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Research Article

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Metformin-3-hydroxyflavone, A New Schiff Base Complex Modulates the Activities of Carbohydrate Regulatory Enzymes in High Fat Diet Fed-Low Dose Streptozotocin Induced Type 2 Diabetes in Experimental Rats

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ABSTRACT

T2DM is a multifaceted endocrine disorder arises due to insulin resistance coupled with insufficient secretion of insulin from the pancreatic β-cells. Its prevalence is increasing alarmingly worldwide due to genetic and environmental factors. Among the various oral antidiabetic drugs widely used for the treatment of T2DM, metformin is considered as the first line of treatment to T2DM and backbone for combination therapy. However, at higher doses metformin induced lactic acidosis in addition to vitamin B12 deficiency. In order to circumvent the toxicity of metformin at high doses, an attempt has been made to synthesize a new metformin-3-hydroxyflavone complex. The Schiff base complex synthesized was characterized by various spectral studies such as FT-IR, Mass, ^{1}H NMR and ^{13}C NMR. The effect of oral administration of metformin-3-hydroxyflavone complex at a concentration of 20 mg/kg.b.w./rat/day to high fat diet fed-low dose STZ induced type 2 diabetes in rats was evaluated. The biochemical alterations such as fasting blood glucose, hemoglobin, glycosylated hemoglobin, plasma insulin and c-peptide observed in the diabetic rats were restored to near normal after treatment with the Schiff base complex. Additionally, the complex treatment regulates the activity of carbohydrate and glycogen metabolizing enzymes. The efficacy of the complex was comparable with metformin which was administration at a relatively high dose of 50 mg/kg.b.w./rat/day. The data obtained evidenced the regulatory properties of metformin-3-hydroxyflavone complex in maintaining normoglycemia in experimental type 2 diabetes.

Keywords: Type 2 diabetes mellitus; High fat diet; Spectral characterization; Metformin-3-hydroxyflavone; Carbohydrate metabolizing enzymes

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INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder arises due to an autoimmune destruction of insulin producing β-cells of pancreas (T1DM) or the development of resistance to endogenous insulin at various degrees in association with insufficient secretion (T2DM) [1]. Historically, the prevalence ratio for type 1diabetes to type 2 diabetes has been 1:20 and the mortality is up to five times higher in diabetic than in non-diabetic individuals. According to recent reports the prevalence of DM is expected to rise to 366 million by the year 2030 [2]. The increasing knowledge of the etiology and the molecules involved in β-cell functions provides several new concepts in the development of drugs for the treatment of T2DM and its complications. However, most of the currently prescribed drugs in the clinical practice such as sulfonylureas, biguanides, α-glucosidase inhibitors and insulin either do not succeed in restoring normoglycemia or fail after a variable period of time in most diabetic patients. Additionally, the adverse effects include hypoglycemia and weight gain associated with the use of sulfonylureas or insulin; weight gain, edema, heart failure and fractures associated with thiazolidindiones; or gastrointestinal side effects, lactoacidosis, vitamin B12 deficiency and weight loss with metformin [3]. Thus, an ideal treatment for

T2DM capable of reversing insulin resistance and β-cell dysfunctions in most treated persons and prevent or reverse long term complications is necessary.

Among the various oral antidiabetic drugs widely used for the treatment of T2DM, metformin is considered as the first line of treatment to T2DM and backbone for combination therapy [4]. Ever since metformin was developed as an antihyperglycemic drug by Sterne in the 1950s, its efficacy, security profile, metabolic actions and its ability to be associated with other antidiabetic agents have been evaluated and are being extensively prescribed by the clinicians for the treatment of both T1DM and T2DM [5]. Metformin is relatively well tolerated and the hypoglycemic episodes do not occur in therapeutic dosing as it has no effect on the insulin secreting pancreatic β-cells. Metformin acts primarily at the liver by reducing glucose output and secondarily by augmenting glucose uptake in the peripheral tissues, chiefly in skeletal muscle. Inhibition of mitochondrial respiration has also been proposed to contribute to the reduction of gluconeogenesis since it reduces the energy supply required for this process [6]. Thus, the antihyperglycemic effect of metformin is demonstrable only in diabetic patients that too in the presence of insulin [7].

Intestinal absorption of metformin reaches the peak level between 1 and 3 hours after an oral dose and the maximum blood concentration at a steady state was found to be 4 µg/ml. Metformin does not appear to be metabolized in human and its bioavailability at a therapeutic dose reaches 50-60% [8] but may drop as the dose is increased due to the decrease in the absorption [9]. Metformin is rapidly excreted by the kidneys and the clearance rate is about four times the creatine clearance with normal renal function [10,11]. Although monotherapy with metformin is often initially effective, glycemic control deteriorates in many cases which require relatively high doses of metformin or an addition of a second agent. However, metformin treatment at relatively high doses was associated with metabolic acidosis characterized by increased serum lactate and beta-hydroxybutyric acid levels and decreased serum bicarbonate and urine pH [12,13]. Additionally, gastrointestinal tract complications are reported in at least in 10% of the diabetic patients treated with metformin [14]. A reduction in vitamin B12 absorption can also occur after a long period of metformin treatment [15]. All noninsulin antidiabetic drugs, when added to maximal metformin therapy, are associated with significant HbA1c reduction but with various degrees of weight gain and hypoglycemic risk [16]. Hence, metformin treatment should be avoided in diabetic patients whose renal functions is impaired and in those patients in whom oxygenation, tissues perfusion or liver functions are severely compromised [17]. Despite a number of combinations of metformin is currently available [18], none is found to be ideal due to hypoglycemic episodes and/or undesirable side effects.

