Original Article

Identification of Ascorbic Acid and Gallic Acid as Novel Inhibitors of Secreted Frizzled-Related Protein for the Treatment of Obesity-Induced Type 2 Diabetes

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Abstract

Type 2 diabetes mellitus (T2D) has been reported as major public health issue rising at an alarming rate worldwide, and obesity is the leading risk factor for the development of T2D. Secreted frizzled-related protein 4 (*SFRP4*) released with inflammatory mediators from adipose tissues constrains the exocytosis of insulin containing granules from the pancreatic islets that leads towards the development to T2D. The significant overexpression of *SFRP4* in diabetic patients and its involvement in islet dysfunction suggest its critical role in the development of diabetes. Thus, this study was designed to explore the potential of ascorbic acid (AA) and gallic acid (GA) against *SFRP4* for the treatment of diabetes. Molecular docking approach was used for the prediction of binding interactions of AA and GA at the active pocket of *SFRP4*. Docking analysis indicated strong binding interactions of AA and GA to the amino acid residues at the active site of *SFRP4*. A significant reduction in the level of *SFRP4* was observed in transfected cells treated with AA and GA. For the evaluation of the cytotoxicity of AA and GA against HepG2 cells, MTT assay was performed. The results of MTT assay demonstrated that AA and GA are non-cytotoxic towards HepG2 cells at concentration of 15 μM. The oral administration of AA and GA to diet-induced obese mice caused significant reduction in body weight, blood glucose level, and *SFRP4* expression. The results of this study suggest that AA and GA have potential for the treatment of obesity-induced T2D.

Keywords

biomarker, diet-induced diabetic mice, HepG2 (NT), high fat diet, molecular docking

Introduction

Diabetes mellitus, a metabolic disorder with elevated blood glucose levels, is growing vastly with high mortality and economic burden worldwide, posing it to be a health threat of the 21st century. About 6.4% of the world population is suffering currently from diabetes, with 4.6 million deaths reported each year.[1] Worldwide 463 million people corresponding to

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9.3% of world's population are reported to be diabetic by International Diabetes Federation in 2019.[2] About 90% of diabetic individuals have type 2 diabetes (T2D) influenced by obesogenic lifestyle factors. Incidence of T2D is expected to rise to about 8% corresponding to 454 million individuals by the year 2030 while 548 million people are expected to be diabetic till 2045.[2] The pathophysiological mechanisms are not completely revealed, causing a major hurdle in development of effective therapies. Currently, employed treatments did not cure the disease or complications nor protect the β-cells against detrimental diabetic environment. Complete understanding of pathophysiology and prevention are the significant challenges to date.

Overweight is due to caloric abundance and sedentary lifestyle leading to insulin resistance and T2D. Due to slow-onset, most patients remain undiagnosed for years, and associated complications also appear when diagnosed.[3] The development of T2D is proposed due to various physiological mechanisms, including dysfunction of mitochondria and β-cells, oxidative stress, glycol-lipotoxicity, insulin resistance, and inflammation.[4] Adipose tissues (ATs) play critical roles in the homeostasis of glucose and lipids. Besides serving as a passive energy source, AT also act as endocrine organs and release different cytokines that play an essential role in the pathogenesis of T2D.[5] About 300 adipokines secreted by AT exhibit altered expression in obesity, leading to chronic inflammation, consequential inflammation of pancreatic islets, and reduced insulin sensitivity.[6] Hyperglycemia and elevated free fatty acid levels also trigger the release of proinflammatory mediators like interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), IL-1β, and IL-1 from pancreatic islets that also reduce insulin secretion due to β-cell dysfunction.[7] Various molecular signaling pathways, including Wnt signaling, act as key switches to promote lipid accumulation and inhibition of free fatty acids release leading to obesity-induced T2D. Increased production of nitric oxide and IL-1β also reduce insulin secretion by altering the Wnt pathway.[8]

The Wnt signaling antagonist-secreted frizzled-related protein 4 (*SFRP4*) is correlated with other inflammatory mediators that are released from adipose tissues numerous years before the onset of diabetes, which in turn causes β-cell dysfunction and reduces insulin secretion.[5,9,10] SERP4 is a member of the SFRP family, which is composed of 5 different proteins that modulate Wnt signaling by binding to the Wnt proteins and frizzled receptors. The release of *SFRP4* is induced by IL-1β which reduces the expression of voltage gated calcium channels in islets and so leads to membrane hyperpolarization and hinders exocytosis of insulin containing granules.[9] By playing a critical role in adipogenesis, adipose tissues work as primary sites to produce *SFRP4* that regulates adipogenic differentiation.[11,12] Obese individuals have a high level of *SFRP4* in their systemic circulation which increases the chances of developing T2D within the next few years by 5 times. *SFRP4* can thus act as a biomarker for the detection of early diabetes up to several years before its clinical onset.[5,9,10]

Various therapeutically important compounds and novel drugs are extracted from medicinal plants,[13] and during numerous *in vitro* and *in vivo* studies for the reduction of obesity and T2D,[14] these phytochemicals were proved to be particularly potent. Molecular docking algorithms were used in the first step towards identifying potential phytochemicals that could work against *SFRP4*. Both ascorbic acid (AA) and gallic acid (GA) exhibited good interactions with the predicted *SFRP4* structure, and thus, to explore their roles as modulator for the expression of *SFRP4*, they were tested further through *in vitro* and *in vivo* tests. These compounds also exhibit a potent role as inflammation suppressors and antioxidants in diabetes.[15,16] This means that through early identification and the targeting of high-risk individuals, these compounds could affect islets and the pancreas' β-cells to reduce obesity-induced T2D. Therefore, they could be studied for the regulation of *SFRP4* expression.

