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Cinnamon ethanolic extract mitigates salinity stress and activates the antioxidant defence system in cucumber seedlings

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ABSTRACT

Salinity stress is one of the most causative abiotic stress of crop losses. This study aims to improve the growth of cucumber seedlings under salt stress conditions by applying different concentrations of cinnamon ethanolic extracts. The effects of foliar treatments with 50, 500, 1000 and 2000 mg / L of the ethanolic extract of cinnamon bark on cucumber seedlings watered with tape water or 2000 ppm NaCl were investigated. The results indicated that salt stress resulted in the reduction of cucumber seedling's fresh and dry weight and a significant increase in the level of lipid peroxidation and % of electrolyte leakage. Also, salt stress resulted in a significant increase in the activities of antioxidant defence enzymes, including peroxidases (POD) (EC 1.11.1.7), ascorbate peroxidase (AS-PX) (EC 1.11.1.11), and SOD (EC 1.15.1.1) by 97%, 19%, and 31% respectively in addition to significant reduction in catalase (CAT) (EC 1.11.1.6) activity by 22 % compared to control. Foliar application with all examined concentrations of ethanolic cinnamon extract improves the fresh and dry weight in a concentration-dependent manner and significantly reduces lipid peroxidation and electrolyte leakage. The highest fresh and dry weights were recorded in salt-stressed plants treated with 1000 and 2000 ppm of cinnamon extracts. Treatments of non-stressed cucumber seedlings with different concentrations of the cinnamon alcoholic extract resulted in significant increments in the activities of POD, AS-PX and SOD. Moreover, treatment of salt-stressed seedlings with ethanolic cinnamon extract increases the activities of POD, AS-PX, SOD as well as CAT. It can be concluded that the ethanolic extract of cinnamon mitigates the oxidative stress induced by NaCl and activates the antioxidant enzymes under salinity stress conditions.

***Keywords*:** salt stress, cucumber, lipid peroxidation, electrolyte leakage, antioxidant enzymes, cinnamon ethanolic extract.

Introduction

Long-term use of water containing a high level of salt and excessive use of inorganic fertilizers resulted in the accumulation of salt in soil and led to soil salinity (Chen *et al*., 2004). The high level of salt in water or soil reduces plant growth, especially salinity sensitive plants. Salinity has dramatically decreased the growth and development of several plant species and reduced their productivity (Singh, 2015; Oukarroum *et al*., 2015). Cucumber (*Cucumis sativus*) is one of the essential vegetables produced either in open fields or in the greenhouse in Egypt and is highly affected by salinity stress. The adverse effects of salinity on a plant's growth and productivity are due to osmotic stress, ion toxicity, and imbalance in ions equilibrium. Also, salt stress-induced oxidative stress can affect cellular important molecules and photosynthesis and accelerate plant senescence (Hasanuzzaman *et al*., 2012). Salt stress resulted in the accumulation of reactive oxygen species (ROS), including singlet oxygen, superoxide radicals, hydrogen peroxide and hydroxyl radicals. The accumulation of ROS could cause damage to important molecules inside plant cells, such as nucleic acids, proteins, and lipids (Gill and Tuteja, 2010; Mittler *et al*., 2004). Salt tolerance plants resist salinity induced oxidative stress by reducing the cellular level of ROS by either antioxidant enzymes or non-enzymatic antioxidants (Apel and Hirt, 2004; Mittler, 2002). Antioxidant enzymes include peroxidase (POD), ascorbate peroxidase, superoxide dismutase (SOD), and catalase (CAT) (Evelin and Kapoor, 2014). Non-enzymatic antioxidants involve ascorbate, glutathione, α-tocopherol, flavonoids, and carotenoids can scavenge ROS and induce plant resistance against salt stress (Foyer and Noctor, 2011). There is a

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positive correlation between plant antioxidative capacity and the level of salt tolerance (Ruiz-Lozano *et al*., 2012).

