A proteomics approach to identify the differential protein level in cardiac muscle of diabetic rat

Dhanaraj Karthik1, Ravichandran Vijayakumar2, Kalalingam Pazhanichamy3 and Sivanesan Ravikumar1

1Department of Biotechnology, PRIST University, Vallam, Thanjavur, Tamilnadu, India; 2Aquatic Animal Health and Environment Division, Central Institute of Brackishwater Aquaculture-CIBA (ICAR), Chennai, Tamilnadu, India; 3Department of Biotechnology, Periyar Maniammai University, Vallam, Thanjavur, Tamilnadu, India

Background: Cardiovascular proteomics investigation reveals the characterization and elucidation of the novel therapeutic targets and strategies to prevent the development of heart failure associated diabetic complications by using 2DE and MS. Methods: The experimental animals were made diabetic with a single intraperitoneal injection of alloxan (150 mg/kg of bw). Albino rats were randomly divided into four individual groups: Group-I control (n=6), group-II alloxan-induced diabetic rats, untreated (n=6), group-III (n=6) and group-IV (n=6) alloxan-induced diabetic rats were treated with aqueous and ethanolic extracts of Cynodon dactylon for 15 days, respectively. Animals were euthanized to collect the heart tissues and blood samples. 2DE sample preparation, gel running and staining (n=6; each group) were performed at the same time to avoid variation. The result of six gel images from each group were analyzed and evaluated as one match set with 2D software (P<0.05). Results: The above experiment revealed two up-regulated proteins in group-II i.e. NTF4 and ETFB. Conclusions: NTF4 is a neuro-protective agent for neuro-degenerative diseases. It will prevent diabetic secondary complications, such as diabetic polyneuropathy and cardiomyopathy. ETFB is active in the mitochondria, the energy-producing centres in the cells. It is clear from the experiment that because of up-regulation of ETFB more energy is available and the electron transfer for heart during diabetes is possible, what leads to reduce the oxidative stress and free-radical formation. The up-regulated proteins reduced CVD that occurred just before overt hyperglycaemia due to administration of C. dactylon. This approach established the preliminary reference map for decoding cellular mechanisms linked between pathogenesis CVD and diabetes.

Key words: diabetes mellitus, cardiac muscle proteome, 2D electrophoresis, MALDI-TOF-MS, phylogenetic analysis

INTRODUCTION

Diabetes is a chronic disorder caused by impaired insulin secretion from the pancreatic beta cells. In India, there were about 32 million diabetic patients in 2000 and by 2030 this number is going to increase to 80 million. The characteristic primary symptom for diabetes is hyperglycaemia, and secondary symptoms are retinopathy, nephropathy, cardiomyopathy, coronary heart disease (CHD) and stroke (Garber & Neely, 1983). There are many types of oral hypoglycaemic agents (sulfonylureas and thiazolidinediones) along with insulin for the treatment of diabetes, but they are linked to their own side effects (Langtry et al., 1998). The Diabetes Control and Complications Trial (DCCT) research group stated that the control of blood glucose is an effective strategy in clinical complications of diabetes mellitus, but even an optimal control of blood glucose cannot prevent complications in diabetes, which suggests that an alternative therapeutic approach is needed. In recent years, the use of natural medicine has increased worldwide and it may be used (Prince et al., 1998) as an alternative treatment against different diseases. However, in most of the cases the efficacy of this traditional medicine treatment is yet to be proved. Thus, the study of herbal medicine against diabetes is of great importance in the management of diabetes mellitus without any side effect. There are many plants that have anti-diabetogenic effects, such as Opuntia streptacantha Lem, Trigonella foenum graecum L., Monardica charantia L., Ficus bengalensis L., Polyscias senega L., Gymnema sylvestre R., Allium sativum, Citrullus colocynthis, etc. Cynodon dactylon is one among them (Santosh et al., 2008). C. dactylon L. (Pers.) (Family: Poaceae) is a herbal plant commonly known as 'Arangamul' (Tamil name: ஆரங்கமுழி) in Tamilnadu, India. C. dactylon is found in almost all warmer parts of the world. It is a short C4 grass, which is rhizomatous, stoloniferous and water-stress tolerant (Bethel et al., 2006). The various extracts of this plant have been reported to have anti-microbial (Ahmed et al., 1994), anti-inflammatory, anti-cystitis (Uncini & Tomei, 1999), anti-diuretic (Atmani et al., 2009), anti-hypertensive, anti-viral, anti-hysteria, anti-psychotic and anti-gonorrhoeal (Auddy et al., 2003) activities. C. dactylon is used by traditional healers for purifying blood, anuria, and in conjunctivitis, diarrhoea, gonorrhoea, itches and stomach ache (Muthu et al., 2006). Its root and rhizomes are used as a treatment for depression, vomiting, cough, epilepsy and haemorrhage (Miraldi et al., 2001). Recently, it has been reported that the plant possesses a protective effect against streptozotocin-induced hepatic injury in rats (Singh et al., 2008). It was ascertained recently, that C. dactylon has anti-diabetic, hypolipidaemic and anti-oxidant properties, and contains seven major compounds in aqueous extract and 6 major compounds in ethanolic extract.

