Argyreia Nervosa Facilitates GLUT4 Translocation to the Plasma Membrane by Promoting IRAP and VAMP-2 Genes in STZ-Induced Type 2 Diabetic Rats

Sumedha Balaji[1], V. Vishnu Priya[2*], Selvaraj Jayaraman[3], R. Gayathri[4], Kavitha.S[5]

[1]Saveetha Dental College and Hospitals Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India
[2*]Department of Biochemistry, Saveetha Dental college and Hospitals Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India
[3]Department of Biochemistry, Saveetha Dental college and Hospitals Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India
[4]Department of Biochemistry, Saveetha Dental college and Hospitals Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India
[5]Department of Biochemistry, Saveetha Dental college and Hospitals Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

Abstract: Background Argyreia nervosa is a perennial vine which is originally found in the Indian subcontinent and was later introduced to various areas world wide such as Hawaii, Africa and the Caribbean. It is commonly called an elephant creeper. GLUT 4 mechanism is an example of cascade effect and is responsible for the control of blood sugar levels of a living organism. It mainly occurs in the skeletal muscles and adipose tissue. IRAP and VAMP-2 are the genes which are expressed in response to the GLUT-4 mechanism and are used to evaluate the blood glucose levels in an organism.

Aim and Objective

This study was aimed to determine if *Argyreia nervosa* facilitates GLUT4 translocation to the plasma membrane by promoting IRAP and VAMP-2 genes in STZ-induced diabetic rats.

Materials and Methods

The healthy male Wistar albino rats were made to fast overnight and the next morning were injected with streptozotocin to induce diabetes in them. And then the plant extract was fed to them to check if it helped in reducing diabetes. The animals were grouped into 4 groups consisting of four rats in each group consisting of 6 animals. Group 1: Control; Group 2: STZ-induced diabetes; Group 3: Diabetes+*A. Nervosa;* Group 4: Control+*A.nervosa* for 30 days. At the end of the treatment period, animals were anesthetized, serum was collected, skeletal muscle was dissected out and used for the assessment of various parameters. Fasting blood glucose, serum insulin, mRNA expression of IRAP, VAMP-2 and GLUT 4 mRNA expression were carried out.The data were statistically analyzed using One-Way-ANOVA followed by Duncan's multiple range test were used to check the statistical significance and considered at the levels of $p<0.05$.

Results: STZ-induced rats showed an increased fasting serum glucose and serum insulin level compared to normal control rats ($p<0.05$). However, type-

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2 diabetic rats treated with A.nervosa extract significantly reduced hyperglycemia and hyperinsulinemia effectively. mRNA expression of molecules involved in the GLUT4 translocation in the skeletal muscle were also studied by Real Time-PCR. Results of mRNA expression showed that GLUT4 mRNA expression was significantly reduced in STZ-induced rats. Similarly, IRAP and VAMP-2 mRNA levels were also found to be significantly reduced due to STZ-induced hyperglycemia. *A. nervosa* treatment brought back to the normal levels of GLUT4, IRAP and VAMP-2 effectively.

Conclusion

It is concluded from the present findings that *A. nervosa* reduces diabetic complications by facilitating the expression of molecules involved in the GLUT4 translocation such as IRAP, VAMP2 mRNA expression in the gastrocnemius muscle. Hence, *A. nervosa* could be used as a therapeutic drug candidate for the treatment of diabetes mellitus and associated complications.

Keywords: Novel method, Argyreia nervosa, IRAP, VAMP2, GLUT4, type-2 diabetes, drug development, Innovative technique.

1. INTRODUCTION

Argyreia nervosa is a perennial vine which grows primarily in the Indian subcontinent and was later introduced to Hawaii, Africa and the Caribbean. The common name of this plant is elephant creeper and it is widely known for its aesthetic and medicinal value and also for its entheogenic properties (1), (2). This vine contains many alkaloids such as ergines, ergometrine,lysergol and lysergic acid. The seeds of *Argyreia nervosa* contain ergot alkaloids in varying concentrations. The root of this plant has been in use in Indian ayurvedic medicinal treatment. Argyreia nervosa belongs to the phylum spermatophyta and to the subphylum angiospermae and to the family dicotyledonae. This plant mostly grows in the tropical rainy and monsoon climate.

