**Original Research Article****DOI: 10.26479/2019.0502.27****IN SILICO CHARACTERIZATION OF VISFATIN AND ITS ADDITIVE EFFECT WITH PHYTOCHEMICAL HESPERETIN ON INSULIN SYNTHESIS****Bhawna Kumari, Mohit Singh, Pradeep Kumar Rajput, Umesh C. S. Yadav\***

Metabolic Disorders and Inflammatory Pathologies Laboratory, School of Life Sciences,  
Central University of Gujarat, Sector 29, Gandhinagar, Gujarat, India.

**ABSTRACT:** Visfatin, an adipocytokine, is associated with obesity-induced diabetes and has been characterised in different animal models using diverse technologies. Discovered in 2004, visfatin is released by adipose tissue and has been implicated in various health complications. However, not much is known about this protein's physicochemical characteristics and computational attributes. The regulatory roles of this protein have been inconclusive and confusing in studies. Here, we have characterised visfatin using an *in silico* approach. Computational analysis of visfatin has revealed its amino acid sequences, isoelectric point (pI), stability and transmembrane regions. The new set of information has introduced many different characteristics making identification and further investigation of this protein easier for the investigators. Further, we have investigated its insulin mimetic effects in combination with a known phytochemical Hesperetin on beta TC-6 cells. The results depict the possibility of Hesperetin as a naturally-derived molecule which may promote insulin secretion along with visfatin showing a combinatorial effect. These results may be crucial for developing a therapeutic approach during hyperglycaemia and diminished insulin response.

**KEYWORDS:** Visfatin; Hyperglycaemia; Hyperinsulinemia; Adipocytokines; Hesperetin.

**Corresponding Author: Dr. Umesh C. S. Yadav\* Ph.D.**

Metabolic Disorders and Inflammatory Pathologies Laboratory, School of Life Sciences,  
Central University of Gujarat, Sector 29, Gandhinagar, Gujarat, India.

Email Address: umeshyadav@cug.ac.in

**1. INTRODUCTION**

Diabetes is a lifestyle disease which has shown alarming increase in its incidence rate in the past few decades [2]. In India, it has attained an epidemic proportion in recent times. The disease

incidence has increased due to change in the lifestyle and food habits of people consuming energy-rich diet and leading a sedentary life resulting in increased obesity and metabolic syndrome. Obesity is one of the leading causes of diabetes. Out of many links connecting the obesity and diabetes, the increased level of visfatin in circulation is one of the prominent causative factors [3, 4]. Visfatin is an adipokine predominantly secreted by visceral fat which has been shown to increase during obesity [5]. Visfatin was identified for the first time in 2004 and since then many research articles have implicated visfatin as one of the important causes of diabetes [6-9]. A basal level of visfatin concentration is maintained in the body which regulates the growth of pancreatic beta cells [10]. However, in the presence of increased levels of visfatin due to obesity or metabolic disorder, its impact is not clearly known. A research study has investigated and performed characterization of visfatin and shown the connection between gene expressions in chicken development to have a theoretical basis for learning visfatin functions [11]. Further, visfatin has been characterised in various organisms and found to play important role in different pathophysiological and metabolic pathways, however the comparative study of the protein structure and amino acid sequences between the visfatins of different animals is not available. Flavonoids, found in many different types of vegetables and fruits, have been associated with possible health benefits. Hesperidin, a citrus glycosidic flavonoid, displays such biological and pharmacological important characteristics as anti-inflammatory, anti-proliferative, lipid-lowering and anti-oxidative [12]. Hesperetin, aglycone form of hesperidin, is a well-known phytochemical that stimulates insulin secretion and exhibits hypoglycaemic effect [13]. Hesperetin also decreased the liver and serum cholesterol levels that could be probably because of the reduced activities of liver HMG-CoA reductase; acyl CoA cholesterol acyltransferase and amplified levels of faecal cholesterol [14]. A recent study has investigated the antihyperglycemic effects of Hesperetin against streptozotocin (STZ)-induced diabetic rats. Hesperetin showed antihyperlipidemic and antioxidant effects and hence can serve as therapeutics against diabetes in STZ rats [15]. The study indicated that Hesperetin could inhibit enzymes involved in glucose metabolism, prevent the progression of insulin resistance and normalize plasma glucose and insulin levels [12]. This synthetic drug, apart from generating a hypoglycaemic effect, has also been found to exhibit hypo-lipidemic and anti-obesity effect [13]. Hesperetin shows protective effect in diabetes by antioxidant properties against liver and kidney tissues [15]. In the present study, we have performed comparative study of the protein structure and amino acid sequences of visfatin from different animals by *in silico* method and performed wet lab experiments to validate its impact on pancreatic beta cell line. Further. we have investigated the effects of Hesperetin which is an aglycone form of naturally occurring hesperidin, in combination with visfatin and examined their effects with respect to insulin secretion.