Abbreviations: CHD, coronary heart disease; DCCT, Diabetes Control and Complications Trial; DC, Diabetic cardiomyopathy; C. dactylon, Cynodon dactylon

e-mail: dravinikesh@yahoo.co.in (S. Ravikumar), kar07bio@gmail.com (D. Karthik)
extract. Anti-diabetic activity of C. dactylon is related to the presence of 2-propenoic acid and tetra-methyl-2-hexadecen-1-ol (Karthik & Ravikumar, 2011a, b).

Diabetes is associated with profound changes in cardiac metabolism characterized by modulation of glucose transport, glycolysis, glycogen synthesis, lipid metabolism, protein synthesis, growth, contractility and apoptosis in cardiomycocytes by insulin (Abel, 2005). The impairment of shared insulin signalling pathways in metabolic and cardiovascular tissues contributes to reciprocal relationships between insulin resistance and endothelial dysfunction (Kim et al., 2006). Diabetic cardiomyopathy (DC) is an underlying aetiology of heart failure (~30% of all type 1 diabetic patients). The pathological mechanisms associated with DC are still not understood (Lam et al., 2006). The ultimate goal of the study is to identify and define possible causative agents involved in the disease outcome. According to the World Health Organization (WHO), cardiovascular disease resulting in cardiac dysfunction and heart failure is among the leading causes of morbidity and mortality in developed countries (Schott et al., 2010). Although the science of genomics has formed the basis of our understanding of the cellular and molecular mechanisms underlying cardiovascular disease, many downstream processes remain still largely unknown. Further development in proteomic technologies will not only allow the monitoring of changes in protein expression, but also together with genomic data, promise to be valuable in broadening our knowledge of the pathogenic pathways that lead to heart failure (Lien et al., 2006). Proteome analyses related to ischaemic/reperfusion and mitochondrial signalling in the myocardium have been used to identify alterations in mitochondrial signalling mechanisms and changes associated with stress response, as well as energy metabolism in mitochondria (White et al., 2005; Yan et al., 2004). Hamblin et al. (2007) characterized cardiac proteome changes as an indicative of DC, and this study may be useful to assess diagnostically the efficacy of anti-oxidant therapies as indicative of DC, and this study may be useful to assess diagnostically the efficacy of anti-oxidant therapies as treatments against diabetes mellitus complications involving the heart problems. Proteomic approaches have been documented in many studies related to clinically useful biomarkers of disease and treatment response (Anderson, 2005; Cruz-Toptete et al., 2011).

Proteomic studies can be applied to a range of biological systems including animal models and/or cell culture systems to answer the desired research question. In this study, we review the current status of proteomic technologies and describe how it is applied to unmask the pathophysiological mechanisms associated with diabetes-related cardiomyopathy.

MATERIALS AND METHODS

Chemicals. Alloxan was purchased from Sigma chemicals (St Louis, Mo, USA). Ampholyte was purchased from Fluka (St Louis). Protein marker was obtained from Fermentas (St Leon-Rot, Germany). α-cyano-4-hydroxycinnamic acid was obtained from Bruker Daltonik (Bremen, Germany). Unless otherwise stated, all other chemicals used were from Sigma–Aldrich (St Louis).