Phytochemicals are ecologically derived plant secondary metabolites which protect them against environmental stress such as UV radiation, pollution, high temperature, extreme cold, drought, flood, tissue damage and microbial attacks [19]. Ethanopharmacological reports evidenced that these secondary metabolites are known to play a central role in alleviating the primary and secondary complications of human ailments such as cancer, diabetes and atherosclerosis [20]. Among the various phytochemicals, flavonoids are known for their wide range of pharmacological as well as beneficial effects on the maintenance of human health care due to their wide range of antioxidant properties [21]. Amongst flavonoids, flavones are known to chelate the metal ions with great affinity owing to the presence of α-hydroxycarbonyl group and their ability to quench the free radicals [22]. The synthetic 3-hydroxyflavones were found to have significant antidiabetic properties by pleiotropic and multimodal suppression of insulin resistance and enhancement of glucose uptake by skeletal muscles [23]. In view of the above, we have made an attempt to synthesize a new metformin-3-hydroxyflavone complex and to evaluate its antidiabetic properties as well as its role in regulating the activities of key enzymes associated with glucose homeostasis in high fat diet fed-low dose STZ induced experimental type 2 diabetes in rats.

MATERIALS AND METHODS

Chemical

Metformin hydrochloride, 3-Hydroxyflavone and STZ were procured from Sigma-Aldrich, St. Louis, USA. Ultra-sensitive ELISA kit for rat insulin and C-peptide was purchased from Crystal Chem Inc. Life Technologies, India. All the other reagents used in the present study were of analytical grade.

Analytical Instruments

IR spectral studies were carried out in the solid state as pressed KBr pellets using a Perkin-Elmer FT-IR spectrophotometer in the range of 400-4000 cm^{-1}. The mass spectrum of the complex was obtained using Jeol Gcmate. The ^{1}H NMR and ^{13}C NMR data were obtained at 300 MHz and 500 MHz, respectively, using a BrukerAM-500 instrument. The spectral analysis data were recorded without any modification for instrumental characteristics.

Synthesis of Metformin-3-hydroxyflavone Complex

3-Hydroxyflavone (0.005 mol, 1.2 g) is dissolved in 25 ml of methanol and stirred in hot condition till the formation of a clear solution. To this warm solution, metformin hydrochloride (0.005 mol, 0.6458 g) dissolved in methanol (25 ml) was added in a drop wise manner. The mixture is refluxed with continuous stirring over a water bath for six hours. The resultant coloured product (L) was washed with water and methanol, dried under vacuum and recrystallized from methanol.

Experimental Animals

Male Albino Wistar rats weighing around 160 to 180 gm were procured from the Tamilnadu Veterinary and Animal Sciences University, Chennai, and were housed under standard husbandry conditions (12 ± 1 h light and dark cycle, relative humidity 55% ± 10%). The animals were fed with a balanced diet (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. The rat pellet diet is composed of 55% nitrogen-free extract, 21% protein, 5% fat and 4% fiber (w/w) with sufficient levels of vitamins and minerals. The experimental design was conducted according to the ethical norms approved by the Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines, for the examination of experimental pain in conscious animals (IAEC NO: 03/10/12).

Induction of Experimental Diabetes

The rats were allocated into two dietary regimens by feeding either normal pellet diet (NPD) or high fat diet (HFD) for 2 weeks of dietary manipulation. HFD contains powdered NPD-365 g/kg, lard-310 g/kg, casein-250 g/kg, cholesterol-10 g/kg, vitamin and mineral mix-60 g/kg, DL-methionine-3 g/kg, yeast powder-1 g/kg, and NaCl-1 g/kg [24]. After 2 weeks of dietary manipulation to induce insulin resistance, Group 2, Group 3, group 4 rats were injected with a single low dose of STZ (35 mg/kg b.w./rat); control rats (Group 1) fed with NPD were injected intraperitoneally with the same volume freshly prepared cold citrate buffer (pH-4.5, 0.1 mol/L). On the third day after STZ injection, rats having fasting blood glucose levels ≥ 250 mg/dl were considered as diabetic rats and chosen for further studies.

Acute Toxicity and Dosage Fixation Studies

Acute toxicity studies were performed in normal rats. Graded doses (10, 25, 50 and 100 mg/kg b.w./rat as per OECD guidelines (423) for testing of chemicals) of metformin-3 hydroxyflavone complex in aqueous suspension was administered orally. The rats were observed for four weeks following administration. The change in food consumption, fluid intake, psychomotor activities, body weight gain, changes in skin, fur, eyes, salivation, diarrhea and lethargy were continuously monitored. Macroscopic examinations were also performed on vital organs. Similarly, the dosage fixation studies were carried out by administering graded doses of metformin-3-hydroxyflavone complex (5, 10, 20 and 50 mg/kg b.w./rat/day) for 30 days to determine the dose-dependent hypoglycemic effect in high fat diet fed-low dose STZ induced diabetic rats by monitoring the fasting blood glucose levels periodically.

Experimental Protocol

The animals were divided into four groups each comprising of a minimum of six animals as follows:

Group 1 : Control rats.

Group 2 : HFD-STZ (35 mg/kg b.w./rat) induced diabetic rats.

Group 3 : Diabetic rats treated with metformin-3- hydroxyflavone complex (20 mg/kg.b.w./rat) for a period of 30 days.

