

# **EVALUATION OF PROTECTIVE ROLE OF *SALACIA OBLONGA* AND MANGIFERIN IN TOBACCO SMOKE AND GLUCOSE-INDUCED TOXIC CONDITIONS**

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**DOCTOR OF PHILOSOPHY**

By

**SUJATA BASU**



**Department of Biotechnology**

JAYPEE INSTITUTE OF INFORMATION TECHNOLOGY  
(Declared Deemed to be University U/S 3 of UGC Act)  
A-10, SECTOR-62, NOIDA, INDIA

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## ABSTRACT

“Tobacco smoking” and “high blood glucose level” are two of the widely explored toxic agents inducing oxidative stress (OS) in cells of various organs/tissues of the body including: skeletal muscles, pancreas, kidney and nervous tissue etc., thereby worsening the condition of diabetes (a multi-targeted disorder) and post diabetic complications. Present study is an attempt to evaluate the cytoprotective efficacy, molecular mechanism, and antiapoptotic properties of methanolic-aqueous extract of the plant *Salacia oblonga* (SOE) along with its active principle Mangiferin, against the toxicity caused by tobacco smoke concentrate (TSC) and high glucose (Glc), individually, and in combinations. This experimental plan was to mimic the situation of skeletal muscle in normal human and diabetic patient addicted to tobacco smoking. To achieve various objectives of this goal, L6 (rat skeletal muscle) cell line was exposed to these agents [0.5% and 1%TSC (24h); 10 mM and 30 mM Glc (24h); their combinations] and was examined for various parameters. The results have shown that tobacco smoke and/or high glucose could alter the redox state of the cells and led towards cell death. Exposure of L6 cells to these stressors resulted in significant production of superoxide ( $\cdot\text{O}_2^-$ ) [indicated through increase in % NADPH consumption from 22% (Control) to 90% (1%TSC), 98% (30 mM Glc) and complete consumption (1%TSC+30 mM Glc) and, nitric oxide ( $\cdot\text{NO}$ ) [0% (Control) to 64% (1%TSC), 95% (30 mM Glc) and 426% (1%TSC+30 mM Glc)] radicals, which damaged the membrane lipid bilayers at the whole cell and organelle (mitochondria) levels, increased the leakage of lactate dehydrogenase (LDH) from cells [0% (Control) to 312% (1%TSC), 310% (30 mM Glc) and 406% (1%TSC+30 mM Glc), caused DNA damage, and revealed both apoptotic and necrotic features. In response to the induced OS, an increase in Superoxide dismutase (SOD) [100% (Control) to 202% (1%TSC), 238% (30 mM Glc) and 270% (1%TSC+30 mM Glc)] enzyme activity and Glutathione (GSH) [100% (Control) to 142% (1%TSC), 457% (30 mM Glc) and 424% (1%TSC+30 mM Glc)] content was observed. These antioxidants are known to scavenge the  $\cdot\text{O}_2^-$  and  $\cdot\text{NO}$  radicals in the cells. On pre-treatment of cells with the optimized dose of SOE (15  $\mu\text{g}/\text{ml}$ , 3h) and Mangiferin (1 $\mu\text{M}$ , 3h) individually, minimal enhancement in SOD activity occurred but, significant increase in GSH content [142% (1%TSC) to 628% (1%TSC+10SOE) and 1332% (1%TSC+1 $\mu\text{M}$  Mang.); 273% (10 mM Glc) to 922% (10 mM Glc+1 $\mu\text{M}$  Mang.)] was observed in comparison to TSC and Glc treated cells. Further, on exposure of cells to increasing doses of TSC+Glc, the extent of cellular damage was exaggerated much more than their individual exposure. Pre-treatment with SOE or Mangiferin also prevented DNA damage

and upregulated the anti-apoptotic protein Bcl-X. The stressor-induced alteration in mitochondrial membrane permeability transition was prevented on pre-treating the cells with SOE and Mangiferin, thus preserving the mitochondrial structure and function, as observed. Optimized doses of Mangiferin showed better ability in maintaining the oxidant-antioxidant balance through modulation of the above parameters in comparison to SOE and prevented cellular damage and death to a better extent. Taken together, these findings suggest that, SOE and Mangiferin protects rat skeletal muscle cells against reactive oxygen and reactive nitrogen species (ROS/RNS) by enhancing the level of antioxidants: SOD and GSH, maintaining mitochondrial membrane permeability, preventing necrosis by maintaining plasma membrane integrity, and inhibiting the events of apoptosis through upregulation of Bcl-X. Thus, through the present study *Salacia oblonga* and Mangiferin are proven to be beneficial for scavenging free radicals and preventing cell death in tobacco smoke and glucose induced toxicity in skeletal muscle cells. Mangiferin is found to possess better beneficial effects than SOE. Thus, it is suggested that, Mangiferin can be further studied deeper to analyze its molecular mechanism, its specific receptors/targets and, can be developed further as formulation for the management of diabetes, especially for tobacco smokers.

# Thesis – Chapter 1

## INTRODUCTION

Tobacco smoking is a major risk factor for developing diabetes and is also reported to further deteriorate this disease condition [1]. The present study was undertaken to analyze the toxic effects of tobacco smoke and high glucose in normal and diabetic conditions in rat skeletal muscle cell line (L6) and to investigate if, the plant *Salacia oblonga* and its active constituent Mangiferin could prevent the toxicity caused by the above said stressors along with analyzing their mechanism of action.