GLUT4 (glucose transporter type 4) is the insulin mediated glucose transporter primarily found in the adipose tissue and striated muscles. The first evidence for the glucose transporter gene was provided by David James in 1988. This mechanism was commonly observed in skeletal muscles, adipose tissue and cardiac muscle. The GLUT-4 mechanism is an example of cascade effect where the binding of a ligand to a membrane causes a cellular response in return.

Streptozotocin is an antineoplastic agent that is particularly toxic to the beta cells of the pancreas of mammals. It is used in medicine to treat cancers occurring in the langerhans cells of the pancreas and in medical research for producing an animal model in rats for studying hyperglycemia and Alzheimer's. Streptozotocin is approved by the FDA for using it as a drug for treatment of metastatic cancer of pancreatic islet cells (3), (4).

IRAP gene is Insulin-regulated aminopeptidase and it is one of the genes which are expressed to assess antiglycemic activity. It is a membrane bound protein with broad tissue distribution and dual functions. It is the companion protein for insulin responsive glucose transporter in specialized vesicles or endosomes or the cell surface. The main function of IRAP gene is intracellular cell trafficking for the N-terminal and the intraluminal domain is responsible for the aminopeptidase activity to trim peptides (5). VAMP-2 gene is encoded by the Vesicle associated membrane protein 2. This gene is thought to participate in neurotransmitter release at

a step between docking and fusion. Heterozygous mutations in VAMP2 gene causes a neurodevelopmental disorder with hypotonia and autistic features. This gene is known to interact with RABAC1,SNAP-25,SNAP-23,STX1A and STX4 (6)
Type 2 diabetes mellitus is a defect in the way the body regulates and uses glucose as a fuel. It is a long term chronic condition which results in too much glucose circulation in the bloodstream. Hyperglycemia can lead to disorders of the circulatory, nervous and immune system. There is no such cure for diabetes mellitus type 2 but losing weight, eating well and exercising can help to regulate the defect (7). If proper diet and exercise are not enough to regulate the blood sugar level then the patient might also have to take diabetes medications or insulin injections. Symptoms of diabetes mellitus 2 include increased thirst, frequent urination, increased hunger, intended weight loss, fatigue, blurred vision, slow healing sores, frequent infections, etc (8).

2. MATERIALS AND METHODS

Chemicals

The entire chemicals and reagents used in this research were of molecular and analytical grade acquired from sigma chemical company and SISCO research laboratories (Mumbai, India)

Collection of plant materials

The species will be verified at Anna Siddha hospital in Chennai, Tamil Nadu, using Argyreia nervosa root powder obtained from a pharmacy.

Extract preparation

The roots of Argyreia nervosa powder were soxhlet extracted with 70% ethanol. The extract was then filtered with Whatman no. 1 filter paper and the solvent evaporated at reduced pressure by using a Rotary evaporator apparatus to get a viscous mass, which was then stored at 4°C until used (Kokate 2001).

Wistar rats are an outbred strain of albino rats belonging to Rattus norvegicus. This strain was developed at the wistar institute in 1906 hence the name. These strains of rats are just used for medical research purposes and is notably the first rat strain developed to function as a model organism at a time where laboratories used the common mouse found in homes for research. Now it is the most popular rat strain used for laboratory research (9) (10). These rats are characterized by their wide head, long ears and whose tail length is always lesser than the body length. Studies have shown that *A.nervosa* has hypoglycemic effects but the molecular mechanisms underlying the antidiabetic potential are not known (11). Our team has extensive knowledge and research experience that has translate into high quality publications (12–21))((22–31).
In this study, we showed the possible mechanisms by which *A.nervosa* could regulate insulin signaling by facilitating the GLUT4 translocation via mediating IRAP and VAMP-2 molecules.

Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics committee (BRUTAL/SDCH/SIMATS/IAEC/04-2022/109). Healthy adult male Wistar albino rats of Wistar strain (150–180 days old weighing 180–200 g) were used in this study and maintained in clean polypropylene cages at the Biomedical Research Unit and Lab Animal Center (BRULAC), Saveetha Dental College & Hospitals, Saveetha Institute of Medical & Technical Sciences, Chennai – 600 077, Tamil Nadu, India, under specific humidity (65 ± 5%) and temperature (21 ± 2°) with constant 12 h light and 12 h dark schedule. The standard pellet diet (Lipton India, Mumbai, India) was provided with clean drinking water in ad libitum.