Cinnamon (*Cinnamomum sp*.) is a traditional spice used as a food ingredient. Cinnamon bark is rich in polyphenols, which exhibit antioxidant activity (Singh *et al*., 2007). Cinnamon alcoholic extract contains phenolic acids, including gallic acid, *p*- hydroxybenzoic acid, salicylic acid, *p*-hydroxybenzaldehyde, protocatechuic acid, syringic acid, vanillic acid, vanillin, caffeic acid, quercetin, tannic acid, chlorogenic acid, ferulic acid, p-coumaric acid, cinnamic acid, sinapic acid and eugenol were identified as the phenolic constituents in cinnamon (Helal *et al*., 2014; Klejdus and Kováčik, 2016). Phenolic compounds which exist in the ethanolic extract of cinnamon have several roles in the cells, such as reducing agents, metal chelators and singlet oxygen quenchers. Also, cinnamon phenolic compounds showed antioxidant activities and have been studied for substitution of synthetic antioxidants (Talaz *et al*., 2009). The main objective of this study is to evaluate the ability of the ethanolic cinnamon extract to induce salt tolerance in cucumber seedlings. In addition, the effect of the application of different concentrations of cinnamon extracts on the oxidative status and the activities of antioxidant enzymes was investigated.

Materials and Methods

Plant material:

Cucumber seeds (*Cucumis sativus* var. Vivasun) were obtained from Rijk Zwaan Egypt LLC., Giza, Egypt. Seeds were cultivated in pots containing washed sand.

Cinnamon ethanolic extract preparation:

Cinnamon bark (*Cinnamomum cassia*) was obtained from the local market and ground to a fine powder. Cinnamon ethanolic extract was prepared by macerating 50 g of cinnamon powder in 500 ml ethanol 80% (v / v) (500 ml) in a one-litre flask at room temperature for 12 hours., then the mixture was filtrated by Whatman No. 1 filter paper. The extraction with ethanol was repeated three times, then the filtrate was collected, and ethanol was evaporated at room temperature. Four cinnamon alcoholic extracts were prepared with concentrations of 50, 500, 1000, and 2000 mg/L.

Cucumber seedlings treatments:

One hundred cucumber seedlings were divided into two groups: i) non-Stressed group: irrigated with nutrient solution dissolved in tap water, ii) salt-stressed group irrigated with the same nutrient solution plus 2000 mg NaCl / L. Each group were divided into five groups; the first group acted as nontreated control, and the other four groups were treated with 50, 500, 1000, and 2000 ppm cinnamon ethanolic extract. After two weeks of treatments, plant samples were collected and used for measurement of fresh weight, dry weight, electrolyte leakage, lipid peroxidation and antioxidant enzyme extraction.

Biochemical analysis:

Determination of lipid peroxidation

The level of lipid peroxidation was measured by the determination of malondialdehyde (MDA) in fresh cucumber seedling tissues, as described by Heath and Packer, 1968. One gram of fresh seedling tissue was homogenized in 5 ml of 0.1 % (w/v) TCA. The homogenate was centrifuged at 10000 g for 5 min, and then 4 ml of thiobarbituric acid (0.5% in TCA 20%) was added to 1 ml of the supernatant. The mixture was heated to 95°C for 30 min and then quickly cooled in the ice bath. The contents were centrifuged at 10000 g for 15 min, and the absorbance of the supernatant was measured at 532 nm. The MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm-1. MDA content is expressed as μmol. g^{-1} fresh weight (FW).

Determination of electrolyte leakage:

Electrolyte leakage in different treated seedlings was assessed as described by Dionisio-Sese and Tobita (1998). Plant discs (1g) were immersed in 10 mL of deionized water for 4 hours then electrical conductivity was measured by using an EC meter (EC1). Then the cucumber leaf discs were

subjected to a freeze-thaw cycle and kept for 4 hours; then, electrical conductivity was measured. % of electrolyte leakage was calculated as the following equation.

$$Electrolyte\ leakage = \frac{EC_1 \text{ X } 100}{EC_2}$$

Measurement of enzymatic activities:

Preparation of crude enzyme extract

Cucumber seedlings was homogenized in a volume of of chilled Potassium phosphate buffer (100 mM, pH7.0) containing 0.1mM EDTA and 1% polyvinyl pyrrolidone (PVP) (w/v) at 4ºC with extraction ratio 1:4 (w:v). Homogenate was squeezed through four layers of cheesecloth, and the extract thus obtained was centrifuged at 15,000× g for 15 min at 4º C. Supernatant was used to measure the activities of POD, CAT, SOD, AS-PX. The protein content in the enzyme crude extracts was measured according to Lowry *et al.* (1951).