### 1. Diabetes mellitus

#### 1.1 Diabetes mellitus and its types:

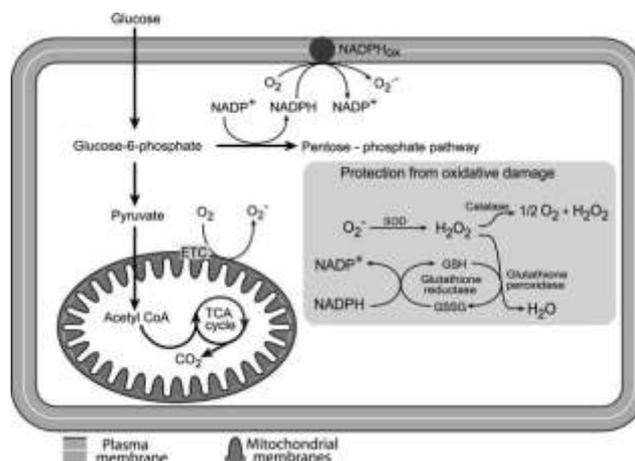
“Diabetes mellitus” is a metabolic disorder wherein the patient is unable to produce or utilize insulin properly in the body, thus leading to a state of chronic hyperglycemia [2]. It can be classified into three main types – Type 1 diabetes (insulin-dependent diabetes mellitus or IDDM), Type 2 diabetes (non-insulin dependent diabetes mellitus or NIDDM), and gestational diabetes. Among them, Type 2 diabetes can go unnoticed and undiagnosed for years. Multiple factors responsible for diabetes are: insulin resistance, impaired insulin secretion, reduced insulin-mediated glucose uptake and utilization, metabolic/physiological state of cells utilizing glucose, makes the treatment of Type 2 diabetes complicated with one drug - one target approach [3]. A multi target-oriented approach has to be obtained, which is difficult to attain with artificially synthesized, target-oriented single molecule based strategy [3]. As time passes, these hyperglycemic people develop late onset diabetic complications like retinopathy, neuropathy, nephropathy and microvascular diseases. In worst cases, all these might sometimes also result in development of blindness, loss of memory, kidney failure and gangrenes. This is largely responsible for increasing rate of morbidity and mortality of the diabetic patients worldwide [2].

#### 1.2 Statistical scenario:

An estimated worldwide figure of 143 million people is suffering from diabetes [3]. India ranks first in the list of top ten countries with highest number of estimated diabetes cases for 2000 (31.7 million), with an estimated rise of up to 79.4 million by 2030 [4].

### 1.3 Hyperglycemia and oxidative state of a biological system:

In a normal subject, glucose obtained from the breakdown of complex carbohydrates after food intake, gets absorbed into the blood stream through intestine, and is either stored as glycogen in liver (or fats in the adipose tissue) or utilized through several metabolic pathways for generation of ATP (the energy molecule). Glucose gets broken down through glycolysis pathway followed by tricarboxylic acid cycle that generates reducing equivalents (Figure 1). The latter enters the mitochondrial electron transport chain (ETC) for the production of ATP. During normal/abnormal mitochondrial ETC (Figure 1), reactive oxygen species (ROS) (mainly superoxide radical  $\cdot\text{O}_2^-$ ) are produced from molecular oxygen that creates a state of OS inside the cell. Presence of mitochondrial and cytoplasmic repair enzymes on their respective locations helps the cells in regaining the balance between the levels of oxidants and antioxidants (Figure 1) by inhibiting the initiation and propagation steps of free radical reactions [5].



**Figure 1:** Fate of glucose inside a cell and its relation to sites of Reactive Oxygen Species (ROS) production and the antioxidants in action [6]

During a chronic increase in blood glucose level, the mitochondria gets overloaded with pyruvate and this leads to generation of more and more  $\cdot\text{O}_2^-$  through the ETC [6-7]. This generates OS and further multiplies the events involved in development and progression of diabetes and its complications [7].

Besides these, in hyperglycemic patients, it has been proven that an increase in blood glucose level also triggers a non-enzymatic reaction between glucose and proteins, lipids as well as, nucleic acids, thereby producing “advanced glycation end products (AGEs) [7]. AGEs can attack several other proteins leading to increased ROS formation as well as, alteration of structure and function of antioxidant enzymes, and finally aggravating OS in diabetics [6].

#### **1.4 Tobacco smoking and hyperglycemia – the link:**

Tobacco smoking is reported to be one of the several agents responsible for the occurrence of diabetes. Scientific evidences analyzed through a literature survey of experimental studies available for the past 15 years on diabetes mellitus and tobacco smoking, strongly indicates towards a causal link between tobacco smoking and hyperglycemia [1, 8-9].

##### **1.4.1 Prevalence and habit of tobacco smoking:**

India is home to approximately 275 million tobacco users [10]. Today, numerous “smoking tobacco products” are available in the world market which includes cigarillos, cigars, bidis, and kreteks [11]. Bidi (India specific non-filtered hand-rolled cigarette) smoking is the most common form of tobacco smoking [11] in rural India, as well as among the low income category of urban Indian population. “Bidi” is a form of hand-rolled cigarette containing flaked tobacco that is secured inside a sun-dried Temburni or Tendu leaf with a cotton thread. The number of bidis smoked per day, duration of smoking and the age of initiation are some of the key factors that determine the mortality rate in a bidi smoking population [11].

##### **1.4.2 Chemistry of tobacco smoke:**

Cigarette smoke is composed of organic and inorganic compounds like ethylene oxide, acrylamide, carbon monoxide, nitrogen oxides and hydrogen cyanide [12]. Numerous types of free radicals and oxidants present in tobacco smoke are collectively called as “Reactive Oxygen Species” (ROS) and “Reactive Nitrogen Species” (RNS). Few of them include ROS like superoxide ( $\cdot\text{O}_2^-$ ), hydroxyl ( $\cdot\text{OH}$ ) and peroxy ( $\cdot\text{RO}_2$ ), and RNS like nitric oxide ( $\cdot\text{NO}$ ), nitrogen dioxide ( $\cdot\text{NO}_2$ ) and peroxy nitrite ( $\text{ONOO}^\cdot$ ) [13]. The toxic compounds of tobacco smoke as discussed above, gets absorbed into the blood stream from where they reach and enter into various organs of the body like pharynx, lungs, heart, pancreas, liver, kidney, skeletal muscles etc., thus causing toxicity in all exposed tissue and organs [14].

##### **1.4.3 Relation between tobacco smoking and Type 2 diabetes:**

Active smoking has been proven to be a risk factor associated with glucose intolerance, impaired fasting glucose, and Type 2 diabetes [1]. Tobacco smoking and hyperglycemia are two individual agents that have been reported to generate toxic amount of free radicals [7, 15] in the body. Cigarette smoke exposure has been reported to alter the muscle metabolic and functional stability by promoting muscle catabolism [16] through a large number of oxidants [17]. Skeletal muscle is the major site of glucose utilization in humans, accounting for 80% of

total body glucose uptake. Excess oxygen consumption during exercise might lead to production of increased amount of free radicals and their production is controlled to quite an extent, through an inbuilt protective mechanism of the skeletal muscle cells [5] in normal conditions. Heavy smoking additionally adds up to the number of ROS radicals in the skeletal muscle and disturbs the balance between oxidants and antioxidants. Moreover, the mitochondria-dependent oxidative capacity of skeletal muscle is directly linked with insulin sensitivity. The OS, thus generated due to tobacco smoking has been reported to induce insulin resistance in humans due to this interplay among various biochemical events including damaged mitochondrial genetic makeup, thereby affecting glucose metabolism [18].

