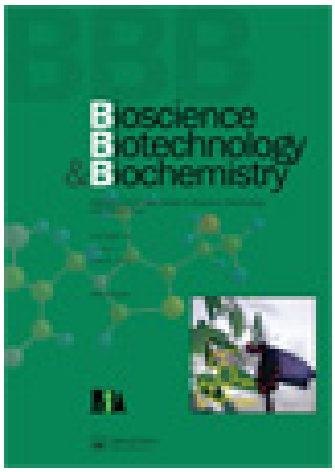

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Salt stress-induced protein pattern associated with photosynthetic parameters and andrographolide content in Andrographis paniculata Nees

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Salt stress-induced protein pattern associated with photosynthetic parameters and andrographolide content in *Andrographis paniculata* Nees

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Andrographis paniculata **is a multifunctional medicinal plant and a potent source of bioactive compounds. Impact of environmental stresses such as salinity on protein diversification, as well as the consequent changes in the photosynthetic parameters and andrographolide content (AG) of the herb, has not yet been thoroughly investigated. The present study showed that the salinity affects the protein pattern, and subsequently, it decreased the photosynthetic parameters, protein content, total dry weight, and total crude extract. Exceptionally, the AG content was increased ($p \leq 0.01$). Moreover, it was noticed that the salinity at 12 dS m^{-1} led to the maximum increase in AG content in all accessions. Interestingly, the leaf protein analysis revealed that the two polymorphic protein bands as low- and medium-sized of 17 and 45 kDa acted as the activator agents for the photosynthetic parameters and AG content. Protein sequencing and proteomic analysis can be conducted based on the present findings in the future.**

Key words: *Andrographis paniculata*; andrographolide content (AG); leaf protein; net photosynthetic rate; salinity stress

King of bitters (*Andrographis paniculata* Nees.) is a medicinal herb from the family Acanthaceae that is rich in bioactive compounds.[1)] High-salinity stress causes alterations in various biochemical and physiological responses of plants and cause adverse effects on all plant processes, including photosynthesis, growth, and development.[2)] The effects of salinity on mature plants of *A. paniculata* have been studied in relation with macro- and micro-nutrients accumulation,[3)] as well as the morphological and physiological responses.[4)] One of the metabolic reactions by which higher plants encounter the high osmolarity of salt is the accumulation of compatible organic solutes such as soluble carbohydrates, amino acids, proline, and betaines.[5–7)] Proline is the most common compatible solute, which provides protection to the cell membrane, was found in some accessions (AC) of *A. paniculata*.[8)]

The protein profiling study is an important alternative measurement of gene expression in plants in response to various abiotic stress conditions. Therefore, it is necessary to examine the salt stress at the molecular level, and investigate the relationship between biomarkers (such as protein bands) with physiological, biochemical, and phytochemical changes. Many proteins undergo post-translational modifications which play an important role in their activity and subcellular localization.[9,10)] In this research, we demonstrate the impact of salinity on some photosynthetic parameters, AG content, and protein profiling in *A. paniculata*. In addition, the present article aims to get insights into the changes in osmotic composition of the plant associated with the salt acclimation.

Results

Effects of salinity on photosynthetic parameters

The results indicated that salinity levels (SL) significantly affected all AC in terms of the measured photosynthetic parameters (Table 1).

The interaction of SL × AC was significant in terms of chlorophyll *b* (Chlo*b*), net photosynthetic rate (NPR), AG content, and stomatal conductance (COND). The Chlo*a*, Chlo*b*, F_v/F_m, NPR, and

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Abbreviations: AG, andrographolide; Chlo*a*, chlorophyll *a*; Chlo*b*, chlorophyll *b*; COND, stomatal conductance; F_v/F_m, chlorophyll fluorescence; NPR, net photosynthetic rate; TCE, total crude extract; TDW, total dry weight; σ_G^2, genetic variance; σ_P^2, phenotypic variance; h_B^2, broad-sense heritability.

Table 1. Analysis of variance of protein, photosynthetic parameters, and AG content in the nine AC of *A. paniculata* under salinity stress.

		Mean square								
Source	df	Chlo *a*	Chlo *b*	F_v/F_m	NPR	COND	Protein	TDW	TCE	AG
Salinity (SL)	3	4723.96**	845.57**	0.04**	515.01**	0.733**	1.919**	2197.17**	40.82**	7.68**
Accession (AC)	8	182.33**	38.37**	0.03**	10.23**	0.008**	0.228*	51.58**	0.87**	1.30**
SL × AC	24	56.666^{ns}	28.73**	0.01^{ns}	3.22**	0.007**	0.122^{ns}	12.898^{ns}	0.15^{ns}	0.38**
Error	64	42.07	11.66	0.01	0.32	0.002	0.118	12.72	0.35	0.14

Notes: Chlo *a*: chlorophyll *a* (μg g^{-1} FW), Chlo *b*: chlorophyll *b* (μg g^{-1} FW), F_v/F_m: chlorophyll fluorescence, NPR: net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), COND: stomata conductance (mol m^{-2} s^{-1}), protein (mg g^{-1} FW), TDW: total dry weight (g), TCE: total crude extract (g) and AG: andrographolide content (%).
$^{**}p \le 0.01$.
$^{*}p \le 0.05$.
^{ns}(non-significant).

