ORIGINAL ARTICLE

Preventive Effects of *Enicostemma littorale* Blume Extract against Dexamethasone Induced Insulin Resistance in Wistar Rats

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Abstract:

Background: Insulin resistance is a pathological condition in which physiological levels of insulin produce only subnormal biological responses. In such instances, β cells of the pancreas produce more insulin but the cells in other body tissues are resistant to insulin leading to hyperinsulinemia, and subsequent hyperglycemia further results in metabolic syndrome. *Aim and Objectives:* This study was aimed to evaluate the hydro-alcoholic extract of *Enicostemma littorale* (ELE) in preventing dexamethasone 8mg/kg induced insulin resistance. *Materials and Methods:* Thirty-six male Wistar rats were categorized into Group I plain control that received distilled water, Group II dexa control that received dexamethasone 8mg/kg. Group III and Group IV received dexamethasone 8 mg/kg along with Metformin 500 mg/kg and 1g/kg respectively. Group V and VI received dexamethasone along with ELE 2.5 g/kg and 3.5 g/kg respectively. At the end, the fasting and repeated blood samples were collected at 30, 60 and 120 min for the estimation of post Intraperitoneal Glucose Tolerance Test (IPGTT), serum glucose and insulin. Data were utilized for the assessment of the homeostasis model of assessment for insulin resistance and sensitivity, Gutt, Matsuda, fasting glucose to insulin ratio and disposition indices. *Results*: All insulin sensitivity indices were worsened in dexa control group as compared to plain control ($P<0.05$). ELE 3.5 g/kg significantly lowered fasting glucose and insulin compared to metformin 1 g/kg ($P<0.05$), and significantly prevented the fall of insulin sensitivity indices compared to dexa control group ($P<0.05$). Glycosuria and ketonuria were also absent in ELE 3.5 g/kg group. *Conclusion:* ELE 3.5 g/kg showed efficacy in preventing insulin resistance evidenced by improved insulin sensitivity indices comparable with that of metformin 1 mg/kg.

Keywords Hyperglycemia, Hyperinsulinemia, Dyslipidemia, Metformin, Surrogate Indices

Introduction:

Insulin Resistance (IR) is a pathological condition in which physiological levels of insulin produce only subnormal biological responses. In such instances, β cells of the pancreas produce more insulin but the cells in other body tissues are resistant to insulin leading to hyperinsulinemia and, subsequent hyperglycemia further results to Metabolic Syndrome (MetS) [1]. IR is broadly categorized into hepatic and peripheral insulin resistance [2]. The central obesity includes IR in the liver, resulting in improper insulin signalling in hepatocytes by stimulating Suppressor of Cytokine Signalling (SOCS), other kinases which include

tyrosine phosphorylation, c-Jun N-terminal Kinase (JNK) and Protein Kinase-C (PKC). Hepatic IR further can cause impaired glucose transportation into hepatocytes by insulin and contributes to hyperglycemia [3]. The peripheral insulin resistance results from altered intrinsic cell-pathways of mitochondrial dysfunction, oxidative stress and Endoplasmic Reticulum (ER) stress; whereas alterations in adipokines and fatty acids levels and the presence of inflammation in metabolic tissue are the dominant extrinsic mechanisms that modulate peripheral actions of insulin [4]. Partial fall of Integrin Linked Kinase (ILK) demonstrate possible peripheral vascular resistance which can be further confirmed by increased Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index, decreased Quantitative Insulin-sensitivity Check Index (QUICKI) values and minor response to an exogenous bolus injection of insulin during Insulin Tolerance Test (ITT) [5].