Induction of type-2 diabetes

Diabetes was induced in rats by a single intraperitoneal administration of STZ (55 mg/kg) dissolved in 0.1 M citrate buffer, pH 4.5. 48 hours later, blood samples were collected and glucose levels were estimated to confirm the development of diabetes. The rats that showed hyperglycemia (blood glucose level > 250 mg/dl) were selected for experimental study (Shiv 2010).

Experimental design

Animals were grouped into 3 groups of six animals each and treated oral administration for 15 days.

Group I – Normal rats

Group II- diabetic rat

Group III - diabetic rat + oral administration of *Argyreia nervos*a 500 mg/kg b.wt/day

Group IV - normal rat + oral administration of *Argyreia nervosa* 500 mg/kg b.wt/day

Fasting blood glucose (FBG)

After the overnight fasting, the blood glucose was estimated using On-Call Plus blood glucose test strips (ACON Laboratories Inc., USA). From the rat tail tip, the blood was collected and the results were expressed as mg/dl.

Fasting serum insulin

Serum insulin was assayed using ultrasensitive rat insulin ELISA kit obtained from Crystal Chem Inc (Illinois, USA). The range of detection is 0.1–64 ng/ml. The percentage cross reactivity of insulin antibody to rat insulin was 100%. The intra-assay coefficient of variation was ≤10.0% and inter-assay coefficient of variation was ≤10.0%. Results were expressed as mIU/ml.

Gene expression analysis

Total RNA isolation, cDNA conversion and real-time PCR

Using a TRIR kit (Total RNA Isolation Reagent Invitrogen), total RNA was isolated from control and experimental samples. In brief, to 100 mg of fresh tissue, 1 ml of TRIR was added and homogenized. The content was transferred to a microcentrifuge tube instantly and 0.2 ml of chloroform was added, vortexed for 1 min then kept at 4°C for 5 min. Later, the contents were centrifuged at 12,000 ×g for 15 min at 4°C. The aqueous phase (upper layer) was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 S and placed on ice for 10 min. After centrifugation of the content at 12000 ×g for 10 min at 4°C, the supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol by the vortex. The isolated RNA was estimated spectrometrically by the method of Fourney et al. (1988). The RNA concentration was expressed in micrograms (µg). By using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2 µg of total RNA as stated in the manufacturer's protocol. To perform real-time PCR, the reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), Forward and reverse primers of the target gene and house-keeping gene, water and β-actin (the primer sequences were listed in Table 1) in total volume of 45 µl expect the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 µl of control DNA for positive control, 5 µl of water for negative control and 5 µl of template cDNA for samples were taken and reaction mixture (45 µl) were added. 40 cycles (95°C for 5 min, 95°C for 5 s, 60°C for 20 s and 72°C for 40 s) was set up for the reaction and obtained results were plotted by the PCR machine (Stratagene MX 3000 P, Agilent Technologies, 530 l, Stevens Creek Blvd, Santa Clara CA, 95051) on a graph. Relative quantification was calculated

from the melt and amplification curves analysis.

List of primers used in this study

Gene name	Primer sequence	Reference
IRAP	Sense: 5′-CCA GAT GTG GTA GAT TTA GCC-3′	Han
	Anti-sense: 5′-CTG CCT GTA GCC TGT TGC-3′	
Vamp 2	Sense : 5′- GCA TCT CTC CTA CCC TTT CA-3′	Dias
	Anti-sense: 5′- TTT AGG GGT CTG AGG GTA CA-3′	
GLUT4	Sense: 5′-CCT TGC CTC GCT GCT GTA-3′	Bayat
	Anti-sense: 5′-CCT CCT GCC TTA GTT GGT CA-3′	
Rat β-actin	Sense primer: 5′ - GAC GTT GAC ATC CGT AAA GAC C-3′	Prasad 2022
	Anti-sense primer: 5′ - TGC TAG GAG CCA GGG CAG TA-3′	

Statistical analysis

The data will be analyzed statistically and ONE-WAY- ANOVA will be used followed by Dencan's multiple range test will be used to check statistical significance among groups. The significance will be considered at the levels of $P<0.05$.

