4. Discussion

Dietary flavonoids are increasingly suggested to have the capacity of modulating the complex mechanisms involved in pathology of chronic diseases such as cancer, cardiovascular disease, diabetes, hypertension, immune and neurodegenerative disorders as well as general aging processes (Patil et al., 2003, Patel et al., 2007). In U.S., the daily dietary intake of mixed flavonoids is estimated to be in the range of 500 to 1000 mg (Kühnau, 1974), but can be as high as several grams in those supplementing their diets with flavonoids or flavonoid-containing herbal preparations such as ginkgo biloba, Pycnogenol 227 (Horphag Research Ltd., Guernsey, UK), or grape seed extract. The emerging scientific support for potential health claims and identification of active functional ingredients in leafy food plants, traditional medicinal plants and bio-prospective endemic plants, need to be appropriately balanced by addressing any toxicological concerns and mechanisms of action (Mennen et al., 2005; Sang et al., 2005; Sutherland et al., 2006). In this vein, the toxicity of selected flavonoids was investigated. The present work was designed to provide the insight about relative safety of the flavonoids; their effects on liver and kidney and reveal gene expression signatures related to hepatotoxicity at an early stage following acute dosing of selected flavonoids by using microarray technology as a predictive tool. Microarray technology is preferably used in the development of a global understanding of gene expression abnormalities that contributes to different toxicities and progression of degenerative changes, for differentiating between responsive genes in acute and chronic toxin exposure, and in delineating cell-specific gene expression patterns. Gene expression analysis along with classical parameters of toxicity increased the understanding significantly about dose specific toxicity.

However, earlier reports on selected flavonoids (Apigenin, Genistein and Quercetin) confirm their Pharmacological nature, the toxic effects are unequivocal. These reports are apparent to only their mutagenic, genotoxic, cytotoxic, pro-oxidant and moreover carcinogenic behavior. Present study addressed certain dose specific effect of selected flavonoids on liver and kidney of Swiss Albino mice. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) are established biomarkers of liver damage. From the damaged liver tissue they are subsequently released and their concentration is increased in the circulation which is an indicative of liver tissue injury (Ozer et al., 2008). Apigenin at 25 and 50 mg/kg, Genistein at 125 and 250 mg/kg, and Quercetin at

500 and 1000 mg/kg did not produce any detectable damage in both serum and liver histology.

A significant increase in the serum level of ALT, AST and ALP were found in higher dose group of 100 or 200 mg/kg bw Apigenin, 500 or 1000 mg/kg bw Genistein and 1500 or 2000 mg/kg bw Quercetin treated animals as compared to control. In higher treatment groups (1500 and 2000 mg/kg) of Quercetin, estimated LDH was found to increase significantly. Increase in hepatic biomarkers is in agreement with the earlier report following treatment of pyrogallol that significantly damaged the hepatic tissues in rats following intraperitoneal administration at 100 mg/kg dose (Gupta et al., 2002). Galati et al., 2006 also reported that plasma ALT level increased up to 4-fold in CD-1 mice following intra-peritoneal injection of flavonoids; EGCG (120 mg/kg), propyl gallate (170 mg/kg), gallic acid (500 mg/kg) and tannic acid (120 mg/kg) after 24 hr. Moreover, throughout the experiment food and water was *ad libitum*. The reason for permitting food and water throughout the experiment was to simulate normal physiological state and to obviate chances of affecting gene expression with altered physiology during fasting. Normal histo-architecture of the liver of mice in lower dose groups of flavonoids further supports the serum biochemical findings and suggest that acute exposure of mice to flavonoids doses do not induce hepatotoxicity. Hydropic changes along with ballooning and degeneration of hepatocytes are the signs of damaged liver and confirm the adverse effects of flavonoids on the liver of mice following 100 or 200 mg/kg Apigenin, 500 or 1000 mg/kg Genistein and 1500 or 2000 mg/kg Quercetin doses. The biochemical and histological results indicate that Apigenin, Genistein and Quercetin do not produce hepatic tissue damage at lower doses in their respective treatment groups used in this study following single acute treatment. However, different tissue responses may be produced if lower doses are administered for longer durations.