### **1.5 A possible association between free radicals, antioxidants and stress associated signaling pathways - in non-diabetic smokers, diabetic non-smokers and diabetic smokers**

The free radicals present in/generated from tobacco smoke, along with nicotine brings about adverse effects on several organs of the body including the skeletal muscles [14]. Generation of free radicals in the cells due to high glucose (as discussed in section A3) has also been implicated in the onset of OS inside the cells. This ROS mediated induction of OS has been shown to interfere with several pathways including cell signaling pathways, apoptosis pathway and gene expression as well [7] thus, triggering a vicious cycle of OS, in several pathological conditions.

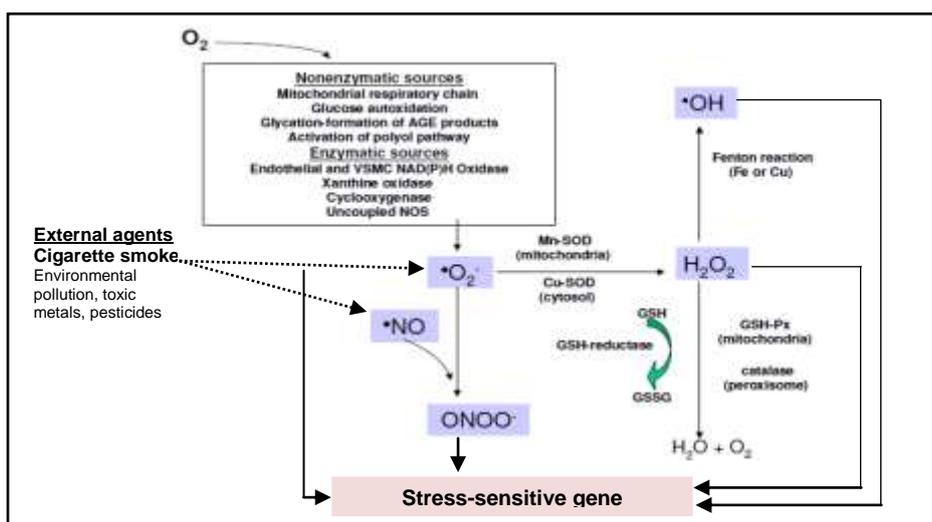
Both tobacco smoking and hyperglycemia are known to generate OS inside the cells of the body (Figure 2) through:

Enzymatic mode of action:

- Increase in enzyme activities: nitric oxide synthase (NOS), NADPH oxidase and Xanthine oxidase that produces  $\cdot\text{O}_2^-$  radical,
- Alteration of mitochondrial respiratory chain function (as discussed in A3).

Non enzymatic mode of action:

- Increase in nonenzymatic glycosylation,
- Glucose autoxidation,
- Activation of polyol pathway.



**Figure 2:** Various pathways leading to generation of highly reactive free radicals (highlighted in grey) in diabetes. Oxygen gets converted to superoxide via activated enzymatic and non-enzymatic pathways. It is then dismutated to  $H_2O_2$  by Superoxide dismutase (SOD) and, finally converted to  $H_2O$  by Catalase and Glutathione peroxidase (GSH-Px).  $H_2O_2$  can also get converted to hydroxyl radical ( $\cdot OH$ ) through Fenton reaction. GSH is regenerated by Glutathione reductase. Also,  $\cdot O_2^-$  reacts rapidly with  $\cdot NO$  to form  $ONOO^-$ .

### 1.5.1 Nitric oxide radical synthesis pathway:

The  $\cdot NO$  radical obtained from nitrogen dioxide present in cigarette smoke reacts rapidly with peroxy radicals to form alkoxyl radicals, thereby triggering lipid peroxidation in cells. This alters the structure and fluidity of biological membranes, and affects their functional capacity [7].

While analyzing the role of ROS in altered gene expression, scientists have reported that  $ONOO^-$  radical mediates activation of nuclear transcription factor (NF)- $\kappa B$  which further increases  $\cdot NO$  formation [5] and the cycle continues. Several reports have also indicated the upregulation of Nox isoforms Nox1 and Nox2 in diabetes [6]. Thus, an overload of ROS and RNS along with an absence/lack of endogenous antioxidant compensatory mechanism to abolish them, leads to activation of several other stress-sensitive intracellular signaling pathways by  $\cdot O_2^-$  and  $\cdot NO$  and other ROS/RNS [6-7].

**1.5.2 Superoxide radical and the mitochondria:** During hyperglycemia, NADPH oxidase and mitochondrial respiratory chain plays a key role in generating  $\cdot O_2^-$  radicals [7]. Studies have shown the existence of cross talk between mitochondria and NADPH oxidase [19]. The  $\cdot O_2^-$  radicals thus generated, gets scavenged by the antioxidant enzyme Superoxide dismutase (SOD) at both the locations, in cytoplasm as well as, in mitochondria. The activity of this enzyme has been found to have variations in the results obtained by various scientists wherein it was decreased or increased or showed no change in several diabetic models [7].

During attack by free radicals like  $\cdot\text{O}_2^-$  and  $\cdot\text{NO}$ , occurrence of loss of mitochondrial membrane integrity leading to altered membrane phospholipid structure, mainly Cardiolipin, opening of mitochondrial permeability transition pore which leads to release of cytochrome c has been observed. All these events trigger apoptosis and finally cell death [20].

Apoptotic pathways in skeletal muscle involve activation of initiator and effector caspases during maintenance of a balance between pro- and anti-apoptotic proteins [21]. Besides this, an altered functional ability of mitochondria is found to be linked with skeletal muscle insulin resistance [22]. Hence, in Type 2 diabetics exhibiting characteristic insulin resistance, there is a definite occurrence of several defects in mitochondria. The OS also activates serine/threonine kinase cascades that further phosphorylate the insulin receptor (IR) as well as, the family of IR substrate (IRS) proteins [6, 18], leading to insulin resistance and defective glucose uptake in cells.

