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Effects of ionic and heavy metal stress on secondary metabolites accumulation in calli of *Ajuga Bracteosa* L.

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Abstract

Ajuga bracteosa L, a medicinal plant that contains numerous phytochemicals, is becoming endangered worldwide due to the lack of knowledge and overexploitation. Research efforts are necessary for its conservation, preservation, and secondary metabolite production. In this study, an attempt was made to develop calli from this plant and evaluate the effects of abiotic stresses, i.e., NaCl and $Cd(NO_3)_2$, on the relative growth rate, total phenolic contents (TPC), proline contents, sugar contents, total flavonoid contents (TFC), alpha-amino acids, and antioxidant activity of *A. bracteosa* at the cellular (calli) level. NaCl and Cd $(NO_3)_2$ reduced the biomass production in terms of relative growth rate (RGR) but enhanced metabolite accumulation. The highest total phenolic contents (40µg/g), proline contents (0.9µg/g), total sugar contents (90mM/g), and total flavonoid contents (5mg/g) were recorded at 150mM NaCl, while the maximum total antioxidant activity (900µM/g) was measured at 50mM NaCl. On the other hand, the lowest amino acid contents (5µM), phenolic contents (27µg), and proline contents (0.5µg) were found at 50mM NaCl. Similarly, the highest level of $Cd(NO_3)_2$ stress (8mM) resulted in insignificantly increased production of phenolics (22µg/g), proline (0.9µg/g), total sugars (92mM), alpha-amino acids (7µM), and total antioxidant activity (850µM). This study not only provides an ex-situ conservation strategy for a highly exploited medicinal herb but can also be used as a commercial production methodology for valuable secondary metabolites.

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Introduction

All plants either cultivated or wild ones having active ingredients, which are used for therapeutic purpose, are called medicinal plants. The classification of plants into medicinal plants, poisonous plants, arable plants, ornamental, weeds etc. is always based on a human element and reflect a certain attitude, a purpose or a goal or economic interests (Imre, 2012). In broad sense, any plant is considered as medicinal herb, including fruits, vegetables, and arable plants. Humans depend on plants for various purposes like, food, medicines and shelter (Morgan *et al*., 1981). Now days due to overexploitation, overgrazing and lack of knowledge lot of plants species becomes endangered (Sher *et al*., 2008). Round about 10% flora and 709 plants in Pakistan are endangered (Shinwari *et al*., 2010), among these 580 are flowering plants (Khan *et al*., 2011). From medicinal plants approximately 25% of modern medicines are directly or indirectly derived. Distributions of these plants are worldwide but they are mostly abundant in tropical regions. These plants are abundant in Himalaya and upper Gangetic plans (Singh *et al*., 2006; Israili *et al*., 2009).

Ajuga bracteosa L, a medicinal plant is locally called Butey, in English "Bungle", in Sansikrit "Nilkanthi" and in Kashmir "Jan-I adam". In Northern areas of Pakistan due to its sour taste, it is called kauri booti (Jan *et al*., 2014). *A. bracteosa* contains numerous phytochemicals such as flavonol glycosides, iridiod, glycosides, neo-clerodane diterpenoids, phytoecdysones and ergosterol-5, 8-endoperoxide (Castro, 2011; Kayani *et al*., 2016). From *A.bracteosa* wall, phenolic compound Ajuganane which is a new compound along with three other compound 7-hydroxy-3,6,3',4'-tetramethoxyflavone, 3,4'-dihydroxy-3,6,7-trimethoxyflavone and urasolic were isolated (Hussain *et al*., 2012). Mostly *A. bracteosa* are used for treatment of hepatitis in Taiwan (Hsieh *et al*., 2011). It is used for the treatment of gout, palsy, amenorrhea and rheumatism (Kaithwas *et al*., 2012). For treating malaria, it is also used in many countries (Chandal and Bagai, 2011). *A. bracteosa* is also used for treatment of different diseases like, neuro inflammatory diseases, pneumonia, gout and hepatitis in Asian countries (Nisar *et al*., 2014).

In India the plant leaves, flowers and barks are used for treatment of diabetes, cancer and malaria etc. (Pal *et al*., 2014). Leaves extracts are used for headache, throats and ear infection, acne, pimples and also used as blood purifier etc. (Bisht *et al*., 2013). Plant extract is also used against gastric ulcer, protozoa infection, liver fibrosis, hypoglycemia, anti-inflammatory, anti-cancer, anti-arthritic, anti-plasmodial, immunoregulatory and insecticidal. The root juice of plant is used for treatment of dysentery and diarrhea; leaves are used as a substituent for quinine for treatment of fever (Pal and Pawar, 2011).

