Diabetes mellitus

The World Health Organization estimates that 171 million people over the world have diabetes and that number is expected to double by 2030 (Wild et al. 2004). It’s one of the most common chronic diseases in the world, and it’s getting more common every day. According to an estimate (Anjana et al. 2011), in the year 2011, 62.4 million people are suffering with diabetes. In Maharashtra state alone, 6 million individuals are estimated to be suffering with diabetes (Anjana et al. 2011). Fig. 1 shows a map depicting spread of diabetes across Indian states. Sadly, India is fast becoming the “diabetes capital of the world” (Mohan et al. 2007).

Figure 1: Map showing spread of diabetes in Indian population across different states. (Source: Mohan et al. (2007)).
The major reason behind pandemic spread of diabetes is changed lifestyle of the population. It is said that “genes load the gun; but lifestyle pulls the trigger” (Bray 2004). Lack of exercise, stress, overindulge of fast food and obesity etc. are some of the possible reasons that precipitate diabetes. Diabetes is a disorder of sugar metabolism, specifically the blood sugar known as glucose. Glucose is produced when the body breaks down carbohydrates in the food. Glucose is essential for survival as it provides fuel for the brain; it’s required to manufacture proteins; it’s what we use to make energy when we need it. With the help of insulin, a hormone made by the pancreas, glucose is stored in the liver, muscles, and fat cells waiting to be converted to fuel as necessary.

Figure 2: Main symptoms of diabetes. Diabetes induced hyperglycemia is one of the reasons behind these symptoms (source: http://royalayurveda.com/diabetes/).

The full name of the disease is actually diabetes mellitus which literally means “passing through honey sweet.” Fig. 2 shows main symptoms of diabetes mellitus. One of the first outward manifestations of diabetes is the need to pass large amounts of sugary urine. And for thousands of years, observers have noticed that diabetics’ urine smells (and tastes) particularly sweet. In the past Chinese physicians actually diagnosed and monitored
diabetes by looking to see whether ants were attracted to someone’s urine. In diabetics, the process through which insulin helps the body use glucose is broken, and the sugar in the blood builds up to dangerously high levels.

Unmanaged, these abnormal blood sugar levels can lead to rapid dehydration, coma, and death. Even when diabetes is tightly managed, its long term complications include blindness, heart disease, stroke, and vascular disease that often lead to gangrene and often needs amputation of legs in diabetics.

Cyclodextrins

Cyclodextrins are cyclic oligosaccharides consisting of 6, 7 or 8 α-D-glucopyranose units in which the glucose units are linked by α1-4 linkage. The eight-member ring is called γ-cyclodextrin. Cyclodextrins form inclusion complexes with the hydrophobic guest molecules of appropriate size. Cyclodextrins, cyclodextrin derivatives, inclusion complexation process and characterization of complexes are widely discussed by Szejtli (1996).

Why γ-cyclodextrin?

Previous literature shows that attempts have been made to prepare cyclodextrin inclusion complexes with quercetin. For example, Zheng et al. (2005) have described preparation and stability of quercetin-β-cyclodextrin inclusion complexes. A spray dried inclusion complex of quercetin with β-cyclodextrin has been described by Borghetti et al. (2009). According to the FAO/WHO Joint Expert committee on Food Additives recommendations (JECFA 1995), the daily oral dose of β-cyclodextrin should not be beyond 6 mg/kg body weight. A small part of β-cyclodextrin is absorbed from the gastro-intestinal tract and may cause irreversible damage of the kidneys. All quercetin-β-cyclodextrin complexes described in literature exceed this daily intake of 6 mg/kg. Therefore, oral quercetin formulations containing β-cyclodextrin are not feasible.
The obvious solution to this problem is to replace \( \beta \)-cyclodextrin with another safer cyclodextrin. \( \gamma \)-Cyclodextrin is more tolerated than \( \beta \)-cyclodextrin when administered orally (JECFA 1995). The oral LD\(_{50}\) of \( \gamma \)-cyclodextrin in rats has been shown as high as 8 g/Kg. United States Food and Drug Administration have awarded GRAS (Generally Recognized as Safe) status to \( \gamma \)-Cyclodextrin (Rowe et al. 2005). The GRAS status means the substance can be used as an excipient in drug products and foods. Gamma cyclodextrin is known to have largest internal molecular cavity amongst all cyclodextrins (Li et al. 2007). The larger internal cavity makes it easier to form inclusion complexes with \( \gamma \)-cyclodextrin (Li et al. 2007).

