Chapter – 2

Literature review

2.1. Diabetes and mitochondria

2.1.1. Diabetes

Diabetes is a group of metabolic disorder characterized by increased level of glucose due to insufficient insulin production by pancreas or insulin resistance (IR) (American Diabetes 2009). Around 347 million people are suffering from diabetes worldwide. WHO projects that diabetes death will increase by two-fold between 2005 and 2030 (WHO 2013). Diabetes can be divided into two main forms based on its etiology such as type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) as shown in Figure 2.1 (WHO 1999). T1DM is an autoimmune disorder occurs due to destruction of the insulin secreting pancreatic β-cells resulting in insulin deficiency and this type accounts for 5–10% of all diabetic cases (American Diabetes 2009). T2DM is the most common form of diabetes and affects over 90% of diabetic patients (WHO 2013). T2DM is characterized by impaired insulin release in pancreatic β-cells and IR in target tissues such as muscle, liver and adipose tissue (WHO 1999).

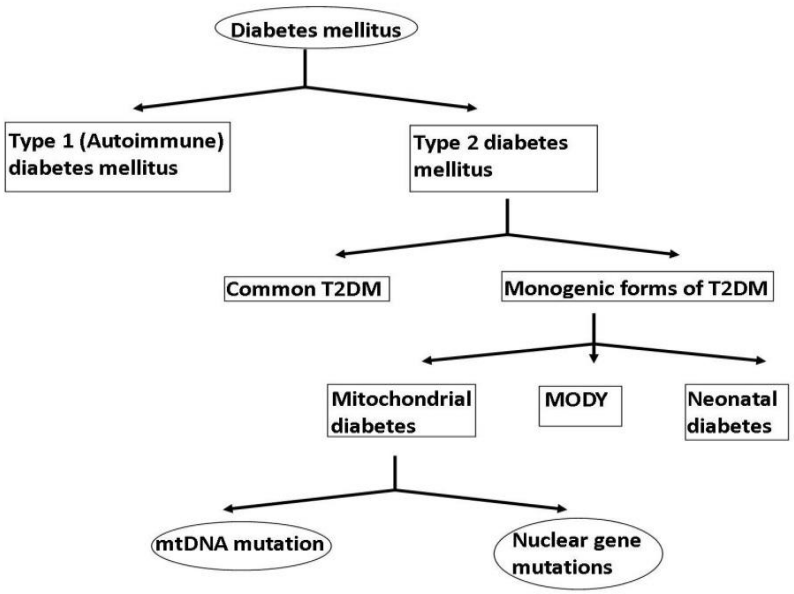


Figure 2.1: Types of diabetes based on pathophysiology

2.1.2. Mitochondria

The research on mitochondria was started back in 18th century. Particularly, in 1890, Altman discovered mitochondria when he identified cellular components and he named them as "bioblasts". Later in 1898, Benda termed the name "mitochondrion" as a singular form of mitochondria (Barnett and Robinow 2002). The Greek meaning of mitochondrion is mitos-thread and chondros-granule (vander-Giezen 2011). The presence of protein and lipid were observed by Meves in 1908. Further, Meves and Regaud identified the presence of genes in mitochondria. Mitochondrial role in respiration was observed by Kingsbury in 1912. Mitochondrial role in the oxidation of metabolites and in energy conversion was carried out by several researchers during the period 1948-1951 (Ernster and Schatz 1981). Since then, several research works are being carried out on mitochondria. Mitochondrial role in the production of ROS and its treatment are the major concern for biologists and pharmacists in

the treatment of oxidative stress induced diseases such as cancer, diabetes and neurodegenerative diseases.

Mitochondria are highly dynamic, semi-autonomous and motile in nature, which undergo frequent shape changes. They are found in every cell in the human body. Mitochondria are morphologically and functionally self-governing which do not depend on cells function. Mitochondrial distribution in cells is diverse and maintained by the fusion and fission balance (Watala 2012). Fission and fusion process is necessary for maintaining mitochondrial integrity, electrical and biochemical connectivity, mitochondrial turnover and segregation and protection of mtDNA (Detmer and Chan 2007). In addition, fusion process is essential for functioning of mitochondria and any disturbance to the process leads to loss or changes in mitochondrial membrane potential (Sivitz and Yorek 2010).

Mitochondria consist of inner and outer membrane both are made up of phospholipid bilayer. The inner membrane encloses and convolutes into the mitochondrial matrix, forming cristae which carries the enzymes needed for oxidative phosphorylation (OXPOS) which is also called as electron transport chain (ETC), a main metabolic pathway that happens in mitochondria (Krauss 2001). The outer membrane is widely permeable to ions and larger molecules whereas inner membrane is less permeable (Krauss 2001).

2.1.3. Mitochondria involvement in diabetes

The most important function of mitochondria is the production of ATP molecules (Tarasov et al. 2012). Other functions include fatty acid oxidation, generation of ROS with important signalling functions, control of cytoplasmic calcium and synthesis of all cellular Fe/S clusters, regulation of protein cofactors which are essential for cellular functions, heme biosynthesis, and amino acid metabolism (Quijano et al. 2016).

Production of ATP molecules from glucose occurs by the involvement of three processes which include glycolysis, TCA cycle and ETC (Figure 2.2). Briefly, glucose molecules present in blood are transported across the cellular membrane by glucose transporters (GLUT) primarily by GLUT-1 and secondarily by GLUT-2 and are rapidly phosphorylated by the enzyme, glucokinase (Bell et al. 1990). Through glycolysis pathway, glucose is converted into pyruvate in cytosol. Out of which, 90 % of pyruvate is transported from cytosol to mitochondria by pyruvate transporters (German 1993). Pyruvate molecules are converted into acetyl CoA molecules by TCA cycle and are readily oxidized to CO_2, water, ATP and reducing equivalents such as NADH and FADH. These reducing equivalents shuttle electrons to the ETC (Fernie et al. 2004). When these reducing equivalents shuttle electrons to ETC, an electrochemical gradient of 150-200 mV negative to cytosol occurs and produces ATP molecules (from ADP + Pi) which is driven by proton movement back through ATP synthase (or complex V) (Newsholme et al. 2007).

