Insulinotropic Activity of Methanolic Extract of *Mesua ferrea* Linn.

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Abstract: *Mesua ferrea* flowers have been used in the Indian traditional medicine for treating various diseases. In present study, the *Mesua ferrea* flowers methanolic extract (MFME, 200 mg/kg bw) was studied for anti diabetic activity and underlying mechanisms for its activity. In Streptozotocin induced diabetes rats model, MFME treatment enhanced plasma insulin levels by 69.53% (P<0.0001) than the diabetic control rats. In diabetic rats, MFME treatment significantly restored body weight and blood glucose levels to normal. In Insulin release studies on MIN6 beta cells, insulin release was potentiated in a dose dependent manner of extract and glucose. The insulin stimulatory effect of MFME was stimulated by 3-isobutyl-1-methyl xanthine, Glibenclamide and elevated extracellular calcium. In distinction, the stimulatory effect was inhibited with diazoxide, nifedipine, EGTA and K^+ depolarized media. These results indicate MFME anti diabetic activity might be a result of insulin secretagogue effect through beta cell physiological pathways.

Keywords: Diabetes, Insulin secretagogue, MIN6 - β cells, K_{ATP} channel, Calcium, Nifedipine.

INTRODUCTION

Mesua ferrea Linn. (Guttiferae) is a tree commonly known as cobra's saffron. The plant is widely spread over in countries India, Sri Lanka, Thailand, Cambodia, Malaysia, Myanmar, Philippines, Singapore and Vietnam. The flowers of *Mesua ferrea* L. have been traditionally used as a medicine in the Ayurveda for treating fever, itching, nausea, leprosy, dysentery, skin diseases, bleeding piles, metrorrhagea, menorrhagea, excessive thirst, and sweating cough, dysentery and liver diseases [1]. Stamens of the flowers contain phytoconstituents like flavanoids (mesuaferrone A and B), cyclohexadiane derivatives (mesuaferrol, mesuanic acid) and other compounds (β-amyrin, β-sitosterol). There are several reports on the biological activities of the *Mesua ferrea* flowers include anti oxidant and hepatoprotective activities, immunomodulatory activity and anti convulsant activity [2]. These reported activities of *Mesua ferrea* flowers prompted us to evaluate the anti diabetic activity along with mechanisms involved.

Currently, various classes of oral anti diabetic agents like sulphonylureas, biguanides, thiazolidin-ediones, meglitinides etc., are used in the treatment of type 2 diabetes [3]. Sulphonylureas are the insulin secretagogues drugs acts *via* K^+-ATP channel dependent way, where glucose metabolism in pancreatic β cells increases intracellular ATP/ADP ratio and leads to closure of K^+-ATP channels. This closure depolarizes β cell membrane and stimulates insulin release through Ca^{+2} influx by opening voltage gated Ca^{+2} channels [4].

The present study was aimed to test *Mesua ferrea* flowers methanolic extract for the anti diabetic activity in *in vivo* by using Strepozotocin induced diabetes rat model and mechanisms of anti diabetic activity was investigated *in vitro* by using mouse insulinoma pancreatic β cells (MIN6 β cells).

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Streptozotocin (STZ), Methanol, fetal calf serum, glucose, glutamine, penicillin, streptomycin, diazoxide, 3-Isobutyl-1-methylxanthine (IBMX), calcium chloride, potassium chloride, ethylene glycol tetra acetic acid (EGTA) and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) reagents were procured from Sigma Aldrich (Bangalore, India). MIN6 beta cells were provided by National Centre for Cell Science, Pune. Glibenclamide and nifedipine were the gift samples provided by Symed Labs Ltd (Hyderabad, India). All the other chemicals used were of analytical grade.

Plant Material and Extraction

Mesua ferrea dry extract powder was provided as a gift sample by Kuber impex limited, India. The extract powder was exhaustively extracted with methanol by using cold percolation method. The prepared *Mesua ferrea* methanolic extract (MFME) was subjected for filtration and concentrated under reduced pressure, followed with freeze drying.