## **2. MATERIALS AND METHODS**

### **2.1 Collection of amino acid sequences of visfatin of different animals**

Visfatin sequences of different animals including Human, Mouse, Rat, Pig, Chick, Goldfish and Croceine croaker were collected from public protein database, SwissProt in FASTA format for this study [16].

### **2.2 The amino acid content**

Amino acid contents such as count and frequency were calculated using CLC workbench [17].

### **2.3 The physicochemical parameters**

Physicochemical factors like isoelectric point (pI), molecular mass and aliphatic indices were analysed using the CLC workbench [17].

The calculation of grand average hydropathy (GRAVY) [18], aliphatic index [19], instability index etc [20,21] was done by Expasy's ProtParam server.

### **2.4 Biological properties**

The Transmembrane region, average of hydrophobicity, soluble and non-soluble membrane protein and sequence of N terminal and C terminal region were identified using SOSUI servers [22].

### **2.5 Prediction of secondary structure**

Self-optimized prediction method with alignment (SOPMA) was used to predict the secondary structure of the protein [23].

### **2.5 Cell culture conditions and treatment**

Beta-TC6 ( $\beta$ TC cell line) cells were purchased from NCCS (Pune, MH, India). Cells between passages 25-36 were cultured in DMEM- supplemented with glucose 18.5mM, 1% penicillin, streptomycin, and amphotericin B cocktail, 10% heat-inactivated foetal bovine serum (FBS) prior to treatment and used for the experiments. For experiments, cells were seeded in 6-well plates (for mRNA expression) or in 96-well plates (for cell viability assays). Cells were starved for 16 h in the starvation media containing 2.2 mM glucose and no FBS, and were incubated with different concentration of either recombinant visfatin (ng/mL) or Hesperetin ( $\mu$ M) or their combination (Axxora, Nottingham, UK) for the durations indicated.

### **2.6 Semi quantitative and quantitative PCR**

Approximately  $4 \times 10^5$  beta-TC6 cells seeded in 60-mm dishes, serum-starved for 18h and stimulated with specified doses of visfatin and Hesperetin for 24 h. Cells were washed with chilled PBS, total RNA was isolated by Trizol method as per supplier's manual (Invitrogen, Bengaluru, India). The cDNA was synthesised, PCR was performed for Insulin and 18s rRNA transcripts in thermal cycler using 96-well PCR reaction plate using following primer sequences:

Insulin:

(F) 5'-TACGGGGTTTGTGAAAGGAG-3'

(R) 5'- CATTGATAGCTGGGCCCTTA -3'

18S-rRNA:

(F) 5'-GTTGGTGGAGCGATTTGTCT-3'

(R) 3'-GAACGCCACTTGTCCCTCTA-5'

The conditions for PCR cycle were as following: denaturation for 3 min at 95°C followed by 30 cycles of 20 sec at 94°C for denaturation; 20 sec at 57°C for annealing; and 30 sec at 72°C for extension, followed by 72°C for 5 min for final extension. After amplification, the samples were loaded in Real Time PCR for quantitative analysis. Fold difference in m-RNA abundance for the Insulin genes was calculated using the equation  $2^{-\Delta\Delta C_t}$ , assuming the amplification efficiency of two [24].

## 2.7 Fluorescence Microscopy

Beta-TC-6 cells were cultured on tissue culture coverslips and stimulated with visfatin and Hesperetin as mentioned above. The cells were incubated for definite time point and the cells were fixed with 4% buffered paraformaldehyde by incubation for 1 h and permeabilized with 0.1% triton-X100 and stained with the phalloidin stain according to the supplier's protocol.

