with their genetic bases, especially using current genomic and proteomic approaches, would definitely ameliorate our understanding of the relationship between organisms and their environment. However, although fast development of, and more expedite access to, genomic tools have enhanced a look of research for the genetic bases of a wide range of biologically related processes in a diverse array of organisms, studies on algae are still scarce (Stanley et al., 2005 ; Contreras-Porcia et al., 2011). Real-time PCR is one of the most sensitive and reliably quantitative techniques for analysis of gene expression. It is well known that the synthetic antioxidants, such as butylated hydroxyl anisol (BHA) and butylated hydroxyl toluene (BHT), are commonly used to preserve food quality mainly by prevention of oxidative induced deterioration of lipids constituent. But they contribute to some negative health side effects, according to the published literature (Santoso et al., 2004; Lobo et al., 2010). Therefore, there is a growing interest on the discovery of natural antioxidants. At present, marine algae are becoming the focus for targeting effective antioxidants towards oxidative stress in human body due to the presence of diverse natural products with unique structures possibly caused by extreme marine environment (Ryu et al., 2009; Bouhlal et al., 2011). Marine algae are considered to be a rich source of antioxidants (Devi et al., 2011; Kelman et al., 2012). Furthermore, marine algae have also been used as a source of novel functional foods with potential nutritional benefits (Santoso et al., 2004 ; Figueiredo et al., 2016), because they rich in vitamins, minerals, dietary fiber, protein, and various functional polysaccharides (Kuda et al., 2005). Antioxidant activity of marine algae is being intensively investigated due to the currently growing demand from the food and pharmaceutical industries, where there is interest in anti-aging and anticarcinogenic natural bioactive compounds (Ganesan et al., 2008; Murugan and Iyer, 2012). But the exposure of marine algae to continuously and extreme fluctuations in abiotic conditions for example, temperature, salinity, pH, and heavy metal pollution could affect their efficiency as a source of natural antioxidant compounds. Therefore, study of abiotic stress- induced changes in this efficiency become important requirement.

In this study, the green alga *Ulva linza* that inhabits marine environment of Alexandria City was examined under cadmium metal stress. This was to get insight into the metal-induced prospective changes in the potential commercial uses of this alga as natural antioxidative resource for food, medicine, pharmaceutical, and cosmetic industries. *Ulva linza* was exposed to different elevated concentrations of Cd. The growth rate, photosynthetic efficiency, protein content, lipid peroxidation, and H_2O_2 content were quantified as an indication of cellular damage that may be induced by exposure to Cd. Subsequently, the regulation of proline, antioxidant enzymes and non enzymatic antioxidants were determined to evaluate their possible role in the struggle against Cd toxicity. Furthermore, in order to linking the physiological responses to metal stress with their genetic bases, a real time

PCR-based assay was performed to determine the transcript abundance of antioxidant enzymes genes. Quantification the total antioxidant activity of the investigated alga was also performed.

Materials And Methods:-

Algal Collection, Seawater Sampling and Experimental Setup

Healthy thalli of *U. linza* were collected from about one and half meter depth of Mediterranean Sea shore of Alexandria, Abu-Qir district. Alga was transported to the laboratory in a cooler at 5–7 °C. Thalli were extensively rinsed with filtered seawater. Seawater stocks were filtered through 0.2 mm pore size membrane filters and stored in darkness at 4°C until used. The cleaned thalli were acclimatized to laboratory conditions by culturing in glass jars containing filtered seawater gently bubbled with air and maintained under a 12:12 h light: dark cycle, 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white cool fluorescent lamps at 25 °C \pm 2 for 24 h. Following the acclimatization period, thalli with known weight (10 g FW) were transferred to other glass jars containing 1 L of filtered seawater supplemented with 50, 100, 200, 300 and 400 μM of $\text{CdCl}_2 \cdot \text{H}_2\text{O}$ for 3 days under the same conditions of the acclimatization period described earlier. Controls were cultivated in seawater without metal additions. There were three replicates per treatment.

Determination of Bioconcentration Factor

The bioconcentration factor (BCF) was calculated using the equation: $\text{BCF} = C_b / C_i$ where, C_b is the concentration of the metal in the dry macroalgal biomass and C_i is the initial concentration of the metal in the feed solution (DeForest et al., 2007). The heavy metal content in algal tissue was measured according to a modified procedure of that described by Mamboya et al.(2007) as follows: 0.1g of the oven dried algal sample was placed in conical flask with 3 ml of 60% hydrochloric acid and 10 ml of 70% nitric acid. The conical flask was then placed on a laboratory hot plate for digestion until the white fume evolving from the conical flask turned brown. The digest was allowed to cool and then filtered through a Whatman's filter paper. The filtrate was then made up to 25 ml using distilled water and analysed for heavy metal concentrations using Atomic Absorption Spectrophotometer (Perkin Elmer 2380).

