POSSIBLE ANTIDIABETIC MECHANISM OF SALACIA OBLONGA IN DIABETIC SMOKERS: IN VITRO INVESTIGATION WITH RINm5F AND L6 CELL LINES

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ABSTRACT

Bidi smoking is the most common form of tobacco smoking in rural India. Tobacco burning during smoking generates tremendous amount of toxic “free radicals” that induce oxidative stress in the body. Antioxidants inhibit free radical generation and have been used against degenerative and inflammatory diseases. The present study investigates the in vitro cytoprotective and antioxidant potential of the methanolic-aqueous extract of Salacia oblonga (SOE) in preventing cell death induced by tobacco smoke concentrate (TSC). RINm5f (rat insulinoma cell line) and L6 (rat skeletal muscle cell line) cells were exposed to 0.5 - 5% TSC for 24 hrs. Analysis through MTT assay showed that 0.5% and 1% TSC caused almost 50% cell death. Pre-treatment of both the cell lines with 10 and 15 µg/ml SOE for 3 hrs showed cytoprotective effect against the TSC induced toxicity. Microscopic analysis of the treated and control groups confirmed the above findings. The plant extract showed reductive ability and superoxide radical scavenging activity in cell-free system as well. Thus, our study suggests that the antioxidant potential of SOE might be attributed to the observed cytoprotective role. Further investigations are needed to unveil the mechanism of action of SOE at the molecular level.

KEYWORDS: Bidi; tobacco; smoking; Salacia; RINm5f; L6.
INTRODUCTION
Cigarette smoking has been reported to have a dose-dependent relationship with the incidence of diabetes.[1] Type 2 diabetes depicts an inadequate insulin production by the pancreas or, inability of the body (like the peripheral tissues) to respond to the secreted insulin. High blood glucose level generates oxidative stress (OS) through formation of reactive oxygen species that alter several bio-molecules in the body.[2] Bidi (tobacco rolls) smoking is the most common form of tobacco smoking in India. Each puff of tobacco smoke contains approximately $10^{17}$ oxidant molecules.[3] This tremendous amount of harmful short and long-lived oxidants or free radicals generated from tobacco smoking also increases OS in the cells by attacking the proteins, DNA, lipid molecules and, their dysfunction leads to cell injury.[4] Besides this, hyperglycemia induced OS and activation of stress-sensitive pathways ads up to late complications of diabetes.[5] Several herbs have been explored for their antidiabetic potential as a better alternative to blood glucose lowering drugs. Among them, many species of Salacia have been used for thousands of years to treat diabetes. Salacia oblonga (Family: Celastraceae) is a woody climber distributed in Southern India and countries like Sri Lanka. It has been reported to possess antioxidant, anti-inflammatory and antidiabetic properties. [6] The roots, root bark and aerial parts of this plant have been extensively used in traditional medicine for the treatment of diabetes.[7] The present study aims to investigate the possible cytoprotective effects of SOE against OS induced by tobacco smoke in RINm5f (rat pancreatic) and L6 (rat skeletal muscle) cell lines, respectively. Further, the cellular microscopic analysis was carried out in SOE pre-treated group that was further exposed to toxic doses of TSC. The free radical scavenging potential of Salacia oblonga extract in a cell-free system has been analyzed through Reducing Power Assay and Superoxide Radical Scavenging activity assay. SOE was found to possess considerable antioxidant potential in an oxidatively stressed cellular environment.

MATERIALS AND METHODS
Chemicals required: Potassium ferricyanide, ferric chloride, trichloroacetic acid, gallic acid, ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were purchased from Central Drug House Pvt. Ltd., New Delhi, India. RPMI-1640 and DMEM were purchased from Sigma-Aldrich Pvt. Ltd., USA. Nitroblue tetrazolium (NBT), 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fetal bovine serum were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India.
Plant extract: The methanolic-aqueous extract (powder) of *Salacia oblonga* was purchased from Natural Remedies Pvt. Ltd., Bangalore, India.

Antioxidant activity measurement of *Salacia oblonga*: In our previous study, we have characterized SOE through various phytochemical analysis methods. Our data showed the presence of phenolics, flavonoids and tannins in the plant extract. Further, the *in vitro* antioxidant activity of the methanolic-aqueous extract of *Salacia oblonga* was analyzed through Reducing Power assay and Superoxide Radical scavenging activity assay.

Reducing Power assay: The Ferric Reducing Power of plant extract was carried out by FRAP method. 10 – 100 μg/ml of the plant extract was mixed with phosphate buffer (0.2M, pH 6.6) and 1% potassium ferricyanide, followed by incubation for 20 min at 50˚C. The reaction mixture was then rapidly cooled in ice and, 10% trichloroacetic acid was added in order to stop the reaction. The mixture was vortexed and centrifuged at 250 x g for 5 min. The supernatant was collected and 0.1% FeCl₃ was added, mixed and incubated for 30 min at room temperature. The absorbance of blue color solution obtained was measured at 700 nm and, the activity was reported as O.D. values in comparison to reference standard Gallic acid.

