



Standardization of 1- and 2-D protein profiling of *Haloxylon* species: Basis of proteome analysis of haloxerophytes

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One and two dimensional electrophoresis is a powerful technique for separation of proteins from complex biological sources. These are effective techniques of gene expression analysis at translational level especially for the species of which genome is not yet sequenced. High resolution electrophoresis separation of proteins from complex biological systems is crucial in protein science. Three protein extraction methods, such as Phenol, Multi detergent and TCA acetone were analyzed in the present investigation to find out appropriate protein extraction method amenable to one dimensional (1-D) and two dimensional (2-D) protein profiling of *Haloxylon recurvum* and *H. salicornicum*. Plant species selected for the present investigation are major components of saline and arid ecosystem and valuable source of food additives, nutritional ingredients, herbal medicines and fuel in the region. Phenolic components present in these plants are major hindrances for extraction of pure proteins. High concentrations of good quality proteins represented by clear, intense and more bands/spots in 1-D and 2-D gels were extracted with Phenol extraction method in comparison to TCA acetone and Multi detergent methods. This is possibly the first report on extraction and purification of proteins amenable to 1-D and 2-D proteome profiling of *Haloxylon* species of Indian arid zone, and forms a basis for further comparative proteome analysis of these two contrasting species with respect to salinity and drought tolerant ability for prospects.

Keywords: Food additives, *Khar*, Phenolic components, Protein extraction, Proteome analysis

Eukaryotic genes have many functional facets, due to translational and post translational processes, which form different types of proteins under various stages of development and environmental conditions from single gene¹. One and two dimensional electrophoresis techniques are the basis of translational products analysis of complex biological processes that occur under any given condition²⁻⁴, such as abiotic stress⁵⁻⁸, wounding⁹, water deficiency¹⁰⁻¹² and peroxidase activity¹³. These are strong, cost effective and a valuable technique for studying gene expression at protein level¹⁴⁻¹⁷ for species of which genome is not yet sequenced¹⁸. The protein profiles have also been useful for species as well as varietal identification¹⁹. The 2-D electrophoresis is utilized for identification of differentially abundant proteins during the fruit ripening process of the two varieties of tropical mango, *Mangifera indica*²⁰. Proteome analysis by 2-D electro-phoresis is also utilized for identification of differentially expressed proteins in trunking and non-trunking sago palm leaf tissues²¹. High resolution electrophoresis separation of

proteins from complex biological systems is crucial in protein science. Sample preparation is an important step in 2-D analysis, but is also difficult due to co-extraction of non-protein components that interfere in the processes^{22,23}. Presence of non-protein contaminants like, phenolics, polysaccharides, lipids, gummy substances and other secondary metabolites makes sample preparation troublesome in plants¹⁵.

Haloxylon recurvum and *H. salicornicum* of family Amaranthaceae have economical and ecological significance in Indian arid zone. *H. recurvum* thrive well in saline habitats of Pachpadra, and Phalodi region of Jodhpur, whereas *H. salicornicum* are keystone species of sandy areas of Jaisalmer, India. *H. recurvum* (Moq) Bunge ex Boiss is an under shrub perennial plant, locally known as Khar and is a source of crude sodium carbonate- Barilla or Sajji-khar and used against internal ulcers. The ash of the plants is used as a soap substitute for washing clothes in rural areas²⁴. The ash extract (Sajji-khar) is also used as an important ingredient of Bikaneri Bhujia and Papad of Rajasthan, India²⁵. *H. salicornicum* (Moq) Bunge ex Boiss is also an under shrub perennial plant locally known as Lana. It is a source of fuel, fodder and food additives²⁵. The plant contains alkaloids and oils^{26,27}.

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Here, we tested three protein extraction methods such as Phenol, TCA acetone and Multi detergent to find out appropriate protein extraction method amenable to one dimensional (1-D) and two dimensional (2-D) protein profiling of *Haloxylon* species of Indian arid zone.

Materials and Methods

Plant materials

Shoot tips of *H. recurvum* and *H. salicornicum* were collected from the nursery of PBMB Laboratory, Department of Botany, Jai Narain Vyas University, Jodhpur, India. These were immediately frozen in Liquid Nitrogen (LN). These frozen plant materials were crushed in LN to make fine powder for extraction of protein.

Protein Extraction

Three protein extraction methods were used in the present investigation with slight modification.