Similarly, different medicinal activity of *A. bracteosa* like, antibacterial, astringent and anthelmintic is considered in folk medicine. In KPK, an ethno botanic survey of 92 medicinal plants was done, in which *A. bracteosa,* due to its high medicinal value (antijaundice, antiasthma, ant cough and cooling agent) listed at the top (Ibrar *et al*., 2015). Studies investigated the anticoagulant, anti-depressant, anti-oxidant an inflammatory activities of *A. bracteosa*. *A. bracteosa* adventitious root also contains Gallic acid, Rutin, Catechin, Myricetin and apigenin (Ali *et al*., 2018). Due to over exploitation of medicinal plants from last decades' important plants are at the risk of extinction. *A.bracteosa* has rated as an endangered flowering medicinal plant species belongs to family L miaceae. It contains 170 genera of medicinal herb, and is mostly found in temperate regions (Hafeez *et al*., 2017).

Plant tissue culture an important component of biotechnology, generally used for micro propagation, production of virus free plants, rapid multiplication of clones, suspension culture, embryo culture and organ culture etc. One of the most important uses of this technology is to induce and multiply callus culture for extraction of secondary metabolites (active ingredients) (Hussain *et al*., 2011). Abiotic stresses like, salt, temperature, light, drought and heavy metals etc. affect secondary metabolites production

like phenol, proline, flavonoid and amino acids etc. (Akula and Ravishankar, 2011). However, in some plant species heavy metals alter the nature of secondary metabolites (Nasim and Dhir, 2010).

The current study was designed with the following objectives:

1. To induced and multiply callus culture of *A. bracteosa*.

2. To determine the antioxidant activity and secondary metabolites i-e phenols, flavonoids, proline, amino acids, total sugar contents in response to ionic and heavy metal stress (NaCl and $Cd(NO_3)_2$).

Materials and methods

Collection of plant

The research work was conducted at the Institute of Biotechnology and Genetic Engineering, Agricultural University of Peshawar KPK. Plants were collected from different regions of Swat (Pakistan) in the month of September.

Experimental design

For callus induction and multiplication Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962) was used. From a month old healthy plant of *A. bracteosa,* under control condition, different explants of leaves and shoot were collected. For sterilization purpose, explants were washed with tap water for 15minutes, followed by washing with 5% bleach for 5times, after that the explants were rinsed using sterilized distilled water for 5 minutes and inoculated on sterilized MS media containing Kinetin (0.25mg/L) and 2, 4- D (2.0mg/L). Explants were incubated for induction in incubator, at 27°C for 28 days.

Stress treatments

After callus induction and multiplication, to study the effect of various elicitor on induction of callus, callus was pretreated with two ionic elicitor NaCl and $Cd(NO_3)_2$ at varying concentrations NaCl (0, 50mM, 100mM and 150mM) and $Cd(NO_3)_2$ (0, 4mM and 8mM), following culturing of explants on MS media with same condition as described above. Callus were harvested on week 4 after stress treatments and, on analytical balance, fresh weight (g) of callus were taken. In labeled paper bags fresh samples of calli were stored, followed by oven dried at 60°C, and then weight (g) was recorded.

2.4 Relative Growth Rate

Relative growth rate (RGR) of calli was calculated by the following method described by (Shah *et al.*, 1990). Relative growth rate = (log final weight - log initial weight) / time (weeks).

Extraction methodology

For sample preparation, following method explained by (Usman *et al.*, 2020) was used, sample prepared by this method were used for metabolites analysis. Extraction buffer Methanol: Chloroform: Water (12: 5: 1) were used. In liquid nitrogen 0.5g of calli was frozen. In 5mL of extraction buffer MCW (12:5:1) calli were homogenized. At 5000rpm, the homogenate was centrifuge for 5 minutes, and supernatant was transferred to another test tube. The pellet was re-extracted in 5mL buffer and supernatant were pooled with the previously separated supernatant. 2.5mL of chloroform and 4ml of water were added to the extract. The extract was mixed until formation of two layers; upper methanol-water layer and lower chloroform layer. After formation of two layers, the extracts were covered, and then stored at 4°C in dark. The upper layer was used for analysis of metabolites.

Physiological parameter

Using spectrophotometry, analyses for various secondary metabolites (phenolics, proline, sugar, flavonoids etc.) were carried out.

Test for total Phenolic Content

Using well established protocol of (Nazir *et al.*, 2020) samples were extracted and checked for total phenolic contents. In a test tube diluted sample and 7ml distil water was added. In a test tube, Folin Ciocalteu reagent of 500 µl was added, after 3minutes sodium carbonate solution was added. The absorbance was measured at 760 nm, as blank distil water was used. For calibration galliac acid was used.

To calculate total phenolics, following equation were used

TP (mg Gallaic acid $^{eq}/_{kg}$ calli) = (ΔA – b) × F/ a × E

Test for Proline

Total proline contents were measured by the well-established method describe by (Bandeh *et al.*, 2008). Sample of 1ml was taken in a test. Ninhydrin of 1.5mL and 1.5mL Glacial acetic acid was added in a same test tube. In water bath the tubes were heated for 45minutes and then cooled to room temperature. To each tube 5mL of toluene was added. Tubes were vortexes, for 30 minutes and upper layer was used for measuring the absorbance. Absorbance was checked at 515nm.Different concentrations of L-Proline were used as standard.