Serum creatinine (CRT) and blood urea nitrogen (BUN) are established biomarkers for kidney damage. In lower treatment groups of Apigenin (25 and 50 mg/kg), Genistein (125 and 250 mg/kg) and Quercetin (500 and 1000 mg/kg), there were no change observed in the level of CRT and BUN. A significant rise in the level of Creatinine and BUN indicating nephrotoxicity was observed in groups treated with 1000 mg/kg of Genistein as compared to controls. However, no change in histology of kidney was found. It might be due to the nature of the treatment. As the treatment was

of acute nature and the autopsy was done after 24 hr. Previous investigators also reported the fact that, altered level of serum markers do not always correlate well with preclinical histological changes (Ozer et al., 2008). These biomarkers were unchanged in the group of Apigenin while a non-significant increase was observed in higher treatment groups of quercetin (1500 and 2000 mg/kg). These biochemical findings may show the affect of availability of food at all times during the course of study and/or species differences. The rationale for observed difference in CRT and BUN level is based on the observation that most toxicity studies include withdrawal of food before the administration of drug in an attempt to aggravate the toxic outcomes (Lucas et al., 2000). The histo-architecture of the kidney of mice following Apigenin, Genistein and Quercetin treatments were found to be unaltered at all doses.

Previous studies have indicated the generation of reactive oxygen species (ROS) and oxidative stress following Apigenin treatment (Chan et al., 1999; Galati et al., 2002; Morrissey et al., 2005; Miyoshi et al., 2007). Fivefold increase in ROS level in the PBMCs of 100 and 200 mg/kg Apigenin treated animals as compared to control indicated the damage in liver may be due to ROS which can damage essential biological molecules like proteins, DNA and lipids. Membrane lipids are among the first to succumb to deleterious effects of ROS and measurement of lipid peroxidation is a commonly used index of increased oxidative stress and subsequent cytotoxicity. It is believed that lipid peroxidation is initiated by the attack of a free radical on fatty acid or fatty acyl side chain of any chemical entity (de Zwart et al., 1999) and is regarded as one of the basic mechanisms of tissue damage caused by free radicals. TBARS assay was used for the quantification of the end-products of lipid peroxidation, to be specific, malondialdehyde (MDA). Increase in the concentration of thiobarbituric acid reactive substances (TBARS)/ MDA in liver and kidney of mice treated with higher doses of flavonoids (100 or 200 mg/kg of Apigenin, 500 or 1000 mg/kg of Genistein and 1500 or 2000 mg/kg of Quercetin) was observed. However, in lower dose groups of Apigenin (25 or 50 mg/kg), Genistein (125 or 250 mg/kg) and Quercetin (500 or 1000 mg/kg), LPO level was comparable to control. These findings establish the dose specific oxidative stress generation in mice liver and kidney treated with flavonoids.

Reduced glutathione i.e. GSH, a non enzymatic antioxidant pool in the body that is synthesized in cytoplasm of liver cells and then distributed through circulatory system into different organs and subcellular compartments is considered as first line of

