Online resource 1

Materials and methods

In silico studies

Collection of data and preparation

To determine the phyto-chemicals present in *Gymnema sylvestre* leaf extract having antidiabetic activity, the Indian Medicinal Plants, Phytochemistry and Therapeutics 2.0 (IMPPAT 2.0) database was used. The phytochemicals having antidiabetic activity, such as Gymnemagenin, Gymnemic acid I, Gymnemic acid IV, Gymnemic acid VII, Lupeol, Quercitol, and stigmasterol, were selected and the SuperPred Prediction database (https://prediction.charite.de/) and Swiss TargetPrediction (https://www.swisstargetprediction.ch/) were utilised to predict possible targets of the selected phytochemicals. Subsequently, therapeutic targets related to diabetes were gathered from the Genecards database, excluding those with a Relevance score below 1 (https://www.genecards.org/). The relevant targets downloaded from both databases were combined, and duplicates were removed to identify the potential targets. Additionally, the online tool available at (https://bioinformatics.psb.ugent.be/webtools/Venn/) was utilised to create a Venn diagram in order to identify the core targets shared between "*Gymnema sylvestre* leaf extract" and "Diabetes".

Development of the Protein-Protein Interaction Network

To construct protein-protein interaction (PPI) network, we used the STRING database (https://string-db.org/). PPI networks are valuable for understanding protein functions and their interrelationships [1]. On the STRING platform, we input the core target proteins using "Multiple proteins" option and selected "*Rattus norvegicus*" as organism. The required score was set to the highest confidence (0.900). This process resulted in the construction of the PPI network. Cytoscape (v3.8.0) was employed to create a visual PPI network and identify the hub genes [2]. The ranking system of node connect degree (Degree) was used to identify the top 10 hub genes. Each method was applied to screen the top 10 genes. These ranking methods are available in Cytoscape's Cytohubba plug-in [3].

Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

We utilised Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to understand functions of the core targets and the key pathways

involved in *Gymnema sylvestre* leaf extract's action against diabetes. These analyses were conducted using online tool called ShinyGO 0.82 (https://bioinformatics.sdstate.edu/go/). The GO functional enrichment analysis categorised results into biological processes, cellular components, and molecular functions, which were presented in a column chart. Higher count values indicated a stronger association with the core targets. The results of KEGG pathway enrichment analysis were displayed using bubble diagrams and histograms, with statistical significance determined at $p<0.05$.

Molecular docking

Molecular docking studies were conducted using AutoDock Tools version 1.5.7 to investigate binding affinity between selected phytochemicals and key chemokine signalling pathway receptors. The three-dimensional crystal structures of the target proteins C-C chemokine receptor type 2 (CCR2), C-X-C chemokine receptor type 4 (CXCR4), C-X-C chemokine receptor type 7 (CXCR7), and C-C chemokine receptor type 5 (CCR5) were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/). Corresponding ligand structures, including Gymnemagenin, Lupeol, and Stigmasterol, were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in SDF format and converted to PDB format for docking using OpenBabelGUI. Prior to docking, all protein structures were pre-processed by removing water molecules and heteroatoms, adding polar hydrogen atoms, and assigning Gasteiger partial charges. The prepared protein structures were then saved in PDBQT format for use in docking simulations. Gymnemagenin was docked with CXCR4, CXCR7, CCR2, and CCR5; Lupeol was docked with CXCR4, CCR2, and CCR5; and Stigmasterol was docked with CXCR7, CCR2, and CCR5. Molecular docking was performed with a binding energy threshold of ≤ -5.0 kJ·mol^{-1}, indicating favourable interactions. The resulting protein–ligand complexes were visualised using BIOVIA Discovery Studio Visualizer for two-dimensional interaction mapping and UCSF ChimeraX for three-dimensional structural representation.

In vivo experimental study

Place of study

The experiment on rats was conducted at ICAR-IVRI, Bareilly, India. The conceptualisation, draft preparation, statistical analysis, and editing of the manuscript were carried out at ICAR- NRCM, Nagaland, with contributions from all authors.