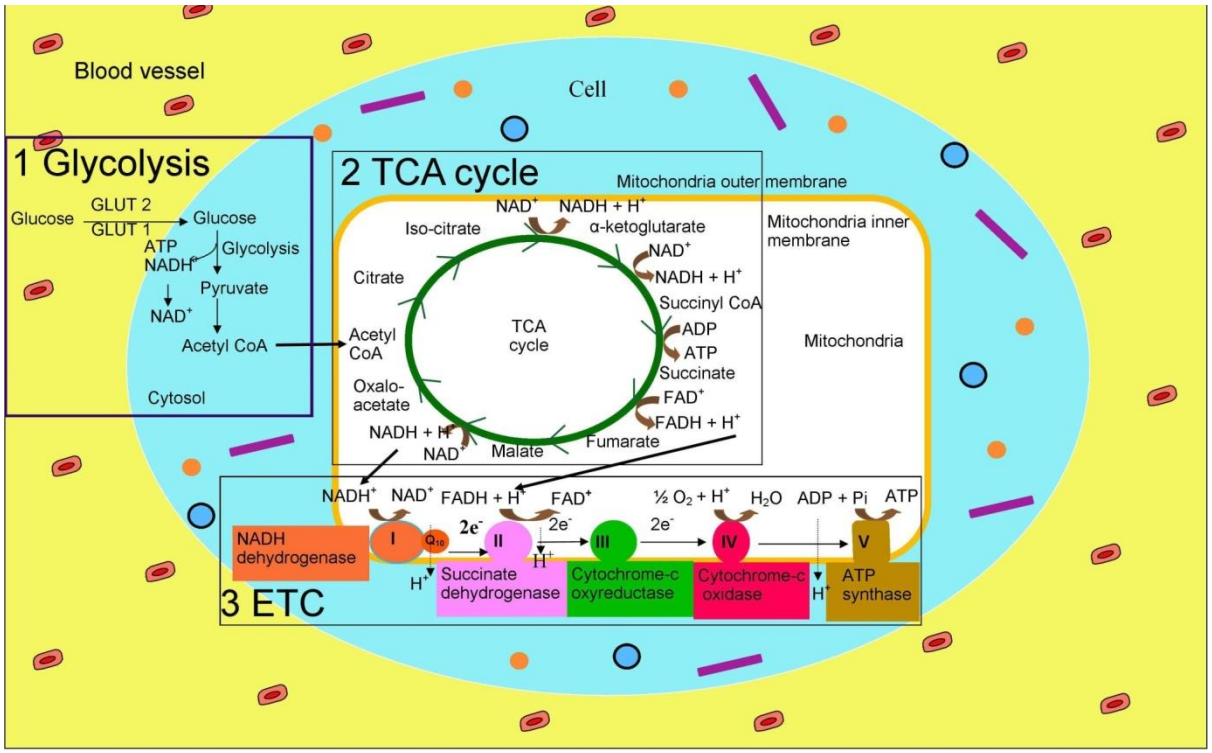


Figure 2.2: Diagrammatic representation of ATP production from glucose: 1. Glycolysis: Glucose is entering cell from blood by GLUT 2/1 and through glycolysis process, glucose is converted into acetyl CoA. 2. TCA cycle: Acetyl CoA enters mitochondria of cell, undergoes TCA cycle and liberates reducing equivalents such as NADH and FADH. 3. ETC: NADH and FADH are utilized by five complexes of ETC, resulting in the production of ATP with the liberation of ROS. (Abbreviations: ATP: adenosine triphosphate, GLUT: glucose transporter, TCA: tricarboxylic acid, ETC: electron transport chain, NADH: nicotinamide adenine dinucleotide, FADH: flavin adenine dinucleotide and ROS: reactive oxygen species.

In ETC, five complexes are involved in the production of ATP molecules (Figure 2.3). Complex I (NADH dehydrogenase or NADH-ubiquinone reductase) converts NADH to NAD^+, NAD^+ is further converted into $FMNH_2$ by flavin mononucleotide and $FMNH_2$ gets oxidized through semiquinone intermediate present in complex I (Weiss et al. 1991). Through this conversion, four H^+ ions liberate and translocate from mitochondrial matrix to mitochondrial intermembrane space with transfer of two electrons (e^-) to complex II through iron-sulfur clusters (Piekna 2000). Complex II (succinate dehydrogenase) oxidizes succinate

molecules (intermediate of TCA cycle with the use of FAD as coenzyme, three iron-sulfur clusters and cyto-chrome b_{560}) into malate and liberates reducing equivalents (e^-) that are shuttled to complex III via ubiquinone (Lodish et al. 2000). In complex III (cytochrome c reductase), two electrons received from complex II and are shuttled to complex IV (Lenaz and Genova 2010). Complex IV (cytochrome-c oxidase or cytochrome A3) is a transmembrane complex that receives electrons, translocates four protons per pair of electrons and reduces oxygen to water with the liberation of two protons (H^+) (Malmström 1989). Liberated protons are utilized by complex V (ATP synthase or F_1F_0 ATPase) by proton gradient which creates a transmembrane potential, driving force for the production of ATP from ADP (Lodish et al. 2000). Produced ATP will be transported to cytosol by ADP exchange and used for various biological events that require energy (Liu and Chen 2013).

Among the five complexes, complex I and III are the two main sources for the ROS generation (Fato et al. 2009). During the process of ETC, produced ROS have an impact on other reactive species generation i.e. reactive nitrogen species (RNS) inside mitochondria (Trachootham et al. 2008).

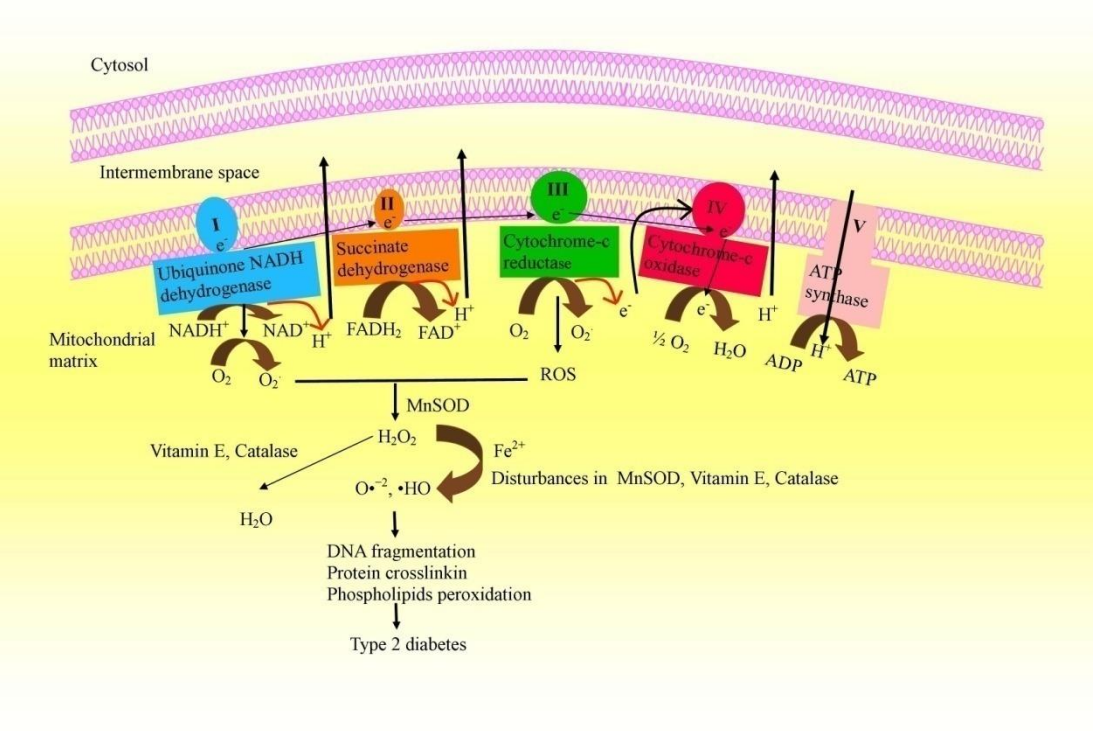


Figure 2.3: Antioxidant defence mechanism in mitochondria and its involvement in type 2 diabetes

Produced ROS and RNS are enzymatically converted to hydrogen peroxide by manganese superoxide dismutase (MnSOD) within mitochondria (Turrens 2003). Glutathione peroxidase, an enzyme present in cytoplasm helps in reducing hydrogen peroxide into water molecules (Dunning et al. 2013). In addition to glutathione peroxidase, presence of vitamin E and catalase, two other antioxidants present in the inner mitochondrial membrane and in peroxisomes, respectively, act as antioxidant defence mechanism for the elimination of ROS produced in mitochondria (Turrens 1997). When the rates of H_2O_2 generation exceeds than its removal in case of diabetes, H_2O_2 accumulation occurs which results in the production of highly reactive ROS in the presence of Fe^{2+} ions (Valko et al. 2007). This causes increased mitochondrial membrane potential which leads to elevated ATP

production but with reduced electron transport capability (Hüttemann et al. 2012). Further, increased ROS levels result in generation of other reactive species such as peroxynitrile, reactive carbonate radical and singlet oxygen which also have an great impact on diabetes (Figure 2.4) (Bhattacharya 2015).

2.1.4. Impact of increased ROS on diabetes

Mitochondrial diseases differ from other diseases because of their nuclear mutation and different dynamics with respect to their DNA. Tissues with high ATP requirement such as neural tissue, β-cells and renal cells are more affected by an ATP shortfall than tissues with low ATP requirement (e.g. bone) (Berdanier 2007). In β-cells, higher levels of glucose lead to increased production of reducing equivalents and causes increased ATP production in mitochondria (Green et al. 2004). This increased ROS level have an impact on diabetes by several mechanisms such as increased ATP to ADP ratio, generation of 8-hydroxydeoxyguanosine, activation of mitogen-activated protein kinases and increased RNS generation (Sifuentes-Franco et al. 2017).

