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TITLE:

ASSESSMENT OF THERAPEUTIC POTENTIAL OF HESPERIDIN AND PROTEOMIC RESOLUTION OF DIABETES MEDIATED NEURONAL FLUCTUATIONS EXPEDITING ALZHEIMER'S DISEASE

RUNNING TITLE:

Hesperidin and Diabetes Associated Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD) is a type III diabetes mellitus (DM). Prognosis and early stage diagnosis of AD is essentially required in diabetic patients for avoiding extensive irreversible neuronal damages. Also, simple medication regimes including therapeutics for maintaining glucose levels and simultaneous resistance to neuronal damages are quintessential. In the present study, secretome and hippocampus proteome modulations were investigated for serum based markers having correlations with DM mediated neurological alterations which extend to AD. Concurrently, the therapeutic effect of hesperidin on DM and DM mediated neurodegeneration was investigated. Twenty one male Wistar rats were separated into three groups namely: healthy control, diabetic (65mg/kg STZ i.p., single) and diabetic administered with hesperidin (STZ i.p. + 50mg/kg hesperidin orally, four weeks). The secretome and hippocampus proteome profiling was accomplished by two dimensional electrophoresis, and proteins showing differential expression were characterized by MALDI-TOF MS PMF and validated by relative expression analysis. APO A-IV and secretory AGK were found to have prognostic and/or diagnostic potentials in detecting early stage of DM associated AD. A novel protein *'WajidSaima_Diabetes protein or WSDP* was found to have probable role in the neural homeostasis. Proteomic resolution manifests the therapeutic potential of hesperidin in DM and DM mediated neuronal fluctuations has successfully been determined. Our study emphasizes on DM mediated neuronal fluctuations that expedites as AD.

KEY WORDS

Alzheimer's disease; Diabetes; Proteomics; Mass Spectrometry; Hesperidin; Relative expression

INTRODUCTION

Diabetes mellitus (DM) is a frequently reported ailment[1-5]. The global incidence of DM in the year 2000 was 171 million and by 2030 total number of cases have been estimated to increase up to 366 million [6]. In India, the prevalence of DM was 31.7 million (2000) and by 2030 total number of cases have been realized to increase up to 79.4 million [7]. In accordance with an Indian study on diabetes, conducted by the Indian Council of Medical Research (ICMR-INDIAB, 2011), approximately 62.4 million people in India were suffering with DM and within two decades the numbers of sufferers are expected to rise over 100 million [8]. Diabetes mellitus is a complex ailment comprising of heterogeneous disorders arising due to insulin deficiency, impaired insulin action or both, resulting in hyperglycemia and glucose intolerance [9].

In the medication regime of DM, chemical drugs are available specifically targeting hyperglycemia, causing lowering of the blood glucose levels to an extent that the normal or near to normal blood glucose levels are achieved in DM patients[10, 11]. Antidiabetic drugs have multiple ill effects on key body organs, to wit, heart, brain, kidneys, eyes, liver, etc [10, 11]. These pernicious side effects being irreversible increase health complexities in diabetes patients leading to incorporation of the medicine for secondary diseases in the existent treatment regime. Current antidiabetic drugs are inefficient and have multiple ill effects [12]. Development of simple medication systems including therapeutics for maintaining glucose levels in normal range and simultaneous resistance of secondary damages to body organs is quintessential. Hesperidin (HP), an abundant flavonone glycoside found in citrus fruits, are involved in plant defense mechanisms [13, 14]. HP reduces cholesterol [15], blood pressure [16] and bone density loss [17]. HP carries significant anti-inflammatory and analgesic ramifications [18, 19]. HP exhibits anticancer activity [20] and can penetrate the blood-brain barrier [21]. HP possesses antioxidant and neuroprotective attributes on the brain tissue against the hyperglycemia mediated diabetic oxidative damage in STZ-DM rat models [14]. HP oral administrations significantly diminish the elevated levels of oxidative stress and neurotoxicity biomarkers induced during STZ mediated diabetes [14]. Conjointly, depleted activities of both enzymatic and non-enzymatic antioxidants get restored [14]. Antidiabetic attribute of HP mediated by its antihyperglycemic and antidyslipidemic efficacies have been reported in STZ-DM rodent models [14, 22, 23]. But its potential in controlling diabetes associated neuronal complications needs exploration.

Neurological impairments in DM is a sign of ongoing neurodegenerative pathologies [24]. Several prospective studies have perceived DM as a perilous factor for cognitive functions decline and eventuation of Alzheimer's disease (AD)[25]. Researchers have evaluated the threat of developing AD to be approximately double in DM [26]. Type 1 diabetic patients are at an increased risk of damaged cognitive functions; additionally, type 2 diabetic patients have increased the peril of developing AD in their middle and later lives [27]. Conjointly, pre-diabetic condition called as borderline diabetes has been found to aggrandize the threat of developing dementia and AD [28]. The risk of Alzheimer's disease (AD) development in DM condition is so strong that it has been categorized as the type III diabetes mellitus (DM) [29]. Both AD and DM have interlinked pathologies [30, 31].

Deposition of amyloid occurs in target organs; namely, Aβ and tau in AD brains, and amylin in pancreatic islets of type-2 diabetes [32]. Moreover, insulin deficiency in STZ mediated DM mouse results in hyperphosphorylation of tau proteins [33-35]. Also, in AD condition, insulin signalling and glucose metabolism pathways become blemished in brain. [36-38]. The molecular links existent between them needs enlightenment. Increased load of insoluble Aβ (amyloid beta) plaques, soluble Aβ oligomers, and neurofibrillary tangles are existent in AD brain [31, 39-43]. Aβ, a peptide containing 39–43 amino acids, comes into existence after the sequential cleavage of amyloid precursor protein (APP) by beta and gamma secretases; the cellular functional operations conducted by APP in a healthy brain are still enigmatic [31, 44-46]. The neurofibrillary tangles constituting hyper-phosphorylated 'tau' proteins, are damaging to neural tissues [47]. Detection of such proteinaceous plaques and tangles in the neural tissue is a late stage diagnostic strategy for AD. By the time plaques and tangles are visible, significant irreversible neural damages have already been done [24, 48]. Development of serum based methods may be beneficial in prognosis and early stage diagnosis of AD in diabetic patients.

Streptozotocin (STZ) is a glucosamine-nitrosourea compound [14]. STZ is being used for treating cancers of the Islets of Langerhans (U.S. Food and Drug Administration) [13, 49-52]. The colossal pertinence of STZ is found in diabetes investiture of animal models for experimental inquisitions [53]. Intraperitoneal (i.p.) administration of STZ is used for induction of DM in rodent models, by the virtue of its toxic attributes for insulin-secreting beta cells [14]. Intracerebroventricular (i.c.v.) administration of STZ causes neurological pathologies having similarities with those observed in sporadic AD, developing animal models for conducting studies on the early patho-physiological

alterations prevalent in AD [54-57]. In the present study, therapeutic effect of HP in DM and DM associated neurological complications was investigated. Concurrently simultaneous modulations in secretome and hippocampus proteome of STZ-DM rat model were investigated for serum based biomarkers having correlations with neurological alterations extending into AD. The objectives comprised of (i) evaluation of the protective role of HP in STZ-DM by blood glucose monitoring, (ii) evaluation of the protective role of HP in STZ-DM mediated neuronal damages by proteomic techniques and validation by relative gene expression using Real Time PCR (iii) to find secretome based biomarkers corresponding to STZ-DM neuronal fluctuations expediting into AD.

METHODS

Ethics Statements

The experimental plan was approved by Institutional Animal Ethics Committee (Hamdard University). The study was conducted in accordance to the guidelines given by Committee for the Purpose of Control and Supervision of Experiments on Animals (Ministry of Environment & Forests, INDIA).

Treatment Schedule

Twenty one male Wistar rats weighing between 270 and 300 g were procured from the Central Animal House Facility of Jamia Hamdard (Hamdard University). Animals were acclimatized for a week before starting the treatment. They were kept in an environment with temperature 25 ± 2 ^{0}C, relative humidity at 45-55 % and at a photoperiod of 12 h light/dark cycles. Standard pellet rodent diet and water was provided to animals *ad libitum*. One month diabetic animal models were prepared according to Parvez *et. al.* (our group) [14]. Twenty one animals were divided into three groups with seven animals per group as followed:

i) Group I (control): Non diabetic. Animals were administered orally with normal saline.

ii) Group II (toxicant): Diabetic. Animals were administered intra-peritoneally with single injection of STZ (65 mg/kg b. w.).

iii) Group III (toxicant + protectant): Diabetic administered with HP. Animals were administered intra-peritoneally with single injection of STZ (65 mg/kg b. w.). After accomplishing hyperglycemic state animals were daily administered orally with HP (50 mg/kg b. w.) for continuous four weeks.

STZ was dissolved in sterile milliQ water and HP (50 mg/kg b.w.) was dissolved in 0.5%w/v sodium carboxymethyl cellulose (CMC) solution. The administered doses of STZ and HP were based on previously published reports [14, 58, 59]. Hyperglycemic state is accomplished after three days from STZ i.p. administration [14]. Hence, establishment of diabetes was assured by blood glucose assessment after 3 days from STZ administration, and the animals maintaining blood glucose levels significantly higher than control were considered diabetic. The treatment schedule culminated on the 31st day from STZ administration.

