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Effect of Salt and Osmotic Stresses on the Activity of Some Antioxidant Enzymes and Biochemical Traits in *Catharanthus roseus*

Solaf Alaakel¹

Ayman Shehada AL-Ouda^{1*}

Youssef AL-Ammouri²

¹Faculty of Agriculture, Damascus University, Syria .

²Syrian Private University, researcher in National Commission of Biotechnology, Syria.

*Corresponding author: soaloakel@gmail.com, aymanalouda@gmail.com, uosephjo@gmail.com

*ORCID ID: <https://orcid.org/0000-0002-4640-7514>, <https://orcid.org/0000-0001-8260-426X>, <https://orcid.org/0000-0002-1233-1144>

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Abstract:

The experiment has been carried out in the Syrian National Commission of Biotechnology, during the growing season 2018/2019, to study the effect of abiotic stresses (salinity and osmotic stresses) on the activity of some antioxidant enzymes and biochemical traits in *Catharanthus roseus*. The experiment has been laid according to (CRD) with three replications. The seeds have been sterilized by NaOCl solution (0.5% v/v), then planted on MS medium. Plantlets have been moved to MS medium enriched with NAA (1 mg.L⁻¹) and BA (2 mg.L⁻¹). The callus has been initiated from leaves using MS medium containing NAA (1 mg L⁻¹) and KIN (2 mg.L⁻¹). After 60 days, callus has been transferred to MS medium supplemented with different concentrations of PEG 6000 (-0.2, -0.3, -0.4, -0.5 MPa), and NaCl (25, 50, 75, 100 mM) in succession as stimulating agents. The results show that the top value of solutes leakage has been in the salt and osmotic treatments (28.04 and 26.98% respectively) compared with the control (8.563%). MDA content has significantly been higher in salt stress (102.3 $\mu\text{mol.g}^{-1}$ FW) followed by the osmotic stress treatment (79.41 $\mu\text{mol.g}^{-1}$ FW), while it was significantly lower in the non-stressed treatment (37.76 $\mu\text{mol.g}^{-1}$ FW). An increase in the proline content occurred in both the stress treatments (4.623, 4.243 mmol.g⁻¹ FW, respectively) compared with the control (2.477 mmol.g⁻¹ FW). The activity of antioxidant enzymes (CAT, APX, and SOD) have significantly been higher in salt stress treatment (506.9, 12270.02 mol.min⁻¹.mg⁻¹ protein, 191.4 U.mg⁻¹ protein respectively), followed by osmotic stress (259.4, 7106.22 mol.min⁻¹.mg⁻¹ protein, 65.60 U.mg⁻¹ protein, respectively), while it has been significantly lower in control (126.9, 1800.38 mol.min⁻¹.mg⁻¹ protein, 36.03 U.mg⁻¹ protein, respectively).

Key words: Abiotic stresses, APX, CAT, MDA, Proline, SOD.

Introduction:

Catharanthus roseus (L.) is an ornamental and medicinal perennial subshrub which is native and endemic to Madagascar, which belongs to the Apocynaceae family (1). It is cultured as an ornamental plant in parks and promenade for its diverse colored flowers (pink, red, white, purple) and ever blooming nature (2). It contains a lot of alkaloids and also phenolic compounds as minor metabolites (3) having antimicrobial, anti-cancer, and anti-virus activity (4). Physiologically it produces pivotal antineoplastic alkaloids such as vincristine and vinblastine, which are mainly available in the leaves, in addition to antihypertensive alkaloids (reserpine ajmalicine, and serpentine) which are concentrated in roots

(5,6). Vinblastine (VLB) and vincristine (VCR) are considered powerful anticancer medicines, especially for children leukemia (7). These natural products are present only as small constituents of the complex mixture of about 130 alkaloids biosynthesized by specific plant parts depending on the nature of these compounds. The concentration of such effective constituents are found to be very limited, so it might take about 500 kg of dried leaves or roots to extract just 1 g of VLB, keeping in mind that the isolation process is very tedious, laborious and costly due to the large number of alkaloids (8). These characteristics make the alkaloids of a great importance goal for production by biotechnological tools. *C. roseus* plants are