Estimation of Daily Growth Rate

The daily growth rate (DGR) of alga as a percentage of fresh weight increase per day was calculated according to the equation: $\text{DGR} = [(W_t / W_0)^{1/t} - 1] \times 100$ (Coll  net al., 2003 ; Kumar et al., 2010). Where W_t is the fresh weight at time t, W_0 is the initial fresh weight and t is the time in days.

Determination of Photosynthetic Efficiency

The maximal quantum yield of PSII in the dark-adapted state is expressed as a ratio of variable to maximal chlorophyll (Chl) fluorescence (i.e. F_v/F_m) of the samples was measured by Chl fluorometer, Aqua Pen-P AP-P 100 (Photon Instruments System, Czech Republic). Before Chl fluorescence readings were taken, thalli were adapted for 15 min in the total darkness to complete re-oxidation of PSII electron acceptor molecules (Han et al., 2008).

Determination of Protein, Proline, H_2O_2 and malondialdehyde Content

Total protein was extracted by adding 10 ml of 0.5N NaOH to about 100 mg of the oven-dry algal material and left overnight. The extract was completed to known volume with distilled water (Rausch, 1981). The protein was assayed according to modified Lowry method that given by Hartree (1972). Proline content was determined spectrophotometrically following the ninhydrin method of Irigoyen et al.(1992). Hydrogen peroxide was measured by homogenizing the algal tissue (50 mg) in an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) (Lee & Shin, 2003). The homogenate was centrifuged at 12,000 g for 15 min. An aliquot of 0.5 ml of the plant extract was added to 0.5 ml of 10 mM-potassium phosphate buffer (pH 7) and 1 ml of 1 M potassium iodide. The absorbance of the supernatant was measured at 390 nm. The level of lipid peroxidation was measured according to the thiobarbituric acid (TBA) test, which determines the malondialdehyde (MDA) as the end product of the lipid peroxidation reaction (Heath and Packer, 1968).

Assays of Antioxidant Enzymes Activities

For assays of SOD, APX, CAT and GR, algal thallus samples (0.2 g FW) were ground in a liquid nitrogen frozen potassium phosphate buffer (K_2HPO_4 50 mM, EDTA Na_2 1mM, pH 7) using a mortar and a pestle. The homogenate was centrifuged (10,000g, 15 min, 4 °C) and the supernatant was used for spectrophotometric determination of enzymatic activity (Nguyen-Deroche et al., 2012). Activity of SOD was assayed by measuring the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) (Beauchamp and Fridovixh, 1971). The activity of APX was assayed according to Nakano and Asada (1981). The CAT activity was determined by consumption of H_2O_2 in

absorbance at 240 nm according to method of Aebi (1984). The GR activity was assayed according to Foyer and Halliwell (1976), with minor modifications as described by Azevedo Neto et al. (2006).

Determination of Ascorbate and Glutathione concentrations

The levels of AsA and dehydroascorbate (DHA) were determined as described by Ratkevicius et al. (2003). GSH and GSSG contents were extracted and determined according to the fluorescence spectroscopy method of Hissin and Hilf (1976).

Quantitative Real-Time PCR Detection of Gene Expression

Total RNA was extracted using total RNA kit I Q easy TM plus plant Korea. The Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit is used for synthesis of first strand cDNA from RNA templates. Expression of the SOD APX, CAT and GR genes were determined by RT PCR; Real MOD™ GH Green Real-time PCR Master Mix (2x). The relative expression levels of the target genes (SOD APX, CAT and GR) in the investigated alga treated with different concentrations of Cd for 3 days were determined on the basis of normalization with the constitutive gene (Actin gene) using specific primers for amplification of both target and reference genes were designed as follows: The forward and reverse primers for real-time PCR were 5'TGCACGCCGAAGGACATA3' and 5'CCAAAGCCATTGTGAATCGAG3' for SOD, 5'GTTTCAGGCAGGCAGCA3' and 5'ATTCGCATTGTTCTGGAATC3' for APX, 5'GAATACCTTGACCAAAGTGGTT3' and 5'GTAAGTGCAGTCTACGTCG3' for CAT and 5'GATTTAGGCCAGGCGGA3' and 5'TCATTTCATCTGATCATATAACAGAACCC3' for GR and 5'TGTTCCCCGGAATTGCTGATAGAATGAGC3' and 5'TTGAAAGTGCTGAGAGATGCCAAAATGGAG3' for Actin (Wu et al., 2009). The following amplification program was used: 94°C for 5 min (1 cycle), 94°C for 30 Sec., 58 – 62°C for 30 Sec. (40 cycles).