In the present study, an inclusion complex of quercetin with \( \gamma \)-cyclodextrin was prepared. The antihyperglycemic activity of quercetin-\( \gamma \)-cyclodextrin inclusion complex was then compared with pure quercetin and a positive control, glibenclamide. A novel chick embryo model was developed and used for antihyperglycemic studies.

**Cyclodextrin-guest inclusion complexation**

The beta and gamma cyclodextrins and several of their derivatives are unique in having the ability to form molecular inclusion complexes with hydrophobic drugs having poor aqueous solubility. These cyclodextrin molecules are versatile in having a hydrophobic cavity of size suitable enough to accommodate the lipophilic drugs as guests; the outside of the host molecule is relatively hydrophilic. Thus, the molecularly encapsulated drug has greatly improved aqueous solubility and dissolution rate (Martin et al. 1993).

Cyclodextrin complexation improves the stability of drugs in a formulation resulting in longer shelf life. They are available in highly pure forms and widely used as pharmaceutical excipients for increasing solubility, bioavailability, stability. Cyclodextrin inclusion complexes have gained gradual acceptance by various regulating bodies (Otero-Espinar et al. 2010). The cyclodextrins can be considered as an empty cavity of molecular size.
When the cavity is filled with a guest molecule, it is called an “inclusion complex”. The molecules in the inclusion complex are maintained in their position by physical forces alone, without covalent binding (Szejtli 1996). Moreover, the dissociation-association equilibrium is probably the most characteristic feature of the host-guest association (Szejtli 1996).

**Mechanism of cyclodextrin inclusion**

Since the cavity of cyclodextrin is hydrophobic, the inclusion of a molecule in the cyclodextrin cavity is basically a substitution of the water inside the cavity with a less polar substance. The substitution of water from the cavity with a more non-polar guest is energetically favorable for both cyclodextrin and the guest (Szejtli 1996). The “driving force” in the complexation is due to combination of different effects depending on the specific guest and cyclodextrin. The effects can be hydrophobic interaction, *van der waals* interaction, hydrogen bonding, dipole-dipole interaction and release of “enthalpy-rich” water (Wiebe *et al.* 2008).

**Why quercetin?**

Quercetin (Fig. 3) belongs to the chemical class of flavonoid and is widely distributed in vegetables and plants. It has been demonstrated to possess a wide array of biological effects that are considered beneficial to health, including antioxidant, free radical scavenging, anticancer, and antiviral activities (Formica & Regelson 1995). Highest concentration of quercetin is found in red grapes (Fig. 4). Previous literature shows antidiabetic and antihyperglycemic activity of quercetin in rats (Asp *et al.* 2006).
Figure 3: Structure of quercetin.

Figure 4: Red grapes are fruits having high concentration of quercetin. Improvement of membrane permeation of quercetin and thereby enhancement of its bioactivity was objective of present study.

Quercetin itself (aglycone quercetin), as opposed to quercetin glycosides, is not a normal dietary component. Quercetin is sparingly soluble in water, which has limited its absorption upon oral administration. In addition, it is chemically unstable, especially in aqueous alkaline medium (Makris & Rossiter 2000), although acidic conditions can offer it some protection against degradation. The compound is also known to undergo extensive metabolism in the gut and the liver following absorption (Chen et al. 2001), and the resulting metabolites still retain some biological activity (Manach et al. 1998). All these problems lead to an extremely low oral bioavailability of quercetin (based on the unchanged quercetin) in human (Gugler et al. 1975). Quercetin is available in different dosage forms: powder,
tablet and capsules. Some representative products are shown in Figs. 5A, 5B and 5C.

Quercetin is one of the cheapest flavonoids available in the market. In the present study, it was postulated that if membrane permeation of quercetin is improved, higher bioavailability can be achieved. It was further postulated that higher bioavailability would result in increased antihyperglycemic activity of quercetin, thereby reducing the dose required for activity.