defense against oxidative insult (Meister and Anderson, 1983). GSH plays a crucial role in both scavenging ROS and detoxification of drugs and chemicals. Therefore, perturbation in the redox status of GSH can not only impair cell defense against toxic compounds, but also result in enhanced oxidative stress and tissue injury (Meister and Anderson, 1983). In 25 and 50 mg/kg Apigenin treated groups, no significant change in the ratio of GSSG and GSH was observed. Apigenin treatment at higher doses (100 and 200 mg/kg) decreased the reduced glutathione content and increased the ratio of GSSG and GSH in liver. Similar observations were documented by Kachadourian and Day, 2006 in PC3 cells following Apigenin treatment. As GSH is the functional anti-oxidative system in physiological conditions in tissues, its depletion indicates oxidative stress. GSH depletion might be due to its direct involvement in scavenging ROS in the process of neutralization and subsequent protection of essential thiol groups from oxidation. Total glutathione content in higher treatment groups 500 mg/kg ($p< 0.05$) or 1000 mg/kg of Genistein and 1500 or 2000 mg/kg (p value= 0.0238) of Quercetin were decreased significantly. Depletion of GSH may be due to its conjugation to reactive products, rather than its oxidation. Earlier studies have also pointed out the conjugation of Quercetin intermediates with GSH results the formation of mono-GSH and bis-GSH conjugates (Galati et al., 2001). It has been observed that no significant alteration in total glutathione content in liver and kidney of lower treatment group animals of Genistein (125 or 250 mg/kg) and Quercetin (500 or 1000 mg/kg).

Having established dose specific potential of flavonoids in imparting oxidative stress and subsequent toxicity in murine liver and kidney it was imperative to study the status of antioxidant enzymes. Activity and expression of major antioxidant enzymes; SOD, CAT, GR, GST and GPX were measured. SOD accelerates the conversion of superoxide radical (O_2 free radical) to H_2O_2 (Fridovich, 1989) while CAT scavenges H_2O_2 and convert it to water (Shull et al., 1991; Mates et al., 1999). GR, GST and GPX are glutathione dependent major phase-II drug metabolizing enzymes involved in combating against oxidative stress. Apigenin treatment significantly reduced the activity of SOD in mice liver and kidney at higher doses (100 or 200 mg/kg). This may be due to less production of mRNA transcript subsequently proteins of SOD. As SOD catalyze the dismutation of superoxide into oxygen and hydrogen peroxide forming the important antioxidant defense in all cells exposed to oxygen, its decrease infers excessive ROS generation. A significant increase in CAT and GPX activity in the liver

of 200 mg/kg Apigenin and kidney of 100 or 200 mg/kg Apigenin treated animals clearly indicated the H_2O_2 generation, as CAT decomposes H_2O_2 to water and oxygen. The alterations in CAT and GPX were not significant in the liver of 100 mg/kg Apigenin treated animals which may be due to more turnover of CAT and lower level of GPX in the mammalian cell (Goodsell, 2004). GR activity was found unaltered in the liver while increase in mRNA level in higher doses was quite obvious. In contrast, activity and expression of GR in kidney of higher treatment groups were reduced. mRNA level and activity of GST were decreased in liver and kidney of higher treatment groups of Apigenin. The decrease of GST along with SOD and the change in the transcript level of GR indicate severe insult to the liver tissue following acute exposure of Apigenin at higher doses. Activities and expression of SOD, CAT, GPX, GR and GST in liver and kidney of 25 and 50 mg/kg Apigenin were comparable to control.

Higher doses of Genistein (500 and 1000 mg/kg) decreased the activity and expression of SOD in liver and kidney of mice that may be the consequences of decreased de novo synthesis of enzymes (Limaye et al., 2003) characterized by reduction in transcript and protein level or irreversible inactivation of enzymes from increased free radical production resulting from Genistein metabolism (Kono and Fridovich, 1982; Blum and Fridovich, 1985). Likewise SOD; CAT and GPX activities and mRNA expression were decreased in liver and kidney of higher treatment groups (500 and 1000 mg/kg) as compared to control. CAT is solely responsible for the destruction of H_2O_2 while GPX has a wide spectrum of activity and also reduces lipid peroxides. In the liver and kidney of higher doses of Genistein, the decrease in GR and GST activities was not very apparent but decrease in transcript levels of these enzymes was quite significant. The decrease in expression level of SOD, CAT, GPX, GR and GST might be the effect of generation of ROS and/or development of oxidative stress in the higher doses of Genistein (500 and 1000 mg/kg) resulting finally in the decline in enzyme synthesis (Limaye et al., 2003). Moreover, the decrease in GR along with decrease in total GSH content suggests the overall reduction in GSH/GSSG ratio, an index of tissue oxidative stress (Meister and Anderson, 1983; Werner and Cohen, 1993). Decreased ratio of GSH/GSSG may shift the biological system towards different biological states, such as proliferation, differentiation, apoptosis and necrosis.