Estimation of Antioxidant Activity

To evaluate the antioxidant activity of investigated *Ulva linza* the methanolic extract of algal samples were prepared according to the modification of the method described by Han et al. (2008). The antioxidant activity of extracts was evaluated by using the stable 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) according to a modification of the method of Bandoniene et al. (2002). The free radical scavenging activity (FRSA) of the tested samples was calculated as a percentage of DPPH discoloration using the following equation proposed by Molyneux (2004).

$$\text{FRSA \%} = \frac{\text{AbsB} - \text{AbsS}}{\text{AbsB}} \times 100$$

Where, Abs B is the absorbance of blank and Abs S is the absorbance of sample

Statistical Analyses

All experiments were performed in three replicates. The data are presented as the mean \pm SD. Data were analyzed using SPSS statistical software. Statistical analyses were performed by one way analysis of variance (ANOVA). Significant differences between means were tested by the least significant difference (LSD) at $P \leq 0.05$.

Results:-

After 3 days exposure to different metal concentrations, Data in Table (1) clearly demonstrate an increase in Cd accumulation in *Ulva linza* by increasing the metal concentration. Cd concentration in control treatment was negligible or below the detection limit. The BCF of Cd by *U. linza* attained a peak at 300 μM Cd above which no further increase in BCF was observed.

Moreover, Treatment with 50 μM Cd did not change DGR of *U. linza* compared to its control. The DGR was significantly decreased as Cd concentration increased. Exposure to 100, 200, 300 and 400 μM Cd caused decline in DGR by 14, 25, 36, and 49%, respectively.

With reference to the control, results in Table (1) show that no obvious significant differences in Fv/Fm at 50 and 100 μM Cd was noticed, while it showed declining trend by 19, 32 and 48% at 200, 300 and 400 μM Cd, respectively.

It was noteworthy that varying concentrations of Cd had a pronounced effect on protein content of *U. linza*. In relative to the control, the lowest studied Cd concentration (50 μM) did not cause significant change in protein content of *U. linza*, while the Cd concentrations of 100, 200, 300 and 400 μM decreased protein content by 9, 15, 22 and 28%, respectively.

On the other hand, as the concentration of Cd increased, there was a conspicuous increase in proline content of the investigated alga. At lowest studied Cd concentration, proline content of *U. linza* increased by 1.3 fold of the control, whereas it rose sharply when Cd concentration exceeded 50 μM and attained a peak of 4.2 fold of the control at 300 μM .

U. linza showed a modest increase in H_2O_2 content upon exposure to studied Cd concentrations. In relative to the control, a maximum H_2O_2 content was 39% at the highest studied Cd concentration. Taking into consideration that the alga did not show significant difference in H_2O_2 content between 50 and 100 as well as between 200 and 300 μM Cd treatments.

The lipid peroxidation, indicated by the level of MDA content, in the investigated alga was generally increased upon exposure to all Cd concentrations and reached the maximum (48%) at 400 μM Cd. Moreover, no significant difference regarding to MDA content between 50 and 100 μM Cd treatments.

In order to investigate the responses of their reactive oxygen scavenging systems following exposure to Cd for 3 days, the activities of several antioxidant enzymes were measured (Table 2). The results demonstrate an increase in SOD activity in a metal concentration-dependent manner. Since compared to the control, SOD activity of *U. linza* increased by 12% at 50 μM Cd and continued to increase up to 96% at 400 μM Cd. A considerable increase in APX activity by 49% and 67% compared to the control at 50 and 100 μM Cd, respectively. Then it increased reaching up 147, 191 and 160% compared to the control at 200, 300 and 400 μM Cd, respectively. As compared to the control, low Cd concentrations (50 and 100 μM) did not significantly change the CAT activity of *U. linza*, whereas it was enhanced sharply and progressively with increasing of Cd concentration >100 μM . Since the alga achieved significant increases in CAT activity by 86, 129 and 193% at 200, 300 and 400 μM Cd, respectively. The GR activity of *U. linza* increased upon exposure to Cd in all treatments and attained a peak at 200 μM Cd. As compared to the control, increase of GR activity was 26, 52, 220, 98 and 39% at 50, 100, 200, 300 and 400 μM Cd, respectively.