Blood glucose monitoring

Fasting blood glucose levels were evaluated on 4th, 18th and 31st day from STZ administration, which respectively corresponds to 1^{st,} 15th and 28th day of HP treatment that further corresponds to conditions of no HP treatment, two and four weeks of HP treatments respectively. Results were expressed as mean ± Standard error (SE). All data were analyzed using analysis of variance (ANOVA) followed by Tukey's test; values of $p<0.05$ were considered significant. All the statistical analyses were performed using Graph pad Prism 5 software (Graph Pad Software Inc.).

Proteomic analysis

Serum samples: On completion of the treatment schedule i.e. 31st day from STZ administration, 500µl peripheral blood was collected from all animals. Blood samples were incubated at RT for 20mins followed by centrifugation at 8000xg for 20mins at RT. The clear yellow colored supernatant depicting serum samples were collected. Subsequently, the serum samples were made depleted of abundant proteins using Albumin and IgG Depletion Kit (Merck). Crude and depleted serum samples were stored at -80°C. Protein concentrations of the depleted samples were determined by Bradford micro-assay [60].

Tissue samples: After blood withdrawal, animals were anesthetized and sacrificed by cervical decapitation. Hippocampus was dissected out, rinsed in chilled normal saline and expeditiously frozen in liquid nitrogen followed by stored at -80 °C until used. Tissue samples were homogenized in urea lysis buffer (8 M urea, 65 mM CHAPS, 65 mM DTT, 2 M thiourea, 33 mM Tris and 6 mM PMSF [61]) using Polytron PT3100 homogeniser. The homogenate was centrifuged (9,600xg for 10 mins) and supernatant was collected and stored at -80°C. The proteins

concentrations of supernatant fractions extracted from hippocampus tissues were estimated by Bradford micro-assay [60].

One-Dimensional Gel Electrophoresis

Complex protein samples prepared from peripheral blood and hippocampus were resolved on 12%–SDS-PAGE in accordance to the protocol developed by Laemmli [62]. The depleted serum samples containing 20µg proteins were mixed with 2x protein loading buffer (10ml 1.5M tris HCl pH 6.8, 6 ml 20% SDS, 30 ml glycerol, 15 µl β-mercaptoethanol, 1.8 mg Bromophenol Blue, 39 ml water) and heated at 70° C for 10 minutes prior to loading in the gel. Likewise, 20 µg of hippocampus tissue proteins were mixed with 2x protein loading buffer. In case of tissue proteins no heat treatment was given. Samples were electrophoresed at a constant current (100–120V) in the running buffer Tris–glycine-SDS (pH 8.3). After electrophoresis, proteins were visualized by standard protocols of silver staining [63].

Two-Dimensional Gel Electrophoresis

100µg of proteins (depleted serum or hippocampus tissue proteins samples) were mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer Phramalyte (pH 4–7), and 1% bromophenol blue [61]) giving the final volume of 200 µl. The 11 cm IPG strips (pH 4–7) were incubated with rehydration buffer protein premix (passive rehydration) for 12 h. Rehydrated strips were subjected to isoelectric focusing at 250 V for 20 min, followed by 5000 V for 2 h and then kept for 15,000 Vh [61]. After isoelectric focusing, the strips were equilibrated in equilibration buffer I for 15 mins followed by equilibration buffer II for another 15mins. The composition of equilibriation buffer I was 6 M urea, 75 mM tris pH 8.8, 29.3% glycerol (v/v), 2% SDS (w/v), 0.002% bromophenol blue (w/v) and 2% DTT (w/v) [61]. Equilibration buffer II comprised of 6 M urea, 75 mM tris pH 8.8, 29.3% glycerol (v/v), 2% SDS (w/v), 0.002% bromophenol blue (w/v) and 135 mM IAA [61]. Equilibrated IPG strips were placed on 12% homogenous SDS polyacrylamide gel (14 x16 cms) and sealed with low melting point (1%) agarose for second dimensional gel electrophoresis at a constant current (180 V) in a running buffer (Tris–glycine-SDS, pH 8.3) [61]. The two dimensional gels were silver stained using standard protocols [61]. The spot patterns obtained were analyzed using PD Quest 2D analysis software (Bio-Rad) and the spots of interest were characterized by MALDI TOF MS Peptide Mass Fingerprint [61, 64].

MALDI TOF MS Peptide Mass Fingerprint

The selected protein spots were excised from the gels and slices were diced into small pieces followed by destaining 15 mM potassium ferricyanide and 50 mM sodium thiosulphate for 10minute intervals (3-4 times) until the gel dices turned translucent white. The gels slices were dehydrated using acetonitrile and Speedvac for complete dryness. The gel pieces were rehydrated with DTT and incubated for an hour. After incubation the DTT solution was removed. The gel pieces were now incubated with Iodoacetamide for 45min. The supernatant was removed and the gel was incubated with ammonium bicarbonate solution for 10min. The supernatant was removed and the gel was dehydrated with acetonitrile for 10min and Speedvac till complete dryness. The trypsin solution was added and incubated for 16 hrs at 37°C. The digest solution was transferred to fresh microcentrifuge tubes. The gel pieces were extracted thrice with extraction buffer and the supernatant was collected each time into the microcentrifuge above and then Speedvac till complete dryness. The dried peptide mixture was suspended in Tris acetate buffer (20 mM, pH 7.5) [64]. The peptides obtained were mixed with HCCA matrix in 1:1 ratio and the resulting 2 µl was spotted onto the MALDI plate. After air drying the sample was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument and further analysis was done with FLEX ANALYSIS SOFTWARE Version 3.2 for obtaining the PEPTIDE MASS FINGERPRINT. The masses obtained in the peptide mass fingerprint were submitted for MASCOT server for Peptide Mass Fingerprint search in "NCBInr" database for protein characterization [61]. The fixed modification(s) and variable modification(s) considered during analysis including residue specificity of trypsin (the protein digesting enzyme), respectively comprised of carbamidomethylation at cysteine and oxidation at methionine. The Peptide Mass Tolerance for precursor ions varied for each spots as ± 150 ppm (SSP 6708), ± 500 ppm (SSP 7418), ± 100 ppm (SSP 7122), and ± 70 ppm (SSP 2107); no contaminants were excluded from the from the PMF data. The significance threshold score/expectation value for accepting individual spectra was selected as $p<0.05$. Protein score was -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 59 were selected to be significant ($p<0.05$).The number of missed and/or non-specific cleavages permitted during analysis lied between 0-2.

Validation by Real Time PCR

Dissected hippocampus tissues were stored at -70°C in the RNA later (Ambion) solution. Total RNA was isolated using Mini Surespin Total RNA isolation Kit (Fermentas) including on-column DNase treatment. The isolated

samples were quantified on NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Incorporation). The genomic DNA contamination was checked by PCR with RNA samples and β-actin primers (targeted for 1,234 bp amplicon) in the reaction composition of 50ng RNA, 1X Taq buffer, 1U Taq polymerase, 1.5mM $MgCl_2$, 0.4mM dNTPs, 0.4µM β-actin Forward primer and 0.4µM β-actin Reverse primer. The thermal cycling conditions performed on Thermo Scientific Arktik thermal cycler (Thermo Fisher Scientific Incorporation) were (i) 96°C for 5 mins (initial denaturation) (ii) 35 cycles at 96°C for 1 min, 58°C for 1 min, 72°C for 1 min (iii) Final extension (72°C for 10 mins). The amplification products were analyzed by horizontal gel electrophoresis on 1.5% agarose 1X TAE buffer system followed by visualization on UV gel documentation system (AlphaImager HP System, Protein Simple). For cDNA synthesis, 0.5 µg of normalized RNA were reverse transcribed using RevertAid First strand cDNA synthesis kit (Fermentas). The integrity of cDNA was verified by PCR with β-actin specific primers resulting in 207 bp amplicons. The reaction mixture constituted of 1µl of cDNA, 1X Taq buffer, 1U Taq polymerase, 1.5mM $MgCl_2$, 0.4mM dNTPs, 0.4µM β-actin Forward primer and 0.4µM β-actin Reverse primer. The thermal cycling conditions were (i) 95°C for 5 mins (initial denaturation) (ii) 35 cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min (iii) Final extension (72°C for 10 mins). Quantitative PCR was performed on the in a Rotor-Gene Q real-time PCR cycler (Qiagen) using SYBR Green dye. All reactions were performed in duplicates. The total reaction volume of 25µL contained 12.5 µL of Maxima SYBR Green qPCR Master Mix (Fermentas), 0.4µM each primer (table 3), 1µl of diluted cDNA, and 9.5 µl of nuclease free water. The thermal cycling conditions were (i) 95°C for 10 mins (initial denaturation) (ii) 45 cycles at 95°C for 30s, 60°C for 30s, 72°C for 60s (iii) followed by melt curve analysis at temperature range of 72°C to 95°C. The raw data were analyzed with the Rotor-Gene Q series software 1.7 (Qiagen). The Ct values were obtained using a constant threshold value for all the genes examined. Relative gene expression was quantified with the comparative Delta Ct ($2^{-\Delta\Delta CT}$) method [65]. Control group was used as the calibrator. HPRT1 served as the normalizer gene [66].

