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Ficus racemosa **leaves**

FR1   **FR2**   **FR3**
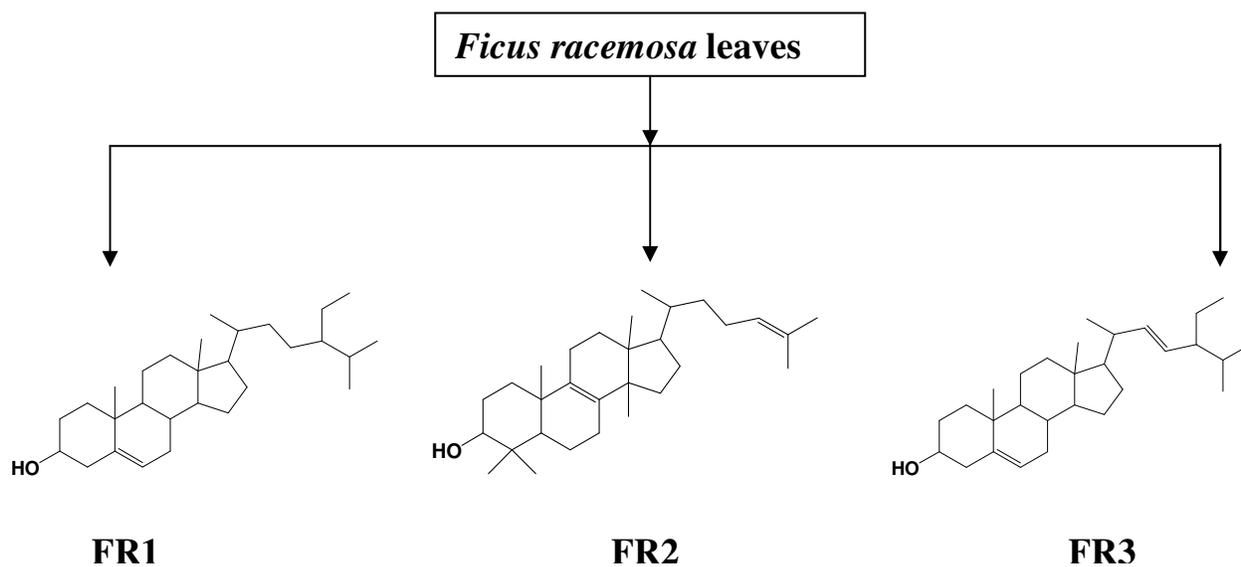
> - **Isolation and Structure confirmation through NMR and MS**
> - **Antidiabetic and hypolipidemic activities using streptozotocin induced albino rats**
> - **Toxicological assessments**
> - **Mechanistic study to PPAR$_\gamma$ through molecular docking**

Antidiabetic effects of isolated sterols from *Ficus racemosa* leaves

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Abstract

The present study was undertaken to evaluate the antidiabetic, hypolipidemic, and toxic effects of isolated sterols from *Ficus racemosa* (FR) leaves using streptozotocin induced diabetic rats. Diabetes was induced by administration of streptozotocin (50mg/kg) intraperitonially to albino rats. Three sterols were administered once in a day for a period of seven days at dose of 100 mg/kg body weight. Blood glucose and body weight changes were measured at different (1st, 3rd, 5th, and 7th) days of experiment. Serum lipid profile, hepatic biomarker enzymes levels were measured and various antioxidant parameters in liver, pancreas were also determined at the end of experiment. Our results collectively suggested that oral administration of sterols significantly reduced blood glucose level and restored body weight. Sterols also reduced serum lipid parameters and improved (high density lipoprotein) HDL as compared to diabetic control group, signifying hypolipidemic action. They increased glutathione and various enzyme levels in pancreas at the same time. Various oxidative stress parameters in liver were decreased after sterols administration with respect to diabetic control rats. Three sterols possessed antidiabetic, antioxidant, and hypolipidemic activities in STZ induced diabetic rats, which supported the use of FR as a supplement for future drug design perspective.

Running Title:

Antidiabetic Effects of sterols from *Ficus racemosa*

1. Introduction

Type II diabetes mellitus (also known as non-insulin-dependent diabetes mellitus) is the fourth leading cause of death in worldwide which is also a major contributor to blindness and chronic renal failure. The disease is linked to several long-term complications like retinopathy, nephropathy, neuropathy, hypertension, atherosclerosis and hyperlipidemia [1,2]. It affected an estimated 366 million people in 2011 which expected to increase up to 552 million in 2030 [3]. Several synthetic drugs available for diabetes which is cost effective and highly toxic to liver and kidney [4,5]. Moreover, there is an interest to develop safer drugs and it should be of lesser side effects. India is the richest source of natural drugs and different parts of medicinal plants with isolated materials have been found to be effective against diabetes [6]. According to World Health Organization (WHO), antidiabetic agents from natural origin are the richest source for diabetes therapy in Indian Traditional System of Medicine [6].