For all Cd treatments, the results in Table (2) show that *U. Linza* exhibited noticeable increase in SOD transcript reaching the maximum at 400 μM . This increase was 1.40 fold of the control at 50 μM Cd and remained the same up to 200 μM Cd. Afterward, it elevated to 2.05 and 2.73 fold at 300 and 400 μM Cd, respectively. The APX transcript of *U. linza* was stimulated markedly by all studied Cd concentrations and attained a peak (3.6 fold of the control) at 200 μM Cd. Taking into account that there was no significant difference between 300 and 400 μM Cd regarding to APX gene transcript of *U. linza*. Stimulation in CAT transcript with increasing of Cd concentration was observed in *U. linza*. The alga showed increase in CAT transcript graded from 1.16 fold of the control at 50 μM Cd to 4.31 fold of the control at 400 μM Cd. The results indicated a conspicuous increase in the GR transcript of *U. linza* upon exposure to all studied Cd concentrations. This increase attained a peak of 4.15 fold of the control at 200 μM Cd.

Data in Table (3) displayed the AsA, DHA and AsA/DHA ratio of *U. linza* after exposure to various concentrations of Cd for 3 days. AsA content of *U. linza* was significantly increased by treatments with 50 - 400 μM Cd. This increase ranged from 31 to 63% and attained a peak at 200 μM Cd. The DHA content also increased but its increment was less than that of AsA. Where, it was not affected by the lowest Cd concentration (50 μM Cd) and it rose gradually by 13, 24, 29 and 36% over the control with increasing of Cd concentration from 100 to 400 μM . The ratio of AsA/DHA for *U. linza* achieved increase of 28, 33, 31, 7% over the control when the alga exposed to 50, 100, 200 and 300 μM Cd, respectively, while its value did not change significantly at 400 μM Cd.

Moreover, the results indicated a steady slight decrease in GSH content of *U. linza* by 50-300 μM Cd treatments. This decrease elevated to a significant value (21%) at the highest Cd concentration (400 μM). Cd concentrations did not significantly affect the GSSG content of *U. Linza* except for 18% decline at 300 μM Cd. Similarly, the GSH/GSSG ratio of *U. linza* was not significantly influenced by any of Cd concentrations, except the highest one (400 μM), which decreased it by 23% than control.

The results clearly demonstrated that Cd-induced stimulation of antioxidant activity of *U. linza*. For the range of Cd concentration studied (50 - 400 μM), *U. linza* exhibited a considerable increase in its antioxidant activity increased to 1.84 fold of the control at 300 μM Cd.

Discussion:-

The bio-concentration factor is a useful parameter to evaluate the potential of plants for accumulating metals and this value was calculated on a dry weight basis (Raskin et al., 1994). It is known that marine macroalgae can accumulate heavy metals to a large extent (Baumann et al., 2009 ; Jiang et al., 2013). Results showed that the accumulation of Cd by treated the alga was parallel to increasing its concentration in the culture medium. It is known that algal cell walls are often porous and allow the free passage of molecules and ions in aqueous solutions (Hope and Walker, 1975). In addition, the constituents of the cell wall provide an array of ligands with a mosaic of functional groups capable of binding various metallic ions. Indeed, it has been shown that many metal binding mechanisms are involved in the biosorption process; these include ion exchange, complexation, coordination, and microprecipitation (Chen et al., 2002 ; Flores-Garnica et al., 2013). In view of the structure complexity of the algal surfaces, it is possible that several of these mechanisms occur simultaneously to varying degrees, depending on the biosorbent and the aqueous environment. Cd is a non- essential element for growth, the plants may actively exclude or sequester such to minimize the metal toxic impact (Xia et al., 2004 ; Williams and Salt, 2009).

Growth rate is an important way for expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it. In the present study, the DGR of *U. linza* exposed to Cd for 3 days generally decreased with increasing of metal concentration. Heavy metal ions could interrupt routine metabolic processes by competing for the protein binding sites, activate enzymes and various biological reactive groups, causing poor or no growth (Arunakumara and Xuecheng, 2008 ; Narula et al., 2015). At the same time, the decrease in growth rates of algae may be attributed to the use of energy for activation of adaptation mechanisms and repair of damage induced by metal stress. It could be suggested that heavy metal ions can inhibit the growth of algae in different ways, which depend on the species, the metal types and the conditions in the growing media.