RESULTS

Monitoring of blood glucose levels

Prior to the 1st HP dose, group I showed normal fasting range (70-100mg/dL); groups II and III had average blood glucose levels >70-100mg/dL and <126mg/dL, implying establishment of hyperglycemia. On the 15th day of HP

administration, group II showed average blood glucose 132 mg/dL (>126mg/dL), significantly higher than group I, indicating the development severe hyperglycemia and DM; on contrary, group III ranged in normal limits, indicative of HP role in controlling hyperglycemia and preventing STZ-DM in group III animals. On the 28th day of HP administration, group II displayed average glucose value 145mg/dL (>126mg/dL), a gradually overwhelming DM condition. Group I and group III possessed normal fasting blood glucose levels, indicating persistent ameliorative attribute of HP for STZ-DM in group III animals (figure 1).

Proteomic analysis

One-dimensional gel electrophoresis

Depleted serum profiles showed two bands (‘a’ and ‘b’) differing in stain intensity and band thickness (white arrow) (figure 2A) which were lower in group II than groups I and III. Hippocampus tissue profiles showed more differences (figure 2B); group III profile resembled group I, both significantly differing from group II. Stain intensity and band thickness were lower for bands ‘a’, ‘b’ and ‘c’ while higher for bands ‘d’, ‘e’ and ‘f’ in group II profile (figure 2B) than groups I and III.

Two-dimensional gel electrophoresis

In case of both depleted serum (figure 3) and hippocampus tissue (figure 4) proteins, group I profiles shared similarities with group III while both differed significantly from group II; indicating that a single STZ low i.p. dose significantly affects the secretome and hippocampus proteome. Additionally, such induced modulations can be restored to normal by regular intake of low doses HP orally. Figure 5, shows PD Quest software generated image of master gels containing all spots obtained in groups I, II and III. Differential spots selected for further analysis included serum proteins ‘SSP 6708’ and ‘SSP 7418’, and hippocampus proteins ‘SSP 7122’ and ‘SSP 2107’ (table 1). Spot intensities of ‘SSP 6708’ and ‘SSP 7418’ were lower in group II than groups I and III. Spot intensities of ‘SSP 7122’ were higher in group II than groups I and III while the spot ‘SSP 2107’ was present in group II only (table 1). The protein intensities of the selected spots calculated by the PD quest were SSP 6708: 43,640.84 INT*Area (Control); 13,050.26 INT*Area (STZ); 45,859.16 INT*Area (STZ+HP). SSP 7418: 191,653.44 INT*Area (Control); 1,572.06 INT*Area (STZ); 22,656.49 INT*Area (STZ+HP). SSP 7122: 896.37 INT*Area

(Control); 26,638.75 INT*Area (STZ); 7.04 INT*Area (STZ+HP). SSP 2107: 0.00 INT*Area (Control); 158.33 INT*Area (STZ); 0.00 INT*Area (STZ+HP).

MALDI-TOF MS Peptide Mass Fingerprint

MALDI-TOF MS Peptide Mass Fingerprint characterized (table 1) 'SSP 6708' as apolipoprotein A-IV precursor of *Rattus norvegicus* (accession Id 114008); 'SSP 7418' as mitochondrial acylglycerol kinase of *Rattus norvegicus* (NP_001120969.1); 'SSP 7122' as PREDICTED: hypothetical protein of *Rattus norvegicus* (XP_001067747.1) and 'SSP 2107' as tubulin beta-2A chain (T beta-15) of *Rattus norvegicus* (P04691). Table 2 contains the list of all peptide sequences, including any deviations from expected cleavage specificity. In table 1, SSP 6708 which corresponds to Apolipoprotein A-IV had approximately similar theoretical and experimental molecular weights. SSP 7418 which corresponds to Acylglycerol kinases had experimental molecular weight lower than the theoretical weight. It may be due to cleavage of signal and propeptides [67-70]. It may also be due to alternate splicing of full length AGK precursor RNA resulting in the shorter reading frame [67]. SSP 7122 which corresponds to Hypothetical protein had the experimental molecular weight higher than the theoretical weight such difference may be attributed by post translational modifications most likely glycosylation, which has its impact on electrophoretic mobility [67-69]. SSP 2107 which corresponds to Tubulin beta 2A chain showed the experimental molecular weight lower than the theoretical weight. This spot may contain fragments of Tubulin beta 2A chain instead of complete protein, indicating the higher rates of degradation the full length Tubulin beta 2A chains (~50KD) in the hippocampus of diabetic rats [67].

Validation of Differential Expression Proteins

The PCR reactions with RNA samples and β-actin specific primers produced no amplification, hence verifying absence of genomic DNA in the isolated total RNA. The cDNA samples were verified by amplifying 207bp β actin amplicons (Figure 6). Real Time PCR was performed for relative expression analysis of AGK, APO A-IV, TUBB2A and HYPO genes, in the hippocampus tissue of STZ-DM rats and HP treated STZ-DM rats in comparison with that of control healthy rats as calibrator samples. Figures 7 shows the amplification plots and melting curves generated during Real Time PCR. As shown in figure 8, the AGK and APO A-IV genes were down regulated in STZ-DM rats. The expression increased for both the genes in the HP treated STZ-DM rats as compared to STZ-DM rats. The expression of HYPO gene was up-regulated in STZ-DM rats while its expression was reduced in the HP

299 treated STZ-DM rats. The relative expression pattern for AGK, APO A-IV and HYPO genes was consistent with
300 2DE proteomic results while the expression pattern of TUBB2A showed a different trend. The expression of
301 TUBB2A in STZ-DM rats was down-regulated while its expression was found to be increased in HP treated STZ-
302 DM rats.

303 **DISCUSSION**

304 Hippocampus, a region in brain, responsible for learning and supports memory is affected by oxidative damages [71].
305 Hyperglycemia promotes oxidative stress and resulting damages increases complications [14]. In the hippocampus
306 proteome, Tubulin β 2A, showed presence only in STZ-DM rats, while it was absent in the HP treated group similar
307 to control. In our study the experimental molecular weight of TUBB2A chain was much lower than its theoretical
308 weight. Such reduction in the size may be due to fragmentation or cleavage of full length chain [67]. Significantly
309 changed expression of TUBB2A chains has been linked to both type 2 diabetes mellitus and Alzheimer's disease [72].
310 The protein spot was lower than 20KD and may indicate higher rates of degradation in STZ-DM rats compared to
311 HP treated and control groups. The regulated degradation pathways exist for tubulin *via* cofactor E-like or parkin[73].
312 The degradation of beta tubulin has been implicated in the pathogenesis of Alzheimer's disease. Cleavage of
313 tubulins and actins has been reported to cause axonal degenerations [74]. The reduction in expression of full length
314 beta tubulin chain exists in AD [75]. Transgenic AD model rats expressing Swedish mutant human APP proteins have
315 down-regulated tubulin beta chain A in the hippocampus proteome [76]. APP proteins are involved in familial AD [74,
316 75, 77]. The down-regulation of tubulin beta 2A is associated with DM [72]. Also, low levels of tubulin beta have been
317 reported in the hippocampus of AD patient [72]. Diabetes patients are at increased risks for developing Alzheimer's
318 disease [14]. Diabetes patients have decreased risk of Parkinson's disease [78]. The oxidative stress built in diabetes is
319 responsible for AD pathogenesis [79-83]. The cellular oxidative stress is involved in neuronal degeneration in living
320 animals [81] and strong relationship exists between oxidative stress and built up of amyloid plaques in the neural tissue
321 [82, 84-88]. DM mediated oxidative stress is associated with increased risk of developing AD [89-91]. DM and AD shares
322 insulin deficiency and have linked pathologies [31, 92-94]. Thus in the present study increased levels of degraded
323 hippocampal TUBB2A chain in combination reduced mRNA levels within STZ-DM rats may indicate that DM
324 neuronal fluctuations have ultimately expedited as AD; hence may reflect the establishment of AD in STZ-DM rats.
325 The experimental i.c.v. STZ administration has been known to produces early patho-physiological changes of AD
326 [14-16, 57]. The i.c.v. administration of STZ interferes with brain energy metabolic pathways, reducing the usage of

glucose in brain and functioning of glycolytic key enzymes, ultimately lowering the concentration of ATP and creatinine phosphate [95-97], causing neuron death. On the contrary, i.p. STZ administration induces dose-dependent DM [53] by its explicit toxicity towards beta cell of pancreas. STZ enters beta cells by GLUT2 glucose transporter and causes DNA alkylation leading to cell death. Additionally, STZ fructifies oxidative stress leading to diabetes associated neuronal degenerative ambiguities [14, 98]. In the present study, proteomic analysis mediated detection of AD related changes was obtained in DM rat model developed by Parvez et al. (our group) [14]; hence, further studies may be performed for analyzing the potential of this designed rat model to serve as the experimental model for studying the pathologies of DM mediated AD.