Animals

Male Wistar rats weighing about 180-200 g were procured from Sanzyme Ltd (Hyderabad, India).

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Animals were housed at 25 °C, relative humidity of 45-55% under 12 hrs natural light, dark cycle with unlimited access to food and water. Throughout the experimental period, the rats were fed with balanced pellet diet with a composition of 5% fat, 21% protein, 55% nitrogen-free extract and fiber (w/w) with adequate mineral and vitamins. The entire experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the guidelines of Committee for Control and Supervision of Experimentation on Animals, Government of India on animal experimentation.

Acute Toxicity Studies

The MFME acute toxicity studies were preformed on normal rats as previously described by Lorke *et al.* [5]. MFME treated four rat groups were administered separately with 0.25, 0.5, 1.0 and 2.0 g/kg body weight (bw)) and control group received vehicle alone (CMC 0.5%; 1ml/kg bw). The animals were observed for toxicity symptoms and behavioral changes for a period of 24 h.

Glucose Tolerance Test (GTT) in Normal Rats

Glucose tolerance test was performed in normal male Wister rats to determine the optimized dose of MFME, as discussed elsewhere [6]. Overnight fasted rats were randomly divided into five groups (n=6). Glucose (2 g/kg bw) was given to all rats by oral gavage and treated according to their respective grouping, naïve group animals received only glucose. Blood samples were collected and analyzed for blood glucose at 0, 30, 60, 90 and 120 min after glucose loading [7].

Animal Treatment

A freshly prepared solution of streptozotocin (STZ, 50 mg/kg bw) in 0.1 M citrate buffer (pH 4.5) was used in diabetes induction by intra peritoneal administration to the overnight fasted rats. Group I (Naïve) animals were administered with only 0.1 M citrate buffer. To prevent STZ induced hypoglycemic shock the animals were supplemented with 10% glucose solution for 48 h. A week after induction, blood glucose levels of the rats were estimated by glucose oxidase method [7]. The animals with blood glucose above 250 mg/dL were treated as diabetic animals. Diabetic animals were divided into three groups (n=8): Group II served as diabetic control, while group III and group IV received MFME (200 mg/kg bw) and glibenclamide (5 mg/kg bw), respectively, for a period of 4 weeks.

Body Weight and Biochemical Estimations

For all the rats, on the first (1st day) and last (28th day) days of the study body weight, plasma glucose and plasma insulin levels were evaluated. On 7th and 15th days of treatment, body weight and plasma glucose levels were determined. Rat insulin ELISA kit (Crystal chem. inc., Illinois, USA) was used in determination of plasma insulin concentrations.

Cytotoxicity Assay (MTT Assay)

Cell viability assay was performed as discussed elsewhere with few modifications [3]. Briefly, cell viability of MIN6 - β cells was assessed by seeding 30,000 cells per well in 96 well plates and allowed to attach for overnight. Then the cells were treated with MFME at a concentration range of 5-1000 µg/mL and standard drug, glibenclamide (5-100 µM/mL), allowed for 72 hrs incubation. Followed by incubation, to each well a 20 µL solution of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) (5 mg/mL) was added and incubated for 4 hrs. After that, 100 µL of dimethylsulphoxide (DMSO) was added to each well and aspirated to dissolve the formazan crystals formed by reduction of MTT. Further, plates were shaken for 10 seconds for uniform mixing followed by read for absorbance at 570 nm by using Multiskan Ex microplate reader (Thermo scientific, USA).