### **1.6 Diabetes and Ayurveda (anti-hyperglycemic medicinal plants)**

Since the oral diabetes medicines carry numerous long term side effects, a vast majority of anti-diabetic Ayurvedic formulations are being preferred all over the world. Being natural in origin, these herbs are believed to be safe, economical and so are becoming an alternative therapy or treatment for diabetes. “World ethnobotanical information about medicinal plants” reports about almost 800 plants used in the control of diabetes mellitus. Herbs are rich in various types of phytoconstituents which are found to be majorly responsible for different biological activities. These secondary metabolites majorly fall in the category of Phenolics, Flavonoids and Tannins. Majority of the phytochemicals in plants are known to capture the free radicals inside a biological system by donating hydrogen atoms or electrons, thus neutralizing them and decreasing the load of OS in cells [3, 23] but, decreasing OS is not the only way the phytoconstituents might work. There may be several other specific targets for each of the phytoconstituent of the plant through which they may help to treat a particular diseased condition.

Since, diabetes is a “multi target disease” and individual therapeutic agents of modern medicine fail to act on the overall impaired diabetic metabolic system; a better treatment approach considering medicinal plants and their formulations with multi-target action may seem to be a better solution. These types of phytocompounds from single or multiple herbs might have unique ability to control the blood glucose level near normal or completely under control, and there is a need to explore the mechanism of action of these therapeutic agents

having single or a combination of phytoconstituents. It has also been seen that even a single phytocompound obtained from the plants can have multiple therapeutic targets.

The plant chosen for the present study is *Salacia oblonga* which is a very well known antidiabetic plant. The following description justifies this choice for the present study.

**1.6.1 The plant - *Salacia oblonga*:** Among several herbs of medicinal importance, antidiabetic and antioxidant plant *Salacia oblonga* (SO) has been chosen for our research work.

- **Taxonomical and geographical distribution:** *Salacia oblonga* belongs to the family Celastraceae [24]. It is a woody climber with pale yellow and light brownish coloured branches. The plant is commonly known as “Saptachakra” in Hindi. It is widely distributed in India, Sri Lanka, China, Vietnam, Malaysia, Indonesia, Thailand, Brazil, and Western peninsula [25].
- **Chemical constituents:** Among several phytochemicals present in SO, the main active constituents include Salacinol, Kotalanol, Kotalagenin-16-acetate and Mangiferin [24].
- ***In vitro*, *in vivo* and clinical studies with plant/plant extract:** Results from various studies carried out on the glucose lowering, antioxidant potential and other related activities of different extracts of SO are as follows:
  - a. The petroleum ether extract of the root bark of SO has been reported to decrease the blood glucose level as well as, formation of lipid peroxides in streptozotocin-induced albino rats [26]. They also reported a significant decrease in formation of lipid peroxidation products and an increase in antioxidant enzymes: Superoxide dismutase, Catalase and Glutathione Peroxidase in the heart tissue of diabetic animals [27].
  - b. The glucose lowering effect of ethanol and water extract of SO (240 mg and 480 mg) in Type 2 diabetic patients after ingestion of a high carbohydrate meal was reported by Williams *et al.* [28]. The extract also significantly lowered the postprandial insulin response in patients.
  - c. The methanolic extract of roots and stems of SO dose-dependently decreased the high postprandial glucose level in hyperglycemic rats induced by maltose, sucrose or starch by inhibiting the intestinal  $\alpha$ -glucosidase activity [29].
  - d. The rat lens-derived aldose reductase activity has been reported to be inhibited by the ethyl acetate soluble portion of aqueous-methanolic extract of roots of SO [29].

This study also reported the  $\alpha$ -glucosidase inhibitory activity of water soluble portion of the extract in sucrose- and maltose-loaded rats.

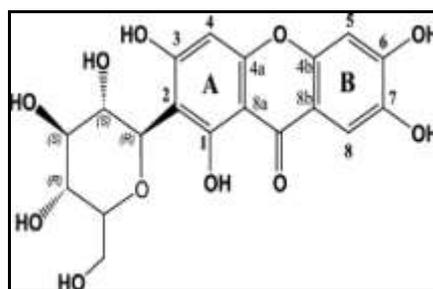
- e. The methanolic extract of root bark of SO has been found to prevent oxidative stress induced by the mutagenic agent cyclophosphamide in rats by elevating GSH level and decreasing lipid peroxidation [30].

The above reports suggest that the plant *Salacia* is a powerful antioxidant and is useful in controlling a diabetic condition. As diabetes is found to be linked with OS and, smoking is found to be linked with increased production of ROS as well as an increased risk of diabetes, it was postulated that, this plant may also be useful in conditions where tobacco smoke is the major cause for initiating or deteriorating this disease condition. No scientific evidence exists till date analyzing this plant for its antioxidant potential/protective potential in tobacco smoke and tobacco smoke+high glucose induced oxidatively stressed state in skeletal muscle model system, investigating its mechanism of action.

### 1.6.2 *In vitro* and *in vivo* studies with Mangiferin:

- **Chemical and physical properties**

Mangiferin is a polyphenolic compound ( $C_{19}H_{18}O_{11}$ ) and a glucoxanthone (C-2- $\beta$ -D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone; also known by the name C-glucosyl xanthone) (Figure 3) [31]. It is a white to light yellow powder with a molecular weight of 422.35 g/mol [32]. The melting point (anhydrous) of Mangiferin has been reported to be 271°C and it is a heat-stable molecule [31]. It is soluble in solvents such as DMSO, water and methanol.



**Figure 3:** Structure of the polyphenol Mangiferin

- **Biological activities**

In India, Mangiferin rich plants are widely used to treat immunodeficiency diseases like arthritis, diabetes, hepatitis, and cardiac disorders [31]. Several experimental evidences have reported the antioxidant and antidiabetic [31, 33], and anti-inflammatory [34] properties of Mangiferin. It has been found to induce synthesis of the antioxidant Glutathione (GSH) in an

oxidatively stressed cell [34]. Mangiferin decreased the blood glucose level by increasing the level of insulin as well as, activity of enzymes of pathways involved in glucose metabolism [35]. Mangiferin has also been reported to reduce the blood glucose level through its inhibitory action on glucose absorption from intestine of rats thus, proving its pancreatic and extra-pancreatic targets of action. In streptozotocin-induced diabetic rats, Mangiferin decreased the blood glucose and glycosylated hemoglobin levels, and it increased the levels of insulin and hemoglobin [35]. It has been reported to protect the D (+) galactosamine mediated oxidatively stressed kidney tissue in rats [33]. The structural make of Mangiferin makes it a highly bio-available molecule during oral administration [31].