Plant collection and extract preparation. Fresh plants of C. dactylon were collected in October from the herbal garden of PRIST University, and were identified and authenticated by botanist Rev Dr S John Britto SJ, Director, The Rapinet Herbarium and Centre for Molecular Systematics, St Joseph’s College (Autonomous), Tiruchirappalli, Tamil Nadu, India. The voucher specimen of herbarium has been deposited at the Rapinet Herbarium and its number is RHCD02. Leaves of C. dactylon were shade dried at room temperature. Dried plant materials were subjected to size reduction to a coarse powder by using dry grinder. A total of 450 g of powder was packed into reflection unit apparatus and boiled for 10 h and 450 g of powdered plant material was subjected 70% ethanol in distilled water (v/v). The two different resulted extracts were filtered and concentrated by rotary evaporator under reduced pressure and a low temperature.

Experimental procedure. Healthy male adult Wister rats (Rat: Rattus norvegicus) weighing 130–150 g were selected for the study. The study was carried in accordance with the rules and regulations laid by the Institutional Animal Ethics Committee (IAEC no.: 743/03/abc/CPCSEA dt 3-3-2003-approval no.: PhD2/2009-2010). The animals were housed with free access to food and water. Group I, controls (n=6) were fed with normal diet. Albino rats were made diabetic by a single intra-peritoneal (i.p) injection of alloxan dissolved in normal saline at a dose of 150 mg/kg body weight (n=30). The animals were considered diabetic if their blood glucose values were >200 mg/dl on the third day after being injected with alloxan (i.e. after 72 h). After the induction of diabetes, they were randomly divided into individual groups: Group II, alloxan-induced diabetic rats, untreated (n=6); Group III, alloxan-induced diabetic rats, treated with aqueous C. dactylon leaves extract at a dosage of 450 mg/kg b.w. daily/orally for 15 days (n=6); and Group IV (n=6) alloxan-induced diabetic rats, treated with ethanolic extract of C. dactylon leaves at a dosage of 450 mg/kg b.w. daily/orally for 15 days. After 15 days of treatment, the animals were euthanized to collect whole heart tissues and blood samples with heparin (10 IU). The blood samples were centrifuged at 6000 rpm; 4°C for 10 min and plasma were collected and then rapidly stored at ~80°C. Plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA; Mercodia) before and after the induction of diabetes (alloxan) and after the C. dactylon treatment.

Sample preparation. Myocardial tissue of control and diabetic rats and both treated groups were immediately frozen in liquid nitrogen and stored at ~80°C until use. Each set of animal heart samples (six samples from each group) were processed in parallel through protein extraction (TCA/acetone method), 2DE electrophoresis (2DE) and staining to avoid differences from sample handling. Frozen myocardial tissues taken from control (n=6), and diabetic rats (n=6), and diabetic rats treated with aqueous C. dactylon leaves (n=6), and diabetic rats treated with ethanolic extract of C. dactylon leaves (n=6) were homogenized in 1X PBS with protease inhibitor. The homogenate was centrifuged at 10000 rpm for 10 min h at 4°C. The pellet was discarded, and the protein concentration of the supernatant was determined by using Bradford method (Bradford, 1976). Two milligram of protein sample was mixed with 1:1 ratio volume of 10% TCA/acetone solution and kept for overnight at 20°C. Then, centrifuged at 10000 rpm for 15 min at 4°C in a micro-centrifuge and washed with 1 ml ice-cold acetone four times after discarding the supernatant. The pellet was re-suspended with 200 µl of lysis buffer (85 mM Tris pH 6.8, 0.2% SDS).

2DE/imaging/protein identification. Isoelectric focusing (IEF) (O’Farrell, 1975) was carried out with 18 cm rod gel in pH 3–10 and gel contains 3 g urea, 7.125 ml distilled water, 2.44 ml acrylamide (28.38% acrylamide, 1.62% bis-acrylamide), 0.750 ml carrier am-
phytates 3/10, 0.375 ml NP-40, 34.625 μl ammonium persulphate (10%) and 12.5 μl TEMED. At the cathode side, 350 μg of the samples were loaded and electrophoretic conditions of the rod gels during the IEF were 200 V for 15 min, 300 V for 30 min and 400 V for 16 h. After focusing red gels were equilibrated in 6 M urea, 75 mM Tris/HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue for 30 min in buffer containing 8 mg/ml DTTF followed by incubation for 30 min in buffer containing 20 mg/ml iodoacetamide. The 2DE was performed with 12% polyacrylamide gels in 250 V for about 4 h. The analytical gels were stained with Coomassie blue. The protein Mr and pI were estimated by using SDS/PAGE protein standard with a molecular mass range from 10 to 170 kDa and a pH range from 3 to 10. The gel image were captured by gel doc image system and analysed by Melanie Viewer 7 2D software. The altered proteins were excised, and the identification by mass spectrometry (MALDI-TOF-MS) is described below.