Ficus racemosa (FR) belongs to the family Moraceae, is distributed in India (from Himalayan region to Southern India), Southeast Asia and Australia [7,8]. Various pharmacological actions had been reported with this plant which includes antioxidant, antidiabetic, anti-inflammatory, antidiarroheal, antipyretic, antibacterial, antifungal, antifilarial, hypolipidemic, hepatoprotective actions [9-10]. β-Sitosterol had been isolated from leaves and steam bark previously which have potent Antidiabetic activity [7]. The other isolated materials from leaves of FR plants are flavonoids, triterpenoids, alkaloids, tannins and triterpene (gluanol acetate and racemosic acid) [7].

To the best of our knowledge, no detailed antidiabetic studies had been performed previously with sterols isolated form leaves of FR in streptozotocin (STZ) induced diabetic rats. Therefore, the main objective of this study is to investigate the role of isolated sterols from leaves of FR in curing diabetes. To achieve this goal, three sterols had been isolated (FR1, FR2

and FR3) from petroleum ether extract of FR leaves. Again, antidiabetic, hypolipidemic actions and toxicological responses of these materials were determined in STZ induced rats. Later, binding affinities to peroxisome proliferator activated receptor gamma (PPAR$_\gamma$) through docking studies were performed to investigate the mechanism of action of isolated materials.

2. Materials and methods

2.1. Materials

Disodium ethylene diamine tetra acetic acid (EDTA), disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium citrate and trichloroacetic acid were procured from SD Fine Chemicals, Mumbai, India. 5, 5-dithiotris-2-nitro benzoic acid, tris buffer, sodium carbonate, glacial meta phosphoric acid, sodium potassium tartarate and sodium chloride were purchased from Loba Chemicals, New Delhi, India. All other chemicals were obtained from Himedia, Mumbai, India. All the solvents and chemicals were of analytical grades with 99% purity and in house distilled water was used throughout the experiment.

2.2. Plant materials

The fresh leaves of FR were collected during the month of July from Lucknow, Uttar Pradesh, India and authenticated by Department of Horticulture, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow. A voucher specimen had been deposited for future reference (60/SM/DAPS/BBAU/14). The plant materials were air dried under shade, powdered and extracted with petroleum ether (60-80^{0}C) with Soxhlet apparatus by successive solvent extraction method (20 h). Finally, the extracted samples were evaporated by using rotary vacuum evaporator (IKA, Germany). The final yield was 12% and petroleum ether extract of FR was used for further studies.

2.3. Isolation of sterols

The petroleum extract of the leaves of FR on chromatographic resolution over silica gel (60-120 mesh) yielded FR1 to FR3. The elution solvent was petroleum ether (60-80^{0}C) and ethyl acetate in a ratio of 95:5 (FR1), 90:10 (FR2) and 50:50 (FR3) and recrystallized using same solvents. The structures were elucidated on the basis of 1D NMR (nuclear magnetic resonance spectroscopy) and MS (mass spectroscopy).

2.4. Experimental animals

Healthy adult Albino Wistar rats (125–150 g) were used for the study and protocol was approved by Institutional Animal Ethical Committee (Approval No. UIP/IAEC/2014/FEB/10). Rats were housed in polypropylene cages in standard environmental conditions (temperature 25±5^{0}C, relative humidity 55±10%). All the animals were acclimatized in laboratory condition for 7 days. The rats were fed on a standard pellet diet and had free access to water during acclimatization.

2.5. Induction of Hyperglycemia

Hyperglycemia was induced in albino Wistar rats by single dose of STZ (50 mg/kg, intraperitoneally) reconstituted in normal saline after overnight fasting. On 5th day after STZ administration, the blood sample was collected through tail vein puncture and blood glucose level was measured using one touch select Glucometer (Johnson & Johnson, India) strips. Rats with fasting blood glucose level 250 mg/dL were considered for hyperglycemic condition [11].

2.6. Experimental design

Rats were randomly divided in to six groups (n=6). Group I served as normal control and received vehicle orally (N control) (0.25% carboxy methyl cellulose [CMC], 2 mL/kg body weight). Group II served as diabetic control, received 0.25% CMC (1 mLl/kg body weight) (D control). Group III, IV, V and VI were given glibenclamide (G, 10 mg/kg), FR1 (100 mg/kg),

FR2 (100 mg/kg) and FR3 (100 mg/kg) orally, respectively. All these dosages were administered after 5th day of STZ administration (except N control) and were given for seven days. Body weight and blood glucose was measured with strips on 1st, 3rd, 5th, and 7th day of treatment. On 8th day, blood was collected for further biochemical estimations. Animals were sacrificed by cervical decapitation and organs like pancreas and liver were dissected out and rinsed with ice cold saline and stored at -20°C for further studies.