Test for Sugar Contents

Total sugar contents were determined by the method of (Redillas *et al.*, 2012). Sample of 1mL was taken in a test tube. Distilled water 1mL; 5mL conc. Sulphuric acid and 1mL of l5% phenol were added. After shaking for 10minutes, in water bath tubes were heated at 25°C and measured the absorbance at 490nm. Different conc. of D-glucose was used as standard.

Test for Total Flavonoids

Total flavonoid contents were measured through the method described by (Balestrasse *et al.*, 2003). In a test tube 2mL sample was taken. Added 0.6ml $NaNO_2$ (5%) in the same test tube. After shaking 0.5mL of Al $(NO_3)_3$ (10%) and 3mL of NaOH (4.3%) was added. Test tube were vortexes and wait for 15minutes. Rutin 2mg/ml (70:30) v/v (ethanol- water) with various concentrations was used as standard.

Test for Total Alpha Amino Acids

Total Alpha Amino Acids were determined through a well-established protocol described by (Paek *et al.*, 1988). In a test tube 1mL sample, 2mL hydrazine sulphate solution, 4mL Ninhydrin solution and 1ml distilled water were added. In water bath tubes were heated, and cooled in ice bath at room temperature. Two ml Ethanol: Water was added. On a vortex mixture tubes were vortexes, OD was measured at 570nm.L-glutamic acid with different concentration were used for calibration.

Test for Antioxidant Activity

Through modified Trolox equivalent antioxidant activity method (TEAC) described by (Van den *et al.*, 1999), antioxidant activity was found. In a test tube 3800µL ABTS solution was taken, to the same test tube 200µL sample was added. Tubes were vortexed, and OD was checked at 734nm. Absorbance was checked at 734nm. ABTS solution (1900µL) was added with 100µL of Phosphate Buffer Saline (PBS) for Blank. Different concentrations of ascorbic acid were used for calibration. Following formula was used to calculate antioxidant activity.

Statistical analysis

Statistix 10 was used to calculate data for various biochemical qualities, and vegetative parameters (SPSS, Inc, Chicago, IL). Significant differences were found at various P values (p0.05), as well as standard deviation and mean separation.

Results

Callus induction and multiplication

Callus were induced and multiplied on MS media (Fig. 1). Calli were developed in three stages. Stage 1, explant inoculation of *A. bracteosa*, stage 2, callus initiation and stage 3 callus multiplication.

Relative growth rate (RGR)

Relative growth rate under salinity stress of *A. bracteosa* callus is presented in (Fig. 2a). Different concentration of NaCl (0.0 mM, 50mM, 100mM and 150mM) affected the relative growth rate of *A. bracteosa* callus, the RGR of calli decreased gradually with increasing NaCl stress.

Data recording the relative growth rate of calli are given in (Fig. 2b). Calli in the absence of Cd $(NO_3)_2$treatment showed the highest growth; however, at different concentration of Cd $(NO_3)_2$ at (4mM and 8mM), the (RGR) of calli decreased significantly.

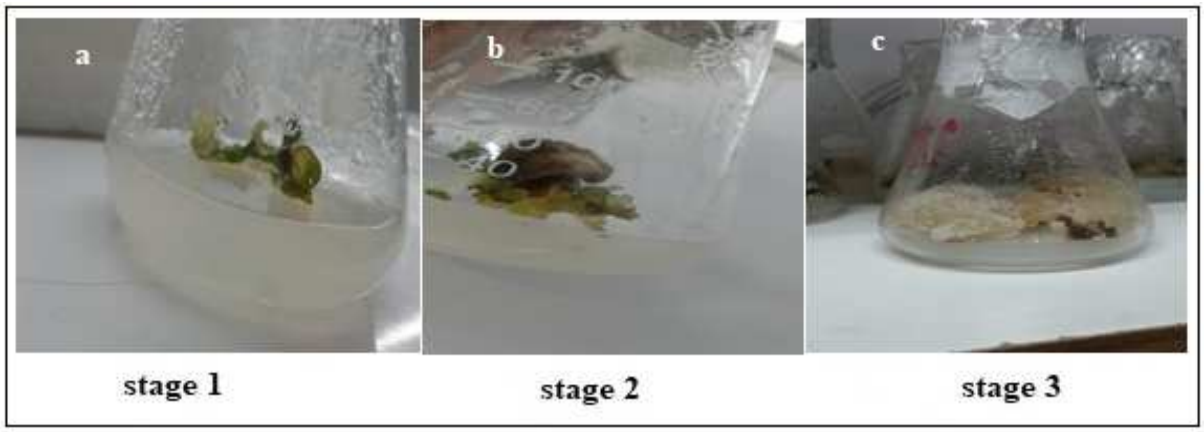

Fig. 1. Developmental stages of *A. bracteosa* L. callus.