Study conditions

The rats were housed in plastic cages according to European Standards, at room temperature (22±3°C), 50% average relative humidity, central ventilation (15 air changes/ hour) with a 12:12 light cycle, and fasted for 12 hours. The composition of the control diet (Standard rat chow diet: Group N) was protein (25%), carbohydrates (40%), fat (5%), fibre (3%), and other constituents like vitamins and minerals.

Chemicals and kits

Streptozotocin [N-(methylnitrosocarbamoyl)-α-D-glucosamine] was purchased from Sigma Aldrich (St. Louis, MO, USA) and prepared in a 100 mM citrate solution (pH 4.5). Analytical kits were obtained from Coral Clinical Systems, Goa, India. Leptin, Adiponectin, and Insulin levels were measured with the Invitrogen Rat Leptin ELISA Kit, the Invitrogen Rat Adiponectin Rapid ELISA Kit, and the Rat Insulin ELISA Kit, respectively, procured from Thermo Fisher Scientific (Waltham, MA, USA). All analyses were done according to the manufacturer's instructions.

Preparation and Evaluation of Hydro-Ethanolic Extract from *G. sylvestre* leaves

Fresh leaves of *Gymnema sylvestre* (Retz.) R.Br. ex Sm. were collected from ICAR–Indian Veterinary Research Institute, Izatnagar, Bareilly district, Northern India, during February-March and identified and authenticated by the Botanical Survey of India (Voucher No. CNH/1-1/2020 Tech 14). The scientific name was validated using https://www.plantsoftheworldonline.org and cross-checked with https://mpns.kew.org/mpns-portal (accessed 09 March, 2026) to ensure conformity with the latest taxonomic classification. The leaves were washed, air-dried, and powdered. A hydro-ethanolic extract was prepared using a 1:1 aqueous: ethanol solvent system, filtered, and evaporated with a rotary evaporator. The extract was then dried in vacuo and stored at 4°C. Before the study, to determine the half maximal effective concentration (EC_{50}) of the *G. sylvestre* leaf extract, 40 adult male obese albino Wistar rats (body weight 280-290 grams) were divided into 5 groups. Dextrose (2 mg/kg body weight) was administered orally, and five doses of the extract (100, 150, 200, 250, 300 mg/kg) were given to the respective groups. Blood glucose levels were monitored hourly for 6 hours using glucometer strips (Accu-Check, Bombay, India). The dosage that reduced blood glucose by approximately 50% within 1-2 hours was identified as the EC_{50}. This extract (EC_{50}) was administered orally for 90 days via gastric gavage.

Induction of obesity and metabolic alteration

Experimental rats treated groups were fed for 4 months with a feed mixture of corn oil and other dietary requirements, a high-fat diet (HFD) formulated *in-house*. The HFD comprised 58% fat, 25% protein, and 17% carbohydrate as a percentage of total kilocalories along with vitamins and minerals. Induction of insulin resistance in rats was carried out as previously reported, by feeding the HFD for a minimum of two weeks, followed by injection of Streptozotocin (STZ) into the rats [4].

Confirmation of obesity

Obesity status was assessed after 15 weeks of feeding using body mass index (BMI), where calculation of BMI is based on body weight and length. The length of each rat was measured from the tip of the nose to the anus. In adult male Wistar rats, a normal BMI ranges from 0.45 g/cm² to 0.68 g/cm² [5], with obesity defined as a BMI exceeding 0.68 g/cm² [6]. Rats in the HFD groups that did not reach the obesity threshold after 16 weeks (4 months) were excluded from the study. However, all experimental rats successfully attained the target BMI and were included in the analysis.

Induction of pancreatic beta cell dysfunction

The STZ injection caused the dysfunction of β cells in the pancreas [7]. Feeding of HFD to rats with pancreatic dysfunction resulted in hyperglycemia, glycosuria, hypercholesterolemia, increased triglycerides, ketonemia, and ketonuria [8]. Therefore, the HFD/STZ-treated rats simulated the natural disease progression and metabolic alterations specific to T2DM patients [7].