Insulin Secretion from MIN6 - β Cells

MIN6 - β cells were maintained in DMEM supplemented with 10% FCS, 2mM glutamine, 10,000 units/mL of penicillin and 10 mg/mL of streptomycin with culture conditions of 37 °C and 5% CO_2. MFME extract stock solution was prepared by dissolving in DMSO and further working solution were prepared by using KRB. Insulin secretion assay was performed as reported with few modifications [8, 9]. Study was conducted by seeding about 30,000 MIN6-β cells per well under naïve, basal and hyperglycemic conditions where media contains KRB only, 3mM glucose and 11.1 mM glucose, respectively. Extract at a sub maximal dose (1000 µg/mL) and standard drug, glibenclamide (10 µM) were used in the studies. Unless stated, the experiment was conducted at 11.1 mM glucose concentration.

The insulin releasing mechanism of MFME was ascertained in the presence of diazoxide (0.5 mM) (K^+-ATP channel opener), IBMX (100 µM) (phosphodiesterase inhibitor) and Glibenclamide. Role of K^+-ATP channel and calcium was evaluated in the

presence of Calcium chloride (1.28 mM), EGTA (1 mM), Nifedipine (20 µM) and KCl (30 mM, Depolarising concentration). After an hour of incubation period, supernatant from each well was collected and centrifuged (4000 g, 5 min, 4° C). Clear supernatant after centrifugation was stored at -20 ° C until determination of insulin concentrations by ELISA.

Statistical Analysis

All the values are expressed as mean± SD. One-way or two-way ANOVA was used upon suitability. The significance of difference was assessed by using the Dunnett's post hoc test or Bonferroni test. Values with $P<0.05$ were considered to be significant. Graph Pad Prism (Graph Pad Software, San Diego, CA) was used for all statistical analysis.

RESULTS

Acute Toxicity Studies and Glucose Tolerance Test

Acute toxicity studies of prepared MFME extract at doses of 0.25, 0.5, 1.0 and 2.0 g/kg bw were proven safe without any toxicity symptoms, behavioral changes and mortality.

Glucose tolerance test of MFME at 200 mg/kg bw dose was effectively reduced blood glucose levels than the doses of 50 and 100 mg/kg bw (Table **1**). Administration of MFME (200 mg/kg bw) led to a significant ($P<0.001$) reduction in blood glucose levels after 30 min, when compared with vehicle treated naïve animals. Thus, the dose of 200 mg/kg bw was used for the *in vivo* anti diabetic and insulin secretion studies.

Body Weight and Biochemical Estimations

The effect of MFME (200 mg/kg bw) on the body weight on 1, 7, 14, 21 and 28th days of study in the STZ induced diabetic rats were shown in Figure **1**. On end day of the study, MFME treated rats shown a loss of 9.35% in their initial weight; where diabetes control animals lost 21.41% their initial day body weight by end day of study. Administration of MFME resulted into a significant ($P<0.001$) restoration in the bodyweight of the animals in comparison with the diabetes control animals.

Table 1: Oral Glucose Tolerance Test of MFME at Different Doses

	0 min	30 min	60 min	90 min	120 min
Naïve	75.26±1.65	131.39±2.77	104.26±2.12	89.59±1.81	83.97±2.12
Glibenclamide (5 mg/kg)	70.35±1.84	104.17±1.77	86.35±1.38	76.56±1.55	70.29±1.32
MFME (50 mg/kg)	82.02±1.71[@]	141.24±2.95[#,@]	107.73±2.25[@]	100.48±4.97[#,@]	89.32±1.86[@]
MFME (100 mg/kg)	81.85±2.5[@]	129.57±3.97[@]	103.81±3.18[@]	97.07±4.47[*,@]	88.98±2.72[@]
MFME (200 mg/kg)	81.09±3.36[@]	120.37±3.49[#,@]	95.09±3.06[#,@]	86.22±3.97[@]	83.59±2.93[@]