Glucocorticoids (GC) are known to cause insulin resistance which may result in complications like diabetes mellitus, hyperlipidemia and other cardiovascular abnormalities. GC induced IR also results from peripheral and hepatic components where insulin action fails to suppress hepatic glucose production and stimulation of peripheral glucose utilization [6]. This reflects the reduction in insulin induced glucose uptake by the skeletal muscle which is a primary site for glucose disposal [7]. In the management of insulin resistance associated diabetes mellitus, two insulin sensitizer groups are widely available; Thiazolidinediones and Biguanides but, adverse effects are the determining factors in the long term care. Some indices of Insulin Sensitivity (IS) and IR are in practice which, include simple ratios such as I/G ratio and products of insulin and glucose levels at single time points or integrated over time during an Oral Glucose Tolerance Test (OGTT) or Intraperitoneal Glucose Tolerance Test (IPGTT) as proposed by Perley *et al.* [8] and Yalow *et al.* [9]. More complex indices of insulin sensitivity or insulin resistance have been suggested by several investigators such as Matthews *et al.* (HOMA) [10], Gutt *et al.* (ISI0, 120) [11], Matsuda *et al.* (ISI composite) [12] Fasting Glucose to Insulin Ratio (FGIR) [13]. The above methods have been correlated well with more rigorous but laborious measurement of insulin sensitivity such as steady-state plasma glucose method [14], the commonly used intravenous (i.v) glucose tolerance test and minimal model method [15-16] or the gold-standard euglycemic clamp method [17-18]. With this background**,** the current hypothesis was undertaken to evaluate, substantiate and compare the degree of dexamethasone mediated insulin resistance and comparison of its prevention with *E. Littorale* Extract (ELE) and Metformin (MET) treatments by determining fasting glucose and insulin and sensitivity indices.

Material and Methods:

This experimental study was carried out at Department of Pharmacology, KS Hegde Medical Academy, Mangalore, Karnataka, India during January 2015.

Experimental Animals

Thirty-six male Wistar albino rats weighing around 230-270 g were chosen for this study. Prior to the study, all the animals were housed and maintained at 22-24°C temperature, under a 12-h light: 12-h dark cycle *ad libitum*. This study obtained the approval from the Institutional Animal Ethics Committee (AEC/29//2011) and all procedures were conducted according to the revised guidelines of CPCSEA Act, 1960 India.

Grouping of animals

All animals selected for the study were divided into five treatment groups and one plain control group of six animals in each (Table 1).

Table 1: Grouping of Animals (n=6)

Group I	Plain Control (PC)
Group II	Dexa 8 mg/kg (DC)
Group III	MET 500 mg/kg + Dexa 8 mg/kg
Group IV	MET 1 g/kg + Dexa 8 mg/kg
Group V	ELE 2.5 g/kg + Dexa 8 mg/kg
Group VI	ELE 3.5 g/kg + Dexa 8 mg/kg

MET: Metformin, ELE: Enicostemma littorale Extract, Dexa: Dexamethasone

Plant material and extraction

Several herbs are quoted for the management of diabetes mellitus in Ayurveda and *Enicostemma littorale* is one of them. It was procured from SDM Ayurvedic Pharmacy, Udupi, Karnataka. The plant was identified and authenticated by SDM Research Center, Udupi, Karnataka, India. The voucher specimen (No. SP-73: 8/3/2013) was preserved for future reference. Blume of 500 g was dried in shade was weighed and placed in a round bottom flask. Five litres of an equal mixture of distilled ethanol (approximately 95%) and distilled water (1:1) was added and allowed to stand for 24 h. Contents were filtered and the extract was concentrated by distillation and solvent was removed by evaporation on a water bath. It was completely dried under vacuum. The percentage of dried extract with reference to the sample was 20.07%. The extract was picked up and stored in a cool and dry bath, which was further employed in the study.

Drugs and Doses

The doses were selected based on a pilot study conducted in a small group (two rats per group) of animals. Dexamethasone was procured from Cadila Pharma and doses of 8 mg/kg body weight/day were chosen and administered intraperitoneally (i.p). The solution of hydroalcoholic extract of *E.littorale* was prepared freshly everyday according to body weights of each rat with distilled water and was given at oral doses of 2.5 g/2 ml and 3.5 g/2 ml to the respective treatment groups. A pure form of MET was purchased from Sri Mahalakshmi Chemicals, Hyderabad. Doses of 500 mg/kg and MET 1g/kg were given orally to the respective groups. The plain control was given normal saline i.p followed by equal volume of distilled water by oral route.