2.1.4.1.Increased ATP to ADP ratio

Increased ROS levels enhance ATP to ADP ratio in cytoplasm and causes closure of ATP-sensitive K^+ channels (K_{ATP}), which decreases hyperpolarization of outward K^+ flux resulting in depolarization of plasma membrane (Fridlyand and Philipson 2004). Thus, allowing influx of extracellular Ca^{2+} ions which rapidly increases intracellular Ca^{2+} ion levels and causes activation of protein kinases and mediates exocytosis of insulin (Lanner et al. 2006). Further increase in intracellular Ca^{2+} levels can stimulate mitochondrial generation of ROS (Gorlach et al. 2015). Also, due to low levels of free radical detoxification and

redox-regulating enzymes in β-cells, damage to mitochondria and cell death occur (Newsholme et al. 2007).

2.1.4.2.**Generation of 8-hydroxydeoxyguanosine**

In addition to the increased ATP to ADP ratio, 8-hydroxydeoxyguanosine (8-OHdG) generation also has an impact on oxidative stress induced diabetes (Araki and Nishikawa 2010). When oxidative damage occurs to DNA in mitochondria by enzymatic cleavage, it causes 8-hydroxylation of guanine base and produces 8-OHdG. 8-OHdG was 16-fold higher in mitochondria DNA than in nuclear DNA in live rats (Nishikawa and Araki 2007).

2.1.4.3.Activation of mitogen-activated protein kinases

The next mechanism by which diabetes occurs by increased ROS levels is by activation of mitogen-activated protein kinases (MAP kinases)**.** In oxidative stress induced diabetic and increased free fatty acid conditions, activation of MAP kinases occurs by phosphorylation in the presence of c-Jun NH(2)-terminal kinase (JNK) (Tikoo et al. 2008). MAP kinases are responsible for cellular proliferation, differentiation, development, inflammatory responses and apoptosis and they are inactive in normal conditions (Kashihara et al. 2010).

2.1.4.4.Increased RNS generation

Increased ROS level also generates RNS particularly peroxynitrite. Peroxynitrite can damage DNA which in turn activates nuclear enzyme, poly(adenosine diphosphate (ADP)-ribose) polymerase and causes intracellular accumulation of NAD^+. Thus, resulting in decreased rate of glycolysis and decreased ETC and finally, decreased ATP production (Ceriello 1999).

2.1.4.5.Other mechanisms

Lifestyle habits such as intake of high-calorific food and low physical activity and smoking can increase the prevalence of obesity, diabetes and insulin resistance. Further, several environmental toxins are responsible for mitochondrial dysfunction (Heindel et al. 2017). Arsenic, persistent organic pollutants (POPs) and antiretroviral drugs are the environmental toxins which affect mitochondria function. Both organic and inorganic forms of arsenic enter the body through contaminated food or water. Organic form of arsenic can excrete in urine whereas inorganic form accumulates in adipose tissue and causes type 2 diabetes (Lim et al. 2010, Park et al. 2013). POPs are pesticides that are present in the food chain. Also, POPs are resistant to biodegradation and have longer half-lives (several months to years) (Lee and Jacobs 2015). POPs include bisphenol-A, 2,3,7,8-tetrachloro-dibenzo-p-dioxin, organochlorine, atrazine and 3-nitropropionic acid affect the mitochondrial function (Heindel et al. 2017).

2.1.5. Different conditions of diabetes

Diabetic conditions caused by increased ROS level are of two types i.e. microvasular diseases and macrovasular diseases (Cade 2008).

2.1.5.1.**Microvascular diseases**

Microvascular diseases happen in the small blood vessels in the body such as capillaries. Chronic hyperglycemia initiates microvascular conditions by increased ROS or production of advanced glycation end products or abnormal activation of protein kinase C and rennin-angiotensin system (Rask-Madsen and King 2013). Three main forms of diabetic

microvascular diseases include diabetic nephropathy, diabetic retinopathy and diabetic neuropathy.

2.1.5.1.1. Diabetic nephropathy

Both T1DM and T2DM have the serious complication of diabetic nephrophathy which is progressive in nature. Diabetic nephropathy accounts for leading cause of chronic kidney disease leads to kidney replacement therapy and increases cardiovascular mortality (Ghaderian et al. 2015). The main symptoms of diabetic nephropathy include microalbuminuria (or advanced stage), glomerular membrane thickening, glomerular hyperfiltration, tubular sclerosis and finally kidney failure (Lim 2014). Activation of phospholipase A2 by protein kinase C causes hyperperfusion and hyperfiltration of kidneys. In chronic condition of diabetes, renal vasoconstriction and increased deposition of extracellular matrix occur, which result in hypertension and nephrosclerosis (Noh and King 2007).

2.1.5.1.2. Diabetic retinopathy

Retina needs higher level of oxygen for normal activity which makes retina more prone to ROS (Kowluru and Chan 2007). Diabetic retinopathy can affect peripheral retina, macula or both. It is the leading cause of blindness in diabetic patients. Diabetic retinopathy is seen in both type 1 diabetes and type 2 diabetes and also in patients with insulin resistance. Further, diabetic retinopathy is associated with higher body mass index and hypertension (Cade 2008). Symptoms of diabetic retinopathy include vascular closure, loss of pericytes, growth of new blood vessels in retina and vitreous, macular edema, protection against increased ROS and thickening of leaky blood vessions (Fong et al. 2004). In diabetic condition of retinopathy, levels of malondialdehyde and sulfhydryl proteins were found to be

less in sub retinal region with increased level of vasoactive prostanoids (Kowluru and Chan 2007).

2.1.5.1.3. Diabetic neuropathy

Nearly 50 % of the diabetic patients have some form of peripheral neuropathic condition (Cohen et al. 2015). Different forms of diabetic neuropathy include autonomic neuropathy, cardiovascular autonomic neuropathy and distal symmetric polyneuropathy (Bansal et al. 2006). It is difficult to find the target responsible for nerve damage. Different conditions of diabetic neuropathy include thickening of axons and basement membrane, loss of pericytes and decreased capillary blood flow (Pop-Busui et al. 2017). Intracellular accumulation of NAD^+ by increased peroxynitrite ions produces ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase, this causes endothelial dysfunction leads to reduced neural perfusion and capillary occlusion (Ceriello and Testa 2009).

2.1.5.2.Macrovascular diseases

Macrovascular diseases happen in large blood vessels in the body such as arteries and veins. Chronic diabetic condition leads to accumulation of fat and blood clots in large blood vessels. Increased ROS level oxidise low-density lipoprotein (LDL). Oxidised LDL cannot be recognized by the LDL receptor instead taken up by scavenger receptors present in macrophages (foam cell formation) and atherosclerotic plaques (Cominacini et al. 2000). Macrovascular complications of diabetes include coronary disease in heart (artherosclerosis), peripheral artery disease and cerebrovascular disease (Cade 2008). Myocardial infarction is the major coronary disease caused by prolonged diabetes. Also, chronic diabetes can cause congestive heart failure (Pálsson and Patel 2014). Peripheral artery disease can be

characterized by the occlusion of lower-extremity arteries which results in limb disability. It is reported that diabetes is the major cause of lower-extremity amputation (Thiruvoipati et al. 2015). Chronic diabetes can lead to stroke independent of age and leads to neurological defects and disability. Further, it increases the risk of atherosclerosis in both intracranial and extracranial carotid arteries (Cade 2008, Ergul et al. 2012).

2.2. **Drug selected for the study**

Most of the synthetic drugs available for treating diabetes come with side effects as listed in the Table 2.1. Nowadays, the research on herbal drugs is tremendously increasing to avoid the side effects associated with synthetic drugs.