**MS analysis (MALDI-TOF-MS).** Proteins of interest were excised, de-stained with 10 mM ammonium bicarbonate solution containing 50% (v/v) acetonitrile and dried in a Speed Vac centrifuge. The polypeptides were reduced (10 mm DTT, 100 mM ammonium bicarbonate for 30 min at 56°C), alkylated (50 mM iodoacetamide, 100 mM ammonium bicarbonate for or 30 min in dark room temperature) and digested with sequencing grade modified trypsin (10 ng/μl in 5 mM ammonium bicarbonate). After incubation for 5 h at 37°C, the reaction was stopped by adding 1 μl of 1% TFA. The digested peptides were directly mixed with 1:1 ratio of MALDI matrix, i.e. 10 mg/ml CHCA in 50% acetonitrile and 0.1% (v/v) trifluoroacetic acid and allowed to dry in open air. Tryptic peptides were analysed with a MALDI-TOF mass spectrometer (Bruker-Daltonics, Germany) in positive mode. The samples were analysed in a MALDI-TOF mass spectrometer equipped with linear positive mode 25kV, laser shots 150 [337 nm, 50 Hz, N2 laser (Bruker-Daltonics, Germany)] and mass spectra were acquired using the flexanalysis software. Spectra were analysed using the m/z program and contaminant peaks (keratin and trypsin) were removed manually. The remaining peaks were then matched with the theoretical peptide masses using MASCOT (Matrix Science, http://www.matrixscience.com). The search was performed against *Rattus* database with considered 0.1Da peptide tolerance, one missed cleavage allowed, carbamidomethyl (C) of cysteine (fixed modifications) and oxidation of methionine (variable modifications). Molecular Weight Search (MOWSE) scores from database interrogation above 30 (P<0.05), number of matched ions, number of matching ions with independent MS/MS matches, percent protein sequence coverage, and correlation of gel region with predicted MW and pI were collectively considered for each protein identification (Perkins et al., 1999).

**Alignment and phylogenetic analyses of identified proteins.** Multiple sequence alignments were performed with clustalX (2.01) and the output of the alignments was formatted by clustalX. The phylogenetic tree was calculated using Neighbor-Joining method (NJ method) integrated in clustalX, then visualized with Treeview 32 with 1000 bootstrap replicates (Kumar et al., 2004).

**Statistical analysis.** Data were expressed as the mean ± S.E.M. and were statistically evaluated using analysis of variance (ANOVA). The value P<0.05 was considered statistically significant.