Superoxide Radical scavenging activity: The Superoxide Radical scavenging activity of the plant extract was determined as per standard method. Briefly, 20 – 200 μg/ml of the plant extract was mixed with phosphate buffer (20 mM, pH 7.6), EDTA (0.1M) and nitroblue tetrazolium (NBT, 57 µM). The reaction mixture was mixed and incubated for 10 min in dark at room temperature. This was followed by addition of 2 mM riboflavin for generation of superoxide anions, and the reaction mixture was further incubated for 15 min. The blue formazan thus formed was measured at 570 nm. The Superoxide Radical scavenging activity of the standard Ascorbic acid and the plant extract was calculated using the formula:

\[
\% \text{ Inhibition} = \left( \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \right) \times 100
\]

Cell lines and culture conditions: The RINm5f (rat pancreatic β cells) and L6 (rat skeletal muscle) cell lines were obtained from National Centre for Cell Sciences, Pune, India and cultured by standard method.\[11\]
Tobacco smoke concentrate (TSC) preparation: Branded bidi rolls (tobacco rolls used in rural India, brand not to be disclosed) were market purchased. The tobacco smoke concentrate (TSC) was prepared by passing the complete vapour phase of bidi smoke obtained from complete combustion of three bidis over 3 ml of incomplete medium in a compact set-up. The concentrate was made fresh for every experiment and used within 2-3 hrs for various experimental studies.

Effect of TSC on the viability of RINm5f and L6 cells: Cell viability was assessed by MTT {3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide} assay using a microplate reader (Bio-Rad, USA). 5 x 10^4 cells/well (RINm5f and L6 cells) were seeded in a 96-well cell culture plate and cultured overnight at 37ºC (5% CO₂ and humidified atmosphere) prior to treatment. The cells were exposed to TSC (0.5 – 5%) for 24 hrs in order to determine its toxic dose causing 50% cell death. The absorbance of the violet formazan formed was obtained at 570 nm and calculated for percentage cell viability in comparison to control.

Effect of SOE on TSC-induced cytotoxicity in RINm5f and L6 cells: In order to determine the safe dose range for SOE, both the cell lines were seeded and cultured as mentioned above. The overnight culture was incubated with SOE (5 – 75 μg/ml) for 3 hrs followed by determination of cell viability through MTT assay. Further, the cytoprotective effect of SOE in TSC-induced toxicity in RINm5f and L6 cells were determined through pre-treatment of cells with respective doses followed by TSC exposure. The results were analyzed from three independent experiments.

Morphological examination of SOE and TSC treated RINm5f and L6 cells: On exposure of RINm5f and L6 cells to 15 μg/ml SOE for 3 hrs, and 1% TSC for 24 hrs, individually and in combinations, the cell morphology was examined under an inverted microscope (20X magnification). Microphotographs were taken using a digital camera (Sanyo Electric Co. Ltd.).

Statistical analysis: All the data are expressed as mean ± Standard deviation (S.D.) of number of experiments (n = 3) and tested using one-way ANOVA. The values were considered significant at P < 0.01.
RESULTS

Reductive ability of *Salacia oblonga* and the standard Gallic acid

The reducing power of methanolic aqueous extract of *Salacia oblonga* (10 – 100 µg/ml) was found to increase with an increase in concentration. Similar effect was observed with the standard Gallic acid (Figure 1). Thus, a positive linear relationship in the reductive ability of both was obtained.

![Figure 1: Reductive ability of *Salacia oblonga* extract and the standard Gallic acid. The data represent the absorbance obtained due to reduction of Fe$^{3+}$ to Fe$^{2+}$ by each of them. All data are expressed as mean ± SD (n = 3). A positive linear relationship in reductive ability was observed between the plant extract and the standard](image1)

Figure 2: Superoxide Radical Scavenging activity of *Salacia oblonga* extract and the standard Ascorbic acid. The data represent the percentage inhibition on superoxide radicals. All data are expressed as mean ± SD (n = 3). The plant extract showed an IC$_{50}$ value of 191 µg/ml and that of standard was found to be 115 µg/ml

![Figure 2: Superoxide Radical Scavenging activity of *Salacia oblonga* extract and the standard Ascorbic acid. The data represent the percentage inhibition on superoxide radicals. All data are expressed as mean ± SD (n = 3). The plant extract showed an IC$_{50}$ value of 191 µg/ml and that of standard was found to be 115 µg/ml](image2)
Superoxide Radical scavenging activity of *Salacia oblonga* and standard Ascorbic acid

As shown in Figure 2, decrease in absorbance at 570 nm with an increase in concentration of plant extract indicates an increase in consumption of superoxide generated in the reaction mixture and hence, an increasing Superoxide Radical scavenging ability of the plant extract. The plant extract exhibited an IC\textsubscript{50} value of 191 µg/ml and the standard Ascorbic acid exhibited an IC\textsubscript{50} value of 115 µg/ml.