Table 2.1: List of available antidiabetic drugs with their side effects

Class	Examples	Side effects
Sulphonyl ureas	Tolbutamide Chlorpropamide Glipizide	Hyperinsulinemia, hypoglycemia weight gain and GIT* upset
Meglitinide analogues	Repaglinide Nateglinide	Hypoglycemia, weight gain and drug interactions.
Biguanides	Metformin	Metallic taste, GIT disturbances (nausea, vomiting, diarrhea) and lactic acidosis
Thiazolidinediones	Pioglitazone	Fluid retention, weight gain and headache
α-Glucosidase inhibitors	Acarbose Miglitol	GIT disturbances: flatulence, diarrhea, abdominal pain, bloating and increase in liver enzyme levels.
Dipeptidyl peptidase-4 (DPP-4) inhibitors	Sitagliptin	GIT disturbance and nasopharyngitis

* GIT- gastrointestinal tract

Herbal drugs as single phytochemicals or as extracts are known for their mixed pharmacological actions with the benefit of no or less harmful side effects. Nowadays, the research on herbal drugs is tremendously increasing to avoid the side effects associated with synthetic drugs. Several advantages of using herbal drugs over synthetic drugs include single plant can be used to treat various diseases, herbal drugs are not incorporated with chemicals, herbal drugs come with no warnings, self-administration is possible, drug addiction is not observed, readily available in almost all areas and less expensive. Herbal drugs are being used as extracts or as phytochemicals. Several herbal extracts possessing both antidiabetic activity and antioxidant activity are being used for the treatment of oxidative stress induced diabetes (Nasri et al. 2015). Antioxidants present in herbal extracts usually give electrons for neutralization of free radicals with simultaneous hypoglycemic effect (Lobo et al. 2010).

The availability of synthetic drugs is more in the market than herbal drugs in spite of their effective pharmacological actions and little or no side effects. The presence of several phytochemicals in a single plant or extract causes difficulty in carrying out qualitative and quantitative analysis. For qualitative and quantitative analysis, there is a need of reference compounds for each phytochemicals present which results in higher cost of the experiment. Also, due to unavailability of pharmacokinetic parameters of most of the phytochemicals in the current scenario limits the use of natural products.

The use of extracts pose several advantages over phytochemicals such as mixture has proven clinical effectiveness even the active constituent is unknown, if one or more active components of an extract are known, further substances may be responsible for an optimal effect and most of the extract contains structurally similar compounds, which may increase the effectiveness.

2.2.1. Ficus religiosa L.

Ficus religiosa (Figure 2.5) is a sacred tree and native to India. It grows up to elevations of 5,000 ft (1,524 m) and it belongs to the family, Moraceae. In the Indian culture, *Ficus religiosa* has got great religious importance. It has been mentioned in several ancient literatures such as arthasastra, puranas, upanisads, ramayana, mahabharta and bhagavadgita. In Ayurveda, *Ficus religiosa* belongs to the class of rasayana (rejuvenators, antioxidants and stress reliever). More than 800 species and 2000 varieties of genus, *Ficus* have been reported. Of which, *Ficus religiosa* (pipal tree), *Ficus benghalensis* (banyan tree) and *Ficus carica* (anjir tree) are the most common with wide pharmacological properties. *Ficus religiosa* L. possesses strong anti-oxidant activity and it is included in several ayurvedic formulations for the treatment of diabetes, epilepsy, inflammatory conditions, microbials, gout, stomatitis, leucorrhea, ulcers and against several microbes. Phytoconstituents present in the bark, leaves, fruits, latex and decoction of *Ficus religiosa* L. are responsible for its pharmacological actions (Singh et al. 2011).

Figure 2.4: *Ficus religiosa*: (A) Tree; (B) Leaves; (C) figs; (D) stem bark; (E) root

Taxonomy of *Ficus religiosa* L.

Family **:** Moraceae (mulberry family)

Latin name **:** *Ficus religiosa* L.

Common names **:** Peepal tree, bo tree, beepul tree, sacred tree

Nomenclature **:** As it is planted beside temples and people worship this tree, it has the species name, *religiosa*.

Traditional Uses **:** In Ayurveda, *Ficus religiosa* belongs to the class of rasayana (rejuvenators, antioxidants and stress reliever). Nalpamaradi tailam, chandanasavam, nyagrodhadi churna, saribadyasavam, pancha valkaladi tailum and pancha valkala kashaya are the commercially

available ayurvedic formulations of *Ficus religiosa* (Pandit et al. 2010; Ambika and Rao, 1966).

Morphology **:** *Ficus religiosa* has heart shaped leaves and bright green in colour. Fruits are epiphytic, hidden within figs with drooping branches. The bark is flat or slightly curved, outer surface is covered with crustose lichen. Inner surface is smooth and yellowish to orange brown in colour.

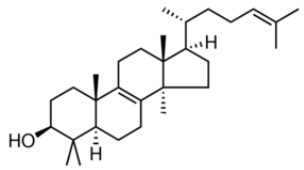
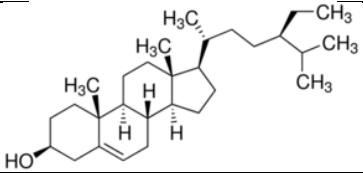
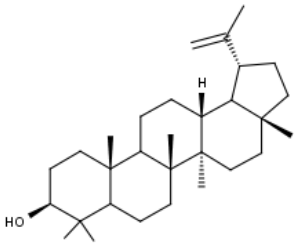
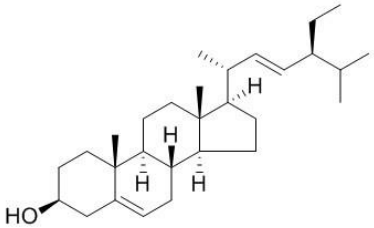
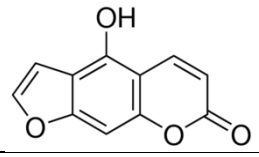
Physical constants **:**Total ash 7.86 % w/w, acid insoluble ash 0.41 % w/w, alcohol soluble extract 7.21 % w/w and water soluble extractive 15.76 % w/w17.

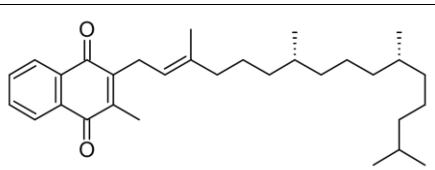
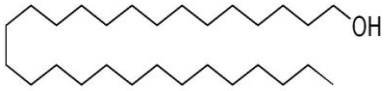
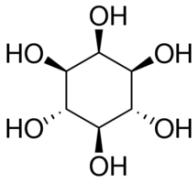
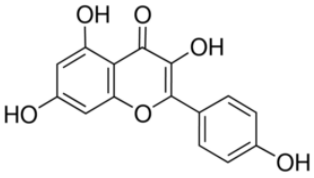
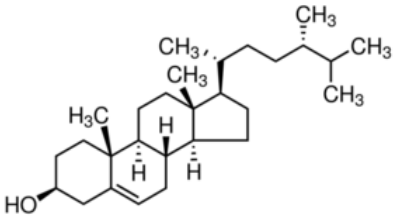
Phytoconstitutents : Stem bark of *Ficus religiosa* L. is reported to contain β-sitosterol, β-sitosteryl-d-glucoside, stigmasterol, campesterol, lupeol and lanosterol. Collectively called as phytosterolins, root bark is reported to contain Vitamin K_1, n-octacosanol, Methyl oleonate and Lupen-3-one , Furanocoumarins (bergapten and bergaptol), fruits contain Amino acids, flavonoids (myricetin, quercetin and kaempferol), phenolic components and volatile compounds (undecane, tridecane, tetradecane, (Z)-3-hexenol, monoterpenes), leaves were found to have Phytosterolins, long-chain hydrocarbons and aliphatic alcohols and triterpene alcohols. The structures of important phytoconstituents are illustrated in Table 2.2

Clinical uses : Phytoconstituents present in the bark (stem bark, root bark), leaves, fruits, latex and decoction are useful in the treatment of

various diseases such as diabetes, cancer, epilepsy, inflammatory conditions, gout, stomatitis, leucorrhea, ulcers, wound healing, acetylcholinesterase inhibitory activity, and against bacterias and microbials (Pandit et al. 2010, Sing et al. 2011).

