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EXPLORING PHYTOCHEMICAL CANDIDATES TARGETING SFRP4: A PROMISING APPROACH FOR DIABESITY TREATMENT

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ABSTRACT

The simultaneous presence of both obesity and diabetes in an individual is known as diabesity. Type 2 diabetes mellitus is a global chronic condition characterized by an increase in blood sugar levels caused by insufficient pancreatic insulin production and in the development of diabetes type 2 obesity plays an important role. Wnt signaling is an evolutionarily conserved system that regulates a wide range of activities during embryonic development, including cell differentiation, proliferation, and growth. Wnt signaling can be influenced by a variety of antagonists. By specifically targeting secreted frizzled-related protein4 (SFRP4) that is released from white adipose tissues and results in the increased production of adipokines into to the blood obesity can be controlled. Therefore, identification of SFRP4 inhibitors from phytochemicals which shows maximum anti-obesity and anti-diabetic activity is necessary. Molecular operating environment (MOE) software was used for protein docking to observe association between protein and compound that can be used as anti-obesity drug. Six to eight-week mice were used in this experiment that were randomly divides into two different studies. For 10-12 weeks, 2 control group was fed on normal diet (ND) while the 12 groups were fed on High fat diet (HFD). After induction of obesity induced diabetes, the mice were treated with the phytochemicals for 3 weeks and their role in reducing blood glucose level and obesity was determined by observing blood glucose level and body weight. The blood sample was collected in properly labeled tubes. Enzyme linked immunosorbent assay (ELISA) was used for quantification and detection of protein in serum. The correlation of body weight reduction and diabetes with SFRP4 level indicates their relation. One-way ANOVA on Graph pad prism was used to compare the mean and standard deviation of each group for identification of their significance level ($p < 0.05$). The Serum SFRP4 level significantly increased after HFD. Coumarin, Trehalose, Thymol and Ferulic acid showed significant difference to diabetic, non-diabetic and standard treatment group ($p < 0.05$) while exhibiting non-significant difference among them. In conclusion, treatment of Coumarin, Trehalose, Thymol and Ferulic acid significantly decreased the body weight, glucose level and SFRP4 level. These compounds could be potential candidate to be used as anti-obesity and anti-diabetic drug.

Key Words: Type 2 Diabetes, Obesity, Wnt Signalling, SFRP4

INTRODUCTION

The term diabesity was first Introduced in 1973 by Sims et al. to signify the coexistence of obesity and type 2 diabetes (T2D) within an individual [1-3]. Diabetes is one of the most serious public health problems of the twenty-first century, particularly in low- and middle-income nations [4]. Diabetes mellitus is a chronic disease worldwide caused by an increase in blood sugar levels due to a small secretion of pancreatic insulin [5]. A combination of two words Diabetes Mellitus (DM) is derived from the Greek words "diabetes," which means "to pass through," and "mellitus," which means "sweet or honeyed" [6]. The global prevalence of T2DM in 2019 was about 463 million worldwide rising day by day to approximately 578 million by 2030 and by 2045 the prevalence would be 750 million [7]. Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM) are the two most common subtypes of diabetes, both of which are caused by faulty insulin production (T1DM) and/or insulin action (T2DM). T1DM affects children and adolescents, but T2DM is considered to afflict middle-aged and older people who suffer from chronic hyperglycemia as a result of poor lifestyle and nutritional choices [8]. Obesity is a metabolic syndrome that increases the chances of developing type 2 diabetes mellitus (T2DM) [9]. As the rate of obesity increases then the incidence of obesity-linked complications like improper glucose tolerance and type 2 diabetes mellitus also enhanced [10, 11]. The most commonly used measure of adiposity is body mass index (BMI) shows a deep association between insulin resistance and diabetes. Obese people have increased concentrations of cytokines hormones, pro-inflammatory markers, factors that are involved in developing insulin resistance, and also non-esterified fatty acids. The pathogenesis in the developing process of diabetes is due to the functional impairment of β-islet cells of the pancreas, which results in failure of maintaining blood glucose levels. The onset of diabetes becomes more predictable if the functional impairment of pancreatic β-islet cells results from insulin resistance. The weight gain and mass of the body are the main factors in the onset and increased risk of T1DM and T2DM [12]. Many signaling molecular pathways, Wnt signaling are included that play a major role in the accumulation of lipid and free fatty acid inhibition that release leading to obesity-linked diabetes. Increased production of cytokine IL-1β reduces secretion of insulin by altering the Wnt pathway and it is responsible for SFRP4 secretion [13]. The Wnt signaling mechanism is controlled by a feedback system that involves different Wnt pathway components. SFRP4 belongs to the secreted protein family unit and shares a sequence with frizzled receptors in the Wnt signaling pathways [14]. High levels of SFRP4 or its overexpression result in adipocyte dysfunction, resistance to insulin, obesity, and improper secretion of insulin in T2D patients [15]. SFRP4 is a T2D biomarker that was found several years before the condition was diagnosed clinically [16]. Obesity, T2D, and other metabolic diseases linked to increased SFRP4 levels can be prevented by inhibiting the development of mature adipocytes from precursor cells. Early identification of beta-cell dysfunction biomarkers in high-risk people may aid in the prevention of Obesity and T2D development [17]. Patients with obesity and diabetes-related problems face a growing medical burden, which has the potential to stifle global economic growth in the near future. This demonstrates that the current healthcare system is woefully unable to deal with the growing worldwide effects of diabetes and obesity, necessitating the urgent development of new and better solutions [18]. Traditional medicine based on plant extracts has been shown to be less expensive, more clinically successful, and have less side effects than contemporary medicines [19]. Plant-based chemicals have gotten a lot of interest for treating diabetes and other illnesses because of their low cost and lack of renal, gastrointestinal, or hypertensive adverse effects [20]. Molecular docking algorithms are used to estimate the affinity and activity of drug candidates for targeted proteins based on its binding orientations [19]. The phytochemicals Trehalose and Coumarin exhibiting good result in diabetes case and Ferulic acid & Thymol exhibiting good result in obesity against SFRP4 protein, and thus to find-out their role as modulator for the SFRP4 Expression they were tested further in vivo and in vitro. These findings suggested that these phytochemicals could affect beta cells of pancreas and glucose metabolism directly and decrease SFRP4 expression.

MATERIALS AND METHODS

Screening of compounds

A total of 150 herbal plants of diabetes and obesity were selected from previously existing literature and their phytochemicals were downloaded by ChemIDplus and PubChem database in .sdf format. These phytochemicals act as potential inhibitors [21, 22].

SFRP4 structure determination

The amino acid sequence of the SFRP4 protein was retrieved from the National Center of Biology (NCBI) by using accession number CAG46532.1 and this sequence was used for the determination of 3D structure prediction. This accession number was obtained from protein databases that is accessible at https://www.ncbi.nlm.nih.gov/protein. The 3-Dimensional structure of protein SFRP4 in human was analyzed by the use of homology modeling-based method because SFRP4 crystal structure was not present in the protein data bank (PDB). The 3-D structure of SFRP4 protein was predicted through use of SWISS-MODEL server and it was visualized through UCSF Chimera workbench [23, 24].

Structure Evaluation

The 3-dimensional predicted structure was identified by the use of bioinformatics online tools or software like Ramachandran plot and also Z-score was assessed through using proSA-web [25, 26] for confirmation of backbone along with the total quality of the predicted model. Furthermore, the confirmation of the SFRP4 protein structure was done by ERRAT [27].

Protein structure optimization for docking

SFRP4 protein 3-dimensional structure was optimized by using molecular operating environment (MOE) software [28]. The total water molecules were deleted, addition of hydrogen atoms, ionization energy level was attained through 3D protonation. Minimization of the molecular energy was done by regulating some parameters such as force field: 0.05 and chiral constraint: MMFF94x, gradient: current geometry.

Ligand optimization and data base construction

Plant's chemical structure of all the phytochemical constituents were attained through PubChem that was opened in molecular operating environment [29]. The optimization of ligands was done by using 3-Dimensional protonation along with minimization of energy by use of force field MMFF94X and a gradient of 0.05. The optimized obtained ligands were saved into .mdb format for further interaction.

Binding site prediction

The prediction of binding site or active site of SFRP4 protein was identified through studying previously stated literature. Interacting site or active site includes glutamate 63, glutamate 61, methionine 112, tyrosine 62, leucine 64, leucine 111, histidine 117, tyrosine 115 and tyrosine 119 close to the N-terminal [1, 25, 30, 31].