## 2.8 Statistical analysis

Statistical analysis for PCR was performed using one-way Analysis of Variance followed by Post Hoc test. Cell viability experiments data were analysed by Analysis of Variance followed by Turkey's post-hoc test. The  $p < 0.05$  was considered significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Collection of visfatin sequences

For the sequence of visfatin proteins of different animals public protein database Swisprot was screened for keyword 'visfatin'. The system generated seven visfatin protein sequences including Human (*Homo sapiens*), Mouse (*Mus musculus*), Rat (*Rattus norvegicus*), Pig (*Sus scrofa*), Chick (*Gallus gallus*), Goldfish (*Carassius gibelio*) and Croceine croaker (*Larimichthys crocea*). From this we retrieved all the seven organism's visfatin sequences by arbitrary choice and prepared as a non-redundant set of information (see supplementary figure 1). These sequences were collected in FASTA layout and utilized for further study. The organism's name with its accession number has been represented in table 1.

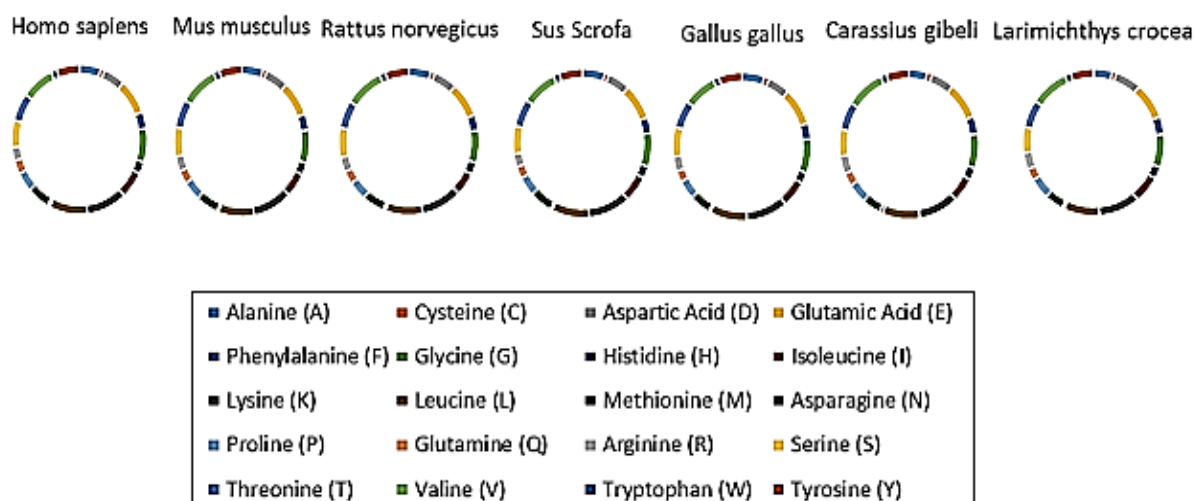
**Table 1:** The SWISPROT accession number of the organisms (with respective scientific names) used for accessing the sequences of visfatin protein.

S. No.	Organisms	Accession number
1	<i>Homo sapiens</i>	P43490
2	<i>Mus musculus</i>	Q99KQ4
3	<i>Rattus norvegicus</i>	Q80Z29

4	<i>Sus scrofa</i>	Q52I78
5	<i>Gallus gallus</i>	Q58I02
6	<i>Carassius gibelio</i>	A9LRS6
7	<i>Larimichthys crocea</i>	S5MI21

### 3.2 Characterization of amino acid composition and physicochemical and Biological properties

The result of primary examination suggested that most of the amino acid composition of visfatin were almost similar in all the seven organisms, consists of nearly similar hydrophobic and hydrophilic composition in nature. Majority of the sequences were rich in tyrosine (Y) amino acids and contained almost equal proportion of tryptophan (W). The presence of cysteine (C) indicated the possibility of disulphide bridges ("S-S" bonds) in visfatin (Fig 1).



**Figure 1:** The schematic diagram depicting the amino acids composition of visfatin protein in different organisms

The protein characteristics study tools and formulae are available on ExPASy well described by Gasteiger E. et al. in 2005 [1]. At the pI, the overall charge on the protein becomes zero. On protein's specific pI, they become stable and compact. The pI value of visfatin as determined by us was below 7, which specifies acidic nature of visfatin. The calculated pI would be appropriate for evolving a suitable buffer for purifying visfatin using isoelectric focusing.