Table 2.2: Structures of phytoconstituents present in *Ficus religiosa* L.

S.No.	Phytochemical	Structure
1	Lanosterol	
2	Sitosterol	
3	Lupeol	
4	Stigmasterol	
5	Bergaptol	

6	Vitamin K1	
7	n-octacosanol	
8	Inositol	
9	Kaempferol	
10	Campesterol	

2.3. Targeted drug delivery to mitochondria

Drug targeting is the delivery method that delivers drug to the specific site of action which improves the drug concentration at the target site where the action occurs and avoids its distribution to other parts of the body, thus reduces the drug side effects. Targeting are of many types; direct targeting, passive targeting, magnetic targeting, targeting using a specific vector molecules. In direct targeting, a drug is directly applied onto the area where its effect is needed; passive targeting improves drug accumulation in the areas where leaky vasculature occurs. Magnetic targeting needs the help of a magnet; a magnet kept on the skin attracts the drug molecules present in the systemic circulation and produces its effect. Targeting using a

specific vector molecule is in the trend for several decades. Specific vector molecules may be of targeting molecules or ligands or peptides which have increased affinity towards the target area.

Mitochondrial targeting can be achieved by conjugation of targeting moieties with anti-oxidants/anti-diabetic drugs, nanoparticulate technology (micelles, liposomes, polymeric nanoparticles, gold nanoparticles, biosensor tethered nanoparticles loaded with anti-oxidants/anti-diabetic drugs) by conjugating targeting moieties with polymers used for encapsulating drugs or by surface modifying drug loaded nanoparticles with targeting moieties and bifunctional targeting and anti-oxidant effect are effective in the treatment of diabetes. Mitochondrial targeting by nanoparticles have been performed either by conjugating targeting moieties with carriers used or by surface modifying drug loaded nanoparticles with targeting moieties (Figure 2.6) (Das et al. 2013).

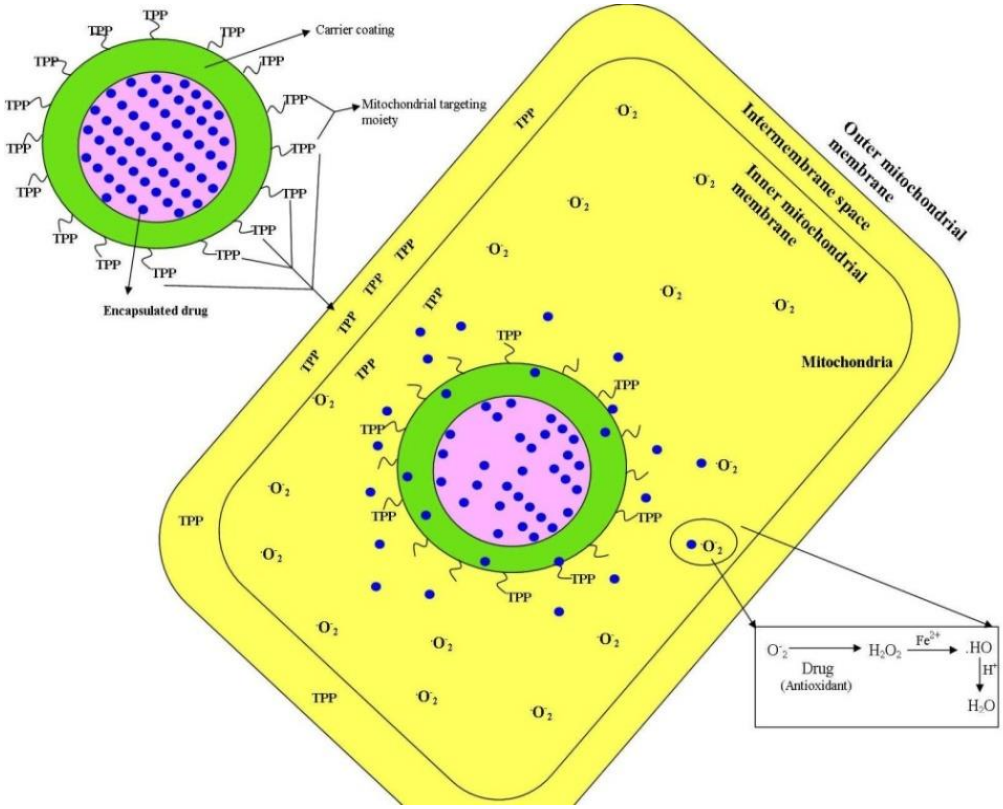


Figure 2.5: Schematic representation of mitochondrial targeting of nanoparticles loaded with antioxidant

2.3.1. Mitochondrial targeting moieties

There are different targeting moieties available for targeting mitochondria. They include lipophilic cations, dequalinium, cationic fluorescent dyes (rhodamine 123), peptides (SS-peptides), MKT-077 and Sk-compounds (Table 2.3).

2.3.1.1.Lipophilic cations:

Lipophilic cations are the most widely used mitochondrial targeting moiety. Lipophilic cations are also known as penetrating ions. Phenyl residues present in lipophilic cations are responsible for charge delocalization and charge screening. Charge delocalization

contributes to the permeability of lipophilic cations (Trendeleva et al. 2012). Lipophilic cations can pass the phospholipid bilayer because of net positive charge which is independent of the charge to accelerate equilibration times (Ritchie 1984, Murphy and Smith 2007). In addition to the net positive charge, the plasma membrane potential negative charge of about 30 to 60 mV also assist the penetration of these lipophilic cations (about 90%) into mitochondria from the extracellular fluid (Smith et al. 2012). Lipophilic cations can selectively accumulate mitochondria of several hundred folds which is driven by the plasma and mitochondrial membrane potentials due to the larger membrane potential ($\Delta\psi m$, −150 to −170 mV, negative inside) (Porteous et al. 2010).

The selective uptake of lipophilic cations by mitochondria significantly amplifies the efficacy and specificity which also reduces the harmful side reactions (Smith et al. 2003). Examples of lipophilic cations include triphenylphosphonium (TPP), N-methyl-4-phenylpryidinium (MPP), tetraphenylphosphonium cation, (4-iodobutyl) triphenylphosphonium cation, (4-thiobutyl)triphenylphosphonium cation, tetraphenylborate anion, methyltriphenylphosphonium cation, (2-hydroxyaminovinyl)-triphenylphosphonium (Frantz and Wipf 2010). N-methyl-4-phenylpryidinium (MPP) is one of lipophilic cations which accumulates into mitochondria by the difference in mitochondrial membrane potential and inhibits complex I of the respiratory chain in mitochondria leading to the toxicity of complex I (Murphy and Smith 2000). Effective uptake of lipophilic cations by mitochondria showed improved efficacy *in vivo*. Upon oral administration of lipophilic cations, mitochondrial uptake within tissues was found to be effective. Porteous et al. (Porteous et al. 2010) proved that lipophilic cations can be delivered rapidly to mitochondria *in vivo* following iv administration.

2.3.1.2.**Rhodamine 123:**

Rhodamine-123 is a cationic fluorescent dye. It is widely used to visualize mitochondria selectively within cells and revealed as clusters of organelle in the perinuclear region of src-transformed cells (Murphy and Smith 2007). The mechanism of mitochondrial penetration of rhodamine is same as that of lipophilic cations (Wang et al. 2010). Surface modification of liposomes by rhodamine 123 were found to have higher mitochondrial uptake (Biswas et al. 2011). Rhodamine 123 is reported to be non-cytotoxic up to 10 µg/ml concentrations (Baracca et al. 2003).

2.3.1.3.**Dequalinium:**

Dequalinium is a derivative of amphiphilic quinolinium. It contains two positive charges and a C-10 aliphatic side chain. Dequalinium also accumulates into mitochondria by differences in the mitochondrial membrane potential (Wang et al. 2010). Dequalinium conjugated with polyethylene glycol-distearolyphosphatidyl ethanolamine was used for mitochondrial targeting for the delivery of resveratrol liposomes (Wang et al. 2011). The mitrochondrial efficiency of targeted liposomes in A549 cells and A549/cDDP cells were estimated with the use of confocal laser scanning microscopy. Results revealed that mitochondrially targeted coumarin liposomes with the help of rhodamine 123 were selectively accumulated into the mitochondria with evidently bright yellow fluorescence whereas free coumarin or coumarin liposomes were not observed in the cells (Wang et al. 2010).