Unlike to Apigenin and Genistein; 1500 and 2000 mg/kg Quercetin treatment increased the SOD at protein and mRNA level in liver and kidney of mice. This results a significant increase in the activity of SOD in highest treatment group (2000 mg/kg). It has been found that a significant decrease in the activity of CAT and non significant increase in the activity of GPX in liver of higher treatment groups of Quercetin (1500 and 2000 mg/kg). Increase in the activity of SOD results the higher production of H_2O_2 that may be further neutralized by GPX as CAT activity was lesser in the higher treatment groups. GPX also converts H_2O_2 to water and oxygen by using GSH, it further supports the depletion of GSH in higher treatment groups (1500 and 2000 mg/kg). However in kidneys of higher treatment groups (1500 and 2000 mg/kg), an increase in activities of both enzymes CAT and GPX were observed that was significant for GPX and non significant for CAT. GR and GST activities were found to decrease in the liver of higher treatment groups (1500 and 2000 mg/kg). In 1000 mg/kg Quercetin treatment group GST activity was significantly increased. Quercetin increased the activity of GR significantly in the kidney of highest treatment group (2000 mg/kg). Alteration in the activity of GST in the kidney of mice was not significant, however an obvious decrease in GST mRNA level was observed at higher doses (1500 and 2000 mg/kg). Sahu and Gray, 1997 reported the flavonoid induced concentration-dependent decrease in GST activity. GST protects cells against toxicants by conjugating them to GSH, thereby neutralizing their electrophilic sites, and increasing their solubility and aiding excretion from the cell (Habig et al., 1974).

Alterations in antioxidant enzyme activities and expression with subsequent generation of oxidative stress by selected flavonoids further compelled to study the expression of one of the most important protein Hsp70, 70 kilodalton heat shock protein. Hsp70 is an important part of cell's machinery found to be involved in the regulation of cell proliferation and differentiation and can also be induced by heat stress, hypoxia, glucose starvation, and exposure to arsenite, heavy metals, or amino acid analogs (Hosokawa et al., 1990). It has been found that a dose dependent reduction in Hsp70 expression at mRNA and protein levels in liver and kidney of mice treated with Apigenin and Genistein were observed. A subtle increase in the expression of Hsp70 was observed in 1000 mg/kg treatment group of Quercetin while Hsp70 expression was reduced significantly in liver and kidney of higher treatment groups (1500 and 2000 mg/kg). Studies have shown that HSP synthesis has been blocked

following the treatment of Quercetin in different conditions (Elia and Santoro, 1994). Gupta et al., 2007 convincingly demonstrated a direct relation of Hsp70 with ROS generation in Drosophila. Dose related decrease in Hsp70 expression is an indicative of involvement of heat shock and stress pathway leading to induction of apoptosis (Nylandsted et al., 2000).

mRNA expressions of antioxidant enzymes both in kidney and liver do not fall in complete agreement with the pattern of changes observed in their protein activity. It can be speculated that this may be an affect of inherent biochemical and molecular machinery within the cell interior that disturbs the harmony between the transcription and translated products. Apigenin, Genistein and Quercetin affected the activities and expression of antioxidant enzymes in a much unrelated way. This might be due to the regulation of oxidative stress pathways by compound or their metabolites or free radicals generated by them or different tissue responses following the exposure of flavonoids. For further clarification, whole genome expression analysis was performed on the liver tissue following Apigenin, Genistein and Quercetin treatments to identify the differentially expressed genes and their involvement in different pathways. High throughput expression profiling facilitates prediction of toxicity and interpretation of mechanism of toxicity based on distinct gene expression changes. The simplest approach to identify genes of potential interest through several related experiments is to search for those that are consistently either up or down-regulated. Differentially expressed genes in mice liver at statistical criteria (i.e. $p< 0.05$ and fold change> 2) were studied. This increased the statistical confidence in detection of important genes and cellular processes with a probable role in initiation and propagation of toxicity as well as those possibly involved in regeneration during the early phase of tissue response. Furthermore, inherent biological variations among the members of same group were reduced by pooling RNA samples. Technical means of error were corrected by performing dye swap experiments and normalizing the microarray data. This ensured the detection of probes corresponding to significantly deregulated genes with a high statistical confidence following drug treatment. Furthermore, validation of microarray findings using quantitative real time-PCR substantiates these results, which is the most sensitive and accurate method of validating microarray based differential expression of genes (Chuaqui et al., 2002). Therefore, identification of genes with similar expression changes, at such a high stringency, both in direction and magnitude