Induction of diabetes by Streptozotocin (STZ) administration

To induce diabetes, the rats were subjected to a 12-hour fast (water provided) before administration of STZ. Streptozotocin was dissolved in ice-cold 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally @ 35 mg/kg body weight, which is a low dose used for the induction of type 2 diabetes. The non-diabetic control animals received only citrate buffer intraperitoneally.

Determination of diabetes

The diagnosis of diabetes was established through comprehensive clinical examination and serum analysis. Diabetic animals demonstrated increased polyuria, polyphagia, and polydipsia compared to control group (standard diet and HFD). Animals injected with streptozotocin were markedly diabetic, as indicated by elevated fasting serum glucose levels. Fasting plasma glucose levels were determined, where the initial glucose levels were

comparable across all groups. After 72 hours of the STZ injection, blood samples from animals were tested for glucose levels. A plasma glucose level of ≥ 300 mg/dl was considered to be the onset of diabetes in rats. Serum insulin concentrations were assessed via a radioimmunoassay kit, with rat insulin serving as the reference standard. To evaluate insulin resistance, the Homeostasis Model Assessment for Insulin Resistance (HOMA-R) was calculated using the formula: HOMA-R = fasting glucose (mmol/L) × fasting insulin (μU/mL) / 22.5 [9].

Experimental design

The study was conducted at ICAR-IVRI, Bareilly, India, with prior permission from the Institute Animal Ethics Committee. Adult male Albino Wistar rats (IVRI2CQ strain) of 16 weeks of age, with a body weight of 280-290 grams, were procured from the Laboratory Animal Resources (LAR) section of ICAR-IVRI, Bareilly and acclimatized in the laboratory for 7 days. The body weight and food and water consumption of the animals were measured daily. The rats (n=40) were randomly divided into 5 groups, *viz.,* standard diet (N), high-fat diet (A), HFD+STZ (B), insulin-treated (C), and extract-treated (D) of 8 animals each. The treated animals were individually housed in separate cages to minimise physical activity while maintaining visual, auditory, and olfactory interactions with other rats throughout the diet-induced obesity experiment. Both the HFD and standard rat chow diets were formulated and standardized by the Division of Nutrition at the Institute of ICAR-IVRI, Bareilly.

Evaluation of the Efficacy of *G. sylvestre* Leaf Extract on the Amelioration of Adipose Tissue Dysfunction

Various parameters were evaluated throughout the experiment to assess the efficacy of *G. sylvestre* leaf extract. Body Mass Index (BMI) was measured weekly using body weight (g) divided by length squared (cm^2) [10]. Urinalysis was performed by housing rats in individual metabolic cages for 6-8 hours to collect urine, with glucose levels assessed semi-quantitatively using Benedict's reagent and ketone bodies detected using Ornialysis® test strips. Blood analyses were conducted on Days 0, 45, and 90 by collecting samples from retro-orbital venous plexus using heparinised micro-hematocrit capillaries. Using different capillary tubes for different parameters, blood was collected from rats, which were under light ether anaesthesia using ether-soaked cotton in a chamber. Plasma glucose and hematological parameters were analyzed using sodium fluoride and K2 EDTA vacutainers, respectively, while serum for lipid and thyroid profiles was separated by centrifugation at 3500 rpm and stored at 4°C. Biochemical analyses of blood included glucose estimation using GOD-POD [11], and triglycerides measurement with GPO/PAP method [12]. Furthermore, Total cholesterol using CHOD/PAP [13], Low-density

lipoprotein cholesterol (LDL-C) using Direct Enzymatic Method [14], High-density lipoprotein cholesterol (HDL-C) using Direct Enzymatic Method [15], Very-low density lipoprotein cholesterol (VLDL-C) were also measured [16]. The assessment of T_3, T_4, and TSH was done by using ELISA as described previously [17]. PCR was performed with gene-specific primer sets using SSO fast Evagreen Supermix (Bio-Rad Laboratories). For this purpose, the 18sRNA gene was used as a housekeeping control. Gene-specific primers were designed such that every set had a 57°C annealing temperature. Analysis of qPCR data was done following the methodology described previously [18].