The hippocampus protein, corresponding to the HYPO (XP_001067747.1) lacks functional recognition. The availability of HYPO in the NCBI protein database was generated by the rectitude of computational scrutiny of the genomic DNA of *Rattus norvegicus* using protein prediction tools. The only procurable information (NCBI) presents HYPO as a stretch of 64 aa. We have named this hypothetical protein as '*WajidSaima_Diabetes protein'* (WSDP) signifying that the protein was experimentally first reported in DM condition in the STZ-DM rat model. This protein was present in all three groups reflecting its involvement in neuronal homeostasis. The expression of WSDP enhances in DM and associated AD.

The two secretomic or serum proteins APO A-IV and sAGK showed disparate levels in STZ-DM rat. Apo A-IV has association with triacylglycerol-rich lipoprotein and plays a role in lipid absorption, transport, and triacylglycerol-rich lipoprotein cellular internalization via many different mechanisms [99]. APO A-IV improves glucose homeostasis by augmenting insulin secretion [100]. The apoA-IV containing pre-b-migrating particles are present in cerebrospinal fluid [31]. The apoA-IV 2 phenotype has been associated with increased susceptibility for the development of AD [31]. APO A-I down regulation is associated with poorly controlled DM [101]. APOA-IV has been linked to type 2 DM (The GeneCards Human Gene Database; http://www.genecards.org/cgi-bin/listdiseasecards.pl?type=full#). APO A-IV plays an important role in brain metabolism and genetic reduction of APO A-IV inflates extracellular amyloid-β peptide (Aβ), provoking neuron loss in the brain, accelerating spatial learning deficits and increased mortality [102]. Polymorphism in APO A-IV genes serves as a risk factor for depression [103]. APO A-IV interacts with neurotoxic oligomeric $A\beta_{42}$ [104]. Studies by Kronenberg and coworkers have associated low APO A-IV with coronary artery disorders [105]. The diminished serum levels of APO A-IV reported in our study were obtained in STZ-DM rats. Also its reduced expression was observed in the hippocampus of the STZ-DM rats. These observations propose a key role

of APO A-IV in DM and associated AD. Apolipoproteins play a well-established role in the transport and metabolism of lipids within the Central Nervous System and are critical for healthy brain functions [106]. Apolipoproteins are present in the human cerebrospinal fluid [106]. The expression of brain apolipoproteins is significantly altered in several brain disorders for example, the late-onset AD is linked to apolipoprotein E (apoE) [107]. APOA4 Gln360His polymorphism has association with risk of coronary artery calcium progression in type 1 diabetes patients [108].

The mAGK, a multiple substrate lipid kinase, phosphorylates both mono and diacylglycerol to form lysophosphatidic acid (LPA) and phosphatidic acid (PA), respectively [109]. The LPAs activate diverse groups of G-protein-coupled receptors that are widely expressed to regulate decisive cellular functions [110]. They have been implicated in development, regulation of the cardiovascular, immune and nervous systems, inflammation, arteriosclerosis and cancer [110]. The increased levels of mAGK in the vitreous fluid of eye have been reported in retinopathy in DM rat model and serve as clinical biomarker for retinopathy in the DM patients [111]. The mAGK has cellular (mitochondrial) locations with 47.195KD molecular weight (UniProt). In the present work, a protein similar to mAGK was isolated from the secretome of all three groups; its experimental molecular weight ranged between 29KD and 20.1KD, significantly lower than the theoretical molecular weight of mAGK. These observations may signify presence of less bulky AGK isoform in the serum, hence may be named as the '*secretory AGK (sAGK)*'. In *Rattus norvegicus* the gene for AGK is located on chromosome 4 and comprises of 16 exons (Ensembl Genome Browser). The isoform sAGK might have originated by alternate splicing from full length AGK precursor RNA resulting in the shorter reading frame. The sAGK may have role in maintaining lipid homeostasis. On contrary, sAGK shows different expression profiles, STZ-DM rats exhibited expression lower than controls, further the low expression level was simulated in HP administered STZ-DM rats.

AD being most prevalent form of dementia constitutes more than 60–80% of reported cases[112, 113], affecting more than 27 million persons worldwide and by the year 2050 number of patients are expected reach 86 million [114]. AD involves loss of synapses and neurons and reduction in brain volume [114] causing deterioration to cognitive functions, tarnishing personal-social lifestyle and eventually death [39, 43, 115]. Early and definitive diagnosis of Alzheimer's disease (AD) can lead to a better and more-targeted treatment and/or prevention for diabetes patients [89, 114]. In the

prognostic and diagnostic biomarkers of AD, the serum based methods represents a more non-invasive, inexpensive and acceptable sources for repeated measurements than the cerebrospinal fluid [89]. The identification of peripheral biomarkers would enable presymptomatic detection of AD and would be valuable for monitoring the efficacy of disease interventions during clinical trials [114]. We suggest that reduction in APO A-IV and sAGK may serve as a diagnostic marker for DM and DM associated AD and in addition it has a therapeutic potential. Further studies on sAGK may enlighten involved lipid homeostatic pathways and their pathological alterations causing DM. Conjointly, the role of sAGK in DM associated neurodegeneration and AD may be investigated for an insight. The WSDP seems to have an important role in DM mediated AD and possesses high potentiality as a therapeutic target.

Anti-diabetic drugs are targeted towards controlling of glucose levels in the normal range ultimately aimed at preventing hyperglycemia [116]. The oxidative stress generated during DM is not taken into consideration by such drugs [117]. HP along with controlling the normal glucose levels, has been recognized to overcome the oxidative stress developed in STZ-DM rat model[14]. These dual properties of HP are important in preventing or managing DM and associated neurological complications [14]. HP may be useful in developing drugs with dual antidiabetic and AD treating attributes [31]. Prolonged STZ induced hyperglycemia enhances blood brain barrier permeability, exposing neural tissue to potentially pernicious agent [118]. HP crosses blood brain barrier [119] and restores oxidative damages [14]. In the present study, a daily oral dose of HP (50mg/Kg b.w.) to STZ-DM rats efficiently maintained glucose levels in the normal range while untreated STZ-DM rats suffered with progressively increasing hyperglycemia and diabetes. Along with maintaining blood glucose levels in the normal range, HP treatment was found to restore normal secretome and hippocampal proteome profiles. Interestingly, this restorative effect was more pronounced in hippocampal proteome.

CONCLUSIONS

Diabetes mellitus possesses an appreciably significant association with AD. Persistent hyperglycemia and oxidative stress in diabetes leads to numerous changes in the secretome and proteomic profiles of hippocampus tissue. Hesperidin possesses a strong candidature as a potential therapeutic drug for controlling and treating diabetes mellitus as well as preventing and treating diabetes mellitus associated Alzheimer's disease. Hesperidin is capable of

controlling hyperglycemia; conjointly its administration can restore normal proteomic expression profiles in the diabetic conditions. Early detection of diabetes mellitus associated Alzheimer's disease is a pre-requisite for preventing irreversible cognitive loss and neural impairments. APO A-IV and secretory AGK may serve as diagnostic markers for early stages detection of diabetes mellitus associates Alzheimer's disease. The predicted Hypothetical protein (XP_001067747.1) earlier unrecognized, was found to have an important role in maintaining neural homeostasis and was named as 'WSDP' signifying that the protein was experimentally first reported in the diabetic condition and its experimental molecular weight lies between 29KD and 20.1KD. The serum proteins (APO A-IV and secretory AGK) and hippocampus proteins (WSDP and Tubulin beta 2A) scrutinized in this work may serve as therapeutic targets for development of efficient treatment regime for diabetes mellitus and associated Alzheimer's disease. The current study established the capability of regular oral dose of hesperidin in diminishing oxidative stress and associated proteomic alterations in the hippocampus and serum of STZ-induced diabetes rat model. Our results strongly suggest that hesperidin may play an important role in restoring the neuro pernicious complications induced by diabetes mediated oxidative stress.

ABBREVIATIONS

aa: Amino acids AGK: Acylglycerol Kinase; mAGK: Mitochondrial acylglycerol kinase; sAGK: Secretory AGK; AD: Alzheimer's disease; Aβ: Amyloid β; APO A-IV: Apolipoprotein A-IV; b.w.: Body weight; CNS: Central Nervous System; DM: Diabetes mellitus; WSDP: WajidSaima_Diabetes protein; DTT: Dithiothreitol; HCCA: α-Cyano-4-Hydroxycinnamic acid; HP: Hesperidin; HYPO: Hypothetical protein accession Id *XP_001067747.1*; i.c.v: Intracerebroventricular; i.p.: Intraperitoneal; KD: Kilo Dalton; kg: Kilogram; mg: Milligram; mins: minutes; PMSF: Phenylmethanesulfonyl fluoride; RT: Room Temperature; SE: Standard Error; SSP: Standard Spot Parameter; STZ: Streptozotocin; STZ-DM: Streptozotocin mediated diabetes mellitus; Tris: Tris(hydroxymethyl)aminomethane; TUBB2A: Tubulin beta-2A chain

CONFLICTS OF INTEREST

None

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FIGURES

Figure 1. Effects of hesperidin on blood glucose level at different intervals in control and STZ induced experimental diabetic rats. Values are mean ± SE, n = 7, P < 0.05. *Significantly different from untreated control and #significantly different from diabetic control.