2.7. Estimation of Blood glucose, liver glycogen level and serum lipid profile

2.7.1. Blood glucose and liver glycogen

Blood glucose levels were measured through one touch select Glucometer strips. Liver glycogen level was estimated using anthrone method with slight modifications [12]. Glycogen extraction was performed by boiling 1.0 g of fresh liver tissue in a 3 mL of 30% potassium hydroxide KOH solution. Then, glycogen was precipitated from the extract by adding 95% ethyl alcohol. After separation by centrifugation, the glycogen precipitate was treated with 95% sulfuric acid containing 0.2% anthrone to give a green color and absorbance measured at 620 nm (optical density, OD) and compared with standard. Glycogen concentration was calculated by application of the formula:

Anthrone concentration = [(OD of test X Volume of extract) / (OD of standard X gm. of tissue)] X Concentration of standard x100 x 1.11

2.7.2. Serum lipid profile

Serum lipid concentrations were determined spectrophotometrically using lipid profile kit (Erba Diagnostics, India). Total cholesterol in serum was measured via series of enzymatic reaction where cholesterol ester hydrolase hydrolyzed cholesterol to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). This H_2O_2 formed color complex with 4-aminophenazone and was measured

spectrophotometrically at 500 nm. The color intensity was found to be directly proportional to cholesterol concentration. Serum triglycerides produced fatty acids and H_2O_2 in presence of lipase and the measurement was similar to cholesterol measurement.

High density lipoprotein (HDL) estimation was performed where sulfated α-cyclodextrin and lipoproteins reacted with HDL to form HDL unesterified cholesterol, followed by cholestenone and H_2O_2. This H_2O_2 reacted with 5-aminophenazone and absorbance was measured at 600 nm. The formation of H_2O_2 was directly proportional to HDL concentration in plasma. Low density lipoprotein (LDL) and Very low density lipoprotein (VLDL) were calculated using Friendewald's Formula [13].

LDL (mg/dl) = (TC – HDL – TG)/5

VLDL (mg/dl) = TC – HDL – LDL

2.8. Determination of oxidative stress parameters

The oxidative parameters like thiobarbituric acid reactive substances (TBARS) [14], protein carbonyl (PC) [15] were measured in liver. Other parameters like, superoxide dismutase (SOD) [16], tissue catalase (CAT) [17], glutathione (GSH) [18] level were estimated in pancreatic tissue in the similar experiment. Total protein content of each sample was measured using Barford reagent where bovine serum albumin (BSA) was used as standard.

2.8.1. Liver thiobarbituric acid reactive substances (TBARS)

1 mL of 10% (w/v) tissue homogenate, 0.5 mL of 30 % trichloroacetic acid (TCA) and 0.5 mL of 0.8% thiobarbituric acid were taken in a test tube and covered with aluminum foil and kept in a shaking water bath for 30 min at 80°C. After 30 min, tubes were taken out and kept in ice-cold water for 15 min. They were then centrifuged at 3000 rpm for 15 min. The absorbance of supernatants was read at 540 nm at room temperature against appropriate blank which consisted

of 1.0 mL distilled water, 0.5 mL of 0.8% TBA solution, and 0.5 mL of 30% TCA solution. The amount of malonaldehyde (MDA) present in a sample was calculated according to the following equation:

nM of MDA/mg of protein = (V × OD at 540 nm) / (0.56 × protein concentration), where V is final volume of the test solution.

2.8.2. Liver protein carbonyl (PC)

150 µL of 10% tissue suspension in water was transferred into a clean tube and precipitated using 500 µL of 10% TCA solution. The tubes were centrifuged subsequently at 13,000 rpm for 2 min and the supernatant was removed. Each cell pellet was incubated with 500 µL of 0.2% diphenyl picryl hydrazine for 1 h with constant vortexing at every 5 min interval. 50 µL of 100% TCA solution was added, vortexed and centrifuged at 13,000 rpm for 5 min. All the supernatant was removed and the cell pellet was washed with 500 µL of ethanol: ethyl acetate (1:1) mixture three times. Finally, the cell pellet was dissolved in 1000 µL of 6.0 M guanidine hydrochloride and absorbance was measured at 360 nm wavelength of light.

2.8.3. Pancreas superoxide dismutase (SOD)

100 µL of cytosolic supernatant (10%) was added to tris-hydrochloric acid buffer, pH 8.5. The final volume of 3 mL was adjusted with the same buffer. At last, 25 µL of pyrogallol was added and changes in absorbance were recorded at 420 nm in each one minute interval for 3 min. The increase in the absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD. One unit of SOD is described as the amount of enzyme required causing 50% inhibition of pyrogallol auto-oxidation per 3 mL of assay mixture and is given by the formula:

Unit of SOD / mg or protein = [100 × {(A-B)/ (A×50)}]/ mg of protein, where A = Change in absorbance per minute in control and B = Change in absorbance per minute in test sample.