Materials and Methods

Molecular Docking

Molecular operating environment (MOE) software was used to identify interactions of active compounds with structurally important residues of *SFRP4* for their potential role to modulate and/or inhibit *SFRP4* expression as described earlier.[10]

Cytotoxicity Determination by MTT Assay

A549 and HepG2 cells were a kind gift from Cell and Molecular Biology Lab, Department of Zoology, Government College University, Faisalabad. Both cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotic for cytotoxicity determination of penicillin-streptomycin were seeded in 96 well plates and placed in CO_2 incubator (S-Bt Smart Biotherm of Biosan UK) for 24 hours at 37°C. Stock solution was prepared by adding AA and GA in DMSO, applied to each well at a concentration of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.9, and .97 μM in triplicates for 48 hours at 37°C and 5% CO_2 concentration. To each well, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated again for 4 hours. After discarding media, 150 μL of DMSO was added to the cells and kept at room temperature for 15 minutes, and absorbance was measured at 490 nm at Multimode Reader (Thermo Scientific, USA).[17]

Testing of Compounds in Transfected Cells

Total RNA was extracted from human adipose tissues by TRIzole reagent (Catalog No. 10760055-1), and cDNA was synthesized using revert aid First strand cDNA synthesis kit of Thermo Fisher Scientific (Catalog No. K1622). The full-length human *SFRP4* gene was isolated using gene-specific primers (forward 5′-ATCGCGGATCCATGTTCCTCTCCATCC-3′ and reverse 5′-ATCGCTCGAGCTACACTCTTTTCGGG-3′). Both PCR

product and mammalian expression vector pcDNA3.1+ were restriction digested with XhoI (ER0691) and BamHI (Catalog No. ER0051) and ligated by T4 ligase.[18,19] The product was then transformed in TOP10 cells by heat shock method and cultured on an ampicillin plate overnight at 37°C overnight. A single transformed colony was cultured in LB media to isolate plasmid by using GeneJET plasmid miniprep kit (Catalog number K0502). The isolated plasmid was confirmed by sequencing and restriction digestion. Transfection was done using Lipofectamine 2000 (Catalog # 11 668 019) with opti-MEM Reduced serum medium according to the manufacturer's instructions.[20] The cells were then incubated at 37°C and 5% CO_2 humidified incubator for 18–48 hours. Medium was changed after 8 hours. Transfected cells were applied with AA and GA dissolved in DMSO at a 4, 75, and 125 μM final concentration. Ascorbic acid (AA) and gallic acid (GA) at final concentrations of 4 μL, 75 μL, and 125 μL to elucidate their role in inhibition of *SFRP4* overexpressed by transfected pcDNA3.1+(*SFRP4*) plasmid. The non-transfected cell line HepG2 (NT) was used as negative control, while the transfected cell line without any treatment (TS) was used as a positive control. Then ELISA was performed to assess the level of *SFRP4* protein.[21]

Testing on Animal Model

A high caloric diet–induced diabetic mouse model was used to evaluate the effects of AA and GA *in vivo*. Before conducting an experiment, the procedure was approved by animal ethics committee of GCUF. Male albino mice of 5–6 weeks age weighing 18–22 g were obtained from animal house of Department of Pharmaceutical Sciences, Government College University, Faisalabad, Pakistan. The mice were housed at room temperature range 24 ± 3°C with 12 hours light/dark cycle and 45 ± 5% relative humidity in polypropylene cages at well ventilated animal house of Physiology Department, GCUF. Animals had free excess to food and water and left for 1 week to acclimatize the environment before experiment.

Diabetes induction and treatment in mice: Mice were divided into 7 groups (n = 8 each group). Table 1 summarizes the details of groups and treatments. The mice in non-diabetic control group were fed on normal chow, while a high fat diet (HFD) (60% calories from fat) was given to the mice to induce diabetes. After 12 weeks of HFD treatment, body weight and blood glucose level were checked from the tail vein. The mice with body weight above 40 g and blood glucose level above 140 mg/dL were used for experiment.[22] 2 concentrations (i.e., high and low) of AA and GA were given orally to mice for 21 days. The mice were dissected, and blood samples were collected properly in labeled serum collecting tubes to collect serum. Serum *SFRP4* level was determined by ELISA kit of fine Test Catalogue No. EM1725 by Wuhan Fine Biotech Co., Ltd. China.[23] Tissue samples were stored immediately in liquid nitrogen to study inhibitor activity at transcription level.