Figure 2. One dimensional SDS page profile of samples collected from animals on the 31st day from STZ administration. A: Albumin and IgG depleted serum proteins; B: Hippocampus tissue proteins. Lanes corresponds as the following: M- protein molecular marker, C- group I (control), T- group II (toxicant), T+P- group III (toxicant + protectant). White arrows indicates the bands that are up/down regulated in group II (toxicant) on comparison with the group I (control) and group III (toxicant + protectant).

Figure 3. Two dimensional gel profiles of proteins from albumin and Ig G depleted serum samples. A= Group I (Control), B= Group II (Toxicant) and Group III (Toxicant + Protectant). Protein bandsp: 1 (66 KD), 2 (43 KD), 3 (29 KD) and 4 (20.1 KD) constitutes protein molecular markers.

Figure 4. Two dimensional gel profiles of proteins isolated from hippocampus. A= Group I (Control), B= Group II (Toxicant) and C=Group III (Toxicant + Protectant). Protein bands: 1 (66 KD), 2 (43 KD), 3 (29 KD), 4 (20.1 KD) and 5 (14.3 KD) constitutes protein molecular markers.

Figure 5. Analysis of two dimensional gels of serum and hippocampus proteins employing PD Quest software. Each spot was assigned a unique SSP (Standard Spot Parameter) number by the software. An image of master gel automatically produced by PD quest software showing all the proteins present in group I (control), group II (toxicant) and group III (toxicant + protectant) depleted serum samples. C = group I gel, T = group II gel, T+P = group III gel.

Figure 6. cDNA integrity check by PCR amplification with β-actin specific primers. The cDNA samples used were C = Control group, T = Toxicant group, T+P = Hesperidin treated group. M is the 100bp DNA ladder.

Figure 7. The amplification plots (A) and melting curves (B) generated during Q-PCR.

Figure 8. Differential expression of AGK, APO A-IV, TUBB2A and HYPO genes in toxicant (STZ) group and Toxicant + Protectant (STZ+ Hesperidin) group.

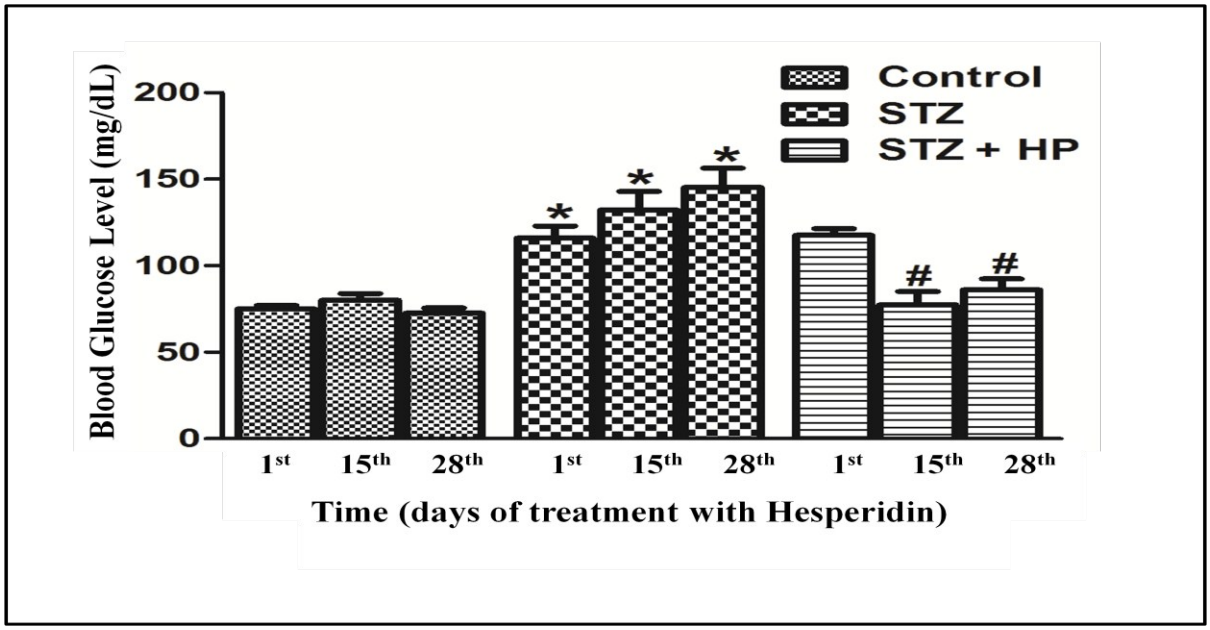


Figure 1.

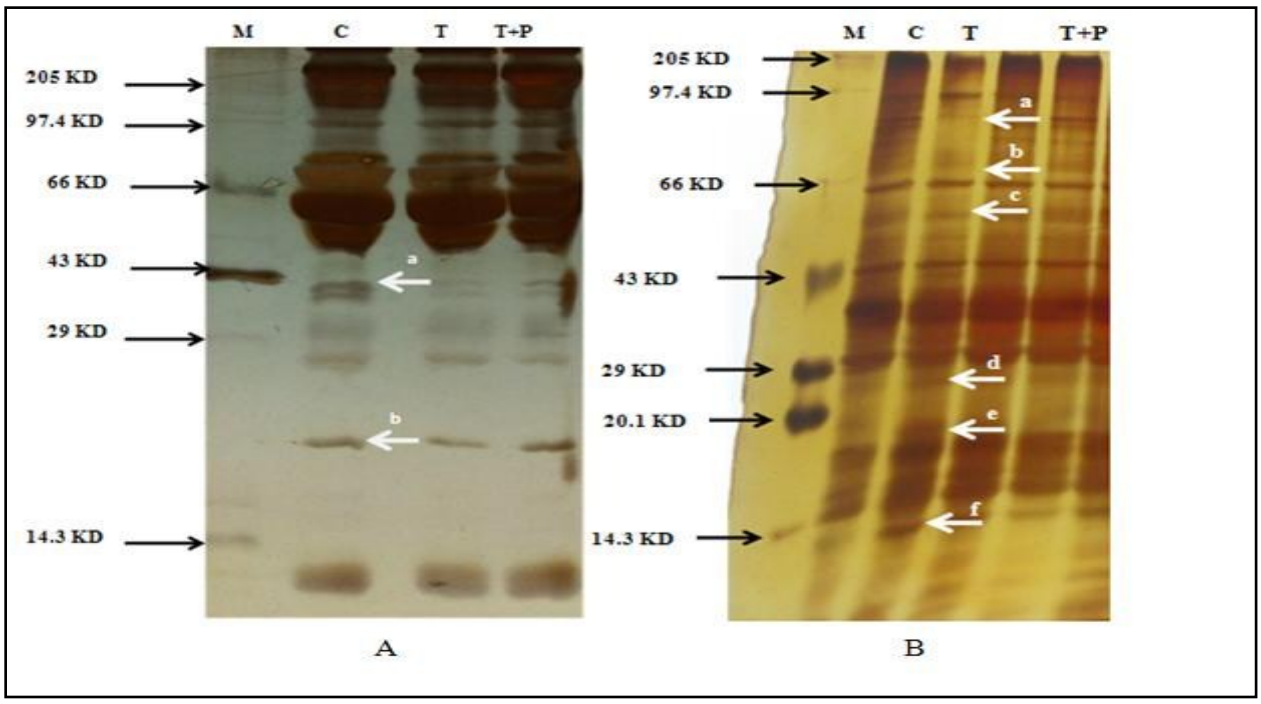


Figure 2

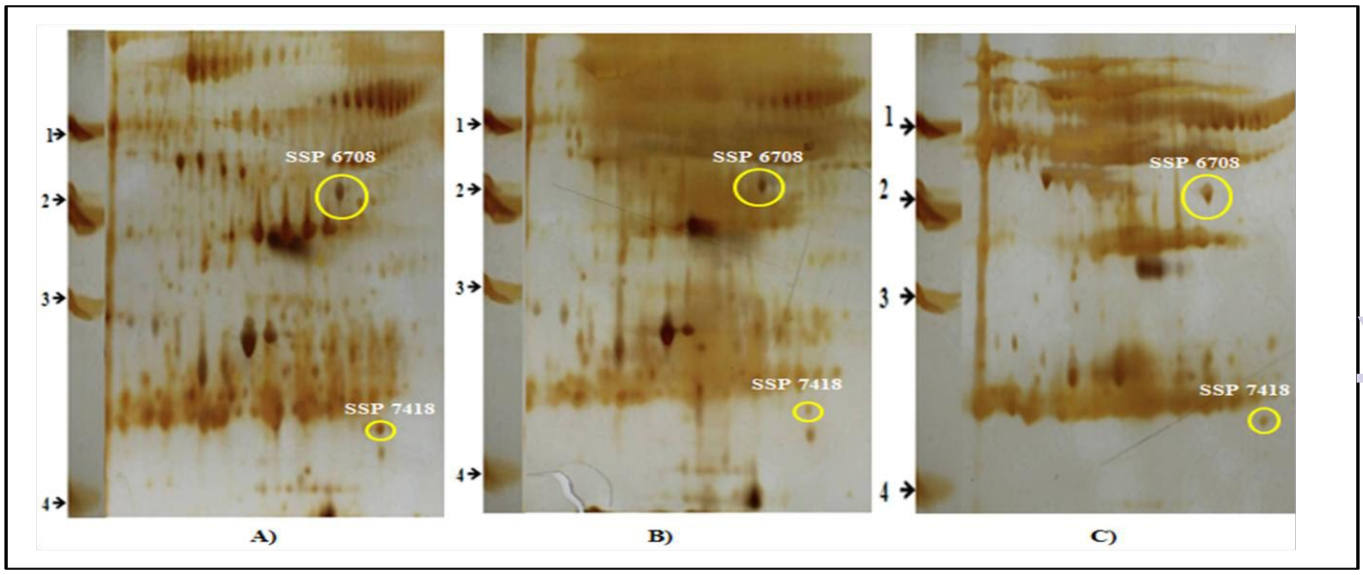


Figure 3.