Study design

All animals in all groups received respective treatment doses daily throughout the study period (12 days). Groups II to VI received dexamethasone treatment from day 7 to day 12 whereas plain control received normal saline i.p from day 7 to day 12. Each rat was allowed to have 100 g of standard food pellets and 100 ml of water daily up to 11th day evening, followed by overnight fasting with free access to water alone. On 12th day morning, drugs were given two hours prior to collecting blood by retro-orbital sinus puncture method. Blood samples were centrifuged (4000 rpm/20 min) and the serum was collected for biochemical estimations (fasting glucose, insulin and post IPGTT values).

IPGTT

After 16 h of fasting, blood samples from all the animals were collected, followed by the administration of glucose i.p (2 g/kg b w). The

samples were collected again at intervals of 30 min, 60 min and 120 min and then processed for glucose and insulin levels [19].

Estimation of serum glucose:

Glucose Oxidase-Phenol Amino Phenazone (GOD-PAP) method was employed to determine the serum glucose. The values were measured as mg/dL and were presented as Mean ± SD [20].

Estimation of serum insulin:

ELISA insulin estimation kit which is ultra-sensitive for rats [7] was bought from Crystal Chem Labs, New Delhi. A high range assay was conducted (1-64 ng/ml) to obtain the insulin values with the provided reagents and serum samples. Micro plates coated with the antibody reagent and marked 'A' were fixed to the Elisa frame. Each well was filled with 95 µl of sample diluent that was marked 'G' and five µl of the sample. The micro plate was kept in incubation for the period of 2 hours at 4°C. After the incubation period, each well was washed for five times with wash buffer solution. Anti-insulin enzyme conjugate, 100 µl per well was dispensed and the microplate was kept in incubation for half an hour at the room temperature. Later, each well was washed for seven times with wash buffer solution. Enzyme substrate solution marked 'E', 100 µl per well was dispensed. Then, the micro plate was again incubated for 10 min at room temperature in a light free area. The enzyme reaction stop solution marked 'F', 100 µl per well was added to stop the enzyme reaction. With the help of standard curves optical density values were obtained. The optical density values were converted to its original insulin values (µU/ml) by using linear regression equation in MS Excel 2013 version. The data were presented as Mean±SD. The insulin (ng/ml) values were converted into µU/ml by using standard conversion factor 23.98.

Calculation of Indices:

HOMA assessment was used to determine the degree of hepatic IR and IS respectively while the Disposition Index (DI) and Glucose To Insulin Ratio (FGIR) were used to understand the improvement in glucose intolerance and glycemic variability with the treatment. The Gutt and Matsuda Indices were determined to assess the improvement in peripheral and whole-body insulin resistance respectively.

HOMA-IR = $Fasting\ insulin \times Fasting\ glucose \div 405$

HOMA-IS = $10000 \div fasting\ insulin \times fasting\ glucose$

Disposition Index (DI) = $(I_{30}\text{-}I_0) \div (G_{30}\text{-}G_0) \times ISI_{(Matsuda)}$ [22]

Fasting Glucose to insulin ratio (FGIR) = G_0/I_0

Gutt Index= $ISI_{0,\,120} = \frac{500+(G0 - G120)\times 0.19\times b\ w}{120 \times Gmean \times \log(Imean)}$

Matsuda Index $ISI_{(Matsuda)} = \frac{10000}{\sqrt{G0\times I0\times Gmean\times Imean}}$

Statistical Analysis

Data were showed as Mean ± S.D. The statistical analysis was performed using One-way ANOVA followed by Scheffe multiple comparison post-hoc test. Statistical significance was assumed at the 5% level and $P<0.05$ as significant.