Values indicate mean ±SD. Data was analyzed by two Way ANOVA followed by Bonferroni test (n = 6); #P < 0.001, *P < 0.01 as compared to naïve animals at respective time points. [@]P < 0.001comapred with Glibenclamide control at respective time points.
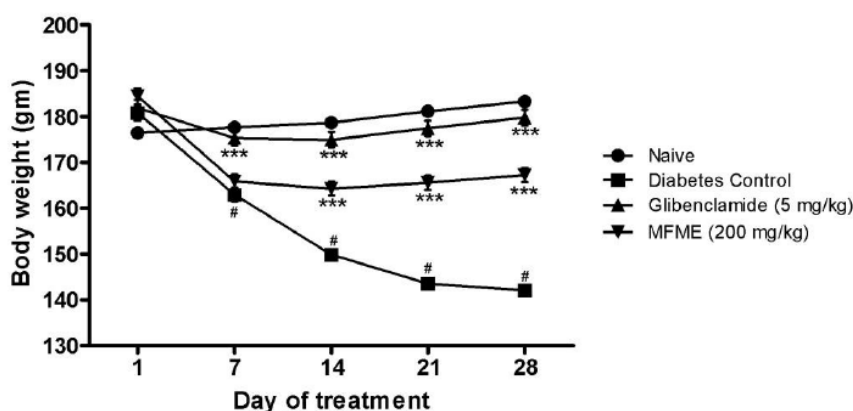

Figure 1: Effect of MFME extract (200 mg/kg) and standard drug glibenclamide (5 mg/kg) on body weight in STZ induced diabetes model in rats. (Data was analyzed by two Way ANOVA followed by Bonferroni test (*n* = 6); [#]*P* < 0.001 as compared to naïve animals, [***]*P* < 0.001, as compared to diabetes control group at respective time points).
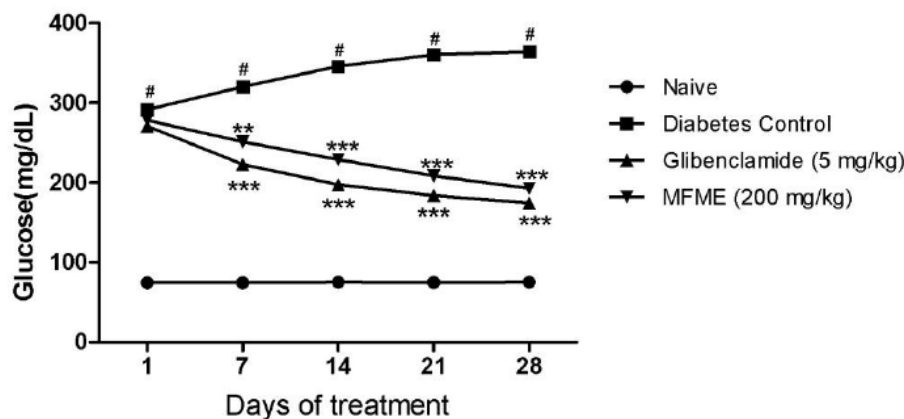
Figure 2: Effect of MFME extract (200 mg/kg) and standard drug glibenclamide (5 mg/kg) on blood glucose levels in STZ induced diabetes model in rats. (Data was analyzed by two Way ANOVA followed by Bonferroni test (n = 6); [#]$P < 0.001$ as compared to naïve animals, [***]$P < 0.001$, as compared to diabetes control group at respective time points).

In comparison with naïve animals, the significant increase in the blood glucose levels were observed in the STZ treated diabetic rats (Figure **2**). MFME (200 mg/kg bw) treatment led to a significant (P<0.001) lowering of hyperglycemia by 30.67% from the blood glucose level of 278.32±4.47 mg/dL on first day of study. On 28th day, the standard drug, glibenclamide reduced blood glucose levels by 35.51% (p<0.01) of blood glucose levels on 1st day. The treatment of diabetic rats with standard drug, MFME reduced hyperglycemia significantly.