Enzyme-Linked Immunosorbent Assay

ELISA was performed to find the expression of *SFRP4* in serum. For this purpose, mouse *SFRP4* ELISA kit of fine Test catalogue No. EM1725 by Wuhan Fine Biotech Co., Ltd. China was used as per the instruction manual.[28]

Real Time PCR: *SFRP4* expression in the pancreas and adipose tissues was determined at the transcription level using quantitative real time PCR (qRT-PCR). The total RNA was extracted and used to synthesize cDNA by following conditions described earlier. To amplify cDNA, Maxima SYBER Green/ROX qPCR master mix (2X) (catalog Number K0221) was used for 40 amplification cycles in qRT-PCR (CFX96 Touch Real-Time PCR detection system). The set of primers used by Bai et al.[29] was employed for amplification of *SFRP4* and β-actin. Cycling parameters include 95°C for 3 min, 94°C for 30 sec, 55°C for 30 sec, 72°C and 40 cycles for melt curve 95°C for 30 sec, 60°C for 1 min and 65°C for 10 sec. Melting curve analysis was carried out at the end of each cycle to ensure single amplicon. The relative level of *SFRP4* and β-actin was calculated from the same sample by using $2^{-(\Delta\Delta Ct)}$ method.[30]

Statistical Analysis

The data was presented as mean ± standard deviation (SD). Statistical difference was determined by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test using GraphPad Prism 9 (Graph Pad Software Inc., San Diego, USA). $P < .05$ was considered statistically significant.

Table 1. Animal Groups and Treatments.

Sr No	Group description	Diet	Treatment	Reference
1	Non-diabetic control	Normal chow	No	[24]
2	Diabetic control without treatment)	HFD	No	[24]
3	Diabetic control with standard treatment	HFD	Metformin (300 mg/kg)	[24]
4	Diabetic with AA low dose	HFD	Ascorbic acid (60 mg/kg)	[25,26]
5	Diabetic with AA high dose	HFD	Ascorbic acid (120 mg/kg)	[25,26]
6	Diabetic with GA low dose	HFD	Gallic acid (10 mg/kg)	[27]
7	Diabetic with GA high dose	HFD	Gallic acid (20 mg/kg)	[27]

Enzyme-Linked Immunosorbent Assay (ELISA).

Results

Molecular Interactions

Docking analysis indicates the resonant interactions of AA and GA to the amino acid residues in the active pocket of the predicted *SFRP4* structure (Figure 1). The RMSD values and interacting amino acid residues of *SFRP4* are given in Table 2.

Cellular Cytotoxicity

Both AA and GA are cytotoxic at higher concentrations. The percent cell viability of AA (Figure 2(a)) and gallic acid (Figure 2(b)) indicated that these have minimal effects on cellular integrity when applied at low doses (.97 µM, 1.9 µM, 3.9 µM, 7.81 µM, and 15 µM) while about 50% reduction in cell number is observed at 500 µM concentration.

Ascorbic acid at 4 µL concentration non-significantly reduced the *SFRP4* expression as compared to the positive control ($P > .05$), while a significant difference was observed when compared to the NT cells ($P < .0001$). A highly significant difference was also observed when all 3 doses were compared to each other ($P < .001$). Maximum inhibition was observed at 125 µL AA concentration that also exhibited a significant difference ($P < .01$) from the NT control. A significant reduction in level of *SFRP4* was revealed by gallic acid treatment at a final concentration of 4 µL, 75 µL and 125 µL