In the present study, Fv/Fm was used to indicate the influence of metals on the photosynthetic efficiency of the investigated alga. Since the measurement of Fv/Fm provide a first insight into changes of the photosynthetic apparatus upon the action of the metals (Sbihi et al., 2012) and can reveal the mechanisms implicated in metals toxicity (Miao et al., 2005). In general, the Fv/Fm of *U. linza* decreased gradually with increasing of Cd concentration. It is known that heavy metals could solely affect the photosynthetic apparatus by irreversibly binding the components of photosynthetic electron transport chain. For instance, Cd can substitutes Mg in the center of chlorophyll molecule leading to termination of photosynthesis activity by forming non-fluorescent inactive metals substituted chlorophyll (Sharma and Dietz, 2009 ; Dietz and Pfannschmidt, 2011).

The pattern of metal effect on protein looked like that was observed on growth and photosynthetic efficiency, where in general, the effect negatively escalated as much as the concentration increased. The decline in protein represents the growth inhibition occurred. In addition the reduction of protein content may be attributed to the shortage of carbon skeleton resulting from low photosynthetic rate (Afkar et al., 2010 ; Carfagna et al., 2013). It is also likely that the heavy metals may cause fragmentation of protein due to toxic effects of ROS, which led to reduced protein content.

The reactive oxygen species and lipid peroxidation are the well known indices for determining the degree of oxidative stress and considered main contributors for growth retardation (Blokina et al., 2003). In the present study, increase in H₂O₂ and MAD (end products of lipid peroxidation) contents with increasing Cd concentration was obvious for *Ulva linza*. This suggests that the toxic effect of heavy metals is probably exerted through free radical generation. In higher plants, heavy metals induce generation of superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]), and singlet oxygen (¹O₂), collectively termed ROS, and exert a variety of damaging effects, also called oxidative stress (Devi and Prasad, 1998). In contrast to higher plant, algae and cyanobacteria have not been studied extensively with regard to generation of free radicals under stressed conditions. The ROS can rapidly attack all types of biomolecules such as nucleic acids, proteins, lipids, and amino acids (Hu et al., 2007 ; Yadav, 2010). This leading to irreclaimable metabolic dysfunction and cell death when the ROS metabolism cannot cope the increased amounts of the formed ROS.

Heavy metals are implicated in oxidative injury as a result of the formation of ROS. The protective mechanisms adopted by plants to scavenge free radicals and peroxides include several antioxidant enzymes and antioxidant substances (Collén et al., 2003; Ahmad et al., 2010).

Studies on the regulation of antioxidant enzymes at the molecular levels are limited in macroalgae. De Oliveira e Silva et al., (2012) showed an increase in the catalase gene expression by RT/PCR analysis in the group treated with free phenolic acid (FPA) fractions from *Halimeda opuntia*, suggesting the inductor effect of these compounds on the enzyme genes. Hou et al., (2018) presented a new issue related to nanoparticles the impact of which is not known and their use in industry is increasing. Researchers report that inhibition of *Chlorella* sp. algae influenced by zinc nanoparticles has been observed.

So in the attempts to answer the question, whether studied metal can induce the gene expression of antioxidant enzymes, the genes SOD, APX, CAT and GR were cloned from *U. linza* and transcript abundance in response to Cd was examined.

Amongst various enzymes involved in the abolition of ROS, SOD can be considered as a key enzyme. SOD being a fundamental component of antioxidative defense system in plants. It converts $O_2^{\cdot -}$ to H_2O_2 at a very quick rate (Alscher et al., 2002). The increase in SOD activity might be attributed to the elevated production of superoxide radicles, thus resulting in activation of existing enzyme pools or up-regulated expression of the gene (Elbaz et al., 2010). To ensure the possibility, analyzing the transcripts of Fe-SOD coding one of SOD isoforms in algae were done. The expression of Fe-SOD in *U. linza* was up-regulated by Cd exposure and these results suggest that SOD can be regulated by Cd at molecular level in addition to enzymatic activity.

Cellular H_2O_2 generated by SOD mediated reaction is highly toxic and must be tightly controlled at low levels. In plants, a number of enzymes regulates intracellular H_2O_2 levels. CAT and APX are considered the most dominant enzymes that catalyze H_2O_2 to H_2O or the other non-toxic products (Nakano and Asada, 1981; Del R  o et al., 2006).