Group 4 : Diabetic rats treated with metformin (50 mg/kg.b.w./rat) for a period of 30 days.

Biochemical Studies

The levels of fasting blood glucose [25], hemoglobin [26] and glycosylated hemoglobin [27] were estimated. Insulin and C-peptide levels were estimated using Ultrasensitive ELISA kits. Urine sugar was detected using urine strips.

Carbohydrate Metabolizing Enzymes

A portion of liver tissues from the control and experimental groups of rats were dissected and washed immediately with ice-cold saline and were homogenized in 0.1 M Tris–HCl buffer (pH 7.4) for the assay of key enzymes of carbohydrate metabolism. The homogenate was centrifuged at 10,000 rpm to remove the debris and the supernatant was used as enzyme source for the assays of hexokinase [28], pyruvate kinase [29], glucose-6-phosphate

dehydrogenase [30], glucose-6-phosphatase [31], fructose-1, 6- bisphosphatase [32], glycogen synthase [33], glycogen phosphorylase [34] and lactate dehydrogenase [35]. Another portion of wet liver tissue was used for the estimation of glycogen [36] content in control and experimental groups of rats.

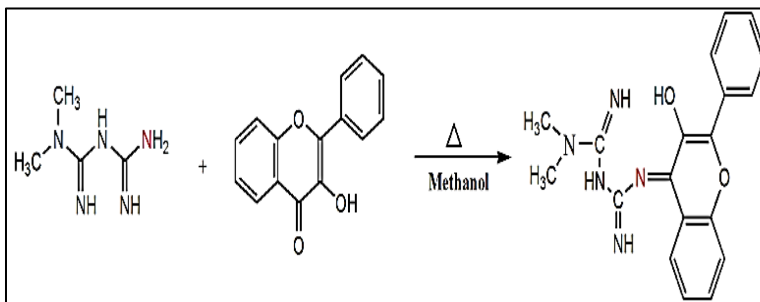
Statistical Analysis

The values are expressed as mean values of six rats in each group ± SEM. Data analysis was done with SPSS 16 student software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed with least significance difference (LSD). The value of $P<0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Synthesis of Metformin-3-hydroxyflavone

The Schiff base ligand (L) was synthesized from the coordination between 3-hydroxyflavone and metformin by a molar ratio method. The Schiff base ligand (L) was formed as a yellow-colored product (Scheme 1).



Scheme 1: Structure of metformin-3-hydroxyflavone

Characterization of Metformin Hydrochloride, 3-hydroxyflavone and Metformin-3-hydroxyflavone Complex

The IR spectral data obtained for metformin hydrochloride, 3-hydroxyflavone and Schiff base L are presented in Figures 1- 3 respectively. Mass spectrum analysis of Schiff base ligand L is shown in Figure 4. The ^{1}H NMR spectra of metformin, 3-hydroxy flavone and Schiff base ligand (L) were presented in Figures 5- 7, respectively. In free metformin, the free amine exhibit ν(NH_2) a peak around 3162 cm^{-1}. The bands in the region of 3386 cm^{-1} and 3271 cm^{-1} are due to the presence of N–H stretching. The sharp peaks in the region of 2824 cm^{-1} are due to the presence of methyl groups coordinated to nitrogen group and the nitrogen connected with carbon and hydrogen. The peak around 1159 cm^{-1} is due to the presence of carbon–nitrogen stretching as depicted in Figure 1 [37].