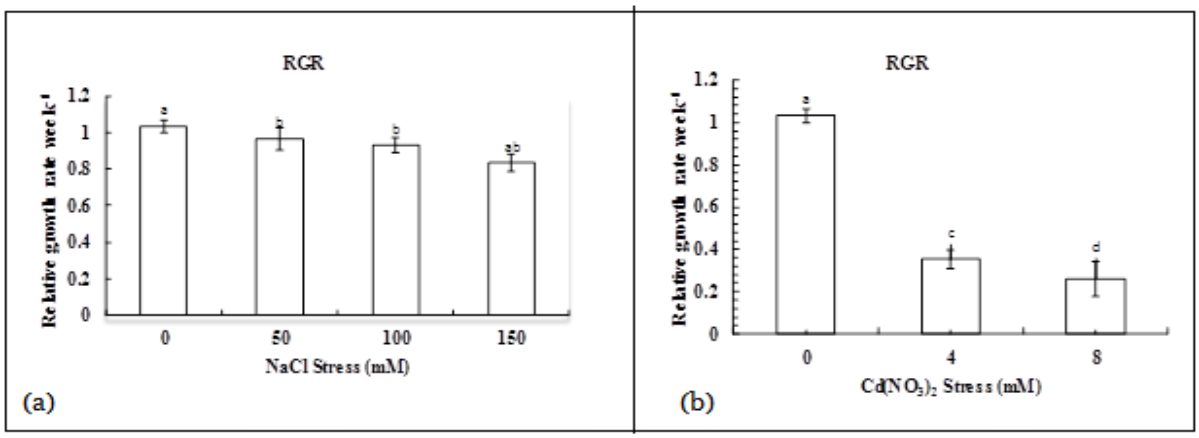

Fig. 2. a) The effect of salinity stress on the relative growth rate (RGR) of *A. bracteosa* calli. Data presenting in graph are means of four replicates ± SE, b) The effect of $Cd(NO_3)_2$ on the (RGR) of *A. bracteosa*calli. Data presenting in graph are means of four replicates ± SE.

Total phenolic Content

The effect of NaCl concentration on the total phenolic content (TPC) of *A. bracteosa* callus is presented in (Fig. 3a). Total phenolic contents showed gradual increase with increasing salinity level. ANOVA showed a significant ($P<0.05$) effect of stress treatment of NaCl. Total phenolics of calli are given in (Fig. 3b). Calli in the absence of $Cd(NO_3)_2$treatment accumulated the highest total phenolics content; however, at low concentration of $Cd(NO_3)_2$ (4mM) total phenolics content decreased followed by a slight increase at 8mM. ANOVA showed significant ($P<0.05$) effect of stress treatment.

Total Proline Content

Total proline content of calli is presented in (Fig. 4a). Different NaCl concentration (0.0mM, 50mM, 100mM and 150mM) affected the total proline contents of *A. bracteosa*, the total proline content significantly enhanced with increasing level of NaCl. ANOVA showed significant ($P<0.05$) result. Total proline of calli is given in (Fig. 4b). Total proline contents gradually increased with increasing Cd $(NO_3)_2$stress. A significant ($P<0.05$) effect of stress treatment is shown by ANOVA.

Total Sugars

Sugar contents of *A. bracteosa* calli are presented in (Fig. 5a). Control calli accumulated the lowest sugar contents; however, sugar contents gradually increased with increasing level of NaCl stress. ANOVA showed a highly significant ($P<0.05$) effect of stress treatment of NaCl. Total sugars of calli are given in (Fig. 5b). Control calli accumulated the least amount of total sugar followed by a gradual increase at (4mM and 8mM) Cd $(NO_3)_2$ stress. Significant ($P<0.05$)

effect of stress treatment showed by ANOVA.

Total Flavonoids contents

Total flavonoids of calli are presented in (Fig. 6a). Total flavonoid contents of *A. bracteosa* first decreased at (50mM and 100mM) followed by an increase in NaCl stress with respect to control. Significant ($P<0.05$) effect of stress treatment showed by ANOVA. Total flavonoid contents of calli are given in (Fig. 6b). Calli underCd $(NO_3)_2$ stress accumulated highest total flavonoid contents; however, Cd (NO3)2 at (4mM and 8mM) depleted total flavonoid contents. Significant ($P<0.05$) effect of stress treatment showed by ANOVA.