The main hydrogen peroxide-detoxification system in plant chloroplasts is the ascorbate-glutathione cycle. In this cycle APX is a key enzyme uses AsA as specific electron donor to reduce H_2O_2 to water (Asada, 1992). As CAT is absent in the chloroplast, so degradation of H_2O_2 is catalyzed by APX in chloroplasts. Moreover APX has a higher affinity for H_2O_2 than CAT so it plays a more substantial role in the H_2O_2 scavenging during stress (Wang et al., 1999; Gill and Tuteja, 2010). The enhancement of APX transcripts in *U. linza* upon exposure to Cd supported the regulation of APX activity at the transcriptional level. This explains that the Cd-triggered antioxidative capacity might be responsible for the removal of excessive H_2O_2 .

Catalase is one of the most important components of plant protective mechanisms that catalyzing H_2O_2 to H_2O and O_2 via two-electron transfer (Srivalli et al., 2003; Wang et al., 2008) hence, prevent the generation of OH^{\cdot} and protect proteins, nucleic acids and lipids against ROS (Imlay and Linn, 1988). CAT provides an energy efficient mechanism to degrade H_2O_2 , thereby it removes H_2O_2 without consuming cellular reducing equivalents such as NADPH (Mallick and Mohn, 2000).

Glutathione reductase is another key enzyme in the ascorbate-glutathione cycle that protects cells against oxidative damage. GR is a flavo-protein oxidoreductase, which catalyses the reduction of GSSG to the sulphhydryl form GSH, employs NADPH as a reductant (Jim  nez et al., 1997). GR activity is important to maintain a high GSH/GSSG ratio, being substantial to the functioning of the glutathione-ascorbate cycle and phytochelatin synthesis (Cobbett, 2000), which in turn are responsible for the tolerance of the species to contamination by heavy metal (Benavides et al., 2005). The activity and transcript of GR in this work were consistently reflecting the transcriptional regulation of GR activity by studied metal.

Since antioxidant enzymes are the first strategy in plants to detoxify heavy metals and scavenge ROS, the low molecular weight non-enzymatic antioxidants such as ascorbic acid, glutathione and proline, are considered as the second strategy.

AsA plays a great role in minimizing the damage caused by the oxidative process. This is carried out by its synergetic action with other antioxidants (Foyer and Noctor, 2005). A fundamental role of AsA in the plant defense system is to protect metabolic processes against H_2O_2 and other toxic derivatives of oxygen. AsA can function essentially as reductant and scavenges many types of free radicals. AsA can directly scavenge $O_2^{\cdot -}$, H_2O_2 and OH^{\cdot} and regenerate tocopherol from tocopheroxyl radical, thus providing membrane protection (Smirnoff, 1996; Shao et al., 2007). In addition, it is the most important reducing substrate for H_2O_2 detoxification in ascorbate-glutathione cycle (Smirnoff, 1996; Moucheshi et al., 2014). Moreover, the AsA/DHA ratio, an important indicator of the redox

status of cells, is one of the first signs of oxidative stress (Foyer et al., 2006). In present study, the regeneration of AsA leading to maintenance of AsA/DHA ratio larger than or equal to control.

GSH is a key component in the metal scavenging due to the high affinity of metals to its thiol (-SH) group and as a precursor of phytochelatin (PCs), which are responsible for controlling cellular heavy metal concentration by forming heavy metal complexes (Yadav, 2010). Besides metal homeostasis, GSH is considered an important antioxidant because it is a key ROS scavenger and major cellular redox buffer, in addition to its primary antioxidant capacities; it acts as a substrate for the regeneration of other essential antioxidants (Foyer and Noctor, 2005 ; Bashandy et al., 2010). The GSH can act directly as a free radical scavenger by reacting with other ROS, $^1\text{O}_2$, $\text{O}_2^{\cdot-}$, and OH^{\cdot} . In addition, GSH can form non-enzymatic conjugates with a great variety of reactive electrophiles, hence protect the cellular macro molecules (Larson, 1988 ; Asada, 1994). Furthermore, GSH contributes in the control of H_2O_2 levels in the ascorbate-glutathione cycle through the regeneration of AsA (Smeets et al., 2009). In this way, GSH/GSSG ratio plays an important role in indicating cellular redox status (Smeets et al., 2005) and cellular responses under environmental stress conditions (Foyer and Noctor, 2011 ; Seth et al., 2012). Therefore, availability of glutathione (i.e., a high GSH/GSSG ratio) is very necessary for plants exposed to oxidative stress such induced by heavy metals (Israr et al., 2006).