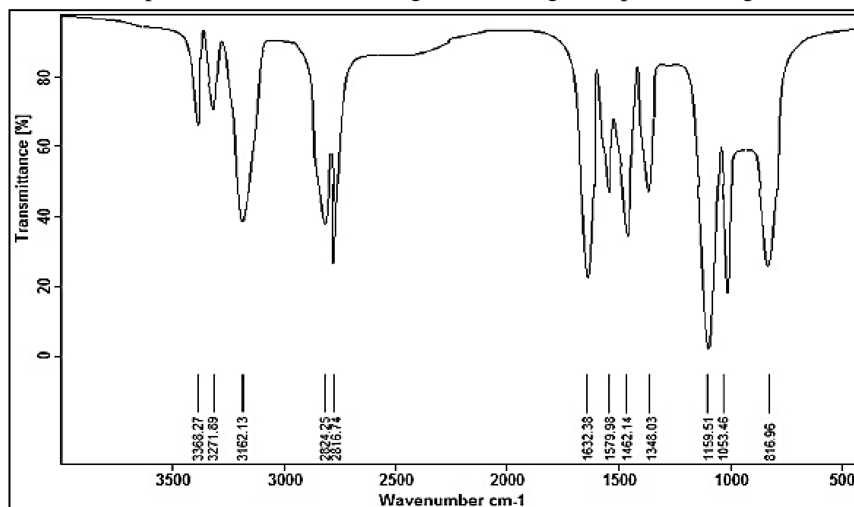


Figure 1: The IR spectrum of metformin

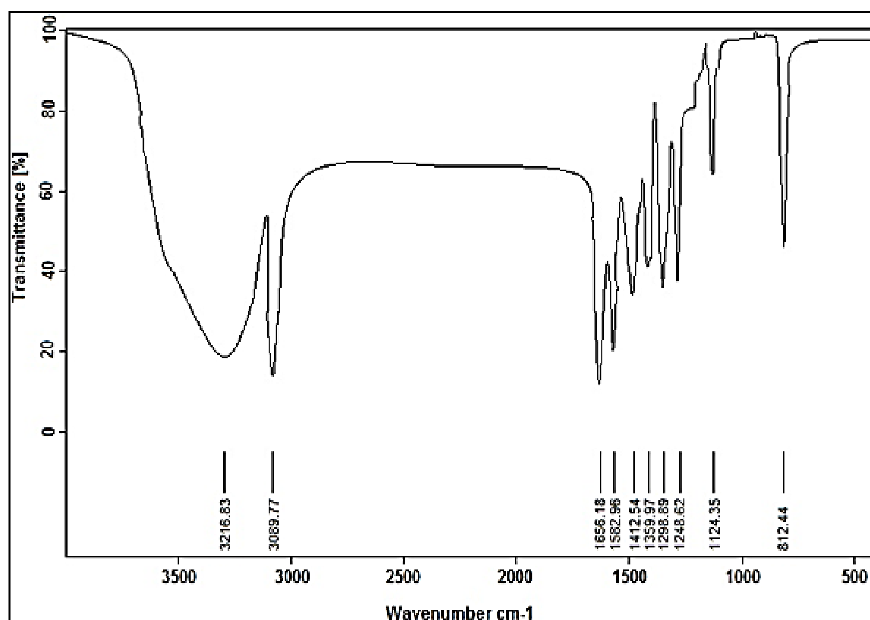


Figure 2: The IR spectrum of 3-hydroxyflavone

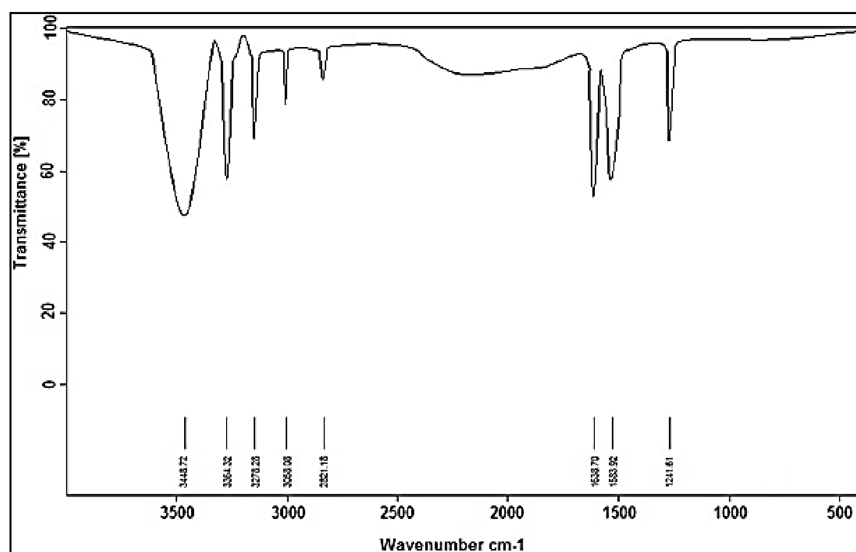


Figure 3: The IR spectrum of metformin-3-hydroxyflavone

Similarly, metformin- free 3-hydroxyflavone exhibited various peaks. In the free 3-hydroxyflavone, the peak around 3216 cm^{-1} is due to the presence of the hydroxyl group present in the aromatic ring. The peak around 3089 cm^{-1} is owing to the presence of C–H stretching. The peaks around 1656 and 1582 cm^{-1} is due to the presence of carbonyl and C=C groups. The peaks corresponding to C–O and C–O–C were found in the range of 1359 to 1248 cm^{-1} [38].

The formation of the Schiff base ligand L has been confirmed by infrared spectroscopy. IR bands obtained at 1638 cm^{-1} clearly evidenced the presence of an imine functionality which is due to the condensation of 3-hydroxyflavone with metformin. IR band at 3162 cm^{-1} for the NH_2 stretching was not observed which clearly indicate that the primary amine functionality of metformin has successfully condensed with the carbonyl functionality of 3-hydroxyflavone. The other peaks correspond to various functional groups did not show any alterations which result in the formation of Ligand L. The molecular ion peak [M+] at m/z = 349 confirms the molecular weight of the Schiff base ligand $C_{19}H_{19}N_5O_2$. The peaks at m/z = 273, 245, 195, 167, and 125 correspond to the various fragments of $C_{13}H_{15}N_2O_2$, $C_{11}H_{11}N_5O_2$, $C_7H_9N_5O_2$, $C_7H_9N_3O_2$, and $C_6H_7NO_2$ respectively. This confirms the molecular structure of the ligand.

