Chapter 7

Summary and Conclusions
INTRODUCTION

Strawberry (*Fragaria X ananassa* Duch.) is a hybrid species and an important berry crop which is cultivated worldwide for its fruits. Strawberries have been reported to possess high antioxidant activity due to presence of polyphenols and flavonoid compounds like anthocyanins, flavonols, ellagitannins etc. These phenols and flavonoids are excellent antioxidants and effectively inhibit free radicals.

It has been reported that consuming polyphenols either in the form of fruits or vegetables increases insulin activity/sensitivity and decreases the risk of type 2 diabetes. Some flavonoids are actively involved in improving disease conditions by activating glyoxalase 1, removing advanced glycation end products (AGEs), reducing oxidative stress, lowering blood glucose levels etc. This may in turn prevent further complications of diabetes. Significant antioxidant and anti-inflammatory functions have been associated with strawberries as they are good sources of phytochemicals, polyphenols, flavonoids and anthocyanins. Anthocyanins also protect pancreatic beta cells from glucose induced oxidative stress.

The present thesis consists of the biochemical analysis of two cultivars viz. *Sweet Charlie* and *Camarosa* for their antioxidant and radical scavenging activity, phenol and flavonoid content and evaluation of various antioxidant enzymes from fruits at progressive developmental stages. Flavonoid biosynthesis pathway was studied at molecular level. Expression profiles of genes involved in flavonoid biosynthesis pathway were studied at nine fruit developmental stages of two commercially important cultivars. The mature fruits were also evaluated for its nutraceutical potential such as antihyperglycemic activity at biochemical, histological and molecular levels.

METHODOLOGY

The strawberry fruits were collected from Mahabaleshwar and Panchagani region after getting consent from the farmers. These regions are main producers of strawberry in India. Strawberry fruits were collected during progressive developmental stages of ripening. The flowers were tagged on the day of anthesis and fruits were harvested every alternate day (considered as day(s) after flowering - DAF). The fruits were immediately frozen in liquid nitrogen and they were kept at -80°C till further analysis. Information regarding the farming practices was also collected from farmers.
OBJECTIVES

1) To study flavonoid content and antioxidant potential of strawberry cultivars during fruit development.

2) To study changes in expression levels of genes from flavonoid biosynthesis pathway during strawberry fruit development.

3) To study effect of strawberry extracts on nicotinamide-streptozotocin induced diabetes in Wistar rats.

Following objectives were addressed in the present study:

1) To analyze the strawberry fruits during progressive stages of maturation, for antioxidant and antidiabetic potential and antioxidant enzymes.

   The collected fruits were subjected to different in vitro anti-oxidant and anti-diabetic assays. Variations in the activities of enzymes such as catalase, peroxidase and polyphenol oxidase were also evaluated during progressive stages of fruit development.

2) To study the expression of genes involved in flavonoid biosynthesis pathway during fruit development.

   Literature about flavonoid biosynthesis genes were searched online using available databases. The sequences of the genes were downloaded and subjected to in silico identification of conserved regions. The conserved regions of the sequences were used for primer designing. RNA was isolated from frozen fruits by using commercially available kits or reagents, as per the manufacturer’s protocol. cDNA was synthesized using commercially available kits. Normalized concentrations of cDNAs were used for expression studies. The expression pattern(s) were studied using Real Time PCR with SyBr green chemistry.

   The full length anthocyanidin reductase gene was cloned using specific primers. Amplicons were eluted from the gel using extraction kit and were ligated in the pGEMT vector. The vectors were transformed in the competent E. coli cells. The overnight grown colonies were selected by blue/white screening and recombinant colonies were cultured in LB media. The cultures were subjected to plasmid isolation. The quality and integrity of plasmid DNA was checked on agarose gels and the plasmid DNA was subjected to restriction digestion for confirmation of insert. Plasmid DNA from the cultures, showing presence of insert, was sequenced. The
obtained sequence was subjected to in silico analysis for its similarity and conserved domain search.

3) To assess the in vivo effect of strawberry extract on streptozotocin induced diabetes in Wistar rats.