Results:

Fasting serum glucose and insulin

The anti-hyperglycemic and anti-hyperinsulinemic effects of ELE 2.5 g/kg, 3.5 g/kg, MET 500 mg/kg and MET 1g/kg were exhibited in Fig. 1. The doses of 3.5 g/kg and 2.5 g/kg of ELE significantly decreased the fasting glucose and insulin levels compared to DC ($P<0.05$), but in-significant compared to MET groups (MET 500 mg/kg & MET 1 g/kg) ($P>0.05$). The differences in mean

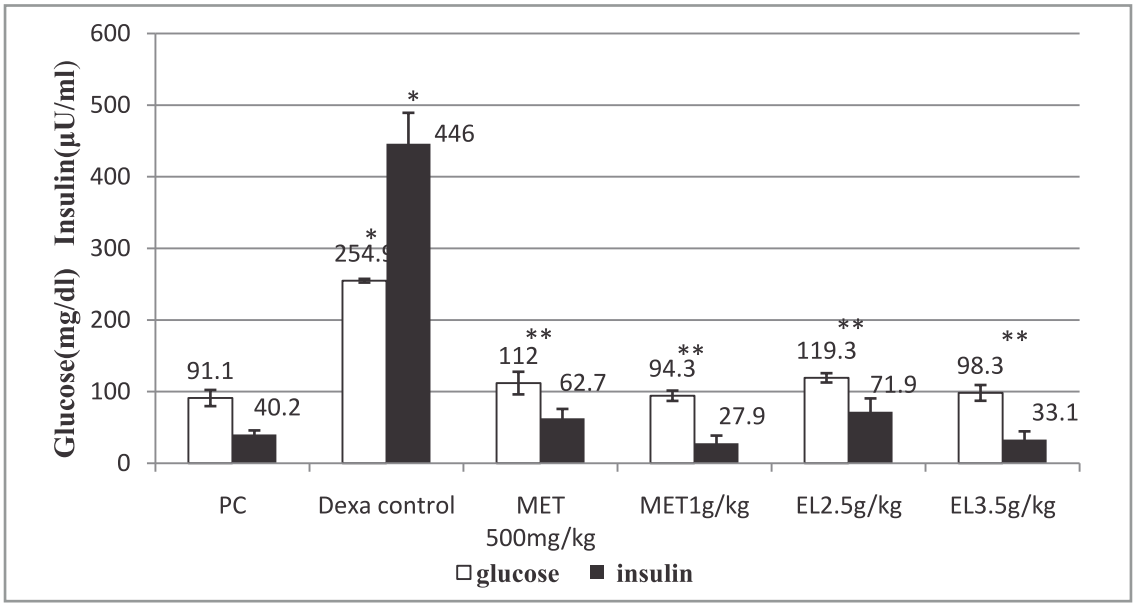
values of glucose and mean values of insulin between MET 500 mg/kg and MET 1 g/kg groups are statistically significant (P<0.05). The ELE 2.5 g/kg and ELE 3.5 g/kg groups were also exhibited statistically significant differences (P<0.05) in mean values of glucose and mean values of insulin between them.

Surrogate indices for the assessment of insulin resistance and insulin sensitivity

The degree of hepatic insulin resistance was calculated and assessed by HOMA-IR and IS and displayed in Fig. 2. The rise of HOMA-IR was significantly halted with ELE 3.5g/kg and it conserved the sensitivity to insulin (HOMA-IS) compared to DC (P<0.05). Although MET 1g/kg reduced HOMA-IR value and spared HOMA-IS value significantly compared to ELE 3.5 g/kg (P<0.05), the difference in HOMA-IR was non-significant between low and high doses of ELE (P>0.05). As shown in the fig. 3, ELE 3.5 g/kg significantly conserved FGIR compared to DC (P<0.05). Both MET 1 g/kg and ELE 3.5 g/kg treatments significantly improved the DI compared to DC (P<0.05). However, this difference was non-significant between ELE doses (Fig. 4). ELE 3.5 g/kg significantly conserved the IS as evidenced by improved Gutt and Matsuda indices compared to DC and the MET groups (P<0.05) (Fig.5). Although, this improvement was non-significant when MET 1 g/kg was compared with ELE 3.5 g/kg group (P>0.05).