repeatedly exposed to abiotic stresses such as low temperatures, heat, salinity, water deficit, oxidative stress, and nano- metal toxicity and UV radiation (9-11), which produce free radicals like Reactive Oxygen Species (ROS) in plant tissues, which are toxic for membrane system and biological macromolecules. Water and salt stress are major production-limiting factors of agricultural crops throughout the world, which often lead to a remarkable decline in plants growth and productivity (12). During water deficit and salt stress many morphological and physiological features and properties are affected, changes include reduction of relative water content (RWC), diminished leaf water potential (Ψ_w), and turgor loss (13), in addition to formation of reactive oxygen species (ROS), which are extremely harmful (14). Abiotic-induced ROS are accumulated in many cell organelles include a wide variety of oxygen-radicals, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), Singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2) (15). Due to their highly reactive nature, they destroy lipids, cell membrane, proteins and causes DNA mutation in the absence of any protective mechanism (16). Malondialdehyde (MDA) is considered as an indicator of lipid peroxidation, which generally form when the targeted abiotic stresses (osmotic and saline) are applied *in vitro* conditions (17,18). Plant usually develop many adaptive strategies to withstand abiotic stresses, including the accumulation of compatible organic osmoprotectants, such as glycine betaine (GB), mannitol, sorbitol, trehalose, and proline, which is considered to be the most important one (19,20). Plants usually keep control of the production of ROS by various enzymatic and non-enzymatic antioxidant defense systems include the superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) enzymes (21). From the technological point of view, it was revealed that *C. roseus* plant cells can be successfully cultured to produce the required alkaloids in quite enormous amounts (22). Large scale production could be achieved via either manipulation of *in vitro* cultures, to improve the selection efficiency and development of high producing cell lines and optimization of culture conditions (23), or through genetic alteration, which includes the overexpression of some step-limiting enzymes in alkaloid biosynthesis pathways (11). Several results demonstrated that inducing agents (NaCl and PEG-6000), have fruitfully been applied to enhance alkaloid production. The main aims of this work are to study the effect of PEG-6000-induced osmotic stress and NaCl-induced salinity stress as stimulating agents

on some biochemical traits and to assess the relevance of some antioxidant enzymes in alleviating the detrimental effects of such abiotic factors in *Catharanthus roseus* L callus.

Materials and Methods:

The research work has been conducted in the laboratories of National Commission for Biotechnology (NCBT), Faculty of Agriculture, Damascus University, Syria, during the growing season of 2018-2019. Seeds have been purchased from Syngenta flowers company. They have been superficial sterilized with ethanol 70% (v/v) for 1 min then with the solution of sodium hypochlorite (NaOCl) (0.5%) for 5 min and rinsed with sterile distilled water three times for five minutes each. Seeds have been germinated on solid MS medium (24). The resulting plantlets have been transferred to MS medium supplemented with Naphthalene acetic acid NAA (1 mg.L^{-1}) and Benzyl adinine BA (2 mg.L^{-1}) (25), in order to obtain a sufficient number of plants. The callus has been initiated from leaves using MS medium supplemented with the best hormone combination which has been obtained from previous results of the same research work (1 NAA and $2 \text{ Kinetin mg.L}^{-1}$) and some vitamins such as myo-inostol (80 mg.L^{-1}) and casein (500 mg.L^{-1}) (Table 1 and Fig.1) (26). Callus stocks have been grown on the same initiated medium for 60 days, then callus has been transferred to MS medium supplemented with different concentrations of (PEG-6000) (-0.2, -0.3, -0.4, -0.5 MPa), and NaCl (25, 50, 75, 100 mM), both of the stimulating agents have been added in succession to the culture medium with an interval period of 21 days between each and every subculture. The formed callus has been used to measure the investigated biochemical and physiological traits.

Table 1. The composition of MS medium used in callus induction.