3. RESULTS

Effect of A.nervosa root extract on fasting blood glucose and fasting serum insulin in STZ-induced experimental type-2 diabetic rats

STZ-induction significantly raised fasting blood sugar levels and serum insulin concentration ($p<0.05$) when compared with control suggesting that STZ-causes hyperglycemia and hyperinsulinemia in experimental rats (Table 1 & 2). However, oral administration of A.nervosa for a period of 30 days, reduced hyperglycemia and hyperinsulinemia significantly and this study clearly indicates that A.nervosa plays an important role in mitigating diabetes mellitus.

Table 1

FBG				
Mean	77	197.5	105.5	86
Std. Deviation	7.071	10.61	7.778	8.485
Std. Error	5	7.5	5.5	6

Table 2

Serum insulin				
Mean	37.5	84.5	59	52.5
Std. Deviation	3.536	7.778	5.657	4.95
Std. Error	2.5	5.5	4	3.5

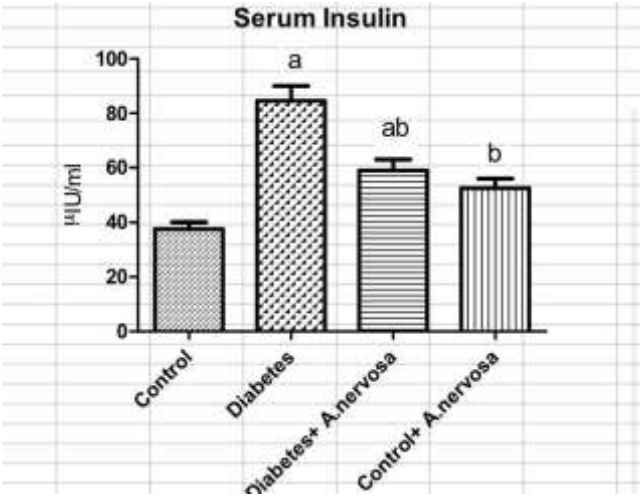

Table 1 & 2 and Figure 1: Effect of A.nervosa root extract on fasting blood glucose and fasting serum insulin in STZ-induced experimental type-2 diabetic rats. Each bar represents the mean±SEM of six animals (n=6). Significance at p<0.05, a-compared with control, b-compared with STZ- induced type-2 diabetic rats. Blood glucose concentration was given as mg/dl concentration while serum insulin has been expressed as micro international unit per ml.X-axis indicates group while Y-axis represents concentration of glucose given as mg/dl concentration while serum insulin has been expressed as micro international unit per ml.

Effect of *A.nervosa* root extract on GLUT4 mRNA expression in the skeletal muscle of STZ-induced experimental type-2 diabetic rats

Since glucose is a polar molecule, it has a difficult time diffusing through the hydrophobic plasma membrane. Specific carrier molecules called glucose transporters are involved in glucose absorption into tissues (GLUTs). These transporters have 12 transmembrane domains and facilitate glucose diffusion down a concentration gradient without requiring energy. The GLUT family consists of 13 members that can be classified into three subclasses based on structural similarities. Among which GLUT4 protein is responsible for the transport of glucose from circulation into adipose tissue and muscle cells. In this study, diabetic rats showed a significant decline in the GLUT4 mRNA levels in cytosolic and plasma membranes (Table 3 and Fig. 2). However, *A.nervosa* treatment significantly raised its level in type-2 diabetic rats.