2.3.1.4.**MKT-077**:

MKT-077 is a highly water soluble rhodacyanine analogue and it is structurally related to rhodamine 123. MKT-077 accumulates mitochondria due to higher mitochondrial

membrane potential (Modica-Napolitano et al. 1996). MKT-077 exhibits cytotoxicity in tumour cell lines that have higher mitochondrial membrane gradients not in lower gradients through mitochondrial damage and ATP depletion which results in decreased mitochondrial oxygen consumption (Propper et al. 1999, Chunta et al. 2012).

2.3.1.5.Sk-compounds:

SKQs consist of a TPP-linked plastoquinone moiety which had higher permeability in *in vitro*, *in vivo* and *ex vivo* models (Roestenberg et al. 2012).

2.3.1.6.SS-peptides:

SS peptides are rapidly taken up into cells, reaches a steady-state concentration in minutes, and have a sequence motif that targets them to mitochondria in an energy-independent and nonsaturable manner (Armstrong 2007). SS peptides target mitochondria selectively partition into the inner mitochondrial membrane which is independent of membrane potential. SS peptides uptake is not self-limiting and nontoxic and hence, mitochondrial depolarization does not happen even at 1 mM concentrations (Frantz and Wipf 2010). Tyrosine residues present in SS-peptides are responsible for free radical scavenging property (Heller et al. 2012).

2.3.1.7.$_{D}$-(KLAKLAK)$_2$ pro-apoptotic peptides and analogues:

$_{D}$-(KLAKLAK)$_2$ peptide is a cationic amphipathic α-helical forming peptide. $_{D}$(KLAKLAK)$_2$ can disrupt the mitochondrial membrane which are negatively charged and results in cell death (Ko et al. 2009).

2.3.1.8.Mn-porphyrin-oligopeptide conjugates:

Recently, conjugated manganese metalloporphyrin with signal oligopeptide (Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Lys-Gly-Cys-S-spacer-porphyrin) was used for targeting mitochondria and found to be effective in targeting mitochondria (Murphy 1997).

2.3.1.9.Cyclosporin A peptide:

The anticancer agent cyclosporine A (CsA) can be used for targeting mitochondria. Treatment with CsA would benefit from a mitochondria-specific drug carrier system (Heller et al. 2012).

2.3.1.10. Gramicidin S-based conjugates

Gramicidin S-based conjugates consist of three portions, a portion containing electron, a portion containing ROS-scavenging activities and a portion having targeting efficiency. The portion with targeting efficiency promotes selective accumulation within mitochondria (Frantz and Wipf 2010). By accepting one-electron, nitroxide radicals are converted to the corresponding hydroxylamine. Hydroxylamines act as effective ROS scavengers (Fink et al. 2007).

1 **Table 2.3: List of mitochondrial targeting moieties**

S. No.	Targeting moieties	Properties	Toxic effects	References
1	Triphenylphosphonium	Accumulates mitochondria by differences in mitochondrial membrane potential	--	Porteous et al. 2010
2	Dequalinium	Accumulates mitochondria by differences in mitochondrial membrane potential	Globular change and perinuclear distribution	Wang et al. 2010
3	Rhodamine 123	Altered mitochondrial transmembrane potential	--	Biswas et al. 2011
4	MKT-077	Altered mitochondrial transmembrane potential	Hypomagnesemia	Chunta et al. 2012
5	Cyclosporin A peptide	Low binding to the mitochondrial membranes but higher accumulation in mitochondrial matrix was observed.	Mitochondrial damage	Heller et al. 2012
6	SS-peptide	Triple positive net charge at physiological pH values is responsible for its penetrating in to mitochondria.	--	Szeto 2008

2.3.2. Mitochondria targeted nanomedicines for diabetes treatment

Summary of different mitochondria targeted drug delivery systems for the treatment of diabetes is given in Table 2.4.

2.3.2.1.**Polymeric nanoparticles**

Polymeric nanoparticles prepared by using suitable biodegradable or biocompatible polymers have wide application in drug targeting to mitochondria (Singh and Lillard Jr 2009). One of such examples is targeting 2,4-dinitrophenol, an anti-obesity drug to mitochondria as nanoparticles with the help of conjugated polymer, poly (D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol) - TPP polymer (PLGA-bPEG-TPP). This polymer was prepared by conjugating PLGA-b-PEG-OH / PLGA-COOH with TPP. From the results, it was concluded that targeted 2,4-DNP NPs suppressed the adipocytic differentiation of 3T3-L1 cells at a low concentration (1 or 4μM) and didn't show cytotoxic effect which may be helpful in the management of obesity (Das et al. 2013).

In a different study conducted by Samadder et al. (Samadder et al. 2013), insulin loaded poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles, Upon administration of insulin and its nano-form, level of calcium ion was reversed along with reduction in ATP/ADP ratio. The mitochondrial membrane potential was re-established up on administration of insulin and NIn and this result was also supported by the data obtained from flow cytometric study.

2.3.2.2.Biosensor-tethered nanoparticles

Prow et al. (Prow et al. 2008) developed biosensor-tethered nanoparticles using antioxidant response element (ARE) to study their use in the expression of reporter genes to specifically detect mitochondrial oxidative stress (MOS) in diabetes. The bleb site of diabetic

animals had many more EGFP positive cells and most of these cells were RPE whereas in case of non-diabetic animals, very few EGFP positive cells were observed. This suggests that non-diabetic retinas did not have highly activated ARE systems. From these results, authors have concluded that ARE-tethered nanoparticles were capable of detecting the cellular response to the diabetic condition in the eye.

2.3.2.3.Liposomal drug delivery

Recently, mitochondrial targeting signal peptide (MTS) was reported as a mitochondria targeting moiety by Yamada and Harashima (Yamada and Harashima 2013) through liposomal nanocarrier system. MTS can selectively deliver drugs, proteins and linear DNA to mitochondria. MTS was first conjugated with 1, 2-Dioleoyl-sn-glyceo-3-phophatidyl ethanolamine (DOPE), conjugated MTS-DOPE was then used for preparing liposomes using egg yolk phosphatidyl choline and cholesterol. MITO-Porter was integrated into liposomes for delivering drug to mitochondria. From the results of mitochondrial binding activities of isolated mitochondria, it was found that MTS enhances the mitochondrial binding of MTS-modified liposomes which was dependent on MTS-concentration.

Table 2.4: Summary of different mitochondria targeted drug delivery systems for the treatment of diabetes

S. No.	Drug delivery system	Drug used	Targeting moiety used	Observed pharmacological effects	Toxicity	Reference
1	PLGA nanoparticles	2,4-dinitrophenol	TPP	Significant reduction in lipid accumulation and suppressed adipocytic differentiation of 3T3-L1 cells at a low concentration (1 or 4μM).	Not cytotoxic in 3T3-L1 cells.	Das et al. 2013
		Insulin	TPP	Reversal of diabetes in arsenic induced diabetes.	-----	Samadder et al. 2013
2	Gold nanoparticles	Cy5-labeled thiol-modified oligonucleotides	Rhodamine 123	Penetration into the deep layers of islets, reversal of diabetes and maintenance of steady body weight.	No change in mitochondrial integrity.	Vega et al. 2010
3	Nanoparticle-delivered biosensor	-----	Oligonucleotide	ARE-tethered nanoparticle was capable of detecting the cellular response to the diabetic condition in the eye.	-----	Prow et al. 2008
4	Liposomal nanocarrier	-----	Mitochondrial targeting signal peptide	Enhanced mitochondrial binding and activity of MTS-modified liposomes.	-----	Yamada et al 2012

TPP – triphenylphosphonium, MPP – mitochondrial membrane potential, α-CEHC - 2,5,7,8-tetramethyl-2-(29-carboxyethyl)-6-hydroxychroman, SkQ1 - 10-(6'-plastoquinonyl)decyltriphenylphosphonium, PLGA - poly (D,L-lactic-co-glycolic acid), Cy5-labeled thiol-modified oligonucleotide - 5' -Cy5-GAG CTG CAC GCT GCC GTC AAA AAA AAA A-SH-3' and EGCG - (−)-epigallocatechin-3-gallate.

