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Formulation and evaluation of SGLT2 inhibitory effect of a polyherbal mixture inspired from Ayurvedic system of medicine

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ABSTRACT

Background and aim: The ingredients viz., *Artemisia roxburghiana, Cissampelos pareira, Stephania glabra, Drimia indica, Roylea cinerea, Tinospora sinensis* and *Curcuma longa* of the present formulation are used to treat diabetes in the Indian traditional medical system. Adopting the concept of multiple herbal mixtures for better therapeutic effects from the ancient Ayurvedic text Sarangdhar Samhita, the present study aimed to develop a polyherbal formulation (PHF) of seven herbs and to evaluate its sodium-glucose cotransporter protein-2 (SGLT2) inhibitory effect on type 2 diabetic rats.

Experimental procedure: Streptozotocin (STZ) (60 mg/kg) and nicotinamide (NAM) (120 mg/kg) were intraperitoneally administered to induce type 2 diabetes in Wistar rats. The animals were divided into 5 groups viz. normal control, diabetic control, positive control (dapagliflozin at 0.1 mg/kg) and two test groups (PHF at 250 and 500 mg/kg). Various parameters including blood glucose, serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), bilirubin, triglycerides and creatinine were measured.

Results and conclusion: The treatment with PHF (250 and 500 mg/kg) showed a significant ($p < 0.05$) decrease in blood glucose levels by 56.37% and 58.17%, respectively. The levels of SGOT, SGPT and bilirubin were significantly reduced in PHF-fed diabetic rats. Histopathological examination revealed no major changes in the treated groups as compared to the normal control. The molecular docking study showed strong binding of β-sitosterol, insulanoline, warifteine, dehydrocorydalmine, taraxerol acetate, lupeol, corydalmine and luteolin to SGLT2 protein. The present study concludes that PHF has promising antidiabetic activity via inhibiting SGLT2 protein without showing any adverse effects.

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1. Introduction

Diabetes mellitus (DM), a metabolic chronic disorder, is one of the major health issues that reached its alarming stage and affected around 500 million people worldwide irrespective of their genders. This number is projected to reach 700 million by the year 2045.[1] Out of the total diabetes cases, almost 90% belong to type 2 DM which is mainly managed by different oral hypoglycemic drugs including metformin, glipizide, acarbose and miglitol. However, the rest 10% cases belong to type 1 DM which needs insulin therapy to manage the glucose level.[2] The global expenditure on diabetes and its related complications was estimated to be $760 billion in the adults' population of which almost 70% of expenditure belongs to the aged population of 50 years and above.[3]

Almost 90% of the existing traditional medicines worldwide are mainly obtained from medicinal plants and are commonly used in their crude forms.[4] The most advantage of herbal treatment is its high therapeutic value with fewer adverse effects. Importantly, most of the herbal remedies including spices can be used without consulting any practitioner because their use is based on traditional knowledge that transfers from one generation to another. It is evident that the remedial values of medicinal plants are due to the presence of their secondary metabolites such as alkaloids, flavonoids, steroids and terpenoids.[5] In addition, primary metabolites like carbohydrates, proteins, vitamins and lipids also played a significant role in human health mainly in body-building and energy production.

The ethnobotanical knowledge had reported more than 800 medicinal plants particularly useful in DM.[6] Most of these plants are also found in India and still in traditional practice in rural areas.[7] For the past two decades, the medicinal plants are extensively studied for their efficacy against DM for their scientific validation and also to find out an alternate for the existing antidiabetic drugs which are known for their serious side effects.[8] Based on the recent findings, these plants are capable to reduced blood glucose level via different mechanisms including improvement in insulin secretion and glucose utilisation, activation of insulin receptors, regeneration of pancreatic β-cells and inhibition of α-glucosidase, α-amylase and sodium-glucose transport protein 2 (SGLT2).[9,10] In addition to using as crude, purified molecules like berberine, curcumin, mangiferin, stevioside and capsaicin obtained from these plants have also been investigated for their antidiabetic effect and found more effective than their source extracts.[11]

Inhibition of SGLT2, a protein responsible for 80–90% glucose reabsorption in the kidney, is presently one of the key targets in the treatment of type 2 DM and diabetes-associated complications like diabetic kidney disease.[12–14] However, possible side effects like urinary tract infections, foot amputations and kidney damage by existing SGLT2 inhibitors viz. canagliflozin, dapagliflozin and empagliflozin are a matter of serious concern.[15,16] Hence, herbal medicine with high efficacy and the least adverse effect can be developed as a substitute for existing SGLT2 inhibitory drugs to treat type 2 diabetic patients. In this direction, a formulation was developed with seven traditional antidiabetic herbs viz. *Artemisia roxburghiana* (aerial parts), *Cissampelos pareira* (roots), *Stephania glabra* (tubers), *Drimia indica* (tubers), *Roylea cinerea* (aerial parts), *Tinospora sinensis* (stems) and Curcuma longa (rhizomes) to evaluate its SGLT2 inhibitory effect.

Artemisia roxburghiana Wall. ex Besser (Compositae) is used as a traditional medicine in India for diabetes, rheumatic arthritis and malarial fever.[17,18] Earlier scientific studies revealed its antiprotozoal, anthelmintic, anti-inflammatory and antidiabetic potential. Various secondary metabolites such as taraxerol acetate, lupeol, friedelan-3β-ol, friedelin, betulinic acid, betulin, apigenin, artemisinin, apigenin-7,4-dimethyl ether, α-selinene, α-copaene, α-gurjunene, α-eudesmol, curcumene, caryophyllene oxide, β-selinene, germacrene D, β-eudesmol, δ-cadinene, bicyclogermacrene, artemisinin, scopoletin and quercetin have been reported from this plant.[19] *Cissampelos pareira* L. (Menispermaceae) is a popular traditional remedy for ulcers, wounds, arthritis, asthma, cholera, diarrhoea, cancer, inflammation, snakebite, malaria, rabies and diabetes.[20] Its antidiabetic, antimalarial, antiparasitic, antiulcer, antidiarrhoeal and antivenom activities have been scientifically validated in different experimental models. Chemical analysis of its roots showed the presence of bulbocapnine, corydine, dicentrine, norrufescine, insulanoline, isochondrodendrine, warifteine, pelosine, cissampareine, insularine, cissampentin, hayatine, hayatinin, dehydrodicentrine, cycleanine, reserpine, sepeerine, berberine, cycleanine, corytuberine, pareitropone, cissamine, laudanosine and milonine.[21] *Stephania glabra* (Roxb.) Miers (Menispermaceae) is also used traditionally in asthma, tuberculosis, dysentery, diabetes, cancer, fever and inflammation in many Asian countries.[22,23] Similar to *C. pareira*, it is also rich in alkaloidal constituents like roemerine, reticuline, palmatrubine, dehydrocorydalmine, corydalmine, jatrorrhizine, stepharanine, capaurine, magnoflorine, stepholidine, n-desmethylcycleanine, tuduranine, tetrahydropalmatine, columbamine, laurifoline, stepharine, corynoxidine and palmatine.[24] It is evident from the literature that tubers of this plant showed strong antidiabetic, antipsychotic, antibacterial and antihypertensive effects. The bulbs of *Drimia indica* (Roxb.) Jessop (Asparagaceae) are popular for their Ayurvedic properties in cardiac protection, GI disorders, asthma, leprosy, skin problems and diabetes.[25,26] Cichoriol A, urgineol A-F, anhydroscilliphaeosidin, proscillaridin A, scillaren A, scilliphaeoside, scillarenin, β-sitosterol, luteolin, stigmasterol, campesterol, kaempferol and quercetin are the common constituents reported from this plant. Pharmacological studies on the bulbs revealed anticancer, antidiabetic, antimicrobial, anti-inflammatory and bronchodilator action on different models.[27]