Table 3

Glut4				
Mean	1	0.385	0.8	0.95
Std. Deviation	0	0.09192	0.1273	0.05657
Std. Error	0	0.065	0.09	0.04

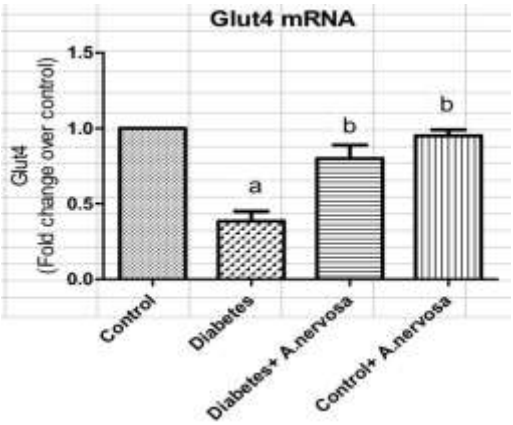

Table 3 and Figure 2: Effect of A.nervosa root extract on GLUT4 mRNA expression in STZ-induced experimental type-2 diabetic rats. Each bar represents the mean±SEM of six animals (n=6). Significance at p<0.05, a-compared with control, b-compared with STZ- induced type-2 diabetic rats. mRNA expression has been represented in fold change over control. X-axis indicates groupings while Y-axis represents mRNA expression of GLUT4 representing in-fold change considering healthy control animals.

Effect of A.nervosa root extract on IRAP and VAMP-2 mRNA expression in the skeletal muscle of STZ-induced experimental type-2 diabetic rats

Insulin regulated aminopeptidase (IRAP) is a type II transmembrane protein with broad tissue distribution initially identified as a major component of Glut4 storage vesicles (GSV) in adipocytes (Table 4, 5 and Fig 3 & 4). In the present study, STZ-induced type-2 diabetic rats showed a significant reduction in the mRNA expression of IRAP and VAMP-2 compared with control. Treatment with A.nervosa root extract, showed a significant increase in the mRAN levels whose effects were found to be near to that of the control animals ($p<0.05$) suggesting that A.nervosa has a significant role over the GLUT4 vesicle translocation.

Table 4

IRAP				
Mean	1	0.45	0.75	0.85
Std. Deviation	0	0.07071	0.07071	0.07071
Std. Error	0	0.05	0.05	0.05

Table 5

VAMP-2				
Mean	1	0.465	0.695	0.965
Std. Deviation	0	0.06364	0.06364	0.0495
Std. Error	0	0.045	0.045	0.035