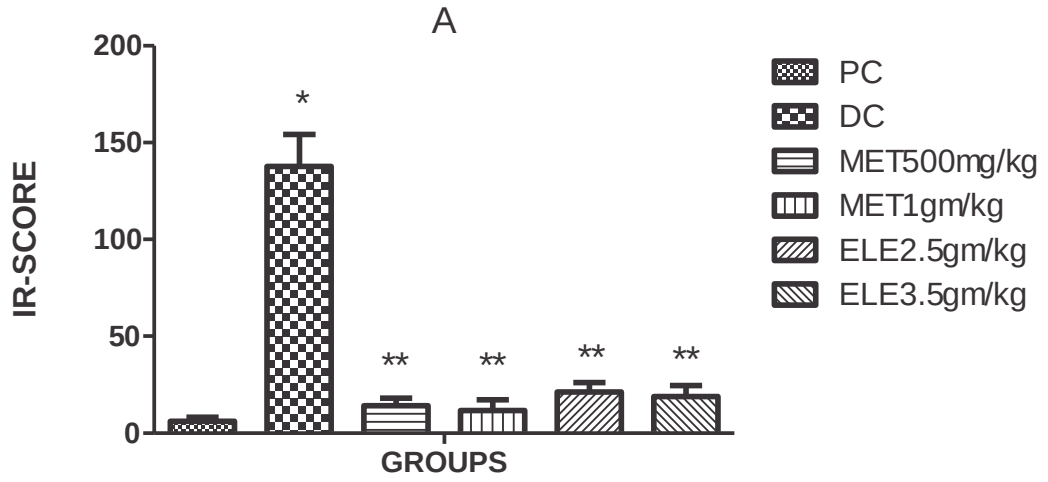
Effect of ELE on dexamethasone induced glycosuria and ketonuria

DC group showed moderate and severe glycosuria and ketonuria. MET and ELE treatments effectively prevented the glycosuria and ketonuria compared to DC as evidenced by the absence of glucose and ketones in urine samples (Table 2).

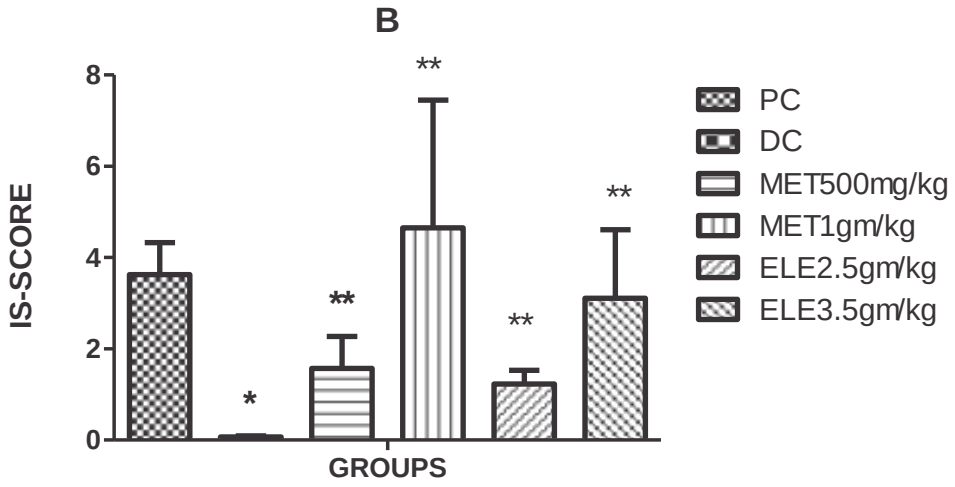


*Note: *= significant at the level of 5% (P= < 0.05), *= significant compared to control, **=significant compared to DC, ***= significant compared to MET.*