**Effect of SOE and TSC on cell viability of RINm5f and L6 cells**

To analyze the safe dose range of SOE for RINm5f and L6 cells, both the cell lines were treated with SOE (5 – 75 µg/ml) for 3 hrs and the number of viable cells was assessed. As shown in Figure 3, treatment of RINm5f cells with SOE up to 20 µg/ml dose brought about an increase in cell viability more than the control. Further increase in dose to 75 µg/ml decreased the cell viability to almost 64%. In contrast to pancreatic cells, a slight increase in cell viability was observed in L6 cells treated with SOE up to 15 µg/ml. Further increase in dose to 75 µg/ml did not show any significant alteration in cell viability of rat skeletal muscle cells, when compared to control. This study shows that pancreatic cells are more sensitive to SOE and these cells could attain higher cell viability than L6 cell line in comparison to control. The safe dose range of 15 – 20 µg/ml SOE was considered for further study in both the cell lines.

![Figure 3: Effect of SOE on the viability of RINm5f and L6 cells. The cells were treated with SOE (5 - 75 µg/ml, 3 hrs) and the cell viability was assessed through MTT assay. Data were presented as mean ± S.D. of three experiments (no statistical significance was observed between the treated and control groups).](image-url)
However, exposure of both types of cell lines to 0.5 - 5% TSC (Figure 4) for 24 hrs brought about a significant decrease in cell viability, with 5% TSC treatment causing almost 70% and 58% cell death in RINm5f and L6 cells respectively, in comparison to control. Thus, 15 and 20 μg/ml SOE (plant extract) and, 0.5% and 1% TSC were chosen for further studies. Similar to SOE, pancreatic cells were more sensitive than the skeletal muscle cells to tobacco smoke.

![Figure 4: Effect of TSC on the viability of RINm5f and L6 cells.](image)

**Figure 4: Effect of TSC on the viability of RINm5f and L6 cells.** The cells were treated with TSC (0.5 - 5%, 24 hrs) and the cell viability was assessed through MTT assay. The results were expressed as percentage of control value from untreated group. Data were presented as mean ± S.D. of three experiments. (* P < 0.01 versus their respective Control). Increase in TSC dose showed a significant decrease in cell viability in both the cell lines.

**Effect of SOE on TSC-induced changes in cell viability of RINm5f and L6 cells**

As shown in Figure 5, the RINm5f and L6 cells were pre-exposed to SOE and then exposed to TSC in order to investigate the cytoprotective effect of SOE under induced cellular toxicity. 15 and 20 μg/ml SOE exerted significant protection against 0.5% and 1% TSC-induced cytotoxicity in L6 and RINm5f cells. Thus, both these safe doses of SOE regained the cell viability of TSC treated cells and, the effect was statistically significant with respect to TSC treated groups. We also observed that SOE showed better potential in overcoming TSC-induced cytotoxicity in rat skeletal muscle cells in contrast to rat pancreatic β cells.
Figure 5: Effect of SOE on TSC-induced cytotoxicity in RINm5f and L6 cells. The cells were pre-treated with SOE (15 and 20 μg/ml) followed by exposure to TSC (0.5% and 1%). Cell viability was determined through MTT assay and the data were presented as mean ± S.D. of three independent experiments. (* P < 0.01 versus 0.5% TSC; † P < 0.01 versus 1% TSC). An increase in cell viability almost near to control was observed in all the SOE-treated groups in both the cell lines.

Morphological analysis of TSC-induced cytotoxicity in RINm5f and L6 cells and its protection with SOE pre-treatment

Present study is a comparative analysis of effect of treatment with TSC and SOE, individually and in combinations, on the cellular morphology of rat pancreatic and rat skeletal muscle cells. The inverted microscopic analysis showed the cells in the control group to be confluent and closely adhered to the substratum (Figure 6A and 6a). Treatment with 1% TSC resulted in altered cellular morphology and nuclear morphological changes in both the cell lines, but the effect was observed more in RINm5f cells (Figure 6B). Both the treated groups showed decrease in cell number. The TSC exposed RINm5f cells started rounding up with cell swelling and formation of cytoplasmic vacuoles (Figure 6B, white arrow) and nuclear condensation (Figure 6B, black arrow). Besides these, they also showed cell membrane blebbing (Figure 6B, red arrow) and few cells with typical crescent-like appearance, all indicating towards a combined necrotic and late apoptotic cellular changes. On the other hand, the L6 cells exposed to 1% TSC showed abnormal constriction of cellular extensions and shrinking of cells (Figure 6b, white arrow) with condensed nuclei (Figure 6b, black arrow), and few cells with complete loss of epithelial morphology. Treatment with 15 μg/ml SOE (Figure 6C and 6c) showed cell number and morphology similar to control. Besides this,
Figure 6D and 6d showed that SOE pre-treatment helped both the cell lines to overcome the toxic effects of TSC and, the effect was comparable to control. Thus, 15 µg/ml SOE could overcome the toxic effects of TSC treatment in both the cell lines.