Plants have been shown proline accumulation under environmental stress (Delmail, et al., 2011 ; Joseph et al., 2015). The present work thus clearly represents the protective role of proline against metal toxicity, although it is difficult to elaborate the manner in which this role was executed. It might be that proline forms complexes with metal ions thereby protecting sensitive cellular sites from toxic effects. Alternatively, proline might sequester the harmful radicals (Mallick, 2004). It has been suggested to act as a hydroxyl radical scavenger (Smirnov and Cumbe, 1989; Signorelli et al., 2014), and a singlet oxygen scavenger (Matysik et al., 2002). In addition, proline synthesis has been involved in the mitigation of cytoplasmic acidosis and may maintain NADP /NADPH ratios at values compatible with metabolism (Hare and Cress, 1997). Moreover, proline maintains the water balance in the cell, which is often disturbed by heavy metals (Mallick, 2004).

Conclusion:-

Based on the current work, it could be concluded that *U. linza* was effective biosorbent for removal of Cd from aqueous solution. The decreased growth rate, photosynthetic efficiency and protein contents with corresponding increase in lipid peroxidation (MDA content) and H_2O_2 content following the exposure to Cd indicates that the ROS generation confirming the state of oxidative stress.

The antioxidant homeostasis, represented by antioxidant enzymes and non-enzymatic antioxidants is markedly altered by Cd in order to prevent or alleviate oxidative damage. The activities and transcripts of SOD, APX and GR were a consistent reflecting the transcriptional regulation of SOD, APX and GR activities by Cd. This, in general, indicates that the tolerance capacity of alga to Cd depends on an interrelated network of physiological and molecular mechanisms.

According to the results of the present study and since *Ulva linza* has some important aspects, such as their rapid growing along Alexandria sea shore and the possibility of controlling the production of some bioactive compounds by manipulating the cultivation conditions the present study suggests the possibility of using the heavy metal in improving the production of antioxidants from algae.

Table 1:-Data on BCF, DGR, Photosynthetic Efficiency, Total Protein, Proline, H_2O_2 and MDA content of *U. linza* following its exposure to Cd for 3 days (means \pm SD, n = 3).

Cd Conc. (μM)	(BCF)	(DGR) (%)	Fv/Fm	Total Protein ($\text{mg g}^{-1}\text{DW}$)	Proline ($\mu\text{g g}^{-1}\text{DW}$)	H_2O_2 ($\mu\text{mol g}^{-1}\text{DW}$)	MDA ($\text{nmol g}^{-1}\text{DW}$)
0	-	4.158 ^a \pm 0.215	0.770 ^a \pm 0.014	148.079 ^a \pm 5.726	86.383 ^f \pm 4.274	0.734 ^c \pm 0.034	18.250 ^d \pm 1.268
50	0.560 ^c \pm 0.028	4.344 ^a \pm 0.631	0.736 ^a \pm 0.027	139.715 ^a \pm 6.774	113.596 ^e \pm 9.246	0.813 ^{bc} \pm 0.023	20.615 ^{cd} \pm 1.646
100	0.683 ^{bc} \pm 0.049	3.558 ^{ab} \pm 0.387	0.717 ^a \pm 0.036	134.712 ^{ab} \pm 5.152	219.844 ^c \pm 6.587	0.835 ^{bc} \pm 0.047	21.323 ^{cd} \pm 2.150

200	0.770 ^{ab} ± 0.029	3.103 ^{bc} ± 0.184	0.618 ^b ± 0.003	125.008 ^{bc} ± 6.058	274.258 ^b ± 12.441	0.917 ^{ab} ± 0.035	23.291 ^{bc} ± 1.184
300	0.812 ^a ± 0.059	2.624 ^{cd} ± 0.304	0.518 ^c ± 0.052	115.804 ^{cd} ± 3.659	369.065 ^a ± 8.680	0.973 ^{ab} ± 0.043	25.142 ^{ab} ± 1.488
400	0.765 ^{ab} ± 0.064	2.103 ^d ± 0.165	0.393 ^d ± 0.024	105.939 ^d ± 4.648	180.215 ^d ± 9.199	1.023 ^a ± 0.059	27.038 ^a ± 1.735

Table 2:-The activities and transcript of SOD, APX, CAT and GR in Ulvalinzain response to elevated Cd concentrations for 3 days (means ± SD, n = 3).