The comparative amount of a protein that is represented by aliphatic amino acids (e.g. Ala, Ile, Val and Leu) referred to as aliphatic index (AI). Higher AI is associated with increased stability of a globular protein at higher temperature [1].

$$AI = X(A) + a * X(V) + b * X(I) + X(L) \quad (1)$$

Here X(A), X(V), X(I), and X(L) are moles percentage, i.e. (100 x molar fraction) of Ala, Val, Ile,

© 2019 Life Science Informatics Publication All rights reserved

Peer review under responsibility of Life Science Informatics Publications

2019 March – April RJLBPCS 5(2) Page No.377

and Leu amino acid residues, respectively. The terms 'a' and 'b' represent comparative amount of Val residue (a = 2.9) and that of Leu/Ile residues (b = 3.9) compared to Ala. The high aliphatic index of all visfatin sequences from different organisms inferred as their increased stability at a wide range of temperatures. Based upon instability index (I.I.), Expasy's ProtParam describes visfatin as stable protein that has lowest instability index (Instability index <40)(1) (Table: 2).

$$\text{I.I.} = (10/L) * \sum \text{DIWV}(x[i] * [i+1]) \quad (1)$$

Here L = sequence span  $i=L-1$

The term DIWV(x[i] x [i+1]) represents instability value for a given dipeptide beginning at the place i.

**Table 2:** The molecular weight, pI, molecular composition, Instability Index, aliphatic index of visfatin protein observed in different organisms through in silico study.

S. No	Visfatin Protein Organism	Mol. Wt.	Theoretical pI value	Mol. Formula	I. I.	A. I.	GRAVY
1	P43490	55521.16	6.69	C2518H391 0N654O745 S8	35.48	86.31	-0.429
2	Q99KQ4	55446.89	6.69	C2507H389 6N658O748 S7	32.87	85.09	-0.451
3	Q80Z29	55437.90	6.69	C2505H389 3N657O748 S8	31.81	84.30	-0.451
4	Q52I78	55373.92	6.69	C2509H390 1N653O746 S7	33.01	86.11	-0.436
5	Q58I02	55587.13	6.66	C2513H391 2N656O751 S8	35.38	84.58	-0.455
6	A9LRS6	55950.76	6.69	C2534H394 1N663O746 S10	30.89	85.52	-0.420
7	S5MI21	55759.41	6.27	C2528H392 4N658O748 S8	31.98	85.27	-0.449

The GRAVY index of visfatin was found to vary from -0.420 to -0.455. Extremely low value of

GRAVY index in case of visfatin was inferred as its likely hydrophilic nature, that is, the protein could have a superior interaction with water [1]. The SOSUI [21] server identifies trans-membrane regions in a protein (Table: 3). Through this, visfatins of all organisms were found to be water-soluble proteins. There was no transmembrane region found in visfatin sequences from all the organisms examined.

**Table 3:** The GRAVY index and identification of transmembrane region of visfatin protein of different organisms shown

S. No.	Organism	GRAVY	The values indicate that visfatin is a water-soluble protein containing no transmembrane regions in its sequence.
1	<i>Homo sapiens</i>	-0.429	
2	<i>Mus musculus</i>	-0.451	
3	<i>Rattus norvegicus</i>	-0.451	
4	<i>Sus scrofa</i>	-0.436	
5	<i>Gallus gallus</i>	-0.455	
6	<i>Carassius gibelio</i>	-0.420	
7	<i>Larimichthys crocea</i>	-0.449	

### 3.3 Prediction of Secondary structure

Bioinformatics tools were used to predict secondary structure of protein using SOPMA programme. The SOPMA stands for Self-Optimized Prediction Method with Alignment tool which can be used for secondary structure prediction. The primary sequence of protein was subjected to the programme. The SOPMA analysis provided information regarding the secondary structures of Helices, coils and strands of visfatin protein of different organisms. The secondary analysis showed that visfatin contains more alpha helix, beta turn, extended strand, and random coils (Table 4). Conversely, it lacks in 310 helix, Pi helix, Beta Bridge and Bend region.