**RESULTS**

**Plasma glucose and insulin level**

The significant difference between the mean values of insulin and glucose levels between the control and diabetic rats are shown in Figs. 1a and 1b. There was a significant decrease in the levels of plasma insulin and significant increase in the levels of plasma glucose in diabetic rats as compared with control (P<0.05). In this experiment, the plasma insulin levels before the induction of diabetes were same in all groups, whereas plasma insulin levels after induction of diabetes were significantly decreased as compared with control (P<0.05). This indicates that alloxan efficiently destroyed the β cells of pancreas, which in turn decreased plasma insulin (average mean 61.1%), and increased glucose levels, and caused diabetes. The administration of *C. dactylon* leaf extract to diabetic rats resulted in a significant increase in plasma insulin (Group III: 68.4%; Group IV: 60.6%; average mean 64.5%) and significant decrease glucose levels (P<0.05). Treatment with the aqueous extract of *C. dactylon* leaves to diabetic rats resulted in a significant decrease of 7.8% more than ethanolic extract. Based on the validation procedure, we concluded that the plasma insulin and glucose values obtained in this experimental research would be useful for further studies.
In an effort to gain an insight into the molecular mechanisms underlying the pathogenesis of DC, we performed a comparative analysis of the cardiac protein expression profiles of control and diabetic rats following alloxan-induced diabetes using 2DE and mass spectrometry (2-DE/MS). Each set of animal heart samples (six samples from each group) were processed through protein extraction (TCA/acetone method), 2DE and staining simultaneously to avoid differences from sample handling. The resulting six gel images (for each group) were analysed and evaluated as one match set with the 2D software, i.e. Melanie Viewer 7. Average amounts of 350 μg of total proteins from each sample were loaded for 2-DE investigations. On the average, 725 matched spots were detected on each gel and two proteins were differently expressed in alloxan-induced diabetic rats (group II) as compared to control and diabetic treated groups using Melanie Viewer 7 2D software. Figure 3 shows one set of 2DE gels (six samples for each groups), where differentially expressed proteins are marked with arrow and close-up 3D view of differentially expressed proteins. Numbers of spots, average correlation coefficient and mean S.D. for spot intensities from each set of gels are shown in Table 1. As expected average correlation coefficient, this gave a straight line (R^2 0.8). Values are expressed as mean ± S.E.M. of six animals. The value P<0.05 is statistically significance.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total spots</th>
<th>Average cor. coef.</th>
<th>Mean S.D.</th>
<th>P value (P&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n=6)</td>
<td>725</td>
<td>0.81 ± 12.00</td>
<td>0.034</td>
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</tr>
<tr>
<td>Group II (n=6)</td>
<td>723</td>
<td>0.86 ± 15.00</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>Group III (n=6)</td>
<td>727</td>
<td>0.89 ± 14.00</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>Group IV (n=6)</td>
<td>724</td>
<td>0.83 ± 10.00</td>
<td>0.025</td>
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In order to identify the altered proteins, the protein spots were excised from the 2DE gels and analysed by MALDI-TOF-MS. By using in-gel digest and MS with consecutive database search, we identified two differentially expressed proteins. Figure 4a and Fig. 4b show an
example of a MALDI-TOF-MS peptide mass fingerprint of the tryptic digests corresponding to spots 1 and 2. Table 2 gives a detailed overview of these two proteins with respect to observed and expected molecular weight and pI value, and the mode of identification with matched peptides. The MS analysis identified two proteins, i.e. spot 1 is neurotrophins (NTF4), having molecular weight 22.7 kDa and isoelectric point 9.1; and spot 2 is electron transfer flavoprotein beta subunit (ETFB), having molecular weight 27.8 kDa and isoelectric point 7.6. A review of the literature shows that most of these proteins are closely related to cardiac metabolism and function. The cellular locations of these proteins were determined using UniProt, and proteins were associated with many subcellular locations with the largest number associated with the cytoplasm and mitochondria in heart. These results indicate that cardiac function may be modified during type I diabetes. The identified proteins were classified into two groups based on their biological function: (i) Neurotrophic cascade protein (NTF4) and (ii) Electron transport chain cascade protein (ETFB).

Phylogenetic analyses of the NTF4 and ETFB

For phylogenetic analyses, the collected protein sequence of NTF4 and ETFB were aligned with ClustalX software. With the aim to evaluate the results for phylogenetic similarity between rat and other closest species, protein sequences were collected from NCBI protein blast database. The phylogenetic tree was constructed by the NJ method with 1000 bootstrap replicates. The multiple sequence alignments of the NTF4 and ETFB in rat, mouse, human, cattle and house are shown in Fig. 5a. All NTF4 and ETFB protein sequences share a conserved core with C-terminal and core region of each NTF4 and ETFB and varied N-terminal sequences. In this analysis, it is apparent that NTF4 and ETFB protein sequence are conserved with rat and mouse (91.7% similar and 100% similar, respectively). The human and cattle were formed in different node with respect to different percentage similarity. In this division, it is apparent that NTF4 and ETFB of different species are most closely related to rat. The phylogenetic analyses of the NTF4 and ETFB are shown in Fig. 5b.