![Marketed quercetin products](image)

**Figure 5A:** Marketed powder dosage form of quercetin (Source: natrol.com)  
**Figure 5B:** Marketed tablet dosage form of quercetin (Source: holisticheal.com)  
**Figure 5C:** Marketed capsule dosage form of quercetin (source: shopme.com).

**Problems with biological membrane permeation of natural products**

It is not uncommon for manufacturers to identify a natural substance that has been shown to have beneficial properties and undertakes to develop a simple capsule or tablet formulation (Ganesan 2008). However, they typically display little regard, and probably little understanding, of the absorption and bioavailability profile for their product formulation (Zhang et al. 2009). This could be because they assume that all natural substances are bioavailable, but more likely because the cost structure of the bioactive natural product market doesn’t support expensive formulation research.

Notwithstanding this general disregard for the bioavailability of natural products, there are a number of extremely valuable bioactive natural
substances that are poorly soluble in water, not easily absorbed from the intestines, and demonstrate low bioavailability in conventional formulations. One example is coenzyme Q10 (ubiquinone), which has been the subject of intense scrutiny in the United States and Japan (Meng et al. 2012). The importance of achieving and maintaining high blood levels of CoQ10 to achieve a desired clinical effect has been well-documented in the scientific literature (Beg et al. 2010). Another of these extremely valuable substances is quercetin, which is poorly soluble in water and has low bioavailability in powder-based (i.e. hard gelatin capsules and tablets) formulations (Wiczkowski et al. 2008; Reinboth et al. 2010). The importance of increasing biological membrane permeability of quercetin to achieve a desired biological/clinical effect at a lower dose has received little scientific attention, and is virtually unknown in the marketplace.

**Hyperglycemia and its induction**

Several methods have been used to induce hyperglycemia in laboratory animals with variable success and many difficulties. Surgical removal of the pancreas is effective method; however, to induce diabetes, at least 90-95% of the pancreas has to be removed (Lerch et al. 1994). Injection of anterior pituitary extract has been used to induce diabetes with less reliable results (GUPTA et al. 1962). Another method which is more uniformly effective and widely used is the injection of streptozotocin.

**Why streptozotocin?**

Streptozotocin (N-nitro derivative of glucosamine) is a naturally occurring, broad spectrum antibiotic and cytotoxic chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals (Weiss 1982; Szkudelski 2001; Hayashi et al. 2006; Takeshita et al. 2006). Induction of experimental hyperglycemia in the rat using streptozotocin is
very convenient and simple to use (Brosky & Logothetopoulos 1969; Weiss 1982; Ito et al. 1999). Streptozotocin injection leads to the degeneration of the Langerhans islets beta cells (Weiss 1982; Smith et al. 1983; Ikebukuro et al. 2002). Clinically, symptoms of diabetes are clearly seen in rats within 2-4 days following single intravenous or intraperitoneal injection of 60mg/kg streptozotocin (Ganda et al. 1976; Elias et al. 1994).

Why antihyperglycemic activity?

India having the highest number of diabetic patients in the world, the sugar disease is posing an enormous health problem in the country. Calling India the diabetes capital of the world, the International Journal of Diabetes in Developing Countries says that there is alarming rise in prevalence of diabetes, which has gone beyond epidemic form to a pandemic one.

The International Diabetes Federation estimates that the number of diabetic patients in India are more than doubled from 19 million in 1995 to 40.9 million in 2007 (Wild et al. 2004). It is projected to increase to 69.9 million by 2025 (Wild et al. 2004). Currently, up to 11 per cent of India’s urban population and 3 per cent of rural population above the age of 15 have diabetes (Ram et al. 2006). Diabetes affects all people in the society, not just those who live with it. Hyperglycemia is hallmark of diabetes. A reduction in blood glucose levels goes a long way in maintenance of homeostasis and prevents further diabetic complications. Many synthetic drugs such as sulphonylurea are currently used in clinical management of diabetes. The oral antihyperglycemic agents currently used in clinical practice have characteristic profiles of serious side effects (Correia et al. 2008). This leads to increasing demand for herbal products with anti-diabetic activity and less side effects (Yoshioka et al. 1989).
Antihyperglycemic activity of quercetin

Quercetin (a flavonoid), used in doses of 15–50 mg/kg body mass was found capable of normalizing blood glucose level, augmenting liver glycogen content and significantly reducing serum cholesterol and LDL concentration in alloxan induced diabetic rats (Zapolska-Downar et al. 2006). Quercetin has been shown to protect pancreatic β islet cells of against streptozotocin induced damage in rats (Mahesh & Menon 2004; Coskun et al. 2005). Intraperitonial injection of quercetin has been shown to attenuate increased blood glucose levels in streptozotocin treated rats (Vessal et al. 2003). The study also describes attenuation of decreased triglyceride levels in rats (Vessal et al. 2003). The literature shows potential of quercetin as a possible antihyperglycemic agent.