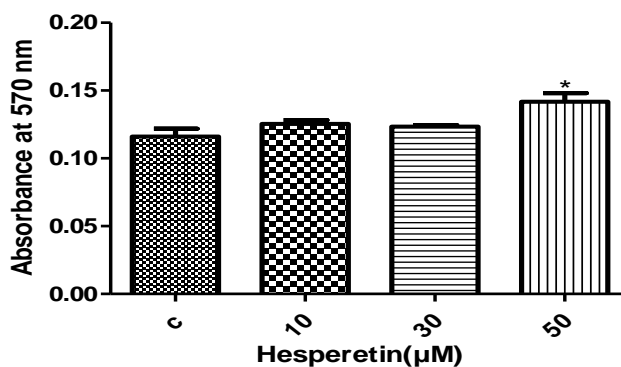
**Table 4:** A representation of secondary structure prediction analysis done by an online tool

SOPMA of visfatin protein in different organisms

Structural parameters	Human	Mouse	Rat	Pig	Chick	Goldfish	Croceine croaker
Alpha helix (%)	38.29	38.90	40.53	40.33	39.55	38.74	39.92
310helix (%)	0	0	0	0	0	0	0
Pi-helix (%)	0	0	0	0	0	0	0
Beta-bridge (%)	0	0	0	0	0	0	0
Extended-strand (%)	18.74	16.70	16.70	16.70	16.84	16.63	16.70
Beta-turn (%)	8.35	9.16	8.76	8.96	8.32	7.91	8.35
Bend-region (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Random-coil (%)	34.62	35.23	34.01	34.01	35.29	36.71	35.03
Uncertain states (%)	0	0	0	0	0	0	0
Other states (%)	0	0	0	0	0	0	0

### 3.4 Effect of Hesperetin on beta cells viability

Many antidiabetic therapies emphasis on improving insulin sensitivity, increasing insulin release, and/or decreasing the blood glucose level. Hesperetin is a flavonoid commonly found in many plant extracted medicines and food, especially the Citrus fruits. First, we assessed the cytotoxic effect of Hesperetin on Beta-TC6 cells by MTT assay after incubation for 24h. Our results showed that there was no significant change in the cell viability up to 30  $\mu$ M concentration of Hesperetin in the treated groups as compared to control. Thus, from this experiment we chose 30  $\mu$ M dose of Hesperetin for further experiments (Fig 2).

**Figure 2.** MTT assay was performed after incubation with different doses of Hesperetin for 24 h.

The bars denote mean  $\pm$  SEM depict cell survival (n=6) and \*\*p=0.0078 Vs. Control.

© 2019 Life Science Informatics Publication All rights reserved

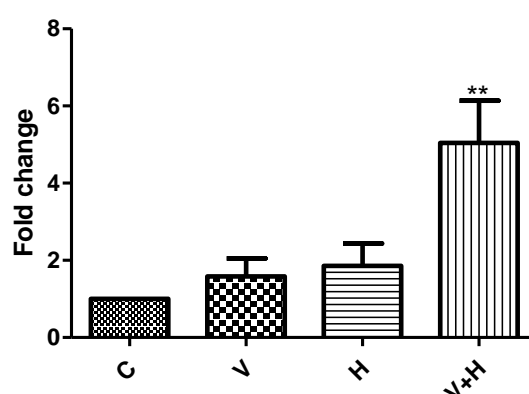
Peer review under responsibility of Life Science Informatics Publications

2019 March – April RJLBPCS 5(2) Page No.380



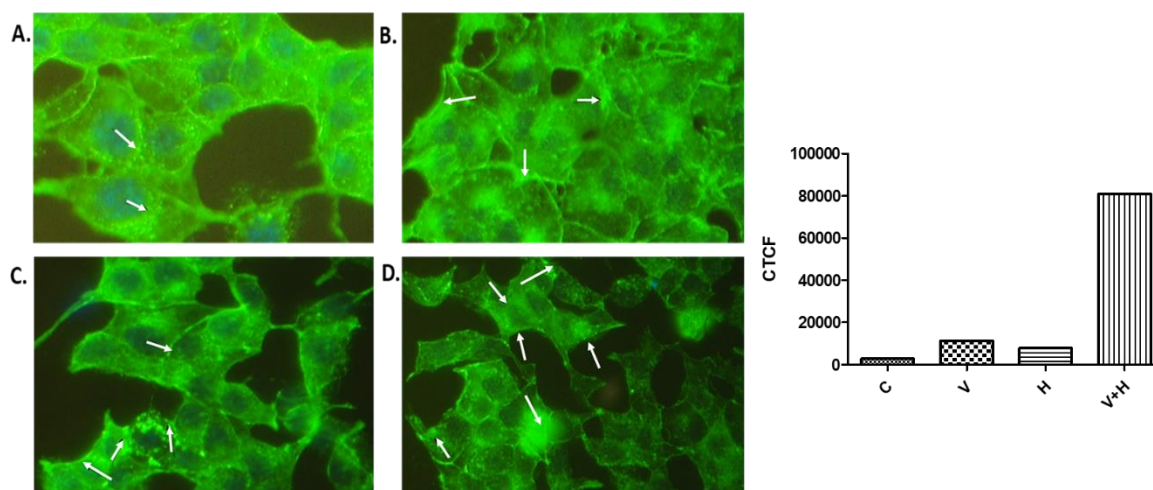
### 3.5 Effect of Hesperetin on visfatin-mediated insulin synthesis and secretion