Molecular docking

The active site or interacting site finder software or tool was used in the molecular operating environment software to identify the amino acid residues present in the binding or active pocket. Ligands database which was saved in .mdb format used to docked against protein SFRP4 by the use of docking algorithm of MOE. There are a lot of factors that are being designed for docking of ligand and protein includes Placement: Triangle matcher, rescoring function 1: London dG, retain: 10, Refinement: Forcefield, Rescoring function 2: London dG and Retain: 10. All the effective ligands that were selected depends upon RMSD values or minimum S-score values along with further interacting studies such as hydrogen bonding and π-π interactions [15, 32].

Establishment of Mice Model for T2DM and Obesity

Animal trials were established for studying the possible effects of selected phytochemicals obtained from the molecular docking result [33].

Animals

Male mice (aged 6–7 weeks, weighing 22-27g) were purchased from the animal laboratory at University of Veterinary & Animal Sciences Lahore, Pakistan. The Ethic Committee of Government College University Faisalabad's approved the study for using animal. The mice were housed in separate cages with unrestricted access to food and water in a room with 24-hour light–dark cycles, and a temperature of 25 °C [34].

Division of mice

The mice were separated into fourteen different groups seven groups for diabetes and 7 for obesity (each with 4 mice) mentioned below in table 1 and 2 body weight (g), Blood glucose level and SFRP4 level were determined for all mice in each group. After 4 weeks of treatment with phytochemicals, the mice were dissected according to the mice dissection protocol to collect blood and tissue sample [35]. The blood obtained was centrifuged after allowing to clot for 3-4 hrs. and the serum was collected in properly labelled Eppendorf's tubes and serum SFRP4 level determined by the used of ELISA kit of Fine test under given manual instructions [36].

Table 1 Experiment Design for Animal (Mice) Model Used for Diabetes

Sr. no.	Group type	Groups	Treatment	Dose Quantity (mg/g of mice)	Dose Administration Method	References
1.	Control groups	Group 1	No Disease, No Treatment	N/A	N/A	
2.		Group 2	Diseased without Treatment	N/A	N/A	
3.		Group 3	Diseased with standard treatment (Metformin)	(250mg/kg/day (125mg/kg twice daily oral dose)—1000mg/kg/day (500mg/kg twice daily oral dose)	Orally	[37]
4.	Drug group	Group 4A	Treatment 1 (Coumarin)	50 mg/kg body weight	Orally	[38, 39]
5.		Group 4B	Treatment 1 (Coumarin)	100 mg/kg body weight	Orally	[38]
6.		Group 5A	Treatment 2 (Trehalose)	2 mg/kg body weight for 4 weeks	Orally	[40]
7.		Group 5B	Treatment 2 (Trehalose)	4 mg/kg body weight for 4 weeks	Orally	[40]

Table 2 Experiment Design for Animal (Mice) Model Using for Obesity

Sr. no.	Group type	Groups	Treatment	Dose Quantity (mg/g of mice)	Dose Administration Method	References
1.	Control groups	Group 1	No Disease, No Treatment	N/A	N/A	
2.		Group 2	Diseased without Treatment	N/A	N/A	
3.		Group 3	Diseased with standard treatment	(250mg/kg/day (125mg/kg twice daily oral dose)—1000mg/kg/day (500mg/kg twice daily oral dose)	Orally	[37]

4.	Drug group	Group 4A	Treatment 1 (Ferulic acid)	25 mg/kg body weight	Orally	[41]
5.		Group 4B	Treatment 1 (Ferulic acid)	50 mg/kg body weight	Orally	[41]
6.		Group 5A	Treatment 2 (Thymol)	20 mg/kg body weight for 4 weeks	Orally	[42]
7.		Group 5B	Treatment 2 (Thymol)	40 mg/kg body weight for 4 weeks	Orally	[42, 43]

Statistical analysis

The final data was evaluated statistically by using one-way ANOVA. All the data was expressed as mean ± standard deviation. The *p* values < 0.05 were considered statistically significant. All analyses were performed by using GraphPad Prism 9.2.

RESULTS AND DISCUSSION

Evaluation of predicted SFRP4 structure

After the prediction of tertiary structure most significant part was structure validation and evaluation to check whether your predicted structure is good or not. Different parameters were used to check the validity of the structure but the most common are the backbone and Z-score confirmation. Confirmation of back bone was done by using Ramachandran plot attained from RAMPAGE and overall quality of the protein model was checked by the Z-score that tells whether the predicted structure found in similar length proteins of native and Z-score are determined by the online tool Swiss model.

Predicted structure analysis by Ramachandran plot

By using the Ramachandran plot which was constructed by PROCHECK the predicted model of SFRP4 was evaluated. SFRP4 predicted model 81.2% residues were in favored regions, 15.9% residues in allowed regions while 2.5% was found in outliner region. As more than 95% of residues of the predicted structure were found in favored and allowed region structure was well validated.