On the basis of above description, the plant *Salacia oblonga* and its individual phytoconstituent Mangiferin, which has been known to have multiple targets of action in cells, have been chosen for the present study. The mechanism of action of the plant extract and Mangiferin, is yet to be explored in a wide manner. Hence, both the extract and this molecule was taken up for our study in order to investigate its efficiency to overcome OS and cell death induced by tobacco smoke and glucose and also to analyze the probable mechanism of action in rat skeletal muscle cell line (L6) model system.

## AIMS AND OBJECTIVES

**2. Aim of the study:** Aim of this study was to investigate and analyze the protective potential of the plant *Salacia oblonga* and its active constituent Mangiferin in tobacco smoke concentrate and glucose induced toxic conditions at cellular, organelle and molecular level in rat skeletal muscle (L6) cell line.

### 2.1 Objectives of the study:

- To characterize the Methanolic-aqueous extract of *Salacia oblonga*: biochemical, phytochemical, and antioxidant potential.
- To standardize the doses for *Salacia oblonga* plant extract, Mangiferin, tobacco smoke concentrate (TSC) and glucose for their safe and toxic ranges.
- To investigate if, *Salacia oblonga* extract and Mangiferin can protect the rat skeletal muscle cell line against the stress/toxicity generated by tobacco smoke and high glucose concentrations.

- To investigate the mechanism of action of *Salacia oblonga* extract or Mangiferin in protection at cellular, organelle and molecular level in oxidatively stressed rat skeletal muscle cells.

## **Thesis - Chapter 2**

Deals with literature survey on the disorder Diabetes mellitus and various antidiabetic medicinal plants, with core focus on *Salacia oblonga* and its active phytochemical Mangiferin.

## **Thesis – Chapter 3**

### **METHODOLOGY**

The phytochemical analysis of Methanolic-aqueous extract of SO was performed in order to characterize and standardize it before investigating the biological activity. This was followed by various biological assays (as mentioned below) that were performed in order to achieve the objectives of this study.

### **3.1 Characterization and standardization of *Salacia oblonga***

This was achieved at two levels of analysis:

#### **3.1.1 Qualitative phytochemical analysis**

- **TLC analysis** using Mangiferin as marker compound – by standard method [36].
- **Chemical and fluorescence analysis** – by standard methods [37].
- **Biochemical tests** for carbohydrates, sterols, terpenoids, alkaloids, phenolic compounds, flavonoids and tannins by standard methods [38].

#### **3.1.2 Quantitative phytochemical analysis**

- Determination of **total phenolic content** – by Folin-Ciocalteu method [39].
- Determination of **total flavonoid content** – by Aluminium Chloride colorimetric method [39].
- Determination of **total tannin content** – by standard method [40].
- Ultra High Performance Liquid Chromatographic (UPLC) analysis.

Further, four different extracts of SO namely Ethanolic, Chloroform, Petroleum ether and Aqueous extracts were prepared, characterized, and compared them with Methanolic extract through HPTLC for relative amount of Mangiferin in each extract.

**3.1.3 High Performance Thin Layer Chromatographic (HPTLC) analysis** using the marker compound Mangiferin.

**3.1.4 The antioxidant property of the Methanolic-aqueous extract of SO** was analyzed through:

- **Reductive ability** of SOE – by FRAP (Ferric-reducing antioxidant power) assay [39]
- **Hydrogen peroxide scavenging activity** – by standard method [39].
- **Superoxide radical scavenging activity** – by NBT (Nitroblue tetrazolium) assay [41].
- **Nitric oxide radical scavenging activity** – by standard method [39].

### **3.2 Preparation and Standardization of Tobacco Smoke Concentrate (TSC)**

a. **Preparation of TSC** – by the method of Lannan *et al.*[15] with slight modifications

b. **Standardization of TSC through:**

- **Spectrophotometric analysis** (O.D. at 260nm; Nicotine content in mg)
- **<sup>1</sup>H NMR analysis** using Nicotine as marker
- **UPLC analysis** using Nicotine as marker

### **3.3 Assessment of toxicity of TSC and high glucose and investigation of protective potential of SOE and Mangiferin against them**

A. **Effects of treatments with TSC, high glucose, TSC+glucose, SOE and Mangiferin, individually and in combinations on cell viability through:**

- a. **MTT assay** – Briefly,  $3 \times 10^5$  rat skeletal muscle cells were treated with different dose ranges (0.5-5% TSC, 10-70 mM Glucose, 5-50 µg/ml of SOE, 0.25-5 µM Mangiferin; SOE and Mangiferin for 3h, TSC and Glc for 24h). Cells in each well were then incubated with 10 µl MTT (Stock: 5 mg/ml) for 3-4h in a 5% CO<sub>2</sub> incubator, maintained at 37°C. 100 µl DMSO was added and the plate was incubated for another 10min in dark at room temperature. Finally, the treatment plate was read in an ELISA plate reader using 570nm filter [42].
- b. **Morphological analysis:** The effect of treatment with TSC, Glucose, SOE, Mangiferin and their combinations on the morphology of L6 cells were analyzed under an Inverted microscope.

c. **Analysis of DNA fragmentation** – To investigate if, the cause of cell death was apoptosis, DNA fragmentation assay was done by standard method [43]. Briefly, DNA was isolated from treated and untreated cells ( $5 \times 10^5$ ). An equal amount (1-5  $\mu$ l; approximately 200 ng) from each DNA sample was then electrophoresed on agarose gel (1% gel containing ethidium bromide) in TAE buffer. DNA ladder was loaded in the first well followed by DNA samples from different treatment groups. Once developed, the gel was taken out and placed on an UV illuminator and the pictures of the gel were captured.

**B. Effects of various treatments on cell membrane integrity of L6 cells through:**

a. **Lactate dehydrogenase (LDH) leakage assay** – In this method, 500  $\mu$ l sodium pyruvate (30 mM), 20  $\mu$ l NADH (6.6 mM), and 250  $\mu$ l Tris-HCl (0.2M, pH7.3) buffer were mixed and incubated at 25°C for 5min. 20  $\mu$ l supernatant from the different cell treatment groups were added to this reaction mixture separately, and the decrease in absorbance (340nm) over time was recorded for 30min [44].

b. Estimation of **lipid peroxidation by Thiobarbituric acid reactive substance (TBARS) assay** – The polyunsaturated fatty acids (PUFA; C<sub>16</sub>, C<sub>18</sub> and C<sub>20</sub> families) get readily oxidized by superoxides, thereby leading to a chain of lipid oxidation reactions which end in formation of MDA (malondialdehyde). In this method, cell lysate (50  $\mu$ g protein) obtained after respective treatments was incubated in 500  $\mu$ l of buffered medium (175 mM KCl and 10 mM Tris, pH7.4) at 25°C for 5min. After incubation, 50  $\mu$ l of this sample was mixed with 450  $\mu$ l TBARS reagent and heated at 80-90°C for 15min. The mixture was then cooled in ice and centrifuged. O.D. of the supernatant was measured at 535nm and the percentage MDA formed was calculated [45].