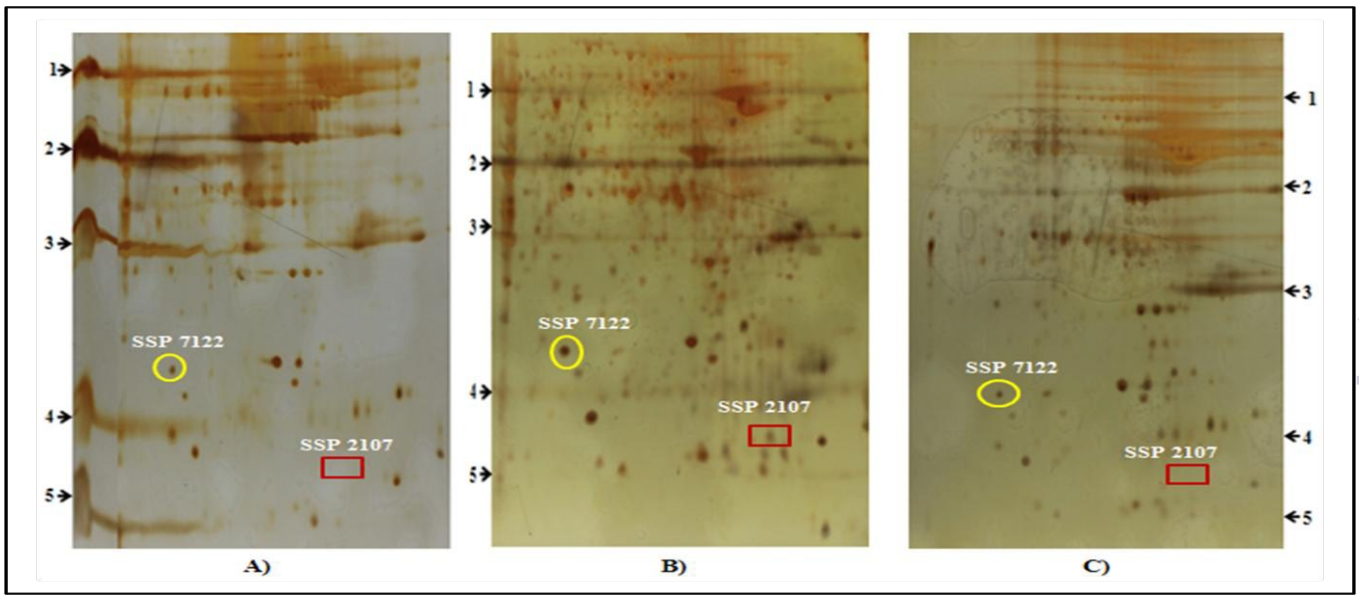


Figure 4.

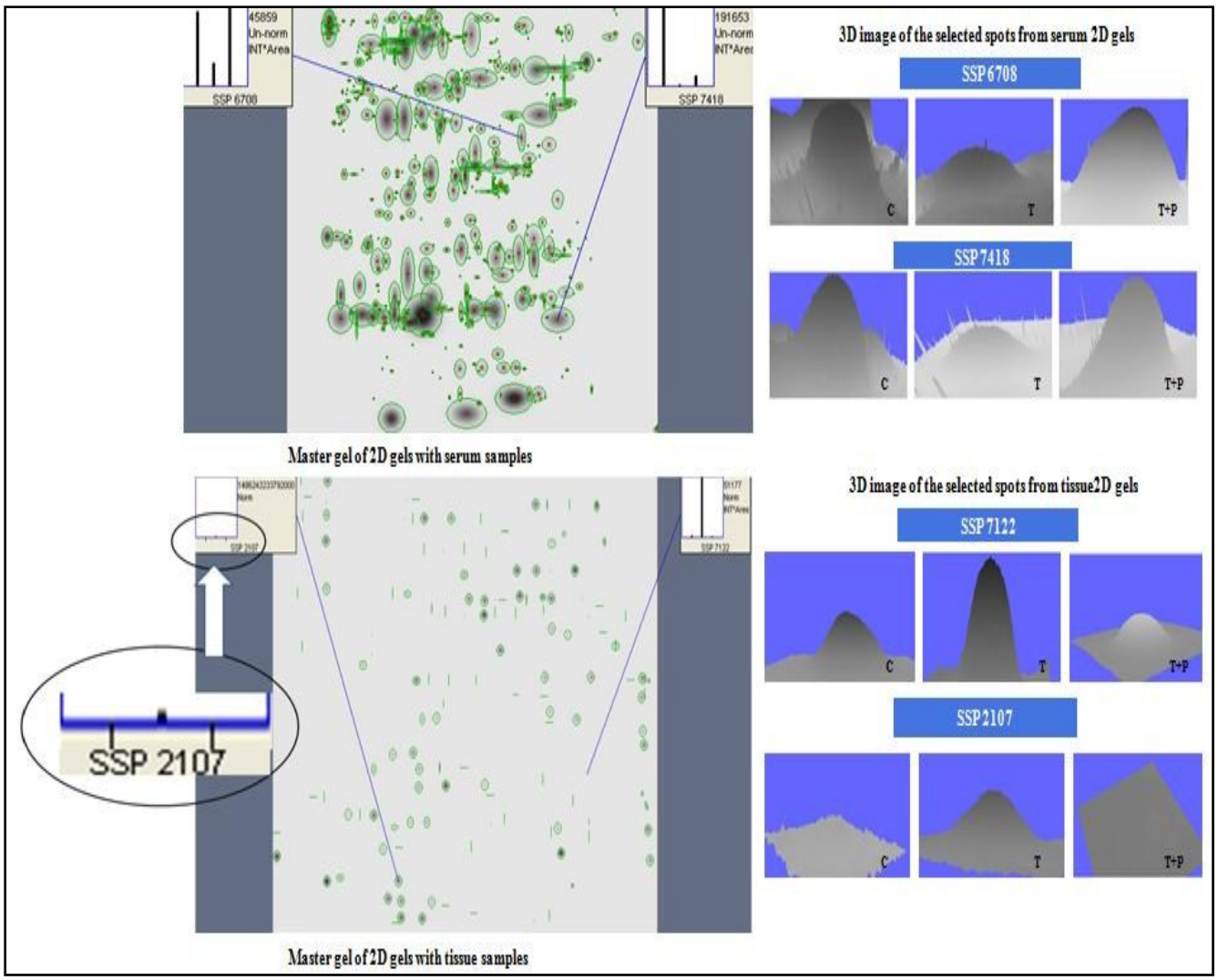


Figure 5.

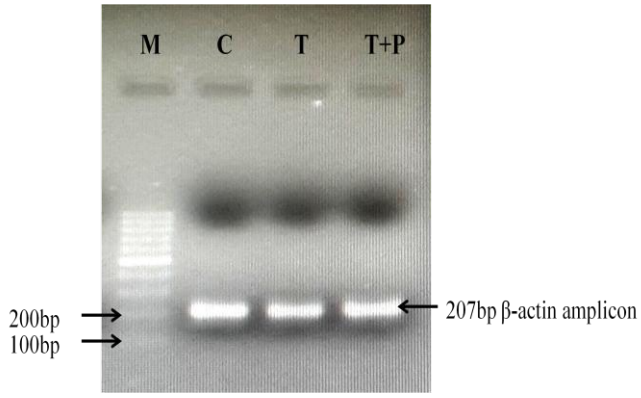
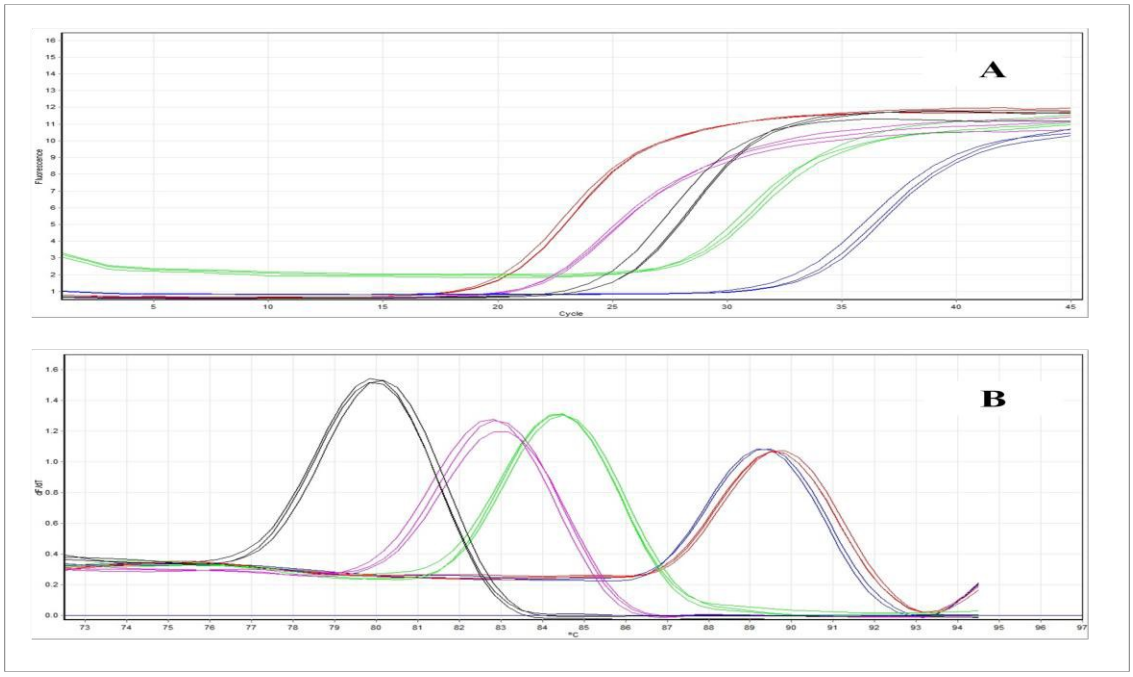


