Original Article

Protective and Curative Effect of *Scoparia dulcis* Leave Extract Against Free Fatty Acid Induced Insulin Resistance in Rat L6 Myotubes

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ABSTRACT

Objective: The objective of the present study was to investigate the insulin sensitizing effect of *Scoparia dulcis* (L) leave extract (SDE) against free fatty acid (FFA) induced insulin resistance in an *in vitro* L6 myotubes.
Materials and Methods: The rat L6 skeletal muscle cells were differentiated to myotubes by treating it with DMEM containing 2% horse serum for 12h. For generating an *in vitro* cellular model of insulin resistant condition, L6 myotubes were treated with FFA (palmitate) for 4h. Insulin resistant L6 myotubes were either pre- or post treated for 1h with SDE and its insulin sensitivity activity was assessed by measuring cellular glucose uptake and the activation status of insulin signalling pathway molecules.
Results: SDE significantly stimulated glucose uptake in L6 myotubes in a dose-dependent manner with maximal effect at 50µg/ml. To investigate the underlying mechanism of effect SDE, we examined the expression and activity of insulin signalling pathway molecules. We found that the SDE treatment notably increased insulin signalling pathway by inducing activatory phosphorylation status of IRS-1 and Akt without altering expression levels of these proteins. The comparative analysis revealed that SDE is more potent than known insulin sensitizer, pioglitazone.
Conclusion: These results suggest that induction of insulin signalling pathway and increased glucose uptake activity of SDE will aid in the treatment of insulin resistance and type 2 diabetes. Future studies on the isolation of bioactive components and a detailed investigation in the animal model of insulin resistance may be promising to find out a novel type 2 diabetes drug.

Keywords: FFA, Insulin resistance, L6 myotube, Glucose uptake,

Scoparia dulcis.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease and around 95% of diabetic patients are diagnosed with type 2 diabetes (T2D)[1]. Menacing increase of T2D is one of the major cause of morbidity and mortality of human beings worldwide and its increased annual rate indicates insufficiency of existing therapeutic options. The pathology of this disease is characterized by the presence of insulin resistance where target cells fail to respond normal circulatory level of insulin and that leads to the development of hyperglycemia[2]. Hence loss of insulin sensitivity in target tissues is the early sign in the progression of this disease which precedes and predicts the development of T2D.

It is now well established that free fatty acids (FFAs) are major player in promoting the loss of insulin sensitivity causing insulin resistance and T2D[3-5]. Several reports demonstrated that increased plasma FFA level contributes to the development of insulin resistance[3,4], while lowering of its level in diabetic subjects improves insulin sensitivity[6]. Incubation of skeletal muscle cells with FFA decreased insulin-stimulated glucose uptake[3]. Reports in this line suggest that FFA disrupts insulin signaling pathway in many facets in insulin target tissues and thus can adversely affect insulin resistance[7,8]. Insulin stimulated glucose uptake is mediated through the activation of insulin signalling pathway that leads to the translocation of glucose transporter, Glut4, from the interior to the cell surface[9]. Any defect in this pathway triggers the development of insulin resistance, which leads to the progression of future T2D. Therefore, amount of insulin stimulated glucose uptake into the peripheral tissues, particularly in skeletal muscle where 75% of postprandial glucose is deposited[10], is an important way to assess insulin sensitivity. Although insulin sensitizers thiazolidinedione group of drugs are effective in improving insulin sensitivity[11-13], however different adverse side effects limits their long term use[14-16]. Hence, the demand for new anti-diabetic compounds continues.

Medicinal plants constitute a common alternative treatment for T2D in many parts of the world[17-21] and that is the reason for thousands of years, plants and their derivatives are being used for treatment of T2D. We have selected *Scoparia dulcis* Linn. (Family - Scrophulariaceae), commonly known as sweet broom weed, in our investigation which has been used traditionally by the ayurvedic practitioners for the treatment of diabetes mellitus[22]. It is an erect perennial herb with serrated leaves and many small white flowers and mainly found in the tropical and subtropical regions. Its anti-diabetic activity has been explored in streptozotocin (STZ) treated animal model of type 1 diabetes (T1D)[23-25] which do not address the pathophysiology of insulin resistance and T2D. These studies only focused the glucose lowering effects of this plant; however their potential mechanism(s) of action have not been clearly elucidated at the molecular level. Since, insulin stimulated glucose uptake in skeletal muscle cells is important for maintaining glucose homeostasis and a decrease in the glucose uptake is one of the key signs of insulin resistance and T2D, the present study was conducted to evaluate the insulin sensitizing effect of *Scoparia dulcis* plant on skeletal muscle glucose uptake and insulin signalling pathway molecule activation.