CdConc. (μM)	Enzyme Activity (Umg ⁻¹ pr)				Relative Transcript Level of Enzyme (Fold of the Initial Control)			
	SOD	APX	CAT	GR	SOD	APX	CAT	GR
0	162.530 ^e ± 7.904	0.253 ^d ± 0.035	0.448 ^d ± 0.052	0.412 ^d ± 0.000	1.000 ^d ± 0.000	1.000 ^d ± 0.000	1.000 ^e ± 0.000	1.000 ^d ± 0.000
50	182.436 ^{cd} ± 16.611	0.376 ^c ± 0.048	0.475 ^d ± 0.088	0.519 ^{cd} ± 0.066	1.399 ^c ± 0.171	1.651 ^c ± 0.126	1.163 ^{de} ± 0.102	1.731 ^c ± 0.158
100	196.138 ^d ± 10.965	0.423 ^c ± 0.045	0.602 ^d ± 0.042	0.628 ^c ± 0.037	1.475 ^c ± 0.113	2.119 ^b ± 0.196	1.511 ^d ± 0.126	3.098 ^b ± 2.772
200	238.724 ^c ± 14.795	0.625 ^b ± 0.05	0.832 ^c ± 0.066	1.319 ^a ± 0.075	1.547 ^c ± 0.160	3.665 ^a ± 2.135	2.056 ^c ± 0.164	4.147 ^a ± 0.199
300	276.541 ^b ± 6.094	0.736 ^a ± 0.000	1.027 ^b ± 0.10	0.817 ^b ± 0.039	2.051 ^b ± 0.156	2.284 ^b ± 0.193	2.827 ^b ± 0.148	2.847 ^b ± 0.185
400	318.437 ^a ± 18.264	0.659 ^{ab} ± 0.060	1.311 ^a ± 0.083	0.574 ^c ± 0.043	2.738 ^a ± 0.200	2.321 ^b ± 0.153	4.314 ^a ± 0.281	1.982 ^c ± 0.201

Table 3:-Levels of AsA, DHA, AsA/DHA ratio and levels of GSH, GSSG, GSH/GSSG ratio and antioxidant activity in Ulvalinzain response to elevated Cd concentrations for 3 days (means ± SD, n = 3).

Cd Conc. (μM)	Ascorbate Pool			Glutathione Pool			Antioxidant activity (%)	
	AsA (μM.g ⁻¹ DW)	DHA (μM.g ⁻¹ DW)	AsA/DHA	GSH (μM.g ⁻¹ DW)	GSSG (μM.g ⁻¹ DW)	GSH/GSSG	BHT	U. linza
0	6.631 ^d ± 0.417	6.907 ^c ± 0.427	0.960 ^c ± 0.087	1.598 ^a ± 0.132	1.182 ^{ab} ± 0.095	1.352 ^a ± 0.093	91.384 ^a ± 2.349	43.013 ^f ± 2.301
50	8.664 ^c ± 0.535	7.041 ^c ± 0.503	1.231 ^{ab} ± 0.116	1.547 ^{ab} ± 0.143	1.132 ^{ab} ± 0.116	1.366 ^a ± 0.107		54.637 ^e ± 2.626
100	9.972 ^{ab} ± 0.304	7.791 ^{bc} ± 0.346	1.280 ^a ± 0.085	1.489 ^{ab} ± 0.126	1.075 ^{ab} ± 0.081	1.385 ^a ± 0.094		69.750 ^{cd} ± 1.526
200	10.814 ^a ± 0.607	8.588 ^{ab} ± 0.717	1.259 ^{ab} ± 0.114	1.438 ^{ab} ± 0.096	1.025 ^{ab} ± 0.090	1.403 ^a ± 0.091		74.400 ^{bc} ± 2.108
300	9.190 ^{bc} ± 0.594	8.932 ^{ab} ± 0.609	1.029 ^{bc} ± 0.089	1.332 ^{ab} ± 0.093	0.966 ^b ± 0.074	1.379 ^a ± 0.081		79.104 ^b ± 3.033
400	8.332 ^c ± 0.564	9.395 ^a ± 0.560	0.887 ^c ± 0.071	1.253 ^b ± 0.122	1.213 ^a ± 0.105	1.033 ^b ± 0.103		63.937 ^d ± 3.520

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