2.8.4. Pancreas catalase (CAT)

Briefly, 10% (w/v) tissue homogenate was prepared using 50 mM potassium phosphate buffer. The homogenates were centrifuged at 10,000 rpm for 20 minutes. 50 μL of supernatant was added to a cuvette containing 2.95 mL of 19 mM/L solution of hydrogen peroxide (H_2O_2) prepared in potassium phosphate buffer. Disappearance of H_2O_2 was monitored at 1 min interval for three times at 240 nm. The tissue catalase activity was calculated as follows:

nM of H_2O_2/min/mg of protein = (ΔA/min × volume of assay) / (0.0719 × volume of sample × mg of protein)

2.8.5. Pancreas glutathione (GSH)

0.2 mL of 10% (w/v) tissue homogenate was taken in a tube and 1.8 mL of distilled water was added to it. Then, 3.0 mL of precipitating solution (1.67 g of glacial meta-phosphoric acid, 0.2 g disodium EDTA and 30 g of sodium chloride in 100 mL distilled water) was added to the above mixture. The mixture was then allowed to stand for approximately 5 min and filtered. 2.0 mL of the filtrate and 1 mL of 0.4% (w/v) 5, 5′-dithiobis- 2-nitrobenzoic acid (DTNB) was added to 8.0 mL of 0.3 M phosphate solution and centrifuged at 13,000 rpm for 1 min. A blank solution was prepared in the above mentioned procedure where tissue sample was absent. The OD was measured at 412 nm. The tissue GSH content was calculated as follows:

GSH (μM/mg of protein) = (310.4 × E_i × OD at 412 nm) / mg of protein, where E_i is the correction factor (0.542).

2.9. Determination of liver function test

Liver function biomarkers like aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were also measured in serum using commercially available kit from Recombinogen Pvt. Ltd, India [19].

2.9.1. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

Working solutions were prepared by dissolving 4 mL of reagent 1 (mixture of L-alanine and α-ketoglutarate for ALT and mixture of L-aspartate and α-ketoglutarate for AST) and 1 mL of reagent 2 (mixture of nicotinamide adenine dineucleotide phosphate, NADP and lactate dehydrogenase, LDH) and kept at 2 to 8°C for future use. The whole assay was performed in cuvette and each cuvette contained 1.0 mL of working solution and 0.1 mL of plasma. This was incubated for 1 min at 37°C and change in optical density ($\Delta A340/min$) was measured per minute for the next 3 min using the UV/VIS Spectrophotometer (Labtronics, Australia). Data were calculated by the following equations:

$\Delta A_{340}/min = [A_{340}$ (Time 2) $- A_{340}$ (Time 1)$] / [$Time 2 (min) $-$ Time 1 (min)$]$

ALT or AST Activity (U/mL) $= \Delta A_{340}/min \times 1746 \times 10^{-3}$

2.9.2. Plasma alkaline phosphatase (ALP)

All groups contained 1 mL buffer substrate and 3 mL distilled water. Later, 0.1 mL distilled water, 0.1 mL phenol and 0.1 mL serum were added to blank, standard and test groups, respectively. All groups including normal control were incubated for 15 min at 37°C. Then, 0.1 mL of plasma was added to control group after incubation. All the tubes were mixed properly and absorbance was measured at 510 nm of wavelength. Plasma ALP was calculated as follows:

ALP activity (U/mL) $= [$A (Test) $-$ A (Control) $/$ A (Standard) $-$ A (Blank)$] \times 7.1 \times 10^{-3}$

Docking studies

Docking studies of isolated material was performed with antidiabetic molecular targets namely PPAR$_\gamma$ (PDB: 2PRG) by using Argus lab, Pyrx virtual screening 0.8 and Autodock 4.0. Before the docking study, the active site domain was identified with the help of DogP Active Site recognizer. Grid box was set according to best configuration of active site of amino acid

sequence. Binding affinities (Kcal/Mol) and count of probable hydrogen bonds were also evaluated.

2.10. Statistical analysis

Statistical analysis was carried out using Graphpad Prism 5.0 (San Diago, CA, USA). All results were expressed as mean± standard deviation (SD). The data was analyzed by one-way ANOVA (analysis of variances) followed by Bonferroni Multiple Comparison Test. For biochemical estimations, statistical significance differences were considered with respect to D control ([a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$).

3. Results

3.1. Structure elucidation of isolated compounds (FR1, FR2 and FR3)

The structures of isolated materials were elucidated by NMR and MS (Supplementary Data Sheet) and FR1, FR2 and FR3 were structurally similar to β-sitosterol, stigmasterol and lanosterol (Figure 1).

3.2. Antidiabetic effect of isolated materials

The isolated materials from FR leaves (FR1, FR2 and FR3) showed dramatic reduction of blood glucose level which was tabulated in Table 1. It is obvious that oral administration of pure compounds reduced blood glucose level after 7 days in rats ([a]$p<0.001$, [b]$p<0.01$). Diabetic rats treated with glibenclamide (G group) reduced highest blood glucose level (from 295 to 181 mg/dL), signified that our experiment had positive direction. FR1 exhibited maximum hypoglycemic effect with reduction of glucose concentration from 277 to 200 mg/dL which was comparable with standard G control group. FR2 and FR3 also showed the reduction of blood glucose level but it was slightly lower than FR1.

3.3. Effect of isolated materials on body weight

As depicted in Table 2, weight loss was observed in D control group (15.4%) till the end of experiment. There was significant improvement in body weight when rats treated with isolated sterols as compared with D control. Minimum improvement was observed for FR2 (~3.6%) whereas FR1 and FR3 showed moderate improvement (~6.2 and ~7.4% for FR1 and FR3, respectively).