**C. Effect of various treatments on mitochondrial membrane integrity:**

a. **10N-nonyl acridine orange (NAO) staining** for analyzing the distribution of mitochondrial membrane phospholipid Cardiolipin – by standard method through fluorescence microscopy [46].

b. **Mitochondrial membrane permeability assay** – A sudden increase in concentration of calcium ions inside mitochondria due to increase in mitochondrial inner membrane permeability leads to mitochondrial swelling which was measured spectrophotometrically at 540nm [47].

**D. Study into the level of oxidative stress – whole cell analysis through:**

**a. Effect on oxidants:**

- **NADPH oxidase assay** – The plasma membrane-bound NADPH oxidase enzyme complex generates superoxide radicals during conversion of NADPH to NADP<sup>+</sup> and the latter is detected spectrophotometrically at 450nm [48].
- **Nitric oxide radical scavenging assay** – The interaction between nitric oxide (NO) and O<sub>2</sub> forms \*NO radicals that further get converted to nitrite ions. The latter forms an azo dye product with Griess reagent which is measured spectrophotometrically at 540nm [42].

**b. Effect on antioxidants:**

- Determination of non-enzymatic antioxidant status – **Glutathione (GSH) assay** – by standard method [47].
- Determination of enzymatic antioxidant status (**Superoxide dismutase - SOD**) through **NBT assay** – by standard method [49].

**E. Study into the level of oxidative stress – analysis at the organelle level through:**

**a. Effect on oxidants:**

- Determination of **nitric oxide production** in the cytoplasmic and mitochondrial fraction - (as mentioned above)

**b. Effect on antioxidants:**

- Determination of **SOD enzyme activity** by **NBT assay** in the cytoplasmic and mitochondrial fraction –(as mentioned above)

**F. Investigation of apoptosis through analysis of expression level of anti-apoptotic marker Bcl-X:**

- **RNA isolation** – by standard method [50].
- **Reverse transcription of RNA** – by cDNA synthesis kit method as per manufacturer's instructions.
- **Semi-quantitative RT-PCR** – by RT-PCR kit method using standard conditions of primers and as per manufacturer's instructions.

## Thesis – Chapter 4

### RESULTS

#### 4.1 Phytochemical characterization and standardization of *Salacia oblonga*

##### 4.1.1 Qualitative analysis:

- a. The phytochemical characterization of Methanolic-aqueous extract of SO through **Thin Layer Chromatography** showed the presence of Mangiferin in it.
- b. **Chemical and fluorescence analysis** of the plant extract showed colour change under visible and UV light, thus indicating the presence of organic and inorganic compounds with fluorescent properties.
- c. **Biochemical tests** carried out on the Methanolic-aqueous extract of SO showed the presence of Carbohydrates, Flavonoids, Tannins, Steroids and Triterpenoids, Phenolics in the Water, Ethanolic and Methanolic soluble portions.

##### 4.1.2 Quantitative analysis:

- a. Various quantitative analyses on the plant extract further confirmed the presence of high amount of Phenolic, Flavonoid and Tannin content.
- b. **UPLC analysis** confirmed the presence of Mangiferin in SOE.
- c. The **percentage yield of five different crude extracts of *Salacia oblonga*** were in the order of Petroleum ether > Water > Ethanol > Methanol > Chloroform extracts, respectively.
- d. **HPTLC analysis** of these five extracts revealed:
  - Numerous coloured, well-defined bands indicating the presence of numerous phytochemicals in *Salacia oblonga*.
  - The marker compound Mangiferin gave a peak with  $R_f$  value 0.12 in all the six lanes in the densitometric scan.
  - The Methanolic and Ethanolic extracts showed greatest similarity in band pattern, indicating the extraction of similar types of phytochemicals in almost equal quantities in both.
    - The **HPTLC chromatogram** showed the presence of Mangiferin in all the five extracts of SO in the concentration sequence: Methanolic > Ethanolic > Chloroform > Water > Petroleum ether, respectively.

Hence, the Methanolic-aqueous extract of *Salacia oblonga* was chosen for the study.

**4.1.3 Antioxidant property of SOE in cell-free system:** Study of **antioxidant potential of SOE** showed that the plant extract possess strong reductive ability as well as, H<sub>2</sub>O<sub>2</sub>, ·O<sub>2</sub><sup>-</sup>, and ·NO scavenging activities.

## **4.2 Investigation of protective ability of SOE and Mangiferin in L6 cell line: Dose optimization and evaluation**

### **4.2.1 Standardization of Tobacco Smoke Concentrate (TSC):**

a. TSC (100%) was prepared by us and its absorbance at 450 nm (O.D. range: 0.7-0.8) was noted.

b. TSC was then standardized through spectrophotometric, <sup>1</sup>HNMR and UPLC analysis with respect to its Nicotine (as marker) content.

**4.2.2 Effect of various treatments on cell viability: MTT assay** showed that 10, 15 and 20 µg/ml SOE and, 0.5 and 1 µM Mangiferin were safe to be used for treatment on this cell line. LD<sub>50</sub> doses for TSC and Glucose (Glc) were found to be 1%TSC and 30 mM, respectively. This toxic effect of TSC, Glc and their combinations was overcome by the above optimized safe doses of SOE and Mangiferin. Mangiferin showed better effect than SOE in above treatment groups.

**4.2.3 Morphological analysis** showed structural abnormalities in TSC- and glucose-treated (individually and in combination) cells thus, suggesting the occurrence of cell death, which was overcome by pre-treatment with SOE and Mangiferin in their respective sets of experiments.

**4.2.4 DNA integrity analysis:** Not much DNA fragmentation but smearing was observed in the samples treated with the toxic doses of the stressors, which indicates that cell death might have included the necrotic pathway.

**4.2.5 Plasma membrane integrity analysis:** Exposure of cells to toxic doses of TSC, Glc and their combinations increased the **LDH enzyme activity in the supernatant** and **lipid peroxidation** of the cellular lipids thus, confirming altered plasma membrane integrity. These effects were reversed by pre-treatment with SOE and Mangiferin, with the latter showing better effect.