However, the therapeutic application of quercetin has been limited due to its low solubility and resultant low permeability (Azuma et al. 2002; Bertrand et al. 2006). Hence attempts to improve its solubility and permeability are needed for realizing true therapeutic benefits of cyclodextrin.

Role of γ-cyclodextrin in hyperglycemia

Since γ-cyclodextrin is an inactive ingredient in many pharmaceutical formulations (Rowe et al. 2005), γ-Cyclodextrin does not show any effect on blood glucose levels by itself. However, inclusion complex formation with γ-cyclodextrin has been known to increase membrane permeation of guest molecules (Jansook et al. 2010). Increased membrane permeation due to inclusion complex formation with γ-cyclodextrin has been assumed to increase biological activity of drugs (Mosher 2007; Jansook et al. 2010). In the present study, complex formation with γ-cyclodextrin was postulated to improve dissolution of quercetin. The improvement in dissolution was further postulated to enhance antihyperglycemic activity of quercetin.
Two possible mechanisms were evaluated for understanding how \( \gamma \)-cyclodextrin improves membrane permeation and antihyperglycemic activity. The first mechanism postulated that \( \gamma \)-cyclodextrin might enhance vascular perfusion enhancing activity of quercetin, thereby increasing membrane permeability of quercetin. The validity of this hypothesis was tested in chapter 3 of the present study. Chick chorioallantoic membrane was used as model biological membrane. Vascular blood perfusion in treated membranes was studied with infrared thermal imaging followed by semiautomatic pixel quantification.

Another postulated mechanism was based on the fact that quercetin occurs in nature as glucosides. Quercetin molecule without attached glucose moieties is called quercetin aglycone. In general, quercetin glucosides have been shown to be more absorbed from intestines (a biological membrane). A previous study has shown that in humans, quercetin glucosides were absorbed within 30 min of ingestion, apparently in preference to quercetin aglycone (Hollman et al. 1995). To explain this, it was proposed that the glucose moiety may enable flavonoid glycosides to be transported by the sodium-dependent glucose transporter 1 (SGLT1) (Hollman et al. 1996). In the present study, it was hypothesized that \( \gamma \)-cyclodextrin might play a similar role as that of glucose moieties in quercetin glucosides and interact similarly with SGLT. This hypothesis was tested by molecular modeling and docking studies performed in chapter 4.

**Chick embryo models**

Rat models are frequently used for preclinical evaluation of new drugs and delivery systems. Mammalian models are costly, time consuming, and difficult to evaluate. One requires to obtain various administrative and ethical permissions before using them. Nowadays, the pharmaceutical field faces an ever-growing demand for making innovative formulations that are able to "Intelligently" deliver modern active compounds (Vargas et al. 2007). The development of high-throughput strategies for the discovery, synthesis, and
screening of drugs, as well as the advances in genomics and proteomics, have resulted in a huge amount of new drug candidates. The increasing interest in the chick embryo as a model in biological and pharmaceutical research is related to its simplicity and low cost compared with mammalian models (Tay et al. 2011). Current laws regulating animal experimentation in the USA, the European Union, and Switzerland allow experimentation with chick embryos without authorization from animal experimentation committees, on the grounds that experiments begin and end before hatching.

The Committee for Purpose of Control and Supervision of Experiments on Animals, also called as CPCSEA in India also has given clarification that chick embryo models do not need any approval from Institutional Animal Ethics Committee or CPCSEA (Tay et al. 2011). The in ovo model forms an alternate system for evaluating the novel drugs in screening procedures of formulation candidates, thus establishing an intermediate step between in vitro cellular tests and preclinical mammalian models.