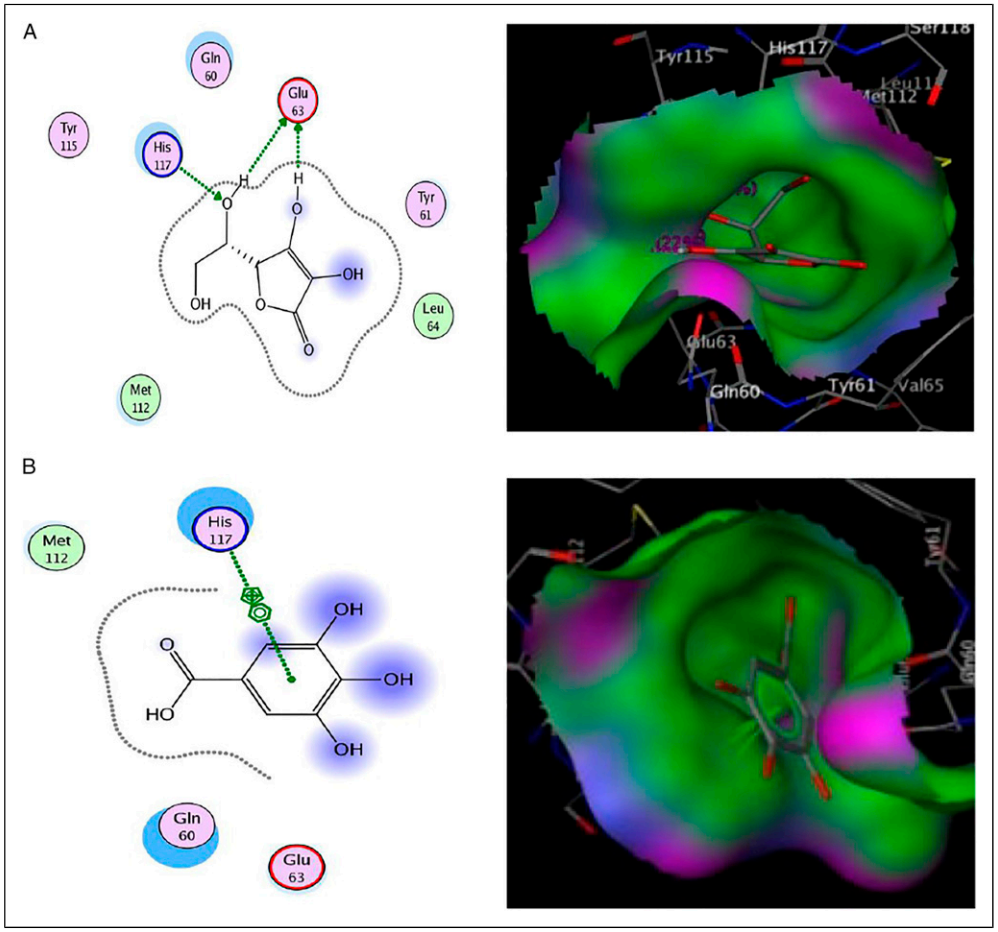

Figure 1. Docking results of ascorbic acid with SFRP4 as receptor protein (a). AA displays interaction with Glu63 and His117 in the active pocket and Gln60, Tyr61, Leu64, Tyr115, Met112 are found in the surrounding region (left). Binding patterns of AA with *SFRP4* (right). (b); Docking results of gallic acid with *SFRP4*. GA interacts with amino acid His117 and the amino acid residues Gln60, Glu63, Tyr61, Met112, and Pro120 are found in the surrounding region (left). Binding patterns of GA with *SFRP4* (right).

Table 2. Docking Interactions of AA and GA with Predicted *SFRP4*'s Active Pocket.

Sr No	Compound	RMSD value	S-Score	Interacting residues	Surrounding residues
1	Ascorbic acid	.7210	−11.0754	Glu63, His117	Gln60, Tyr61, Leu64, Tyr115, Met112
2	Gallic acid	1.5061	−6.618	Glu63, His117	Gln60, Tyr61, ser 118, Pro120

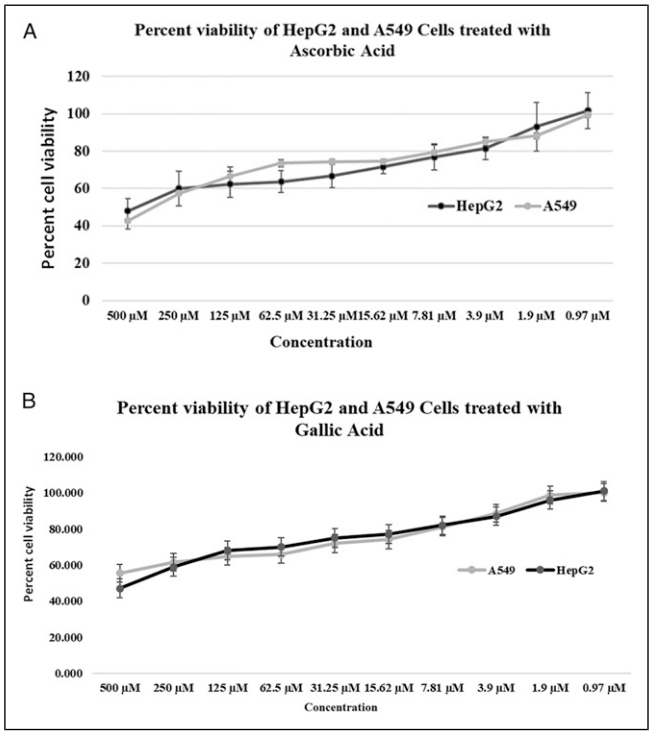

Figure 2. Percent cell viability of HepG2 and A549 cells. (a) Ascorbic acid treatment. The percent cell viability decreases with increasing concentration of ascorbic acid. Data is represented as mean ± SD. (b) Gallic acid treatment. The percent cell viability decreases with increasing concentration of gallic acid. Data is represented as mean ± SD.

when compared to the negative control (NT) with $P < .0001$ for each. However, 4 µL dose significantly reduced expression of *SFRP4* ($P < .01$) while a highly significant reduction was observed at 75 µL and 125 µL ($P < .0001$) (Figure 3).

Body weight: The oral treatment of diabetic mice with low and high doses of AA and GA significantly reduced the body weight of treated mice ($P < .05$). Figure 3(a) represents the effect of ascorbic acid body weight. At the start of animal trial mice, all groups have a non-significant difference in body weight ($P > .05$). Fructose and HFD treatment for 12 weeks significantly increased the body weight ($P < .0001$). Treatment with HFD highly significantly ($P < .0001$) increased the body weight of mice in the diabetic group as compared to a non-significant difference at the start of the experiment ($P > .05$). The AAL dose (60 mg/kg) and AAH doses concentration (120 mg/kg) significantly reduced the body weight from 41.25 ± 1.667 to 36.625 ± 2.445 ($P > .0001$) and 41.375 ± 1.922 to 36.125 ± 2.587 ($P > .0001$), respectively, but a non-significant difference was observed from the normal (ND) and metformin-treated group ($P > .05$). Furthermore, both AAL and AAH dose exhibited a non-significant change on body weight. Gallic acid treatment with low dose (GAL 10 mg/kg) and high dose (GAH 20 mg/kg) significantly reduced the body weight 43.376 ± 1.76 to 34.87 ± 2.4 and 42.5 ± 1.511 to 36.75 ± 2.9, respectively, while metformin treatment also reduced body weight from 42.625 ± 1.922 to 37.125 ± 2.295 having a non-significant difference from the GAL and GAH groups ($P > .05$) (Figure 4). The difference was non-significant ($P > .05$) between ND, D+ ST, GAL, and GAH groups after 4 weeks treatment while a highly significant difference ($P < .0001$) was observed between D and gallic acid–treated groups ($P < .0001$). Table 3 summarizes the mean body weight and standard deviation of each group.