MATERIALS AND METHODS

Reagents and antibodies

All cell culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, USA. Anti-IRS-1 (anti-rabbit), anti-pIRS-1 (Tyr-989, anti-goat), anti-pAkt (Ser 473, anti-rabbit), anti-pAkt (Thr-308, anti-rabbit), anti-Akt (anti-rabbit) and anti-β actin (anti-rabbit) antibodies were purchased from Santa Cruz Biotechnology Inc., California, USA. Alkaline phosphatase conjugated respective secondary antibodies were purchased from Sigma Chemical Co., St. Louis MO, USA. Glucose uptake cell-based assay kit was purchased from Cayman Chemical Company, Ann Arbor, MI, USA. All other chemicals and reagents used were either purchased from Himedia, Mumbai, India or Sigma Chemical Co., St. Louis MO, USA.

Collection of plant leaves and preparation of extract

The *Scoparia dulcis* plant was collected from Tezpur, Assam, India in 2013 and was authenticated by a competent plant taxonomist, Dr. Dipanwita Banik, CSIR-North East Institute of Science & Technology (NEIST), Jorhat, Assam, India and a voucher specimen was preserved in our laboratory. For preparation of extract, the leaves were washed, shade dried and crushed in a grinder and then mixed with ethanol and kept at 150rpm in an orbital shaker for 24h. The mixture was then filtered through Whatman No.1 filter paper and the filtrate was condensed in rota evaporator and was used for the present investigation.

Cell culture

L6 skeletal muscle cell line was procured from the National Centre for Cell Science, Pune, India and Prof. Samir Bhattacharya, Visva - Bharati University, West Bengal, India and was cultured in a similar manner as described by us previously[26]. Briefly, L6 skeletal muscle cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100U/ml) and streptomycin (100µg/ml) in a humidified 5% CO_2 atmosphere at 37°C. Approximately $1x10^6$ cells were plated in each well of the 6-well culture plate and when cells reached 75% confluency the medium was changed to DMEM supplemented with 2% horse serum. After 24h of culture in this medium all the cells were differentiated to myotubes which was used in our experiments.

Cell treatments

Palmitate (FFA) was conjugated with FFA-free bovine serum albumin following the method as described previously[27]. Briefly, palmitate was dissolved in ethanol and diluted 1:100 in 1% FBS-DMEM containing 5% (w/v) bovine serum albumin. Confluent L6 myotubes were incubated for 4h without or with 0.75 mM palmitate. For treatment with the plant extract, cells were either pre- or post-treated for 1h. On termination of incubations, cells were washed twice with ice-cold calcium-magnesium free PBS and harvested with trypsin (0.25%)–EDTA (0.5mM). Cell pellets were resuspended in lysis buffer, vortexed in every 10mins for 30mins and centrifuged for 10min at 10,000rpm at 4°C. Protein concentrations were determined following the method of Lowry *et al* (1951)[28].

Glucose uptake assay

2-NBDG (2-deoxy-2-[(7-nitro-2, 1, 3-benzoxadiazol-4-yl) amino]-D glucose) uptake assay was performed by following a previously described method[29]. Briefly, L6 myotubes ($1x10^6$ cells/ml) were serum starved overnight in glucose free DMEM supplemented with 2% FBS and then treated

without or with plant extract for 1h followed by 4h palmitate (0.75mM) incubation. Cells were then treated with or without 100nM of porcine insulin for 30 min and 10 min prior to the termination of experiment 2-NBDG (100µg/ml) was added to each of the incubations. Cellular uptake of 2-NBDG was measured using a fluorometer at excitation and emission wavelengths of 485 and 535 nm, respectively.