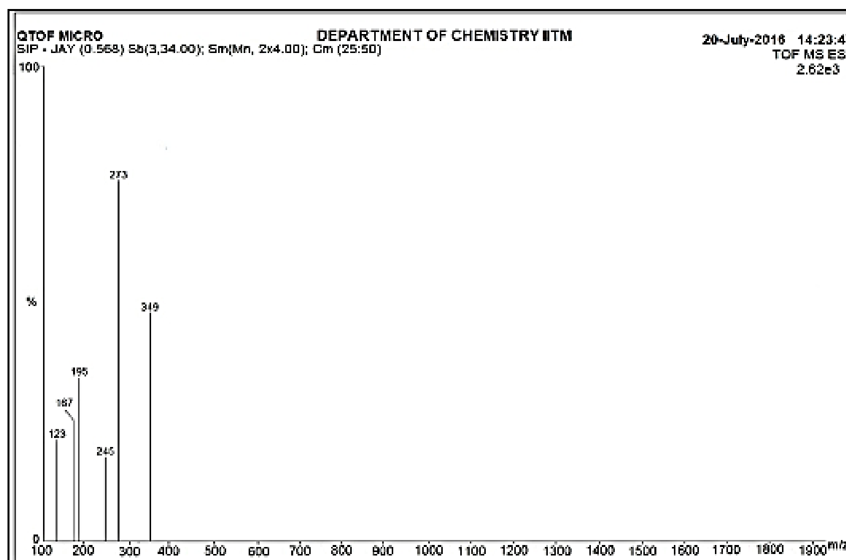


Figure 4: The mass spectrum of metformin-3-hydroxyflavone

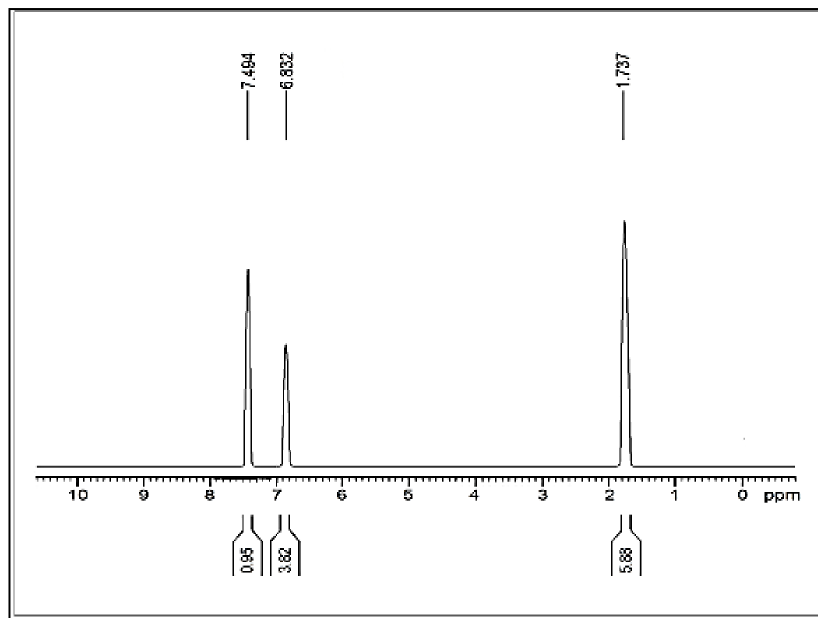


Figure 5: The ^{1}H NMR of metformin

The ^{1}H NMR spectrum of synthesized schiff base ligand was recorded in DMSO-d_6. In the NMR spectrum of the Ligand L, the signal as a singlet in the range δ 1.82 ppm is due to six methyl protons in the metformin. In the aromatic region, a few doublets and in few cases some overlapping doublets/multiplets are observed in the range of δ 7.3–8.5 ppm. The singlet corresponding to phenolic proton was observed at δ 9.35 ppm. The singlets in the region of δ 6.9 and 7.2 ppm were due to the presence of two amide (C=NH) and one C-NH [39,40].

The ^{13}C NMR spectrum of the Schiff base ligand exhibited signals in the region of δ 116–155 ppm corresponding to aromatic carbons. The peak around δ172 ppm is due to the presence of C=N carbon [41]. The methylene carbons were found in the region of δ 35 ppm. The amide carbons were observed in the region of δ 156 and 160 ppm [42].