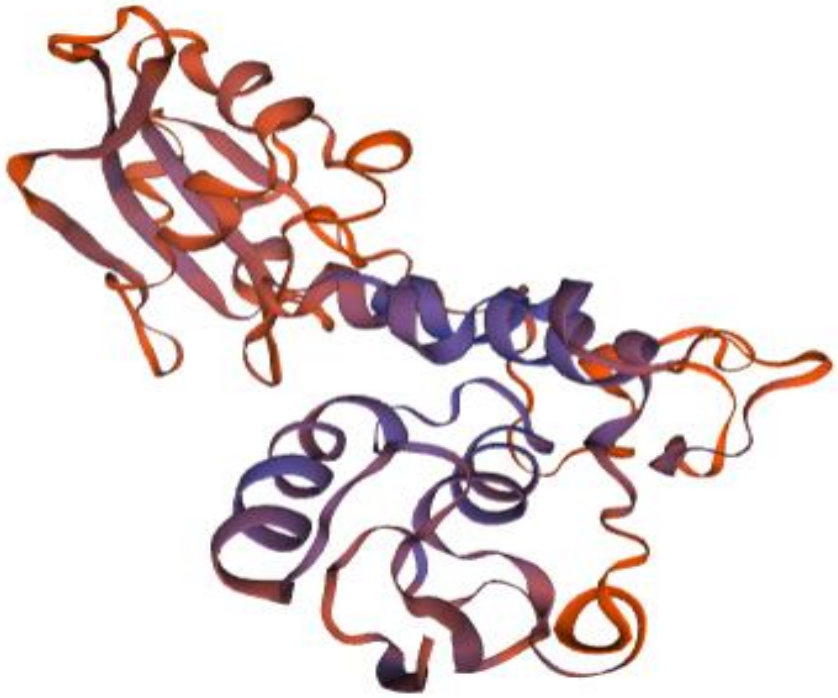


Figure 1: Predicted 3D structure of SFRP4

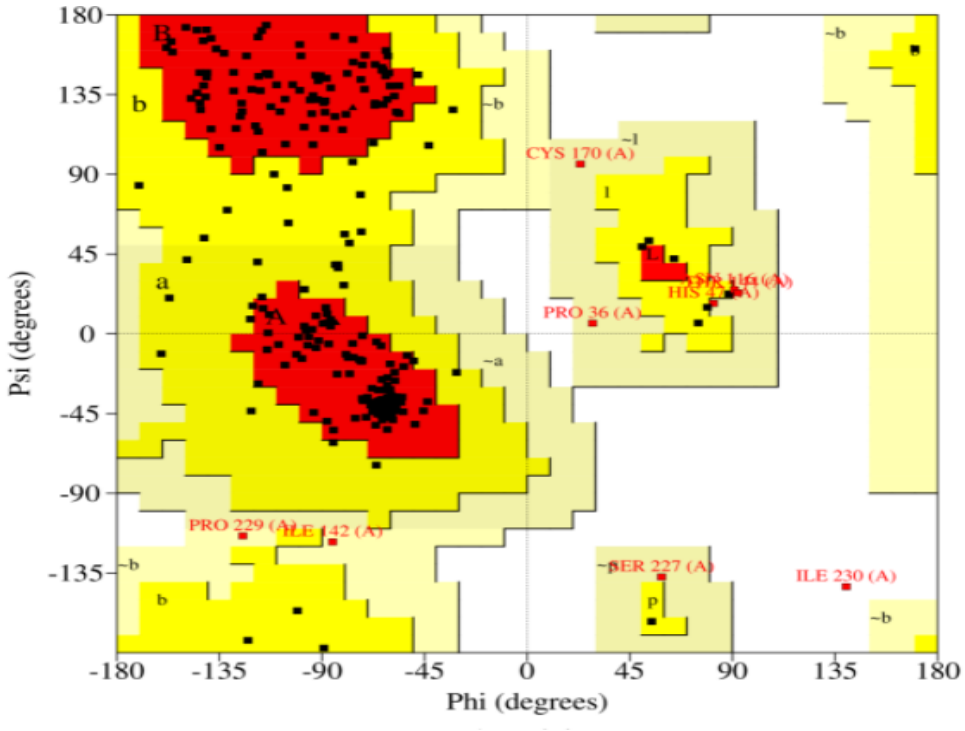


Figure 2: Ramachandran plot of SFRP4 predicted structure

Molecular Docking Interactions

Out of one thousand docked compounds 4 phytochemicals based upon root mean squared deviation (RMSD) were shortlisted two (Coumarin, Trehalose) for diabetes treatment and two (Ferulic acid, Thymol) for obesity treatment. These phytochemicals were found to be deeply bound to the binding pocket of the SFRP4 and showed interactions to amino acid present in binding pocket (Figure 3 and 4). That's way these phytochemicals were used in *in-vivo* studies. The RMSD values and interacting amino acid residues of SFRP4 are given in table 3.

Table 3: Molecular Docking results of phytochemicals

Sr. No.	Ligands Name	S-score	RMSD value	Interacting residues in active pocket	Residues interacting in surrounding region
01	Coumarin	-5.721	1.7804	Ser 118, His 117, Tyr 61	Gln 60, Pro 120, Met 112, Asn 116
02	Trehalose	-6.144	2.4867	Ser 122, His 48, His 48	Ala 57, Tyr 81, Pro 120, Gln 60, Leu 46
03	Thymol	-3.9066	1.8400	His117, Ser118	Met112, Asn116, Tyr61, Gln60
04	Ferulic acid	-4.3897	1.3323	Tyr61, Ser118	Gln60, His48, Pro120, Met112, Ala57, His117

A)

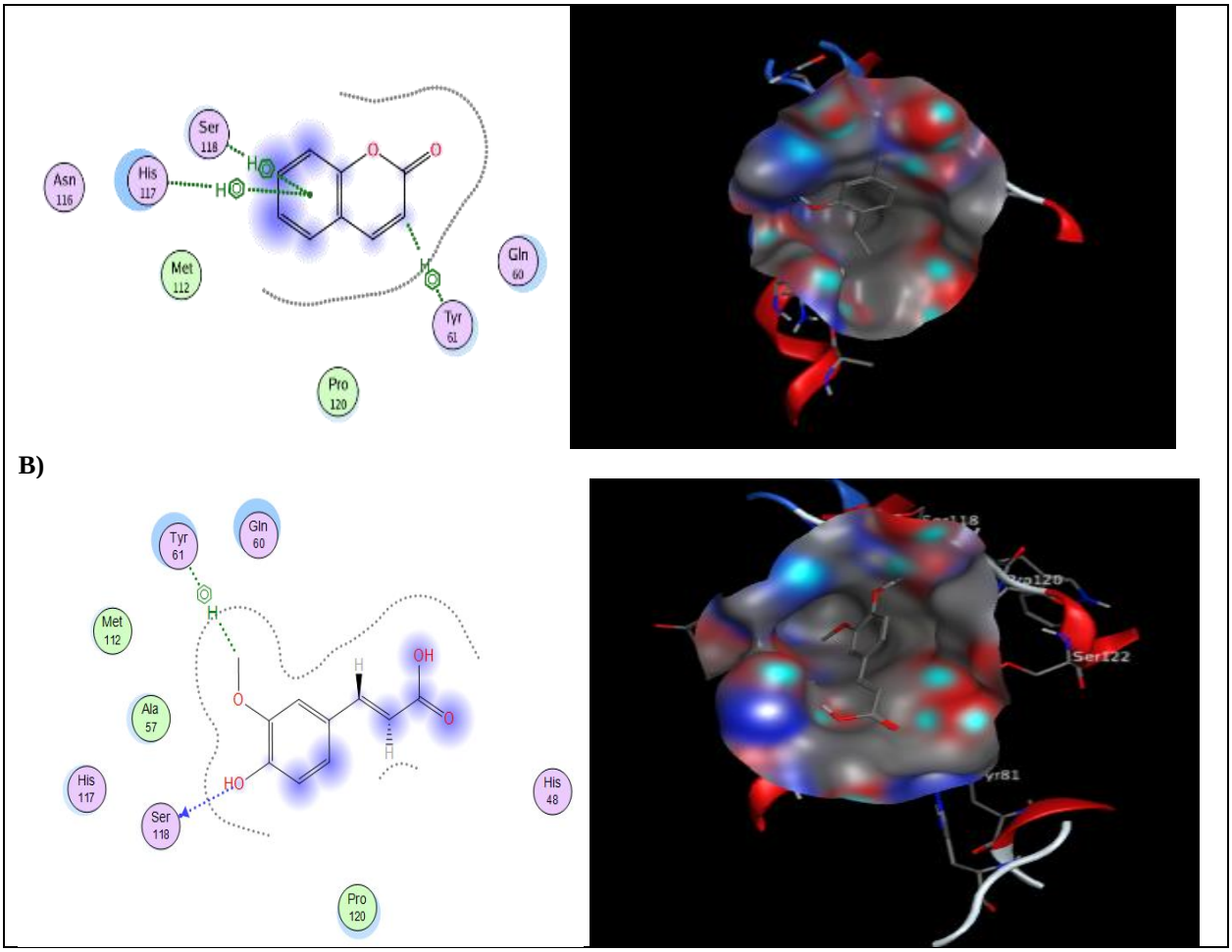
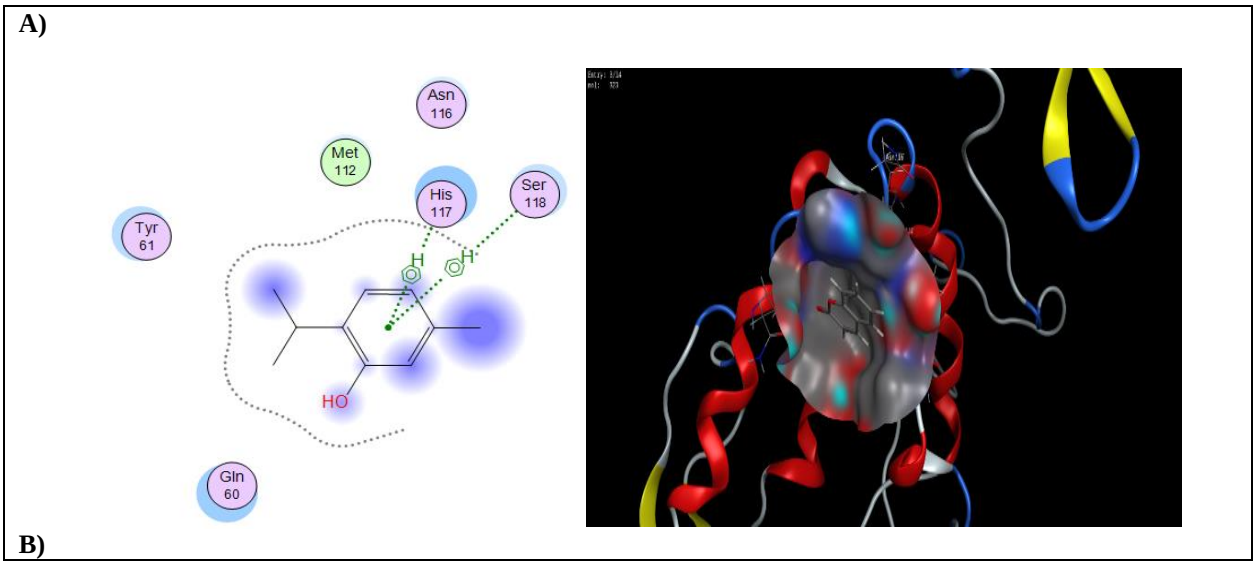


Figure 3: Docking results of Coumarin with SFRP4 as receptor protein (A). In right side Coumarin displays interaction with Ser 118, His 117, Tyr 61 in the active pocket and Gln 60, Pro 120, Met 112, Asn 116 are found in the surrounding region. In the right-side Binding patterns of Coumarin with SFRP4 (B); In left side Docking results of Trehalose with SFRP4. Trehalose interacts with amino acid Ser 122, His 48, His 48 and the amino acid residues Ala 57, Tyr 81, Pro 120, Gln 60, Leu 46 are found in the surrounding region. In right side Binding patterns of Trehalose with SFRP4.