Blood glucose level (BGL): The BGL was significantly increased in the diabetic group compared to the ND group after HFD treatment for 12 weeks ($P < .0001$). Ascorbic acid treatment significantly decreased the BGL at both AAL (207.87 ± 24.95 to 134.25 ± 9.01) and AAH dose (216.5 ± 24.95 to 139.25 ± 8.82) ($P < .0001$). Both doses have a non-significant difference ($P > .05$) from the standard treatment (Figure 5). The BGL after 4 weeks treatment of gallic acid reduced significantly ($P < .0001$) as compared to the D control, and non-significant change was observed from the N group ($P > .05$). Low dose (GAL 10 mg/kg) and high dose (GAH 20 mg/kg) reduced the BGL significantly ($P < .0001$) 216 ± 20.291 to 133.5 ± 10.057 and 218.5 ± 19.011 to 36.75 ± 2.9, respectively. The N, D + ST, GAL, and GAH groups were non-significantly different ($P > .05$). The difference was significant ($P > .05$) between ND and GAL groups ($P < .0001$) and GAH groups after 4 weeks of treatment. The mean BGL is summarized in Table 4.

Serum *SFRP4* level: The level of *SFRP4* in serum significantly increased after HFD treatment for 12 weeks in the diabetic group compared to the ND group ($P < .0001$). Ascorbic acid treatment significantly decreased the serum *SFRP4* level at both AAL 72.663 ± 3.3699 to 37.2041 ± 6.259 and AAH dose 72.718 ± 4.4212 to 39.992 ± 3.475 with a significant difference to the diabetic group ($P < .0001$). The level of *SFRP4* exhibited a significant change by both low and high doses of gallic acid. A highly significant difference was observed among the ND, GAL, and, GAH groups ($P < .0001$). The difference was significant compared to the D group by both GAL and GAH groups ($P < .0001$). The GAL and D + ST represented a significant change ($P = .048$) and highly significant from GAH ($P = .0003$). Both the doses were having a non-significant difference among them ($P = .5231$). The mean *SFRP4* serum levels are summarized in Table 5. The effects of AA and GA on serum *SFRP4* level have been shown in Figure 6.

The fold change in mRNA expression in pancreas and adipose tissues of different tissues is represented in Figure 7. Both AA and GA significantly ($P < .0001$) down regulated the *SFRP4* gene expression in both tissues. In pancreas, significant change was observed ($P < .006$ and $P < .0001$) for low and high doses, respectively.

Discussion

Type 2 diabetes is a chronic metabolic syndrome affecting about 3.2% of world population. About 592 million people contributing to 4.4% of the world population projected to be at risk of

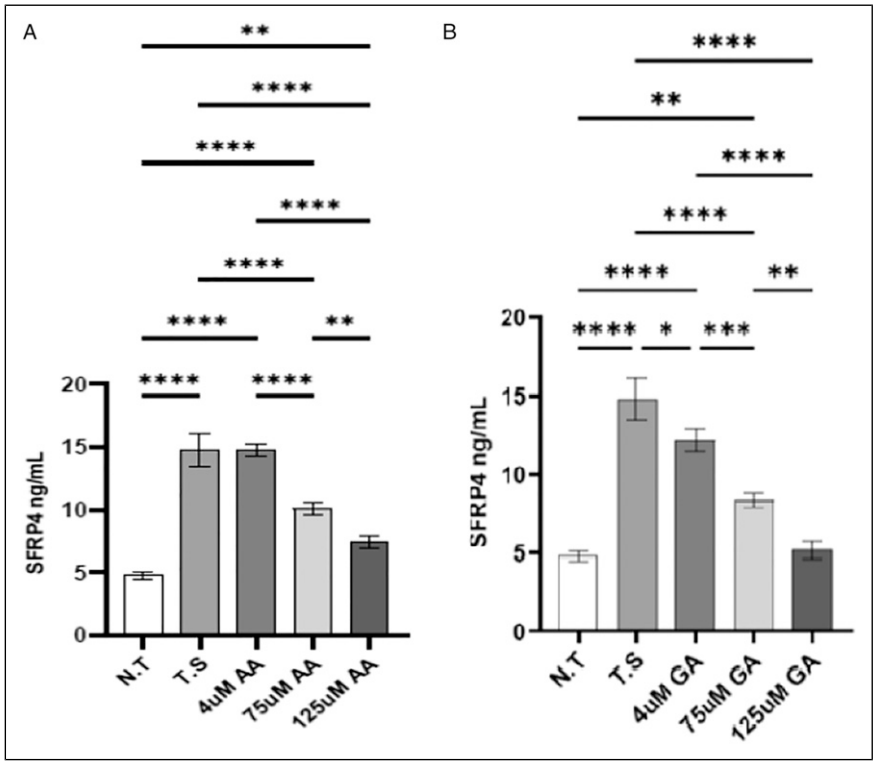

Figure 3. Effect of AA and GA on SFRP4 level. The bars are representing the *SFRP4* concentration in the cell lysate estimated by ELISA. (a) Ascorbic acid; (b) Gallic acid. Statistically significant differences are represented by asterisk ($P < .05$*, $P < .01$**, $P < .001$*** and $P < .0001$****).