We next examined the expression of insulin gene in Beta-TC6 cells at transcription level. We remarkably found that as compared to the control there was increased expression of insulin in the cells treated with Hesperetin (30  $\mu$ M) and visfatin (50 ng/ml) in combination. Hesperetin and visfatin alone also showed increased insulin expression however it was lower than the combinatorial treatment. We observed this pattern of expression of insulin in quantitative RT-PCR. As shown in figure 3, there was nearly 5-fold increased expression of insulin in combined treatment of visfatin and Hesperetin than their individual treatments which were 1.58- and 1.86-fold increase, respectively.



**Figure 3.** The quantitative PCR performed to determine insulin expression in indicated treatment groups. The bars represent fold change showing mean $\pm$ SEM (n=4). \*\*p=0.05 Vs. Control

We next confirmed the effects of two modulators in insulin secretion by performing phalloidin staining, which showed F-actin filaments remodelling, indirectly indicating vesicular secretion of insulin from the cells (Fig 4: A, B, C and D). To quantify this, we performed corrected total cell fluorescence (CTCF) analysis for all the experimental groups, where we found that more F-actin proteins were accumulated near the membrane of the cells when treated with visfatin as compared to control cells (Fig 4: E). There was increase of approximately 4-, 3-, and 29-fold in CTCF when treated with visfatin (50ng/ml), Hesperetin (30), and visfatin and Hesperetin together, respectively. The increase in F-actin remodelling occurred more when treated with Hesperetin and visfatin together than their separate treatment, suggesting the secretory vesicles containing insulin were transported more to the membrane for the release in combined treatment group.



**Figure 4.** The modulation of F-actin remodeling during insulin secretion induced by visfatin or Hesperetin. In the control cells, majority of the F-actin was seen in cytoplasm (A), F-actin was seen more at the membrane when treated with visfatin (B), or with Hesperetin (C) or in Hesperetin + visfatin treated cells (D). The photomicrographs (Magnification 400X) are representative of triplicate experiments (n=3). (E) The phalloidin staining quantification was done using CTCF method and represented as bar diagramme (E).

## DISCUSSION

In the present study we have characterised visfatin secreted by seven different organisms as their sequences are available in public domain. The protein is found to be highly conserved as reported previously. The current availability of high-throughput sequencing programs of protein and genomic sequences has provided us with an array of sequence data to contend with. Identification and characterisation of proteins of interest requires the input from computational tools to prioritise the data. Various computational tools have been exploited to analyse the protein sequence and their physicochemical and biological characteristics and have been reported in various studies [2, 10, 13, & 11]. The information regarding a protein on the parameters such as amount of amino acid residues and number of times they occur in a protein is projected by CLC workbench. Various attempts have been made to identify the sequence similarity and structural analysis of visfatin protein. In the visfatin sequences of canines 96% and 94% similarity was found when compared with that of human and rodent visfatin, respectively [27]. The sequence analysis of visfatin protein of seven different organism's showed presence of high amount of A, D, G, R, N and Y amino acids which coordinates well with the structural analysis of visfatin as an enzyme. These amino acids are important for its catalytic activity and metabolism homeostasis [28]. The study of visfatin of *Rattus norvegicus* revealed that visfatin is a dimeric protein, comprising of antiparallel  $\beta$ -sheet and  $\beta$ -strands and  $\alpha$ -helix region [28]. We found similar results in all the seven organism's visfatin sequence-based structural analyses. Here, we report many new and interesting characteristics including low GRAVY index, hydrophilicity and absence of transmembrane region in the protein [29]. These interesting