**Why chick embryo?**

Next objective was to find whether postulated increase in biological membrane perfusion results in enhancement of antihyperglycemic activity of quercetin. The streptozotocin induced hyperglycemia in chick embryos was used for evaluation of antihyperglycemic activity. The model was selected on the basis of ease of handling and possibility of placing drug on a vascular biological membrane. This way, the system also modeled drug permeation through chorioallantoic membrane (a biological membrane).
To address the issues raised above, the present work is presented through following chapters:

**Chapter 1**

This chapter introduces field of study. Classification, properties and rationale for selection of $\gamma$-cyclodextrin and quercetin has been explained with relevant literature. The chapter describes current issues in bioavailability of quercetin and methods employed in previous literature to overcome them. The chapter also introduces membrane perfusion studies with infrared thermography and docking studies. A brief review of chick embryo as a model for hyperglycemia has been presented. The chapter defines general aims and objectives of the present study.

**Chapter 2**

This chapter describes preparation of solid inclusion complex with $\gamma$-cyclodextrin. A novel, low cost, homemade cyclic voltammetric apparatus was designed and fabricated for estimation of quercetin in hydroalcoholic solutions containing $\gamma$-cyclodextrin. The developed apparatus was used for two purposes: one for optimization of proportion of quercetin to $\gamma$-cyclodextrin for formation of inclusion complex, and another for estimation of quercetin content in the formed complex. Complete formation of the inclusion complex between quercetin and $\gamma$-cyclodextrin was ascertained with differential scanning calorimetry (DSC) and differential thermal analysis (DTA) methods. A novel, low cost, homemade differential thermal analysis (DTA) instrument was designed and fabricated for this purpose.

**Chapter 3**

This chapter describes the study performed to find whether increases in vascular perfusion in chick embryo chorioallantoic membrane were associated with quercetin, $\gamma$-cyclodextrin or inclusion complex. The
chorioallantoic membrane was used in the present study as a model biological membrane. It was hypothesized that increase in vascular perfusion in the biological membrane by γ-cyclodextrin has a role in increase in membrane permeability of quercetin. A CCD web camera was modified by removal of its infrared filter. The images of treated chorioallantoic membrane were captured by the infrared sensitive camera thus produced were processed with advanced thermal imaging software. The vascular perfusion in selected areas of the captured images was quantified with pixel quantification using Adobe Photoshop™.

Chapter 4
This chapter describes molecular modeling and docking studies performed to understand effect of γ-cyclodextrin complexation on active transport of quercetin by sodium linked glucose transporter (SGLT). Quercetin glucosides are transported across intestinal epithelium by active transport. Quercetin is insignificantly transported by SGLT1 and shows lesser intestinal absorption than its glucosides. In silico molecular modeling and docking studies provided an accurate and cost effective method to understand these interactions. X-ray crystal structure of sodium dependent glucose transporter isolated from Vibrio parahaemolyticus (vSGLT) was used as a model for docking studies. The test ligands including quercetin-γ-cyclodextrin, quercetin-3,4'-glucoside, quercetin-3-glucoside, and quercetin were docked in predicted active site of vSGLT.

Chapter 5
This chapter describes studies performed to find antihyperglycemic effect of quercetin-γ-cyclodextrin inclusion complex vis-à-vis quercetin per se against streptozotocin induced hyperglycemia in chick embryos. The study was conducted as three experiments. In experiment 1, normal blood sugar of chick embryos was determined from day 12 to day 14 of incubation.
Experiment 2 was performed for optimization of conditions required to induce streptozotocin induced hyperglycemia in chick embryos. The studies involved optimization of embryo age and development of suction technique for dislodging 14 day embryos from egg shell. The optimization was followed by determination of antihyperglycemic activity of quercetin-γ-cyclodextrin inclusion complex, quercetin dihydrate, and glibenclamide against streptozotocin induced hyperglycemia in chick embryos. Effects of various treatments against streptozotocin induced changes in gross appearance of 17 day old chick embryos were also studied.

Chapter 6
This chapter summarizes studies described in earlier chapters. Major conclusions of each study are described. The interrelationship of various results obtained in separate chapters has been correlated. The chapter comments on social and economic relevance of the study and discusses major contribution of the study to the field of research. The chapter suggests scope for further research; building on results of this study.
REFERENCES