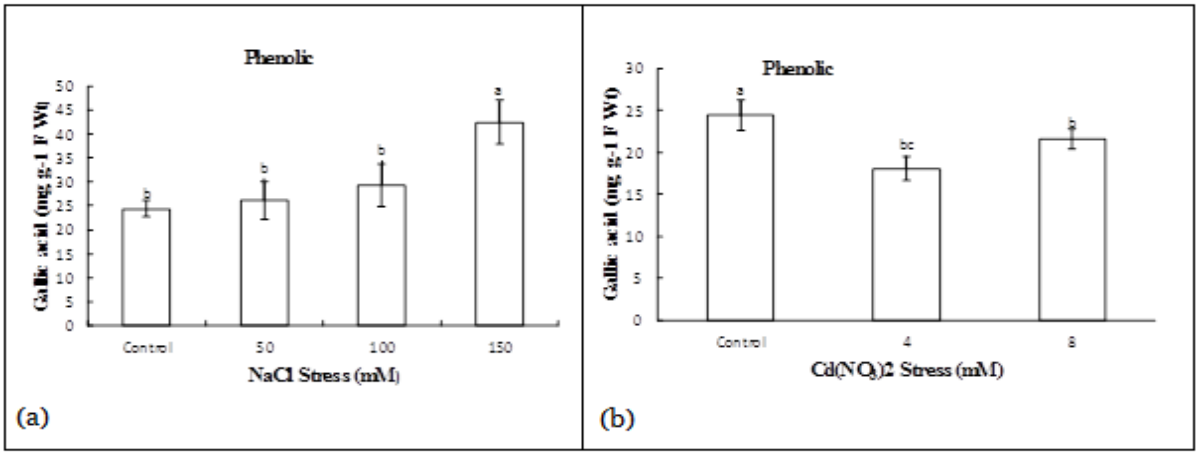

Fig. 3. a) Total phenolics content (µg g^{-1} fresh weight calli) of *A. bracteosa* under salinity stress The data presented in graph are means of four replicates ±SE, b) The effect of $Cd(NO_3)_2$on the total phenolics content (µg g^{-1} fresh weight calli) of *A. bracteosa*. In graph data shows means of four replicates ± SE.

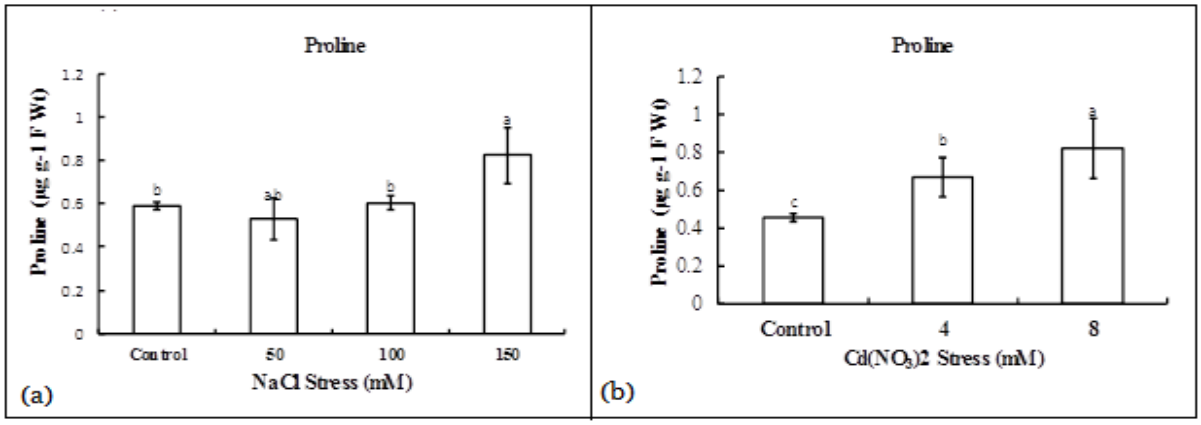

Fig. 4. a) The effect of NaCl stress on the total proline (µg g^{-1} fresh weight calli) of *A. bracteosa*. In graph data shows means of four replicates ±SE, b) The effect of $Cd(NO_3)_2$ on the Proline contents (µg g^{-1} fresh weight calli)of *A. bracteosa*. The data presenting in graph are means of four replicates ± SE.

Amino Acid Contents

Data pertaining to total amino acid of calli is presented in (Fig. 7a). Under control condition total amino acid of *A. bracteosa* increased followed by a gradual increase at (150mM) NaCl. Total amino acid contents of calli are given in (Fig. 7b). Calli in the absence of Cd $(NO_3)_2$ treatments accumulated the highest total amino acid contents followed by a gradual decrease at 4mM and then slightly increased at 8mM. ANOVA shows a non-significant ($P>0.05$) effect.

Total Antioxidant Activity

Total antioxidant activity of *A. bracteosa* callus under salinity is presented in (Fig. 8a). Total antioxidant activity was gradually increased at 50mM followed by

gradual decrease at (100mM and 150mM) compared to control. Total antioxidant activity of calli is given in (Fig 8b). At 4mM Total antioxidant activity gradually increase followed by an increase at 8mMwith respect to control.