3.4. Effect of isolated materials on glycogen content in liver and lipid profile in plasma

Determination of glycogen content in liver is an important parameter to measure hypoglycemic effect of drugs. There was massive decreased in glycogen content in D control (~12 mg/g) than N control (~57 mg/g, Table 3). Improvement in glycogen content was observed during treatment where we found that glycogen content was improved from ~24 to ~32 mg/g during drug treatment. FR1 expressed highest improvement in glycogen content than FR2 and FR3 (Table 3).

We also determined TC, TG, LDL, HDL and VLDL levels in serum separately. Our observation on lipid profile suggested that TC, TG, LDL, VLDL levels were increased and HDL level was deceased in toxic control rats (D control). Oral administration of G and test compounds reduced the TC, TG, LDL, VLDL levels and increased HDL levels. In case of TC content in plasma, it was observed that FR1 significantly reduced TC content (~ 127.7 mg/dL) which was similar to G groups (~ 136.1 mg/dl) than toxic control rats (~ 176.9 mg/dL). Similar effects were observed for FR2 and FR3 but it was slightly lower than FR1 (Table 3).

In the present study, we also observed TG level which was increased in D control group (~ 153 mg/dL) as compared to N control (~ 67 mg/dL). The treatment with FR1 showed significant reduction in cholesterol level (~ 120 mg/dL) which was comparable with standard (~ 107

mg/dL). Both FR2 and FR3 reduced the TG level but this action was less prominent than FR1 (Table 3).

Similar trends were observed for LDL and VLDL where we found that there was a significant reduction of all these parameters with respect to D control group (Table 3). In both cases, FR1 showed highest activity than FR2 and FR3.

HDL level was highly decreased for D group (~ 27 mg/dL) than N control (~ 42 mg/dL). This level was slightly improved when rats treated with isolated materials (~30 mg/dL) which were slightly lower than positive control (G control, [~ 39 mg/dL]) (Table 3).

3.5. Determination of oxidative stress parameters in liver and pancreas

We measured SOD, CAT, GSH levels in pancreas and TBARS, PC levels in liver to investigate the protective action of isolated materials during diabetic condition in rats. As depicted in Table 4, GSH level was dramatically decreased in D treated groups (~18 μM) as compared to N control rats (~68 μM). This level was restored to normal level when rats were treated with isolated materials (~40 μM). SOD levels also decreased in D control rats (~2.7 U) than N control (~7.9 U). This concentration again normalized by both FR1 and G control groups (~6.0 U). Similar observation was observed for CAT assay where we found that this enzyme activity was higher for both positive control and treated groups than D control (Table 4).

Separately, we measured TABRS and PC in pancreas to determine the oxidative stress related toxicity. We observed that both TBARS and PC formation was lower for treated samples than D control. Similar effects were observed for FR2 and FR3 but it was slightly lower than FR1 (Table 4).

The effect of isolated sterols on AST, ALT and ALP levels on plasma were also observed during our experiment. In the D control groups, serum enzyme levels were elevated with respect to N

control. The treatment with sterols and G reduced these enzyme levels as compared to D control (Table 5).

3.6. Docking studies

Docking studies were performed on molecular target of human PPAR$_\gamma$ agonist with ligands (FR1, FR2 and FR3). Their binding affinities (Kcal/Mol) and probable hydrogen bonds were evaluated (Table 6). Docking images of compounds with amino acids involved in binding poses are shown in Figure 2. FR1 exhibited good binding affinities to human PPAR$_\gamma$ (affinity value -12.2 Kcal/Mol and 1, H-bonds) compared than FR2 and FR3 (affinity values -11.3, -9.7 Kcal/Mol, and 1, 1 H-bonds, respectively). All compounds displayed interaction energy $\geq$ -10.0 Kcal/Mole, which signified good PPAR$_\gamma$ binding action.

4. Discussion

Diabetes is a disorder of carbohydrate, protein and fat metabolism which is mainly occurred due to insufficient insulin secretion or insulin resistant. It causes various complications like blindness, liver and pancreatic damage. Diabetes is one of the most clinical and health problems now-a-days [20]. The usage of synthetic drugs has declined due to its potential toxicity to human body. Moreover, the compounds obtained from natural origin are safe, less toxic in nature. Therefore, it is necessary to investigate potential therapeutic agents for the clinical benefits among cancerous patients [20].

From human civilization, natural products served as traditional medicines throughout the world basically Egypt, China and India. Currently, 119 chemicals obtained from 90 plant species are considered as important therapeutics in different countries [21]. In this regards, we isolated three sterols from FR leaves and structures were confirmed by NMR and MS. The isolated materials are structurally similar to FR1 (β-Sitosterol), FR2 (Stigmasterol) and FR3 (Lanosterol).

Later, these compounds were investigated for antidiabetic activity using STZ induced diabetic rats.