Male Wistar rats were procured, after getting ethical permission from Institutional Animal Ethical Committee. The rats were treated with nicotinamide-streptozotocin to induce diabetes and the animals showing stable hyperglycemia were selected for further study. Strawberry fruit extracts (Aqueous, hydro-alcoholic and alcoholic) were administered to animals. After completion of the experiment, the animals were humanely sacrificed and serum was subjected for biochemical studies. Modulations in the gene expression levels were studied from liver tissues of the animals. Histopathological examinations of liver, kidney, brain and pancreas were also performed.

OBSERVATION AND RESULTS

A] Antioxidant activity: Strawberry fruits showed increased in antioxidant activity during fruit development

Various reports are available regarding the antioxidant activity of the enzymes. Antioxidant activity is affected by various parameters such as environment, edaphic factors, farming conditions etc.

Both the cultivars (Sweet Charlie and Camarosa) showed antioxidant activity and carbohydrate hydrolyzing enzyme inhibition activity in vitro, however cv. Sweet Charlie was found to have higher flavonoid content as compared to cv. Camarosa. During fruit development antioxidant, enzymes such as catalase and peroxidase were found to be decreased while polyphenol oxidase activity increased. There was decrease in total phenols and increase in flavonoids with fruit maturation.

B] Gene expression profile: Flavonoid biosynthesis pathway showed two peaks of gene expression, corresponding to flavonoids and anthocynidins biosynthesis

Nine stages of fruit development were evaluated for expression of genes involved in flavonoid biosynthesis pathway. Cloning of three genes namely luecoanthocynidin reductase, flavonol synthase and anthocynidin reductase were
also carried out. Gene sequence of *anthocynidin reductase* (*FaANR*) was submitted to NCBI.

Expression profile of genes involved in flavonoid biosynthesis exhibited two peaks during fruit development. The proanthocyanidin synthesizing genes (*FLS, LAR* and *ANR*) were expressed at early stages of fruit development (before DAF 6 and 8). Pigment synthesizing genes (*DFR* and *ANS*) from flavonoid biosynthesis pathway were expressed late in the fruit development. Some genes like *CHS* and *GTS* were expressed throughout development. The flavonoid biosynthesis pathway showed two distinct patterns of gene expression. The first part comprises of flavonoid biosynthesis whereas the second part of the pathway corresponded to accumulation of pigments in fruits. The expressions were drastically changed before/at the transition stages of the fruit. The expression of genes was also found to be depending upon the cultivars and environmental conditions.

C] Animal experiment: Strawberry exhibits antihyperglycemic and normolipidemic activity

Diabetes was induced by single high dose of the streptozotocin after nicotinamide treatment in Wistar rats. Aqueous, hydroalcoholic and alcoholic strawberry extracts were administrated orally to diabetic rats daily for four weeks. Treatment of strawberry extracts improved lipid profile, liver functions and serum creatinine along with significant increase in anti-oxidant status in diabetic rats. qRT-PCR from liver tissue after intervention with strawberry extracts, showed down regulation of several fatty acid synthesis genes, transcription factors [such as Sterol Regulatory Element Binding Transcription factor (SREBP) and Nuclear Factor-κB (NFκβ)] and inflammatory markers [like Interleukin 6 (IL6) and Tumor Necrosis Factor-α (TNF-α)]. Strawberry extracts also up-regulated liver Peroxisome Proliferator Activated Receptor Gamma (PPAR-γ). Histological examination confirmed the nephoprotective and β-cell regeneration/protection effects of strawberry extracts. The present study demonstrates that strawberry exhibits antihyperglycemic and normolipidemic activity and also reduces oxidative stress.
The important conclusions from the thesis are as follows:

1. Strawberry fruits showed increased antioxidant activity with fruit development
2. Flavonoid biosynthesis pathway showed two peaks of gene expressions, corresponding to flavonoid and anthocyanidin biosynthesis
3. Strawberry exhibits antihyperglycemic and normolipidemic activity in NIC-STZ induced diabetic rats through its action on transcription factors and inflammatory markers modulating the expression of lipid metabolism genes