Fig. 1: Fasting Glucose and Insulin Values in Study Groups on Day 12



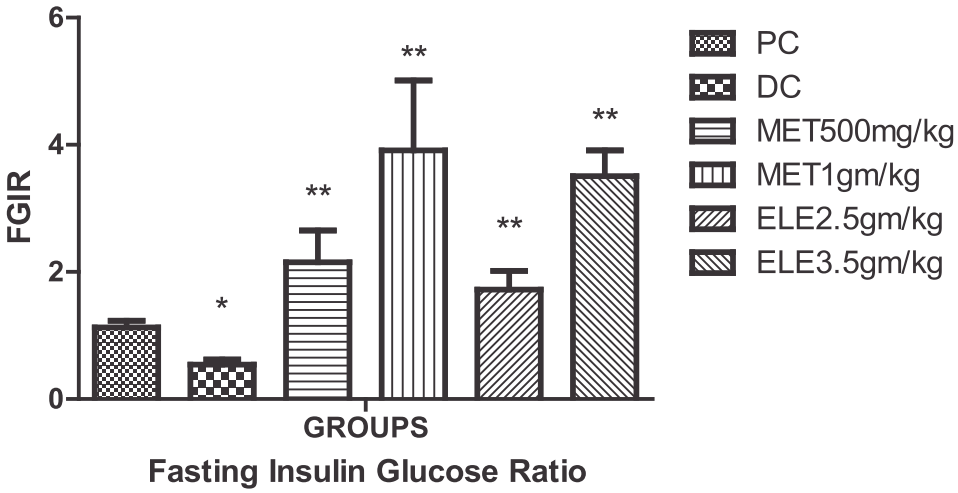
Homeostatic model of assessment for insulin resistance (HOMA-IR)



Homeostasis model of assessment of insulin sensitivity (HOMA-IS)

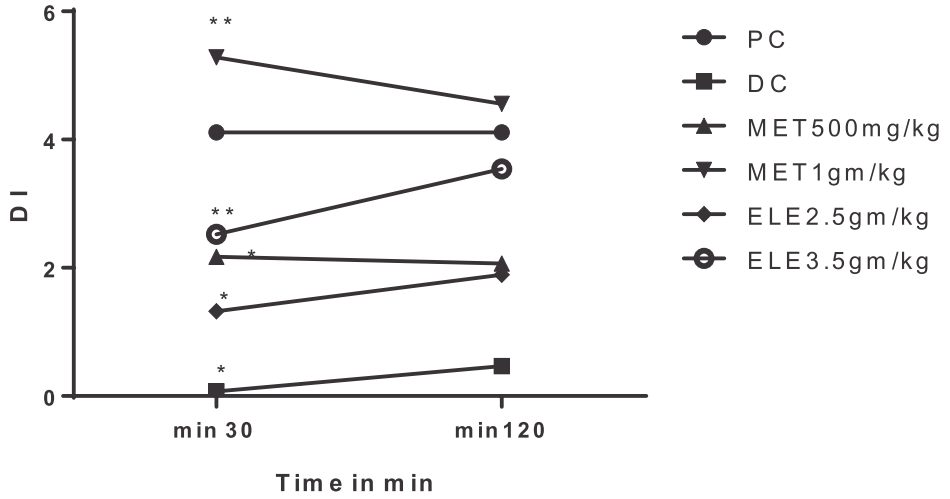
*A: HOMA-IR scores in the study groups, B: HOMA-IR scores in the study groups, *= significant at the level of 5% (P < 0.05), *= significant compared to control, **=significant compared to DC, ***= significant compared to MET.*

Fig. 2: Homeostatic Assessment of Insulin Resistance (HOMA-IR) and Insulin Sensitivity (HOMA-IS) in Study Groups