During the study period, other than naïve group animals the plasma insulin levels reduced significantly (P<0.001) in all groups. When plasma insulin levels on 28th day compared with initial day, treatment with MFME (200 mg/kg bw) significantly (P<0.001) increased plasma insulin levels by 69.53% (0.56± 0.01 ng/mL) in comparison with diabetic control animals (Figure **3**). Whereas, standard drug, glibenclamide enhanced plasma insulin levels by 89.25%. On end

day, the insulin levels were unchanged in naïve group. Therefore, MFME (200 mg/kg bw) treatment reduced hyperglycemia, weight loss and enhanced plasma insulin levels.

Cytotoxicity Assay (MTT Assay)

MFME extract at the tested dose range (5-1000 µg/mL) and standard drug, Glibenclamide at dose range (5-100 µM/mL) has not shown any toxicity on the viability of the cells, upon incubation (Table **2**). Therefore, glibenclaminde and MFME were used at the selected doses for the *in vitro* studies.

MFME Effect on Insulin Secretion from MIN 6 Beta Cells

MFME Effect on Insulin Secretion

In comparison with glucose controls, at basal (3mM) and hyperglycemic (11.1 mM) glucose concentrations insulin secretory activity of the extract was dependent
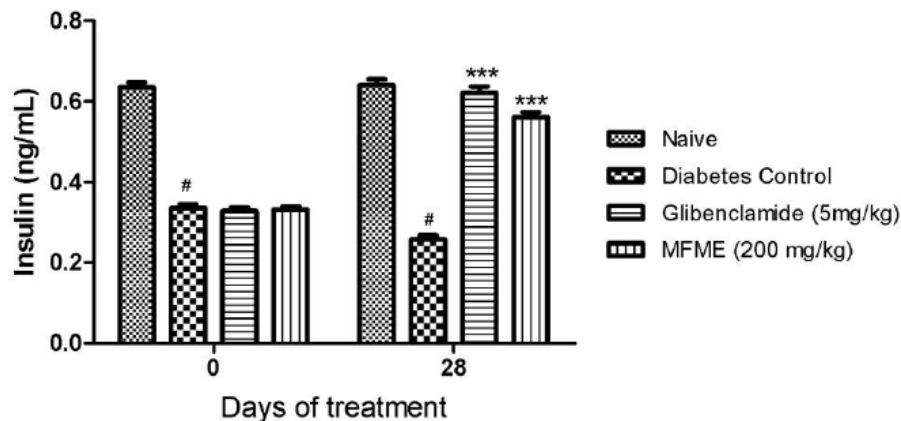

Figure 3: Effect of MFME extract (200 mg/kg) and standard drug glibenclamide (5 mg/kg) on plasma insulin levels in STZ induced diabetes rats on 0th and 28th day of treatment. (Data was analyzed by two Way ANOVA followed by Bonferroni test (n = 6); [#]$P < 0.001$ as compared to naïve animals, [***]$P < 0.001$, as compared to diabetes control group at respective time points).

Table 2:   MIN6 β - Cell Viability Assay of Glibenclamide and MFME at Different Doses

Glibenclamide (µM/mL)					
Concentration	**1**	**5**	**50**	**100**	**Control**
% Viability	97.84±2.56	96.8±1.45	96.32±1.57	95.84±1.18	100.05±0.08
MFME (µg/mL)					
Concentration	**5**	**50**	**500**	**1000**	**Control**
% Viability	99.4±2.24	98.69±1.44	98.02±1.96	97.78±1.46	99.95±0.09

Effect of glibenclamide and MFME on viability of MIN6 beta cells. (The values were expressed as Mean ± SD); (n = 6).

