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Research Article

In vivo Evaluation of Synthetic compound and its derivative for Type II Diabetes Mellitus (T2DM)

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ABSTRACT

We have identified the DPP-IV inhibitor through *in silico* analysis as an anti-diabetic agent, the mechanism reveals that the synthetic compound increases the DPP4 function by degrading GLP-1 activity. This study assesses an antioxidant capacity of synthetic compounds and natural compounds in daily oral administration of 500mg/kg of body weight for 40 days in alloxan induced diabetic rats. The other biochemical tests were performed and shows more significant ($P<0.01$) in alloxan induced diabetic rats. The levels of lipid peroxide in liver of diabetic rats increased signifncantly ($P<0.01$) and catalase, superoxide dismutase, glutathione peroxidase in liver was significantly decreased in alloxan induced diabetic rats, when compared to normal rats. After treatment with the synthetic compound and its derivative were brings back to normal ($P<0.01$) in erythrocytes membrane and liver cell enzyme activities.

Keywords: Diabetes, In silico analysis, Synthetic compound, Antioxidant capacity and Anti-diabetic agent.

Introduction

Diabetes mellitus is a complex metabolic disorder that involves chronic alterations in the carbohydrate, fat, and protein metabolism, basically resulting from an imbalance between the biological action and normal secretion of insulin. It is known in the chronic form by elevated blood glucose concentration; in the untreated state it is accompanied by symptoms of severe thirst, profuse urination, polyphagia, weight loss and stupor[1].

Progressive in nature and characterized by high circulating levels of glucose resulting from insulin resistance and impaired insulin secretion, type 2 diabetes mellitus causes significant morbidity and mortality and results in considerable burden on health care resources. (http://www.who.int/mediacentre/factsheets/fs312/en/index.html).

Current treatment strategies for type 2 diabetes mellitus have only limited long-term efficacy and tolerability given the progressive nature of the disease[4]. A breakthrough was the discovery that postprandial plasma glucose concentrations are regulated by the incretin hormones glucagon-like peptide (GLP) 1 and glucose-dependent insulinotropic peptide (GIP), which sensitize β-cells to glucose stimulation, leading to increased intracellular cAMP concentrations in β-cells and an accelerated and augmented insulin response to absorbed glucose[16]. The insulinotropic actions of GLP-1 and GIP are glucose-dependent; therefore, the risk of hypoglycemia is minimized. In healthy subjects, the insulinotropic effect of GLP-1 accounts for 50 to 70% of the insulin response to an oral glucose load, but the associated "incretin effect" is often markedly reduced in patients with type 2 diabetes[16]. This quick inactivation process leads to an apparent half-life of 60-90s for GLP-1 (7-36) and there is evidence that less than 50% of released active GLP-1 (7-36) can reach circulation because of this natural degradation mechanism[12]. It is therefore apparent that a DPP4 inhibitor can prevent degradation of and lead to potentiation of GLP-1, which will further improve glucose and insulin homeostasis[19].

A good number of medicinal plants also been compared with the synthetic compounds for hypoglycemic activity in both normal and diabetic animals (Iwu, 1980). Some of these have been found to lower the blood glucose level in normal and mildly diabetic animals[17].

The protection of cell against free radical can be accomplished through enzymatic and non enzymatic means. Superoxide dismutase (SOD), catalase and glutathione peroxidase are considered to be primary antioxidant enzymes since they are involved in the direct elimination of active oxygen species[13]. Gluathione S – transferase and glutathione reductase are secondary antioxidant enzyme which helps in the detoxification of reactive oxygen species by decreasing the peroxide levels and maintaining a steady supply of metabolic intermediate like glutathione and reduced NADPH for the primary antioxidant enzymes [10].

Materials and Methods

Preparation of Extract from *Tinospora Cordifolia*

The dried powdered stem of *Tinospora cordifolia* was allowed to pass through SS sieve (20 mesh). It was defatted by treating with petroleum ether (60-80°) and then extracted to exhaustion (Soxhlet) with methanol[17]. The solvent was removed under vacuum to get the some solid mass. The extract was dissolved in physiological saline solution and given orally to diabetic groups and normal groups of (control) 500 mg/ kg of body weight[2]. Doses selected were comparable to what has been generally used in investigating pharmacological activities of herbal extracts[10].

Collection of Synthetic compound from Chemical Library

The drug like compounds was collected from Interbioscience (IBS), Moscow, Russia. This library contains a various types of molecules *viz.*, natural, synthetic and synthetic scaffolding. About 3000 synthetic compounds with diverse functional groups were taken for this analysis. After identification of perfect synthetic compound with a good binding affinity from **Fig. 1 Synthetic compound** the protein taken for studies, that was compound synthesized and methylated shown in Scheme I. Also, that compound was also taken for further analysis [5].