Immunoblotting

Immunoblot analysis was performed by following the method described previously[26]. Briefly, cell lysates (60µg protein) were subjected to 10% SDS/PAGE and transferred on to Immobilon-P PVDF membranes (Millipore, Bedford, MA) with the help of Semi-Dry trans-blot apparatus (GE Healthcare). Membranes were first blocked with 10% non-fat dried milk in TBST buffer followed by probing with primary antibodies (pAkt^{Ser473}, pAkt^{Thr308}, Akt, pIRS-1^{Tyr989}, IRS-1) and subsequently detected using ALP (alkaline phosphatease)-conjugated goat anti-rabbit IgG or mouse anti-goat IgG. The protein bands were visualized using 5-bromro 4-chloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

MTT Assay

The MTT assay was performed by following the method described previously[30]. In brief, L6 myotubes ($1x10^5$ cells/ml) were seeded into 96 well microtiter plates. The next day, medium replaced with DMEM containing SDE at various concentrations and incubated for 24h. After 24h, media was removed and replaced with complete media. MTT (20µl of 5 mg/ml in PBS) was added to each well of the plates and incubated further for 5h. The formazan crystals formed were dissolved in 100µl of acidic isopropanol (0.04 M HCl in absolute isopropanol) after aspirating the medium and incubated further for 30min at 37ºC. Cytotoxicity was measured spectrophotometrically at 570 nm with a microplate reader. Absorbance values were blanked against acidic isopropanol and the absorbance of cells exposed to medium only (without any treatment) were taken as 100% cell viability (control).

Statistical analysis

All data were derived from at least three independent experiments and statistical analyses were conducted using Sigma Plot 8.0 software. Data were analyzed by one-way analysis of variance (ANOVA), where the F value indicated significance, means were compared by a post hoc multiple range test. All values were means ± SEM. A level of $p<0.05$ was considered significant.

RESULTS

Effect of SDE on FFA induced insulin insensitivity

For searching anti-diabetic activity from medicinal plants of North-East India, we have selected *Scoparia dulcis* plant (Figure 1) based on the traditional knowledge in its use against diabetes mellitus. In order to observe the insulin sensitizing effect, *Scoparia dulcis* plant extract (SDE, 100µg/ml) was either pre- or post- incubated for 1h in L6 myotubes treated with FFA (palmitate, 0.75mM) for 4h in absence or presence of insulin (100nM) and 2-NBDG (100µg/ml). Insulin effected 3-fold induction of 2-NBDG uptake in comparison to control cells while FFA significantly inhibited insulin-stimulated 2-NBDG uptake. Both pre- and post-treatment of SDE showed considerable induction of glucose uptake in presence of FFA suggesting that its effect may be due to the suppression of FFA inhibition (Figure 2). SDE pre-incubation showed greater activity in preventing FFA induced reduction of insulin stimulated glucose

uptake by L6 myotubes in comparison to post-treatment (Figure 2). Since loss of insulin sensitivity by FFA that leads to insulin resistance is the major problem in T2D to be addressed, our assay system reflects the validity of such determination.

Dose dependent effect of SDE on insulin sensitivity

In order to observe the dose dependent effect of SDE, L6 myotubes were pre- or post- incubated for 1h with varied concentration of SDE (25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml). Cells were incubated with FFA (palmitate) for 4h without or with insulin in presence of 2-NBDG. Both pre- and post- incubation of SDE showed dose dependent suppression of FFA inhibition, peak of which was observed at 50µg/ml (Figure 3).

Effect of SDE on FFA induced impairment of insulin signaling

It is well known that stimulation of insulin signaling pathway leads to the glucose uptake in insulin target cells. Binding of insulin to its receptor causes phosphorylation of tyrosine residues that activates the signaling pathway. Phosphorylated insulin receptor tyrosine kinase recruits and phosphorylates insulin receptor substrate-1 (IRS-1) and amplifies the signal which leads to the activation of a key kinase, Akt/PKB, that regulates the movement of glucose transporter 4 (Glut4) from cytosol to the plasma membrane. Activation of Akt depends on the phosphorylation of its Thr308 and Ser473 residues which plays a critical role in insulin stimulated glucose uptake in skeletal muscle and adipose tissue. Several reports indicate that FFA mitigates insulin-stimulated glucose uptake in skeletal muscle cells through the deactivation of IRS-1 and Akt. To observe the IRS-1 and Akt phosphorylation status in response to SDE is therefore a highly relevant assay system to assess insulin activity. In order to observe whether FFA induced impairment of insulin signaling pathway could be prevented or waived by SDE, we incubated L6 myotubes without (Con) or with insulin (Ins) or insulin plus FFA (Ins + FFA) in absence or presence of pre- or post- SDE treatment. Insulin signaling was augmented by insulin as showing increased level of IRS-1 and Akt phosphorylation while addition of FFA notably attenuated this stimulation. SDE addition reversed FFA inhibitory effect on insulin signaling as enhanced level of phosphorylated IRS-1 and Akt were observed in FFA incubated pre- and post-SDE treated cells (Figure 4).