Chemicals	mg.L ⁻¹	chemicals	mg.L ⁻¹
NH ₄ NO ₃	1.650	MnSO ₄ .4H ₂ O	22.3
KNO ₃	1.9	H ₃ BO ₃	6.2
MgSO ₄ .7H ₂ O	370	ZnSO ₄ .7H ₂ O	8.6
CaCl ₂ .2H ₂ O	440	Na ₂ MoO ₄	0.83
KH ₂ PO ₄	170	CuSO ₄ .5H ₂ O	0.025
Na ₂ .EDTA		CoCl ₂ .6H ₂ O	0.025
FeSO ₄ .7H ₂ O	27.8	KI	0.83
Naphthalene acetic acid	1	thiamine	1
Kinetin	2	sucrose	30000
myo-inostol	80	Agar	7000
Casein	500		



Figure 1. The callus resulting from the applied hormone combination.

Membrane integrity (solutes leakage %): 25 g from callus with 10 ml of distilled water have been put in a test tube in the shaker for 3 hours, the primary absorbance has been defined with spectrophotometer (273 nm). Then test tubes have been transferred to water bath at 95 °C for 30 min, and the final absorbance has been defined. Solute leakage has been calculated from the following formula (27).

(Initial absorbance/final absorbance)*100

Lipid peroxidation: Malondialdehyde (MDA) has been estimated as an indicator of lipid peroxidation according to (28). MDA has been measured by the thiobarbituric acid (TBA) reaction. 0.25 g of the frozen powder samples have been homogenized in 1 ml Trichloroacetic acid (TCA) (0.1 %) (w:v) solution. The homogenate has been centrifuged at 16,000 g for 15 min and 0.5 ml of the supernatant has been added to 1 ml (0.5%) (w:v) TBA in 20% TCA. The mixture has been incubated in a bath of boiling water for approximately 30 min, then the reaction tubes have been placed in an ice bath to stop the reactions. The absorbance has been recorded for each and every treatment at 532 nm. The quantity of MDA–TBA complex (red pigment) has been computed using an extinction coefficient (ϵ) of $155 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (29,30).

Proline content: 500 mg of fresh callus has been uniformly blended in 10 ml of 3% aqueous sulfosalicylic acid, and centrifuged at 3000 g for 10 min, 1 ml of supernatant has been added in a test tube containing 2 ml of glacial acetic acid and 2 ml of acidic ninhydrin. The mixture has been boiled at 100°C for 60 min, then the reaction has been stopped by placing tubes in an ice bath, 4 ml of toluene have been added to the tubes and strongly mixed, and the absorbance has been measured at 520 nm (31). The concentration of proline has been calculated using a standard curve.

Extraction of enzymes: The liquid nitrogen-powdered callus has been homogenized in 1 ml of potassium phosphate buffer, 50 mM (pH =7) containing 1 mM EDTA and then homogenate has been centrifuged at 13000 rpm for 20 min at 4°C. The clear supernatant of the solution containing the enzyme extract has been used to estimate the total protein content using Lowery method (32). To determine the activity of antioxidant enzyme such as SOD, and CAT equal concentration of protein in the treated samples and control have been used. SOD activity has been assayed according to the method of (33). The reaction mixture contains 1 mM riboflavin, 12 mM L-methionine, 0.1 mM EDTA, 50 mM Na_2CO_3 , and 75 mM nitrobluetetrazolium (NBT), with 200 μl crude enzyme extract and 25 mM sodium phosphate buffer (pH 6.8), in a final volume of 3 ml. SOD activity has been assayed by measuring the capacity of the enzyme extract to hamper the photochemical reduction of NBT. Test tubes containing the mixture have been illuminated for 15 min with a fluorescent lamp (120 W) and another set of identical not illuminated tubes served as blanks. At the end of illumination duration, the absorbance has been recorded at 560 nm. The SOD activity of the extract has been expressed as SOD unit $\text{min}^{-1} \cdot \text{mg}^{-1}$ protein. CAT activity has been estimated according to (34). The reaction blend contained crude enzyme extract, 10 mM H_2O_2 and 25 mM sodium phosphate buffer. The decline in the absorbance has been measured for 1 min at 240 nm by spectrophotometer with the extinction coefficient of $39.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. APX activity has been measured according to (35). 100 μl enzyme solution with reaction complex consists of 50 mM phosphate buffer solution (pH=7), 0.25 mM ascorbic acid (AsA), 5 mM H_2O_2 . Changes in samples absorption have been recorded at 290 nm wavelength for 3

minutes, enzyme activity has been measured by using the $2.8 \text{ mM}^{-1}\text{cm}^{-1}$ extinction coefficient.