COND were significantly decreased under salinity conditions, especially at the extreme salinity situation (12 dS m^{-1}) compared with those of control plants (Fig. 1).

The chlorophyll contents (*a* and *b*), F_v/F_m, NPR, and stomatal conductance at high concentration of NaCl (12 dS m^{-1}) were significantly reduced by 56.23, 60.56, 11.10, 86.82, and 85.00%, respectively. The NPR and COND showed a decreasing slope of 3.06 μmol CO_2 m^{-2} s^{-1} and 0.12 mol m^{-2} s^{-1} per SL (4 dS m^{-1}), respectively (Fig. 1). This might be due to the high concentration and long duration of salinity. Among AC, the highest amounts of Chlo*a* (44.43) and Chlo*b* (16.38) were observed in the AC 11265 and 11228, whereas the lowest amounts of Chlo*a* (31.73) and Chlo*b* (11.49) were recorded under the same conditions in the AC 11216 and 11329, respectively (Table 2). The highest amounts of NPR and COND (6.52 μmol CO_2 m^{-2} s^{-1} and 0.22 mol m^{-2} s^{-1}) were detected in accession 11249, whereas the lowest amounts of NPR and COND (3.61 μmol CO_2 m^{-2} s^{-1} and 0.15 mol m^{-2} s^{-1}) were detected in accession 11228 (Table 2).

In all the AC, Chlo*a*, Chlo*b*, F_v/F_m, NPR, and COND were reduced with the salt treatments. The greatest decrease in chlorophyll contents, NPR and COND, was observed at 12 dS m^{-1} of salt stress. However, accession 11249 was treated with a high level of salinity, but the NPR maintained higher than others. Moreover, the plants grown under extreme SL showed pigment damage, low F_v/F_m, NPR reduction, and exhibited growth inhibition. Even though, net photosynthetic and stomatal conductance were reduced in response to salt stress, some AC (like 11249) showed less reduction in photosynthesis than sensitive ones (such as 11228 and 11306). The results indicated that stomatal conductance limited the photosynthetic capacity of the leaves in the NaCl-treated plants. All in all, these results suggested that the accession 11249 may have a better protection mechanism against reactive oxygen species, and could be applied as an indicator in salt-tolerance screening program of this herb.

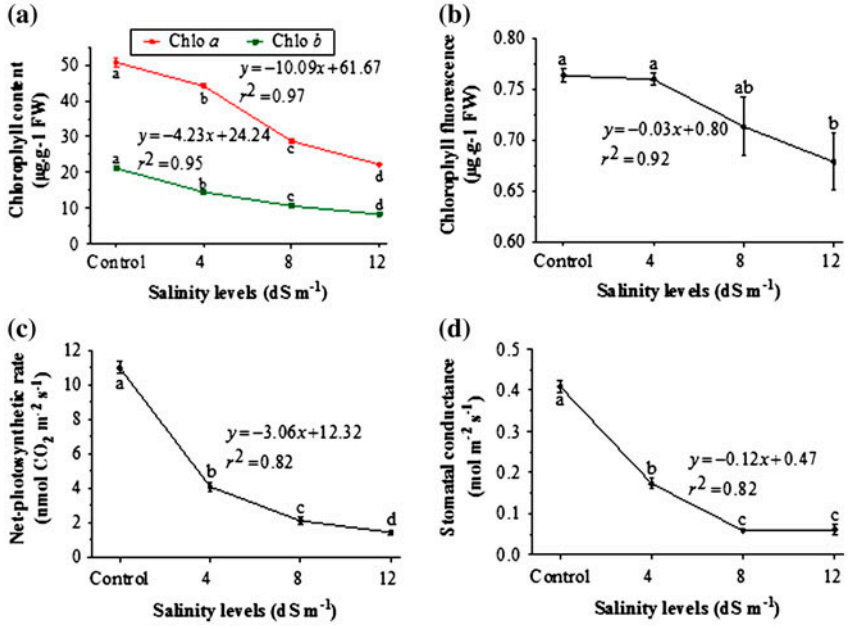

Fig. 1. The effect of SL on the photosynthetic parameters in *A. paniculata*.
Notes: Chlorophyll *a* and chlorophyll *b* (a), F_v/F_m (b), NPR (c), and stomatal conductance (d). Increasing SL led to decrease in chlorophyll content. Vertical bars represent SE for three samples, and different letters indicate significant differences among the SL using Duncan's multiple comparison test at $p \le 0.01$.