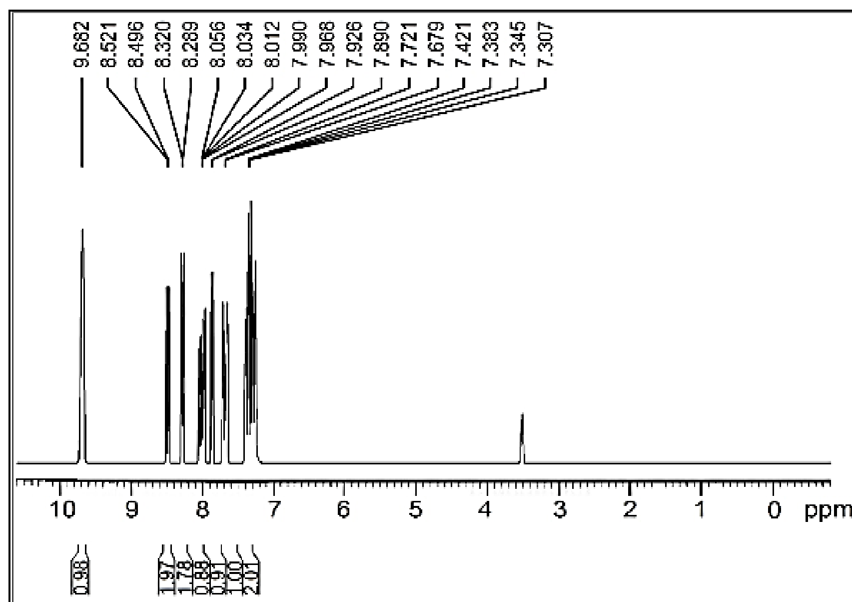


Figure 6: The ^{1}H NMR of 3-hydroxyflavone

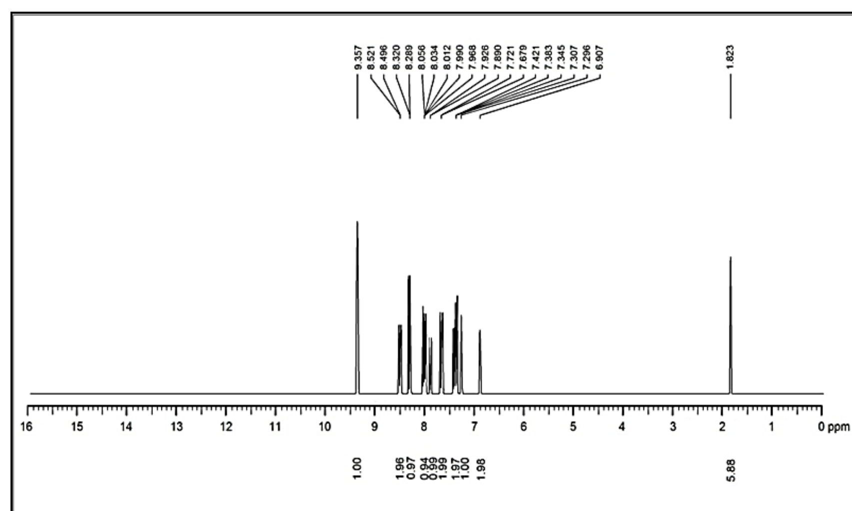


Figure 7: The ^{1}H NMR of metformin-3-hydroxyflavone

On examining the data obtained through various spectral studies of the Schiff base to that of metformin hydrochloride and 3-hydroxyflavone, it was found that the spectrum of the complex showed data similar to that of individual components with slight modifications in some regions due to the coordination between the metformin hydrochloride and 3-hydroxyflavone [43]. The main shifts account for the complex deformation is in the N=CH stretching region. The spectral data obtained for metformin as well as 3-hydroxyflavone are in accordance with several reports available in the literature [44,45].

Type 2 diabetes is strongly associated with defective regulation of hepatic glucose metabolism, involving elevated glucose production in euglycemic conditions. Liver plays a major metabolic process in balancing the uptake and storage of glucose through glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis [46]. It also has a unique role in the regulation of blood glucose in the post absorptive state.

Studies on Biochemical Indices

Table 1 depicts the antidiabetic effect of metformin-3-hydroxyflavone complex on the levels of fasting blood glucose, hemoglobin, glycosylated hemoglobin, plasma insulin and C-peptide. The levels of fasting blood glucose and glycosylated hemoglobin were significantly elevated than the normal range in diabetic group of rats.

Table 1: The levels of fasting blood glucose, glycosylated hemoglobin (HbA1c), plasma insulin, C-peptide and urine sugar in control and experimental groups of rats

Groups	Blood glucose	HbA1c	Insulin	C-peptide	Urine sugar
Control	95.74 ± 5.04	4.42 ± 0.39	15.32 ± 0.27	0.26 ± 0.02	Nil
Diabetic control	268.15 ± 9.87^{a*}	9.77 ± 0.53^{a*}	7.27 ± 0.43^{a*}	0.11 ± 0.07^{a*}	+++
Diabetic + Schiff base complex	117.67 ± 4.67^{b*}	6.21 ± 0.72^{b*}	12.56 ± 0.56^{b*}	0.17 ± 0.01^{b*}	Nil
Diabetic + Metformin	109.46 ± 5.31^{b*}	5.75 ± 0.87^{b*}	13.12 ± 0.45^{b*}	0.19 ± 0.01^{b*}	Nil

Units are expressed as mg/dL for blood glucose, % hemoglobin for HbA1c, μU/mL for plasma insulin and pmol/mL for plasma C-peptide; +++ indicates more than 2% sugar. Results are expressed as mean ± SEM [n = 6]. One-way ANOVA followed by post hoc test LSD was done. Values are statistically significant at $^{*}p<0.05$. The results were ^{a}compared to control rats and ^{b}compared to diabetic rats.

The elevated levels of fasting blood glucose and glycosylated hemoglobin were normalized upon oral treatment with Schiff base complex to type 2 diabetic rats for 30 days of experimental period. The levels of plasma insulin and C-peptide in the diabetic rats were markedly reduced when compared with control rats whereas these altered levels were significantly improved in diabetic rats treated with metformin-3-hydroxyflavone complex. Oral administration of Schiff base complex improved the plasma insulin and c-peptide levels to near normalcy in diabetic rats.

High fat diet fed-low dose STZ induced rats showed increased levels of fasting blood glucose levels and glycosylated hemoglobin. The observed decrease in the level of hemoglobin in insulin resistant experimental type 2 diabetic rats might be due to the increased formation of glycosylated hemoglobin. Glycosylated hemoglobin determination remains the standard golden marker for the assessment of glycemic control in diabetic patients. Insulin and c-peptide levels were significantly decreased in diabetic rats due to the destruction of pancreatic β- cells. The observed glucose lowering effect of the Schiff base complex was associated with significant enhance in the levels of serum insulin and c-peptide, suggesting that metformin-3-hydroxyflavone complex may induce the release of insulin from the remnant pancreatic β- cells. The antidiabetic properties of metformin-3-hydroxyflavone complex may be through activation of insulin secretion or due to improved transport and utilization of blood glucose to the peripheral tissue.