Protein content: 200 μm of the protein sample has been added to 1.5 ml of Bradford reagent and then incubated for 30 minutes at $25 \pm 2 \text{ C}^\circ$. The absorption of the sample has been measured using a spectrophotometer at a 595 nm wavelength. The protein concentration of the samples has been determined by a standard curve of bovine serum albumin (BSA) protein (36).

Experimental design and statistical analysis: The experiment has been designed according to completely randomized design (CRD) with three replications, where Mstat-C program has been used to analyze data at 1% level of significance, for both the PEG-induced osmotic stress and NaCl-induced experiments.

Results and Discussion:

Membrane integrity (%): Solutes leakage (%) increases with increasing the level of both PEG-6000 and NaCl in the growth medium without significant difference between them (26.98 and 28.04 % respectively) compared with the non-stressed treatment (control) (8.56%) (Table 2). This could be attributed to the detrimental effects of these two abiotic factors on membrane integrity, causing a disturbance in the membranes stability, thereby increasing their permeability via affecting on the structural proteins and phospholipids, forming porous membranes, so many beneficial organic and inorganic compounds will leach out of the plant cell and some toxic compounds might enter (30,37). In general, plants that can maintain the stability/integrity of the biological membrane under abiotic stresses will be more tolerant, and can recover faster after the stress being alleviated (38).

Table 2. Effect of salt and osmotic stress on membrane integrity (%).

Treatment	Membrane integrity(%)
Salt stress	28.04 a
Osmotic stress	26.98 a
Control	8.56 b
LSD (0.01)	1.815
CV (%)	2.28

Different letters indicate significant differences at 0.01.

Lipid peroxidation: Results show significant differences in MDA content among the treatments. It has significantly been higher under salt stress ($102.3 \mu\text{mol. g}^{-1} \text{FW}$), followed with osmotic stress ($79.41 \mu\text{mol. g}^{-1} \text{FW}$), but it has significantly been lower in the non-stressed treatment (control) ($37.76 \mu\text{mol. g}^{-1} \text{FW}$) (Table 3). Lipid peroxidation (LPO) has been related to damages caused by different

abiotic stresses. Poly-unsaturated fatty acids (PUFA) constitute the main membrane lipid components vulnerable to peroxidation and stressful conditions (39). The raise in LPO can be linked with the buildup of ions to a toxic levels and production of ROS under stresses (40), so MDA can be considered as a good parameter for evaluating the degree of membrane damage or lipid peroxidation under abiotic stresses, which is attributed to the production of ROS (41). The extent of lipid peroxidation has been found to be higher under salinity compared to osmotic stress, which can be interpreted to the specific ion toxic effect, in addition to osmotic effect (19).

Table 3. Effect of salt and osmotic stresses on Malondialdehyde (MDA) content.

Treatment	Malondialdehyde($\mu\text{mol.g}^{-1} \text{FW}$)
Salt stress	102.3 a
Osmotic stress	79.41 b
Control	37.76 c
LSD (0.01)	12.91
CV (%)	4.70

Different letters indicate significant differences at 0.01.

Proline content: An enhance in the proline content has been observed in both the stress treatments (osmotic and salinity) by almost double compared with the control (Table 4). The proline content has been significantly higher in both the salinity and osmotic stress treatments without significant differences between them (4.243 and $4.623 \text{ mmol.g}^{-1} \text{FW}$ respectively), while it has been significantly the lowest in the control ($2.477 \text{ mmol.g}^{-1} \text{FW}$). The accumulation of proline has been noticed in several plant species cultivated under environmental stresses and its possible involvement in adaptive strategies is assumed (42). Proline plays a pivotal role in osmotic adjustment, regulates protein synthesis, protects against enzyme denaturation, acts as a pool of carbon and nitrogen, as well as cytosolic acidity and detoxification of hydroxyl radicals (43). Proline accumulation under abiotic stresses has been often correlated with stress tolerance (44). Accumulation of organic compatible osmolytes decreases the damaging effects of both salinity and osmotic by decreasing the cytoplasmic osmotic potential ($\Psi\pi$), thereby decreasing the water potential (becomes more negative) and increasing as a consequence of the water potential gradient between the plant cells and the surrounding growth media, which will improve water absorption by the roots system (45,46), enabling the plant to regain and maintain their turgor (47,48).