Assay of Peroxidase activity

Peroxidase, POD (E.C 1.11.1.7) activity in enzyme crude extract was determined as described by Herzog and Fahimi (1973). The reaction mixture (3 ml) consisted of 0.25% (V/V) Guaiacol in 10 mM sodium phosphate buffer (pH= 6.0 containing 10 mM H_2O_2). 25 µl of the crude enzyme extract was added to initiate the reaction. The absorption changes at 470 nm were followed by using spectrophotometrically (UV-visible-160A, Shimadzu). The activity was calculated by measuring the absorbance changes at 470 nm per min. A unit of peroxidase activity is defined as that amount of enzyme which causes 0.01 ΔOD. min^{-1}. The specific activity is expressed as (IU. mg^{-1} protein).

Assay of Catalase activity

The determination of catalase (CAT) activity in plant samples was carried out in accordance with Aebi 1974, using a mixture of protein extract, potassium phosphate buffer solution (100 mmol L^{-1}, pH 7.5) and H_2O_2 (30%). After waiting one minute for the decomposition of H2O2, the CAT activity was determined by a spectrophotometer with a wavelength of 240 nm at a temperature of 25 °C.

Assay of ascorbate peroxidase activity

Ascorbate peroxidase, APX (E.C 1.11.1.11) activity was measured as a decrease in absorbance at 290 nm for 1 min using L-ascorbate as standard in accordance with Asada, 1992. The assay mixture 3 ml consisted of 0.5 mM Ascorbic acid, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.0), and 100 µL enzyme extract. A unit of As-PX activity is defined as the decreased of 1µmol of L-ascorbate per min and evaluated by comparison with a standard curve (0.5µmol to 20µmol L-ascorbate). APX activity was expressed as units per mg protein (IU. mg^{-1}protein).

Assay of superoxide dismutase activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured based on the method of Beyer and Fridovich (1987). The enzyme activity was expressed as a unit. mg^{-1} protein.

Statistical analysis

All data are presented as means of five samples ± SD. The results were analyzed by one-way ANOVA using SPSS software according to Stern (1991). The significance of the difference between treatments was tested by Duncan's multiple range test at $P \leq 0.05$.

Results and Discussion

Application of cinnamon ethanolic extract improves cucumber seedlings growth:
Figure (1) indicated that salt stress (2000 ppm NaCl) resulted in a significant reduction in the fresh and dry weight of cucumber seedlings. The fresh weight and dry weight of cucumber seedlings decreased by approximately 27% and 30%, respectively. Treatments of cucumber seedlings with different concentrations of the ethanolic cinnamon extract led to a significant increase in the fresh and dry weight in salt-stressed and non-stressed plants.

