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LEAF PROTEOME ANALYSIS OF *CLEMATIS CHINENSIS:* A TRADITIONAL CHINESE MEDICINE (TCM) BY TWO-DIMENSIONAL ELECTROPHORESIS TECHNIQUE

MUHAMMAD ISHTIAQ[1*], MEHWISH MAQBOOL[1], TANVEER HUSSAIN[1], SHEHZAD AZAM[1] AND YI WANG[2]

[1]*Department of Botany, Mirpur University of Science & Technology (MUST) Bhimber Campus, Bhimber (Azad Kashmir), Pakistan*
[2]*Institute of Bioinformatics, Department of Chinese Traditional Medicines, College of Pharmaceutical Sciences, Zhejiang, University, Hangzhou, P. R. China*
**Corresponding author: Tel. & Fax: +92582892035 E-mail: drishtiaqajk@gmail.com*

Abstract

Leaf proteome of *Clematis chinensis*, a traditional Chinese medicine (TCM) was analyzed by two-dimensional electrophoresis (2-DE) technique. The samples were extracted by phenol-SDS method (PSM) with high protein quantity *i.e.* 2.35±0.345 mg/g (yield/dw). Proteins were visualized by staining of gels by silver stain and CBB. The gel images of each species were compared by Image Master 2D Platinum software for analytical purpose. The 2-DE profile depicted distribution of 1085 spots and out of these only 255 protein spots (23.5%) were common to all analyzed taxa. The visualized protein spots showed p*I* range from 3.0 to 10.0 (pH) and M_r of 7 kDa to 70 kDa. Twelve proteins were exclusively specific to *C. chinensis* when compared with its allies, *C. finetiana* and *C. armandii*, which may be used as biomarkers. Thirteen proteins were up-regulated in *C. finetiana* (0.75-0.95 fold) and twelve proteins in *C. armandii* (1.05-1.66 fold) whilst seven proteins down-regulated (0.66-0.94 fold) in former and three proteins (1.07-1.20 fold) in later one in comparison with *C. chinensis*. Twenty five differential and similar protein spots were picked and analyzed by LC-MS/MS technique. Identified proteins are related to energy metabolism (ATP synthesis), photosynthesis, environmental stimuli, regulating RNA metabolism, growth hormone regulators, evolutionary trends and gene expression. The efficiency and applicability of proteomic approach as biomarker for identification of *C. chinensis* is discussed in its quality control (QC) perspectives. Leaf proteins of *Clematis* plants are explored for the first time by 2-DE technique and debated for their metabolic role.

Introduction

Plants have paramount role in human life for food subsistence or medicinal values. *Clematis* is one of the major recognized genus of Ranunculaceae and it comprises of more than 300 species worldwide, including 147 (93 endemic) in China (Wang, 1999). In China, mostly traditional Chinese medicines (TCMs) comprise of herbs and their products. These TCMs have been used in many countries, having a long therapeutic history over thousands of years (Li *et al.,* 2003; Xu *et al.,* 1996). Even now, there is an increasing trend for the global use of botanical medicines. Usually, TCMs have a composition of a diversity of ingredients and their contents not only vary with cultivar but also due to growing conditions based on geographical origins, harvest time, processing methods and storage duration (Wang *et al.,* 2005). In some cases, poor identification of medicinal plants may lead to use wrong botanic materials in ethnomedicines (TCMs), causing loss of human lives (Zhu, 2002; Shinwari *et al*., 2013). For effective use of medicinal herbs (TCMs), proper authentication regarding their identification and habitat of origin is inevitable to maintain their quality standards for safe use.

The *Clematis* genus (Ranunculaceae) is medicinally very important because many of its plant species are used in different traditional herbal pharmacopeias (Wen *et al.,* 1993; Geng, 1995; Qiu & Zhang, 1999). In particular, *Clematis chinensis* is medicinal plant which has been used in many TCMs to treat many diseases. It has been used to cure biliary tract disorders (Geng, 1995), tumors (Qiu & Zhang, 1999), inflammations (Li *et al.,* 2003; Wang *et al.,* 1998), cardiovascular pains (Ho *et al.,* 1989) and hepatic problems (Chiu *et al.,* 1988). One group of its active bioconstituents (saponin) has been used as analgesic, diuretic, antitumor, anti-inflammatory and insecticidal agent for ages (Xu *et al.,* 1996).

The species *Clematis chinensis* belongs to subsection *Clematis* of the *Clematis* genus and morphologically it is very similar with its allied taxa, particularly with *C. finetinana* and *C. armandii*. It is acknowledged that plants of subsection *Clematis* and subsection *Rectae,* and subsection *Connatae* and subsection *Crispae* are morphologically so closely related to each other that it is difficult to ascertain its identification and systematic position (Wang, 1998; Shinwari, 1998; Ishtiaq *et al.,* 2010). There have been different attempts made by phenetic and chemical analysis based to solve this plethora (Tamura, 1967; Tobe 1974, Ishtiaq *et al.,* 2007b; 2010). Hoot (1995) conducted DNA based research for systematic analysis of *Clematis* genus but hitherto no proteomic attempt has been conducted in this context.

Two-dimensional electrophoresis has proven to be a powerful tool for analyzing complex mixtures of proteins (Farrell, 1975; Yousuf *et al.,* 2006). The resolving power of 2-DE as separation technology has found great utility in proteomics studies of plants (Canovas *et al.,* 2004). A comparative analysis of 2-DE images with mass spectrometric approaches provides quantitative informations concerning changes in protein expression levels that can be used to characterize genotypes, understand gene function and cell responses to environmental stimuli (Thiellement *et al.,* 1999; Vienne *et al.,* 2001; Thiellement *et al.,* 2002; Shah *et al*., 2011). Proteomic approach has been extensively used to establish genetic relationships between species (Bahrman

et al., 1988) and genera (Thiellement *et al.,* 1989) up to tribe level (Zivy *et al.,* 1995). It has also been used in taxonomic studies in order to differentiate closely related taxa (Ndimba *et al.,* 2003), varieties (Maria *et al.,* 2005; Rogers *et al.,* 1999; Lum *et al*., 2002) and populations of different geographical origins (Bahrman *et al.,* 1986).

The purpose of this research was multifarious: firstly to analyze leaf proteome of *Clematis chinensis* by 2-DE technique with its differential expression. Secondly to identify its expressed genome (proteins) as biomarkers for TCMs. Thirdly elaborate role and functions of identified proteins in conjunction with pharmaceutical (QC) importance and phylogenesis of *Clematis* plants.

Material and Methods

Plant material: Three plant species representing subsection *Clematis* (genus *Clematis*); *Clematis chinensis, C. finetiana* and *C. armandii* were collected from Tian Mu Shan Bio Sphere (TMSBR), Zhejiang Province China. Fresh leaves were collected from each species of same age and same direction for proteomic analysis. Only leaves without any stress symptoms were selected, washed with distilled water, blot dried and stored at –80ºC until extraction. Herbarium specimen of each species was prepared and placed in department of Chinese medicine and engineering, Zhejiang University, Hangzhou, P. R. China.