on the extract dose at tested dose range of 5-1000 µg/mL (Figure **4**). The *in vitro* studies were performed with a dose of 1000 µg/mL, since this dose resulted to maximum insulin secretion from MIN6 beta cells in basal and hyperglycemic conditions. In comparison with control, the extract treatment led to a significant ($P<0.0001$) increase in insulin secretion in both the conditions. The MFME significantly ($P<0.0001$) enhanced insulin release under hyperglycemic conditions than the basal conditions at respective doses.
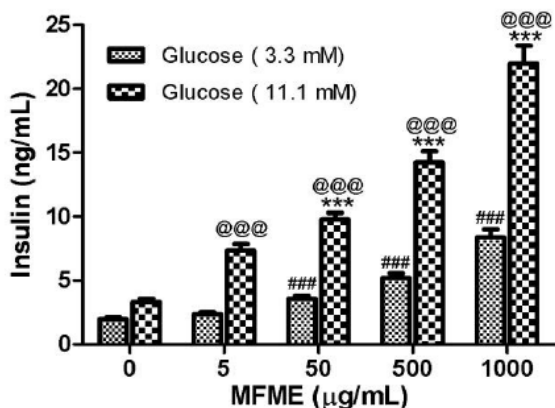

Figure 4: Effect of MFME extract (5-1000 µg/mL) on released insulin levels from MIN6 beta cells upon incubation for 60 minutes. All the values are the means ± SD (n=5) of the insulin released as an effect of response to dose of extract at glucose basal (3.3 mM) and hyperglycemic (11.1 mM) conditions. ### $P<0.001$ respective to control at basal condition. ***$P<0.001$ relative to control at hyperglycemic condition. @@@ $P<0.001$ compared with insulin levels at basal and hyperglycemic conditions at same extract concentration.

Effect of Glucose on Insulin Secretion by MFME

The role of glucose on insulin secretion by MFME was shown in Figure **5**. The extract treatment enhanced insulin secretion significantly ($P<0.001$) in comparison with the control at all the glucose concentrations. Hence, glucose at a sub maximal concentration (11.1 mM) was used in the further studies.

Mechanisms of Insulin Release by MFME

Insulin secretagogue mechanisms of MFME were studied by using insulin release modulators (Figure **6**). K^+-ATP channel dependence of the extract in hyperglycemic condition was demonstrated by using diazoxide. This demonstrated a significant reduction ($P<0.001$) in insulin levels by MFME in the presence of diazoxide (9.478±0.41 ng/mL) than MFME alone (20.396±0.72 ng/mL). MFME extract treatment in the presence of IBMX has significantly ($P<0.001$) increased insulin secretion by 21.89% than the extract alone. In the presence of glibenclamide, MFME significantly ($P<0.001$) enhanced insulin release (32.118±0.72 ng/mL) by MIN6 beta cells in comparison with control (3.328±0.13 ng/mL). However, the treatment of MFME resulted into a comparable insulin release to glibeclamide alone (31.979±0.63 ng/mL).

Role of K^+-ATP Channels and Calcium (Ca^{+2}) on Insulin Secretion by MFME

The MFME extract significantly enhanced insulin release from MIN6 beta cells in the presence of higher calcium levels in incubation media by 42.02% (28.966±0.97 ng/mL, $P<0.001$), in comparison with extract treated control (Figure **7**). This effect of extra cellular Ca^{2+} on insulin secretion was reduced significantly in the presence of nifedipine (20.276±0.54 ng/mL, $P<0.001$), EGTA (19.447±0.67 ng/mL, $P<0.001$) than the presence of external calcium (28.966±0.97 ng/mL). This demonstration of enhanced insulin release in the added calcium in media; reduced insulin release in the presence of nifedipine, EGTA showed the influence of calcium on insulin release from beta cells. Under KCl depolarizes incubating conditions, the MFME effect on insulin release was similar to extract treated control. This indicates the dependence of K^+-ATP channel for the insulin release by MFME.