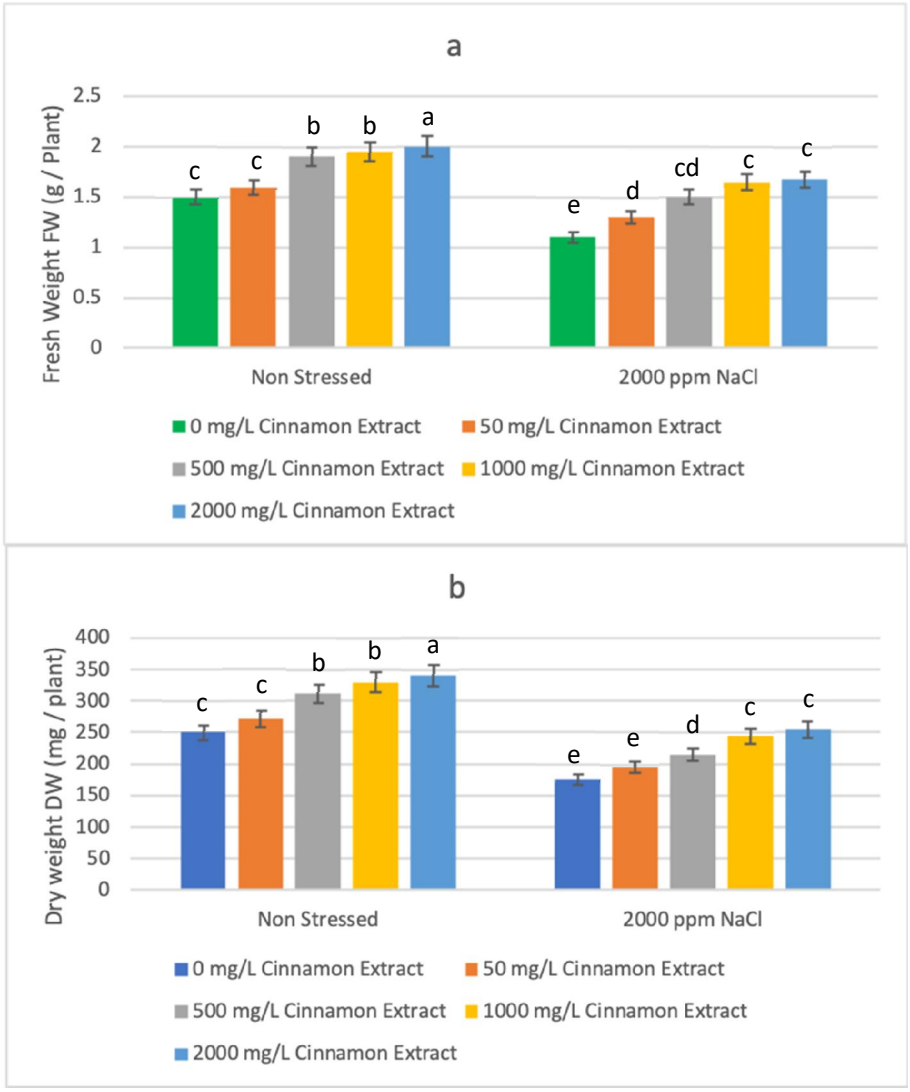

Fig. 1: Foliar application of cinnamon ethanolic extract on cucumber seedling improves fresh and dry weight either in salt-stressed (2000 ppm NaCl) or non-stressed seedlings. The present values are means of five replicated ± SD.

The highest fresh weight was recorded in cucumber seedlings treated with 2000 ppm cinnamon alcoholic extract. The fresh and dry weight increased by 33% and 51.8% after treatment with 2000 ppm cinnamon extract in non-stressed and salt-stressed plants, respectively. The observed reduction in the fresh weight and dry weight of cucumber seedlings as a results of salinity stress treatment could attributed to different types of stresses (Greenway and Munns, 1980): i) osmotic stress, ii) sodium and

chloride toxicity, iii) high level of Na^+:K^+ and iv) salt-induced oxidative stress. Several studies indicated that photosynthetic activity of many crops is reduced under salinity stress conditions (Ashraf, 2004; Dubey and Gairola, 2005). Also, Salinity stress resulted in stomata closure and reduce CO2 uptake, which caused photoxidation and reduce the photosynthetic activity and plants dry weight and inhibits plant growth. (Munns and Tester, 2008). Application of cinnamon extract cause a significant increase in fresh and dry weight of salt stressed cucumber seedlings and non-stressed seedlings. All examined doses improves fresh weight of salt-stressed plants while the dry weight improved only by application of 500, 1000 and 2000 ppm cinnamon ethanolic extract. In non-stressed plants treatment with 500, 1000, and 2000 ppm resulted in increase in the fresh weight and dry weight. The obtained results clearly indicated that cinnamon ethanolic extract contains growth stimulant compounds improving cucumber seedlings fresh and dry weight. Further investigations are needed to understand the nature of these growth stimulant compounds.

Application of cinnamon ethanolic extract reduce lipid peroxidation and electrolyte leakage in salt stressed seedlings:

Figure (2) indicated that treatment of cucumber plants with 2000 ppm NaCl resulted in significant increments in the oxidative stress marker i.e. lipid peroxidation and electrolyte leakage. In non-stressed cucumber seedlings, cinnamon ethanolic extract caused a significant reduction at concentrations of 1000 and 2000 ppm. While electrolyte leakage did not change by application with cinnamon extract. Under salt stress condition, cinnamon extract application resulted in significant reduction in MDA content and % of electrolyte leakage the maximum reduction was recorded in the plats treated with 2000 ppm cinnamon extract.

Several reports indicated that salt stress resulted in elevation in lipid peroxidation due to increase the production of reactive oxygen species (ROS) which acts as signaling molecules during growth, development, and stresses. ROS accumulation can make damage to important molecules and plant cells compartments (Parihar *et al*., 2015). Therefore, growing plants under salinity stress conditions resulted in disturbance of electron transport chain during respiration in mitochondria or light reaction and photophosphorylation in chloroplasts leading to the high production of ROS. When ROS increased up to the limit that the cell can survive with, the level of lipid peroxidation increased and cellular membranes lose their selective permeability. The induction of plant tolerance to salt stress is attributed to activation of enzymatic or non-enzymatic antioxidants. In the present investigation, cinnamon ethanolic extract which contains high level of antioxidant such as polyphenols was applied to induce salt tolerance in cucumber. The observed reduction of MDA content and % of electrolyte leakage can be explained by the high level of antioxidants in cinnamon extract .