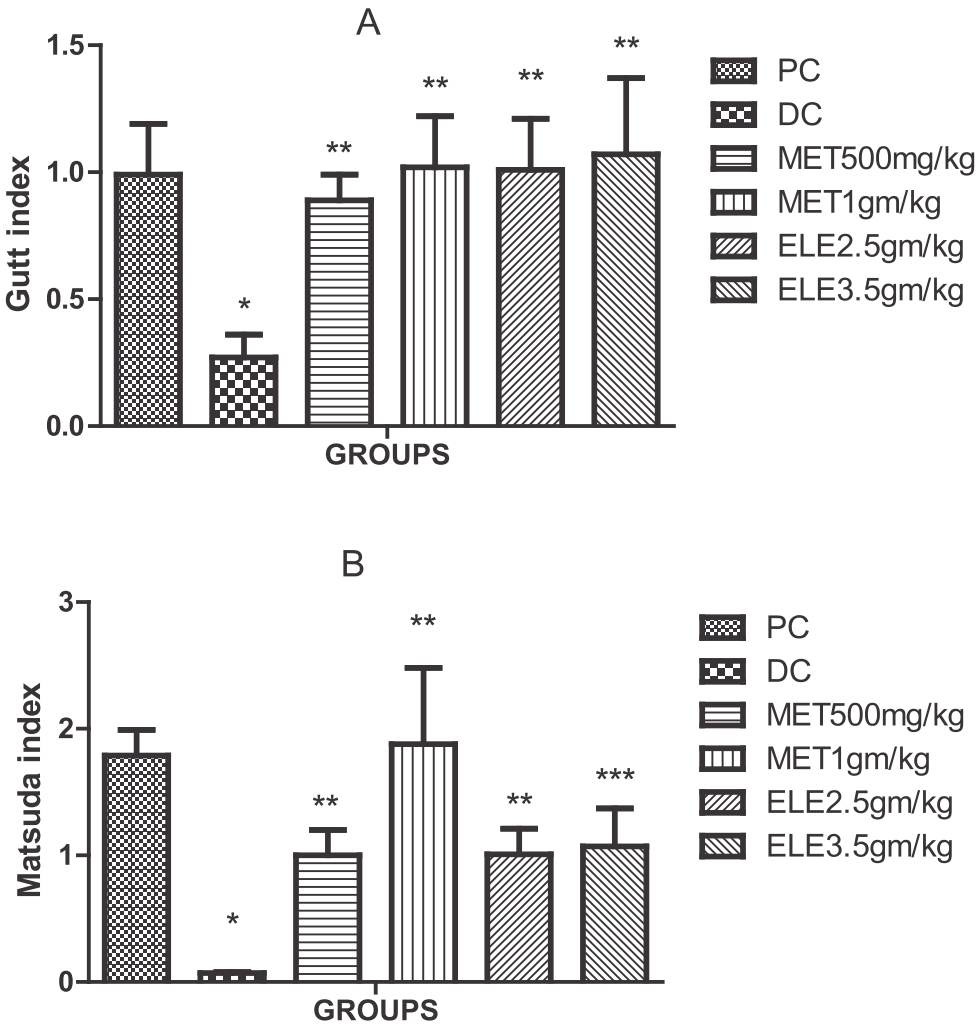
A: Fasting glucose to insulin ratio in the study groups, Note: *= significant at the level of 5% (P= < 0.05), *= significant compared to control, **=significant compared to DC, ***= significant compared to MET

Fig. 3: Fasting Glucose to Insulin Ratio (FGIR) in Study Groups



*= significant *at the level of 5% (P= < 0.05), *= significant compared to control, **=significant compared to DC, ***= significant compared to MET.*

Fig. 4: Post-IPGTT disposition index (DI) at 30 min and 120 min in study groups



*A: Gutt index in the study groups, B: Matsuda index in the study groups, *= significant at the level of 5% (P= < 0.05), *= significant compared to control, **=significant compared to DC, ***= significant compared to MET.*

Fig. 5: Gutt and Matsuda Indices in Study Groups

Table 2: Means of Fasting Urine Glucose and Ketones on Day 12 in Study Groups

Group (n=6)	Urine Glucose (mmol/L)	Urine ketones (mmol/L)
PC	Nil	Nil
DC	76.6 ± 1.2*	10.6 ± 4.1*
MET500mg/kg	Nil	Nil
MET1g/kg	Nil	Nil
ELE2.5g/kg	Nil	Nil
ELE3.5 g/kg	Nil	Nil