Chemicals and materials: Mineral oil, Bisacrylamide (bis), Tris (hydroxymethyl) aminomethane (Tris), Sodium dodecyl sulfate (SDS), Glycine, N,N,NU,NU-tetramethylethyldiamide (TEMED), Ammonium persulfate (APS), Glycerol, ultra pure Urea, protease inhibitor cocktail, 2-D cleanup kit, 2-D Quant Kit were purchased from Amersham Pharmacia Biotech. Acrylamide, Dithiothreitol (DTT), 3-3-1-propane-sulfonate (CHAPS), Coomassie G-250 (ultra pure grade) and Agarose were obtained from Shanghai Biotech. Iodoacetamide (IAA) was purchased from Fluka BioChemika. HPLC-grade acetonitrile was purchased from Merck, Germany. HPLC-grade trifluroacetic acid (TFA) was purchased from Tedia, USA. Formic acid (FA) was purchased from Acros Organics. All other solvents were of analytical grade. The protean IEF cell, Densitometer, versa Doc scanner, ImageMaster 2D Platinum software (Amersham Biosciences) as well as linear immobiline dry strips pH gradient 3-10 (24cm long) were obtained from Bio-Rad, (Hercules, USA); Bromophenol blue, carrier ampholytes, and agrose were purchased from Pharmacia Diagnostics (Uppsala, Sweden).

Protein extraction and sample preparation methods: The leaf proteome of *Clematis chinensis* and other related taxa were extracted according to protocols of Meyer *et al.,* (1998) and Wang *et al.,* (2003) with some modifications. The applied method is described briefly below sections.

Ph-SDS method

Preparation of dry tissue powder: Fresh leaves ca. 5.00g were cut into small pieces by clean scissors and ground in liquid N_2 in a pre-chilled mortar and pestle. The powdered tissue ca. 0.2–0.3 g was resuspended in 1.0–2.0 mL cold acetone in 1.5 or 2.0 mL microtubes. Then it was vortexed thoroughly for 1min and centrifuged at 10,000 rpm using Eppendorf (Centrifuge 5810 R) for 5 min (4ºC). The process was repeated twice. After the initial two washes, the pellet was transferred into a mortar and allowed to dry at room temperature (ca. 20 min). The dried powder was further ground to a finer powder by the aid of quartz sand and then transferred into new microtubes. The powder was sequentially rinsed with cold 10% TCA in acetone until the supernatant became colourless, then it was washed with cold aqueous 10% TCA twice, and finally with cold 80% acetone twice. Each time the pellet was resuspended completely by vortexing, and centrifuged. The final pellet was dried at room temperature and used for protein extraction, or stored at -80ºC for future use.

Protein extraction: Phenol extraction of proteins is based on the protocol described before (Wang *et al.,* 2003) with few modifications. Briefly 0.05–0.1 g of the dry powder of leaf tissue was resuspended in 0.7 mL phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) and 0.7 mL dense SDS buffer (30%sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol) in a 2.0 mL microtube. The mixture was vortexed thoroughly for 3 min and phenol phase was partitioned by centrifugation at 10,000 rpm for 10 min. The upper phase (phenol) was pipetted to fresh microtubes (0.2 mL for 1.5 mL tube, 0.4 mL for 2.0 mL tube). Extraction process was repeated and phenol fractions were mixed. To precipitate proteins, about 5 volumes of cold methanol plus 0.1 M ammonium acetate was added to phenol phase and mixture was stored at -20ºC for one hour. Precipitated proteins were recovered at 10,000 rpm for 10 min (4ºC), and washed with cold methanolic ammonium acetate and cold 80% acetone twice (each). The final pellet was dried and stored at -20ºC until use. Prior to 2-DE run, proteins were dissolved in 100 uL of lysis buffer containing 7 M urea, 2 M thiourea (w/v), 2% CHAPS, 1% Ampholytes pH 3-10 (v/v), (Biorad), 40mM Tris, 10mM Acrylamide.

Protein purification and quantification: In each case, obtained proteins were purified by 2-D clean-up Kit (Amersham Biosciences) and quantified by the Bio-Rad protein assay (Hercules, CA, USA) with bovine serum albumin as standard (Bradford, 1976). Prior to first run, protein extracts were supplemented with trace of bromophenol blue and resolved by two-dimensional electrophoresis.

Two-dimensional gel electrophoresis

IEF in IPG strips: The first dimension was performed on IPG-strips (24cm length, 0.5mm thickness) with non linear gradient from pH 3~10 (Amersham Biosciences) (Rightii, 1990). The rehydration solution contained 7 M urea, 2 M thiourea, 3% CHAPS, 1% ampholytes (pH3-10), 40mM Tris, 10mM acryl amide. Purified protein samples were dissolved in rehydration solution supplemented with 0.02% Bromophenol blue and DTT (2.8mg/ml) was added just prior to use. For analytical run

(to visualize common and differential proteins) 60µg and for preparative runs (to obtain spots for identification with LC-MS/MS) 200ug proteins of each sample were loaded onto dry IEF strips, using the overnight in-gel reswelling method (Berkelman & Stenstedt, 1998). The reswelled IPG strips were subjected to IEF at 20ºC with first rehydration step for 12 hours at 30 V, followed by focusing for 1 hour at 100 V, 1 hour at 200 V, 1 hour at 500 V, 1 hour at 1000 V, 30 min for voltage increasing to 8000 V, and remaining 8000V for 66 kVh on an IPGPhor (Amersham Biosciences).

SDS phase: Focused strips were equilibrated using a first incubation step in equilibration solution (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.8), containing 1% w/v DTT for 15 min, followed by a second incubation step in 2.5% w/v iodoacetamide in the same equilibration solution for 15 min as suggested by Roh (Roh *et al.,* 2004). Equilibrated strips were gently rinsed with SDS electrophoresis buffer and loaded on top of 12.5% w/v vertical SDS-polyacrylamide gels (26×20 cm), prepared using a Bio-Rad Mini Protean II system (Bio-Rad, Hercules, CA, USA), according to Laemmli (Laemmli, 1970). The second dimension separation was performed sequentially with a constant voltage of 5W/gel for 1h, followed by 20W/gel for 6h using the Ettan DALT II system (Amersham Biosciences). A molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences) was used as a molecular size marker on all gels.

Staining of gel images: All gels used for analytical purpose were fixed with solution containing 40% ethanol and 10% acetic acid for over night and stained with silver stain for spot visualization and matching (Shevchenko *et al.,* 1996; Heukeshoven & Denrick, 1985) while preparative gels were fixed with solution containing 40% ethanol and 10% acetic acid for 60 min, and stained with CBB R250 over night to determine their polypeptide sequences by LC-MS-MS approach (Neuhoff *et al.,* 1988).

Proteins gel images and statistical analysis: Sliver-stained gels were scanned using Powerlook 2100XL (Umax) scanner and gel images were analyzed using ImageMaster 2D Platinum software (Amersham Biosciences). In order to minimize the contribution of experimental variations, three separate gels were analyzed for each accession. Those spots displaying the same distribution patterns in three replicates were selected for further analysis. A standard gel was constructed with highest number of spots. All subsequent spot matching and analysis was performed by comparing all gel maps with the standard gel image by 2D Platinum software (Amersham Biosciences) and manually. Gel patterns from each independent analysis were matched together and the relative abundances (%V) of each spot in gel were compared, using student's *t*-test ($p<0.05$). The percent value (%V) represents the pixel density of each spot normalized for the total pixel density from all spots in the same gel. This internal calibration can make the data independent of gel variations, as recommended by software manufactures (Ndimba *et al.,* 2003). All those proteins which were up-regulated or down-regulated in the three samples were studied by statistical analysis (student's *t*-test) with the software. The 2-DE approach thus affords a unique opportunity to investigate the genetic determination of protein amount for several gene products at a time, whether their function is known or not. The M_rs of proteins was determined by co-electrophoresis of standard marker proteins on the gels (Sigma, St. Louis, Mo, USA). The P*I* of the proteins was calculated by migration of protein spots on 24cm IPG (pH 3-10, non-linear; Amersham Biosciences) strips.