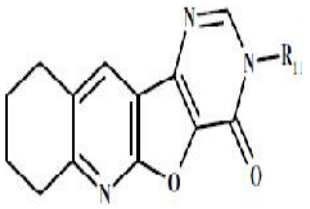

Fig.1 synthetic compound

a) Preparation of the sodium salt of Synthetic Compound (RBMS-01):

50 mg (0.21 mmoles) of synthetic compound RBMS-01 [23] was dissolved into 1.5 mL of dry methanol and a slightly excess equivalent of sodium ethoxide in 2 mL dry ethanol was added slowly. After 2 hrs stirring at RT, the solvent was dried to collect the sodium salt of RBMS-01 [24].

b) N-methyl derivative of Synthetic Compound (RBMS-01):

The sodium salt of Synthetic Compound RBMS-01 was dissolved in 2 mL DMF, and the resulting solution was cooled to 10 ºC and deaerated with nitrogen gas for 10 min. A slightly excess equivalent of methyl iodide dissolved in DMF (1 mL) was cooled to 10 ºC and was added drop wise to the DMF solution of the sodium salt slowly over a period of 5 min. Then the reaction mixture was allowed to rise in temperature gradually to ~60 ºC. The reaction mixture was kept stirring at ~60 ºC for 3 hr. The solvent was removed at reduced pressure, and extraction of the solid product with chloroform gave 0.27 g of crude product after removal of the solvent. The compound was then re-crystalized from chloroform given in Scheme I.

Validation of methylated compound by using *in vivo* analysis.

The selected molecules (Synthetic Molecule, Methylated Synthetic Molecule and Natural Compounds) were validated by *in vivo* analysis. This series of investigations looked at the Wistar rats weighing 200 - 250g used in the experiment and housed in polypropylene cages at room temp. 25° to 30° C and had free access to drinking water and basal diet. The entire animal experiments were done as per the guidelines of Institutional Animal ethical committee. Alloxan was purchased and induced over rats. They were feed with standard laboratory animal feed (Hindustan Lever Ltd., India) and water *ad libitum*. Ethical clearance was obtained from the Institutional Animal Ethical Committee (**Approval No.115/ac/07/CPCSEA**).

Induction of diabetes mellitus

The experimental animal in this model is the male, adult wistar albino rats, weighing 200 - 250g. After a 12-hour fast, the rats were weighed and a solution of 2% alloxan monohydrate (S.D. Fine Chemicals, Mumbai) diluted in saline (0.9%) corresponding to 80 mg of alloxan per kg body weight was administered intraperitoneally in a single dose. Food and water were given to the rats after 30 minutes of drug administration [7,10]. After two week rats blood glucose levels of 200-260mg/dl were used for the study. Blood was taken from eyes (Venous pool) and glucose was estimated by Sasaki method (Sasaki 1972). All the biochemical and chemicals used in the experiment were of analytical grade[21].

Experimental design:
The rats were divided into TEN groups each group in six rats.
Group 1: Normal rats with 0.5 ml of physiological saline.
Group 2: Normal rats with Synthetic compound in 10 mg /kg of body weight once every day up to 6 weeks.
Group 3: Normal rats with methylated synthetic compound in 10 mg /kg of body weight once every day up to 6 weeks.
Group 4: Normal rats with berberine in 10 mg /kg of body weight once every day up to 6 weeks.
Group 5: Normal rats with metformin in 10 mg /kg of body weight once every day up to 6 weeks.
Group 6: Alloxan induced diabetic rats with 0.5 ml of physiological saline.
Group 7: Alloxan induced diabetic rats with Synthetic compound in 10 mg /kg of body weight once every day up to 6 weeks.
Group 8: Alloxan induced diabetic rats with methylated synthetic compound in 10 mg /kg of body weight once every day up to 6 weeks.
Group 9: Alloxan induced diabetic rats with berberine in 10 mg /kg of body weight once every day up to 6 weeks.
Group 10: Alloxan induced diabetic rats with metformin in 10 mg /kg of body weight once every day up to 6 weeks.