Table 2. Mean comparison of the measured characteristics of the nine *A. paniculata* AC (Mean values ± S.E).

Accession	Chlo *a*	Chlo *b*	F_v/F_m	NPR	COND	Protein	TDW	TCE	AG
11179	35.36 ± 2.91	12.13 ± 1.05	0.75 ± 0.01	3.99 ± 1.26	0.17 ± 0.05	2.73 ± 0.13	22.34 ± 2.30	3.15 ± 0.36	1.98 ± 0.21
11216	31.73 ± 4.04	12.49 ± 1.78	0.72 ± 0.02	4.37 ± 1.25	0.15 ± 0.04	2.75 ± 0.16	24.66 ± 2.58	3.23 ± 0.39	1.79 ± 0.19
11228	38.56 ± 3.55	16.38 ± 1.91	0.75 ± 0.01	3.61 ± 0.86	0.15 ± 0.04	2.86 ± 0.10	22.52 ± 2.70	3.15 ± 0.37	1.66 ± 0.12
11249	38.32 ± 3.93	15.82 ± 2.32	0.74 ± 0.02	6.52 ± 1.26	0.22 ± 0.06	2.73 ± 0.18	22.28 ± 2.44	3.23 ± 0.38	2.55 ± 0.20
11264	36.38 ± 4.47	12.39 ± 1.49	0.76 ± 0.01	5.38 ± 0.99	0.21 ± 0.05	2.91 ± 0.11	21.70 ± 2.39	3.05 ± 0.35	1.74 ± 0.29
11265	44.43 ± 3.40	12.88 ± 1.44	0.77 ± 0.01	4.27 ± 1.14	0.17 ± 0.04	2.67 ± 0.15	21.47 ± 2.67	3.06 ± 0.39	1.67 ± 0.17
11266	37.14 ± 4.45	15.25 ± 2.51	0.72 ± 0.02	4.93 ± 1.03	0.16 ± 0.04	3.01 ± 0.09	20.41 ± 2.91	2.83 ± 0.40	1.59 ± 0.21
11306	31.95 ± 3.89	14.24 ± 2.49	0.61 ± 0.08	3.78 ± 1.04	0.16 ± 0.04	2.91 ± 0.10	20.08 ± 3.14	2.77 ± 0.40	1.50 ± 0.16
11329	34.07 ± 4.82	11.49 ± 1.59	0.73 ± 0.01	5.14 ± 1.59	0.19 ± 0.05	3.07 ± 0.09	17.16 ± 2.71	2.41 ± 0.36	1.48 ± 0.16

Notes: Chlo *a*: chlorophyll *a* (μg g^{-1} FW), Chlo *b*: chlorophyll *b* (μg g^{-1} FW), F_v/F_m: chlorophyll fluorescence, NPR: net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), COND: stomata conductance (mol m^{-2} s^{-1}), protein (mg g^{-1} FW), TDW: total dry weight (g), TCE: total crude extract (g) and AG: andrographolide content (%).

Effects of SL on total protein content

The analysis of variance showed that the salinity in different levels could affect the total protein content of *A. paniculata*, significantly. Variation due to SL and AC was highly significant in terms of total protein content under salt stress conditions ($p \leq 0.01$). In contrast, the interaction of SL × AC for total protein content was not significant (Table 1). The results showed that total protein content was negatively correlated to the substrate concentration of NaCl ($p \leq 0.01$) (Fig. 2). Despite this, accession 11266 produced a relatively higher total protein content at high SL (12 dS m^{-1}). However, as a general outcome, a negative slope was established between the total protein content and salinity. Whereas, per 4 dS m^{-1} increase in salinity caused a decrease in protein content up to 0.203 mg g^{-1} FW (Fig. 2).

Effect of salinity on total dry weight

The analysis of variance showed that salinity had a significant effect on total dry weight (TDW). On the other hands, variation due to SL and AC was highly significant ($p \leq 0.01$), while the interaction of SL × AC was not significant in terms of TDW (Table 1). The results showed that TDW was negatively correlated to the substrate concentration of NaCl in the fourth week of salt stress application ($p \leq 0.01$). Four weeks after the salt application, a significant reduction appeared in the growth of all AC. The highest TDW (24.66 g) was observed in accession 11216, whereas the lowest TDW (17.16 g) belonged to accession 11329, under the same condition (Table 2). A decreasing trend was featured for TDW, linearly (Fig. 2). The concentration of 12 dS m^{-1} NaCl reduced the dry weight by 62.80% compared with the control, in a significant manner.