Roylea cinerea (D. Don) Baillon (Lamiaceae) is traditionally used for treating diabetes, malaria, stomachache and skin diseases.[28] The aerial parts of this plant exhibited different pharmacological properties including antidiabetic and anticancer. Its phytochemical profile showed the presence of 4-methoxybenzo[b]azet-2(1H)-one, 3β-hydroxy-35-(cyclohexyl-5′-propan-7′-one)-33-ethyl-34-methyl-bacteriohop-16-ene, stigmasterol, β-sitosterol, β-amyrin and cetyl alcohol.[29,30] *Tinospora sinensis* (Lour.) Merr. (Menispermaceae) is well-known Ayurvedic medicine for Jaundice, fever, rheumatism, gonorrhoea and diabetes.[31] Although it showed diverse pharmacological activities including antidiabetic and antipyretic, its role as an anti-severe acute respiratory syndrome coronavirus 2 (anti-SARS-CoV2) agent as well as a prophylaxis for coronavirus disease (COVID-19) has gained high popularity in India and hence considered as an essential Ayurvedic drug for COVID-19.[32] Columbine, tinosporaside, jatrorrhizine, palmatine, berberine, tembeterine, tinocordifolioside, choline, tinospora acid, tinospora, tinosporin and tinosporide.[33] *Curcuma longa* L. (Zingiberaceae) is traditionally used as a dietary spice and medicinal herb for different infections, wounds, gastrointestinal (GI) troubles and diabetes.[34,35] Several studies reported its multiple pharmacological properties such as anti-inflammatory, antioxidant, antitumor, antibacterial, anticoagulant and antidiabetic activities.[36] It contains curcumin, demethoxycurcumin, bisdemethoxycurcumin, turmerones, furanodiene, ferulic acid, coumaric acid, myristicin and cyclocurcumin as bioactive constituents.[37]

Since no established treatment is yet available for DM, research is continuously going on to find out a permanent solution mainly for type 2 DM. On the other hand, various earlier researches revealed that a single herb is sometimes insufficient to achieve the required medicinal effect. However, the combination of more than

one herb, called polyherbalism, produces enhanced medicinal properties as well as less toxicity, perhaps due to the synergistic effect,[38] and this remains the actual challenge in the development of polyherbal formulation. The herbs used in the present study are used in traditional medicine either as a single herb or in the form of a formulation.[8,39–41] Hence, the present research aimed to develop a polyherbal formulation inspired from Ayurveda to treat diabetes via inhibiting SGLT2 protein.

2. Materials and methods

2.1. Collection and authentication of plant material

The plant samples were collected from the different localities of the Garhwal and Kumaon regions of Uttarakhand Himalaya. The authentication of the plants was done by the Botanical Survey of India, Dehradun and R&D Centre, Uttarakhand Ayurved University, Dehradun. The accession/voucher numbers 118189, 118595, 118596, 118597, UAU211, UAU229 and UAU253 were assigned to *Artemisia roxburghiana* Wall. ex Besser, *Drimia indica* (Roxb.) Jessop, *Cissampelos pareira* L., *Stephania glabra* (Roxb.) Miers, *Tinospora sinensis* (Lour.) Merr., *Roylea cinerea* (D.Don) Baill. and Curcuma longa L., respectively.

2.2. Preparation of extracts

The freshly collected plant parts were thoroughly washed with plenty of tap water to remove impurities and put in the hot air oven at 50 °C for 48 h with occasional turns upside down. The dried and coarsely powdered material was soaked with distilled water and absolute ethanol (1:1) for 72 h (three times, each for 24 h) for complete extraction. The solvent was removed using a vacuum rotary evaporator (Equitron, India) with bath temperature at 50 °C to obtain a dry extract which was then stored at 4 °C until further use.

2.3. GC-MS/MS metabolomics analysis of extracts

GC-MS analysis of the extract was performed on Agilent 7890B gas chromatograph (Agilent Technologies, CA, USA) coupled with an Agilent 5977B mass detector. The sample was injected into GC-MS by an automatic sampler (CN1700443 3 series). HP-5 MS column (5% phenyl methyl polysiloxane; 30 m × 0.25 mm i.d. x 0.25 μm) and helium as a carrier gas were used for metabolites separation. For GC-MS analysis, the extract was dried in an Eppendorf concentrator and resuspended in 100% methanol and further dried in the concentrator, and then directly derivatized with 70 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide for 60 min at 37 °C in a 1.5 mL centrifuge tube followed by centrifugation at 13000 rpm for 10 min. The resulting supernatant was taken out and directly analyzed by the GC-MS system. The injection volume was 1 μL with splitless mode. The injector temperature was set at 280 °C. The oven temperature was initially set at 80 °C for 2 min, then ramped to 220 °C at a rate of 10 °C/min without any hold and further increased at 310 °C at the rate of 20 °C/min held for 10 min and with a solvent delay of 5 min. The column flow rate was 1 mL/min. The conditions for the operation of the mass spectrometer were set as follows: ion source temperature 230 °C, MS Quad temperature 150 °C, electron energy (70eV) and scanning range of m/z, 25—1000 amu. Metabolites were identified by matching the mass spectra of target metabolite (3:1 signal to noise ratio) with the NIST-17 mass spectral library. Metabolite identity was reported only when the matching value of the mass spectra comparison was more than 80%, and an increase in the area of the corresponding peak was observed when spiking the sample with the corresponding pure standard. The identified metabolites were further compared with the available literature on the plant.

2.4. Preparation of polyherbal formulation

The polyherbal formulation (PHF) was prepared by mixing all hydroalcoholic extracts in a definite ratio by following *Bhavprakash Nighantu*, an Ayurvedic classical text.[26] The PHF contained 7.42% *A. roxburghiana* (aerial parts), 22.26% *C. pareira* (roots), 7.42% *S. glabra* (tuber), 0.15% *D. indica* (tuber), 7.42% *R. cinerea* (aerial parts), 29.69% *T. sinensis* (stems) and 25.64% Curcuma longa (rhizomes).

2.5. Experimental animals

Wistar rats with 80—120 g weight were selected for the study irrespective of their sex. The animal studies were conducted at M.M. College of Pharmacy, Ambala with ethical approval No. MMCP/IAEC/55/2019. All rats were acclimatized to the laboratory condition for one week before starting the study. The general behaviour, body weight, and feed-water intake of the rats were observed during the acclimatization period. The animals were maintained as per Control and Supervision of Experiments on Animals (CPCSEA) guidelines, and experimentation was done as per the approved protocol. The rats were kept on a permitted diet and water *ad lib* during the study.