Table 4. Effect of salt and osmotic stress on proline content.

Treatment	Proline content (mmol.g ⁻¹ FW)
Salt stress	4.623 a
Osmotic stress	4.243 ab
Control	2.477 b
LSD (0.01)	1.845
CV	12.99

Different letters indicate significant differences at 0.01.

CAT activity: Results in Table 5 show that abiotic stresses have positive effects on catalase enzyme activity. The highest activity has been found in the NaCl-induced salinity stress followed by PEG-induced one (506.9 and 259.4 mol.min⁻¹.mg⁻¹ protein respectively), while the lowest occurred in the control treatment (126.9 mol.min⁻¹.mg⁻¹ protein). Catalase activity increases under abiotic stresses due to the production of free radicals, where catalase breakdowns H₂O₂ to H₂O and O₂, which are less noxious compounds to prevent the damage of cellular organelles and tissue harms (49). The high activity of this scavenging enzyme compared to the control is probably due to an internal increase in the level of hydrogen peroxide and other ROSSs. One of the most popular ROSSs is O₂⁻, which can be converted to H₂O₂ due to action of SOD (50). The H₂O₂ is formed in the cell as a natural product of the metabolic processes (51), and CAT reduces H₂O₂ into water; therefore, this enzyme is considered as a main defense with respect to H₂O₂. When the production of hydrogen peroxide is elevated as a response, the activity of CAT increases too.

Table 5. Effect of salt and osmotic stress on catalyze activity.

Treatment	CAT activity (mol.min ⁻¹ .mg ⁻¹ protein)
Salt stress	506.9 a
Osmotic stress	259.4 b
Control	126.9 c
LSD (0.01)	49.65
CV	4.44

Different letters indicate significant differences at 0.01.

APX activity: the APX activity has significantly been increased by both the investigated abiotic stress treatments (12270.02, 7106.22 mol min⁻¹ mg⁻¹ protein respectively) as compared with control (1800.38 mol.min⁻¹ mg⁻¹ protein) (Table 6). APX enzyme is reported to be an efficient regulator of ROS, as it contributes deeply to hydrogen peroxide detoxification. It is often used by cells to rapidly eliminate hydrogen peroxide molecules into beneficial molecular oxygen. APX plays a relevant

catalyst of H₂O₂ into less toxic substances in higher terrestrial plants (52).

Table 6. Effect of salt and osmotic stress on APX activity.

Treatment	APX activity (mol min ⁻¹ mg ⁻¹ protein)
Salt stress	12270.02 a
Osmotic stress	7106.22 b
Control	1800.38 c
LSD (0.01)	561.7
CV (%)	2.12

Different letters indicate significant differences at 0.01.

SOD activity: SOD activity has significantly been increased in salt stress followed with osmotic stress (191.4 and 65.60 U.mg⁻¹ protein), while it has been significantly lower in the control (29.47 U.mg⁻¹ protein) (Table 7). Superoxide radicals that are formed as a result of harmful abiotic factors in the plant tissues are changed into H₂O₂ by the SOD enzyme. Surplus quantity of O₂⁻ provokes SOD which converts O₂⁻ to H₂O₂ and the latter is removed by APX and CAT (50). The presence of diverse types of SOD in plant tissues exhibits a maximum tolerance of abiotic stress (48).

Table 7. Effect of salt and osmotic stress on SOD activity.

Treatment	SOD Activity (U.mg ⁻¹ protein)
Salt stress	191.4 a
Osmotic stress	65.60 b
Control	36.03 c
LSD (0.01)	29.47
CV (%)	7.97

Different letters indicate significant differences at 0.01.