2.3.3. **Drug delivery system selected for targeting mitochondria**

Solid lipid nanoparticles (SLN) (Figure 2.7) are particles having nano-size in the range of 1 to 1000 nm and prepared using solid lipids. Liquid lipids are replaced by solid lipids to provide improved technology transfer and scale-up, enhancement of oral bioavailability and reduction in plasma profile variability. Lipids used for preparing SLN are solid at room temperatures. Examples of solid lipids are tristearin, glyceryl monosterate, trilaurin, and trimystrin. SLN were introduced in the year, 1991. SLN have several advantages over traditional colloidal nano-carriers such as biocompatibility of lipids, less toxicity of the excipients used and enhanced stability of the formulation for longer period of time (Muller et al. 2000, Mehnert and Mader 2001).

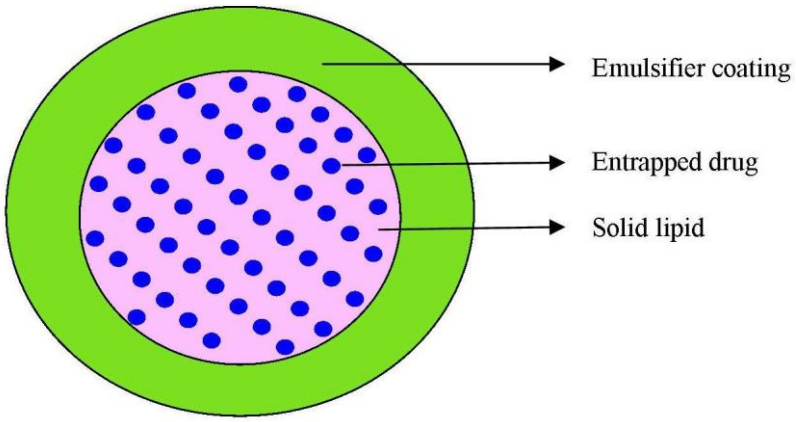


Figure 2.6: Structure of SLN

Lipids used for preparing SLN are listed in the Table 2.5. Emulsifiers used for stabilizing SLN include soybean lecithin, egg lecithin, phosphatidyl choline, poloxamer, polysorbate, sodium cholate, sodium tauracholate and sodium taurodeoxy cholate.

Table 2.5: Lipids used in SLN preparation

Board class	Examples
Triglycerides	Tricaprin
	Trilaurin
	Trimyristin
	Tripalmitin
	Tristearin
	Hydrogenated Coco-glycerides
Hard fat types	Witepsol
	Behanic acid
	Palmitostearate
	Stearic acid
	Palmitic acid
Acyl glycerols	Decanoic acid
	Glyceryl monostearate
	Glyceryl behanate
	Glycerl palmitostearate

Advantages of SLN:

1. Improved stability
2. Enhanced drug entrapment/loading
3. Enhanced bioavailability
4. Improved biocompatibility of lipids
5. Easy scale up
6. Commercial sterilization process can be applied for parenterals
7. Controlled release of entrapped drugs
8. Avoidance of organic solvents
9. Both lipophilic and hydrophilic drugs can be entrapped into lipids in SLN.

2.4.Excipients profile

2.4.1. Triphenylphosphonium

Structure

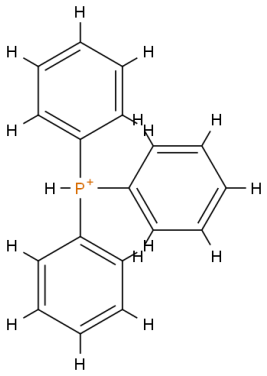


Figure 2.7: Structure of triphenylphosphonium

Molecular formula

$C_{18}H_{16}P$

Molecular weight

263.293 Da

Solubility

It dissolves in non-polar organic solvents such as benzene and diethyl ether.

Description

It exists as relatively air stable, colorless crystals at room temperature.

Few examples of research works employing triphenylphosphonium for mitochondria targeting in the treatment of diabetes are listed in Table 2.6.

Table 2.6: Examples of research works employing triphenylphosphonium for mitochondria targeting in the treatment of diabetes

S. No.	Drug delivery system	Drug used	Targeting moiety used	Observed pharmacological effects	Reference
1	Conjugated system	Ubiquinol	TPP	Reduction in intracellular glucose concentration in retinal capillary pericytes.	Mustapha, Tarr et al. 2010
			TPP	Prevention of tubular injury in db/db mice by regulating mitochondrial function.	Xiao et al. 2017
		Vitamin E	TPP	Prevention of loss of MMP and activation of complex I and aconitase.	Dhanasekaran et al. 2004
		α-CEHC	TPP	Reduction in ROS production in hyperglycemic cells.	Mossalam et al. 2013
		MitoTempol and Mitoquinone	TPP	Protects survival and function of pancreatic β-cell lines under glucotoxic and glucolipotoxic conditions.	Lim et al. 2011
2	PLGA nanoparticles	2,4-dinitrophenol	TPP	Significant reduction in lipid accumulation and suppressed adipocytic differentiation of 3T3-L1 cells at a low concentration (1 or 4μM).	Das et al. 2013
		Insulin	TPP	Reversal of diabetes in arsenic induced diabetes.	Samadder et al. 2013

2.4.2. Glyceryl monostearate

Synonym

Glyceryl stearate, Monostearin

Structure

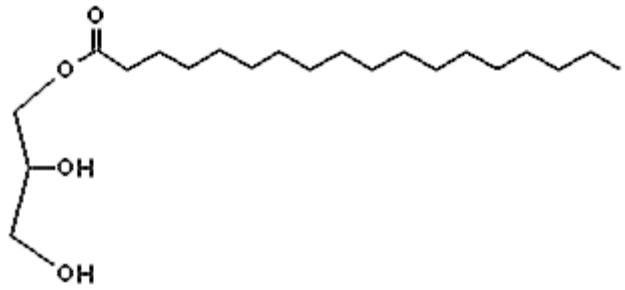


Figure 2.8: Structure of glyceryl monostearate

Chemical name

3-Stearoyloxy-1,2-propanediol; Glyceryl stearate; Alpha-Monostearin; Monostearin; Octadecanoic acid, 2,3-dihydroxypropyl ester; Glycerin 1-monostearate; Glycerin 1-stearate; Glycerol alpha-monostearate; Glyceryl 1-monostearate; Stearic acid alpha-monoglyceride; Stearic acid 1-monoglyceride; 1-Glyceryl stearate; 1-Monostearin; 1-Monostearoylglycerol; 1,2,3-Propanetriol 1-octadecanoyl ester.

Molecular formula

$C_{21}H_{42}O_4$

Molecular weight

358.56 g/mol

Functional category

Emulsifying agent

Description

White or cream colored waxy solid.

Properties

Physical state	:	white powder
Melting point	:	63 - 68 ^{o}C
Boiling point	:	> 100 ^{o}C
Solubility in water	:	soluble in hot water
Solvent solubility	:	soluble in methanol and chloroform mixture
HLB value	:	5.0

Stability and storage conditions

It is stable under ordinary conditions, and should be stored in a well-closed container and protected from light.

Safety

It is generally regarded as an essentially non-toxic and non-irritant material at the levels employed as an excipients.

Handling precautions

Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe dust.

Regulatory status

Induced in the FDA inactive ingredients. Recognized by GRAS status (Rowe et al. 2006).