Comparison of SDE versus pioglitazone on insulin sensitizing effect

It is now well established that thiazolidinedione (TZD) group of drugs including pioglitazone, rosiglitazone showed significant insulin sensitizer effect on adipocytes, but so far, it has not been clarified whether TZD can induce insulin-sensitizing effect on skeletal muscle cells. Although TZDs are clinically very effective, but prolong treatment is found to be associated with serious side effects including congestive heart failure, bladder cancer, fluid retention, peripheral edema and weight gain. To compare the insulin sensitizing activity of SDE and TZD, we incubated L6 myotubes with either SDE (50µg/ml) or TZD (pioglitazone, 10µmol/l) for 1h and then treated without or with FFA (palmitate) for 4h in absence or presence of insulin and evaluated the effect of SDE and TZD on Akt phosphorylation in L6 myotubes. SDE showed significantly greater stimulation on insulin sensitivity than that seen with TZD as indicated by the level of Akt phosphorylation (Figure 5).

Cytotoxic Effect of SDE on L6 myotubes

To observe the cytotoxic effect of SDE, the L6 myotubes were treated for 24h with various concentrations of SDE. MTT result indicates that L6 myotubes incubated with various concentrations of SDE were safe and did not show any toxic effect on cell viability in comparison to control (Figure 6). This result suggests that it could be a viable option for future drug development against insulin resistance and T2D.

DISCUSSION

Free fatty acids (FFAs) play a key role in producing insulin resistance and type 2 diabetes (T2D). Although this disease is threatening the global health and spreading fast, there is yet no suitable medicine that addresses the disease properly. Lipid induced insulin resistance is in the center of pathogenesis of T2D and this problem remains yet unattended so far the available drugs are concerned. Insulin loses its sensitivity in T2D patients due to higher circulatory level of FFA[3-5] and removal of FFA from circulation improves insulin sensitivity[6]. Only thiazolidinediones (TZD) class of antidiabetic drugs can augment greater uptake of FFA into the adipocyte by activating its molecular target peroxisome proliferator-activated receptor gamma (PPARγ) and thereby preventing insulin resistance[31-34]. Recent clinical trial data suggest that long term use of TZD produces several adverse side effects such as congestive heart failure and development of edema[14-16]. Since there is no other drug that targets improvement of insulin sensitivity caused by FFA, requirement of alternative therapeutic choice becomes imminent.

Studies conducted over last several years have shown that plant based therapies have potential to provide relief in T2D without producing harmful side effects[17-21]. To find a viable alternative, we made an extensive search of plant extracts that address the problems of insulin resistance and T2D. Based on the traditional knowledge, we selected *Scoparia dulcis* leaf extract (SDE) in our investigation and observed strong insulin sentitizing activity. Although there are some reports on SDE's antihyperglycemic effect in STZ-induced T1D[23-25] but its effect on insulin resistance and T2D has not been explored. We have shown that both pre- and post- treatment of SDE improves insulin sensitivity in skeletal muscle cells which is lost due to FFA. SDE treatment in L6 myotube did not produce any toxic effect on cell viability. Taken together, SDE would be a potential therapeutic choice to deal with the problem of insulin insensitivity and T2D.

CONCLUSION

The present study showed therapeutic potential of *Scoparia dulcis* leave extract against insulin resistance which support its use in the traditional medicine for the treatment of T2D. Further interaction studies between the plant extract and insulin signalling pathway molecules are needed to identify the precise site of action. Since the ethanolic extract of *Scoparia dulcis* leaves could induce strong insulin sensitivity in skeletal muscle cells, the isolation and a detailed investigation with its bioactive components in different animal models of T2D may be promising to find out novel T2D drugs.

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Figure 1. Plant of *Scoparia dulcis* and its leaves used for extract preparation