In-gel digestion and extraction of peptides: For MS analysis, selected spots were harvested from CBB stained gels manually. The in-gel digestion of spots was performed according to the method by Jensen with some modifications (Jensen *et al.,* 1999). Briefly, protein spots were excised from the CBB-stained gels and were cut into small pieces. Extracted pieces were destained twice using 60µL 200 mMol/L NH_4HCO_3/ACN (50:50 v/v), shrunk by dehydration in 60µL ACN twice then completely dried at 37 ºC for about 20 min. Cystine bonds were reduced with DTT (10 mM in 50 mM ammonium bicarbonate, 56ºC, 60 min) and alkylated with iodacetamide (55 mM in 50 mM ammonium bicarbonate, room temperature, 45 min). The reagents were washed out with 50 mM ammonium bicarbonate and the gel pieces dried in a SpeedVac. The samples were then swollen in a digestion buffer containing 100 m mol/L NH_4HCO_3 and 12.5 ng/µL trypsin (sequencing grade, Roche Diagnostics, USA) at 37ºC for overnight. The supernatant was removed, and the peptides extracted with 5% FA in 50% ACN by sonication at room temperature. The extracts were combined and concentrated in presence of N_2 and, stored at 4ºC until analysis.

Protein identification using LC MS/MS: Mass spectrometric peptide separation and sequencing was performed on an Applied Biosystems QSTAR PULSAR^{TM} quadruple TOF mass spectrometer coupled with LC Packings Ultimate nano HPLC workstation (Amsterdam, The Netherlands). After digestion process, ca. 3.0uL of each concentrated peptide digest was loaded onto an LC Packings C_{18}, 5um, PepMap^{TM} nano-precolumn, washed free of salts with 450 uL of 0.5% (v/v) ACN, 0.05% (v/v) TFA and eluted with a 6 mL linear gradient of 5% (v/v) ACN, 0.05% (v/v) TFA to 65% (v/v) ACN, 0.05% (v/v) TFA through a filter splitter onto an LC Packings C_{18}, 3 um, PepMap^{TM} nano-column for direct infusion at 200 $nLmin^{-1}$ through a nano-spray tip into the mass spectrometer. TOF-MS spectra were collected between the mass range of 100-2000amu throughout the gradient elution and precursor ion selection and product ion spectra were generated using Applied Biosystems BioAnalyst software's fully automated switching and acquisition procedures. The spectra were internally calibrated using two trypsin autolysis peaks at m/z 842.510 and 2211.105. Only multiply charged precursor ions species were selected for fragmentation and peptide sequencing. For protein identification all MS/MS spectra product ion spectra generated from each sample were used in MASCO

(http://www.matrixscience.com) database search of NCBInr database of all Viridiplantae sequences available. For protein identification these parameters were considered: maximum of one missed cleavage peptide was allowed, a mass tolerance of 0.3 Da, and MS/MS tolerance of 0.4 Da were used, and variable modifications such as ribulose biphasphate and other proteins were taken into account. Tryptic autolytic fragments and contaminations were removed from the data set used for database search. The *M*r of each protein computed by the p*I*/*M*r tool with (http://www.matrixscience.com) was compared to the *M*r calculated in the 2-D gel, contributing additional proof of identity of the analyzed protein spot.

Results

Extraction of proteins: The proteins were extracted by phenol SDS method (PSM) according to Wang *et al.,* (2003) with some modifications and 1.0 g of FW leaf of *Clematis* produced 0.2~3.0 g of dry powder, and finally 2.35±00.50 mg proteins were obtained. The extraction was performed on same day and alternative day (s) to visualize experimental variance which produced stabilized readings in the process.

Two DE analyses: The obtained gels from analyses revealed that 1085 (±10) spots by PSM in triplicates. The gel images were with fair background transparency and having maximum number of spots with sharp boundaries. The images demonstrated good repeatability of analytical runs (Fig. 1). A broad distribution range of protein spots was observed with p*I* from 3.0 to 10.0 and a mass (M_r) from 7 kDa to 70 kDa. The three gel pictures depict differential distribution patterns of spots in analyzed samples (Fig. 2). When making comparison among three accessions, it was seen that 13 proteins (1.05-1.66 fold) in *C. finetiana* and 12 proteins (0.75-0.95 fold) in *C. armandii* were up-regulated and 7 proteins (0.66-0.94 fold) in former and 3 proteins (1.07-1.20 fold) in later were down-regulated whilst two proteins were unchanged. The differential profile showed that four proteins were absent in *C. finetiana* gel and eight proteins were absent from *C. armandii*, by comparing with *C. chinensis* individually (Table 1, Fig. 3).

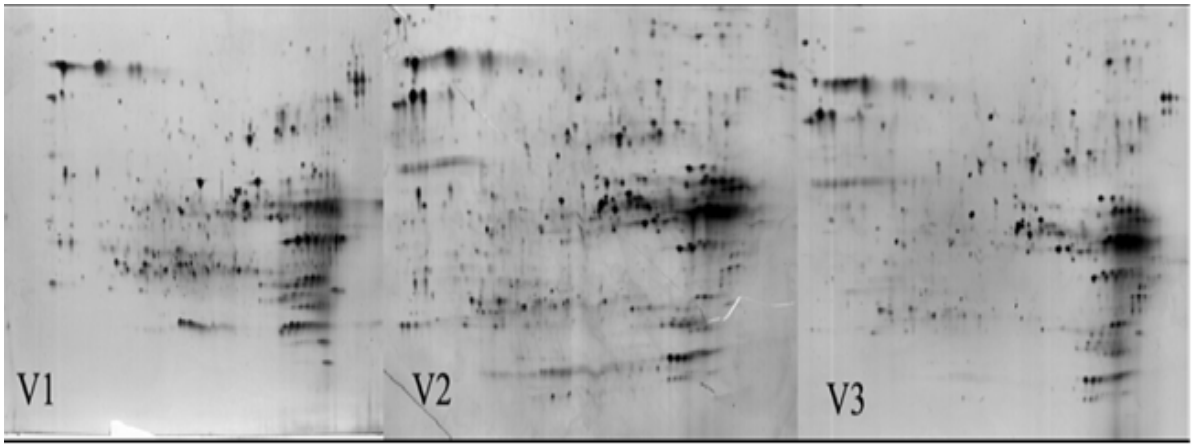

Fig. 1. Proteins extracted from one accession and analyzed by 2-DE, showing repeatability of the experiment and gels were stained with CBB stain.