Figure 6.



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769 **Figure 7.**

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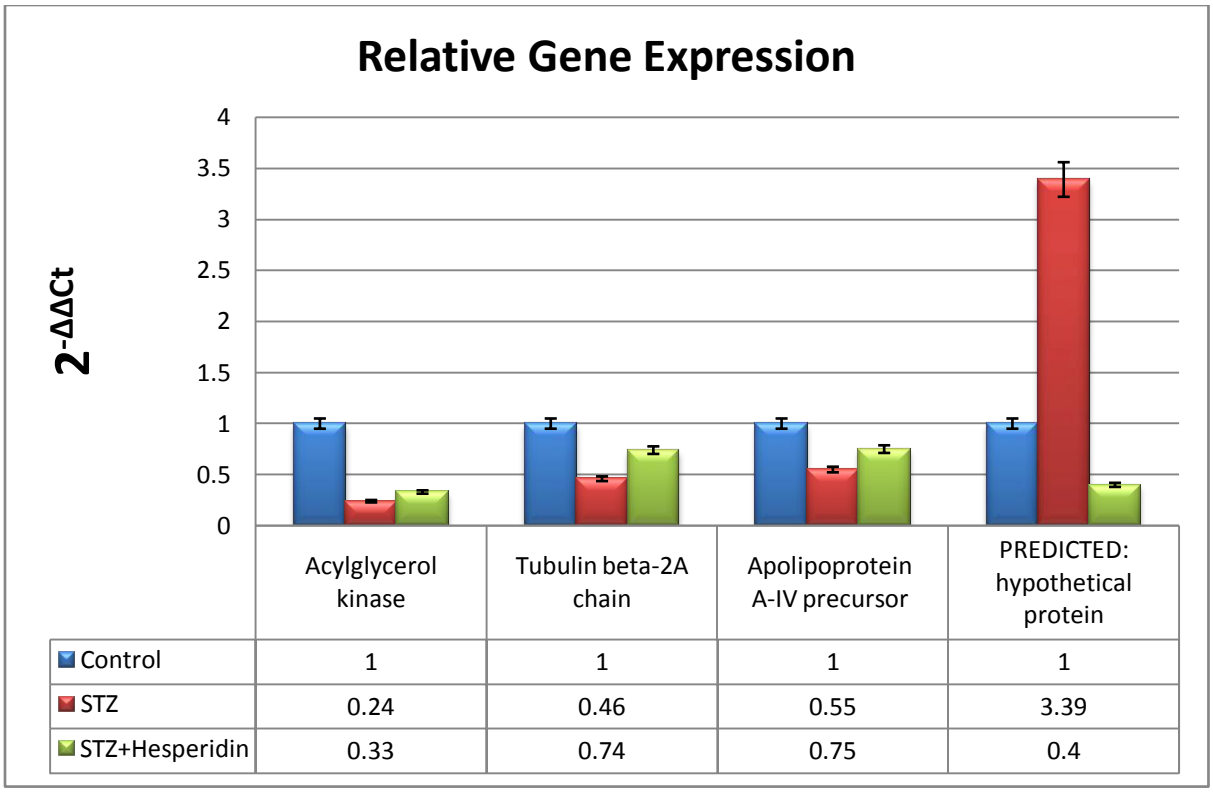

	Acylglycerol kinase	Tubulin beta-2A chain	Apolipoprotein A-IV precursor	PREDICTED: hypothetical protein
Control	1	1	1	1
STZ	0.24	0.46	0.55	3.39
STZ+Hesperidin	0.33	0.74	0.75	0.4

Figure 8.

790 **TABLES**

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792 Table 1. List of proteins SSP selected and their characterization by MALDI-TOF MS.

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SSP No.	Spot Intensity			Proteins (*Rattus norvegicus*)	Accession No.	Score	Queries matched	Expect	Theoretical molecular weight (KD)	Experimental molecular weight (KD)
	Group I (C)	Group II (T)	Group III (T+P)							
6708	↑	↓	↑	Apolipoprotein A-IV precursor	114008	177	16	8e-014	44.429	<66 and >43
7418	↑	↓	↑	Acylglycerol kinase, mitochondrial	NP_001120969.1	64	12	0.015	47.195	<29 and >20.1
7122	↓	↑	↓	Hypothetical protein	XP_001067747.1	61	4	0.033	6.869	<29 and > 20.1
2107	-	+	-	Tubulin beta 2 A or T beta-15	P04691	60	11	0.04	50.361	<20.1 and >14.3

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808 Table 2. List of all peptide sequences, including any deviations from expected cleavage specificity.

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SPOT: SSP 6708							
S. No.	**Observed**	**Mr(expt)**	**Mr(calc)**	**ppm**	**Start - End**	**Miss**	**Peptide**
1	1018.6734	1017.6662	1017.5607	104	156 - 163	0	R.QLTPYIQR.M
2	1261.8066	1260.7993	1260.6463	121	295 - 304	0	K.QLDQQVEVFR.R
3	1287.8163	1286.8090	1286.6466	126	210 - 220	0	K.ATIDQNLEDLR.S
4	1417.9395	1416.9323	1416.7474	131	295 - 305	1	K.QLDQQVEVFRR.A
5	1510.0647	1509.0574	1508.8715	123	80 - 93	0	K.LVPFAVQLSGHLTK.E
6	1546.9603	1545.9531	1545.7432	136	234 - 246	0	K.LNHQMEGLAFQMK.K
7	1733.0628	1732.0555	1731.8726	106	190 - 204	1	K.FNQNMEGLKGQLTPR.A
8	1843.1594	1842.1521	1841.9483	111	205 - 220	1	R.ANELKATIDQNLEDLR.S
9	2025.3182	2024.3109	2024.1055	101	80 - 97	1	K.LVPFAVQLSGHLTKETER.V
10	2061.2857	2060.2784	2060.0538	109	288 - 304	1	K.SLEDLNKQLDQQVEVFR.R
11	2148.3301	2147.3229	2147.0755	115	306 - 324	1	R.AVEPLGDKFNMALVQQMEK.F
12	2379.4164	2378.4091	2378.1503	109	325 - 345	1	K.FRQQLGSDSGDVESHLSFLEK.N
13	2812.7311	2811.7238	2811.3973	116	131 - 154	0	K.LQEHLRPYATDLQAQINAQTQDMK.R
14	2968.8711	2967.8638	2967.4984	123	131 - 155	1	K.LQEHLRPYATDLQAQINAQTQDMKR.Q
15	3309.1745	3308.1673	3307.6936	143	362 - 391	0	K.GSPDQPLALPLPEQVQEQVQEQVQPKPLES.-
16	3437.3078	3436.3005	3435.7885	149	361 - 391	1	K.KGSPDQPLALPLPEQVQEQVQEQVQPKPLES.-
SPOT: SSP 7418							
S. No.	**Observed**	**Mr(expt)**	**Mr(calc)**	**ppm**	**Start - End**	**Miss**	**Peptide**
1	584.3458	583.3385	583.2867	88.9	10 - 13	0	R.NHWK.K
2	637.3290	636.3217	636.3483	-41.71	78 - 82	0	R.TLFEK.N
3	713.4107	712.4034	712.4820	-110.29	277 - 281	2	R.RILRR.L
4	1575.3419	1574.3346	1574.8515	-328.25	304 - 317	0	K.DVQLSTIELSITTR.N
5	1709.0763	1708.0690	1708.8872	-478.76	1 - 13	2	-.MTTFFKTLRNHWK.K
6	2211.2213	2210.2140	2211.0895	-395.94	197 - 215	2	K.GEKEQPVYAMTGLRWGSFR.D
7	2384.0670	2383.0597	2383.1743	-48.07	200 - 220	2	K.EQPVYAMTGLRWGSFRDAGVK.V + Oxidation (M)
8	2399.1303	2398.1230	2398.2930	-70.88	78 - 99	1	R.TLFEKNAAPILHLSGMDVTVVK.T + Oxidation (M)
9	2566.4061	2565.3989	2566.3438	-368.21	40 - 62	2	R.RAACQEAQVFGNQLIPPNAQVKK.A
10	2872.5448	2871.5375	2871.4034	46.7	327 - 351	2	K.EDFMNICIEPDTVSKGDFIIIGSKK.V + Oxidation (M)
11	3582.0028	3580.9955	3582.7109	-478.82	370 - 401	1	R.CTLSLPEGTEGSFSIDSEEYEAMPVEVKLLPR.K
12	3598.0102	3597.0029	3598.7059	-473.21	370 - 401	1	R.CTLSLPEGTEGSFSIDSEEYEAMPVEVKLLPR.K + Oxidation (M)
SPOT: SSP 7122							
S. No.	**Observed**	**Mr(expt)**	**Mr(calc)**	**ppm**	**Start - End**	**Miss**	**Peptide**
1	1179.6898	1178.6825	1178.5947	74.5	56 - 64	0	R.WLWCAFLLA.-
2	2097.1117	2096.1044	2095.9116	92.0	3 - 21	0	R.DPSPHSPGSLEASELCEER.V
3	2210.1958	2209.1886	2209.1199	31.1	33 - 55	1	R.KPRGSALSDSGEAVAGAHLSGSR.W