**= significant at the level of 5% (P= < 0.05), a= Plain control; b=DC; f=MET500mg/kg; g=MET1g/kg; h=ELE2.5g/kg; i=ELE3.5g/kg*

Discussion:

The current study was designed to investigate the potential effects of ELE on dexamethasone induced insulin resistance emphasizing on its prevention in Wistar albino rats. On long-term use, dexamethasone induced insulin resistance irrespective of hyperglycemia, and with hyperglycemia and GLUT-2 positive β cell numbers, glucose transport into cells and insulin response to glucose are reduced [23].

Studies on the anti-diabetic activity of ELE in various models revealed its insulin secretion enhancing property but none of the studies explained its insulin-sensitizing property *in-vivo* and its use in insulin resistance [24]. This work is aimed at evaluating the extent of insulin-sensitizing activity of ELE by reduction in steroid induced IR in comparison with MET. The results in this study have revealed that the insulin sensitizing property of ELE in steroid induced insulin resistance is greater than or equal to that of MET treatments as it improved hyperinsulinemia, hyperglycemia and surrogate sensitivity indices effectively. The rise in hepatic IR with dexamethasone was effectively prevented with ELE, MET treatments as evidenced by improved hepatic IR and IS values. FGIR was appreciably improved by both doses of ELE. Both low and high doses of ELE prevented the fall in DI effectively, but the rise in glycaemic variability was notably controlled well by MET doses. Dexamethasone treatment severely hindered Gutt Index reflecting reduced peripheral glucose uptake into cells while ELE and MET treatments enhanced peripheral uptake of glucose compared to DC. However, the mean difference in improvement of peripheral glucose uptake between MET and ELE was found superior. Matsuda Index for whole body insulin sensitivity was improved by ELE treatment as compared to MET treatment against steroid induced IR.

Few studies have elucidated only the insulin secretagogue action of ELE *in-vivo* and *in-vitro* although, the insulin sensitizing action was only studied with streptozotocin induced diabetes model [25]. As reviewed by Abirami *et al.,* ELE possess hypoglycaemic activity on STZ induced type I DM in rats, and it was believed that ELE increased the insulin secretion [26]. In contrast, findings in the current study indicated reduced insulin levels and prevented hyperinsulinemia and improved dexamethasone induced IR. The prominent insulin sensitizing actions of ELE can be ascribed to the subsequent statements. The basis for positive effects of ELE was put forward by Vaidya *et al*. The presence of major constituent swertiamarin in aqueous, n-butanol and ethyl acetate fractions of ELE, may be responsible for its antidiabetic activity [27]. In support, the findings from the OGTT appeared as a decrease in AUC of glucose and insulin values in type II DM treated groups and improved IS escalated exceptionally with the ELE treatment [25].

The glycosuria and ketonuria were recorded and assessed against dexamethasone induced steroid diabetes model. The results were very encouraging with ELE since the glycosuria and ketonuria were absent in both the groups as it effectively prevented their development. The basis for these positive findings with ELE can be imputed to the enhancement in glucose uptake into tissues which were evidenced by improved Gutt and Matsuda Indices for peripheral and whole-body insulin resistance respectively. Besides, ELE also showed marked correction in DI which may also contribute to the reduction in dexamethasone induced glycosuria and ketonuria.

Conclusion:

ELE 3.5 g/kg has comparable efficacy with that of metformin as evidenced by prevention in the elevation of serum glucose, insulin and surrogate indices against dexamethasone induced insulin resistance. Further, the isolation of its active constituent swertiamarin and its efficacy against steroid induced insulin resistance may give a lead for new potential insulin sensitizer.

Limitations:

This study might require a comparison with gold standard hyperinsulinemic euglycemic clamp method for measuring insulin resistance which we did not perform at that point.

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