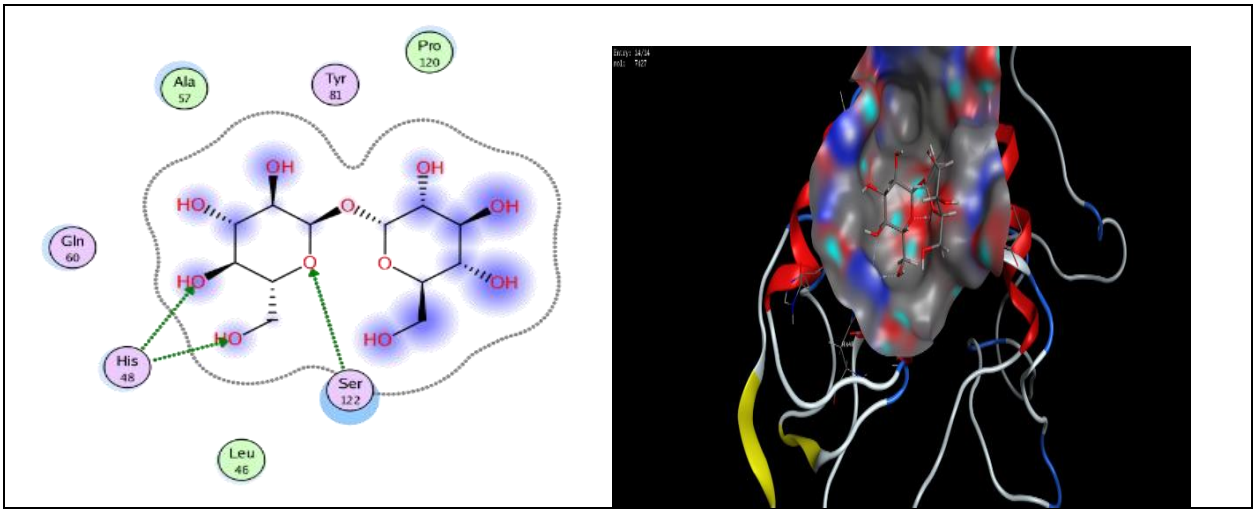


Figure 4: Docking results of Thymol with SFRP4 as receptor protein (A). In right side Thymol displays interaction with His117, Ser118 in the active pocket and Met112, Asn116, Tyr61, Gln60 are found in the surrounding region. In the right-side Binding patterns of Thymol with SFRP4 (B); In left side Docking results of ferulic acid with SFRP4. Ferulic acid interacts with amino acid Tyr61, Ser118 and the amino acid residues Gln60, His48, Pro120, Met112, Ala57, His117 are found in the surrounding region. In right side Binding patterns of ferulic acid with SFRP4.

Study One

Effect of anti-obesity Phytochemicals on Obesity

The changes in body weight, blood glucose level and Serum SFRP4 level of mice was measured after treatment with Ferulic acid and Thymol. The treatment of Ferulic acid and Thymol low and high dose showed a significant reduction in body weight, blood glucose level, and serum SFRP4 level. The SFRP4 serum level was confirmed by the ELISA test.

Effect of Ferulic acid

Ferulic acid showed strong interaction with the binding pocket of the SFRP4 protein. The effect of Ferulic acid treatment was analyzed statistically by significance level ($p < 0.05$).

Body weight

The body weight of mice was significantly increased after the treatment of HFD for 10 weeks. The body weight of each mouse was measured at the initial stage (Week 0). Then HFD treatment was given to mice for 8-10 weeks and again body weight was measured at week 14. Ferulic acid low dose of 25mg/kg and High dose of 50mg/kg was used along with standard treatment of Metformin 50mg/kg. The data was taken at week 0, week 10, and week 14. Then, After the treatment of Ferulic acid body weight was significantly reduced ($p < 0.05$). The Mean and standard deviation of each group's values were measured on Microsoft Excel 2016 to analyze the effect of the treatment of Ferulic acid on the body weight (g) of mice. One-way ANOVA was used to compare the mean and standard deviation of each group to indicate how much one group is significantly different from the other.

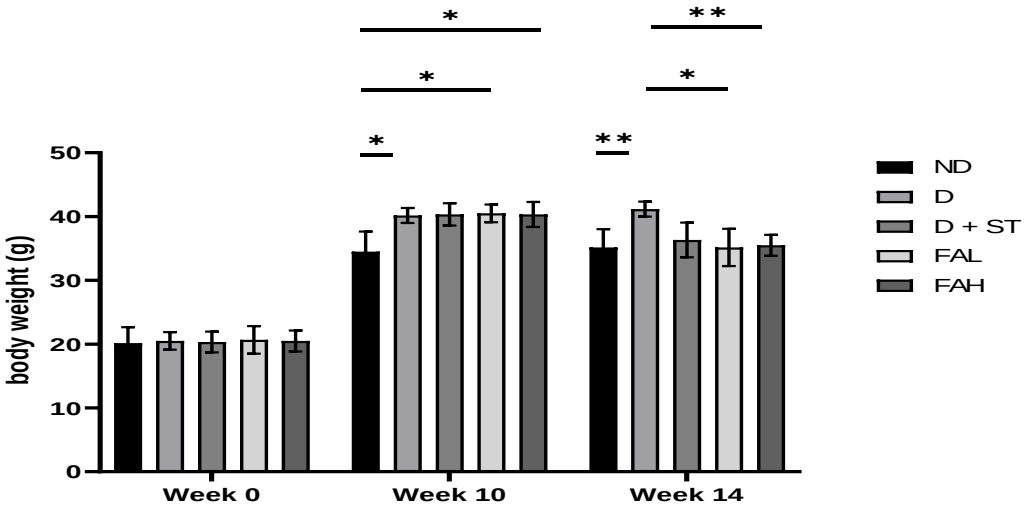


Figure 5: Effect of Ferulic acid on body weight

Graph bars representing non-diabetic (ND), Diabetic control (D), standard treatment (D + ST), Ferulic acid low dose (FAL) and Ferulic acid high dose (FAH). ($p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****).

There was a non-significant difference between groups at week 0 ($p > 0.05$). At week 10, Ferulic acid low dose and Ferulic acid high dose group were significantly different from non-diabetic group ($p < 0.05$). There was a non-significant difference among diabetic group and standard treatment group ($p > 0.05$). Ferulic acid low dose (FAL) with mean and S. D 40.5 ± 1.378 to 35.167 ± 2.926 was significantly different to Diabetic control group ($p < 0.5$) and Ferulic acid high dose (FAH) with mean and S. D 40.333 ± 1.966 to 35.5 ± 1.643 was significantly different to the diabetic control group ($p < 0.01$). Both doses have a non-significant difference ($p > 0.05$) from standard treatment and among them (Figure 5).

Glucose level

Normal blood glucose level after two hours of eating ranges from 120 to 140mg/dL. The blood glucose level was significantly increased after HFD treatment for 8-10 weeks. At initial stages blood glucose level of mice was normal at (Week 0) then after the treatment of HFD blood glucose level of each group were increased significantly (Week 10). After the treatment of Ferulic acid blood glucose level was significantly reduced. The mean and standard deviation was measured on Microsoft excel 2016 and One-Way ANOVA was used on Graphpad prism 9.2 to observe the significance level between different groups ($p < 0.05$).