The Isolation of erythrocytes membrane by Dodge method[6] with a change in buffer according to Quest method[22], Blood collected with EDTA as an anticoagulant and centrifuged at 1500× g for 15 min the produced plasma eliminated and packed cells washed three times with 0.9% saline, the cells lyses by suspending in hypotonic buffer for one hour and centrifuged at 15 000× g for 30 min the supernatant red fluid containing membrane, washed with hypotonic buffer until it became colorless or pale yellow. The membrane solution and liver extracted supernatant were used for the analyses. Superoxide dismutase was assayed according to the method of Misra and Fridovich [18], Catalase was assayed according to the method of Bergmeyer [3], Glutathione Peroxidase was assayed according to the method of Necheles [20], Lipid Peroxide concentrates was determined by thiobarbituric acid reaction as described by Ohkawa [21] Biochemical determinations were carried out using Shimadzu spectrophotometer.

Statistical Analysis
Statistical treatment applied is ANOVA under one way classifications followed by Bonferroni multiple comparison test, changes were considered significant at the *P*-value of <0.01 level of significances. The values are expressed as mean±SD.

Results and Discussions
In the 80 mg/kg alloxan (single IP dose) induced diabetic rats, the potency of the extracts were provided as Metformin > Berberine > Synthetic Compound > Methylated synthetic compound; thus the different extracts had significantly ($p<0.05$) higher blood glucose lowering activities than synthetic compound and its derivative in partially dysfunctional pancreatic β-cells (Table 1).
In the 80 mg/kg alloxan single dose induced diabetic rats; the percentage maximal reduction of blood glucose levels was close to 75% for synthetic compound and having an equal effect with other natural compounds. **Table 1**, shows that an alloxan induced and the extracts may be mainly insulin secretagogues shows the lowering of blood glucose.
The aim of the present study was to assess the antidiabetic and antioxidant potential of berberine from *Tinospora cordifolia* medicinal plant and metformin in alloxan induced diabetes rats. **Table 2** shows the effects of various biochemical tests in diabetic rats were compared to normal. The treatment of oral administration of synthetic *compounds* in diabetic rats significantly decreased and brings back to near normal level. **Table 3** shows the levels of erythrocytes membrane lipid peroxides and catalase activity was significantly increased in alloxan induced diabetic rats. Also, the activities of SOD, glutathione peroxidase was found to be decreased significantly when compared to normal rats.
After administration of synthetic compounds and natural compounds the alteration of erythrocyte membrane lipid peroxidases and activity of catalase, SOD, glutathione peroxidases were reversed and it was close to the normal. However, there was no such alterations exist in the erythrocytes of control group.
Table 4 also shows that catalase, SOD, glutathione peroxidase, lipid peroxide activities in liver of experimental rats. The levels of lipid peroxide in liver of diabetic rats increased significantly and catalase, SOD, glutathione peroxidase in liver was significantly decreased in diabetic rats, when compared to normal rats. The treatment of synthetic compound and its derivative were brings back to normal ($P<0.01$) in the enzyme activities.
The present study states that, an alloxan induced diabetic cause an increased in blood glucose level in rats. The oral administration of synthetic and natural compounds significantly $P < 0.01$ decreased blood glucose in diabetic rats. The increased susceptibility of erythrocytes to lipid peroxidation in diabetes suggested the possibility of increased peroxidative distractions of membrane lipids[11]. It has been reported that the production of lipid peroxide is carried out by free radicals such as SOD, hydroxyl radicals and hydrogen peroxide causing cellular damage[9].

Further, the increased levels of lipid peroxide in diabetic rats indicated the degenerative status in diabetes which was reduced significantly P<0.01 by synthetic compound treatment. Activity of catalase in erythrocyte membrane was increased significantly in diabetic rats, while liver tissues in diabetic rats exhibited a decreased activity [15]. These changes were brought back to significantly P<0.01 near normal after synthetic compound treatment.

Glutathione peroxidase, superoxide dismutase is the enzyme responsible for the destruction of peroxides and has a specific role in protecting tissue against oxidative damage [25]. Decreased level of superoxide dismutase and glutathione peroxidase in erythrocyte membrane and liver tissues of alloxan induced rats these levels are significantly P<0.01 altered to near normal in synthetic compound derivative treated alloxan induced diabetic rats.

Conclusions

It may be concluded that the alterations of impaired blood glucose level status in diabetic condition have been restored to normal by administering synthetic compound and its methylated derivative compound also indicates the protective role of the same. Among these screened compounds the synthetic compound also having activity like natural compounds. In conclusion, these compounds lowered blood glucose level in diabetes.