Activities of Carbohydrate Metabolizing Enzymes

The activities of hexokinase, pyruvate kinase and lactate dehydrogenase in liver tissues were presented in Table 2. These glycolytic enzymes were significantly depreciated in the liver tissues of experimental type 2 diabetic rats. Oral administration of Schiff base complex altered the activities of these glycolytic enzymes to near normalcy which was similar to metformin treated diabetic group of rats. The activity of lactate dehydrogenase in diabetic rats was significantly elevated when compared to control group of rats. The altered activity of lactate dehydrogenase in diabetic rats was restored back to near normal upon oral treatment with Schiff complex and metformin.

Table 3 depicts the effect of metformin-3-hydroxyflavone on the activities of glucose-6- phosphatase, fructose-1, 6-bisphosphatase and glucose - 6 - phosphate dehydrogenase in the liver tissues of experimental group of rats. The significant elevation in the activities of glucose-6-phosphatase, fructose-1, 6-bisphosphatase and a decreased activity of glucose - 6 - phosphate dehydrogenase were noticed in HFD fed- STZ induced experimental type 2 diabetic rats. The altered activities of these enzymes were bringing back to near normal levels by oral administration of metformin-3-hydroxyflavone complex. The effect of metformin-3-hydroxyflavone on the liver glycogen content and the activities of glycogen synthase and glycogen phosphorylase in experimental groups of rats were shown in Table 4. A significant reduction in glycogen level, glycogen synthase activity and a concomitant increase in the activity of glycogen phosphorylase were observed in experimental diabetic rats. Upon oral administration of metformin-3-hydroxyflavone complex as well as metformin to diabetic group of rats restored the level of glycogen and the activities of glycogen synthase, glycogen phosphorylase to near normalcy when compared to control group of rats.

Table 2: The activities of hexokinase, pyruvate kinase and lactate dehydrogenase in liver tissues of control and experimental groups of rats

Group	Hexokinase	Pyruvate Kinase	Lactate dehydrogenase
Control	257.85 ± 0.33	216.26 ± 0.31	221.64 ± 0.34
Diabetic	136.07 ± 0.29^{a*}	126.26 ± 0.23^{a*}	480.6 ± 0.15^{a*}
Diabetic + Schiff base	220.98 ± 0.35^{b*}	195.29 ± 0.19^{b*}	281.02 ± 0.45^{b*}
Diabetic + Metformin	221.28 ± 0.28^{b*}	197.32 ± 0.35^{b*}	284.89 ± 0.21^{b*}

Units are expressed as μmol of glucose-6-phosphate formed/h/mg of protein for hexokinase, mU/mg of protein for pyruvate kinase, μmol of pyruvate formed/h/mg of protein. Results are expressed as mean ± SEM [n = 6]. One-way ANOVA followed by post hoc test LSD was done. Values are statistically significant at $^{*}p<0.05$. The results were ^{a}compared to control rats and ^{b}compared to diabetic rats.

In the glycolytic pathway, the reactions catalyzed by hexokinase (initial step in glycolysis) and pyruvate kinase (final step in glycolysis) are virtually irreversible and hence these enzymes have the regulatory as well as catalytic properties. Dysregulation of glycolysis occurs in conditions of insulin deficiency or resistance, and is attributable to

the inappropriate amount and/or activities of metabolic and regulatory enzymes of glycolysis. Hexokinase plays a major role in the maintenance of blood glucose homeostasis because it is the predominantly expressed in hepatocytes with specific control over the hepatic glucose disposal [47].

Table 3: The activities of hexokinase, pyruvate kinase and lactate dehydrogenase in liver tissues of control and experimental groups of rats

Group	Glucose-6-phosphatase	Fructose1,6-bisphosphatase	Glucose-6 phosphate dehydrogenase
Control	1045.10 ± 0.33	462.13 ± 0.31	505.30 ± 0.34
Diabetic	1995.30 ± 0.29 ^{a*}	794.53 ± 0.23 ^{a*}	255.21 ± 0.15 ^{a*}
Diabetic + Schiff base	1246.40 ± 0.35 ^{b*}	491.29 ± 0.19 ^{b*}	420.16 ± 0.45 ^{b*}
Diabetic + Metformin	1154.60 ± 0.28 ^{b*}	493.38 ± 0.35 ^{b*}	425.17 ± 0.21 ^{b*}

Units are expressed as μmol of Pi liberated/h/mg of protein, μmol of Pi liberated/h/mg of protein, μmol of NADPH/min/mg of protein for glucose-6- phosphate dehydrogenase. Results are expressed as mean ± SEM [n = 6]. One-way ANOVA followed by post hoc test LSD was done. Values are statistically significant at $^{*}p<0.05$. The results were ^{a}compared to control rats and ^{b}compared to diabetic rats.

Table 4: The levels of glycogen content and the activities of glycogen synthase and glycogen phosphorylase in liver tissues of control and experimental groups of rats

Group	Glycogen	Glycogen synthase	Glycogen phosphorylase
Control	60.76 ± 0.26	805.45 ± 0.41	614.91 ± 0.19
Diabetic	24.05 ± 0.30 ^{a*}	504.44 ± 0.40 ^{a*}	870.32 ± 0.35 ^{a*}
Diabetic + Schiff base	43.83 ± 0.21 ^{b*}	711.01 ± 0.33 ^{b*}	673.15 ± 0.34 ^{b*}
Diabetic + Metformin	48.80 ± 0.26 ^{b*}	726.88 ± 0.20 ^{b*}	685.97 ± 0.26 ^{b*}

Units are expressed as mg of glucose/g wet tissue for glycogen, μmoles of UDP formed/h/mg protein for glycogen synthase and μmoles Pi liberated/h/mg protein for glycogen phosphorylase. Results are expressed as mean ± SEM [n = 6]. One-way ANOVA followed by post hoc test LSD was done. Values are statistically significant at $^{*}p<0.05$. The results were ^{a}compared to control rats and ^{b}compared to diabetic rats.