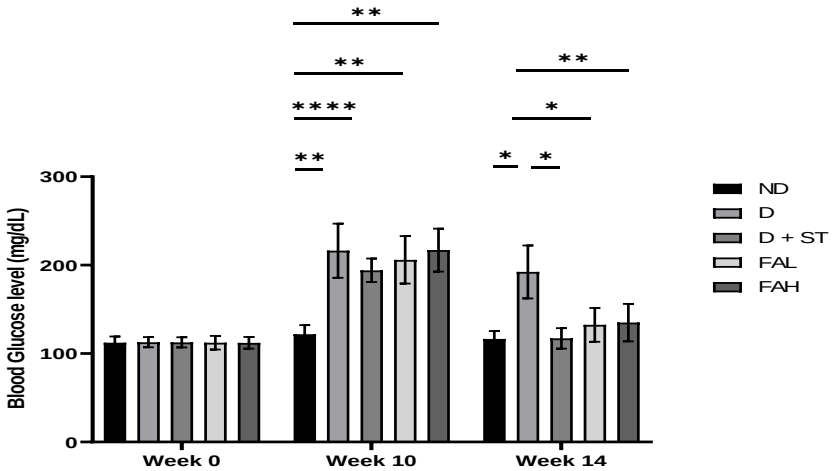


Figure 6: Effect of Ferulic acid treatment on Blood glucose level

Graph bars representing non-diabetic (ND), Diabetic control (D), standard treatment (D + ST), Ferulic acid low dose (FAL) and Ferulic acid high dose (FAH). ($p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****).

There was a non-significant difference among groups at week 0 ($p > 0.05$). After the treatment of HFD for 8-10 weeks' glucose level was significantly increased. At week 10, non-diabetic group was significantly different to diabetic group ($p < 0.01$) and standard treatment group ($p < 0.0001$). Ferulic acid Low dose (FAL) and Ferulic acid high dose (FAH) groups were significantly different to non-diabetic group ($p < 0.01$) while exhibited the non-significant difference among both and to disease control group ($p > 0.05$).

At week 14 after the treatment of Ferulic acid low dose mean and S. D 206 ± 26.802 to 132.5 ± 19.128 was significantly different to diabetic group ($p < 0.05$) and Ferulic acid High dose (FAH) with mean and S. D 217 ± 24.223 to 135 ± 21.128 was significantly different to the diabetic group ($p < 0.01$). The diabetic group is significantly different to non-diabetic and standard treatment group ($p < 0.05$).

Serum SFRP4 level

The serum level of SFRP4 significantly increased after the treatment of HFD for 8-10 weeks. But after the treatment of Ferulic acid low dose and Ferulic acid high dose a significant decline in SFRP4 level was measured. A significant increase in serum SFRP4 level was recorded at week 10. By using ELISA test confirmation of serum SFRP4 was recorded. The mean and standard deviation of each group was measured and One-Way ANOVA was used on Graphpad prism 9.2 to identified the significance level among different groups.

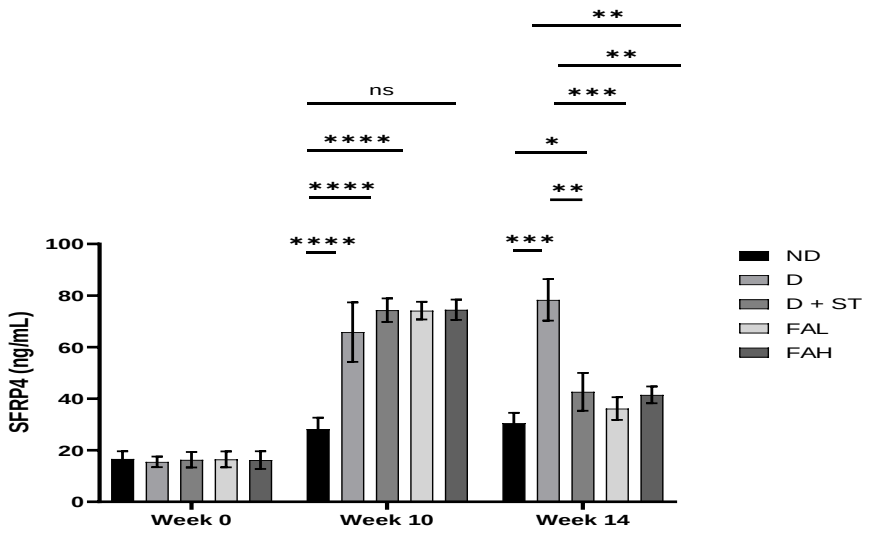


Figure 7: Effect of Ferulic acid treatment on serum SFRP4 level

Groups non-diabetic (ND), Diabetic control (D), standard treatment (D + ST), Ferulic acid low dose (FAL) and Ferulic acid high dose (FAH) are represented. The level of significance indicated by * ($p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****). HFD treatment significantly increased the level of serum SFRP4 ($p < 0.05$).

At week 0 there was non-significant difference among groups. Ferulic acid low dose (FAL), Ferulic acid high (FAH) and standard treatment group were significantly different to non-diabetic group ($p < 0.0001$) while exhibiting non-significant difference among them. Diabetic group was significantly different to non-diabetic group ($p < 0.01$) while having non-significant difference to standard treatment group ($p > 0.05$).

At week 14, Ferulic acid low dose (FAL) group with mean and standard deviation 74.167 ± 3.431 to 36.167 ± 4.401 was significantly different to diabetic group ($p < 0.001$). Ferulic acid high dose (FAH) group with mean and S. D 74.5 ± 3.937 to 41.5 ± 3.271 was significantly different to diabetic group ($p < 0.01$) while exhibiting non-significant difference to FHL ($p > 0.05$). Diabetic group was

significantly different to non-diabetic group ($p < 0.001$) and standard treatment group ($p < 0.01$). Diabetic group was significantly different to FAH ($p < 0.01$).

Effect of Thymol

Thymol showed strong interaction to binding pocket of SFRP4 protein. The effect of Thymol treatment was analyzed statistically by significance level ($p < 0.05$).

Body weight

Thymol is present in the leaves of *thymus vulgaris* showed strong association to SFRP4 protein. Thymol low dose of 20 mg/kg and high dose of 40 mg/kg was used along with standard treatment of drug metformin 50mg/kg. Body weight of each group was measured at week 0, week 10 and week 14. By using Microsoft excel 2016 each group mean and standard deviation was measured and graph bars were made by using One-Way ANOVA on Graph pad prism 9.2 for identification of their significance level ($p < 0.05$). After the treatment of Thymol there was significant change in body weight was analyzed.

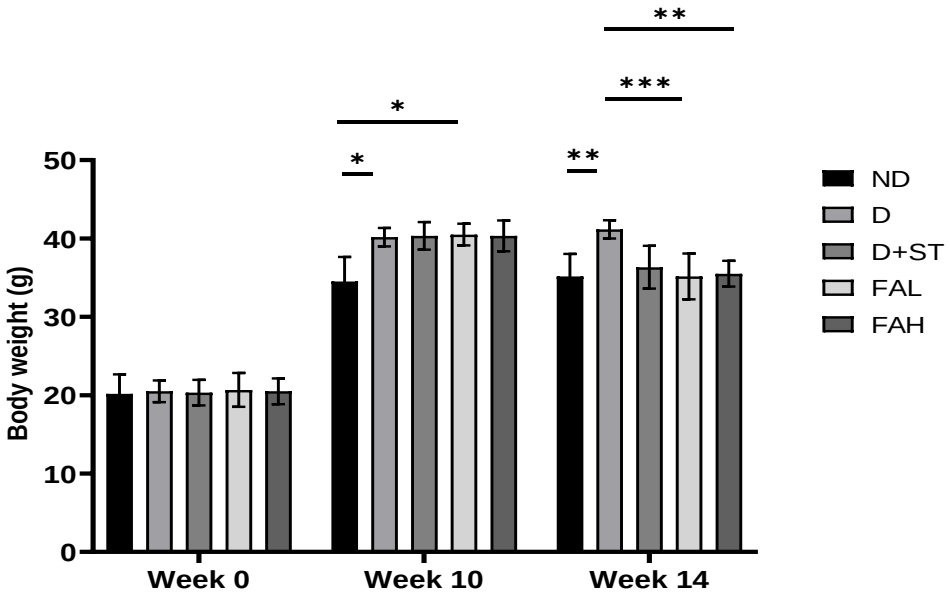


Figure 8: Effect of Thymol treatment on body weight

Groups non-diabetic (ND), Diabetic control (D), standard treatment (D + ST), Thymol low dose (TL) and Thymol high dose (TH) are represented. The level of significance indicated by * ($p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****). HFD treatment significantly increased the body weight ($p < 0.05$).