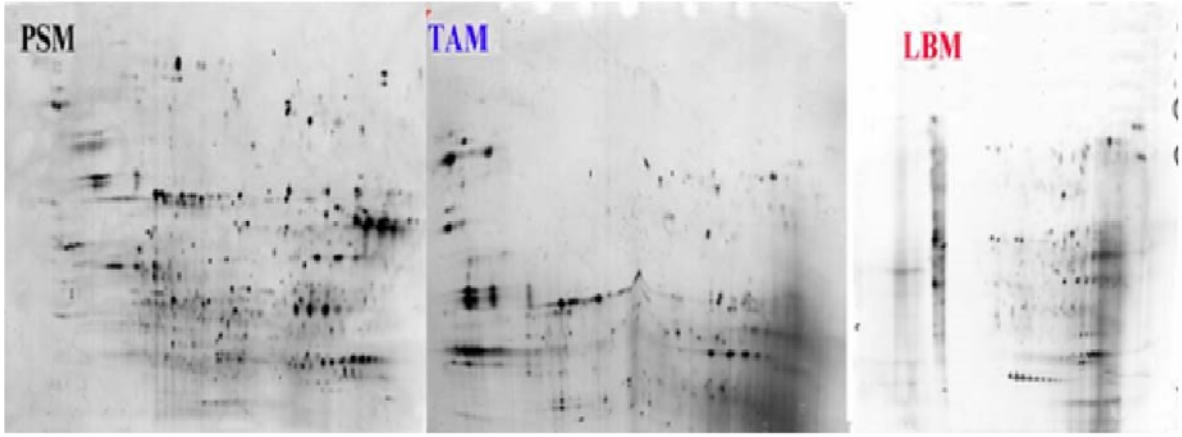

Fig. 2. Three maps of Clematis chinensis obtained by three different protocols; PSM: phenol-SDS method, TAM: TCA acetone method, LBM: Lysis buffer method. The gels were stained with silver stain.

Table 1. Leaf proteins of *Clematis chinensis* identified by LC MS/MS resolved by 2-DE technique.

Spot No.	Variation (Clematis chinensis vs C. finetiana)	Variation (C. chinensis vs. C. armandii)	Experimental pI/ Theoretical pI	Experimental M_r (kDa)/ Theoretic-al M_r (kDa)	Protein identified	Protein source	NCBI Accession number	Sequences	Score	Cover % age
1	↑ (1.14)	↓ (1.07)	5.46/6.81	24.52/15.40	Nucleoside diphosphate kinase	*Lycopersicum esculentum*	gi|14336662	GLVGEIISR GDFAIDIGR GLVGEIISRFEK GLVGEIISRFEK IKPDGVQRGLVGEIISR	79	99.7
2	↓ (0.93)	⊗	6.21/5.44	60.92/25.16	Unknown protein	*Hordeum vulgare*	gi|18652410	LWGKSALDR SGITFATVDIR SGITFATVDIR	46	na
3	↑ (1.36)	↑ (0.84)	5.92/5.87	21.50/22.19	Copper/Zinc superoxide dismutase precursor	*Solidago Canadensis var. scabra*	gi|1944326	GGHELSLSTGNAGK AFVVHELADDLGK AFVVHELADDLGKGGHE LSLSTGNAGGR AFVVHELADDLGKGGHE LSLSTGNAGGR	110	100
4	↓ (0.83)	⊗	4.78/5.25	53.70/16.94	Pathogenesis related protein	*Sorghum bicolor*	gi|58978027	LLPGVDEKDEEVK LLPGVDEKDEEVK EAVTAIFKGAEAYLVANP	43	na
5	↑ (1.05)	↑ (0.89)	5.75/10.82	15.01/15.26	Hypothetical protein	*Oryza sativa*	gi|34901630	SSFQAEVAVLLRR RHEVALVVIVLLDK ANDFPSLAVVPAAARLRP	54	5.85
6	↑ (1.16)	⊗	5.58/6.06	15.20/10.26	Lipoxygenase	*Oryza sativa*	gi|563985	GDSSSDMAAAAAR GDSSSDMAAAAAR GMAEEDPTAEQGK	45	na
7	↓ (0.66)	⊗	5.90/5.15	54.52/55.47	ATP synthase CF1 alpha subunit	*Eucalyptus globules*	gi|60460794	IAQIPVSEAYLGR IAQIPVSEAYLGR EAYPGDVFYLHSR EAYPGDVFYLHSR	332	100
8	↑ (1.05)	↔	8.20/9.77	20.80/7.70	Integrase catalytic region	*Nicotiana tabacum*	gi|87240570	DAALSLDK YGYCKHNPR LRECYVEIIMAMR	38	na
9	↑ (1.66)	↑ (0.75)	6.465/6.40	18.00/11.17	Hypothetical protein	*Arabidopsis thaliana*	gi|12322601	NVLQRIK RGMELGFLGK RGMELGFLGK	62	85.14
10	⊗	⊗	6.18/9.53	22.76/23.8	33 kDa precursor protein oxygen evolving complex	*Lycopersicum esculentum*	gi|19157	RLTYDEIQSK RLTYDEIQSK GGSTGYDNAVALPAGGR DVKIQGIWYAQLE DGIDYAAVTVQLPGGER	61	80.77
11	↔	↔	8.00/10.08	39.45/29.66	Putative maturase	*Aeterella bolanderi*	gi|14336662	YGSNLLTNSINFK YGSNLLTNSINFK FISLNFWQINPYK	56	37.79
12	⊗	⊗	5.25/5.33	55.24/55.43	ATP synthase alpha chain	*Nuphar advena*	gi|69214350	QAQAYR LRELLK FLVQLR ELIIGDR IEQYNR AMKQVAGK QMSLLLR ATQNQLAR KFLVQLR ERUEQYNR	342	100

Table 1. (Cont'd.).