The diabetes caused by STZ is commonly used for experimental purpose as it damages the pancreatic β-cell nucleotide functions via release of nitric oxide (NO). It releases free radical anions which also damages β-cell mitochondrial functions [22]. Based on the above mechanism, diabetes was induced through STZ administration intraperitoneally in our experiment. According to Table 1, FR1 expressed time dependent hypoglycemic effect which was comparable with standard glibenclamide (G control). Both FR2 and FR3 revealed the similar actions but it was slightly lower than FR1 (Table 1). In the above experiment, body weight was also significantly restored during treatment of FR compounds (Table 2). From the above observation, we concluded that all three sterols were active against diabetes. The promising mechanism by which all FR sterols produced antidiabetic activity, might be due to potentiate pancreatic secretion of insulin from β-cells, as there was significant reduction of blood glucose level during treatment. From the above observation, it might be concluded that the mechanism of action of isolated sterols may be similar to standard G.

Diabetes mellitus reduces the normal capacity of liver glycogen via activation of glycogen synthetase, resulting in glycogenesis and this activation appears to increase glucose concentration during diabetes [23]. Treatment with sterols for 7 days significantly improved liver glycogen indicating that glycogen storage was improved during treatment (Table 3). The increase concentrations of TC and TG are the secondary condition during diabetes. Both VLDL and HDL are also increased during diabetes [24]. All FR compounds significantly reduced all these concentrations in STZ diabetic rats, signifying hypoglycemic action. FR1 expressed better activity than FR2 and FR3 in this case (Table 3).

In diabetes, free radicals are formed during degradation of glucose and glycation of proteins [25]. This may lead to lipid peroxidation and protein carbonylation during diabetic state [26,27]. In these circumstances, we performed both TBARS (for lipid peroxidation) and PC assays to measure the oxidative stress condition of pancreas. We observed that all sterols had protective actions and reduced the oxidation of lipids and proteins in pancreas (Table 4). Associated with other assays, there was reduced concentration of SOD and CAT enzymes in D control rats. CAT is most abundant in the liver which is mainly responsible for the catalytic decomposition of H_2O_2 to oxygen and water. Increase in concentration of H_2O_2 in treated samples depicted that there was higher amount of CAT enzyme available in the tissue to decompose the H_2O_2. SOD is responsible for catalytic dismutation of free radicals and decrease superoxide level [19]. The sterols treatment increased the activity of these enzymes and might be responsible to control the free radicals. We also measured the concentration of reduced GSH in liver to find out the toxicity potential of these compounds. GSH plays a major role in the oxidation-reduction process, resulting in the formation of disulfide glutathione (GSSG) [28] during oxidative damage. Elevation of reduced GSH level in treatment groups signified protective action of sterols and was an indication of scavenging activity of sterols (Table 4).

Liver is the vital organ for metabolism and detoxification of xenobiotics. During diabetes and pathophysiological condition, liver cells are necrotized and enzymes leaked into blood stream, resulted an increase concentration of enzymes in liver (ALT, AST and ALP) [29]. Treatment with sterols reduced these enzyme levels in blood, signified hepatoprotective activity of sterols. All these observation suggested that these three isolated sterols had good antidiabetic and antioxidant activities (Table 5).

The mechanism of action of isolated sterols was also an important parameter to be investigated. Docking studies of these sterols were performed to gain an insight regarding the interaction energy using PPAR$_\gamma$ ligand. It is well established that if drug shows interaction energy more than -5.0 kcal/mole to ligand, drug is deemed to have a good affinity to ligand [30]. From the bioinformatics study, it was observed that all three sterols had capacity to bind to PPAR$_\gamma$ (interaction energy $\geq$ -10.0 kcal/mole) (Figure 2, Table 6). FR1 and FR2 showed highest affinity whereas FR3 expressed moderate affinity. This docking study demonstrated that these sterols may have antidiabetic activity potential via binding to PPAR$_\gamma$ ligand. It is also well established that 3-hydroxyl group and double bond are the important pharmacophores for pharmacological action [31]. FR1 and FR2 revealed highest affinities to PPAR$_\gamma$ ligand due to the presence of these two pharmacophores. At the same time, FR3 showed moderate affinity due to absence of double bond. Thus docking studies confirmed that proposed activity of isolated sterols were similar in action to standard drug.

5. Conclusion

In the present study, the hypoglycemic effect of three isolated sterols was observed. All of them were able to restore blood glucose, cholesterol and liver glycogen levels. Pharmacological action via binding to PPAR$_\gamma$ ligand was confirmed through docking studies. They were able to normalize plasma enzyme levels and oxidative stress parameters in both liver and pancreas. Results from our experiment indicated that isolated sterols had good antidiabetic and antioxidant actions. The action of FR1 was similar to glibenclamide. FR2 and FR3 expressed similar action but was slightly lower than FR1. These result served as valuable tools for future antidiabetic drug therapy. Antidiabetic potential of FR1 had already been measured in STZ rats previously [32]. Three sterols were successfully isolated from FR leaves for the first time and

extensively and successfully evaluated for antidiabetic, hypolipidemic and toxicological studies in STZ rats.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Figure legends

Figure 1 Structures of isolated sterols form *Ficus racemosa* leaves (A) FR1 (β-Sitosterol), (B) FR2 (Stigmasterol) and (C) FR3 (Lanosterol).