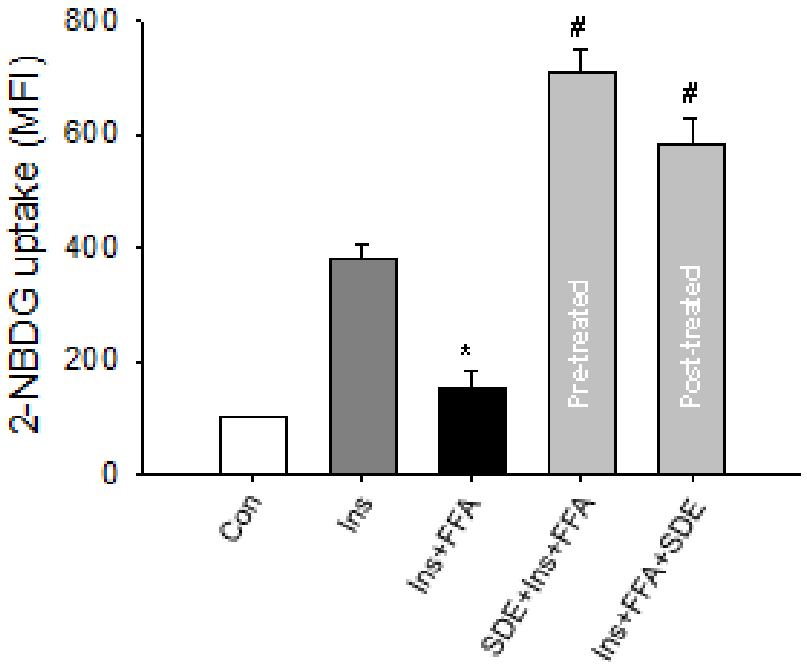

Figure 2. Preventive and curative effect of *Scoparia dulcis* plant extract (SDE) on insulin stimulated 2-NBDG uptake by L6 myotubes in response to the indicated conditions

Each value is the Mean ± SEM of three individual experiments. *p<0.01 vs. Ins, #p<0.001 vs. Ins + FFA.

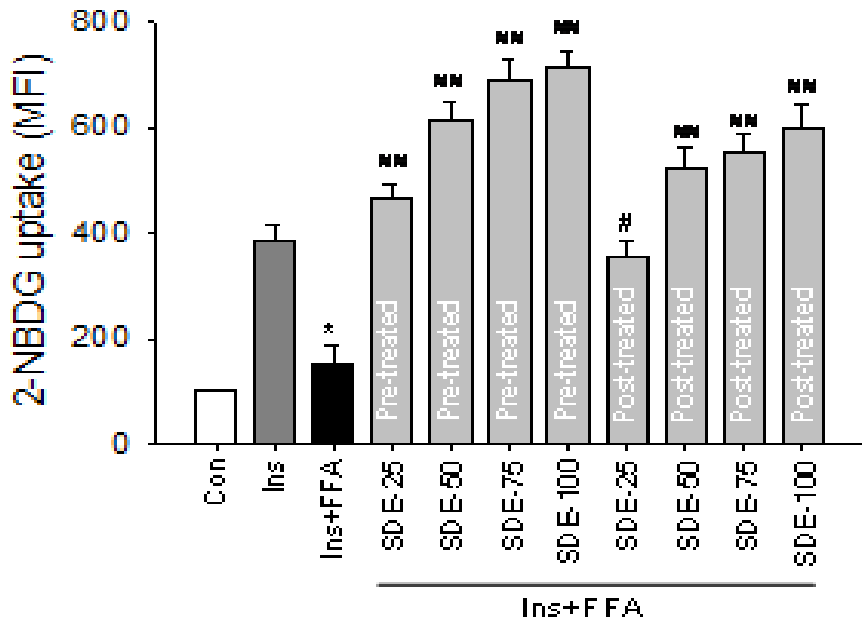

Figure 3. Dose dependent effect of *Scoparia dulcis* plant extract (SDE) on insulin stimulated 2-NBDG uptake by L6 myotubes in response to the indicated conditions

Each value is the Mean ± SEM of three individual experiments. *p<0.01 vs. Ins, #p<0.01, ##p<0.001 vs. Ins + FFA.