Methylation of Synthetic compound from Fig. 1 (Synthetic compound).
Synthesis of 2-(4-Methoxy-phenyl)-6,7,8,9-tetrahydro-2H-11-oxa-2,4,10 - triaza-benzo [b] fluoren-1-one (Methylation of synthetic compound)

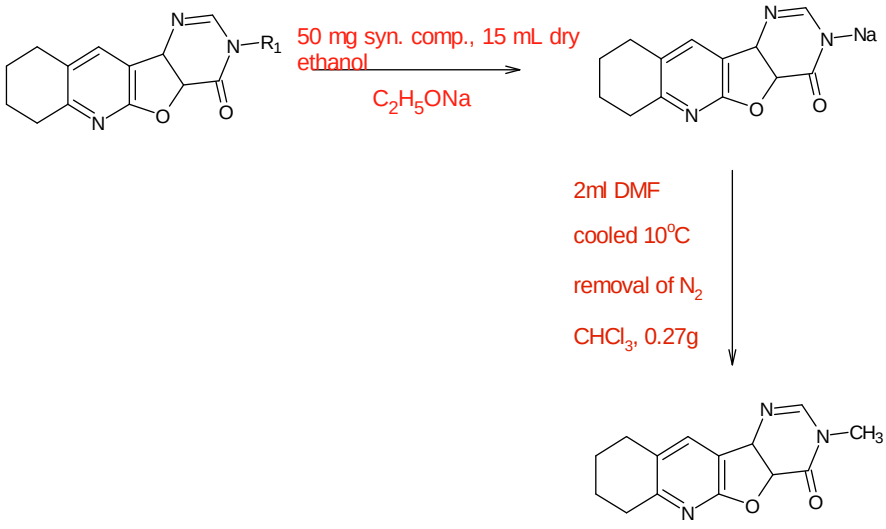

TABLE 1 EFFECT OF SYNTHETIC COMPOUND, DERIVED SYNTHETIC COMPOUND, BERBERINE, METFORMIN ON BLOOD GLUCOSE, BODY WEIGHT AND URINE SUGARS IN NORMAL AND DIABETIC RATS, SYNTHETIC COMPOUND, DERIVED SYNTHETIC COMPOUND, BERBERINE and METFORMIN.

Drugs	Dose	0h	1h	2h	4h	8h	10h	% Max. Red
Normal (No Alloxan)	3ml/kg	215.5±1.4	194.2±2.8	184.9±2.3	171.2±2.9	160.9±2.4	150.9±3.1	70
Synthetic Compound	10mg/kg	220.3±2.5	201.4±4.1	191.3±2.5	178±3.7	165.4±3.9	160.1±1.6	73
Synthetic Compound Methylated	10mg/kg	184.7±1.5	174.1±4.2	164.9±9.2	159.6±4.3	146.4±2.1	138.7±3.0	75
Berberine	10mg/kg	210.6±1.7	199.1±2.4	190.3±1.9	178.2±1.1	168.8±2.3	160.2±2.6	77
Metformin	10mg/kg	218.4±1.8	210.4±1.6	198.1±1.5	190.6±2.4	184.9±1.8	174.8±2.3	80
Distilled Water	3ml/kg							--

TABLE2 EFFECT OF *SYNTHETIC COMPOUND* ON VARIOUS BIOCHEMICAL TESTS IN NORMAL, *SYNTHETIC COMPOUND* TREATED METHYLATED *SYNTHETIC COMPOUND* TREATED AND *COMPARED WITH NATURAL COMPOUNDS* TREATED WITH DIABETIC RATS. VALUES ARE EXPRESSED AS MEAN ± SD FOR SIX ANIMALS IN EACH GROUP.