indicate important biological functions under these circumstances. Differential gene expression analysis was performed for 25, 50 and 100 mg/kg of Apigenin, 125, 250 and 500 mg/kg of Genistein, 500, 1000 and 1500 mg/kg of Quercetin doses so as to get earlier changes at molecular level before the onset of injury at highest dose of 200 mg/kg Apigenin, 1000 mg/kg Genistein and 2000 mg/kg Quercetin.

In mice liver exposed to Apigenin results in differential regulation of 48 probes of which 36 were up regulated and 12 were down regulated. Most of them (*Bnip3l, Neo1, Clca1, Idh3a, Pank2, Prpsap1, Eif5B, Polr2h, Zfp110*) were engaged in the regulation of apoptosis, stress and cell growth. One of the identified genes was isocitrate dehydrogenase (*Idh3a*) that protects cell against oxidative damage (Lee et al., 2002). Isocitrate dehydrogenase has been shown to be more active in producing Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH) than other enzymes in the previous studies (Veech et al., 1969). Down regulation of this gene clearly indicated that the cell undergoes oxidative stress following Apigenin treatment. Interestingly, the simultaneous upregulation of BCL2/adenovirus E1B interacting protein 3-like (*Bnip3l*) (Vande et al., 2000), and Neogenin (*Neo1*) (Matsunaga and Chedotal, 2004) genes that have been reported to regulate apoptosis may involve in the induction of apoptosis in degenerated hepatocytes of Apigenin treated animals in higher dose groups. Apigenin was found to inhibit the hepatoma cell growth by altering the gene expression patterns (Cai et al., 2011). Several studies have also reported that Apigenin induces apoptosis by activating different genes (p53, p21, caspases, TNF-α and IFN-γ) (Zheng et al., 2005, Vargo et al., 2006, Khan and Sultana, 2006). Apigenin was found to upregulated the expression of genes involved in transcription and translation machinery Phosphoribosyl pyrophosphate synthetase associated protein 1 (*Prpsap1*), Eukaryotic translation initiation factor 5B (*Eif5B*), DNA directed polymerase (*Polr2h*), Zinc finger protein 110 (*Zfp110*). Pantothenate kinase 2 (*Pank2*), a mitochondrial enzyme catalyses the first regulatory step of Coenzyme A synthesis was found to be down regulated in the present study. This gene is also responsible for a genetic movement disorder named Pank-associated neuro-degeneration (PKAN). Recent evidence suggests that the silencing of Pank2 gene is directly associated with cell growth reduction and iron deregulation in hepatic cell lines (Poli et al., 2010). Another identified down-regulated gene was calcium-activated chloride channel (*Clca1*) which is integrated to plasma membrane. Its differential regulation in normal,

apoptotic, and transformed mouse cells suggests its function is pro-apoptotic and anti-neoplastic in nature (Elble and Pauli, 2001). Apigenin was appeared to affect the calcium ion homeostasis by modulating the expression of calcium ion binding proteins (Latent transforming growth factor beta binding protein; *Ltbp1* and Dual oxidase 1; *Duox1*). Furthermore, analysis of datasets of animals on MAPPFinder using two criteria either an increase (fold change$\geq$ 2 and $p< 0.05$) or decrease (fold change ≤ 2 and $p< 0.05$) in gene expression, identify GO terms corresponding to various biological processes. This provides further evidences of significant change in gene expression following oxidative stress associated hepatotoxicity. Few gene probes have not been assigned any cellular functions that may have important role in Apigenin induced perturbation in the liver of mice.