Salinity effects on total crude extract and AG content

Analysis of variance showed that different levels of salinity affected the total crude extract (TCE) and AG content, significantly. Variation due to SL and AC were highly significant ($p \leq 0.01$). The interaction of SL × AC was not significant in terms of TCE, while the interaction of SL × AC was highly significant in

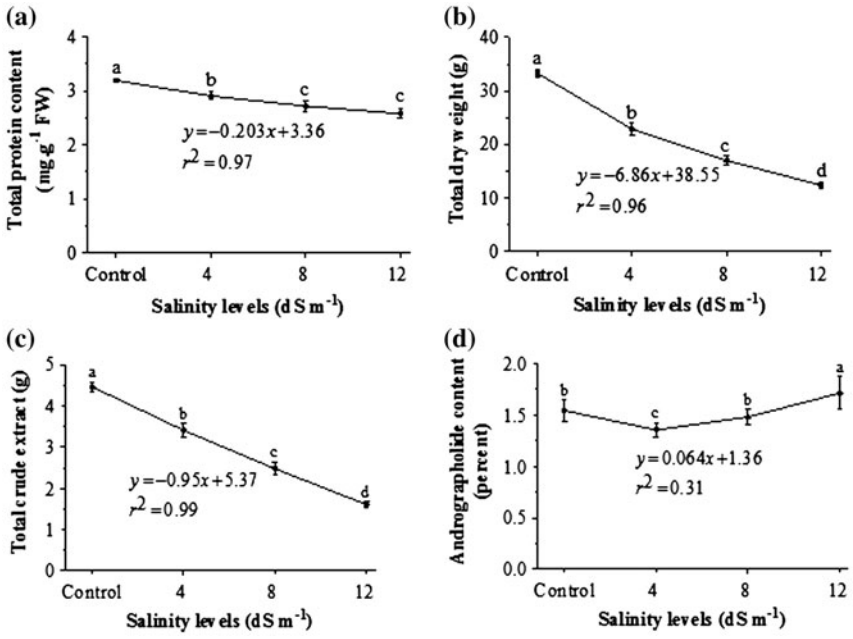

Fig. 2. The effect of SL on the photosynthetic parameters in *A. paniculata*.

Notes: Total protein content (a), TDW (b), TCE (c), and AG content (d). Vertical bars represent standard error of mean (SE) for three samples, and different letters indicate significant differences among the SL using Duncan's multiple comparison test at $p \leq 0.01$.

Table 3. Phenotypic correlation coefficients (r) of the measured traits in the nine *A. paniculata* AC under salinity condition.

	Protein	F_v/F_m	Chlo *a*	Chlo *b*	NPR	COND	TDW	TCE	AG
Protein	1								
F_v/F_m	0.229*	1							
Chlo *a*	0.353**	0.342**	1						
Chlo *b*	0.319**	0.233*	0.769**	1					
NPR	0.461**	0.238*	0.711**	0.681**	1				
COND	0.384**	0.244*	0.681**	0.653**	0.934**	1			
TDW	0.354**	0.364**	0.735**	0.691**	0.788**	0.762**	1		
TCE	0.346**	0.342**	0.731**	0.646**	0.716**	0.693**	0.955**	1	
AG	0.182 ^{ns}	0.095 ^{ns}	0.515**	0.498**	0.654**	0.638**	0.558**	0.590**	1

Notes: Chlo *a*: chlorophyll *a* (μg g^{-1} FW), Chlo *b*: chlorophyll *b* (μg g^{-1} FW), F_v/F_m: chlorophyll fluorescence, NPR: net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), COND: stomata conductance (mol m^{-2} s^{-1}), protein (mg g^{-1} FW), TDW: total dry weight (g), TCE: total crude extract (g) and AG: andrographolide content (%).
$^{**}p \le 0.01$.
$^{*}p \le 0.05$.

terms of AG content (Table 1). The measurements conducted for four weeks after salt application showed that the AG content was positively correlated with the substrate concentration of NaCl, while the TCE was negatively correlated with salinity ($p \le 0.01$) (Fig. 2). Analysis of variance showed high significant differences ($p \le 0.01$) for TCE and AG content among the AC. The highest TCE (3.23 g) and AG (2.55 g) both were found in accession 11249, whereas the lowest TCE (2.41 g) and AG (1.48 g) belonged to accession 11329, under the same condition (Table 2). The trend of TCE and AG content were linear and decreasing (Fig. 2). The concentration of 12 dS m^{-1} NaCl led to a significant reduction in TCE up to 64.10%, and an increase in AG content up to 11.18% compared with the control. The content of AG, as the most important cytotoxic principle in *A. paniculata* varied from 1.48% (in11329) to 2.55% (in 11249), with a mean value of 1.77%. Strong relationships among the studied traits were significantly expressed at the 1% level, except AG with protein and F_v/F_m. Indeed, a high correlation was found between photosynthetic parameters and AG. Simultaneously, strong relationships were detected between NPR, COND, TDW, and TCE (Table 3).