There was a non-significant difference at week 0 ($p > 0.05$). After the treatment of HFD nondiabetic group was significantly different to diabetic group and Thymol low dose (TL) group ($p < 0.05$). At week 14, non-diabetic group was significantly different to diabetic group ($p < 0.01$). Thymol low dose (TL) 39.8333 ± 1.722 to 34.5 ± 1.048 and Thymol high dose (TH) 39.167 ± 2.136 to 34.167 ± 2.401 with a significant difference to the diabetic group ($p < 0.001$) and ($p < 0.01$) respectively. Both doses have a non-significant difference ($p > 0.05$) from standard treatment and among them (Figure 8).

Glucose level

The blood glucose level of mice was significantly increased after the treatment of HFD. Thymol low dose of 20 mg/kg and high dose of 40 mg/kg was used along with standard treatment of drug metformin 50 mg/kg was used. A significant increase in blood glucose level was recorded at week 14 in diseases control group. After the treatment of Thymol blood glucose level was significantly

decreased. Each group mean and standard deviation was measured and Graph bars in figure 9 showed significantly different effect by low dose and high dose of Thymol as compared to standard treatment group.

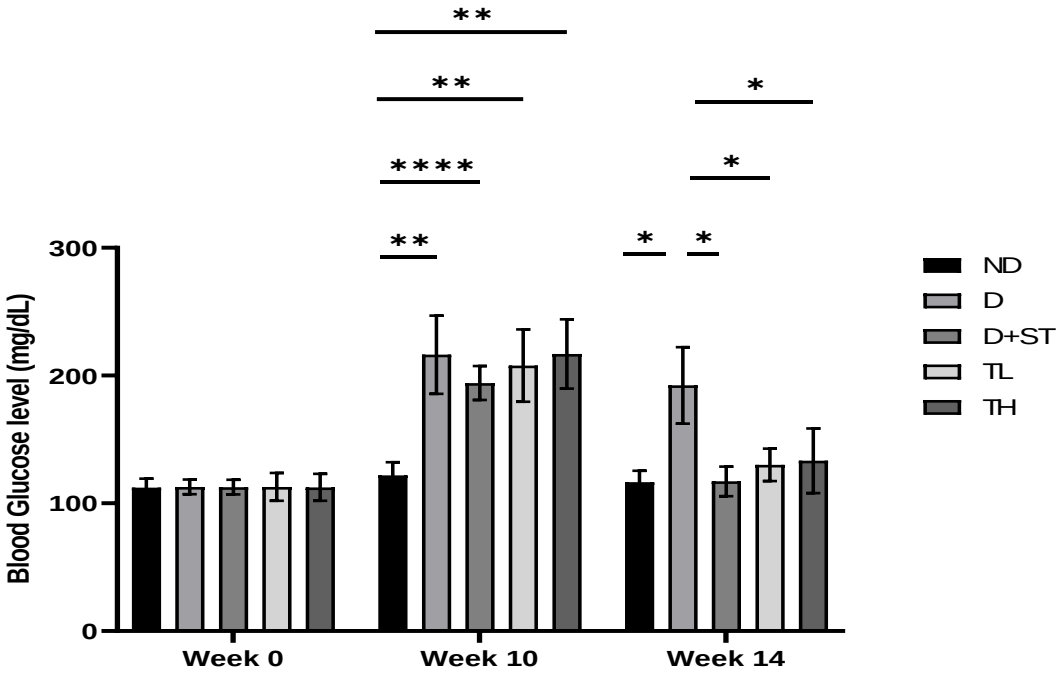


Figure 9: Effect of Thymol treatment on Blood glucose level

Groups non-diabetic (ND), Diabetic control (D), standard treatment (D + ST), Thymol low dose (TL) and Thymol high dose (TH) are represented. The level of significance indicated by * ($p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****). HFD treatment significantly increased the glucose level ($p < 0.05$).

At initial stage (Week 0) there was non-significant difference among groups ($p > 0.05$). After the treatment of HFD Glucose level was significantly increased. Non-diabetic group was significantly different to diabetic group ($p < 0.01$) and standard treatment group ($p < 0.0001$). Thymol low dose (TL) and Thymol high dose was significantly different to nondiabetic group ($p < 0.01$). At week 14 after the Thymol treatment, mean and standard deviation of Thymol low dose (TL) 207.834 ± 28.280 to 130.167 ± 12.687 and Thymol high dose (TH) 216.834 ± 27.095 to 133.334 ± 25.271 with a significant difference to the diabetic group ($p < 0.05$) while exhibited the non-significant difference among both and to standard treatment group ($p > 0.05$) (Figure 9).

Serum SFRP4 level

The Serum SFRP4 level was increased after HFD treatment. Thymol low dose of 20 mg/kg and high dose of 40 mg/kg was used along with standard treatment of drug metformin 50 mg/kg was used. A significant increase in serum SFRP4 level was recorded at week 10 in diseases control group. After the treatment of Thymol SFRP4 level was decreased. Graph bars in Figure 10 were plotted by using One-Way ANOVA in Graph pad prism 9.2 showed the significantly different effect by low dose and high dose of Thymol treatment as compared to standard treatment group ($p < 0.05$).

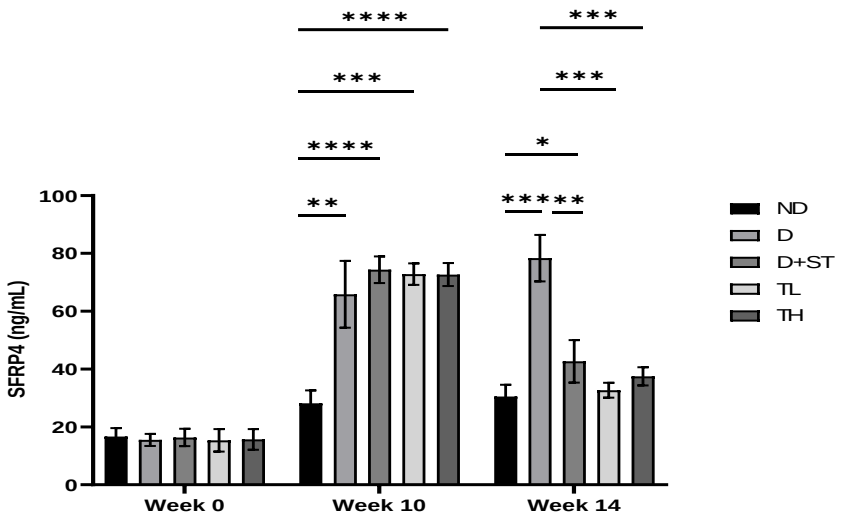


Figure 10: Effect of Thymol treatment on serum SFRP4 level

Groups non-diabetic (ND), Diabetic control (D), standard treatment (D + ST), Thymol low dose (TLD) and Thymol high dose (THD) are represented. The level of significance indicated by * ($p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****). HFD treatment significantly increased the level of serum SFRP4 ($p < 0.0001$).

Data was examined three times at initial stage (Week 0) before trial (Week 10) and after trial (Week 14). There was non-significant difference ($p > 0.05$) among groups at week 0. Diabetic group was significantly different to non-diabetic group ($p < 0.01$) and exhibit non-significant difference to standard treatment group had mean and S. D 65.834 ± 11.531, 28.167 ± 4.445 and 74.334 ± 4.589 respectively. Thymol low dose (TL) was significantly different to non-diabetic group ($p < 0.001$) and Thymol high dose (TH) was significantly different to non-diabetic group ($p < 0.0001$). After the treatment of Thymol for three weeks mean and standard deviation of TL dose 72.834 ± 3.710 to 72.667 ± 3.983 and TH dose 32.667 ± 2.581 to 37.5 ± 3.146 with a significant difference to the diabetic group ($p < 0.001$) while exhibiting non-significant difference among them (Figure 10). And diabetic group was significantly different to non-diabetic group ($p < 0.001$) and standard treatment group ($p < 0.01$). The mean and S. D of Diabetic and non-diabetic group were 78.334 ± 8.014 and 30.5 ± 4.037 respectively.