2.6. Acute toxicity study

The rats were divided into 2 groups of 6 rats per group irrespective of their sex to assess the acute toxicity as per Organisation for Economic Co-operation and Development (OECD) guidelines.[42] Group 1 served as a control and provided a normal diet whereas group 2 is given a single oral dose of PHF at 2000 mg/kg, body weight and also allowed a normal diet. All rats were monitored continuously every hour for the first day and then every day for fourteen days to assess the different parameters including skin, fur, eyes, respiratory pattern, salivation, diarrhoea, urination, tremors, ptosis, relaxation, gait, posture, lethargy, sleep, coma and food/water intake pattern together with other behavioural changes. However, the body weight of the rats was measured after every 4 days.[43]

2.7. Induction of diabetes

The diabetes was induced in overnight fasted rats by a single intraperitoneal injection of 60 mg/kg of STZ (Sisco Research Laboratories, India), freshly prepared in 0.1 M citrate buffer (pH 4.5), 15 min after the intraperitoneal administration of 120 mg/kg of NAM (Sisco Research Laboratories, India), which was prepared in saline solution. The diabetes was confirmed by the elevated blood glucose level determined at 72 h after the STZ/NAM administration. The rats with blood glucose levels of more than 250 mg/dL were considered diabetic and used for the present study.[44]

2.8. Experimental design

The rats were divided into five groups (n = 6) in which the first group comprised of non-diabetic rats whereas the rest of the groups included diabetic rats. The normal control group (G1NC) and diabetic control group (G2DC) did not receive any treatment but were allowed free access to distilled water. The third group (G3S) received standard drug dapagliflozin orally at the dose of 0.1 mg/kg/day in distilled water whereas the fourth group (G4T1) and fifth group (G5T2) received PHF at the oral doses of 250 and

500 mg/kg/day, respectively in distilled water for 28 days. The doses of PHF were determined based on acute toxicity study.

2.9. Biochemical analysis

The blood samples were collected from retro-orbital plexus using ether anaesthesia.[45] Blood collected into the Clot Activator tubes or Fluoride tubes (for glucose test) was stored at 4 °C for 3 h and then centrifuged at 3000 revolutions per minute for 10 min using MiniSpin Plus Microcentrifuge (Eppendorf, Germany) to separate the serum.[46] The isolated serum samples were used to measure the levels of fasting blood glucose (FBG), SGPT, SGOT, total bilirubin, cholesterol, triglyceride, creatinine, uric acid, urea and lactate dehydrogenase (LDH). All the parameters were assessed routinely on the 0th, 7th, 14th, 21st and 28th day using Standard Assay Erba Diagnostic Kits (Erba Semi-Auto Biochemistry Analyzer, UK). In addition, body weight of rats was also measured regularly using digital weighing balance.

2.10. Isolation of organs and their histopathology

At the end of the 28th day, the rats were painlessly sacrificed by cervical dislocation.[47] The liver, kidney and pancreas of each rat were carefully removed for histopathological examination. The organs were washed with normal saline and preserved in a 5% formalin solution (Rankem, India) at room temperature until further use.[48] The histopathological examination of isolated organs was performed by Dr. Lal Pathlabs Ltd., New Delhi, India using a microscope at 10x, 20x, 40x and 100x magnifications.

2.11. Molecular modelling for SGLT2 protein

Due to the unavailability of three dimensional (3-D) structure of SGLT2 of *Homo sapiens*, the sequence of SGLT2 was retrieved from NCBI (accession number- NP_003032.1), and the 3D structure was predicted using the "Easy Modeller" homology modelling module.[49] The generated 3D structures of SGLT2 were subjected to further optimization, which includes minimization, side chain as well as loop refinement. The optimized structure was submitted into the PDBsum server of protein data bank (PDB)[50] to validate the modelled structure.

2.12. Active site confirmation

After modelling, the computed atlas of surface topography of proteins (CASTp) webserver was used to find out the active side in protein and locate delineating, and measuring geometric and topological properties of the modelled SGLT2 structure.[51] The CASTp 3.0 predicted many active amino acid residues which may present in the active site and are responsible for protein-ligand interaction.

2.13. Molecular docking and visualization

Docking was performed to obtain a population of possible orientations and conformations for the ligand at the binding site by using InstaDock open-source software.[52] This software performs the prediction of the bound conformations based on the binding affinity. The grid centre for docking analysis was set to X = −25.462, Y = -43.18, and Z = 61.307, and the dimensions of the grid box were set as $84 \times 110 \times 99$ Å having a spacing of 0.375 Å between the grids points. The virtual screening of compounds was conducted by rigid molecular docking in the active site of SGLT2 protein. A total of 170 compounds for molecular docking study were selected from our previous reports on the individual herbs of the present PHF and other antidiabetic agents.[11,17,19,21,24,27] Molecular interactions between protein-

ligand complexes, including hydrogen bonds and other bonds, were analyzed and depicted using discovery studio software.

2.14. Statistical analysis

All the values are shown as means ± standard error of the mean (SEM). Statistical analyses were performed using one-way ANOVA followed by Tukey's test and analyzed using the GraphPad Prism 8 software. The p values equal to or less than 0.05 were considered statistically significant.

3. Results

3.1. Identification of metabolites

Based on the GC-MS/MS analysis, most intense peaks with highest peak width were characterized from each methyl derivatized extract. The results revealed 2-pyrrolidinone; L-arabitol; α-lyxopyranose; palmitic acid; bisphenol A, bis(trimethylsilyl) ether and acethydrazide, N2-[4-(thiitan-3-yloxy)benzylideno]- as main constituents of the extract of *C. pareira*. *A. roxburghiana* extract was found to contain protocatechoic acid; caffeic acid; arachidonic acid and azelaic acid. Similarly, 2-methylpropanoic acid; 2-hexenedioic acid, bis(-trimethylsilyl) ester; D-tagatofuranose, pentakis(trimethylsilyl) ether; acetin, bis-1,3-trimethylsilyl ether and levoglucosan were characterized from *D. indica* extract. In case of *T. sinensis* methyl derivatized extract, 2-butenedioic acid; 5α-androstan-3β-ol, O-methyl-; ((11,11-dimethyl-8-methylenebicyclo[7.2.0]undecan-4-yl) methoxy)trimethylsilane; 2,5-dichlorohydroquinone; ethanesulfonic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester; syringaresinol and β-epinephrine were found as major constituents. However, the extract of *S. glabra* comprised of cyclopropane, 1-(1-hydroxyethyl)-1-(diethylphosphonyl)-2-methylene-; 5α-androstan-3β-ol, O-methyl-; pyridine-3-sulfonamide, 2,6-dichloro-4-methyl-N-cyclohexyl-; 6H-dibenzo[a,g]quinolizine, 5,8,13,13a-tetrahydro-2,3,9,10-tetramethoxy-; 11-hydroxyetiocholanolone (3α,5α,11β) and 2-{2-oxo-4-[(trimethylsilyl)oxy]pyrrolidin-1-yl}-N-(trimethylsilyl) acetamide as main constituents.

3.2. Acute toxicity

All the animals treated with a single oral dose of PHF at 2000 mg/kg showed normal behavioural, motor, and neuronal functions. No mortality was observed during the experimental period up to 14 days. The monitoring of skin, fur, eyes, respiratory pattern, autonomic nervous system (ANS) characteristics (i.e., salivation, diarrhoea and urination) and central nervous system (CNS) characteristics (i.e., tremors, ptosis, relaxation, gait and posture) in the treated rats remained unaffected. Moreover, the water and feed intake pattern of the rats was regular and consistent during the study period. The changes in body weight of the treated group did not record any substantial changes when compared with the control. Hence, based on the acute toxicity outcome, PHF can be considered safe up to 2000 mg/kg body weight.