Spot No.	Variation (Clematis chinensis vs C. finetiana)	Variation (C. chinensis vs. C. armandii)	Experimental pI/ Theoretical pI	Experimental M_r (kDa)/ Theoretic-al M_r (kDa)	Protein identified	Protein source	NCBI Accession number	Sequences	Score	Cover % age
13	↑ (1.09)	↑ (0.84)	5.05/5.17	39.10/20.81	Chlorophyll a & b binding protein type III	*Alonsoa meridionalis*	gi|7271947	QYFLGLEK QYFLGLEK	46	na
14	↓ (0.85)	↑ (0.87)	6.00/10.02	68.25/35.51	Hypothetical protein	*Oryza sativa*	gi|50934053	ERALVTVDPPVPK ERALVTVDPPVPK LALLAAALMKLFR	55	28.58
15	↓ (0.93)	↑ (0.91)	5.66/5.09	62.20/52.73	ATP synthase beta subunit	*Alloxylon wickhamnii*	gi|3850976	IGLFGGAGVGK VVDLLAPYR SAPAFIQLDTK AMNLEVESNLK	331	100
16	↑ (1.12)	↑ (0.88)	6.92/4.97	20.30/20.12	Unknown protein	*Arabidopsis thaliana*	gi|15236014	LRDSVSR GNLDIFSGR GNLDIFSGR	59	72.21
17	⊗	↑ (0.86)	6.36/6.48	34.50/35.11	Oxygen evolving enhancer protein1	*Bruguiera gymnorrhiza*	gi|9229957	NTSSSTGKITLGVTK NTSSSTGKITLGVTK GGSTGYXNAVALPAGGR HNVVFDEDEIPS	50	na
18	↓ (0.94)	⊗	4.00/4.25	22.70/10.48	Chain A Plastocyanin double mutant G8dL 12 E	*Spinacia oleracea*	gi|48015034	GVDAAK NNAGFPHNVVFDEDEIPS GVDAAK NNAGFPHNVVFDEDEIPS GVDAAK	58	68.1
19	↑ (1.33)	↓ (1.20)	7.93/7.05	30.60/24.77	Chalcone isomerase	*Pisum sativum*	gi|432404	ADKSLATK IKELSGPEYSR MCCSILHHRNPR	42	na
20	↑ (1.08)	↑ (0.85)	6.75/10.08	43.50/29.66	Putative maturase	*Aeterella Bolanderi*	gi|14336662	KGLYQLQYIFK YGSNLLTNSINFK YGSNLLTNSINFK	49	na
21	⊗	⊗	9.00/9.60	50.80/58.20	Integrase catalytic region	*Medicago truncatula*	gi|87240570	ELYGLFR NGKDSIFVVVDR EFVIHSDHESLK	38	na
22	↑ (1.18)	↑ (0.91)	5.66/9.77	17.6/7.70	Hypothetical protein	*Oryza sativa*	gi|50906515	MELGINK FHHYIGSAVWR FHHYIGSAVWRRPSGDL	46	na
23	↓ (0.92)	↓ (1.18)	4.65/8.75	37.5/42.74	Putative immediate early fungal elicitor protein CMPG1	*Oryza sativa*	gi|56785190	AELVAHAAGVAV GKK VWRASPCLSPSFLALYPS VWRASPCLSPSFLALYPS	44	na
24	↑ (1.10)	↑ (0.95)	5.5/9.53	65.8/23.82	Heat shock protein, binding/unfolding protein	*Arabidopsis thaliana*	gi|15240324	MASAAAEVPSYFVCPISL QLNPTIYTS KLSENDYNGAK LSENDYNGAKK	45	na
25	↑ (1.11)	↑ (0.81)	6.4/6.40	58.20/11.17	Caffeoyl Co-enzyme A trunk 2	*Nicotiana tabacum*	gi|3089564	YNLNGAEGAFKPVTEAW EPEPMK ATNGENGR HQEVGHK	34	na

Key: Column 1: Spot numbers from the analysis shown in Fig.2; Column 2: indicates whether protein is increased (↑), decreased (↓), remained unchanged (↔) and exclusively present in *Clematis chinensis* (⊗), digits in parenthesis (show fold change); Column 3: p*I* of polypeptides estimated from the gel according to (Rogers et al.,1999) 5: theoretical p*I* of the MS/MS analysed proteins; Column 6: M_r of the polypeptides estimated from the gel; Column 7:M_r of the MS/MS analysed proteins; Column 8: identified proteins; Column 9: protein source; Column 10: NCBI accession number; Column 11: amino acid sequences of fragments; Column 12: protein score C.I. %, na: not available

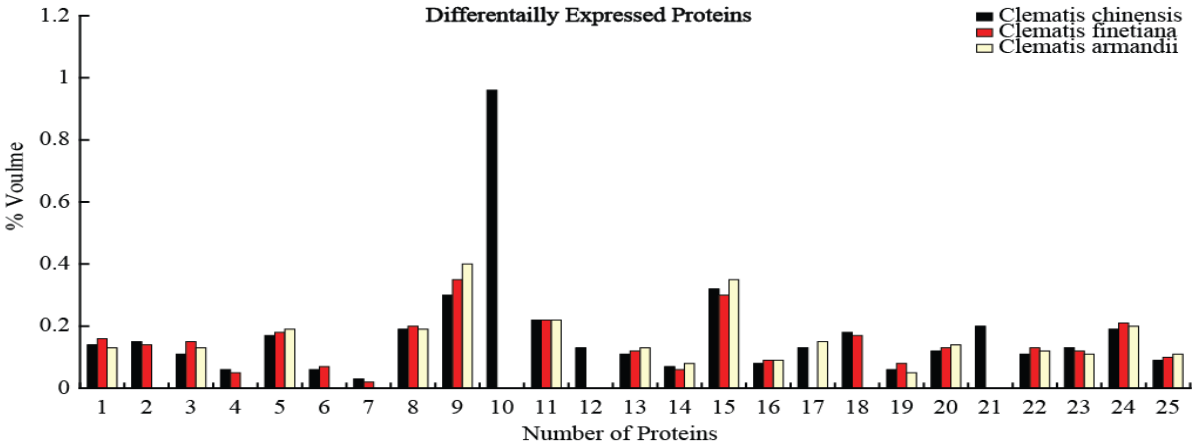

Fig. 3. Summary of differentially expressed proteins in three species, with *Clematis chinensis* as standard reference containing all marked proteins.

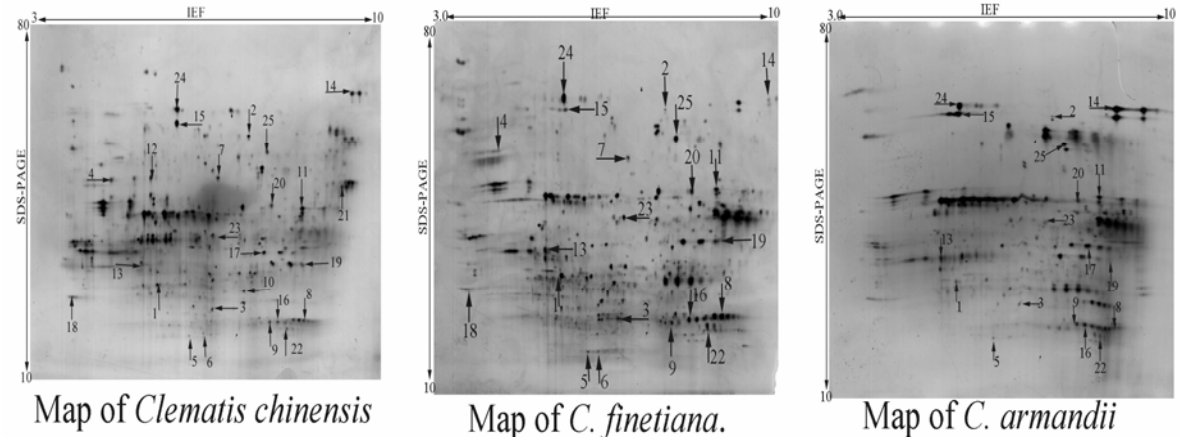

Fig. 4. Representative maps of *Clematis chinensis*, *C. finetiana* and *C. armandii*. Proteins were resolved using a non linear gradient pH 3-10 in first dimension and 12% SDS-PAGE in the second dimension. Marked spots show differential changes in three species.