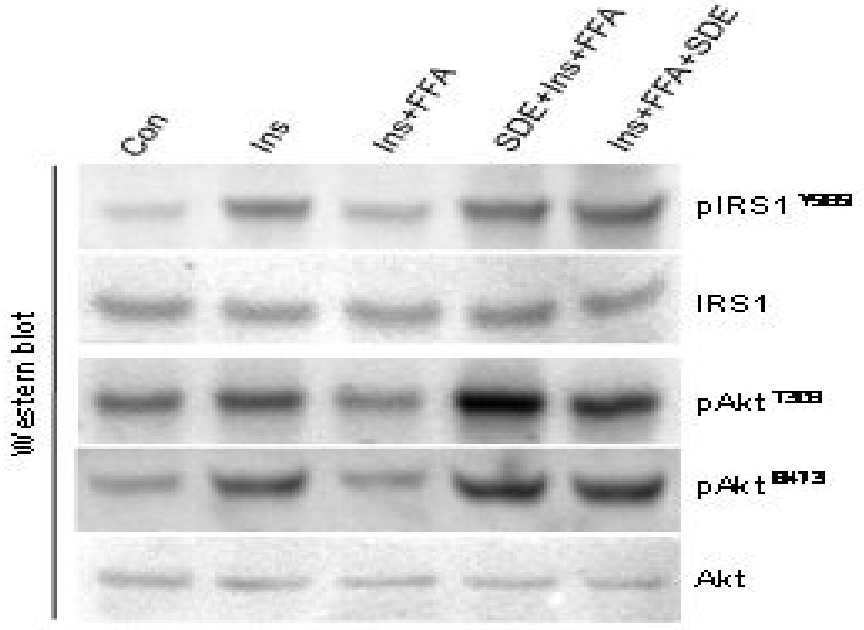

Figure 4. Effect of *Scoparia dulcis* plant extract (SDE) on IRS-1 and Akt phosphorylation. Western blot showing pIRS-1 (Tyr-989) and pAkt (Thr-308 and Ser-473) abundance in L6 myotubes incubated under the indicated conditions. Total IRS-1 and Akt were used as loading control

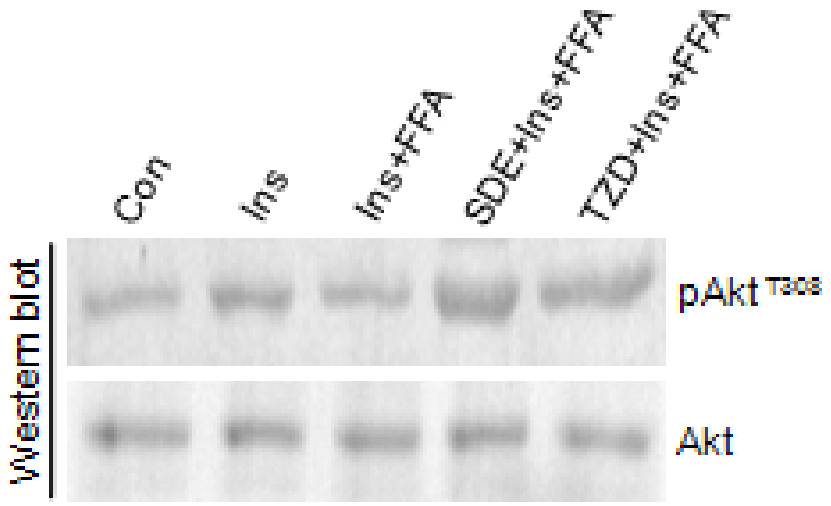

Figure 5. Akt activation status in *Scoparia dulcis* plant extract (SDE) and Pioglitazone treated L6 myotubes. Western blot showing phosphorylated and total Akt level in L6 myotubes incubated under the indicated conditions

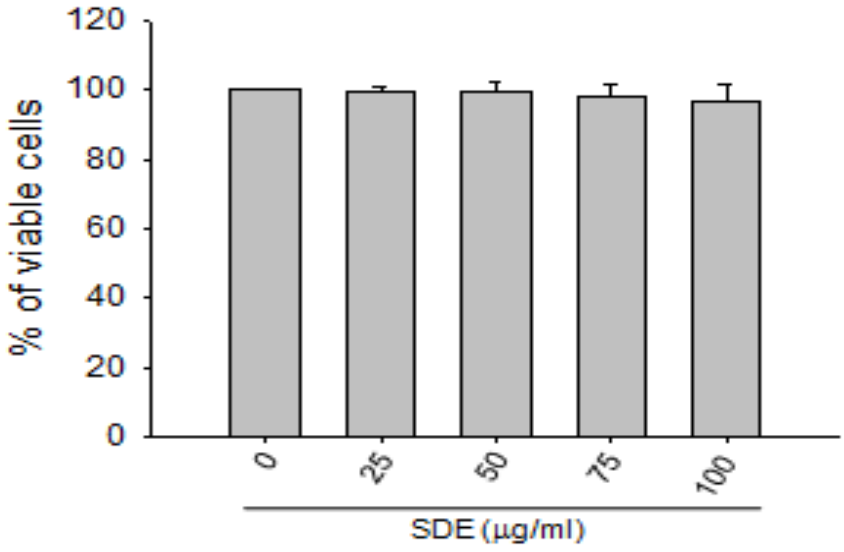

Figure 6. % of viable cells in response to indicated concentrations of *Scoparia dulcis* plant extract (SDE) treated L6 myotubes

Each value is the Mean ± SEM of three individual experiments.