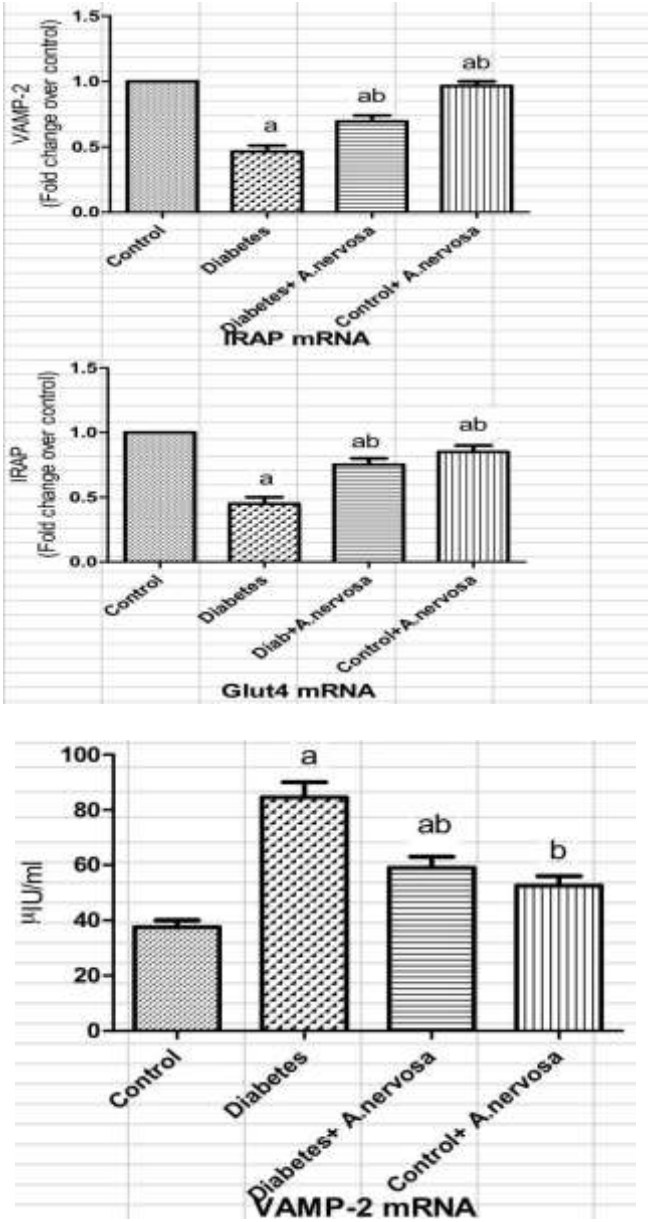

Table 4 & 5 and Figure 3 & 4: Effect of A.nervosa root extract on IRAP and VAMP-2 mRNA expression in STZ-induced experimental type-2 diabetic rats. Each bar represents the mean±SEM of six animals (n=6). Significance at p<0.05, a-compared with control, b-compared with STZ- induced type-2 diabetic rats. mRNA expression has been represented in fold change over control. X-axis indicates groupings while Y-axis represents mRNA expression of VAMP-2 and IRAP represents in-fold change considering healthy control animals.

4. DISCUSSION

From the graph obtained for serum insulin it can be said that for diabetes induced rats the serum insulin becomes very high and on feeding the rats with *Argyreia nervosa* plant extract reduces diabetes in STZ induced diabetic rats and when the control group is fed with the plant extract then also diabetes is reduced. Our team has extensive knowledge and research experience that has translate into high quality publications (32), (33), (34), (35), (36), (37,38), (39), (40), (41), (42).

From the graph obtained for the gene IRAP mRNA, the STZ induced diabetic rats show decrease in the IRAP gene expression and when the rats are fed with the plant extract the IRAP gene expression increases hence facilitating GLUT-4 translocation in the skeletal muscle. Similar results were obtained for the graph obtained for the VAMP-2 gene too, as the IRAP gene. Hence from gene expression analysis it can be determined that both the genes IRAP and VAMP-2 are increased when the STZ induced diabetic rats are fed with the *Argyreia nervosa* root extract hence facilitating the GLUT-4 translocation in the plasma membrane of the skeletal muscle.(43) (44).

From the graph obtained for GLUT4 mRNA, it was observed that for rats with diabetes the GLUT4 mechanism was found to be drastically affected but after feeding the rats with Argyreia nervosa root extract, the GLUT-4 mechanism was again found to happen normal in the skeletal muscle of the rats. (45).

Previous studies show that the plant extract of argyreia nervosa contains 1-triacontanol, epifriedelinol acetate, epifriedelinol and beta sitosterol and hexane extract of the root yielded tetradecanyl palminate, 5,8-oxidotetracosan-10-one and two novel aryl esters along with scopoletin. Even though most of these were said to have antioxidant activity, a prominent hypoglycaemic activity has not still been proven for any of these constituents.(46) No study till now has studied these two genes- IRAP and VAMP2 in relation to their effect on GLUT4 mechanism. From this study we conclude that Argyreia nervosa does facilitate GLUT4 translocation by expressing the genes IRAP and VAMP2 in the plasma membrane of STZ induced diabetic rats and reduces diabetes in them. (47)

5. CONCLUSION

From the above study, it is concluded that the root extract of Argyreia nervosa root extracts potentiates hypoglycemic effects by facilitating the expression of IRAP and VAMP-2 that leads to the translocation of GLUT4 on the plasma membrane of diabetic gastrocnemius muscle and thereby regulated the normoglycemic effects in streptozotocin-induced diabetic rats. Hence, A. nervosa could be considered as a therapeutic drug for the treatment of type-2 diabetes. Moreve, current findings are the first report on the role of A.nervosa on the GLUT4 translocation. Further studies on the human cell line models are warranted in order to prove the potential mechanisms of action of A.nervosa prior to clinical trials.

Conflict of Interest:

The authors hereby declare that there is no conflict of interest in this study.

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Author Contribution:

A) Sumedha Balaji- contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

B) Dr. Selvaraj - contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

C) Dr.V.Vishnu Priya - contributed in study design, guiding the research work, manuscript correction.
D) Dr. Gayathri R - study design, statistical analysis, manuscript proofreading and correction.
E) Dr. Kavitha S - study design, statistical analysis, manuscript proofreading and correction.

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