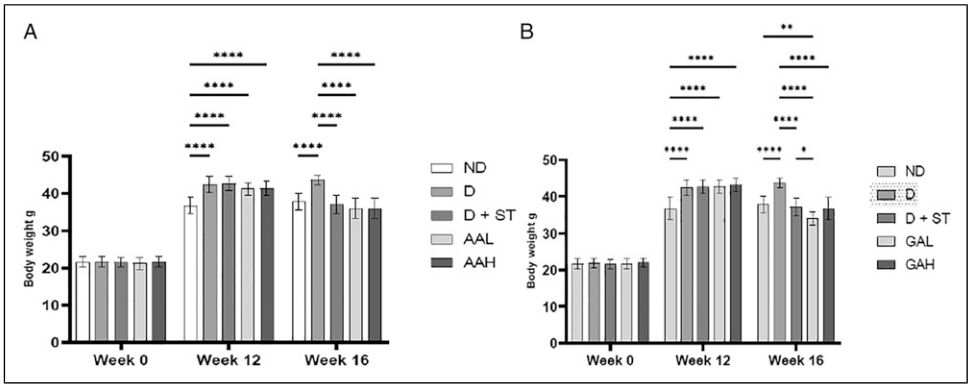

Figure 4. Effect of ascorbic acid and gallic acid on body weight. (a) Graph bars representing non-diabetic (ND), diabetic control (D), Metformin treatment (D + ST), ascorbic acid low dose (AAL) and ascorbic acid high dose (AAH). (b) A significant difference in body weight was observed after gallic acid treatment at both low ($P < .0001$) and high dose ($P < .0001$) with a statistically non-significant difference from the ND and metformin treated group ($P > .05$). The non-significant differences are not mentioned ($P < .05$*, $P < .01$**, $P < .001$*** and $P < .0001$****).

developing diabetes till 2035.[31] Obesity is the primary cause leading to insulin resistance and β-cell dysfunction in T2D patients. To attain an average blood glucose level, the reduced insulin level and increased sensitivity are potential targets and reduce obesity and related ailments.[5] No cure is developed so far, and the incidence is increasing at an alarming rate, posing both health and economic burden worldwide. Furthermore, current therapies have limitations due to adverse side effects.

When looking at treatments for various diseases, including for T2D, cost-effectiveness is a huge factor and thus a great deal of attention is given to the phytochemicals.[10] In addition, these compounds create the least amount of side effects to the hypertensive, renal, or gastrointestinal functions.[32] Various molecular docking algorithms are also employed to target the critical proteins responsible for metabolic malfunctions, such as T2D or cancer, and predict

Table 3. Effect of Ascorbic Acid Treatment On Body Weight Represented As Mean ± SD.

S. No	Group	Dose (mg/kg)	Body weight (g)		
			Week 0	Week 12	Week 16
1	Group1 (ND)	—	21.625±1.41	36.875±2.90	37.875±2.167
2	Group 2 (D)	—	21.75±1.28	42.375±2.133	43.625±1.18
3	Group 3 (D + ST metformin)	50	21.625±1.18	42.625±1.922	37.125±2.29
4	Group 4 (AAL)	60	21.25±1.581	41.25±1.667	36.625±2.445
5	Group 5 (AAH)	120	21.75±1.388	41.375±1.922	36.125±2.587
6	Group 6 (GAL)	10	21.625±1.407	42.75±1.752	34.125±1.807
7	Group 7 (GAH)	20	22±1.309	43.125±1.807	36.75±2.964

The Data Is Represented as Mean ± S.D.

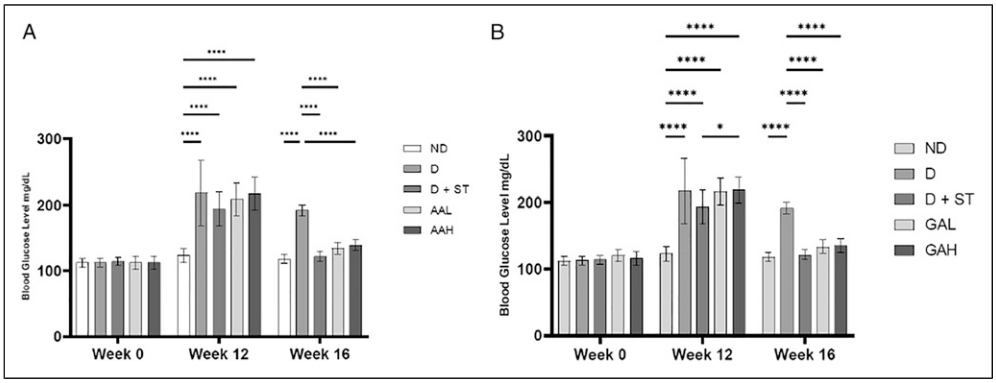

Figure 5. Effect of ascorbic acid on blood glucose level. Graph bars representing groups non-diabetic (ND), diabetic control (D), standard treatment (D + ST), ascorbic acid low dose (AAL) and ascorbic acid high dose (AAH). (a) Treatment with ascorbic acid significantly decreased ($P < .0001$) the BGL while both AAL and AAH exhibited a non-significant difference among both and to D + ST groups ($P > .05$). (b) Gallic acid treatment for 4 decreased the body weight of both groups ($P < .05$*, $P < .01$**, $P < .001$*** and $P < .0001$****).

Table 4. Effect of ascorbic acid treatment on blood glucose level.