Discussion

In the current study calli of *A. bracteosa* L, an endangered plant was developed to study the growth and accumulation of secondary metabolites in response to osmotic and ionic stress (NaCl and $Cd(NO_3)_2$), four levels of NaCl stress (0.0 mM, 50mM, 100mM and 150mM) and three levels of cadmium (0.0 mM, 4mM and 8mM) were used.

In current study the effect of salinity and cadmium on the relative growth rate of *A. bracteosa* calli were studied. Our result showed that with the increase in level of NaCl up to 150mM, the relative growth rate (RGR) of calli decreased. Similar result was found by (Shah *et al.*, 1990) in *Medicago sativa* L, they reported that under salt stress reduction occurred in (RGR) of calli and it showed a positive correlation with metabolites accumulation in calli. Under Cd $(NO_3)_2$) stress the (RGR) of calli significantly reduced. (Kuntal *et al.*, 2017) observed similar result in *tagetes erecta L.*plant they reported that Cadmium in combination with copper reduced the RGR, while enhanced sugar contents.

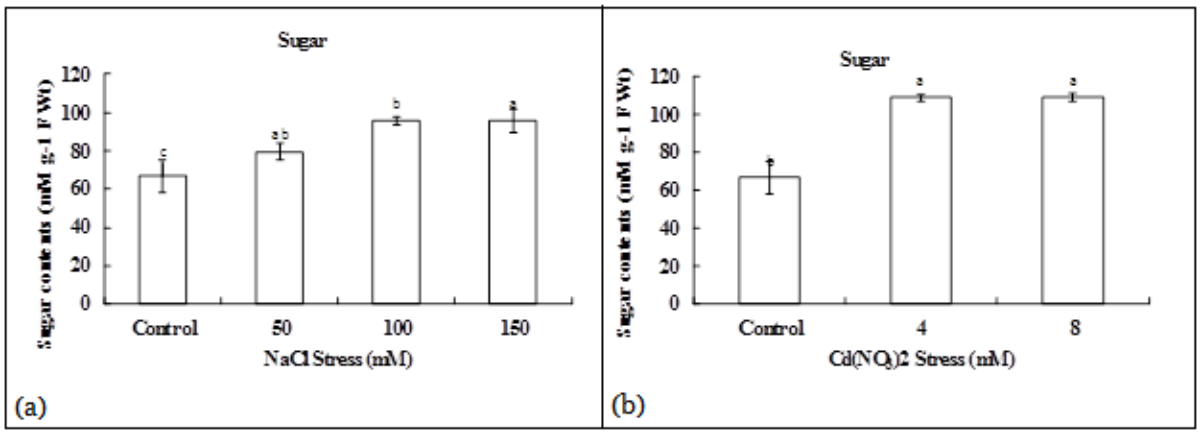

Fig. 5. a) The effect of NaCl stress on the total sugar contents (mM g^{-1} fresh weight calli) of *A. bracteosa*. Data in the graph are means of four replicates ±SE, b) Total sugar contents (mM g^{-1} fresh weight calli)of *A. bracteosa* under heavy metal stress The data presenting in graph are means of four replicates ± SE.

Current result showed that total phenolics contents and total antioxidant activity under salinity stress were gradually increased. Similar results were observed in *Rosmarinus officinal*plant by (Dixon and Paiva, 1995) and Mmdouh *et al.*, 2002) they reported that under salt stress plant synthesize and accumulate phenolics compounds with enhanced antioxidant activity, whereas, antioxidant activity of phenolic compound neutralize the reactive oxygen species (ROS) under environmental stresses including salinity, which ultimately reduced oxidative stress. (Zheng and Wang, 2001; Riadh *et al.*, 2007).

Under heavy metal stress Cd $(NO_3)_2$) phenolic contents and antioxidant activity decreased gradually. Similar results were found by (Cerda *et al.*, 2016) in *vaccinium corymbosum. L.* they reported a positive correlation of phenolics with antioxidant activity.

While (Ibrahim *et al.*, 2017) reported that decrease in antioxidant potential under cadmium stress could be related to reduction in contents of phytochemicals such as, total phenolics, flavonoids and saponin contents.

Under salinity stress, proline contents of callus extract increased. Similar result was found by (Hare *et al.*, 1998) they reported that higher accumulation of proline is an important adaptive mechanism for salt tolerance. Proline reduces toxic ion uptake and act as an Osmolyte to reduce the osmotic potential. Our results also showed similarity with (Bandeh *et*

al., 2008). They reported that in *Rosmarinus officinalis* plant, proline accumulation has been associated with reduction in the adverse effect of salt stress and maintaining leaf turgor (Hong *et al.*, 2000) reported that proline may protect cells against salt stress and act as radical scavenger.