Study Two
Effect of anti-diabetic Phytochemicals on Diabetes
Effect of Coumarin
Body weight

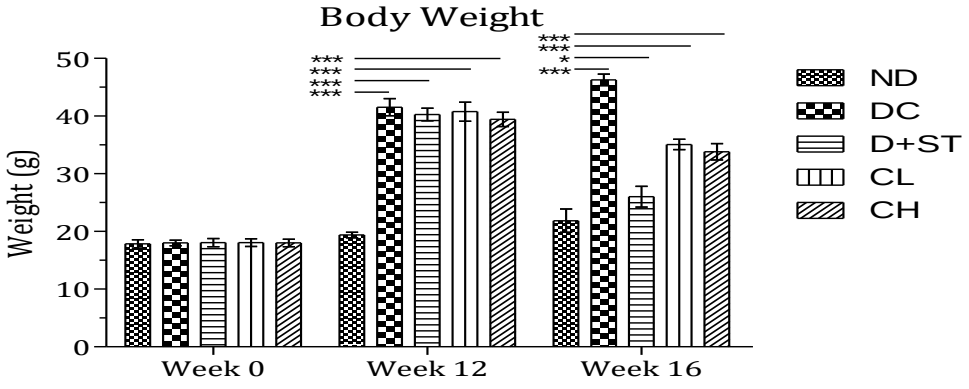


Figure 11: Effect of Coumarin on body weight of mice

The graphs formed by using Graph Pad Prism software. (Graph A) Graphical presentation of body weight of all five groups (ND= Normal Diet, DC= Diseased Control, D+ST= Diseased = Standard treatment, CL= Coumarin Low Dose & CH= Coumarin High Dose) on week 0, week 12 and week 16.

Glucose level

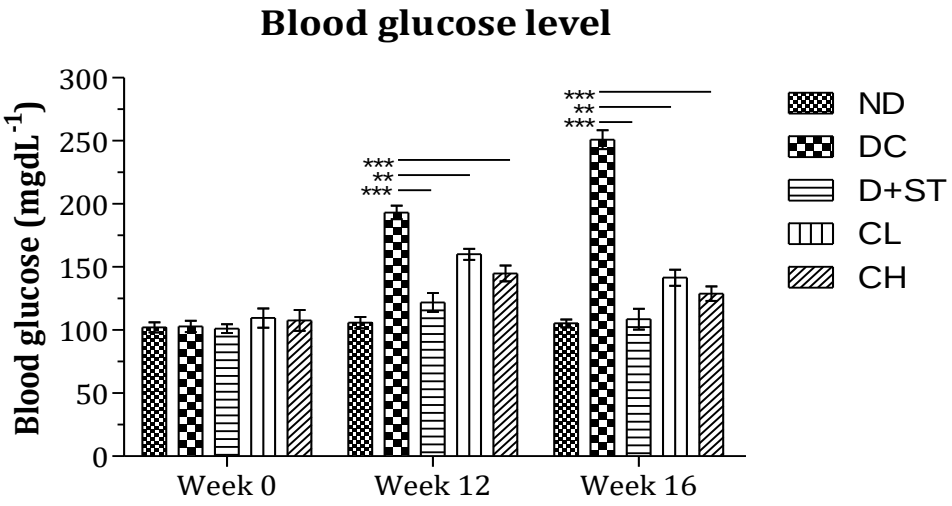


Figure 12: Effect of Coumarin on blood Glucose Level

A graph on week 0, week 12, and week 16, graphs of body weight for all five groups (ND= Normal Diet, DC= Diseased Control, D+ST= Diseased = Standard Treatment, CL= Coumarin Low Dose & CH= Coumarin High Dose) are shown.

Serum SFRP4 level

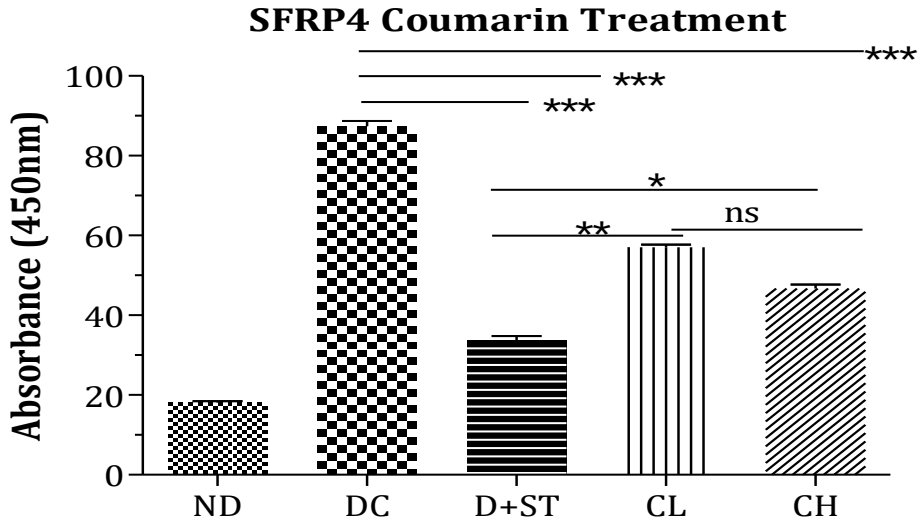


Figure 13: Effect of Coumarin on Serum SFRP4 Level

Coumarin dose were given to group 4A and 4B. Group 4A mice were treated with the low dose of coumarin as calculated earlier in experiment protocol, for 4 weeks. Group 4B mice were treated with

the high dose of coumarin as mentioned above in protocol, for 4 weeks. For four weeks, Group 5A mice were given a Low dosage of trehalose estimated earlier in the experiment methodology. Coumarin show significant (***) P-value that means coumarin significant inhibitory effect against SFRP4 active site. Standard and Low dose of coumarin show significant difference. Also, standard and high dose of coumarin show highly significant value. In contrast of low dose of coumarin and high dose of coumarin significant value is non-significant (ns) as shown in bar graph.

Effect of Coumarin
Body weight

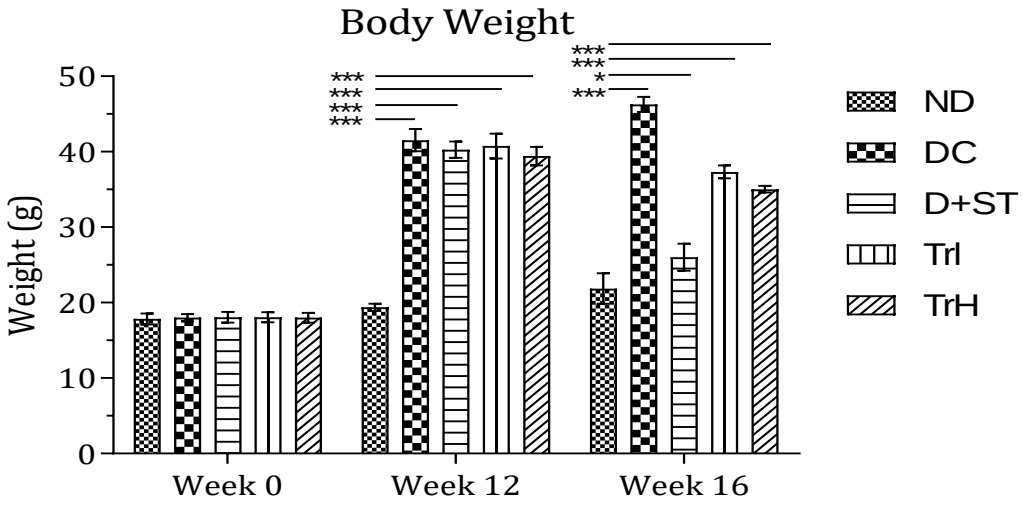


Figure 14: Effect of Trehalose on Body Weight

Graphical presentation of body weight of all five groups (ND= Normal Diet, DC= Diseased Control, D+ST= Diseased = Standard treatment, TrL= Trehalose Low Dose & TreH= Trehalose High Dose), on week 0, week 12 and week 16. Bar graph of body weight show the significant difference between the bars of week 12 and week 16.