Figure 2 Docking images of (A) FR1, (B) FR2 and (C) FR3 with human PPARγ agonist (2PRG), the mesh round circular showed the binding affinity of ligand with amino acids involved in binding poses.

Figure 1

(A)

(B)

(C)

Figure 2

(A)   (B)   (C)
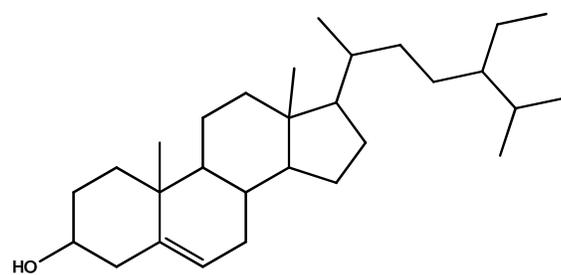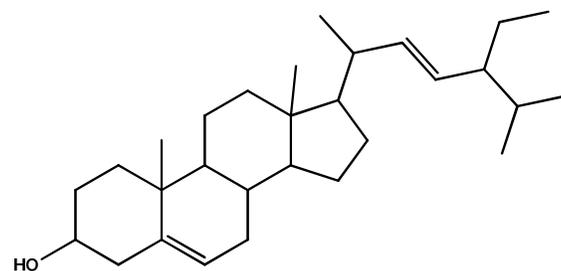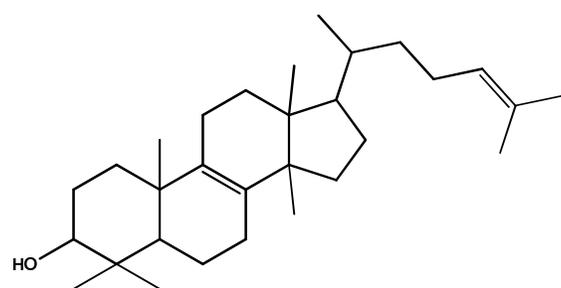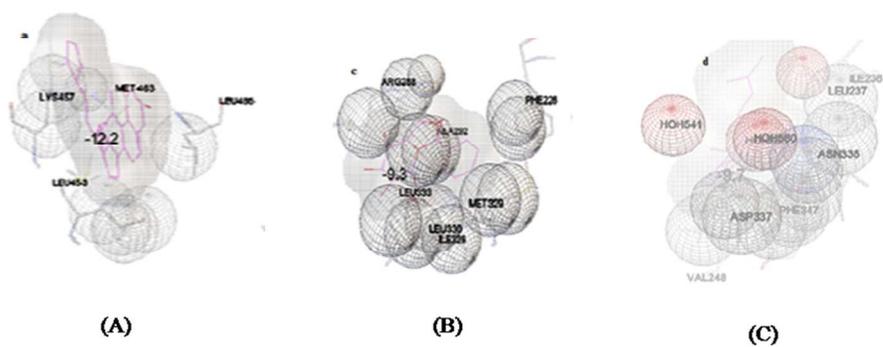
254x190mm (96 x 96 DPI)

Table 1

Effect of isolated materials from *Ficus racemosa* (FR1, FR2 and FR3, orally, 100 mg/kg for 7 days) on blood glucose level (mg/dL) on STZ induced diabetic rats*.

Groups	1st day	3rd day	5th day	7th day
N Control	89.11±5.39	85.75±5.86	93.22±2.44	91.5±3.38
D Control	293.52±9.55	297.66±7.88	302.22±2.37	305.22±9.65
D + G	295.75±6.27	271.5±6.38[a]	220.11±9.24[a]	181.66±5.73[a]
D + FR1	277.5±5.11[b]	260.11±8.55[a]	240.53±3.11[a]	200.33±6.14[a]
D + FR2	291.4±7.74	271.27±5.54[a]	250.15±3.87[a]	218.47±6.11[a]
D + FR3	275.64±7.11[b]	267.77±4.57[a]	250.38±6.52[a]	230.73±6.42[a]

*Data represented as mean±SD (n=6). Statistically significant differences were observed between D control and test groups [one way-ANOVA followed by Bonferroni multiple comparison test; [a]$p<0.001$, [b]$p<0.01$].

Table 2

Effect of isolated materials from *Ficus racemosa* (FR1, FR2 and FR3, orally, 100 mg/kg for 7 days) on body weight (g) in STZ induced diabetic rats*.

Groups	1st day	3rd day	5th day	7th day	% Change in body weight
N Control	140±2	148±6	148±3	148±4	5.71
D Control	142±4	132±5	125±2	118±2	-15.49
D + G	137±2	139±2^c	142±3^a	142±2^a	2.18
D + FR1	134±5^b	135±4	142±4^a	144±3^a	7.46
D + FR2	136±2^c	137±2	141±1^a	141±2^a	3.67
D + FR3	144±2	146±2^a	147±4^a	153±4^a	6.25

*Data represented as mean±SD (n=6). Statistically significant differences were observed between D control and test groups [one way-ANOVA followed by Bonferroni multiple comparison test; ^{a}p<0.001].