Conclusions: In the present study, it has been shown that the treatment of cells with abiotic stresses (PEG and NaCl) caused significant rise of MDA as the end product of LPO and H₂O₂ which is one of the species of ROS. LPO caused disruptions of the cell membrane integrity which, as a result, has affected the internal/external balance of the cells. Thus, in the induction of LPO via ROS generation due to abiotic factors might be another reason of significant reduction of viability. An accumulation of proline has been also observed, which was almost double in the stressed compared with the non-stressed tissues, indicating the relevance of such osmoprotectants in improving tolerance. Also an increase in the activity of scavenging enzymes such as APX, CAT and SOD in *C. roseus* cells has been found, which is in

accordance with previous works studying different abiotic and chemical stress effects on callus tissue (53) and whole plants (54).

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in Damascus University.

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تأثير الإجهادين الملحي والأزموزي في نشاط بعض الأنزيمات المضادة للأكسدة والصفات البيوكيميائية في نبات الونكا

يوسف العموري

أيمن الشحادة العوده

سولاف العاقل

¹كلية الزراعة، جامعة دمشق، سورية.
²الهيئة العامة للتقانة الحيوية، سورية.

الخلاصة:

تُفذت التجربة في الهيئة العامة للتقانة الحيوية في سورية، خلال الموسم الزراعي 2019/2018، لمعرفة تأثير الإجهادات اللاأحيائية (الملوحة، والإجهاد الأزموزي) في نشاط بعض الأنزيمات المضادة للأكسدة، وبعض الصفات البيوكيميائية في نبات الونكا. وضعت التجربة وفق التصميم العشوائي التام، بواقع ثلاثة مكررات. عُقدت البذور بمحلول NaOCl (0.5%)، وزرعت في أنابيب اختبار تحتوي على الوسط المغذي MS، وبعد نجاح الزراعات التأسيسية، نُقلت النباتات إلى أوساط الإكثار المدعمة بالأكسينات NAA (1 مغ.ل⁻¹) والسيتوكينات BA (2 مغ.ل⁻¹). تم استحداث الكالس من وريقات نبات الونكا باستعمال الوسط المغذي MS المدعم بكل من NAA (1 مغ.ل⁻¹) و KIN (2 مغ.ل⁻¹)، بعدها تمّ تعريض الكالس لمستوياتٍ متزايدة من الإجهاد الأزموزي المُصطنع بإضافة سكر البولي إيثيلين جلايكول (PEG-6000) (-0.2، -0.3، -0.4، -0.5 Mpa)، والملحي (NaCl) (25، 50، 75، 100 mM) على التتابع كعاملٍ مجهد. أظهرت النتائج أنّ نسبة الذائبات المتسرّبة كانت الأعلى معنوياً عند تطبيق الإجهادين الملحي والأزموزي (26.98، 28.04% على التوالي) بالمقارنة مع الشاهد (8.563%). وكان محتوى النبات من مركب المالوندي ألدهيد (MDA) الأعلى معنوياً عند تطبيق الإجهاد الملحي (102.3 ميكرومول.غ⁻¹ وزن رطب)، بالمقارنة مع الأزموزي (79.41 ميكرومول.غ⁻¹ وزن رطب)، في حين كان الأدنى معنوياً في معاملة الشاهد المعتمد للمقارنة (37.76 ميكرومول.غ⁻¹ وزن رطب). لوحظ وجود زيادة معنوية في محتوى البرولين عند تطبيق الإجهادين الملحي والأزموزي (4.623، 4.243 ميلي مول.غ⁻¹ وزن رطب على التوالي) بالمقارنة مع الشاهد (2.477 ميلي مول.غ⁻¹ وزن رطب). وازداد معنوياً نشاط الأنزيمات المضادة للأكسدة (SOD، APX، CAT) في معاملة الإجهاد الملحي (12270.02، 506.9 مول.د.مغ⁻¹ بروتين، 191.4 وحدة.مغ⁻¹ بروتين على التوالي) بالمقارنة مع معاملة الإجهاد الأزموزي (7106.22، 259.4 مول.د.مغ⁻¹ بروتين، 65.60 وحدة.مغ⁻¹ بروتين على التوالي)، في حين كان الأدنى معنوياً في الشاهد (1800.38، 126.9 مول.د.مغ⁻¹ بروتين، 36.03 وحدة.مغ⁻¹ بروتين على التوالي).

الكلمات المفتاحية: الإجهادات اللاأحيائية، البرولين، المالوندي ألدهيد، الأنزيمات المضادة للأكسدة.