3.3. Effect on body weight

The weekly measurement of body weight showed no significant changes in diabetic control and standard group. However, the formulation with a higher dose showed a slight increase in the body weight of diabetic rats when compared with the control group. The average body weight of the animals treated with 500 mg/kg was recorded to be 100 ± 8.003 g on the 28th day which was increased from 87 ± 7.278 on a basal day whereas, at a dose of 250 mg/kg, the body weight increased to 100 ± 3.921 from 94 ± 4.416.
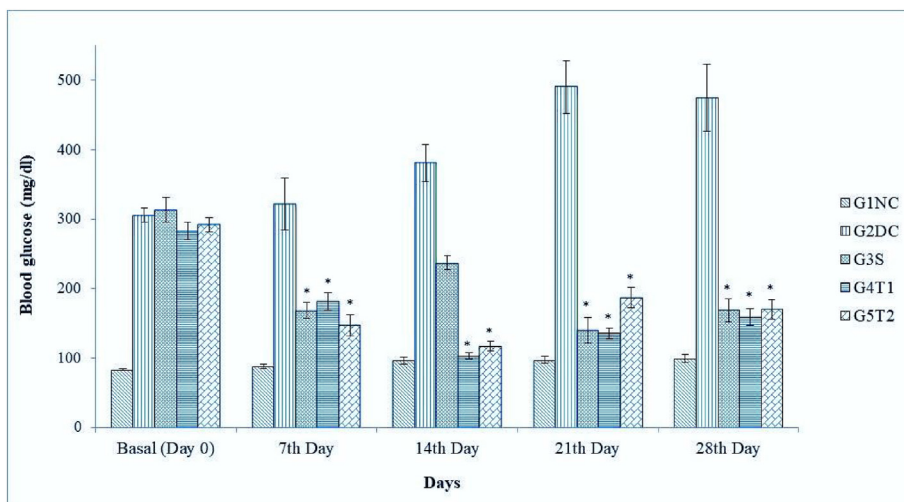
Fig. 1. Effect of PHF on blood glucose level of rats. G1NC (normal control group), G2DC (diabetic control group), standard group (G3S), test group 1 (G4T1) and test group 2 (G5T2); (*) shows significance level with p value of <0.05 when compared to G2DC.

3.4. Effect on blood glucose level

After confirmation of hyperglycemia (fasting glucose level >250 mg/dL), the rats were treated with PHF and standard drug. The results (Fig. 1) showed that blood glucose levels of all treated groups were significantly ($p < 0.05$) decreased on the 7th day of treatment and remains effective till the 28th day when compared to the normal control group. The PHF showed 56.37% and 58.17% reduction in glucose levels after 28 days at the doses of 250 and 500 mg/kg, respectively whereas 53.90% reduction was recorded with 0.1 mg/kg of dapagliflozin.

3.5. Effect on liver function

The STZ-NAM-induced hyperglycemia significantly increased the SGPT and SGOT levels in rats from the 7th to the 28th day. However, there were no significant changes occurred in the levels of SGPT and SGOT in the standard and PHF-treated groups for up to 14 days. Interestingly, 14th day onwards, a significant ($p < 0.05$)

decrease was noted till the end of the experiment (Table 1). Similarly, total bilirubin levels were increased in the diabetic control group during the entire experimental period while the changes measured in the treated groups were found insignificant till 14 days and significantly increased thereafter. Hence, it can be said that the PHF has long-term significant effects on the levels of SGPT, SGPT and total bilirubin in STZ-NAM-induced diabetic rats.

3.6. Effect on lipid profile

The levels of cholesterol and triglycerides in the serum of experimental rats were measured to evaluate the effect of PHF on lipid profile. Their levels were found higher in the diabetic control group when compared to the normal control group. The results (Table 2) revealed a significant ($p < 0.05$) change after two weeks in the levels of cholesterol and triglycerides in diabetic rats treated with both PHF and dapagliflozin when compared to the diabetic control group.

Table 1
Effect of PHF on liver function in experimental rats.

Day	G1NC	G2DC	G3S	G4T1	G5T2
SGPT (U/L)					
Basal (0) Day	28.40 ± 4.69	32.10 ± 5.35	34.69 ± 3.25	32.25 ± 6.16	37.28 ± 0.49
7th Day	31.54 ± 5.77	41.77 ± 9.79	43.30 ± 7.27	36.86 ± 5.29	47.14 ± 8.92
14th Day	31.45 ± 5.05	76.02 ± 16.54	87.51 ± 12.30	54.16 ± 7.80	58.75 ± 7.68
21st Day	33.78 ± 4.74	119.53 ± 8.44	60.76 ± 5.13*	47.28 ± 15.81*	53.51 ± 13.84*
28th Day	32.49 ± 3.36	98.64 ± 11.80	40.28 ± 15.03*	40.39 ± 6.84*	48.31 ± 7.45*
SGOT (U/L)					
Basal (0) Day	89.10 ± 5.60	92.82 ± 10.97	103.47 ± 12.56	101.48 ± 14.86	89.09 ± 4.63
7th Day	86.95 ± 7.84	129.61 ± 6.72	119.05 ± 33.85	116.36 ± 8.33	112.38 ± 3.40
14th Day	92.82 ± 10.37	169.17 ± 8.87	140.74 ± 8.26	137.20 ± 7.92	113.84 ± 10.44*
21st Day	86.03 ± 12.70	147.52 ± 15.57	138.70 ± 13.21	123.69 ± 13.05	101.70 ± 5.89*
28th Day	81.33 ± 10.25	181.20 ± 7.29	119.72 ± 9.46*	115.34 ± 14.68*	118.35 ± 8.37*
Total bilirubin (mg/dL)					
Basal (0) Day	0.66 ± 0.14	0.79 ± 0.16	0.62 ± 0.10	0.83 ± 0.09	0.88 ± 0.10
7th Day	0.87 ± 0.35	0.94 ± 0.16	0.86 ± 0.22	0.91 ± 0.30	0.92 ± 0.13
14th Day	0.68 ± 0.10	0.82 ± 0.22	0.73 ± 0.18	0.89 ± 0.09	0.84 ± 0.09
21st Day	0.88 ± 0.06	0.94 ± 0.09	0.53 ± 0.07*	0.71 ± 0.08*	0.77 ± 0.23*
28th Day	0.87 ± 0.08	1.03 ± 0.22	0.69 ± 0.46*	0.79 ± 0.30*	0.88 ± 0.32*

G1NC (normal control group), G2DC (diabetic control group), standard group (G3S), test group 1 (G4T1) and test group 2 (G5T2); (*) Shows significance level with p value of <0.05 when compared to G2DC.

Table 2
Effect of PHF on lipid profile of experimental rats.