### **4.2.6 Analysis of redox state of cells under various treatment conditions:**

a. **Effect on oxidants:** Under such “TSC and high glucose-induced” oxidatively stressed conditions, we found a significantly high level of **nitric oxide production** and increased **NADPH oxidase enzyme activity** at the whole cell as well as,

mitochondrial level. The effect was more pronounced in TSC+Glc treated groups. The antioxidant activity of SOE and Mangiferin was confirmed in pre-treatment groups through a significant decrease in nitrite production as well as, a decrease in percentage NADPH consumption.

- b. **Effect on antioxidants:** A rise in SOD enzyme activity and GSH content in TSC and high glucose-induced toxic conditions was observed. The decrease in  $\cdot\text{O}_2^-$  and  $\cdot\text{NO}$  radical production in L6 cells, as observed above, might be linked to further increase in activity of **SOD enzyme** and the content of **GSH**. In comparison to SOE, Mangiferin was found to be better in reducing the above toxic effects.

**4.2.7 Effect on mitochondrial membrane integrity:** TSC, Glc and their combinations-induced alteration in mitochondrial membrane integrity and permeability which was overcome on pre-treatment with SOE and Mangiferin, with the latter showing better effect.

**4.2.8 Analysis of expression level of anti-apoptotic marker Bcl-X:** An upregulation of anti-apoptotic protein Bcl-X in all the stressed conditions indicated the involvement of apoptotic pathways as well. So, it can be suggested that cell death observed in all these cases occurred through necro-apoptotic pathways.

## Thesis – Chapter 5

### DISCUSSION

Diabetes is a metabolic disorder characterized by hyperglycemia, wherein either inadequate production or utilization of insulin lead to high blood glucose level in patients. Tobacco smoking has been reported to be a major contributor to the incidence of diabetes. Both tobacco smoking as well as, hyperglycemia has been found to generate tremendous amount of free radicals that induce oxidative stress in cells of affected organs. Several medicinal plants as well as, their active compounds are being explored by various scientists for their antidiabetic and antioxidant potential.

We have chosen a traditionally well known antidiabetic plant *Salacia oblonga* for the present study. A literature survey (from the year 1966 to 2015) was carried out in order to analyze the particular extract of *Salacia oblonga* most studied for its potent antidiabetic and antioxidant potential in the available *in vitro*, *in vivo* and clinical studies. It was found that, besides the Ethanolic extract, Methanolic, Aqueous, and Methanolic-aqueous extracts of SO were studied majorly for analyzing their glucose lowering and antioxidant properties. In the current study,

characterization studies on Methanolic-aqueous extract of SO showed the presence of Mangiferin, as well as many other saturated and unsaturated compounds which might be responsible for the medicinal importance of SO. The plant extract also showed the presence of polyphenols such as; Phenolics, Flavonoids and Tannins which have been reported to act as free radical scavengers, by interfering with free radicals-induced oxidation of lipids and other biomolecules. These polyphenols have been reported to modulate the activity of a wide range of enzymes and cell receptors, thus affecting basic cellular functions like cell cycle, apoptosis etc. [23]. Nadagouda *et al.* [51] have reported the presence of 1.5% Mangiferin in Methanolic extract of root bark of *Salacia chinensis* through HPTLC method, and this is near to our obtained value of 1.2%. The polyphenols content showed statistically significant correlation with the antioxidant activity of SO.

Toxicity studies carried out on skeletal muscle have shown that cigarette smoke induces considerable oxidative damage in them [14, 17]. Besides this, in hyperglycemic patients, high glucose level has been reported to cause OS through formation of ROS. The latter interferes with the apoptotic pathway, gene expression and activates many cell signaling cascades [7]. There are similar indications from our present study wherein we observed a reduction in number of metabolically active functional cells (MTT assay) as well as, occurrence of symptoms of “apoptosis” (microscopic analysis) in them. It thus, indicates towards a halted proliferation pathway or triggering of a death pathway.

Various experimental studies have shown that exposure of cells to toxic agents for longer period lead to generation of free radicals (mainly  $\cdot\text{O}_2^-$ ) from molecular oxygen which then attacks the membrane lipid bilayer, thereby creating a superoxide radical mediated vicious chain reaction [5]. Under diabetic condition and in smokers, the  $\cdot\text{O}_2^-$  has been reported to increase the peroxynitrite and nitric oxide production, thus pushing the cell towards apoptosis [52]. Also, both nitric oxide synthase and NADPH oxidase are key generators of free radicals which modify cellular proteins and initiate redox signaling [53]. In our study, the high amount of OS generated by these stressors in cells was confirmed through increased nitric oxide production and an increase in NADPH oxidase enzyme activity, the latter being considered as an important contributor to OS in skeletal muscle [54]. Besides this, our study also showed an alteration in plasma membrane integrity (leakage of LDH enzyme and increased lipid peroxidation) in cells exposed to toxic doses of TSC, high glucose and their combinations. A combination of the two toxicity inducers i.e. TSC+glucose showed highest toxic effect. Pre-treatment of cells with SOE and Mangiferin kept these cytotoxic effects low, with Mangiferin

showing better effect. Studies carried out by Sellamuthu *et al.* [55] have also shown significant LDH lowering effect of Mangiferin.

In order to restore this oxidant: antioxidant balance, SOD and GSH has been reported to play a strong role [7, 15] and, the observations from our study coincide with it. Exposure of cells to the stressors showed an increase in SOD activity, as well as GSH levels thus, indicating that skeletal muscle has inbuilt capacity to fight against these toxic effects to an extent. Pre-treatment with SOE and Mangiferin caused a decrease in SOD activity at higher doses of TSC+glucose, thereby indicating more utilization of this enzyme under oxidatively stressed condition. Treatment with Mangiferin alone showed an increase in GSH level in cells and this indicates towards its inherent antioxidative property. Mangiferin showed better ability than SOE in scavenging the  $\cdot\text{NO}$  and  $\cdot\text{O}_2^-$  radicals and, simultaneously maintaining high level of GSH under such stressed conditions.