The broad-sense heritability (h^2) of the measured characteristics

As shown in Table 4, the highest broad-sense heritabilities were calculated for NPR (0.97) and AG content (0.90), while the lowest heritability was obtained for protein content (0.66). Therefore, NPR and AG can be considered as a suitable criterion for direct selection in breeding programs of the herb. At the same time, they could be used for assessing the response of the plant to salinity stress.

Leaf protein profiles

SDS-PAGE analysis revealed that the leaf protein patterns of the nine AC of *A. paniculata* were quite identical in control conditions, while these patterns were different under salinity conditions, in which some induction or repression in the synthesis of a few polypeptides happened. The quantity and quality of the proteins in salt-treated samples were lower than the control samples. As shown in Fig. 3, 11 bands with the molecular weight of approximately 15–192 kDa were detected on the SDS-PAGE. It is worth to mention that each of these bands could contain several proteins that further sequence analysis will identify the exact type of protein. According to the SDS-PAGE analysis, proteins' patterns of *A. paniculata* under salt stress conditions were not identical at all. Furthermore, the differences were included in both quantitative and qualitative categories (Fig. 3(b)). Salinity also inhibited the synthesis of two proteins with the size of 45 kDa (protein "X") and 17 kDa (protein "Y").

Based on the presence or absence of the protein "X", the AC were grouped into two separate groups. *T*-test assay indicated significant differences between these two groups in terms of photosynthetic parameters, TDW, and TCE while no significant difference was observed in terms of AG content (Table 5).

However, *T*-test of the two groups of AC based on the presence or absence of the protein "Y" revealed that there were no significant differences between the groups in terms of the photosynthetic parameters and AG content, but at least significant differences between the two groups were detected in terms of TDW and TCE (Table 5). The results also suggested that these two polymorphic protein bands might be related to the photosynthetic parameters and phytochemical components. Interestingly, the mean values of the

Table 4. Components of variance and broad-sense heritability of the measured characteristics in the nine *A. paniculata* AC.

	Characters								
Components	Chlo *a*	Chlo *b*	F_v/F_m	NPR	COND	Protein	TDW	TCE	AG
σ_G^2	182.33	38.37	0.03	10.23	0.008	0.23	51.58	0.87	1.30
σ_P^2	224.4	50.03	0.04	10.55	0.01	0.35	64.30	1.22	1.44
h_B^2	0.81	0.77	0.75	0.97	0.80	0.66	0.80	0.71	0.90

Notes: σ_G^2: genetic variance, σ_P^2: phenotypic variance and h_B^2: broad-sense heritability, Chlo *a*: chlorophyll *a*, Chlo *b*: chlorophyll *b*, F_v/F_m: chlorophyll fluorescence, NPR: net photosynthetic rate, COND: stomata conductance, TDW: total dry weight, TCE: total crude extract and AG: andrographolide content (%).

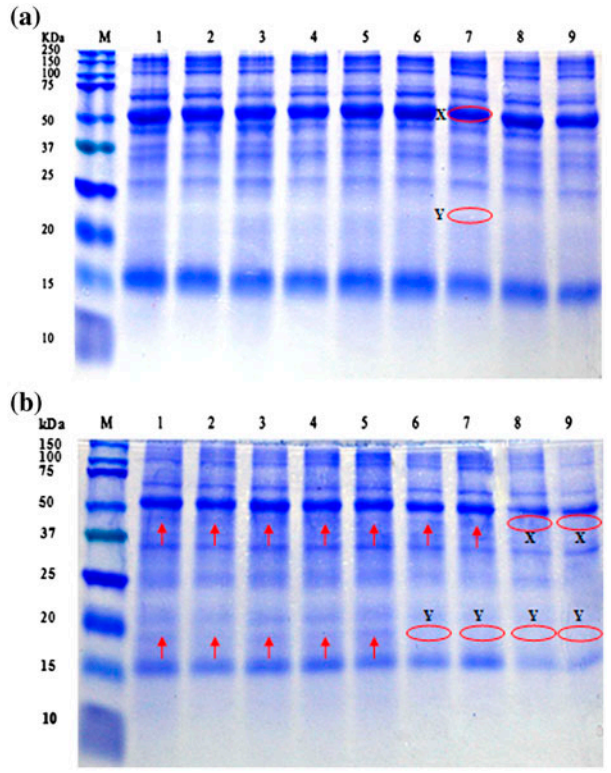

Fig. 3. Leaf protein profiles of the nine *A. paniculata* AC under control (a) and high salinity condition (12 dS m^{-1}) (b) on the SDS-PAGE. "M" represents the protein marker, 1) 11179, 2) 11216, 3) 11228, 4) 11249, 5) 11264, 6) 11265, 7) 11266, 8) 11306 and 9) 11329.