2.4.3. Compritol 888 ATO

It is a marketed product from Gattefosse GmbH (Weil am Rhein, Germany) based on glycerol esters of behenic acid. It is a mixture of glycerol behenic acid with a fraction of 12-18 % mono, 52-54 % di- and 28-32 % triglycerides. The fatty acid fraction consists of greater than 87% behenic acid. (Docosan acid).

Synonym

Glyceryl behenate

Structure

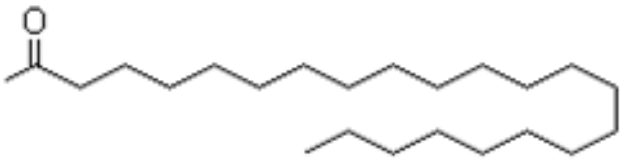


Figure 2.9: Structure of compritol ATO 888

Chemical name

2,3-Dihydroxypropyl docosanoate, docosanoic acid, 2,3-dihydroxypropyl ester, docosanoic acid, ester with 1,2,3-propanetriol, docosanoic acid, monoester with 1,2,3-propanetriol.

Molecular formula

$C_{25}H_{50}O_4$

Molecular weight

414.662 g/mol

Category

Emulsifier

Functional category

Lubricant and a sustained release agent for oral dosage forms

Description

White or cream colored waxy solid

Properties

Melting point	:	69-74 ^{o}C
Boiling point	:	> 100 ^{o}C
Solubility in water	:	soluble in hot water
Solvent solubility	:	soluble in methanol and chloroform mixture

HLB value : 2.0

Density : 0.94g/cm^3

Stability and storage conditions

It should be stored in a well-closed container and protected from light.

Safety

It is generally regarded as a relatively nonirritant and nontoxic material.

Handling precautions

Glyceryl behenate emits acrid smoke and irritating fumes when heated to decomposition.

Regulatory status

GRAS listed (Rowe et al. 2006).

2.4.4. Poloxamer 188

Synonym

Lutrol F 68, Pluronic F 68

Structure

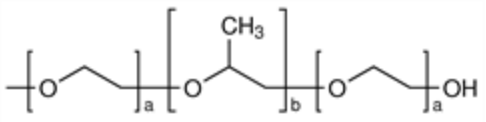


Figure 2.10: Structure of poloxamer 188

Chemical name

Polyethylene-Polypropylene Glycol

Molecular formula

$HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$

Molecular weight

7100 - 8400.00 Da

Functional category

Emulsifying agent

Description

White to off white granules

Properties

Physical state	:	white powder
Solubility in water	:	soluble in water
Solvent solubility	:	soluble in methanol and chloroform mixture
HLB value	:	29.0

Stability and storage conditions

It is stable under ordinary conditions, and should be stored in a well-closed container and protected from light.

Safety

It is generally regarded as an essentially non-toxic and non-irritant material at the levels employed as an excipients.

Handling precautions

Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe dust (Rowe et al. 2006).

2.4.5. Polysorbate 20

Synonym

Armotan PML 20, Capmul POE-l, TW 20, T-MAZ-20, Tween 20.

Structure

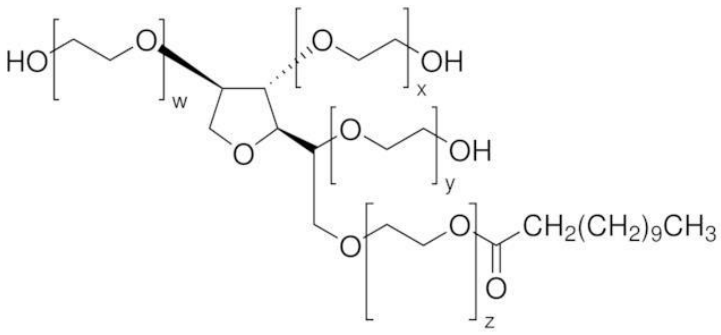


Figure 2.11: Structure of polysorbate 80

Chemical name

Polyoxy ethylene 20 sorbitan mono laurate sorbitan mono decanoate

Molecular formula

$C_{58}H_{114}O_{26}$

Molecular weight

1128 g/mol

Description

It is Yellow oily liquid and having characteristic odour and bitter taste

Properties

Density (g/cm^3) – $1.1 g/cm^3$

HLB Value – 16.7

Solubility – Soluble in ethanol and water. Insoluble in mineral oil and vegetable oil.

Functional category

Emulsifying agent, non-ionic surfactant, solubilizing agent, wetting agent

Uses

It is used to improve oral bioavailability of drug molecule and widely used in cosmetics and food products.

Storage

It should be stored in a well-closed container and protected from light.

Safety

Prolonged storage leads to the formation of peroxides. Stable to electrolytes and weak acids

and bases, Gradual saponification occur with strong acids or bases. It is hygroscopic in nature.

Uses

Polysorbate 20 is widely used in cosmetics, food products, oral, parenteral and topical pharmaceutical formulations and generally regarded as non-toxic and non-irritant materials.

Handling precautions

Eye protection and gloves are recommended (Rowe et al. 2006).

2.4.6. Lecithin

Soybean lecithin contains 21% phosphatidylcholine, 22% phosphatidylethanolamine, and 19% phosphatidylinositol, along with other components

Synonym

Mixed soybean phosphatides; Phosal 53 MCT; Phospholipon 100 H; soybean lecithin; soybean phospholipids; Sternpur; vegetable lecithin.

Chemical name

(2-nonanoyloxy-3-octadeca-9,12-dienoyloxypropoxy)-[2-trimethylazaniumyl)ethyl]phosphinate

Molecular formula

$C_{35}H_{66}NO_7P$

Structure

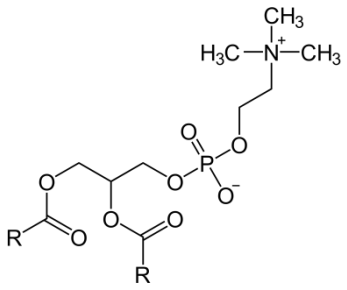


Figure 2.12: Structure of lecithin

Molecular weight

643.887 g/mol

Functional category

Emollient; emulsifying agent; solubilizing agent.

Description

Lecithins vary greatly in their physical form, from viscous semiliquids to powders, depending upon the free fatty acid content. They may also vary in color from brown to light yellow, depending upon whether they are bleached or unbleached or on the degree of purity

Properties

Lecithins are soluble in aliphatic and aromatic hydrocarbons, halogenated hydrocarbons, mineral oil, and fatty acids. They are practically insoluble in water.

Stability and storage conditions

Fluid or waxy lecithin grades should be stored at room temperature or above; temperatures below 108C may cause separation. All lecithin grades should be stored in well-

closed containers protected from light and oxidation. Purified solid lecithins should be stored in tightly closed containers at subfreezing temperatures.

Handling precautions

Lecithins may be irritant to the eyes; eye protection and gloves are recommended.

Regulatory status

GRAS listed (Rowe et al. 2006).

2.4.7. Sodium deoxycholate

Sodium deoxycholate is a bile acid formed by bacterial action from cholate. It is usually conjugated with glycine or taurine. It is used as a choleretic and detergent.

Synonym

Sodium deoxycholic acid; deoxycholate, sodium salt

Structure

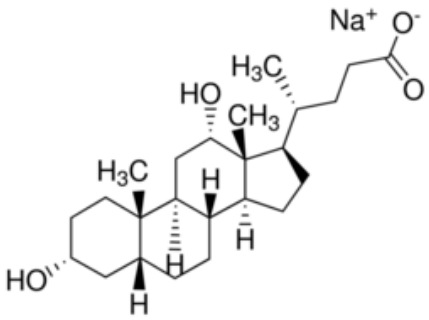


Figure 2.13: Structure of sodium deoxycholate

Chemical name

3, 12-α-Dihydroxy-5β-cholan-24-oic acid, monosodium salt

Molecular formula

$C_{24}H_{39}NaO_4$

Molecular weight

414.562 g/mol

Functional category

Emulsifier

Description

Sodium deoxycholate is an ionic detergent and it comes in a white to off-white crystalline powder form.

Properties

Sodium deoxycholate is soluble in alcohol and acetic acid (Rowe et al. 2006).