Day	G1NC	G2DC	G3S	G4T1	G5T2
Cholesterol (mg/dL)					
Basal (0) Day	36.64 ± 8.83	40.52 ± 4.79	45.15 ± 3.46	39.81 ± 6.90	38.46 ± 9.06
7th Day	41.56 ± 4.77	43.06 ± 1.68	46.76 ± 5.40	50.5 ± 10.98	43.79 ± 4.98
14th Day	41.37 ± 5.31	83.28 ± 9.65	59.85 ± 11.49*	44.13 ± 11.69*	45.39 ± 7.64*
21st Day	37.51 ± 4.81	74.81 ± 15.99	59.86 ± 11.98	44.7 ± 5.27*	48.00 ± 6.92*
28th Day	36.79 ± 12.23	80.05 ± 4.72	56.67 ± 7.29*	40.9 ± 3.59*	45.02 ± 11.02*
Triglycerides (mg/dL)					
Basal (0) Day	37.61 ± 5.32	37.13 ± 2.89	34.81 ± 8.94	40.61 ± 7.00	47.11 ± 57.55
7th Day	37.81 ± 14.86	42.56 ± 9.24	48.07 ± 13.58	43.74 ± 7.39	42.87 ± 3.27
14th Day	44.58 ± 18.51	45.84 ± 10.95	42.30 ± 17.55	49.62 ± 22.68	41.13 ± 17.62
21st Day	40.68 ± 15.58	80.24 ± 22.12	48.47 ± 21.23*	42.55 ± 30.34*	48.20 ± 12.40*
28th Day	47.92 ± 7.80	86.86 ± 13.15	42.06 ± 16.00*	44.47 ± 12.43*	40.74 ± 9.17*

G1NC (normal control group), G2DC (diabetic control group), standard group (G3S), test group 1 (G4T1) and test group 2 (G5T2); (*) Shows significance level with p value of <0.05 when compared to G2DC.

Table 3
Effect of PHF on renal function of experimental rats.

Day	G1NC	G2DC	G3S	G4T1	G5T2
Creatinine (mg/dL)					
Basal (0) Day	0.69 ± 0.02	0.68 ± 0.02	0.70 ± 0.05	0.73 ± 0.03	0.67 ± 0.06
7th Day	0.74 ± 0.14	0.73 ± 0.10	0.73 ± 0.19	0.76 ± 0.10	0.69 ± 0.13
14th Day	0.66 ± 0.05	0.75 ± 0.16	0.70 ± 0.04	0.74 ± 0.05	0.65 ± 0.08
21st Day	0.84 ± 0.17	0.89 ± 0.19	0.80 ± 0.13	0.82 ± 0.07	0.74 ± 0.12
28th Day	0.70 ± 0.02	0.85 ± 0.07	0.70 ± 0.02	0.68 ± 0.05	0.68 ± 0.09
Uric acid (mg/dL)					
Basal (0) Day	2.26 ± 0.38	2.62 ± 0.22	2.21 ± 0.65	2.46 ± 1.12	2.44 ± 0.48
7th Day	3.58 ± 1.90	3.70 ± 0.79	2.81 ± 1.06	2.90 ± 1.83	3.45 ± 0.72
14th Day	2.89 ± 0.78	3.38 ± 0.50	2.91 ± 0.94	2.23 ± 0.49	2.93 ± 0.44
21st Day	2.87 ± 0.29	3.46 ± 0.87	2.48 ± 1.08	3.21 ± 1.09	2.91 ± 0.48
28th Day	2.19 ± 0.52	2.58 ± 0.61	2.48 ± 0.39	2.18 ± 0.53	2.74 ± 1.10
Urea (mg/dL)					
Basal (0) Day	82.89 ± 14.28	70.40 ± 17.99	85.49 ± 12.67	81.87 ± 19.22	80.27 ± 13.20
7th Day	86.12 ± 11.84	80.96 ± 3.45	111.40 ± 3.67	91.87 ± 8.97	82.33 ± 6.49
14th Day	83.47 ± 6.36	80.10 ± 8.80	90.43 ± 3.54	97.93 ± 1.98	88.37 ± 6.71
21st Day	82.47 ± 3.18	88.87 ± 4.01	96.71 ± 8.56	97.81 ± 6.71	85.80 ± 5.69
28th Day	85.87 ± 18.68	118.58 ± 18.12	99.32 ± 11.11	97.55 ± 14.52	92.34 ± 7.60

G1NC (normal control group), G2DC (diabetic control group), standard group (G3S), test group 1 (G4T1) and test group 2 (G5T2); (*) Shows significance level with p value of <0.05 when compared to G2DC.

3.7. Effect on renal function

The present study showed that dapagliflozin and PHF did not produce any significant changes in the levels of creatinine, uric acid and urea in serum of diabetic rats. A slight increase in the levels of urea was although recorded in all treated groups but it was comparatively lower than that of the diabetic control group (Table 3).

3.8. Effect on LDH level

The diabetic control group showed a significant increase in their LDH level on the 28th day when compared to the normal control group. The treated groups also showed an increase in the LDH level but this change was found insignificant (Fig. 2). There was no significant difference in the effect of PHF (at 250 and 500 mg/kg) and dapagliflozin (0.1 mg/kg).

3.9. Histopathology of isolated organs

3.9.1. Histopathology of liver

Microscopic examination of sections revealed the normal histological structure of hepatic lobules in both treated and untreated animals including the normal control group. The hepatocytes were arranged in cords radiating from the central vein and separated by blood sinusoids in all the groups. Mild sinusoidal congestion, as well as congestion of central and portal vein, was seen in G2DC and G5T2 groups similar to the G1NC group, whereas mild congestion of sinusoids was seen in the G4T1 group. Hepatocytes were polyhedral in shape with slightly vacuolated granular cytoplasm and vesicular nuclei in all the groups similar to the normal control group. Focal area of mild degenerative changes with swollen hepatocytes and some cells with karyolitic nuclei were present in G5T2 similar to the G1NC group, whereas the focal area of mild degenerative changes with mild steatosis and some cells with karyolitic nuclei were present in G4T1. Blood sinusoids separating the hepatic cords lined by endothelial cells and kupffer cells were seen in the G3S group only. Bi-nucleated hepatocytes with no infiltration or granuloma were also seen in all the groups (Fig. 3).
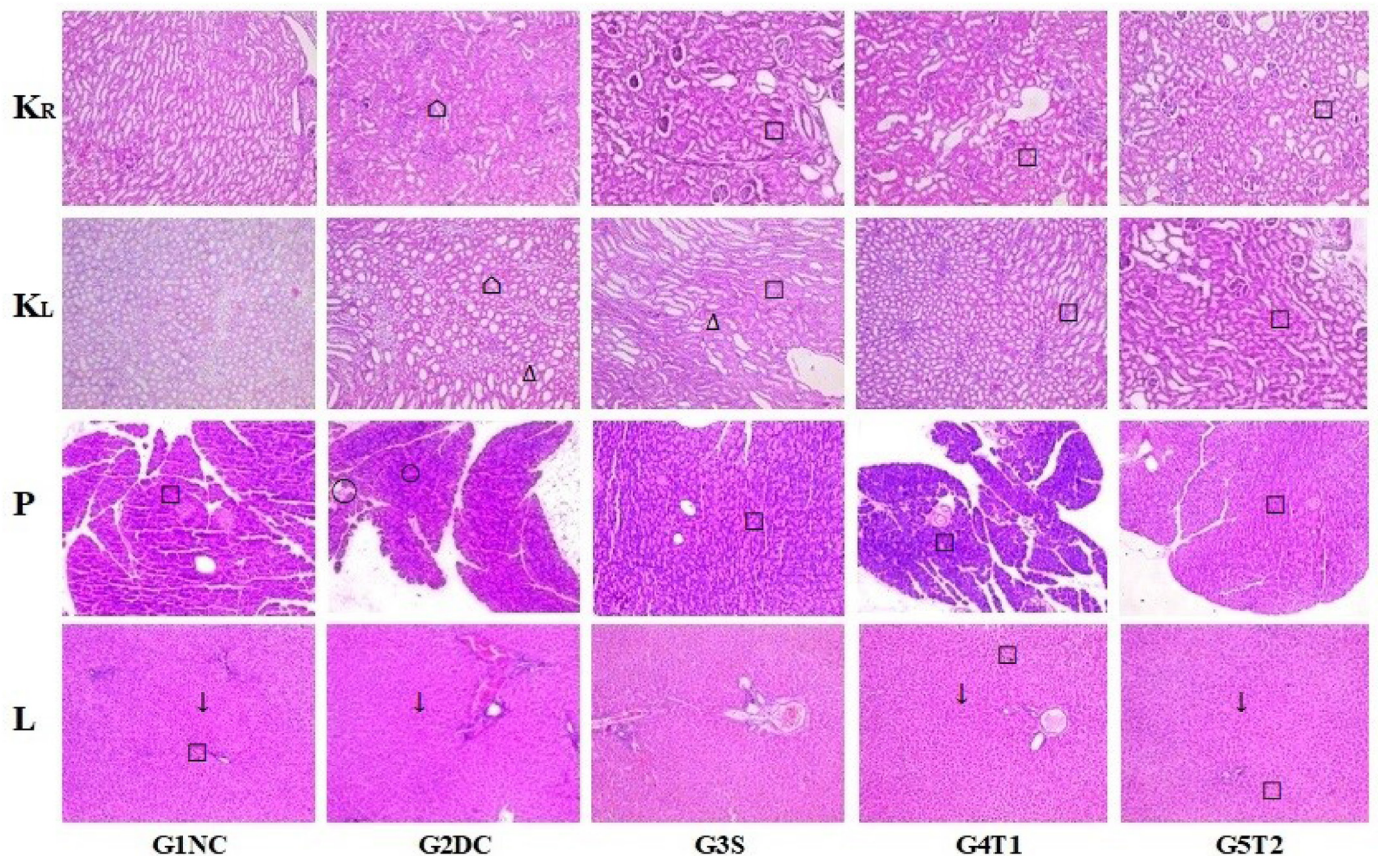
3.9.2. Histopathology of pancreas