Notes: Protein samples were loaded with equal amount of 20 µg. Arrows show the presence of the protein "X" and ovals highlight the absence of the protein "Y".

photosynthetic parameters, TDW, TCE, and AG content in the AC containing the proteins "X" and "Y" were higher than those lacking these two protein bands.

Discussion

Plants growth get stunted under salt stress condition. In line with this, salt stress may lead to (1) stomatal closure and a consequent reduction in the availability of CO_2 in the leaves, (2) changes in cytoplasmic structure, and inhibition of carbon fixation, and finally, (3) exposing chloroplasts to excessive excitation energy.

These in turn can lead to changes in osmotic and leaf water potential, chlorophyll content and chlorophyll fluorescence, CO_2 concentration in the intracellular airspaces of leaves, and reduction in enzymatic activities.[2,11] As a consequence, photosynthetic activities can be inhibited due to the reduced stomatal conductance and low carbon substrate. These effects vary depend on the intensity and duration of the stress as well as the leaf age and the plant species.[12–14] Our results indicated a decrease in photosynthetic parameters, but this reduction varied in different AC. The reduction in stomatal conductance under salt stress condition can be attributed to disturbed water relations.[15] At the same time, these factors are considered as the tools for screening cultivars for salinity tolerance.[2,11]

In general, production of secondary metabolites in plants is regarded as a defense mechanism under different environmental stresses.[16] Similarly, our results indicated that the AG content was positively correlated with increasing the concentration of salt. From this point of view, our upshots are in accordance with the related studies. Reportedly, salt stress increases the polyphenolic compounds in *Zea mays*[17], flavonoids in *Hordeum vulgare*,[18] phenolic contents in *Cuminum cyminum*,[19] menthone in *Mentha pulegium*[20], and alkaloids in *Catharanthus roseus*.[21] Likewise, it is suggested that soluble flavonoids of the vacuole are involved in osmotic adjustment, protecting the cellular structures from oxidative damage. Besides these compounds perform an important role in the enhancement of the plant's tolerance against UV radiation.[22,23] Amusingly, the results of the present research supported the above-mentioned trend, the low levels of salinity decreased the biosynthesis of AG, while the highest levels of salinity (12 dS m^{-1}) led to a significant increase in the AG production (Fig. 2(d)). In another word, however, the feedback of *A. paniculata* to salt treatment was not the same at different levels of salinity, but it agreed with the idea that in the presence of the abiotic stresses, the boost in the production of secondary metabolites happens, and this becomes into a close connection with the self-protection mechanisms of the plants, concurrently.[24] With reference to the available literatures, the genetic aspects of inheritance of the polygenic salt tolerance-related traits have been taken into consideration in the general context of salt tolerance, recently.[25] Heritability of morphological and phytochemical characteristics of *A. paniculat* had been under scrutiny in very recent studies.[25] Two of these researches have been conducted under salinity condition, and the common point of these researches with the present study was recording high broad-sense heritabilities for proline, TDW, total chlorophyll,[8] as well as for AG.[24] As a remarkable evaluation, comprehensive genetic analyses of andrographolides in *A. paniculata* using diallel technique signified moderate

Table 5. *T* test results based on the independent leaf protein samples for photosynthetic parameters and phytochemical components in the nine *A. paniculata* AC.

Protein type	df	Chlo *a*	Chlo *b*	F_v/F_m	NPR	TDW	TCE	AG
X (45 kDa)	25	3.65^{**}	3.81^{**}	2.28^{*}	2.44^{*}	2.48^{*}	2.42^{*}	0.40^{ns}
Y (17 kDa)	25	1.72^{ns}	1.69^{ns}	1.11^{ns}	2.64^{*}	4.17^{**}	3.58^{**}	0.19^{ns}

Notes: Chlo *a*: chlorophyll *a*, Chlo *b*: chlorophyll *b*, F_v/F_m: chlorophyll fluorescence, NPR: net photosynthetic rate, TDW: total dry weight, TCE: total crude extract and AG: andrographolide content (%).

$^{**}p \le 0.01$.