Glucose level

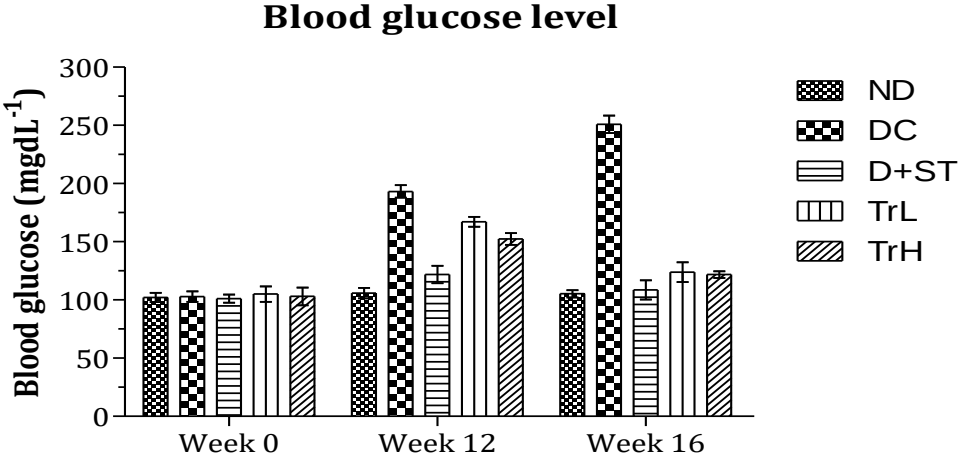


Figure 15: Effect of Trehalose on blood Glucose Level

According to this, on week 0, week 12, and week 16, graphical representations of body weight for all five groups (ND= Normal Diet, DC= Diseased Control, D+ST= Diseased = Standard Treatment, TrL= Treehouse Low Dose & TreH= Trehalose High Dose). The significant change was observed between the bars of week 12 and week 16 is seen in a bar graph of blood glucose level.

Serum SFRP4 level

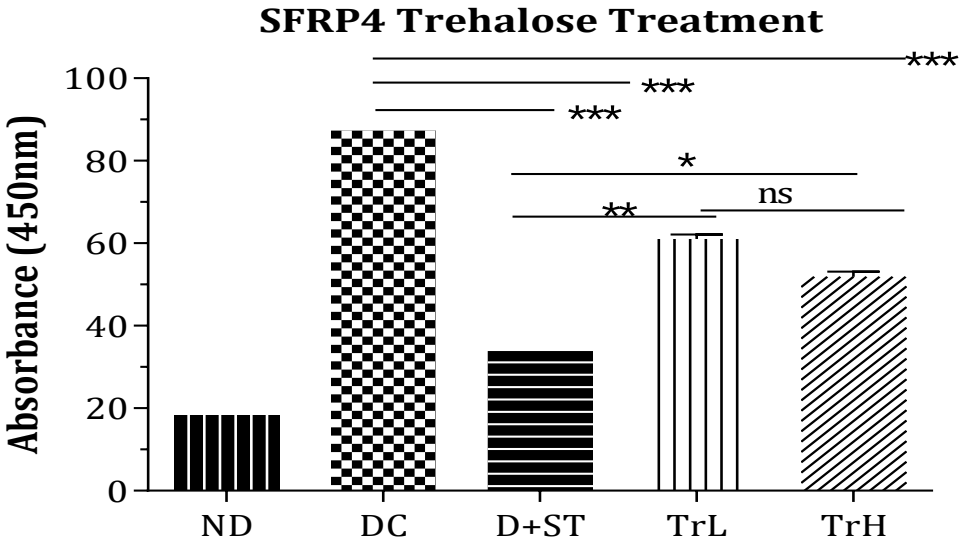


Figure 16: Effect of Trehalose on Serum SFRP4 Level

After four weeks, Group 5B mice were given the high dosage of trehalose described earlier in the procedure. After 4 weeks analysis of body weight a blood glucose level of mice of all groups data were collected and results were produced by applying statistics on obtained data. Mean, SD, and one way ANOVA applied on data and results obtained and analyzed by graphical presentation mentioned below. Trehalose has a substantial (***) P-value, indicating that it inhibits the active site of SFRP4. There is a considerable difference between the standard and low trehalose doses. Trehalose, at both its ordinary and high doses, is also extremely significant. As demonstrated in the bar graph, the significant value for both the low and high doses of trehalose is non-significant (ns).

DISCUSSION

Diabesity represents the simultaneous presence of both obesity and diabetes. Worldwide, the emergence of this concurrent health challenge is raising significant concerns [44]. Diabetes mellitus is a global chronic condition characterized by an increase in blood sugar levels caused by insufficient pancreatic insulin production [45]. T2DM is the leading cause of death worldwide. Chronic T2DM causes damage to the heart, kidneys, nerves, and eyes. The WHO estimates that there is a 170% increase in diabetes cases in developing countries [46]. Pakistan is ranked 10th out of 221 nations in the 2017 International Diabetes Federation (IDF) diabetes atlas, with 7.5 million diabetics aged 20 to 79 [47]. Changes in non-genetic risk factors must be mainly to blame for the recent rise in obesity and diabetes prevalence. Obesity is known as a multifactorial complex disease. It is a metabolic syndrome that increases the chances of developing many severe health complications such as, type 2 diabetes mellitus (T2DM), fatty liver disease, hypertension, steatohepatitis and dyslipidemia [48]. The global incidence of obesity has doubled since 1980 to an alarming rate. Nearly, a third of total population is now categorized as obese or overweight in the whole world [49]. Pakistan is the 9th most obese nation worldwide that is suffering from its prevalence which is affecting people of all age

groups more importantly in females and children as compared to men [50]. In humans, SFRP4 proteins are seen controlling various characteristics functions, like from development of embryo to adults along with various other biological processes. SFRP4 is considered to act as a Wnt signaling antagonists because of their own frizzled like CRD and NLD. SFRP4 also indicated their involvement in the modulation of many fatal disorders like diabetes, obesity, hypertension and cancer etc. [49]. Wnt proteins moved to stimulate of about three different signaling series of events. 1)Planar cell polarity of non-canonical, 2) Canonical Wnts–β-catenin, and 3) Wnt-Ca2+ pathways. However, sFRP4 occurs universally in nearly all types of tissues, it targets genes which depends on context and type of cell [51]. High expression level of secreted frizzled protein 4 is present in adipocytes of obese people and this protein is an antagonist which regulates adipogenesis by wnt signaling pathway. SFRP4 gene expression causes decline in the suppression of gluconeogenesis by insulin. However, hepatic lipogenesis occurred as SFRP4 increases insulin stimulation. SFRP4 stimulated processes might result in a vicious cycle, through which increased rates of de-novo lipogenesis and glycolysis causes hepatic lipid storage and resistance to insulin. Molecular operating environment (MOE) was used for molecular docking. Ferulic acid, Thymol showed good results as an antiobesity phytochemicals whereas coumarin and trehalose showed good interaction to SFRP4 protein as an antidiabetic phytochemical. These fours chemicals were used to evaluate *in-vivo* studies because they were economically less expensive and easily available. Ferulic acid, Thymol, coumarin and trehalose treatment predicts a significant decrease in the SFRP4 protein. Mean and standard deviation of each group was measured and One-Way ANOVA was used on Graphpad prism 9.2 to compare the mean of each group and analyzed their significant level ($p < 0.05$). Initially all the parameters that were required to be evaluated in this research were measured and noted at the initial stage (Week 0) after HFD treatment (Week 10) and after phytochemical treatment (Week 14). One-Way ANOVA was applied on the final data to compare the mean and S. D of each group and measured their significance level ($p < 0.05$). At week 0 there was non-significant difference among all groups ($p > 0.05$). But after the treatment of HFD SFRP4, glucose level and body weight were significantly increased. Ferulic acid, Thymol, coumarin and trehalose low dose and Ferulic acid, Thymol, coumarin and trehalose high dose showed significantly different to Diabetic control group and non-diabetic group ($p < 0.05$) while exhibiting non-significant difference among them. Plants have made the basis for sophisticated arrangements of traditional remedies. According to results of present study we can predict that phytochemicals have antidiabetic and antiobesity impacts that would be further use for prospective medicine of obesity and T2DM.

Conclusion

The current study results indicate that ferulic acid, Thymol against obesity, and coumarin, trehalose against diabetes act as potent antioxidant compounds, these compounds significantly lowered the level of SFRP4 in vivo & in vitro and can be recommended to reduce obesity and diabetes type 2. All these compounds at a lower dose significantly decreased the blood glucose level and weight of the body. These four compounds can be used in future studies to explore the molecular mechanisms involved in obesity-induced T2D and for curing the associated diseases.

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