Table 3

 Effect of isolated materials from *Ficus racemosa* (FR1, FR2 and FR3, orally, 100 mg/kg for 7 days) on glycogen level in liver and lipid profile in serum on STZ induced rats*.

Groups	Glycogen (mg/g)	TC (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
N Control	57.13 ± 4.74	92.24±3.62	67.84±5.33	42.54±2.30	36.13±0.25	13.57±1.07
D Control	12.01 ± 5.33	176.96±9.74	153.64±3.58	27.11±2.42	119.12±6.60	30.73±0.72
D + G	39.63 ± 3.53[a]	127.7±5.11[a]	107.85±3.57[a]	39.48±1.58[a]	66.65±2.81[a]	21.57±0.92[a]
D + FR1	32.19±1.12[a]	136.16±5.47[a]	120.06±3.84[a]	33.69±3.57[b]	78.45±1.13[a]	24.02±1.77[a]
D + FR2	27.51 ±3.84[a]	149.64±5.62[a]	132.53±3.42[a]	29.12±2.96	94.01±1.97[a]	29.61±1.69
D + FR3	24.59±3.25[a]	158.22±5.89[a]	135.53±3.64[a]	30.34±2.22	100.77±2.94[a]	27.11±1.73[b]

*Data represented as mean±SD (n=6). Statistically significant differences were observed between D control and test groups [one way-ANOVA followed by Bonferroni multiple comparison test; [a]$p<0.001$, [b]$p<0.01$].

Table 4

Effect of isolated materials from *Ficus racemosa* (FR1, FR2 and FR3, orally, 100 mg/kg for 7 days) on SOD, CAT, GSH in pancreas and TBARS and PC in liver on STZ induced rats*.

Groups	SOD (U/mg of Protein)	CAT mM H_2O_2 decomposed/min /mg of protein	GSH (µM/mg of Protein)	TBARS (nM of MDA/mg of protein)	PC (µM/mg of protein)
N Control	7.97±1.58	91.75±2.65	68.47±1.99	1.95±0.07	77.19±5.93
D Control	2.75±0.75	37.18±1.68	18.63±2.57	3.24±0.58	185.47±3.28
D + G	6.12±1.05[a]	74.95±1.74[a]	62.45±1.84[a]	2.15±0.34	103.95±3.73[a]
D + FR1	5.83±1.17[a]	65.47±1.85[a]	49.42±3.68[a]	2.27±0.53	136.15±7.01[a]
D + FR2	4.78±0.92[c]	55.65±2.42[a]	42.53±2.43[a]	2.56±0.41	157.62±3.49[a]
D + FR3	3.85±1.14	51.24±1.3	39.11±4.67[a]	2.33±0.45[c]	153.11±2.88[a]

*Data represented as mean±SD (n=6). Statistically significant differences were observed between D control and test groups [one way-ANOVA followed by Bonferroni multiple comparison test; [a]$p<0.001$, [c]$p<0.05$].

Table 5

Effect of isolated materials from *Ficus racemosa* (FR1, FR2 and FR3, orally, 100 mg/kg for 7 days) on AST and ALT in serum in STZ induced rats*.

Groups	AST (U/dL)	ALT (U/dL)	ALP (U/dL)
N Control	52.23±2.33	91.59±4.95	128.95±7.54
D Control	120.86±4.59	164.46±6.53	300.56±3.39
D + G	63.11±2.94[a]	98.23±3.19[a]	191.63±4.49[a]
D + FR1	71.56±4.32[a]	120.96±3.76[a]	232.85±5.52[a]
D + FR2	80.91±3.22[a]	140.27±2.75[a]	239.74±3.22[a]
D + FR3	77.69±4.75[a]	143.85±4.77[a]	248.63±3.64[a]

*Data represented as mean±SD (n=6). Statistically significant differences were observed between D control and test groups [one way-ANOVA followed by Bonferroni multiple comparison test; [a]$p<0.001$].

Table 6

Binding energy of FR1, FR2 and FR3 with human PPARγ agonist. Comparative studies were performed by using three docking softwares.

Ligand	Affinity (Kcal/Mol) By using Autodock	Affinity (Kcal/Mol) By using Argus lab	Affinity (Kcal/Mol) By using Pyrx 8.0	Amino acids involved in interactions	H-bonds
FR1 (β-Sitosterol)	-12.2	-17.73	-11.2	LYS457, MET463, LEU465 ,LEU453	1
FR2 (Stigmasterol)	-11.3	-17.26	-10.7	ASN335,PHE347,ILE236,VAL242	1
FR3 (Lanosterol)	-9.7	-10.4	-8.9	ILE236, LEU237, ASN335, ASP337, PHE347,VAL248	1