Application of cinnamon ethanolic extract activates antioxidant defense enzymes in salt stressed seedlings:

The effects of cinnamon extracts (50, 500, 1000, and 1000 ppm) on the activities of antioxidant enzymes in both salt-stressed and non-stressed cucumber seedling were presented in figure (3). The results clearly showed that salt stress (2000 ppm NaCl) leads to significant elevations in the activities of POD, AS-PX, SOD and a significant reduction in CAT activity. Foliar application of cinnamon ethanolic extracts with dosage of 1000 and 2000 mg / L activated the antioxidant enzymes POD, CAT, and SOD in non-stressed plants. The concentration of 500 ppm of cinnamon extracts increased the activities of POD and SOD in non-stressed plant. All examined concentrations of cinnamon did not affect the activity of AS-PX. Treatment of salt-stressed cucumber seedlings with 500, 1000, or 2000 ppm of cinnamon ethanolic extract significantly increased the activities of POD, CAT, and AS-PX. SOD activity increased in salt-stressed plant by application of 1000 and 2000 ppm cinnamon extract. On the other hand, concentration of 50 mg/L did not cause any significant changes in the activities of POD, CAT, AS-PX and SOD.

Cinnamon extract contains strong antioxidant active compounds (Nanasombat and Wimuttigosol 2011). Also, Su *et al*. (2007), reported that extract of cinnamon contained high phenols content. Cinnamon extracts contains non enzymatic antioxidant and also found to activate antioxidant defensive enzymes as shown in figure (3). Our results concluded that the ethanolic extract of cinnamon mitigates the oxidative stress induced by NaCl and activates the antioxidant enzymes under

salinity stress conditions. The present work proved that the natural antioxidant from plant extracts e.g cinnamon extract can be used as anti- biotic stress and bio stimulant.