4	2384.1013	2383.0941	2383.0532	17.1	1 - 21	1	-.MRDPSPHSPGSLEASELCEER.V
SPOT: SSP 2107							
S. No.	**Observed**	**Mr(expt)**	**Mr(calc)**	**ppm**	**Start - End**	**Miss**	**Peptide**
1	808.3505	807.3432	807.3399	4.16	157- 162	0	R.EEYPDR.I
2	1077.5709	1076.5636	1076.5250	35.8	155 - 162	1	K.IREEYPDR.I
3	1615.9212	1614.9139	1614.8287	52.8	63 - 77	0	R.AILVDLEPGTMDSVR.S
4	1631.9194	1630.9121	1630.8236	54.3	63 - 77	0	R.AILVDLEPGTMDSVR.S + Oxidation (M)
5	1707.8544	1706.8471	1706.8549	-4.60	283 - 297	0	R.ALTVPELTQQMFDSK.N
6	1871.0236	1870.0163	1869.9373	42.2	47 - 62	1	R.INVYYNEAAGNKYVPR.A
7	1959.0514	1958.0441	1957.9745	35.5	104 - 121	0	K.GHYTEGAELVDSVLDVVR.K
8	2087.1149	2086.1076	2086.0695	18.3	104 - 122	1	K.GHYTEGAELVDSVLDVVRK.E
9	2110.1325	2109.1253	2109.0571	32.3	1 - 19	1	-.MREIVHIQAGQCGNQIGAK.F
10	2141.1186	2140.1114	2139.9969	53.5	157 - 174	1	R.EEYPDRIMNTFSVMPSPK.V
11	3102.5552	3101.5479	3101.4003	47.6	20 - 46	0	K.FWEVISDEHGIDPTGSYHGDSDLQLER.I

827 Table 3. List of the primers used in the study

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Genes	Forward Primer	Reverse Primer	Amplicon size	Amplicon sequence
HPRT1	GTCAAGCAGTACAGCCCCAAAATG	AAATCCAACAAAGTCTGGCCTGTA	96bp (cDNA)	GTCAAGCAGTACAGCCCCAAAATGGTTAAGGTTGCAAGCTTGCTGGTGAAAAGGACCTCTCGAAGTGTTGGATACAGGCCAGACTTTGTTGGATTT
Apo A-IV	GCCATCACCGGGACCCAGGC	TCCAGTGGCATTGAGCTGTT	147bp (cDNA)	GCCATCACCGGGACCCAGGCTGAGGTCACTTCCGACCAGGTGGCCAATGTGATGTGGGACTACTTCACCCAGCTAAGCAACAATGCCAAGGAGGCTGTGGAACAACTGCAGAAGACAGATGTCACTCAACAGCTCAATGCCACTGGA
AGK	CACTCTTCAGGAGTGGCCTC	GGGGAGAAGGCATCCTGTGG	168bp (cDNA)	CACTCTTCAGGAGTGGCCTCAGACCCATCAGGCCTCCATCTCTTACACGGGCCCTACAGAGAGACCTCCCATTGGGCCTGAAGATGCTGCTCCCCGGCCTTCTCTGTACAGGAGAATATTACGTAGGCTTGCCTCATTCTGGGCACAGCCACAGGATG CCTTCTCCCC
HYPO	TCGCCTGGGAGCCTGGAGGC	GCCCAAACACCCCATGCCTG	210bp (cDNA)	TCGCCTGGGAGCCTGGAGGCGTCGGAGCTGTGCGAGGAGCGCGTACCCATACGCACTGGGGCGCGGGGTGCAAGAAAGCCACGCGGGTCAGCGTTATCAGACAGCGGAGAAGCCGTCGCGGGAGCGCACTTATCGGGGTCTCGCTGGCTCTGGTGCGCCTTCCTACTTGCCTGATCGGAGTGCGAGCCAGCAGGCATGGGGTGTTTGGGC
TUBB 2A	CGGCGCTAAGTTTTGGGAGG	TATTTGTTGCCAGCAGCTTC	129bp (cDNA)	CGGCGCTAAGTTTTGGGAGGTGATAAGCGATGAGCATGGCATCGACCCCACTGGCAGTTACCATGGCGACAGTGACTTGCAGCTGGAGAGAATCAATGTGTACTACAATGAAGCTGCTGGCAACAAATA
β-actin	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC	1,234bp (gDNA)	CACCCGCGAGTACAACCTTCTTGCAGCTCCTCCGTCGCCGGTCCACACCCGCCACCAGGTAAGCAGGGACGTCGGGCCCAGCGGGCCCCAACTTTACCTTGGCCACTACCTCGCTGCAGGATCGTGAGGAACACTCAGAAGGGACACCGTAGAGGGGTGGAGCGTGGTACCGGGCCGCGGAGCGGACACTGGCAAAGCTTAACTTTCCGCCTAGGGTGTAGAGTGTTTGCAGTCGTATTCCCGCGGTGTAGACACTCGTGGGCACGCTCCTGCTTGGTGCGCGGGGCTTGGGGACACACTAGAGTCGCGGTGTGGGCATTTGGAGAGCCGGTGCGGCTTGCGGGTGTTAAGCCGCATCTGTCCACCTTGAGGGGACACAGTATTGGGAGTCAGGCGTTACAATCACGCTTTGATGGCCTATGGGTCTTTGTCCAAACCGGTTTTGCCCATTCGGCTTGGCGGGCGCGGCGGGGCCGGCTCGGCCGGGTGGGGGCTGGGATGCCATTGCGCGTGCGCGCTCTATCACTGGGCATTGGGGCGCGTGCGCGCTGGGGAGGGAACTCTTCCTCTCCCCCTCTTCCGAGTTAAGAGTTGCGCGTGCGTATTGAGACTAGGAGCGCGGCCGCCCCGGGTTGGGCGAGGGCGGGGCCGTTGCCCGGAAGGGGCGGGGTCGTAGCGGCTAGGGCGCCTGCTCGCGCTTCCTGCTGGGTGTGGTCGCCTCCCGCGCGCGCACTAGCCGCCCGTCGCCTCAGTGTAGGCGGGGCCTGTGCCCGTTTGGGGAGGGGGCGGAGGCCTGGCTTCCTGCCGTGGGTCCGCCTCCGGGCCAGCGTTTGCCTTTTATGGTAATAATGCGGCTGTCCTGCGCTTCCTTTGTCCCCTGAGCTTGGGCGCGCCCCCTGGCGGCTCGAGGCCGCGGCTTGCCGGAAGTGGGCAGGGCGGCAGCGGCTGCTCTTGGCGGCTCCGCGGTGACCATAGCCCTCTTTTGTGCCTTGATAGTTCGCCATGGATGACGATATCGCTGCGCTCGTCGTCGACAACGGCTCCGGCATGTGCAAGGCCGGCTTCGCGGGCGACGATGCTCCCCGGGCCGTCTTCCCCTCCATCGTGGGCCGCCCTAGG

				CACCAGGTAAGTGACCCTTTACTTTGGGAGTGGCAGCCC TAGGGTTTTCTTGGGGGTCGATGCCAGTGCTGAGAACGT TGTTCTCCTCCGCAGGGTGTGATGGTGGGTATGGG
β-actin	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC	207bp (cDNA),	CACCCGCGAGTACAACCTTCTTGCAGCTCCTCCGTCGCC GGTCCACACCCGCCACCAGTTCGCCATGGATGACGATAT CGCTGCGCTCGTCGTCGACAACGGCTCCGGCATGTGCAA GGCCGGCTTCGCGGGCGACGATGCTCCCCGGGCCGTCTT CCCCTCCATCGTGGGCCGCCCTAGGCACCAGGGTGTGAT GGTGGGTATGGG

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