properties indicate that visfatin doesn't bind to the membrane and can reside in cytoplasm and also can circulate in the blood easily due to hidden hydrophobic region and extruding hydrophilic portion of this globular protein. Additionally, the sequence analysis of visfatin revealed the similarity in amino acids composition of the protein and presence of good amount of aromatic amino acids which make it easy to be identified spectrophotometrically. Visfatin is an adipocytokine that is newly identified as a potential link between obesity and diabetes. The protein has been implicated in the pathophysiology of diabetes. Since visfatin is involved in obesity and linked with diabetes we investigated its response with a known insulin mimetic phytochemical Hesperetin. Brown et al., (2010) have investigated the regulatory and insulin inducing role of visfatin in pancreatic beta cell and have demonstrated that visfatin can significantly stimulate insulin receptor phosphorylation and intracellular signalling [10]. We extrapolated their observations in terms of its effect on insulin synthesis. The pancreatic beta cells were induced with visfatin and it was found that they showed increased insulin mRNA synthesis by real-time PCR assays. Further, when Hesperetin was added along with visfatin, the combination showed significantly more insulin synthesis than their individual treatments, suggestive of their additive effect. The actin cytoskeleton plays an important role in regulation of vesicle exocytosis within the cells [30]. In pancreatic  $\beta$ -cells, F-actin cytoskeleton regulates the access of insulin vesicles to the plasma membrane and cells possess shorter actin filaments, which depolymerize readily during insulin secretion in response to glucose. This depolymerisation of actin is the  $\text{Ca}^{2+}$ -dependent actin-remodelling which mainly occurs due to protein gelsolin [31]. In our experiment, we found the similar pattern of remodelling of F-actin filament when treated with Hesperetin and visfatin alone, while in combined treatment group they showed additive effect. This suggested that visfatin and Hesperetin, when given together, were responsible for more insulin secretion as indicated by increased F-actin filament around the cell membrane in the combined treatment group when compared with control.

#### 4. CONCLUSION

Seven different sequences of visfatin protein have been found in public data base and used herein majorly to investigate their physicochemical and biological characteristics by using computational tools, analysis servers and wet lab experiments. Analyses of the primary structure demonstrated for the first time that majority of the visfatin studied here were hydrophilic in nature and likely contained disulphide bridges. Physicochemical characterization studies gave worthy ideas regarding valuable characteristics including pI, A.I., I.I. and GRAVY. These characteristics are important and very crucial and provide vital information regarding the properties of a given protein. We found an additive effect of visfatin with that of Hesperetin in the induction of insulin secretion, which opens the possibility of its use as antidiabetic and probably hypoglycemic effects during diabetes.

**ACKNOWLEDGEMENT**

Bhawna Kumari and Mohit Singh acknowledge the Junior Research Fellowship from Council of Scientific & Industrial Research (CSIR) and University Grant Commission (UGC), Government of India respectively. Pradeep Kumar Rajput acknowledges Junior Research Fellowship from Department of Biotechnology (DBT), Government of India.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**REFERENCES**

1. Gasteiger, E., et al., Protein Identification and Analysis Tools on the ExPASy Server, in The Proteomics Protocols Handbook, J.M. Walker, Editor. 2005, Humana Press: Totowa, NJ. p. 571-607.
2. Tabish, S.A., Is Diabetes Becoming the Biggest Epidemic of the Twenty-first Century? International journal of health sciences, 2007. 1(2): p. V-VIII.
3. Sandeep, S., et al., Serum visfatin in relation to visceral fat, obesity, and type 2 diabetes mellitus in Asian Indians. Metabolism, 2007. 56(4): p. 565-570.
4. Takebayashi, K., et al., Association between plasma visfatin and vascular endothelial function in patients with type 2 diabetes mellitus. Metabolism, 2007 Apr; 56(4):451-8.
5. Nourbakhsh, M., et al., Visfatin in obese children and adolescents and its association with insulin resistance and metabolic syndrome. Scand J Clin Lab Invest, 2015. 75(2): p. 183-8.
6. Romacho, T., et al., Extracellular PBEF/NAMPT/visfatin activates pro-inflammatory signalling in human vascular smooth muscle cells through nicotinamide phosphoribosyltransferase activity, in Diabetologia. 2009 Nov; 52(11):2455-2463.
7. Toruner, F., et al., Plasma visfatin concentrations in subjects with type 1 diabetes mellitus. Horm Res. 2009;72(1):p. 33-7.
8. Malyszko, J., J.S. Malyszko, and M. Mysliwiec, Visfatin, a new adipocytokine, is predominantly related to inflammation/endothelial damage in kidney allograft recipients. Transplant Proc, 2009. 41(1): p. 150-3.
9. Fukuhara, A., et al., Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science, 2005. 307(5708): p. 426-30.
10. Brown, J.E., et al., Visfatin regulates insulin secretion, insulin receptor signalling and mRNA expression of diabetes-related genes in mouse pancreatic beta-cells. J Mol Endocrinol, 2010. 44(3): p. 171-8.
11. Li, Z., et al., Characterization of the visfatin gene and its expression pattern and effect on 3T3-L1 adipocyte differentiation in chickens. Gene, 2017. 632: p. 16-24.
12. Dokumacioglu, E., et al., The effects of hesperidin and quercetin on serum tumor necrosis factor-alpha and interleukin-6 levels in streptozotocin-induced diabetes model. Pharmacognosy