TCA acetone extraction method

One gram finely powdered plant sample was transferred to centrifuge tube containing freshly prepared 5 mL precipitation solution containing 10% (w/v) TCA and 2% (v/v) β -mercaptoethanol (β -ME) in cold acetone and incubated at -20°C overnight. The mixture was centrifuged at 10000 rpm (4°C) for 15 min. The pellet was resuspended in 4 mL acetone containing 0.07% β -ME and kept for 1 h at -20°C . The homogenate centrifuged and pellet was washed 5 times with ice-cold acetone. Final pellet was dried and dissolved in isoelectric focusing (IEF) buffer (9M Urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM dithiothreitol (DTT), 0.2% Biolyts)²⁸.

Phenol extraction method

One gram fine powder of plant sample was added to 3 mL extraction buffer containing 500 mM tris hydrochloric acid (TrisHCl, pH 8.0), 50 mM ethylene di-amine tetra acetic acid (EDTA), 700 mM sucrose, 100 mM potassium chloride (KCl), 2% β -ME and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 10 min on ice with intermittent invert mixing. Equal volume of tris saturated phenol was added and solution was incubated at room temperature (25°C) for 10 min. Homogenate was centrifuged at $5500\times\text{g}$ for 10 min at 4°C . Phenol phase was re-extracted with 3 mL of extraction buffer. Upper phenol phase was recovered in new tube; 4 volume of precipitation solution (0.1M Ammonium acetate in

methanol) was added, invert mixed and incubated at -20°C overnight. Mixture was centrifuged at $5500\times\text{g}$ at 4°C for 10 min. Pellet was washed thrice with precipitation solution and once with acetone. Air dried pellet was dissolved in IEF buffer²⁹.

Multi detergent extraction method

One gram crushed plant tissue was suspended in 3 mL Multi detergent extraction buffer (100 mM dibasic potassium phosphate (pH 7.6), 8M Urea, 1% Triton X-100, 20% glycerol, 0.5M sodium chloride and 2% β -ME and 1.0 mM PMSF). The mixture was shaken at room temperature for 10-15 min and centrifuged at $9500\times\text{g}$ at 4°C for 30 min. To the supernatant equal volume of 10% TCA with 2% β -ME was added and kept overnight for precipitation at -20°C . The mixture was centrifuged at $5500\times\text{g}$ for 30 min to pellet down the protein. The pellet was washed thrice with acetone and dried to dissolve in IEF buffer³⁰.

Quantification and one dimensional electrophoresis (1-DE)

The extracted total protein samples were quantified by Bradford method³¹, using UV-Visible SL 164 (Elico) spectrophotometer. Twelve microgram protein was resolved on 4.5% stacking (pH 6.8) and 10% (pH 8.8) separating gel by ATTO AE-6530m PAGE ($80\times 90\times 1\text{mm}$) unit. Unit was run at 100 V till proteins were in stacking gel and then at 150 V when protein samples were reached in separating gel. Silver stained gels³² were documented in Gel docTM XR+ system (Bio-Rad). The experiments were repeated thrice. Based on distinct protein bands appeared on 1D gel electrophoresis of three extraction method, two were analyzed further for 2-D gel electrophoresis.

Two dimensional electrophoresis (2-DE)

Two dimensional electrophoresis is a powerful technique for the separation of proteins from complex biological sources with high resolution and sensitivity¹². In first dimension, the proteins are separated according to isoelectric point by isoelectric focusing (IEF), and according to molecular weight by sodium dodecyl sulfate (SDS) electrophoresis in the second dimension. Protein was dissolved in rehydration buffer (9M Urea, 4% CHAPS, 20 mM DTT, 0.2% Biolytes) to obtain the final concentration of $0.15\ \mu\text{g}\ \mu\text{L}^{-1}$. A total of 200 μL of rehydration buffer containing protein was poured in rehydration tray. Immobilized pH gradient (IPG) strips (pH 3-10, Bio-Rad) of 11 cm were placed gel side down on rehydration buffer and kept for 14 h at 20°C . IEF was performed at 20°C with constant current of 50 μA /strip

in PROTEAN i12 IEF cell (Bio-Rad). Focusing protocol was set in four steps as (i) 20 min at 250V (rapid); (ii), 1h at 8000V (rapid); (iii) 26000Vh at 8000V (gradual); and (iv) 1500V hold. After focusing, strips were washed using equilibration buffer 1 (6M Urea, 2% SDS, 0.375M TrisHCl (pH 8.8), 20% glycerol, 2% (w/v) DTT) and equilibration buffer 2 (6M Urea, 2% SDS, 0.375 M TrisHCl (pH 8.8) and 20% glycerol) for 20 min each. Finally, strips were washed once in running buffer and mounted on 10% SDS-PAGE (18.5cm×20cm×1mm) for separation of protein spots, using vertical gel electrophoresis (Bio-Rad). Silver stained gels were documented using Gel doc™ XR+ system (Bio-Rad). Spots were analyzed using PDQuest™-Basic 2D analysis software (Bio-Rad).