Parameters	Normal	Synthetic Compound (10 mg /kg)	Synthetic Compound Methylated (10mg/kg)	Berberine treated diabetes (10 mg /kg)	Metformin (10 mg /kg)	Distilled Water (3ml /kg)
Serum protein (g/dl)	**7.3 ± 0.23**	**7.6 ± 0.38**	**6.2 ± 0.54**	**6.8 ± 3.24**	**6.9±2.34**	**7.0±0.42**
Liver tissue protein (mg/g of tissue)	**213.63 ± 2.41**	**227.85 ± 3.84**	**195.2 ± 9.37**	**204.35 ± 4.19**	**209.42 ± 1.12**	**211.32 ± 2.13**
Serum cholesterol (mg/dl)	162.0 ± 1.44	168.0 ± 1.72	243.4 ± 0.24	176.3 ± 2.24	172.4 ± 3.24	**168.3 ± 2.30**
Blood urea (mg/dl)	25.60 ± 2.44	22.32 ± 1.03	49.03 ± 3.24	29.30 ± 0.20	28.23 ± 1.02	**26.60 ± 1.20**
Liver Glycogen (mg/g wet tissue)	46.3 ± 2.29	49.31 ± 1.55	31.48 ± 8.0	40.86 ± 3.07	42.42 ± 2.34	**145.36 ± 1.10**
Glycosylated Haemoglobin (mg/gHb)	0.266 ± 0.004	0.238 ± 0.003	0.779 ± 0.003	0.409 ± 0.002	0.366 ± 0.001	**0.395 ± 0.004**
SGPT	10.31 ± 0.37	9.31 ± 0.26	21.36 ± 0.65	16.13 ± 0.42	14.12 ± 0.32	**13.11 ± 0.12**
GPT	926.15 ± 8.92	936.21 ± 17.85	1048.65 ± 15.11	987.63 ± 16.11	982.43 ± 10.42	**979.38 ± 10.12**
Hexokinase[1]	264.68 ± 0.83	272.63 ± 2.84	115.43 ± 3.46	238.83 ± 2.83	240.73 ± 1.32	**242.36 ± 2.23**
Glucose - 6 – phosphatase[2]	1032.4 ± 0.42	963.3 ± 1.15	1236.8 ± 3.22	1123.5 ± 4.32	1119.3± 3.26	**1052.5 ± 2.34**
Fructose 1, 6 – bisphosphatase[2]	474.14 ± 1.63	456.64 ± 2.32	757.42 ± 3.82	563.56 ± 3.83	542.32 ± 2.13	**504.06 ± 1.32**
Glycogen synthase[3]	810.0 ± 52	818.0 ± 12	473.0 ± 32	746.0 ± 43	762.0 ± 52	**797.0 ± 43**

TABLE 3 SHOWS THE LEVELS OF ERYTHROCYTES MEMBRANE LIPID PEROXIDES AND CATALASE ACTIVITY

Parameters	Normal	Synthetic Compound (10 mg /kg)	Synthetic Compound Methylated (10mg/kg)	Berberine treated diabetes (10 mg /kg)	Metformin (10 mg /kg)	Distilled Water (3ml /kg)
Lipid peroxide [a]	0.34 ± 0.23	0.30 ± 0.25	0.83 ± 0.42	0.46 ± 0.22	0.43 ± 0.23	**0.39 ± 0.13**
CAT[b]	0.165 ± 0.25	0.160 ± 0.02	0.348 ± 0.32	0.221 ± 0.30	0.206 ± 0.12	**0.197 ± 0.13**
SOD[c]	3.19 ± 0.23	3.43 ± 0.32	2.10 ± 0.38	2.94 ± 0.44	2.98 ± 0.13	**3.10 ± 0.42**
Glutathione peroxidase[d]	48.43± 0.41	49.48± 0.52	30.11± 0.73	45.24± 0.63	46.43± 0.42	**47.80± 0.24**

TABLE 4 SHOWS THAT THE ACTIVITY OF CATALASE, SOD, GLUTATHIONE PEROXIDASE, LIPID PEROXIDE ACTIVITIES IN LIVER

Parameters	Normal	Synthetic Compound (10 mg /kg)	Synthetic Compound Methylated (10mg/kg)	Berberine treated diabetes (10 mg /kg)	Metformin (10 mg /kg)	Distilled Water (3ml /kg)
Lipid peroxide [a]	1.44 ± 0.10	1.49 ± 0.19	2.31 ± 0.17	1.86 ± 0.8	1.68 ± 0.4	**1.58 ± 0.10**
CAT[b]	73.61 ± 7.4	74.17 ± 5.6	33.90 ± 4.3	57.50 ± 5.6	61.50 ± 3.2	**68.41 ± 1.2**
SOD[c]	6.31 ± 0.82	6.36 ± 0.97	3.43 ± 0.65	5.85 ± 0.43	6.27 ± 0.42	**6.21 ± 0.12**
Glutathione peroxidase[d]	84.2± 6.4	80.6± 5.2	54.4± 4.8	79.3± 5.4	80.2± 4.2	**81.76± 4.0**

1. µ – moles of glucose - 6 – phosphate formed/h/mg protein
2. n moles of phosphorous liberated/h/mg protein
3. n moles of uridine diphosphatase formed/h/mg protein

a. Lipid peroxide- no of moles MDA/ mg/ Protein.
b. CAT activity is expressed as moles of H_2O_2 decomposed /min/ mg protein.
c. One unit of SOD activity was the amount of protein of protein required to given 50% inhibition of adrenaline autoxidation.
d. Glutothione peroxidase- no of moles of GSH oxidized/min/mg protein

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