- Magazine, 2018. 14(54): p. 167-173.
13. Akiyama, S., et al., Hypoglycemic and Hypolipidemic Effects of Hesperidin and Cyclodextrin-Clathrated Hesperetin in Goto-Kakizaki Rats with Type 2 Diabetes. Vol. 73. 2009.: p. 2779-82.
  14. Bok, S.H., et al., Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methylglutaryl-CoA reductase and acyl CoA: cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. *J Nutr*, 1999. 129(6): p. 1182-5.
  15. Jayaraman, R., et al., Antihyperglycemic effect of hesperetin, a citrus flavonoid, extenuates hyperglycemia and exploring the potential role in antioxidant and antihyperlipidemic in streptozotocin-induced diabetic rats. *Biomedicine & Pharmacotherapy*, 2018. 97: p. 98-106.
  16. Boeckmann, B., et al., The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Res*, 2003. 31(1): p. 365-70.
  17. Smith, D.R., Buying in to bioinformatics: an introduction to commercial sequence analysis software. *Briefings in bioinformatics*, 2015. 16(4): p. 700-709.
  18. Gill, S.C. and P.H. von Hippel, Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem*, 1989. 182(2): p. 319-26.
  19. Guruprasad, K., B.V. Reddy, and M.W. Pandit, Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Eng*, 1990. 4(2): p. 155-61.
  20. Ikai, A., Thermostability and aliphatic index of globular proteins. *J Biochem*, 1980. 88(6): p. 1895-8.
  21. Kyte, J. and R.F. Doolittle, A simple method for displaying the hydropathic character of a protein. *J Mol Biol*, 1982. 157(1): p. 105-32.
  22. Hirokawa, T., S. Boon-Chieng, and S. Mitaku, SOSUI: Classification and Secondary Structure Prediction System for Membrane Proteins. 1998. Vol. 14: p. 378-379.
  23. Sangli, V., s.A., Mundaganur, D.S., and Mundaganur Y.D., Catalase: Phylogenetic characterization to explore protein cluster. 2011. Vol. 1: p. 001-008.
  24. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 2001. 25(4): p. 402-8.
  25. Shaw, K.L., et al., The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein science : a publication of the Protein Society*, 2001. 10(6): p. 1206-1215.
  26. Morin, B., et al., The citrus flavonoids hesperetin and nobiletin differentially regulate low density lipoprotein receptor gene transcription in HepG2 liver cells. *The Journal of nutrition*, 2008. 138(7): p. 1274-1281.
  27. Adeghate, E., Visfatin: Structure, Function and Relation to Diabetes Mellitus and Other Dysfunctions. 2008. Vol. 15: p. 1851-62.
  28. Kim, M.K., et al., Crystal structure of visfatin/pre-B cell colony-enhancing factor 1/nicotinamide

- phosphoribosyltransferase, free and in complex with the anti-cancer agent FK-866. *J Mol Biol*, 2006. 362(1): p. 66-77.
29. Bagag, A., et al., Characterization of hydrophobic peptides in the presence of detergent by photoionization mass spectrometry. *PloS one*, 2013. 8(11): p. e79033-e79033.
30. Porat-Shliom, N., et al., Multiple roles for the actin cytoskeleton during regulated exocytosis. *Cellular and molecular life sciences : CMLS*, 2013. 70(12): p. 2099-2121.
31. Bradbury, J., et al., Actin' roles for glucose in insulin secretion. *Journal of Cell Science*, 2006. 119(10): p. e1005-e1005.