S. No	Group	Dose (mg/kg)	Blood glucose level (mg/dL)		
			Week 0	Week 12	Week 16
1	Group1 (ND)	—	112±6.88	122.75±10.68	117.875±6.68
2	Group 2 (D)	—	112.75±6.71	217.5±48.99	191.5±8.73
3	Group 3 (D + ST metformin)	50	113.875±6.2	193.5±25.71	121.75±7.45
4	Group 4 (AAL)	60	112±9.02	207.87±24.95	134.25±9.01
5	Group 5 (AAH)	120	112.25±9.93	216.5±24.95	139.25±8.82
6	Group 6 (GAL)	60	120±8.502	216±20.291	133.5±10.057
7	Group 7 (GAH)	120	116±10.3372	218.5±19.011	135.38±9.999

The data is represented as mean ± S.D.

the affinity and activity of drug candidate compounds via binding orientations.[33] As a crucial first step in identifying *SFRP4* inhibitors, various phytochemicals taken from potential antidiabetic compounds were docked to *SFRP4* in order to explicate their modulatory role. In the current study, unique binding orientations were exhibited by both AA and GA confined to the active pocket. Furthermore, no π-π stacking were exhibited by the phytochemicals and aromatic *SFRP4* residues. The results are in accordance with Hassan et al.[34] that focused on *SFRP4* for the curing of ovarian carcinoma. Close RMSD values are also represented by AA and GA, while the major amino acids that interacted with both AA are in accordance with the results obtained by Bukhari et al.[35] and include Gln60 and His117. These antioxidant and anti-inflammatory compounds were deeply embedded in the active pocket and showed strong

Table 5. Effect of AA ad GA Treatment on Serum *SFRP4* Level.

S. No	Group	Dose (mg/kg)	Serum *SFRP4* level (ng/dL)		
			Week 0	Week 12	Week 16
1	Group1 (ND)	—	15.757±3.900	29.693±5.169	31.655±5.73
2	Group 2 (D)	—	14.6575±3.937	66.271±11.97	79.04±8.694
3	Group 3 (D + ST metformin)	50	15.647±4.458	72.078±3.699	44.461±8.484
4	Group 4 (AAL)	60	15.75±4.258	72.663±3.699	37.041±6.259
5	Group 5 (AAH)	120	15.148±3.367	72.718±4.212	39.992±3.475
6	Group 6 (GAL)	10	16.871±3.592	72.445±5.009	53.977±7.912ns
7	Group 7 (GAH)	20	16.212±4.566	71.391±5.55	59.322±9.979

The data is represented as Mean ± S.D.

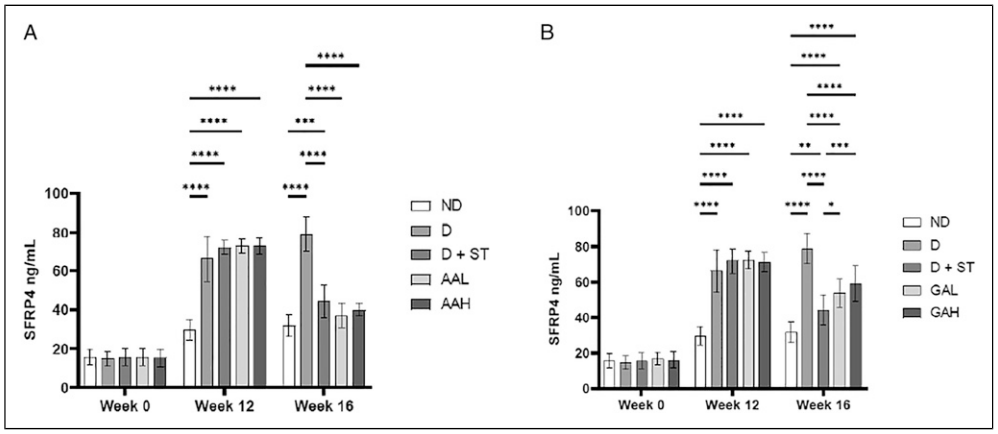

Figure 6. Effect of AA and GA on serum SFRP4 level. The level of significance indicated by *($P < .05$*, $P < .01$**, $P < .001$*** and $P < .0001$****). (a) HFD treatment significantly increased the level of serum *SFRP4* ($P < .0001$). (b) AA treatment both AAL and AAH significantly decreased ($P < .0001$) the serum *SFRP4* level.

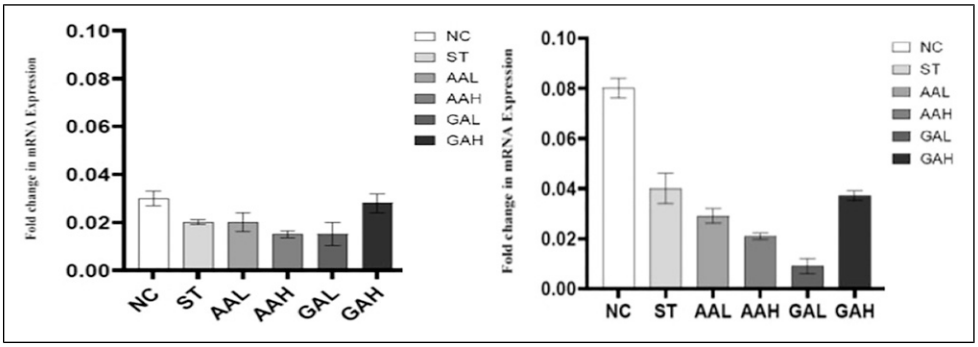

Figure 7. Fold change in mRNA expression level of SFRP4: Relative mRNA expression in pancreas and adipose tissues of treated groups relative to diabetic control (NC: Normal Control; ST: Standard Treatment (Metformin); AAL: Ascorbic Acid Low, AAH: Ascorbic Acid High; GAL: Gallic Acid Low; GAH: Gallic Acid High). The bars are representing mean ± SD.