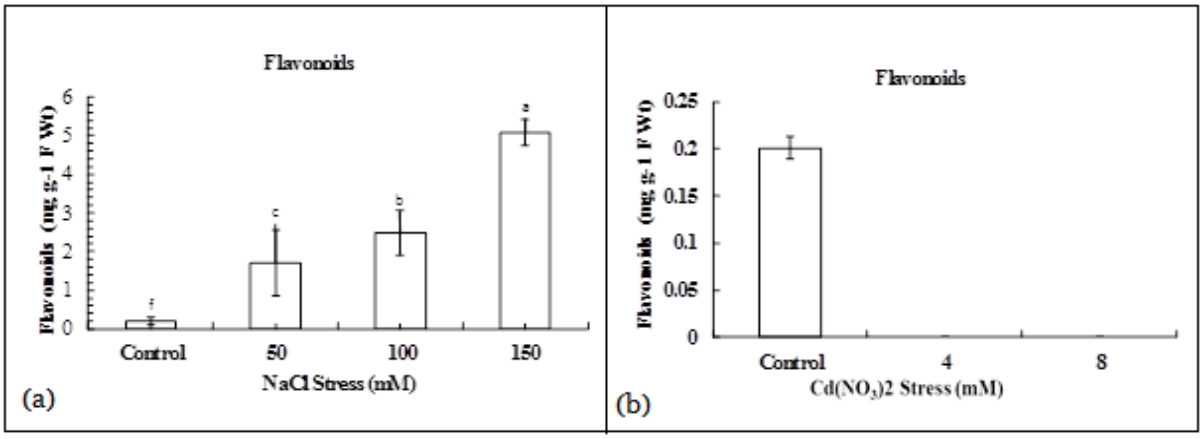

Fig. 6. a) The effect of NaCl stress on the total flavonoid contents (mg g^{-1} fresh weight calli) of *A. bracteosa*. The data presented in graph are means of four replicates ±SE, b) Total flavonoid contents (mg g^{-1} fresh weight calli)of *A. bracteosa* under cadmium stress.

Our results are also in agreement with the result of (El-Esawi *et al.*, 2017) they reported that under salinity condition proline contents increased in rosemary plant. Under Cd $(NO_3)_2$ stress, proline contents increased, similar result was obtained by (Zahra *et al.*, 2019) in *Satureja hortensis L*, stressinduces cellular acidification where as these constrain were reduced by proline accumulation and for recovery provide energy (Parlak and Yilmaz, 2012).

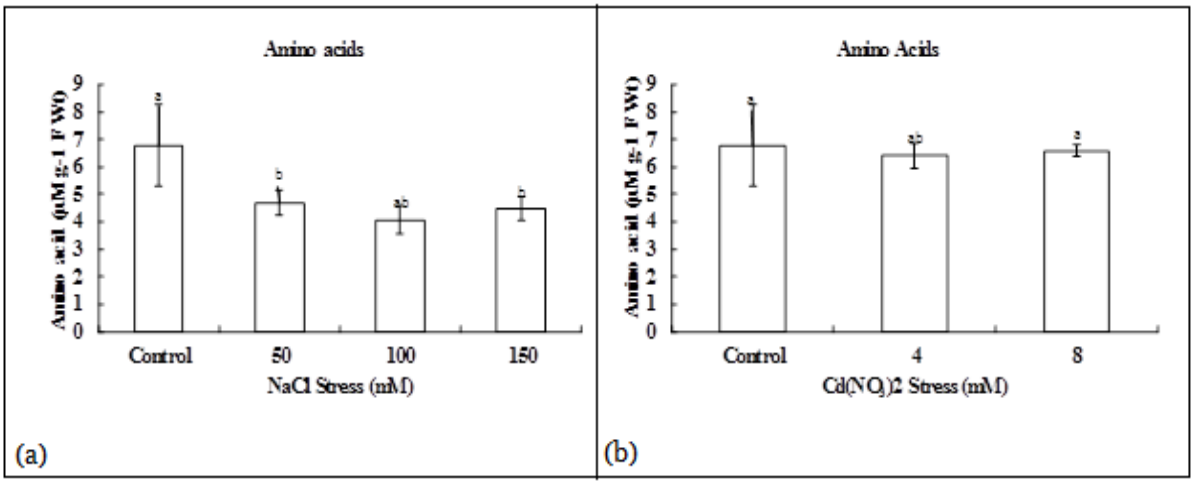

Fig. 7. a) Total amino acid contents (µM g^{-1} fresh weight calli) of *A. bracteosa* under salinity stress The data presented in graph are means of four replicates ±SE, b) Total amino acid contents (µM g^{-1} fresh weight calli) of *A. bracteosa* under cadmium stress. The data presenting in graph are means of four replicates ± SE.