In fact, it acts as a glucose sensor for insulin secretion in pancreatic β-cells [48]. Metfomin-3-hydroxyflavone was found to normalize the altered expression of the enzymes involved in carbohydrate metabolism which may increase the activity of hexokinase in diabetic rats treated with Schiff base complex. Hence a possible elucidation of improved activity of hexokinase is that the oral administration of Schiff base complex activates the gene expression for hexokinase in diabetic rats.

Pyruvate kinase is regulated by its own substrate phosphoenolpyruvate and fructose-1,6-biphosphate an intermediate in glycolysis which both up-regulates pyruvate kinase. The observed decrease in the activity of pyruvate kinase in the liver tissues of STZ induced diabetic rats readily accounts for the decreased utilization of glucose (glycolysis) and increased production of glucose (gluconeogenesis) by liver indicating that these two pathways are altered in diabetes [49]. The observed increase in the activity of pyruvate kinase in the liver tissues upon metformin-3-hydroxyflavone complex treated diabetic group of rats is conceivably the result of an altered endocrine status (stimulation of insulin from the remnant β-cells of pancreas). However, it is assumed that the pyruvate kinase activity is accelerated by metformin-3-hydroxyflavone complex at least partially by facilitating the glycolytic pathway and suppression of gluconeogenesis.

Glucose-6-phosphatase is an important enzyme in regulating the gluconeogenic and glycogenolytic pathways. Glucose-6-phosphatase plays an important role in glucose homeostasis in liver and kidney [50,51]. Streptozotocin has been shown to increase the mRNA expression of glucose-6-phosphatase, which contributes to the increased activity of this enzyme in diabetes mellitus [52]. The activation of this gluconeogenic enzyme is due to the state of insulin deficiency, since under normal condition insulin function as a suppressor of gluconeogenic enzymes [53]. High fat diet fed- low dose STZ induced experimental diabetic rats were administered with Schiff base complex certainly modulated the activities of these enzymes and this might probably be due to an increase in the levels of insulin. Fructose-l, 6-bisphosphatase is also a key regulatory enzyme in the gluconeogenesis pathway. The activity of the enzyme is increased in the state of hyperglycemia in the tissues of liver and kidney of STZ-diabetic rats [54]. Metformin-3-hydroxyflavone inhibits the fructose-1, 6-bisphosphatase activity in the liver tissues of experimental type 2 diabetic rats. Oral administration of Schiff base complex as well as metformin may primarily modulate and regulate the activities of this gluconeogenic enzyme through the inhibition of glycolysis and gluconeogenesis.

Glucose-6-phosphate dehydrogenase (G6PDH), the first and rate limiting enzyme, catalyzes the oxidation of glucose-6-phosphate to 6-phospho gluconate and at the same time reducing NADP+ to generate NADPH in the pentose phosphate pathway (PPP) that supplies reducing energy to the cells. G6PDH deficiency is especially susceptible to free radical damage [55] and show increased risk for diabetes [56]. Several studies have shown that G6PDH activity is decreased in liver and other tissues in diabetes [57]. Impaired activity of G6PDH likely plays a critical role in the pathogenesis of diabetes and treatment with Schiff base complex regulates G6PDH activity through its insulin mimetic activities and/or regulatory action. Glycogen is the storage form of carbohydrate for

virtually every organism from yeast to primates. Most mammalian tissues store glucose as glycogen, with the major depots located in muscle and liver. The French physiologist Claude Bernard first identified a starch-like substance in liver and muscle and coined the term glycogen, or "sugar former" in the 1850s. Glycogen metabolism in hepatic tissue is one of the major metabolic processes involved in glucose homeostasis. Insulin is involved in both acute and chronic regulation of glycogen synthase and phosphorylase, the key regulatory enzymes of glycogen metabolism [58]. Glycogen synthase which catalyses the conversion of glucose-1-phosphate to glycogen is the rate controlling step in glycogen synthesis [59]. Glycogen phosphorylase catalyses the rate-limiting step of glycogenolysis, in which β 1-4 linkages are cleaved for the removal of glucose molecules from the glycogen chain.

Diabetes mellitus is known to impair the normal capacity of the liver to synthesize glycogen. Decreased liver glycogen levels have been reported in diabetic subjects and in some animal models of diabetes, thus suggesting impairment in glycogen synthase and glycogen phosphorylase. A decrease in liver glycogen has been reported in experimental diabetic rats [60]. The reduced glycogen store has been attributed the reduced activity of glycogen synthase and increased activity of glycogen phosphorylase [61]. Oral administration of metformin-3-hydroxyflavone complex exhibits marked glucose lowering efficacy in HFD fed- low dose STZ induced diabetic rats by enhancing glucose transport into various tissues, activating glycolysis and glycogenesis apart from inhibiting glycogenolysis and gluconeogenesis.

CONCLUSION

The coordination between metformin and 3-hydroxyflavone significantly improved the antidiabetic properties of metformin which in turn may be due to the antioxidant properties of 3-hydroxyflavone. Further studies are in progress to understand the molecular mechanisms involved in the regulation of carbohydrate as well as glycogen metabolism by the Schiff base complex treatment.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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