DISCUSSION

The observed significant increase in the level of blood glucose could be related to the destruction of pancreatic beta cells by alloxan. With the supplementation of aqueous and ethanolic C. dactylon extracts for 2 weeks, fasting blood glucose level was significantly different with diabetic rats. Thus, the present study provides substantial evidence on the hypoglycaemic action of aqueous and
Cardiac disease is the leading cause of mortality associated with diabetes mellitus. The causes of heart diseases associated with diabetes are not yet understood and/or mechanisms are still unknown, but the profound reason could be the variation of gene and protein expression (Noble, 2002). Proteomics is a powerful technique that provides a record of intact proteins and can reveal changes in the levels of protein between samples. Therefore, it is likely to provide new insights into cellular mechanisms involved in cardiac dysfunction and new diagnostic and therapeutic markers (Tuñón et al., 2010; Ares-Carrasco et al., 2012). Our results also support the above statement and indicate that cardiac function may be modified during diabetes. The identified proteins were classified into two groups based on their biological function; (i) Neurotrophic cascade protein (NTF4) and (ii) Electron transport chain cascade protein (ETFB).

Neurotrophic cascade protein (NTF4)
Neurotrophins (NTF4) are a family [brain-derived neurotrophic factor (BDNF); neurotrophin-4/5 (NT-4/5), neurotrophin-3 (NT-3) and nerve growth factor (NGF)] of soluble polypeptides, implicated in several functions of the nervous system, including axonal growth, synaptic plasticity, survival, differentiation and myelination. Although originally discovered in the nervous system, many members of the neurotrophin family are expressed in a variety of non-neuronal systems including the cardiovascular, immune, endocrine and reproductive systems (Tessarollo, 1998). NTF4 gene is located on the long (q) arm of chromosome 11 (tessarollo, 1998).

Electron Transfer Flavoprotein Subunit Beta

Figure 5a. Screen display of multiple sequence alignment of different species protein sequence of Neurotrophins and ETFB aligned by NJ method by using ClustalX.

Figure 5b. The phylogenetic tree based on the NJ method by using ClustalX and treeview with 1000 bootstrap replicates. Neurotrophins (a) and ETFB (b).
arm of chromosome 19 at position 13.3. More precisely, NTF4 gene is located from base pair 49,564,396 to base pair 49,568,234 on chromosome 19. These neurotrophins are initially synthesized as larger precursors that are proteolytically cleaved to release the mature neurotrophins. The neurotrophins can be distinguished based on their distinct patterns of spatial and temporal expression as well as their differing effects on neuronal targets (Kaisho et al., 1990). NTF4 binds to specific high affinity receptors of the Trk family proteins (TrkB). Besides the presence of the p75, which binds all neurotrophins with similar affinity (Barbacid, 1993), a receptor tyrosine kinase, that is triggered by its ligand, the BDNF, regulates neuronal cell survival, neurite outgrowth, synaptogenesis and synaptic activity. The up- and down-regulation of NTF4 in diabetic patients cause diabetic polyneuropathy that can lead to atrophy and weakness of distally located striated muscles due to denervation (Andreassen et al., 2009). Defects in NTF4 may be associated with susceptibility to primary open angle glaucoma (POAG) type 1O (GLC1O). POAG is characterized by a specific pattern of the optic nerve and the visual field defects (Liu et al., 2010). NTF4 has been tested as neuroprotective agent for neurodegenerative diseases like cerebral ischaemia (Lindvall et al., 1994). NTF4 is acting as a survival and activation factors for eosinophils in patients with allergic bronchial asthma (Nassenstein et al., 2003). NTF and their receptors are expressed by the developing heart and vessels. In particular, BDNF deficiency reduces endothelial cell-cell contact in the mouse embryonic heart, thus leading to intra-ventricular wall haemorrhage and depressed cardiac contractility (Donovan et al., 2000). Our results also support the above statement based on the up-regulation of NTF4 in diabetic group which leads to reduce the diabetes complications associated with cardiovascular tissues and down-regulation of NTF4 in diabetic-treated groups with C. dactylon which leads to normal cardiac metabolism and function. The possible mechanism related to NTF4 action and prevention of the heart from diabetic complication could be the activation of transcription process in nucleus through NTF4 and MAK pathway; NTF4 and AKT pathway; and NTF4 and PKC.
pathway and regulate survival of neuronal cells as well as cardiac cells.