Total sugar contents (TSC) increases gradually with increase in concentration of NaCl, similar results were reported in *Lavandula multifidi L* by (Garcia-Capparos *et al.*, 2017) at different concentration of NaCl (60, 100 and 200mM) the level of sugar contents in leaves gradually increased. Total sugar contents increased when concentration of $Cd(NO_3)_2$increased, whereas at high concentration their decrease were observed, our results were in agreement with the finding of (Kuntal *et al.*, 2017) in

Tagetes erecta L. at various concentration (6, 18, 24, 30mg/kg) of $Cd(NO_3)_2$, that reduction in total sugar contents occur at high concentration.

Total flavonoids contents (TFC) increased gradually under salinity with respect to control. Similar results in *Schizonepeta tenuifolia* were reported by (Zhou *et al.*, 2018) at 50mM NaCl, level of flavonoids increased, while above 100mM level of flavonoids decreased which is dissimilar to our result, the reason may be the level of salt in medium is not sufficient to alter the flavonoids contents or in current study callus were used while they were used plant. Flavonoids contents depleted at high concentration of cadmium, which are dissimilar to the finding of (Ibrahim *et al.*, 2017), they reported flavonoid contents in *Gynura procumbens L* under stress combination of cupper and cadmium (1.24, 2.1mg). It was reported that reduction in flavonoids occurred when plants were subjected to high level of heavy metal stress, or in combination with different metals (Okema *et al.*, 2015).

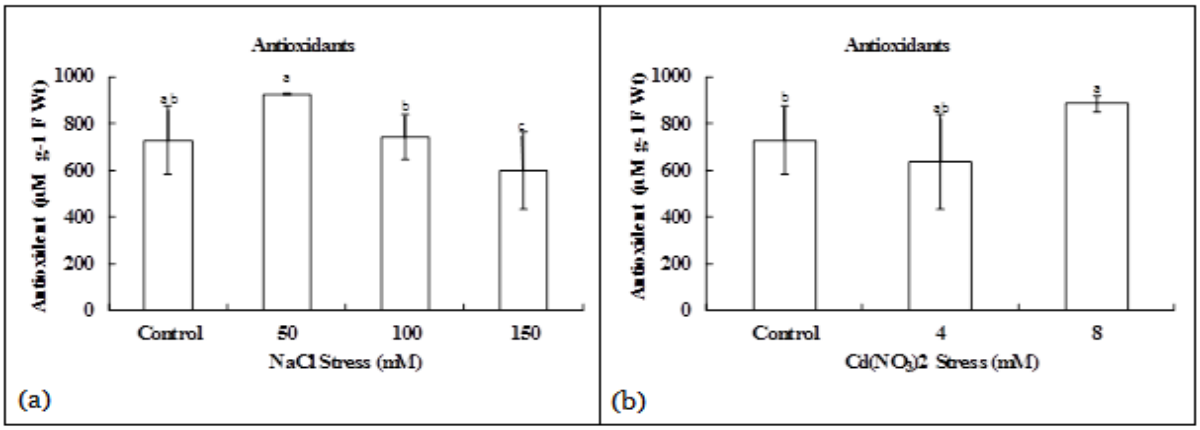

Fig. 8. a) The effect of NaCl stress on the total antioxidant activity (µM g^{-1} fresh weight calli) of *A. bracteosa*. The data presented in graph are means of four replicates ±SE, b) The effect of $Cd(NO_3)_2$on the total antioxidant activity (µM g^{-1} fresh weight calli) of *A. bracteosa*. The data presenting in graph are means of four replicates ± SE.

In our study total α Amino acid contents slightly decreased at different concentration of NaCl (50,100,150mM) with respect to control, similar result was obtained by (Garcia-Capparos *et al.*, 2017) in *Lavandula multifida L.* with increasing salinity total α amino acid contents slightly decreased. Total α amino acid contents under $Cd(NO_3)_2$ stress decreased. (Balesstrasse *et al.*, 2003) found similar results that due to high uptake of cadmium reduction occurred in the amino acid contents was associated with decrease protein synthesis or increase in the rate of protein degradation due to increase protease activity (Palma *et al.*, 2002).

In current study two types of stresses were used NaCl and (Cd $(NO_3)_2$. Gradual increased in the level of NaCl from control to 150mM and $Cd(NO_3)_2$ at 8mM resulted gradual decreased in the (RGR) of calli, which in turn increased the metabolites accumulation like, phenolics, proline, sugar, alpha amino acids and flavonoids etc.

Conclusion

The results obtained during this study concluded that NaCl and Cd $(NO_3)_2$ significantly decrease the RGR and increases secondary metabolites (phenolics, flavonoids, proline antioxidant activity etc.) accumulation. The increase in secondary metabolites is similar to the level found in plants. Therefore, it can be concluded that calli can be used as alternative source for production of secondary metabolites instead of whole plant, an ex-situ conservation strategy for this overexploited medicinal herb.

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Conflict of interest

There are no conflicts of interest declared by any of the authors.

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