The section of G1NC showed normal architecture of the pancreas with very mild degenerative changes. The exocrine pancreas is composed of closely packed acinar cells and arranged into small lobules. Pancreatic lobules are separated by intact intralobular and interlobular connective tissue septa. The islet cells were seen interspersed between the acinar cells. Islets appeared lightly stained than the surrounding acinar cells. However, the histopathology of the pancreas of G2DC revealed moderate to high degenerative changes in both exocrine and endocrine components.
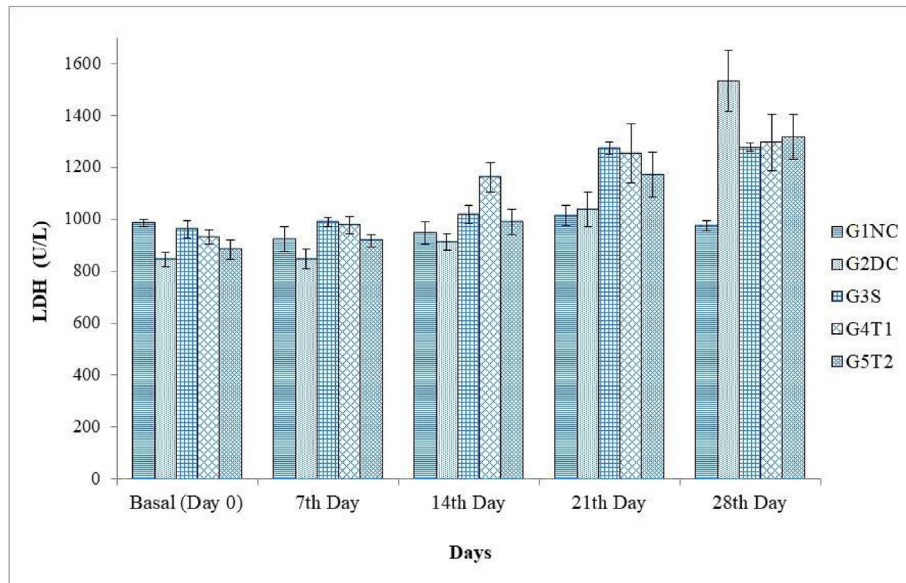
Fig. 2. Effect of PHF on LDH level in experimental rats. G1NC (normal control group), G2DC (diabetic control group), standard group (G3S), test group 1 (G4T1) and test group 2 (G5T2); the results were found insignificant when compared to G2DC.

Fig. 3. Histopathology of right kidney (K_R), left kidney (K_L), pancrease (P) and liver (L) of experimental rats. (↓): Mild sinusoidal congestion; (□): mild degenerative changes; (⌂): moderate degenerative changes; (○) high degenerative changes; () depletion of beta cells; (Δ): desquamation of epithelial linings; G1NC (normal control group), G2DC (diabetic control group), standard group (G3S), test group 1 (G4T1) and test group 2 (G5T2); the results were found insignificant when compared to G2DC.

Acinar cells were swollen and small vacuoles were observed in acinar cells. Interlobular ducts were lined with flattened epithelium. Some lobules showed depletion of islet β-cells. On the other hand, all the treated groups showed mild degenerative changes in both exocrine and endocrine components similar to G1NC. In addition, a few acinar cells swollen with small vacuoles were

observed. Interlobular ducts were lined with flattened epithelium. A small number of lobules in G3S, G4T1 and G5T2 showed depletion of islet β-cells (Fig. 3).

3.9.3. Histopathology of right kidney

Microscopic examination of sections of the right kidney revealed mild haemorrhages present in between intertubular spaces in all the groups. Mildly congested blood vessels were also seen in all the groups. There are mild to moderate degenerative changes of epithelial linings of tubules seen in G2NC whereas all treated groups showed mild degenerative changes similar to the normal control group. Some tubules show desquamation of epithelial linings in G2DC and G3S groups. However, focal interstitial inflammatory mononuclear cell infiltrate were seen in G2DC but not in G3S. Similar to G1NC, no granuloma was seen in the rats treated with standard and PHF (Fig. 3).

3.9.4. Histopathology of left kidney

Microscopic examination of the left kidney showed most histological features similar to the right kidney. G5T2 showed focal mild intertubular haemorrhages. An infiltration of polymorph cells was present in surrounding fibro-fatty tissues in G5T2. Small cystic areas were only present in the G4T1 group. In the G3S group, some of the tubules showed desquamation of epithelial linings and mild expansion of the glomerular cavity similar to the normal control group. Additionally, no infiltration of inflammatory cells or granuloma was seen in the G3S group. G1NC and G2DC showed focal interstitial inflammatory mononuclear cell infiltrates. Focal tubular basophilia, nuclear crowding, and thickened basement membranes were also present in the G2DC group. In the treatment group G4T1, tubules showed necrosis of epithelial linings (Fig. 3).