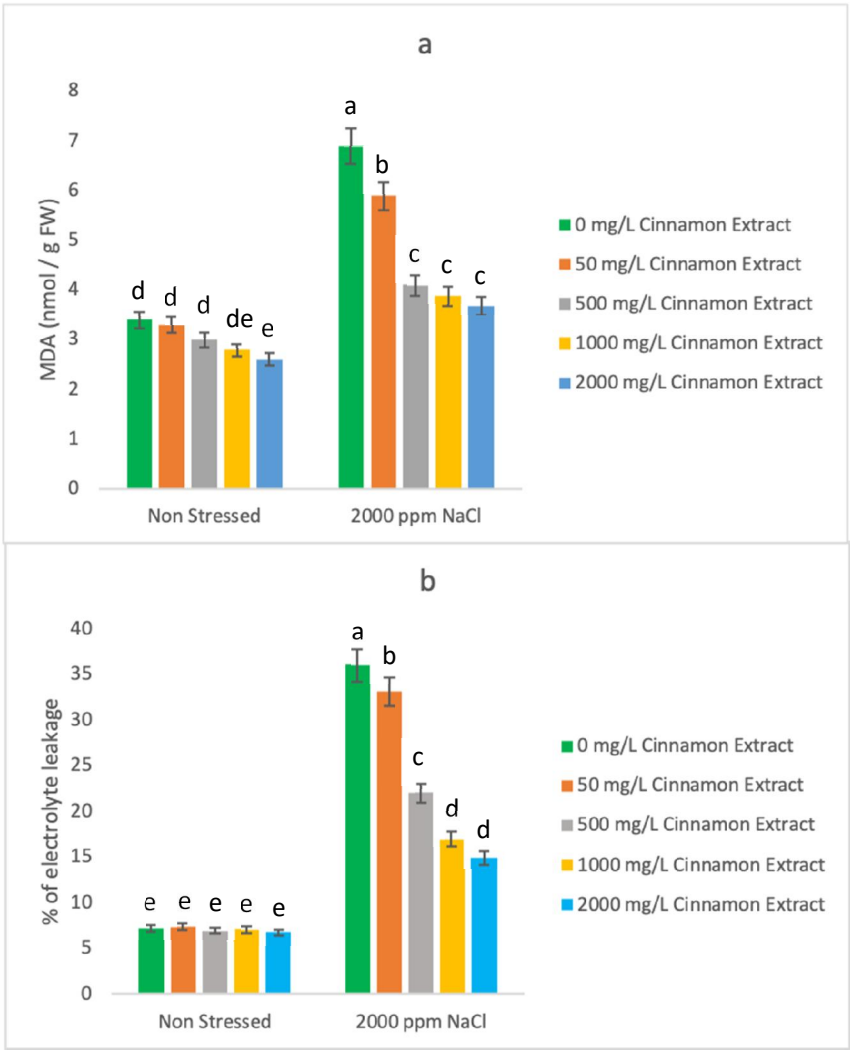

Figure (2): Effect of application of cinnamon ethanolic extract on the level of lipid peroxidation (MDA content nmole / g fresh weight) (a), and the percentage of electrolyte leakage (b) in cucumber seedlings grown under salinity stress condition. Each value presented the means of five samples ± SD. Different letters refers to significant differences at $p \leq 0.05$.

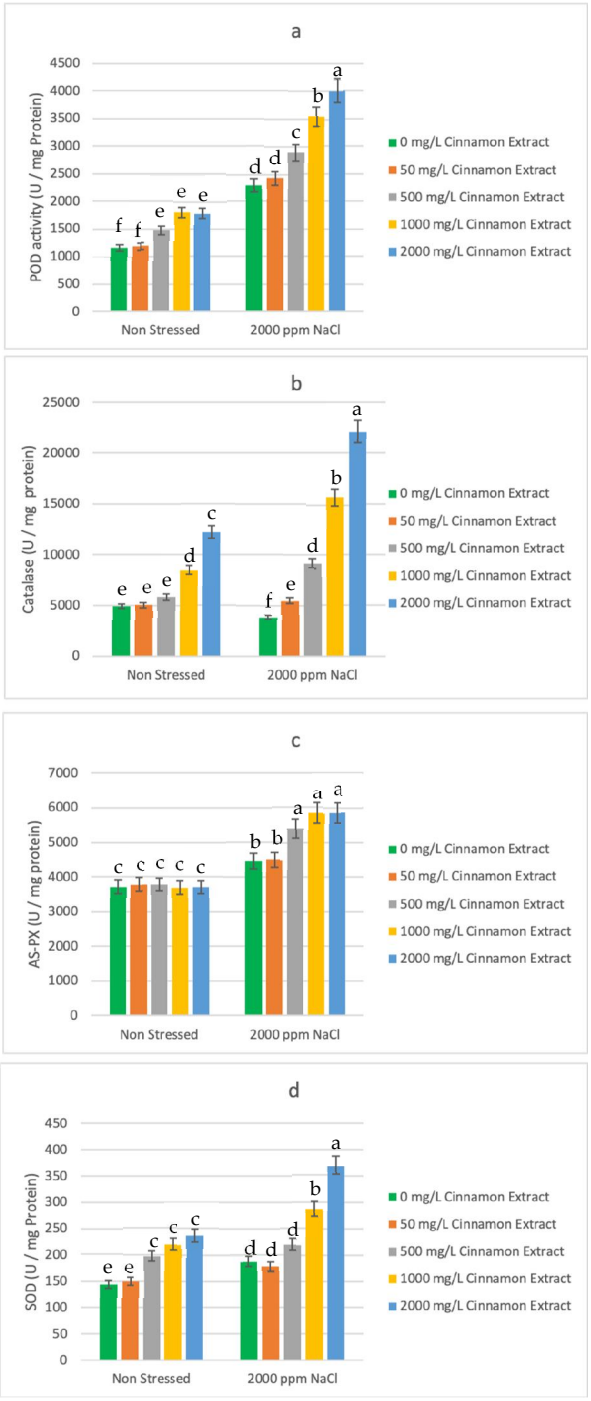

Fig. 3: effect of cinnamon ethanolic extract on the activities of antioxidant enzymes a: Peroxidase (POD), b: catalase (CAT), c: Ascorbate peroxidase (AS-PX), d: Superoxide dismutase (SOD) in non-stressed and salt-stressed (2000 ppm NaCl) cucumber seedlings . Each value presented the mean of five samples ± SD. Different letters refers to significant differences at $p \leq 0.05$

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