$^{*}p \le 0.05$.

^{ns}(non-significant).

broad-sense heritability for AG, while the narrow-sense heritability was suffering from a negative value, under normal condition.[26] However, the basis of the present study is different from the diallel-based experiment, but from the breeding point of view high broad-sense heritability (due to the presence of non-additive components in the formula) implicates the plant capacity for heterosis breeding.[26–28]

The relationship between protein content and proline in *A. paniculata* should be another matter of interest, where investigations prior to this study unveiled proline as a highly heritable trait (h_B^2 = 90%) in *A. paniculata*.[24] On the other hand, heritability estimates are usually categorized as high if values are larger than 50%.[29] Therefore, in spite of a numeric difference between the heritability values of proline (90%) and protein content shown in Table 4 (66%), both of these traits are classified as highly heritable characteristics. This coincidence could be interpreted in such a way that most proportion of the protein content in *A. paniculata* is from those proline-rich proteins.[30–32] This is important as proline plays a substantial role in plant resistance to abiotic stresses.[33]

Furthermore, the present results confirmed high heritability of NPR and AG content. Thus, the underlying genetic mechanisms can be considered as direct criterion for assessment of the plant's responses to salinity stress.

SDS-PAGE analysis revealed that plants grown under NaCl encountered with induction or repression in the synthesis of a few polypeptides. SDS-PAGE analysis also showed identical protein profiles in control samples of the nine AC, though salinity inhibited the synthesis of some leaf proteins such as "X" and "Y" proteins. In accordance with our results, Parida et al.[34] reported the reduction in protein content in *Bruguiera parviflora* under salt treatment. According to Zhang et al.[35], abiotic stresses such as salinity have harmful effects on the structure of proteins and their function in plant cells and increase protein damage. The disappearance of proteins in response to NaCl-based salinity has been observed in wheat[36] and *B. parviflora*, as well.[37] In the present study, the protein amounts of salt-treated plants were decreased in all the AC possibly due to the changes in the ratio of the lipoprotein of pigment–protein complexes and/or chlorophyllase activity. These results are in agreement with the reports complaining that chlorophyll, carotenoids, and protein contents are all decreased due to salinity stress in some plant species.[38]

Existing little literature about the relationships between leaf protein patterns and photosynthetic parameters and phytochemical components are not deniable.[39] The obtained results have a high potential to be combined with different biochemical,[39] molecular[40,41], and morphological[40,42], data and can be used for resolving some critical issues in the herb such as crossability.[40,42,43] Electrophoretic information on the leaf proteins can be specifically matched with the seed protein data[39] of *A. paniculata* to make fruitful decisions on the breeding programs. Consolidation of these data will open new horizons in different fields of interest such as plant physiology and photosynthetic investigations of *A. paniculata*.

Conclusion

Our results showed the indisputable influence of leaf proteins in improving photosynthetic parameters and phytochemical components. Salt stress may affect photosynthetic activity, phytochemical production and their biosynthetic intermediates. On the other hand, the high heritability of the NPR and AG content can be potentially utilized as an effective traits in the next breeding programs to develop salt tolerant varieties of *A. paniculata.* The significant increase in the AG content under salinity condition would be partially compensated by a decline in total biomass. The main purpose of the present study was indeed to find the relationship of leaf protein profiling with photosynthetic parameters and phytochemical components under salinity conditions in *A. paniculata*. Two distinct protein bands with 45 and 17 kDa molecular weights were detected in some AC of *A. paniculata*. These proteins caused the highest records of photosynthetic parameters and phytochemical components in these AC. The obtained data are worthy to be developed toward further analyses.

Materials and methods

Plant material and growth conditions. The seeds of nine different AC of *A. paniculata* were provided by the Agro Gene Bank, Universiti Putra Malaysia. The seeds were sterilized and germinated as described by Talei et al.[44,45]. The seeds were then incubated in a growth chamber under controlled condition adjusted in 14 h d^{-1} photoperiod at 28–30 °C, and relative humidity of 60–75%. The germinated seeds were transferred into the Jiffy media at two initial leaf stage.

Experimental design. The experiment was carried out with a split plot based on a randomized complete block design with two factors and three replicates. The factors were four different concentrations of saline water (control, 4, 8, and 12 dS m^{-1}) in main plots and nine different AC in sub-plots. The 40-day seedlings were transferred from Jiffy pots into the plastic pots containing sand medium. Thirty days after the first transformation (when the plants were almost 70-day old) the plants were exposed to different SL on Hoagland medium. Each plant was separately watered with four levels of saline water, once a day. After three times of irrigation with saline water, the plants were alternately irrigated once with a standard Hoagland nutrient solution. The irrigation process was continued for one month and after that the photosynthetic parameters as well as the total protein contents were measured. All the plants were harvested, and data such as TDW, TCE, and AG contents were measured, afterward.