Results

Maximum yields (mg g⁻¹FW) of total proteins from *Haloxylon recurvum* (5.37 ± 0.15) and *H. salicornicum* (5.04 ± 0.09) were obtained with phenol extraction method (p1) followed by TCA acetone (p2) and multi detergent (p3) extraction methods (Fig. 1). The protein pellet obtained from phenol extraction method was clean and easily soluble in IEF buffer. Slight browning was observed in the protein extracted by TCA acetone and Multi detergent methods.

One and two dimensional protein profiling

More distinct, clear and reproducible protein bands were observed with phenol extraction method (p1) followed by TCA acetone (p2) and multi detergent protocols (p3) in 1-DE gels (Fig. 2). Background smearing was observed with TCA acetone and multidetergent methods. Based on distinct protein bands appeared on 1-D gel electrophoresis of three extraction method, only phenol extraction method (p1) and TCA acetone (p2) were analyzed further for 2-D gel electrophoresis (Fig. 3). Protein spots of 124 to 10 kDa

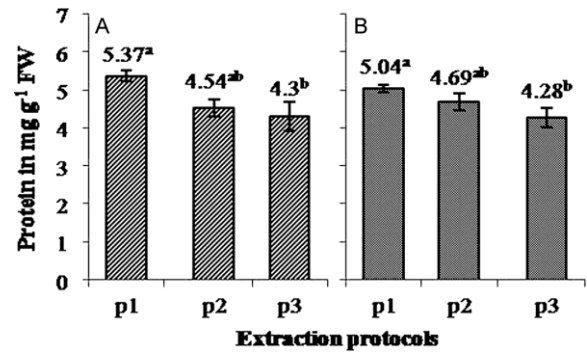


Fig.1 — Total soluble protein of (A) *Haloxylon recurvum*; and (B) *H. salicornicum*; extracted by Phenol (p1), TCA acetone (p2) and Multi detergent (p3) protocols. [The observed data were analyzed by one way-ANOVA and Duncan's multiple range test ($P < 0.05$) and each value represents an average number of three replicates using nine explants. Different letters represents statistically significant differences with standard deviation error bars]

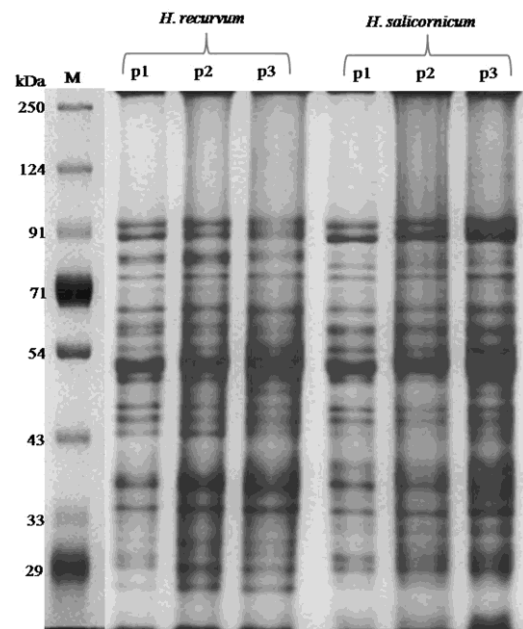


Fig. 2 — One dimensional (1-D) gel electrophoresis of total protein of *Haloxylon recurvum* and *H. salicornicum*; Phenol (p1), TCA acetone (p2) and Multi detergent (p3) protocols

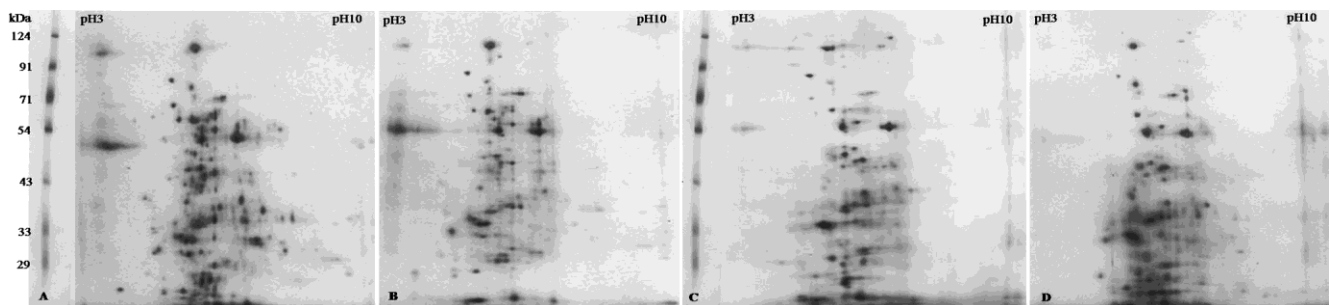


Fig. 3 — Two dimensional (2-D) gel electrophoresis of total protein of *H. recurvum* extracted by (A) Phenol extraction protocol; (B) TCA acetone protocol; and *H. salicornicum* extracted by (C) Phenol extraction protocol; and (D) TCA acetone protocol