Protein identification by LC MS/MS: Some differential and similar (marked) spots from all the accessions were subjected to LC/MS/MS analysis for protein identification (Figs. 4, 5, 6). The number of peptide sequences varied from 3-10 *per* spot and the number of amino acids *per* peptide ranged from 5–23. The results showed that it was problematic to identify certain spots that because they were identified as two different proteins that might be due to localization of different spots I on same p*I* and same M_r (3 spots). Secondly some spots possessed markable difference was observed between theoretical and experimental M_r in 2-DE gel images (8 spots). The results of protein analysis depicted that spot no.1 was differential protein with p*I* 5.46 and molecular wt 24.52 and identified as "nucleoside diphosphate kinase". The gel point no 3 with p*I* 5.92 and molecular wt. 21.50 was recognized as "Copper/Zinc-superoxide dismutase precursor" protein. The gel point no 6 with p*I* 5.20 and molecular wt. 15.58 was confirmed as "lipoxygenase" protein, differentially present in between *C. chinensis* and *C. finetiana* and proving to be biomarker. The image point no 7 with p*I* 5.90 and molecular wt. 55.47 was matched as "ATP synthase CF1 alpha subunit" with protein score (C.I.%) 100 and it was exclusively absent from *C. armandii* (Table 1). The analysis of gel spot no 8 depicted it as 23.8 kDa "precursor protein oxygen evolving complex" protein with credibility (C.I.%) of 80.77 with consistency in all samples (Figs. 5, 6; Table 1). The spot no 12 was recognized as "ATP synthase alpha chain" bearing p*I* 5.25 and molecular wt. 55.43 which was exclusively present in *C. chinensis*. Another protein was identified as "Chlorophyll a & b binding protein type III" with spot location 13 and it had p*I* 5.05 and molecular wt.20.81 and it is up-regulated in both allied species (Fig. 5, 6; Table 1). The protein spot no 25 was found to be "Caffeoyl Co-enzyme A trunk 2" with p*I* 6.04 and molecular wt.11.17 which showed up-regulatory trend (Figs. 5, 6; Table 1). Among other identified proteins were "ATP synthase alpha subunit" (spot 12), "heat shock protein, binding/unfolding protein" (spot 24), "Putative immediate early fungal elicitor protein CMPG1" (spot 23), "hypothetical protein" (spots 9, 14, 22), "ATP synthase beta subunit" (spot 15), "plastocyanin double mutant G8dL" (spot 18), "33 kDa precursor protein oxygen evolving complex" spot (10) that is exclusive representative in *C. chinensis*, "putative maturase" (spot 11) and one "unknown protein" (spot 16). As a demonstration of LC MS/MS analysis, the mass spectrum of "ATP synthase alpha chain" (spot 12) protein is shown (Table 2, Fig. 7).

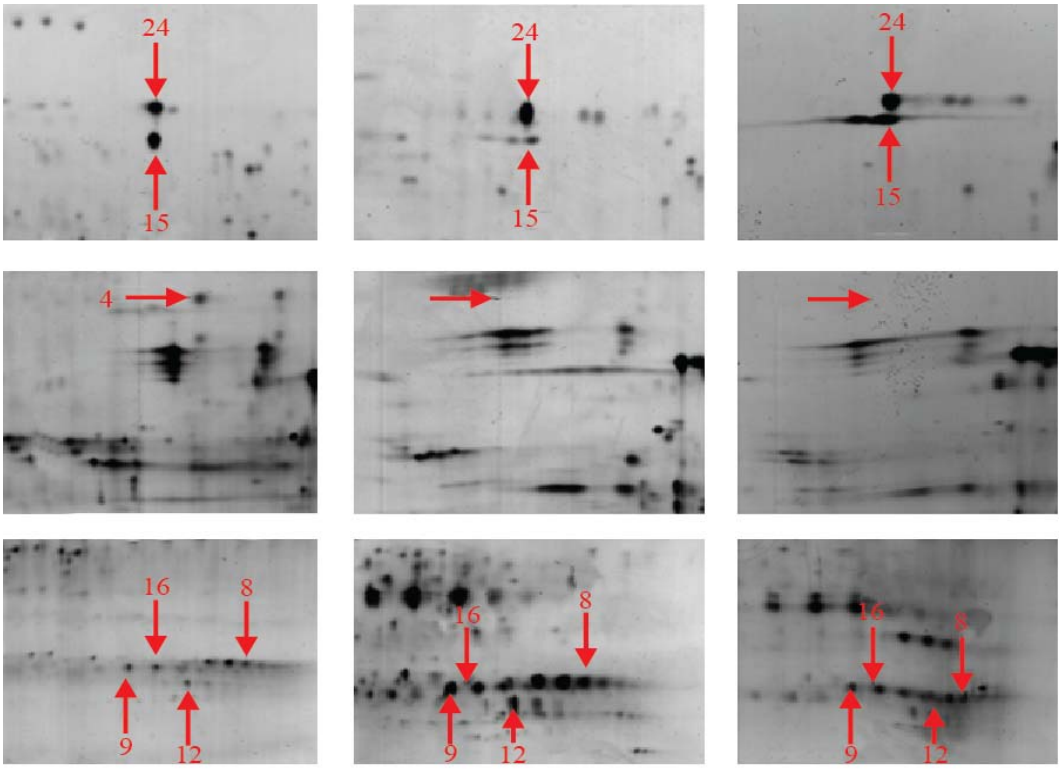

Fig. 5. Enlargement of some regions of D, N, Q gels to highlight some of the differentially expressed protein spots. Arrows in each region point to proteins that were regulated and numbered points indicate proteins identified.

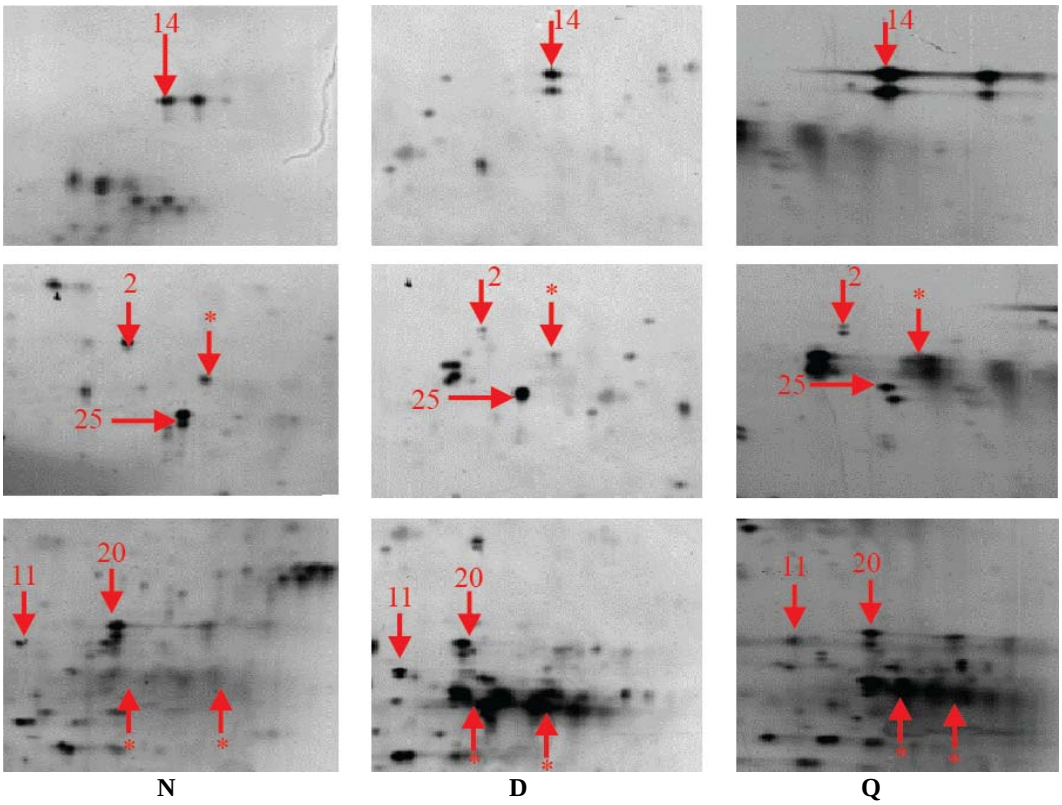

Fig. 6. Enlargement of some regions of D, N, Q gels to highlight some of the differentially expressed protein spots. Arrows in each region point to proteins that were regulated and numbered points indicate proteins identified.