interactions with Gln60 and Glu63 and His117. Both AA and GA also exhibit hypoglycemic activities in both *in vitro* and *in vivo* tests, with minimal side effects due to the already established hypoglycemic and cytoprotective activities.[36-38]

As the first step for *in vitro* studies, the cytotoxicity of these compounds was assessed in 2 cell lines, A549, and HepG2, by MTT assay. Both AA and GA were found to be less cytotoxic when at a low dosage, while with an increased concentration of the compound, the cytotoxicity increased. The cells showed above 60% cell survival rate to different concentrations of AA with minimal cytotoxicity at a lower concentration below 125 μM, agreeing thus with the results of Cho et al.[39] AA's

anti-cancer effect, meanwhile, is reported at a greater concentration (>1 mM) when applied to various cell lines. This means that in the current study, a lower concentration of AA indicates a significantly high percentage of cell viability, while a high dose tends to decrease the survival rate of the cell which is in accordance with the results found by Cho et al.[39] Some studies reported that AA's anti-cancer activity is mediated by oxidative DNA damage,[40] a property that could reduce the oxidative stress induced in T2D and thus causes a reduction in the inflammatory process.

The establishment of the diabetic model included the critical step of identifying the antidiabetic compounds. An increased accumulation of a high calorie diet, including fats, carbohydrates, and any major energy derived from fats, is found to be critical in the establishment of the obesity-induced T2D animal model. In addition, HFD and fructose cause an increase in adipose mass and size. Furthermore, as indicated by the current study and previous studies, an increased intake of fructose causes significant weight gain and an elevation in BGL.[41] This was evident as a significant increase in body weight and BGL was observed ($P > .05$) when comparing the mice fed on a normal diet and those fed on high fat fructose, something which is also in accordance with previous studies.[42] Kelly et al.[43] reported that metformin, in addition to regulating the blood glucose levels, also significantly reduces body weight which is also in accordance with the results of the current study. Metformin has also been found to be effective in reducing BGL, LDL, and cholesterol in aged individuals.[44]

AA is 1 of the most widely used vitamins (vitamin C) and is recommended as an adjunct remedy for hypoglycemia and reduced blood pressure in T2D patients.[45,46] Furthermore, there is a reduction in the levels of IL-1β, TNF-α, and IL-6, all of which also support AA's role in reducing the blood glucose level by decreasing production of the IL-6 that triggers the production of *SFRP4* through adipose tissues.[9] There is a significant increase in the insulin-induced glucose clearance upon the supplementation of AA by an increased synthesis of prostaglandin E1 which ultimately suppresses the inflammatory mediators IL-6 and TNF-α.[47,48] The findings of this study also support the role of AA in down-regulating *SFRP4* mRNA expression in both adipose tissues and the pancreas as the resulting elevated insulin level ultimately leads to an increased glucose metabolism. Furthermore, AA is reported to prevent insulin aggregate formation which thus stops the development of pathological conditions, including diabetes.[49] This study also supports the role of AA in the reduction of T2D by specifically targeting *SFRP4* as well as supporting its role in reducing oxidative stress and related complications.

The 3,4,5-trihydroxybenzoic acid, commonly called gallic acid (GA), is a phytoconstituent responsible for the treatment of a variety of diseases. GA is a strong antioxidant which reportedly modulates reactive oxygen species.[50] Various biological pathways are targeted by GA, and thus, it has multiple targets in the living system, including antidiabetic, nephroprotective, anti-inflammatory, and hepatoprotective, among others. Through PPar-γ and Akt pathways in both animal cell lines as well as in diabetic animal models, Variya et al.[51] reported a strong hypoglycemic activity from GA. In the present study, significant weight loss and glucose level reductions in the blood were observed upon the administration of GA which are in accordance with the previous findings.[51] This study also supports the inhibitory role of GA against obesity-induced diabetes. Diabetes causes other complications in the individuals as well as in the diabetic animal models which are significantly decreased by the administration of GA through altered cellular signaling mechanisms and by changes in the biochemical biomarkers of oxidative stress. The pancreatic β-cells are also protected by the administration of GA which in turn increases insulin sensitivity and its secretion and ultimately leads to a reduced BGL.

In contrast, to the study by Vilapakkam et al.[52] to induce diabetes in animal model by streptozotocin (STZ) injection intraperitonially, the current evaluation employed HFD and fructose treatment to study the effects of 2 doses of GA, that is, 10 mg/kg (GAL) and 20 mg/kg (GAH) as reported previously.[52,53] According to the results reported earlier, a significant reduction in BGL, total cholesterol, and triglycerides was observed. The relative expression of *SFRP4* mRNA was also down-regulated in both pancreas and adipose tissues. To evaluate the role of various phytochemicals as a potential treatment for delaying the onset of T2D, the level of *SFRP4* was reported in diabetic and normal animals.

Conclusion

The results of the current study indicate that both ascorbic acid and gallic acid, in addition to their role as potent antioxidant compounds, significantly reduced the level of *SFRP4 in vitro* and *in vivo* and can be recommended to reduce obesity-induced T2D. Both compounds were found non-toxic to the cells at a lower dose and significantly reduced the BGL and body weight. These observations allow us to explain the contribution of these flavonoids to the antimicrobial activity of polyphenols. These compounds can be employed in future to understand the molecular mechanisms involved in obesity-induced T2D and for curing the associated complications.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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