DISCUSSION

Mesua ferrea has been used in the traditional medicine for the treatment of various diseases.
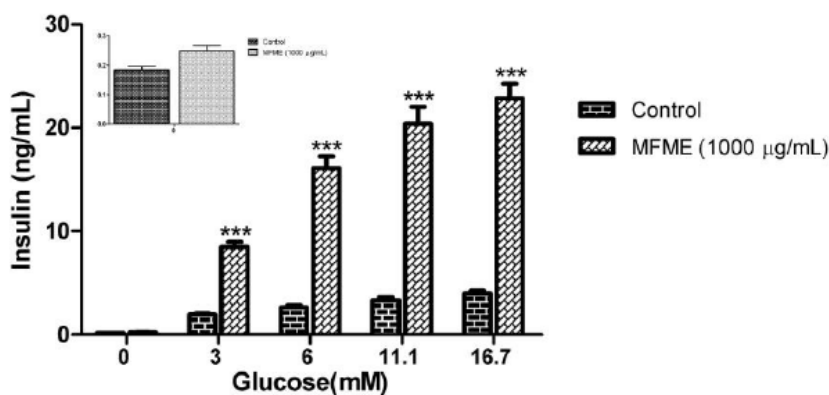
Figure 5: Effect of MFME extract (1000 µg/mL) on released insulin levels) at different glucose levels (0-16.7 mM) from MIN6 beta cells upon incubation for 60 minutes. All the values are the means ± SD (n=5) of the insulin released in response to glucose load. ***P<0.001 relative to control condition. Graph insert shows insulin released at blank (0 mM glucose) with similar axes titles of main graph.
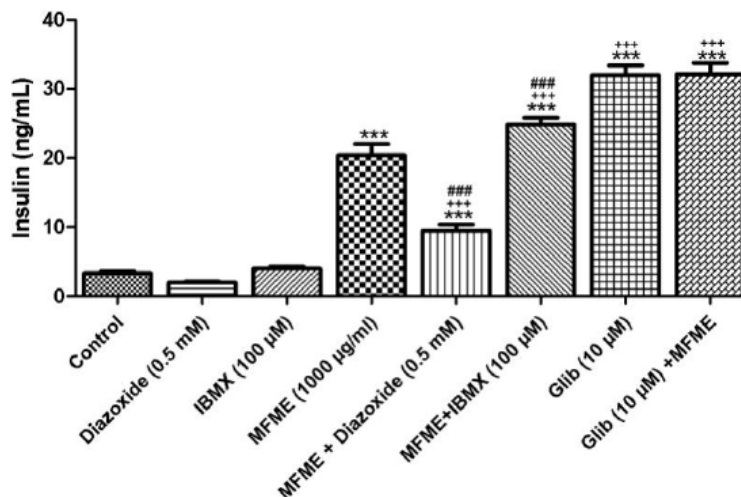

Figure 6: Effect of insulin release modulators (Diazoxide, IBMX and Glibenclamide) on insulin secretion from MIN6 beta cells upon incubation with 11.1 mM glucose for 60 minutes in the presence and absence of MFME (1000 µg/mL). All the values are the means ± SD (n=5). All the data was analyzed by one way ANOVA followed by Dunnet's test. ***P<0.001 in comparison with control. +++ P<0.001, ++ P<0.01 in comparison with the extract. ### P<0.001 in comparison with respective modulator in the absence of MFME.

However, the scientific studies to evaluate its anti diabetic activity and mechanisms of action are not yet reported. Furthermore, the present study indicates insulinotropic effect of MFME leads the antidiabetic activity by acting as an insulin secretagogue.

In acute toxicity studies the given doses of the MFME were found non toxic. In OGTT studies in normal rats, the effective dose of the MFME was determined as 200 mg/kg bw, which led glucose loaded rats into normoglycemic in 120 min. In STZ induced diabetes rats were characterized by loss of bodyweight as an effect of muscle wasting and loss of tissue proteins [10]. However, administration of MFME (200 mg/kg bw) to STZ induced diabetic rats resulted into

improved body weight by improving glycemic control [11]. The enhanced plasma insulin levels and anti hyperglycemic activity of the MFME might have been due to the insulin secretagogue effect of extract on β cells. Gireesh *et al.* [6] reported that the anti diabetic plants are able to enhance circulating insulin levels. In addition, the effect of MFME and standard drug, glibenclamide effect on blood glucose and plasma insulin levels were comparable. This could signify the insulin secretagogue effect of the MFME.