3.10. Homology modelling and active site confirmation

The SGLT2 protein sequence of *Homo sapiens* was retrieved from NCBI, and a basic local alignment search tool-protein (BLASTp) was used to identify homologous templates from the PDB database. BLASTp showed similarities of the SGLT2 query with the crystal structure of secretogranin-2 (SGC2) of *Vibrio parahaemolyticus* (PDB ID 2XQ2 A, 3DH4 and 2XQ2 B), and these structures share 69%, 64% and 63% identity respectively query cover. After that, Easy Modeller software was used for the prediction of the 3D structure of SGLT2 using four structures as templates. The 3-D model of protein also showed 90.5% of the residues in the allowed region of the Ramachandran plot. It demonstrates the stereochemical reliability of the homology template of SGLT2 protein.

The active site (Fig. 4) residue numbers of SGLT2 of *Homo sapiens* are 32, 33, 36, 37, 40, 41, 43, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 65, 66, 69, 70, 72, 73,74, 75, 76, 77, 78, 79, 80, 83, 84, 95, 96, 98, 99, 102, 123, 124, 125, 126,127,129, 130, 136, 139, 142, 143, 145, 146, 149, 150, 153, 154, 157, 158, 161, 184, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 200, 201, 202, 203, 204, 205, 208, 209, 283, 286, 287, 289, 290, 291, 294, 295, 296, 297, 300, 301, 304, 320, 321, 324, 329, 387, 388, 389, 390, 391, 392, 393, 395, 396, 397, 398, 399, 400, 402, 403, 404, 406, 407, 411, 412, 414, 415, 416, 417, 418, 419, 420, 422, 423, 424, 426, 427, 430, 452, 453, 456, 460, 463, 464, 467, 471, 552, 553, 659, 662, 663, 666, 667, 670, 671, 672. Molecular docking was performed with SGLT2 of *Homo sapiens*, specifying the active site using the amino acids area.

3.11. Molecular docking

Phytochemicals of plants were screened by molecular docking against the SGLT2 receptor. Out of 170 compounds, β-sitosterol and insulanoline showed the highest binding energy. The top eight

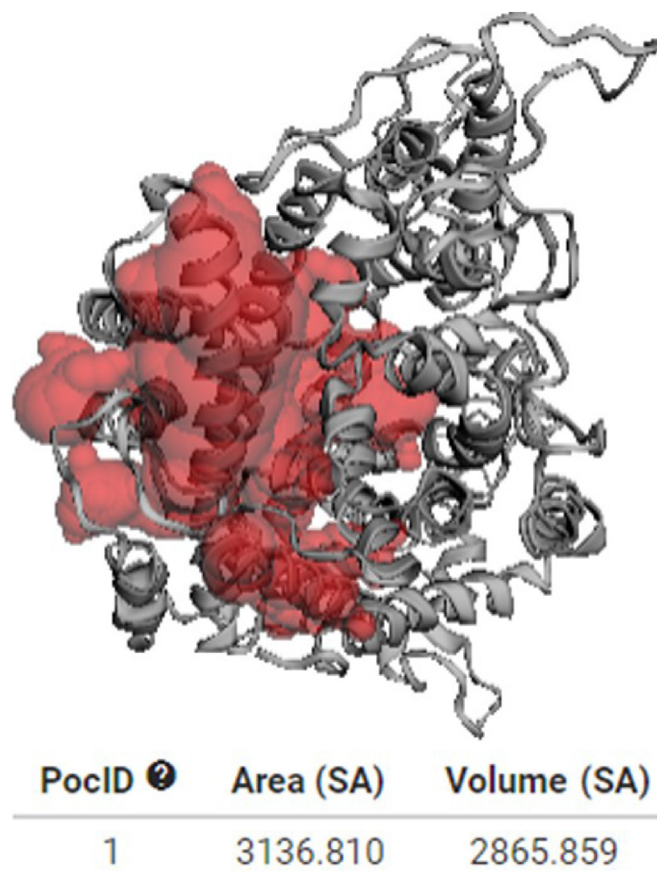| PocID ❓	Area (SA)	Volume (SA)
1	3136.810	2865.859

Fig. 4. Active sites of SGLT2 protein.

active compounds with their docking scores, natural sources, 2D and 3D molecular docking interactions with SGLT2 protein are shown in Fig. 5.

4. Discussion

Based on the global research outcomes to date, it is understood that DM is incurable, although it can be managed with the help of selected medications including modern and traditional medicine.[53] The treatment of DM with herbal medicine is considered a safer approach which has been practised in different traditional systems of medicine since ancient times.[19] Moreover, the treatment with multiple herbs showed better effects with lesser toxicity than a single herb, perhaps due to the synergistic effect of different constituents present in these herbs.[54,55] In the past couple of years, tremendous work has been done on the polyherbal formulations and many of them were found effective in controlling hyperglycemia in different experimental models.[56—58]

The evaluation of toxicity of a plant sample is a key step for conducting pharmacological studies in higher models. Such studies give initial information about the safety profile of a drug candidate and also help in deciding the doses for *in vivo* studies.[59] In the present study, PHF was evaluated for its acute toxicity in rats and based on the outcome of this experiment, the doses for antidiabetic activity were determined. Since the highest doses at 2000 mg/kg did not produce any toxic effect in rats up to 14 days, the oral LD_{50} of PHF can be considered to be higher than 2000 mg/kg.

For the past couple of years, a combination of STZ and NAM is most accepted to develop type 2 DM because STZ causes β-cell damage while NAM partially protects the insulin-secreting cells against STZ
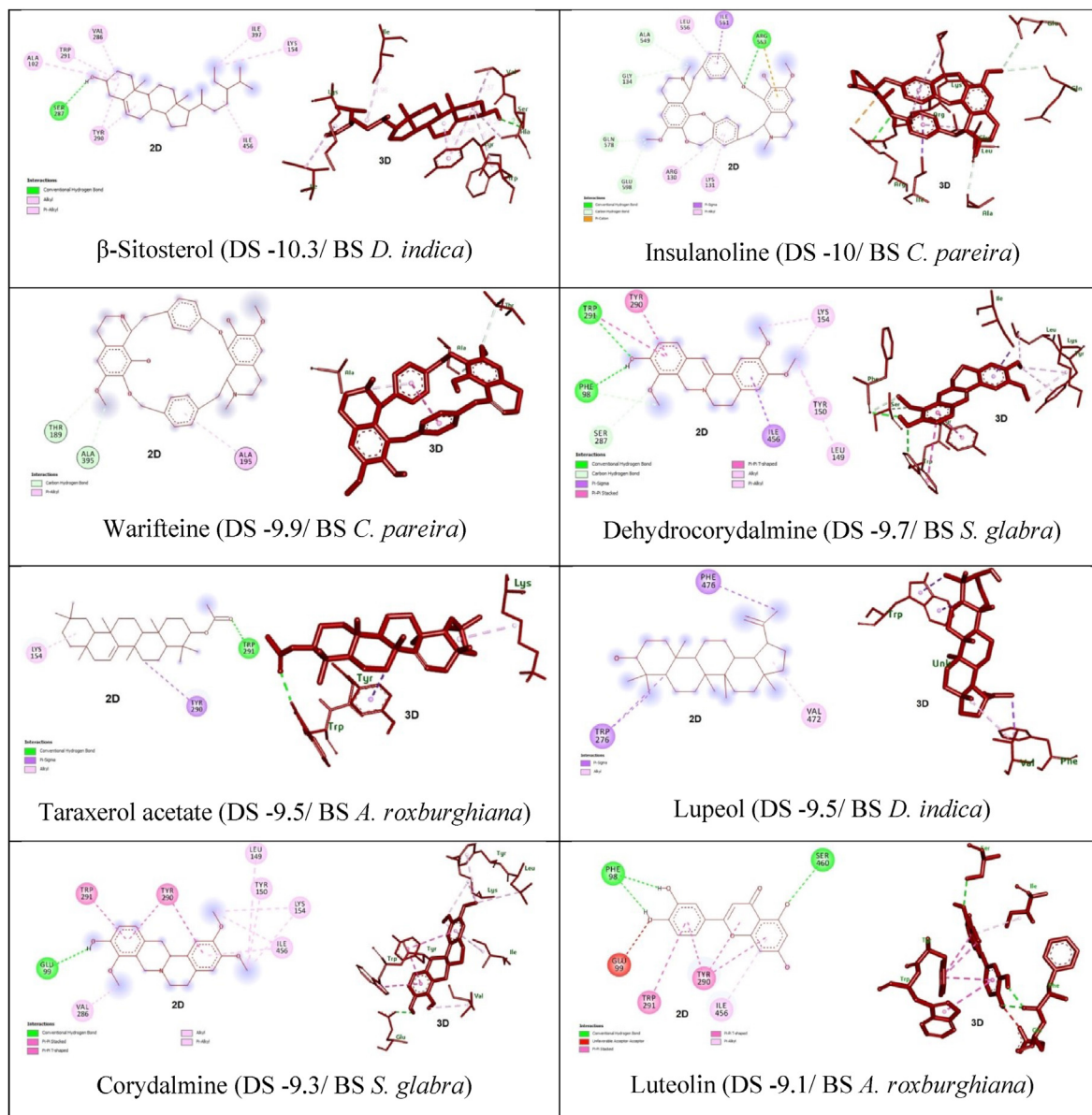
Fig. 5. 2D and 3D molecular docking interactions of bioactive compounds with SGLT2 protein. DS (docking score - kcal/mol), BS (botanical source).