**Electron transport chain cascade protein (ETF)**

ETF part of an enzyme called electron transfer flavoprotein (ETF) is active in mitochondria, the energy producing centres in cells. ETF is involved in the process by which fats and proteins are broken down to produce energy. ETFB gene is located on the long (q) arm of chromosome 19 at position 13.3. More precisely, ETFB gene is located from base pair 51,848,408 to base pair 51,869,671 on chromosome 19 (White et al., 1996). ETF is a soluble mitochondrial flavoprotein. It is a dimer of non-identical subunits. ETF serves as a specific electron acceptor for several dehydrogenases. ETFs consist of αβ heterodimers with a single FAD thought to be bound to the N-terminal region of the β-subunit (Herrick et al., 1994). Both α-subunits (α-ETF, 32 000 molecular weight) and β-subunits (β-ETF, 27 000 molecular weight) were nuclear coded as a precursor (pα-ETF), 30 000 molecular weight larger and synthesized in the cytosol. α-ETF was synthesized to the respiratory chain. Hence, ETF and ETF-B are functionally interdependent and both are required for electron transfer from the flavoprotein dehydrogenases to the main respiratory chain (Henriques et al., 2010). The mutational analysis of ETF genes demonstrated ETFB missense mutation 124T>C in exon 2 leading to production of a truncated α-ETF, no detectable flavoprotein activity was observed. The newly synthesized β-ETF was the same as the mature β-ETF (Ikeda et al., 1986). ETF is a key enzyme in the mitochondrial fatty acid beta oxidation and amino acid degradation pathways, which transfers electrons from at least 12 dehydrogenases via ETF-QO to the respiratory chain. Hence, ETF and ETF-QO are functionally interdependent in the electron transport chain.

In conclusion, proteinomics has emerged as an essential method for decoding cellular mechanisms and tools to investigate signal transduction, metabolic and cellular mechanism, biomarker development, phenotype analysis and pathogenesis of heart disease connected to diabetes mellitus. As per our current and previous studies, we explore the importance of phytomedicine and proteomics to unmask the hypothesis of protein-drug target for effective treatment of diabetes, and this approach facilitates an understanding of the cell functioning in normal and diseased states. With the increasing incidence of diabetes mellitus worldwide, we have investigated anti-diabetic activity, hypolipidaemic and anti-oxidant activities of C. dactylon in alloxan-induced diabetic rats in our previous studies. Based on GC-MS study, seven major phyto-chemical constituents were present in aqueous extract and six major phyto-chemical constituents were present in ethanolic extract. Anti-diabetic activity of C. dactylon is exhibited due to the presence of 2-propenoic acid and tetra-methyl-2-hexadecimal-1-ol. This comprehensive anti-diabetic activity analysis has revealed the identification of potential phytotherapy for diabetes and has given the preliminary map of listed phyto-chemical constituent from aqueous and ethanolic extracts of C. dactylon. The identification of phyto-chemical constituent of diabetes did not provide the complete set for clinical therapeutics. Hence, it is needed to evaluate the phyto-chemical target, i.e. identification and characterization of target proteins for target drugs. The study of phyto-medicine and proteomics may unmask the hypothesis of protein-drug target for effective treatment of diabetes, and this approach facilitates an understanding of the cell functioning in normal and diseased states. Our current studies focus on the identification of differentially expressed proteins in alloxan-induced diabetic rats with the treatment of C. dactylon plant extract. In this current study, analysis of the proteomic in alloxan-induced diabetic rats reveals that the consequence of events for diabetes-associated CVD complication were reduced by the down-regulation of NTF4 with the effect of administration of C. dactylon leaf extract. Interestingly, many of the observed up- and down-regulated proteins reduced CVD and CHD occurred just before overt hyperglycaemia due to administration of C. dactylon leaf extract, and therefore, suggest that diabetes complication may improve CVD management. The combined phytomedicine and proteomics approach established the preliminary reference map for decoding cellular mechanisms and tools of heart disease connected to diabetes mellitus.

**STUDY LIMITATIONS**

Coomassie Blue R and G have become most reputed dye in protein detection (PAGE) due to low cost, ease of use and compatibility with downstream application such as MALDI. The detection limits for Coomassie Blue are 10–12 ng. Coomassie Blue provides a significant response with amount of protein load over a 5–25 fold range of concentration, i.e. protein concentration versus detection sensitivity (Wayne & Patton, 2002).

**Conflict of interest**

The authors are not having any conflict of interest.

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REFERENCE