Several studies have shown that TSC induces apoptosis through mitochondrial mechanisms [56]. Experiments carried out on isolated mitochondria reports that in diabetics, mitochondria play a key role in generation of ROS [57]. This supports our study carried out for the oxidatively stressed mitochondria wherein altered mitochondrial permeability transition (MPT), leading to mitochondrial membrane pore formation and an increased  $\cdot\text{NO}$  production, was observed. But, the SOD activity was found to be increased in cytoplasm more than mitochondria thus, indicating more scavenging of  $\cdot\text{O}_2^-$  produced in cytoplasm as well as, those released from mitochondria due to increased MPT. Mangiferin showed better effect than SOE in decreasing the ROS load in mitochondria also. Besides these, we found an upregulation of anti-apoptotic protein Bcl-X in cells exposed to external toxic stimuli. The observations of our study is supported by Dupont-Versteegden [58] who has reported that, apoptosis is initiated by death-inducing signals such as ROS and RNS, as well as alteration in composition and abundance of Bcl-2 family proteins such as Bax, Bcl-X etc. Thus, in our study, extrinsic stimuli – TSC, high glucose and their combinations were responsible for induction of apoptosis in rat skeletal muscle cells through generation of OS by encompassing probably a mitochondrial apoptotic pathway, leading to Bcl-X upregulation, as well as altered mitochondrial membrane integrity and permeability. It also brought about necrotic changes which might have added up to the process of cell death.

These observations are supported by the fact that, both tobacco smoking and high glucose have a common centre of origin of toxicity induction in cells and this is “generation of high amount of free radicals”. Observations from the current study confirm that exposure of skeletal muscle cells to toxic doses of tobacco smoke and high glucose, individually and in

combination, lead to tremendous amount of cellular OS due to production of free radicals. This might have led to altered mitochondrial structural integrity and an imbalance in oxidant: antioxidant level, triggering cell death, with higher effect observed in TSC+Glc group, thereby indicating that in diabetic smokers, these agents may probably act in a synergistic phenomenon.

This *in vitro* study proved the antioxidant and antiapoptotic potential of SOE and its active compound Mangiferin in tobacco smoke induced oxidatively stressed rat skeletal muscle model. Mangiferin has shown a multi-targeted mode of action in several experimental studies till date. In the present study, it has shown efficiency in regaining oxidant: antioxidant balance in cellular compartments, significantly. Based on its potential properties viz. strong antioxidant, high bioavailability (oral), multi target nature and rapid absorption in the body (by literature), and the observations obtained from our study viz. enhancement of cell viability, maintaining plasma membrane and DNA integrity, lowering  $\cdot\text{O}_2^-$  and  $\cdot\text{NO}$  production (whole cell and mitochondrial level), enhancing cellular GSH content and, suppressing apoptotic events, this compound can be employed to modulate various pathways involved in diseased conditions in order to achieve the desired therapeutic effects and can be taken up further for deeper investigation and drug development.

## Thesis – Chapter 5

### CONCLUSION

From this study we can conclude the following:

- *Salacia oblonga* possess considerable amount of Phenolics, Flavonoids and Tannins.
- Amount of Mangiferin was highest in the methanolic extract of the plant.
- Both SOE and Mangiferin have potential intrinsic antioxidant and radical scavenging activities.
- Tobacco smoke, high glucose and their combinations have deleterious effects on cell viability and oxidative state of the cell.
- Increasing doses of **tobacco smoke, high glucose and their combinations**:
  - a. could alter the cell viability and other parameters in a concentration-dependent manner.
  - b. both necrotic and apoptotic pathways seems to be involved in cell death.

- Both **SOE and Mangiferin could conserve the cell number, morphology and cellular events** individually and in pre-treatment groups, by:
  - a. protecting the cell membrane and DNA integrity,
  - b. preserving the intactness of mitochondrial membrane,
  - c. enhancing the level of antioxidants, and
  - d. maintaining the cellular anti-apoptotic fighting capability by upregulation of the anti-apoptotic marker Bcl-X.
- **Mangiferin showed better effect than SOE** in retaining the normal physiological state by providing antioxidant protection in mitochondria, thereby preventing mitochondria-mediated apoptosis.
- These **events all together maintained the balance between the oxidants and antioxidants** in rat skeletal muscle cells and might have helped in cell survival.
- This study indicates that **both tobacco smoke and high glucose have an almost common mechanism of inducing toxicity in skeletal muscle cells.**
- It supports **the fact** that cells of affected **organs of tobacco smokers, diabetics and diabetic smokers do experience oxidative stress** due to generation of high amount of free radicals. The latter triggers **alteration in various pathways, thereby exerting their toxic effects in a probably synergistic manner.**
- In spite of *Salacia oblonga* extract and Mangiferin - both being good antioxidants, Mangiferin showed better effect in majority of the analysis carried out in this study. This makes **Mangiferin an important target molecule for cure of a multi-targeted disorder like diabetes, both in smokers and non-smokers.**
- In future, study into finding and characterizing the extra/intracellular targets of Mangiferin including: proteins/enzymes, membrane receptors and nucleic acid etc., and their interactions would be useful in understanding its above seen non-specific protective mechanism in skeletal muscle cells.
- In depth study is also needed to track down the pathways involved in the above said activities in future.

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## LIST OF PUBLICATIONS DURING PhD THESIS WORK

### International Journals

1. Sujata Basu, Mamta Pant and Rachana, “Phytochemical evaluation and HPTLC profiling of extracts of *Salacia oblonga*”, *International Journal of Pharmaceutical Sciences and Research*, vol. 4, no. 4, pp. 1409-1418, April 2013.
2. Sujata Basu, Mamta Pant and Rachana, “In vitro antioxidant activity of methanolic-aqueous extract powder (root and stem) of *Salacia oblonga*”, *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 5, no. 3, pp. 904-909, June 2013.
3. Sujata Basu, Mamta Pant, Rachana, “Beneficial effects of *Salacia oblonga* on mitochondrial localization in cells and NADPH oxidase activity in glucose induced cytotoxicity on rat muscle cell line”, *International Journal of Biotechnology and Bioengineering Research*, vol. 4, no. 4, pp. 321-328, 2013.
4. Sujata Basu, Mamta Pant, Rachana, “Possible antidiabetic mechanism of *Salacia oblonga* in diabetic smokers: in vitro investigation with RINm5f and L6 cell lines”, *World Journal of Pharmaceutical Research*, vol. 4, no. 1, December 2014.
5. Sujata Basu, Mamta Pant, Rachana, “Protective effect of *Salacia oblonga* against tobacco smoke-induced DNA damage and cellular changes in pancreatic  $\beta$ -cells,” *Pharmaceutical Biology* (Communicated).

### Books

6. **Book chapter:** Rachana, Thakur S., Basu S., Chapter titled “Oxidative stress and Diabetes”, pp. 241-257, in the book titled “Free Radicals in Human Health and Disease”

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