Table 1 —Total number of protein spots detected from 2-D electrophoresis of *Haloxylon* species using Phenol and TCA acetone extraction protocols

Plant species	Protocol	No. of spots
<i>Haloxylon recurvum</i>	Phenol extraction	221
	TCA acetone	214
<i>Haloxylon salicornicum</i>	Phenol extraction	185
	TCA acetone	181

were found to be distributed mainly within 5-7 pH range in 2-DE. Higher numbers (221 and 185 spots) of well resolved spots were observed respectively from *H. recurvum* and *H. salicornicum* by Phenol method whereas 214 and 181 spots were resolved respectively from *H. recurvum* and *H. salicornicum* by TCA acetone method (Table 1).

Discussion

The plants of arid environment are heavily loaded with various metabolites and makes protein extraction difficult. Phenolic components present in these plants were found to be major hindrances for extraction of pure proteins amenable to 1-D and 2-D electrophoresis. Solubility, quantity and quality of extracted proteins are highly affected by non protein components of the plant system which may interfere during the process. The appropriate protein extraction method was evaluated on the basis of protein yield and quality of 1-D and 2-D profiling. The quantity of protein was found to be greater in Phenol method in comparison to TCA acetone and Multidetegent methods in the present investigation. High yield of clean pellet, dissolved readily in rehydration buffer, was obtained with phenol based extraction method in both the species of *Haloxylon* in the present investigation. Cilia *et al.*³⁰ also reported Phenol extraction protocol most suitable for extraction of cleanest and more soluble pellet. The higher number of clear and distinct protein bands were resolved from protein extracted from Phenol method than TCA acetone and Multidetegent methods. Though TCA is considered effective in precipitating proteins³³ and elimination of proteolytic and other modifying enzymes³⁴, it is effective only for some plant tissues and results in co- extraction of polymeric contaminants in complex tissues², which are difficult to remove with washing³⁵⁻³⁷. Browning of pellet due to polyphenols³⁸ was observed in protein pellets obtained with Multidetegent method in *H. recurvum* and *H. salicornicum*. Due to these contaminants, solubility of pellet was less and resulted in low protein yield in the above two methods. The low

quality 2-Dgels of proteins extracted from TCA acetone could be due to their inability to remove effectively the interfering compounds that precipitates during focusing and results in streaking¹⁴. Tris buffer in Phenol method contained protecting agents, EDTA that chelates metal ions inhibiting metalloproteases and polyphenol oxidases, PMSF inhibits serine proteases, β -ME prevents oxidation of protein and KCl which extracts protein by its salting in effect. Phenol of pH 8.0 is used to ensure nucleic acids partition to buffer phase. Addition of sucrose to Tris buffer lead to phase inversion (phenol phase on top), which facilitates its recovery³⁹. The involvement of all these factors helped in obtaining superior quality protein with the Phenol method. Though TCA acetone method is simpler than the Phenol method but it could not effectively remove contaminants. Phenol extraction protocol was also used for protein extraction from recalcitrant tissues or organs like wood⁴⁰, potato and rapeseed seedlings⁴¹, potato, apple and banana leaves¹⁴, Olive leaf⁴² and tomato, avocado and banana fruits¹⁵. Jellouli *et al.*⁴³ also reported Phenol method to be efficient in protein extraction from leaves and roots of *Vitis vinifera*.

Conclusion

On the basis of quantitative and qualitative results as revealed by 1-D and 2-D electrophoresis in present investigation, we can suggest that the Phenol extraction method is more appropriate for extraction of high amount and quality of protein from *Haloxylon recurvum* and *H. salicornicum* species. Maximum yields (mg g⁻¹FW) of total proteins from *H. recurvum* (5.37 \pm 0.15) and *H. salicornicum* (5.04 \pm 0.09) and higher number of protein spots in 2-DE were obtained with Phenol extraction method (p1) in present investigation. The Phenol extraction method can be applicable for proteome analysis of *Haloxylon* and other recalcitrant plant species of arid environment for prospects.

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Conflict of interest

Authors declare no conflict of interests.

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