Figure 6: Morphological analysis of RINm5f and L6 cells in control and TSC, SOE, and their various combinations (1% TSC for 24 hrs, 15 µg/ml SOE for 3 hrs, and their combinations) exposed groups. After incubation for respective time period, the cells were observed under inverted microscope (20X magnification). B and b: In the above photomicrographs, condensed nuclei and shrunken cytoplasm of the cells are indicated.
by black arrow, whereas intense cytoplasmic vacuolization in RINm5f cells and shrunken cytoplasm are visualized in L6 cells. C and c: The appearance of cells is near to control (A and a). D and d: The SOE pre-exposed cells further treated with TSC are visualized as near to control. Identical results were observed in 4 separate experiments.

DISCUSSION AND CONCLUSION

It has been reported that processed unburnt tobacco contains 3000 compounds and 30 carcinogens whereas, smoke generated from burnt tobacco contains approximately $10^{17}$ oxidant molecules.\textsuperscript{[3]} Both mainstream and sidestream smoke contain harmful proportion of carcinogens like polynuclear aromatic hydrocarbons (PAH), tobacco specific nitrosamines etc., that are reported to bring about drastic changes in several organs of the body including the pancreas, through generation of OS. OS affects the glucose uptake and utilizing ability of cells. Skeletal muscle is responsible for utilization of about 80% of total glucose under insulin-stimulated conditions.\textsuperscript{[13]} An alteration in metabolic functioning of pancreas due to OS can bring about multi-pathway alterations in functioning of several key organs related to it, and skeletal muscle is one of them. Our attempt was to investigate the possible toxic effects arising in TSC-induced oxidatively stressed condition in both RINm5f and L6 cell line. Increase in OS has already been found to be associated with most of the metabolic pathways of pancreatic cells, thus bringing about defect in cellular metabolism, gene expression, insulin production and secretion.\textsuperscript{[14]}

A key finding of the present study is the observation of a significant disturbance in cell viability as well as cell morphology in pancreatic β cells due to their exposure to toxic doses of TSC. Our similar observations in skeletal muscle exposed to tobacco smoke might indicate towards expression of cell death markers in association with increase in OS and cellular toxicity. The metabolic activity of skeletal muscle is also reported to be affected.\textsuperscript{[15]} In this study, we have investigated the two cell lines seperately but it may hold that once the pancreatic and skeletal muscle activities are affected, with lack of insulin, it may further affect skeletal muscle and deteriorate the glucose uptake and other functionality of the diabetic/non-diabetic smoker. If, these observations are found to be true, it is suggested that, this kind of exposure to tobacco smoking may initiate diabetic condition and, may further deteriorate diabetes and its complications as well.\textsuperscript{[16]} Though the toxic effect of TSC on skeletal muscle cells cannot be ignored yet, both the type of cellular changes thus observed, might indicate towards a synergistic crosstalk between these organs in bidi smokers. To deal with such kind of situation, herbal or synthetic products have been analyzed. In the present study, a well known plant for its antidiabetic properties has been investigated.
Several *in vivo* and clinical trials have proven the antidiabetic potential of the genus *Salacia*.\cite{17} SO has antioxidant principles such as phenolic and vitamin compounds which have a positive role in controlling oxidative stress.\cite{8,18} While testing its biological activity in *in vitro* condition with RINm5f and L6, it was found that SOE maintained the cell number and morphology of both the cell lines, similar to control group. So, this is a preliminary study wherein SO was found to increase the cell number and, regain the cell viability and morphology of pancreatic β cells as well as skeletal muscle cells from the toxic effects of TSC. In conclusion, our results indicate towards methanolic-aqueous extract of *Salacia oblonga* to possess the ability to ameliorate the changes in morphology and cell population in TSC-induced OS. Hence, if the cell morphology and number are maintained, the cellular function may also be normal. This study also proves the antioxidant potential of *Salacia oblonga* and its role in overcoming the toxic effects of tobacco smoke. The findings of this study may help in further elucidation of protective mechanism of SO to pancreas/skeletal muscle in TSC-induced toxicity.

**REFERENCES**