Table 2. Protein function and metabolic pathways of 25 spots analyzed by LC MS/MS.

Spot No.	Protein name	Function/ metabolism
1	Nucleoside diphosphate kinase	Respiration, Calvin cycle functions and heat stress (Escobar *et al.*, 2001)
3	Copper/Zinc superoxide dismutase precursor	Photosynthesis and cytochrom b6f to photosystemI (Toshiharu *et al.,* 2003)
4	Pathogenesis related protein	Stress related functions (Benoît *et al.*, 2006)
5 9 14, 22	Hypothetical protein	Regulation of growth hormone (Setsuko *et al.,* 1999) Regulation of microRNA (Rujin *et al.,* 1998) Mitochondrial functions (Notsu *et al.,* 2002)
6	Lipoxygenase	Membrane structure (Benoît, *et al.*,2006)
7	ATP synthase CF1 alpha subunit	Regulates ATP biosynthesis and respiration mechanism (Paul, 1997)
8, 21	Integrase catalytic region	Respiration (Alexandra *et al.,* 2006)
10	33 kDa precursor protein oxygen evolving complex	Photosynthesis system II, (Jansson *et al.,* 2003)
11 20	Putative maturase	Regulation of gene expression of organelle (George & Alan, 2003)
12	ATP synthase alpha chain	ATP biosynthesis and energy metabolism (Jean *et al.,* 2005)
13	Chlorophyll a & b binding protein type III	Photosynthesis (Miguel *et al.,* 2006)
15	ATP synthase beta subunit	ATP biosynthesis and miscellaneous functions (Masasuke *et al.,* 2001)
2, 16	Unknown protein	Chloroplast functions and photorespiration process (Hansson & Vener, 2003)
17	Oxygen evolving enhancer protein1	Photosynthesis (Bahrman *et al.,* 2004)
18	Chain A Plastocyanin double mutant G8dL 12 E	Photosynthesis and ETC system (Jansson *et al.,* 2003)
19	Chalcone isomerase	Flavonoid biosynthesis (Yuan *et al.,* 2006)
23	Putative immediate early fungal elicitor protein CMPG1	Flavonoid biosynthesis (Miguel *et al.,* 2006)
24	Heat shock protein, binding/unfolding protein	Chaperone protein DNAK (Bahrman *et al.,* 2004)
25	Caffeoyl Co-enzyme A trunk 2	Evolutionary trend/Phylogenesis (Hossein *et al.,* 2006)

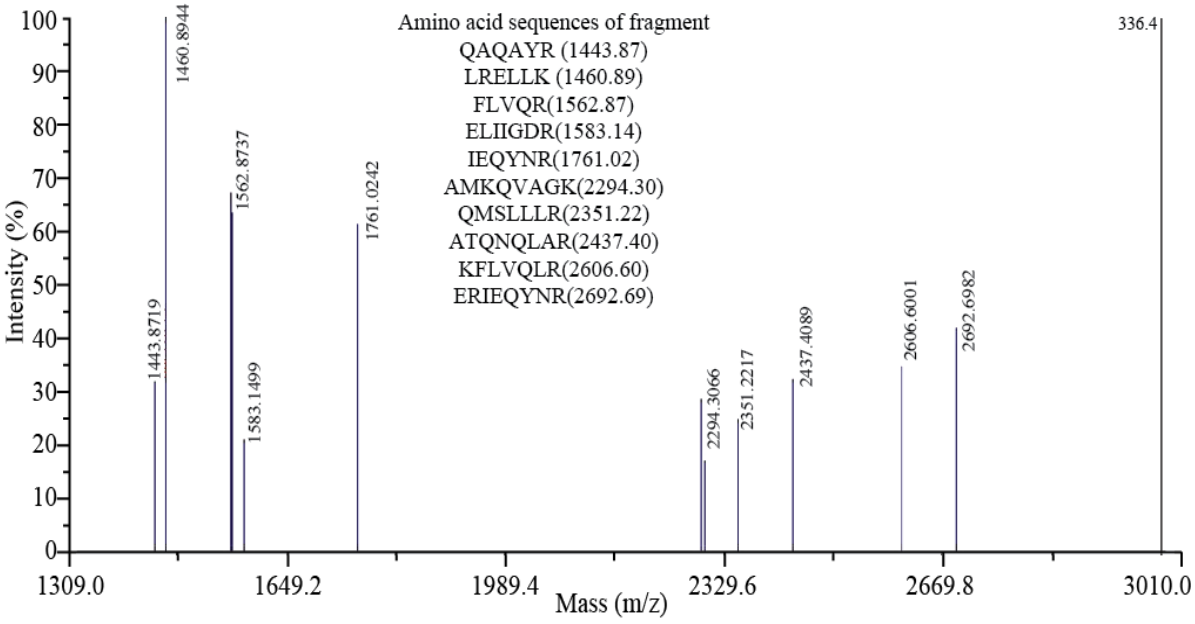

Fig. 7. LC-MS/MS spectra of ATP synthase alpha chain protein isolated from 2-D gel. Out of 14 representative spectra, ten peptides were matched and listed.

Discussion

The leaf proteome of *Clematis chinensis* plant, an important constituent of many traditional Chinese medicines (TCMs) was explored by 2-DE approach to determine differential protein fingerprints for its identification and demarcation. The proteins are called expressed genome of a species and they are proved as good tool for identification and delimitation of any species (Gerber *et al.,* 1997; Susanne *et al.,* 2001).

2-DE approach is very intricate and noxious in its repeatability at different processing steps due to biological and analytical variance. Protein extraction is very important preliminary step for subsequent good results, and different protocols have been optimized for different types of tissues and plants. Protein extraction from plant tissue is often complicatedly due to nonprotein contaminants indigenous to the plant, such as organic acids, lipids, polyphones, pigments, terpenes, etc (Granier, 1988). In conventional methods these contaminants are co-extracted with proteins (Görg *et al.,* 2000; Dennis *et al.,* 2001) and usually these contaminants are more abundant in green tissues than in young seedlings and etiolated material (Granier, 1988). In this work, proteins extracted by Phenol SDS method depicted good gel resolution and with no streaking, even basic polypeptides appeared as round shaped spots up to pH 10 and similar results have been given by Rujin *et al.,* (1998). The results of phenol SDS protein extraction method were well reproducible with high spots density. It may be due to that proteins are well dissolved in phenol phase and separated as upper layer leaving below contaminants in aqueous phase and, phenol also prevents protein degradation pf proteins due to endogenous proteolytic process and similar recommendations for green leaf protein extraction had been presented by different researchers in past (Wang *et al.,* 2003; Meyer *et al.,* 2003).