The *in vitro* experiments conducted on MIN6 beta cells to study the mechanisms of anti hyperglycemic activity of MFME revealed insulin release potentiating effects of the extract. This enhancement in insulin
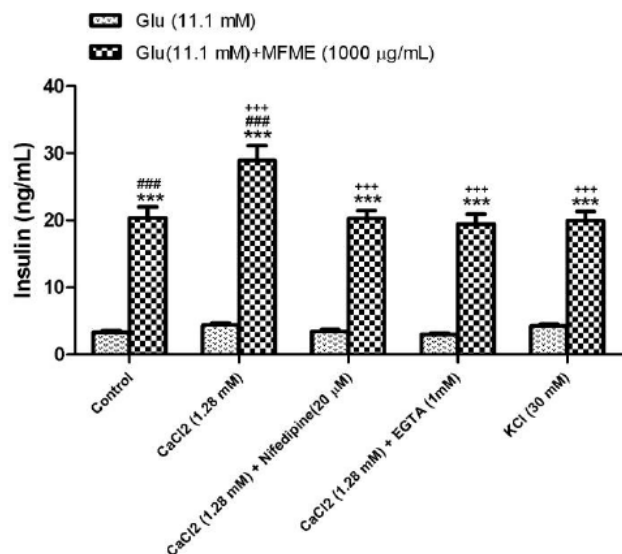
Figure 7: Role of K^+-ATP channels and calcium (Ca^{+2}) on insulin secretion from MIN6 beta cells upon incubation with 11.1 mM glucose in the presence and absence of MFME (1000 µg/mL). All the values are the means ± SD (n=5). All the data was analyzed by one way ANOVA followed by Dunnet's test. ***$P<0.001$ in comparison with control in the absence of extract. ### $P<0.001$ in comparison with control in the presence of extract. +++ $P<0.001$ in comparison with respective control in the absence of MFME.

release was as effect of glucose load in *in vitro* conditions. Hypoglycemia as a result of excessive insulin release is the major limitation with the current treatment of diabetes. The increment in insulin secretion only in hyperglycemic condition implies that MFME would not lead to hypoglycemia [12, 13]. The effects of MFME on insulin secretion in response to higher glucose conditions in *in vitro* indicate that insulinotropic effect of the extract is an effect of β-cell metabolism [14].

Standard class of insulin secretagogue drugs i.e., sulfonylurea's exert their activity by blocking K_{ATP} channels in β cell membrane to depolarize it, which leads to activation of voltage-gated calcium channels to enable Ca^{+2} influx for enhancing intracellular calcium levels. This facilitates the exocytosis of insulin containing granules of β cell. The K_{ATP} channels closure as a mechanism in insulin secretagogue effect of MFME was evaluated by using Diazoxide, which significantly decreased insulin releasing effect of the MFME in the presence of hyperglycemic condition. This suggests the involvement of K_{ATP} channels in the mechanism on insulin release by MFME [15]. The insulin secretagogue effect of the extract in the presence of IBMX denotes the involvement of cyclic AMP generation in augmenting the insulin release. Further, extract did not enhanced insulin release under

depolarizing conditions of KCl (30 mM), which suggest the insulin secretagogue activity if the MFME is a result of depolarization of the β cell [16].

In insulin exocytosis from β cell, role of calcium is clearly evident from the studies conducted in extracellular calcium (1.28 mM) rich media. Where, insulin releases from beta cell by MFME was blocked in the presence of calcium channel blocker, nifedipine and chelating agent EGTA. Thus insulin secretagogue activity of the extract requires presence of calcium and its influx through the β cell membrane [15].

Over all, these *in vivo* and *in vitro* studies concluded that the *Mesua ferrea* flower methanolic extract could act as an insulinotropic agent in the diabetes, by enhancing insulin secretion. Therefore, *Mesua ferrea* could be a probable therapeutic adjunct and natural source for diabetes therapy.

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