Statistical analysis

Statistical analysis was done using IBM SPSS software version 22 (SPSS Inc., Chicago, USA). All parametric values were represented as Mean ± Standard Error of Mean (SE) except for urine glucose and urine ketone bodies (non-parametric data). These non-parametric data are expressed using Score means ± SE. The General Linear Model with Two-way ANOVA was applied, followed by Tukey's test for multiple comparisons between different groups [19]. The level of significance was set at $p<0.05$ for all analyses.

References

1. Athanasios A, Charalampos V, Vasileios T, Ashraf GM. Protein-protein interaction (PPI) network: recent advances in drug discovery. Curr Drug Metab. 2017;18(1):5-10. doi: 10.2174/1389200218011701192048 32.
2. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13(11):2498-2504. doi: 10.1101/gr.1239303.
3. Chin CH, Chen SH, Wu HH, Ho CW, Ko MT, Lin CY. cytoHubba: identifying hub objects and sub-networks from complex interactome. BMC Syst Biol. 2014;8(Suppl 4):S11. doi: 10.1186/1752-0509-8-S4-S11.
4. Reed MJ, Meszaros K, Entes LJ, Claypool MD, Pinkett JG, Gadbois TM, Reaven GM. A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. Metab Clin Exp. 2000;49(11):1390-1394.
5. Novelli ELB, Diniz YS, Galhardi CM, Ebaid GMX, Rodrigues HG, Mani F, Fernandes AAH, Cicogna AC, Novelli Filho JLVB. Anthropometrical parameters and markers of obesity in rats. Lab Anim. 2007;41(1):111-119. https://doi.org/10.1258/0023677077793995

6. Mutiso SK, Rono DK, Bukachi F. Relationship between anthropometric measures and early electrocardiographic changes in obese rats. BMC Res Notes. 2014;7(1):931. https://doi.org/10.1186/1756-0500-7-931
7. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. Pharmacol Res. 2005;52(4):313-320. https://doi.org/10.1016/j.phrs.2005.05.004
8. Shafrir E, Sima AA. Animal Models in Diabetes: A Primer. 1st ed. CRC Press, London; 2003.
9. Madan R, Varghese RT. Assessing Insulin Sensitivity and Resistance in Humans. Endotext. https://www.ncbi.nlm.nih.gov/sites/books/NBK278954/
10. Du Bois D, Du Bois EF. Clinical calorimetry: tenth paper a formula to estimate the approximate surface area if height and weight be known. Arch Intern Med. 1916;17(6_2):863-871. doi:10.1001/archinte.1916.00080130010002
11. Trinder P. Estimation of blood glucose by GOD-POD method. Ann Clin Bio. Chem. 1969a;6:24.
12. Trinder P. Estimation of serum total cholesterol by using chod-pod enzymatic reaction. Ann Clin Biochem. 1969b;6:24.
13. Trinder P. Quantitative determination of triglyceride using GPO-PAP method. Ann Biochem. 1969c;6:24-7.
14. Okada M, Matsui H, Ito Y, Fujiwara A, Inano K. Low-density lipoprotein cholesterol can be chemically measured: a new superior method. J Lab Clin Med. 1998;132(3):195-201. https://doi.org/10.1016/S0022-2143(98)90168-8
15. Naito HK, Kaplan AQ. High-density lipoprotein (HDL) cholesterol. Clin Chem. 1984;1207:1213.
16. Wilson PW, Abbott RD, Garrison RJ, Castelli WP. Estimation of very-low-density lipoprotein cholesterol from data on triglyceride concentration in plasma. Clin Chem. 1981;27(12):2008-2010. https://doi.org/10.1093/clinchem/27.12.2008
17. Agharanya JC. Clinical usefulness of ELISA technique in the assessment of thyroid function. West Afr J Med. 1990;9(4):258-263.
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 2001;25(4):402-408. https://doi.org/10.1006/meth.2001.1262
19. Snedecor GW, Cochran WG. Statistical methods. 8th ed. Iowa State University Press, Ames; 1989.