Determination of photosynthetic parameters. The chlorophyll content of *A. paniculata* leaves were measured using chlorophyll meter SPAD-502 (Minolta Camera Co, Osaka, Japan). The SPAD-502 measures peak chlorophyll absorbance at 650 nm and non-absorbance at 940 nm. Since, the measured area by the SPAD-502 is too small (6 mm^2), thus a minimum of six readings was

averaged. In order to determine the content of chlorophyll *a*, chlorophyll *b* and total chlorophyll, a total of 0.5 g fresh leaf tissue was extracted with 5 mL of 100% acetone. The respective absorptions of the extracted samples were measured using a spectrophotometer (Shimadzu, UV-1201, Japan) at 645 and 663 nm by following the method of Arnon.[46)]

The chlorophyll fluorescence of F_v/F_m was estimated using the fluorescence monitoring system (FMS 2; Hansatech Instruments Ltd, Norfolk, UK) in the pulse amplitude modulation mode as described by Maxwell and Johnson.[47)] Prior to the estimation of F_v/F_m, the leaves were subjected to dark-adapt by using clips for 20 min.[48)] The chlorophyll fluorescence parameters, including initial fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence (F_v), and maximum quantum efficiency of PSII (F_v/F_m) were observed between 9 and 11 am in five individual leaves, under transparent shade condition.

The NPR and stomatal conductance (COND) were measured using the infrared gas analyser (IRGA; model portable photosynthesis system Li 6400, Li-COR® Inc, Lincoln, Nebraska, USA). Before measurements, the Li-6400 portable photosynthesis system was calibrated, and the measurements were carried out at 1000 μmol m^{-2} s^{-1} of photosynthetically active radiation (PPF), 400 μmol/mol carbon dioxide, 30 °C leaf temperature, and 60% relative humidity with the air flow rate set at 500 cm^3/min.[49)] The measurements of the gas exchange were carried out between 9 and 11 am on the fourth leaf from the shoot tip of plants in each treatment under transparent shade condition. The leaf surfaces were cleaned and dried before being enclosed in the leaf cuvette. Data for NPR, leaf temperature (°C) and stomatal conductance (COND) were simultaneously recorded. The operation was automatic, and the data were stored in the LICOR-6400 computer within the console and analyzed by Photosyn Assistant software.

Protein extraction and estimation of total soluble leaf protein contents. The leaf tissue samples of each nine *A. paniculata* AC were ground in liquid nitrogen using pre-cooled mortar and pestle to obtain a fine powder and then homogenized with extraction buffer (20 mM HEPES/KOH pH 7.5, 40 mM KCl, 1 mM EDTA, 10% (v/v) glycerol and 1 mM PMSF) as described by Talei et al.[50)]. The supernatants were collected, and the total protein concentration was determined using the Bradford method.[51)] Bovine serum albumin (Sigma-Aldrich, USA) was employed as a standard at 595 nm using a spectrophotometer (Perkin-Elmer Lambda 25; UV/vis, USA). The protein samples were run on SDS-PAGE separation following the method described in Laemmli.[52)] Fifteen μg of the solubilized protein from each sample was loaded in each lane of the 12% concentrated separating gel. Electrophoresis was accomplished at 100 V over 90 min using a Bio-Rad, mini protein electrophoresis system (Bio-Rad, USA). The observed protein bands were scored using the UVIDoc Analyzer software.

Crude extraction method and estimation of andrographolide (AG) contents. Arial parts of the plants were dried at 55 °C for 72 h, and the dry materials were then ground into fine powder form and were then extracted with a mixture of dichloromethane and methanol (DCM: ME) at the ratio of 1:1. AG (Sigma-Aldrich, USA, purity 98%) was used as a standard sample. Twenty microliter of each filtered sample in three replicates was injected into the HPLC. The HPLC system was operated by Waters™ comprising Waters™ 600 Controller pumps, Waters™ 717plus autosampler injector with a capacity of 96 samples per round.[53)] LiChrocart® HPLCcartridge RP-18 (150 × 4.6 mm, Merck, Germany) was used as the stationary phase. The isocratic mobile phase was prepared with acetonitrile-water (40:60 v/v) and 0.1% (v/v) analytical grade orthophosphoric acid a flow rate of 1 mL min^{-1}.[54)] Detection was performed at 223 nm using Waters™ 486 tunable absorbance detector (photodiode array detector).

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Disclosure statement

The authors declare that they do not have any direct financial relation with the commercial identities mentioned in the present paper that might lead to conflict of interests for any of them.

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