and hence, protects them from permanent damage.[60] The present study found STZ-NAM-induced diabetic model as the most suitable to evaluate the SGLT2 inhibitory effect in rats. The administration of STZ and NAM produces a significant elevation in the glucose levels of experimental rats when compared to the normal group.

The reduction in blood glucose level is the first priority in developing an antidiabetic drug because hyperglycemia can cause various complications including retinopathy, nephropathy and neuropathy.[61] In the present study, the treatment of PHF (250 and 500 mg/kg) significantly ($p < 0.05$) decreased the elevated blood glucose levels in STZ-NAM induced diabetes rats within one week and this effect was also recorded during the entire experimental period when compared with the diabetic control group. The study also found that the effect of PHF was comparable to that of the positive control group at the end of the 28th day. Moreover, body weight is an important parameter to be monitored regularly in the event of diabetes. However, in the present study, no significant changes were recorded in body weight of experimental rats.

The increased levels of lipids, mainly cholesterol and tri-glycerides, in diabetic patients are risk factors for diabetes-associated cardiovascular diseases.[62] It is evident that the risk of developing hypertension is higher in DM patients than in others.[63] Moreover, chronic diabetes plays a key role in developing vascular diseases by increasing the formation of advanced glycation end-products (AGEs), activation of the receptor for advanced glycation endproducts (RAGE), oxidative stress and inflammation.[64] The results obtained from the present study revealed a significant change in the levels of total cholesterol and triglycerides both in test groups and positive control. The maximum reduction was recorded on the 28th day of the experiment.

Similarly, the increased levels of SGPT, SGOT and bilirubin due to liver injury are common conditions mainly in chronic diabetes. This condition can also affect the metabolism of lipids, carbohydrates and proteins and hence causes fatty liver. Moreover, developing liver cirrhosis, hepatocellular carcinoma and oxidative stress were also recorded in the patients with chronic DM.[65] In the present

study, the PHF significantly decreased the levels of SGPT and SGOT enzymes after the 14th day. Moreover, total bilirubin levels were increased in the diabetic control group during the entire experimental period while the changes measured in the treated groups were found significant when compared to G2DC. Hence, it can be said that the PHF has a significant effect on the levels of SGPT, SGPT and total bilirubin in STZ-NAM-induced diabetic rats and as a result, it might play a potential role in the hepatoprotective activity. On the other hand, elevated levels of creatinine, urea and uric acid in the blood are indications of kidney problems. The present study did not find a significant change in the levels of these components when compared to the control group.

The histopathological examination of isolated organs was done to find any internal toxic sign in the treated animals. The sample can be considered safe if its histology matches with normal animals. This study showed that PHF does not cause any harmful effect on the experimental rats when compared to diabetic rats. The results of PHF for liver and kidney function were supported by histopathological analysis. Moreover, the pancreas was also found unaffected after the 28-days of *in vivo* study. Overall, the formulation was found effective and safe for further use.

Dapagliflozin, a synthetic antidiabetic drug, has the potential to reduce blood glucose specifically by inhibiting SGLT2 protein in the renal tubule to block glucose reabsorption. In addition to reducing HbA1c level, this drug also helps in reducing body weight.[66] However, previous studies indicate that the SGLT2 inhibitors may produce adverse effects on the skeleton which results in the weakening of bones in diabetic patients.[67] The present study used dapagliflozin as a standard drug to compare the antidiabetic effect of PHF. The results showed that the values of most of the parameters studied for PHF were comparable to the standard drug. Hence, in view of the potential glucose-lowering effect and safety profile, the PHF was also evaluated for its SGLT2 inhibitory effect via *in silico* method using major bioactives of the herbs present in PHF.

Nowadays, molecular docking studies are most popular among the scientific community for the early inclusion of pharmacokinetics consideration in the process of novel drug discovery with the help of advanced software.[68] The present study identified eight bioactive phytochemicals including β-sitosterol and insulanoline which showed strong binding to the SGLT2 protein with a docking score of more than nine. Since most of the constituents of PHF showed *in silico* SGLT2 inhibitory activity, it can be suggested that the glucose-lowering effect of PHF involved the SGLT2 inhibition pathway. Moreover, the individual molecules having high docking scores can be further studied using higher models.

5. Conclusion

This study concluded that the polyherbal formulation, comprised of 7 herbs viz. *A. roxburghiana, C. pareira, S. glabra, D. indica, R. cinerea, T. sinensis* and *C. longa*, showed significant glucose-lowering effect via inhibiting SGLT2 protein. The levels of SGOT, SGPT, bilirubin, cholesterol and triglycerides were significantly decreased in the PHF-fed rats. The toxicity study, as well as histopathological examination, revealed the nontoxic nature of the formulation. Hence, in view of the antidiabetic effects in animal models, the PHF can be developed as a novel antihyperglycemic agent. However, further studies are needed to consider it for clinical use.

Animal ethical approval

The animal studies were conducted at M.M. College of Pharmacy, Ambala with ethical approval No. MMCP/IAEC/55/2019. The animals were maintained as per Control and Supervision of

Experiments on Animals (CPCSEA) guidelines, and experimentation was done as per the approved protocol.

Authors' contribution

DKS: conceptualized and supervised the work, edited and reviewed the manuscript; AK, ASN, RK and RSD: developed the formulation and conducted the animal experiments; TJ and SC: conducted the molecular docking studies; AK and RS: prepared the first draft of the manuscript; RS and AC: statistically analyzed the data; RBS and SKJ: edited and reviewed the manuscript.

Declaration of competing interest

The authors declared no conflict of interest.

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