The protein gel images comparative analysis demonstrated 25 spots present/absent and 450 spots common in three species: *C. chinensis, C. finetiana* and *C. armandii*. The p*I*s of most selected spots ranged from 3-10 and molecular weight varied from 7-70 kDa. It was found that there was good agreement between experimental and theoretical p*I*s and M_rs for most of the spots which indicated that use of 24cm IPG strips was appropriate for this analysis. However, some spots showed minor differences in p*I*s and M_rs in few cases. This may be due to multifarious reasons; including PTMs of the proteins or incomplete binding and denaturation of SDS during equilibration procedure. It is also possible that the homologous identified from the database represented different protein isoforms with alternative charge and size of proteins (Valeria *et al.,* 2005).

In this systematic analysis of three accessions, common and differential spots were considered for quantitative and qualitative variations which may be used as biomarkers. The qualitative differential profile showed that four proteins were absent in *C. finetiana* and eight proteins were absent from *C. armandii*, by comparing with *C. chinensis* as standard (Table 1) and which could serve as taxonomic fingerprints which corroborates the previous findings (Procida *et al.,* 2003). There was quantitative variation observed with trend of up-regulation and down-regulation in the analyzed taxa. This variation may be due species specific or environmental parameters as these plants are habituated at variable altitudes with dynamic light and moisture availability too (Marques *et al.,* 2001).

As it was a preliminary approach for leaf proteome analysis of *C. chinensis*, so only some selected (25) spots were processed for identification by LC MS/MS technique. Among twenty five analyzed polypeptides, twelve were credibly identified with high C.I.% score and while other showed low score that may be attributed to lack of genome sequencing and unavailability of protein database on *Clematis* genus. Some multiple spots represented similar proteins e.g. spots 5 and 9 which correspond to hypothetical protein. The reason for multiple protein spots might be due to similar gene composition and/or biochemical modifications of translated proteins (Bahrman *et al.*, 2004). Many proteins depicted PTMs which is very common in protein chemistry for performance of functions. Similarly, some proteins (spots 7, 12 and 15) showed oilgomeric structure and/ or composed of different subunits such as ATP synthase with its alpha and beta subunits. Among identified proteins 3 were related to energy metabolism, three were hypothetical and total ten classes of proteins were present. Mostly these proteins were identified by comparing with poaceae, solanaceae and brassicaceae plants' sequences and some scored in other plant families.

Out of the identified proteins, three are related to energy metabolism that assist the plants to acclimatize with dynamic environment. The protein (spot 12) "ATP synthase CF1 alpha subunit" regulates ATP biosynthesis and respiration mechanism which has been confirmed in previous studies too (Paul, 1997). Similarly spot 12 was "ATP synthase alpha subunit" and spot 15 was identified as "ATP synthase beta subunit" are also concerned with energy metabolism and other miscellaneous functions, respectively (Jean *et al.,* 2005; Masasuke *et al.,* 2001). As these *Clematis* plants are liana in habit and they climb around different trees facing light fluctuations and same phenomenon has been ascribed with photosynthetic regulating proteins in plants present in different habitats to cope with light and humidity variability (Montgomery, 2004).

Three polypeptides are identified to be hypothetical proteins concerned with different functions (spots 5, 9, 14) probably encoded by similar gene (Bahrman *et al.,* 2004). One of these proteins (spot 5) is probably concerned with regulation of growth hormone (gibberellin) (Setsuko *et al.,* 1999). Second hypothetical protein (spot 9) is concerned with photo-control of circadian period and regulation of microRNA metabolism (Rujin *et al.,* 1998). The other one (spot 14) is related with regulation of mitochondrial functions (Notsu *et al.,* 2002). Another spot (18) was identified as "chain A plastocyanin double mutant G8dL" related to photosynthesis and ETC system (Jansson *et al.,* 2003). One unknown protein (spot 16) is concerned with chloroplast functions and photorespiration process (Hansson and Vener, 2003) and spot 10 was declared "33 kDa oxygen evolving precursor protein complex" related to photosynthesis system II (Jansson *et al.,* 2003). These

proteins are of great importance for plant survival in fluctuating environment by producing carbohydrates for growth. Spot 17 was "oxygen evolving enhancer protein1", spot 13 identified as "chlorophyll a & b binding protein type III". Protein nucleoside diphosphate kinase (spot 1) is found in inter-membrane space of mitochondria related to respiration, Calvin cycle functions and it is also considered to be involved in heat stress response in pea, possibly as a modulator of the 86-kD protein (Escobar *et al.*, 2001). Another, protein (spot 3) is identified as copper-zinc superoxide dismutase precursor, which is involved in ETC in photosynthesis from cytochrom b6f to photosystemI (Toshiharu *et al.,* 2003). One enzyme, putative maturase (spot 11) is identified and it is probably concerned with regulation of gene expression of organelle in response to the cellular energy state or environmental stimuli (Table 2) (George & Alan, 2003).

From phylogenetic point view, taxonomic delimitations can be assigned between species of *Clematis chinensis*, *C. finetiana* and *C. armandii* by differential expressed proteins by application of two-dimensional electrophoresis approach. There are nine differentially expressed proteins (Table 1) which can be used to characterize the species (*Clematis chinensis*) from its closest taxa (*C. finetiana* and *C. armandii*). These proteins can be good biomarkers at infra-specific level for proper identification of herbs *viz* a *viz* quality control of TCMs. Moreover, this technique can also be applied for solving phylogenetic issues of different plants as well as for authentication of the other medicinal species being used in TCMs (Susanne *et al.,* 2001). Further detailed analysis of more taxa of *Clematis* genus will unravel minor protein expressions which can be taxonomic and physiological markers for their genotype and may explore mysteries of developmental stages or changes in response to environmental stresses of the plants. In this regards, proteomic approach can be a valuable tool to assist in quality control of TCMs and solving complex phylogenetic and biosystematic status of many other plant taxa.

Conclusion

Leaf proteome of *Clematis chinensis* is first analyzed by by 2-DE technique using phenol-SDS extraction method and LC-MS/MS for protein identification. Up to now no protein analysis has been conducted on *Clematis chinensis*. The aim of this work was to determine leaf proteins to facilitate protein expression analysis at inra-species level to distinguish *Clematis chinensis* (tie xie lian) from allies species. 2-DE approach in view of extending to quality control of TCMs, it can be fruitful in proper identification of herbal species, finding their geographical origin and it may help to prevent adulteration of similar species or samples of different geographical origins. Proteomics has become a global approach to conduct research at molecular level for expression studies in last two decades. Application of 2-DE technique to detect, identify and quantify different proteins in various tissues, organelle and species level may constitute an essential step in gathering genomic knowledge for identification and authentication of any plant or its herbal product (Bahrman *et al.,* 2004). The data presented in this research article will constitute a milestone in proteomic characterization of *Clematis* plants and will be aiding step to understand completed gnomic expression. In future, genetic studies could be then be carried out to access gene expression in metabolic pathways to control the quality of herbal medicines which are solely used as medicures and/or being prerequisite raw products of allopathic medicines. Furthermore it will help in explaining of complex plant characters and their genotype-phenotype variation in different climates.

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