Following Genistein exposure mRNA expression in mice liver was assessed with 60,000 unique probes. When less statistically stringent criterion ($p< 0.05$ and 1.2 fold change) was applied 202 differentially expressed probes were identified consisting of 142 up-regulated and 60 down-regulated probes while high stringency ($p< 0.05$ and 2 fold change) identified only 40 differentially expressed probes consisting of 20 up-regulated and 20 down-regulated probes. The most striking finding of this study is massive down regulation of oxidative stress and glutathione metabolism related genes following Genistein exposure. Reactive Genistein metabolites might induce oxidative stress. Despite this, the characteristic induction of nuclear factor, erythroid derived 2, like 2 or other gene expression markers of oxidative stress were not observed. Expression of different isoforms of peroxiredoxins was also reduced. There are six known isoforms of Peroxiredoxins (*Prx*), a thioredoxin-dependent peroxidase, in mammals are present. They play a multifunctional role in the elimination of damaging ROS. PRDX3 is located in mitochondria and over-expressed in different cancers, possibly guarding of emergent tumor cells against apoptosis (Noh et al., 2001 and Kinnula et al., 2002). In breast cancer cells the expression of PRDX3 is more pronounced than normal cells (Huang et al., 2008). The downregulation of PRDX3, by Genistein treatment may decline the ability of mitochondria to neutralize ROS and potentiate the early apoptosis as found in MCF7 cells after exposure of 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2; a derivative of pyrimidine) (Liu et al., 2010). Simultaneous downregulation of Prnp, Stip1 and the genes of heat shock protein family were found. Stip1 provides potential to germ cells to survive in

stress conditions (Mizrak et al., 2006). Stip1 was previously shown to be up-regulated in fibroblasts during viral transformation and to exist in a macromolecular complex with heat shock proteins of HSP 70 and 90 families (Lassle et al., 1997). PrnP (Cellular prion protein) cooperates with Stip1 to regulate SOD activity in PrnP-deficient neuronal cell line (Sakudo et al., 2005). Coordinate down regulation of these genes suggest the augmentation of stress inside cell. Further decrease in total glutathione content of liver in higher treatment groups of Genistein fail to protect from reactive metabolites. This decrease in GSH might be due to the enhanced efflux of glutathione conjugated reactive metabolites of Genistein or decrease in expression of glutathione synthetase gene, one of enzyme involved in GSH biosynthesis. Genistein modulated the expression of defense and MAPK related pathway genes. A simultaneous upregulation of Defensin, alpha, related sequences 4 (DEFA4) was observed. DEFA4 is one of the polymorph of Defensins, small cysteine-rich cationic proteins, and reported to be significantly upregulated in idiopathic pulmonary fibrosis-acute exacerbation (IPF-AEx), a progressive fibrotic interstitial lung disease with a median survival of 2.5-3 years (Konishi et al., 2009). Additionally Genistein invoked the expression of certain immune-modulatory genes of host. Administration of relatively high dose (500 mg/kg bw) of Genistein to mice may have caused nonspecific transcriptional up regulation of interferons (Ifna6), interleukins (Il6) and chemokines receptor (Ccr5) genes. These genes were generally found up regulated during the disease conditions (IL-6 in Intravesical bacillus Calmette-Guerin therapy of superficial bladder cancer; Bevers et al., 1998 and IFN-α6, IFN-α2, IFN-α4, IFN-α5, IFN-α7, IFN-α10 in infection during HIV-1; Lehmann et al., 2009 and CCR5 in infection during Borna disease virus; Rauer et al., 2002). Genistein up regulated the P2RX7 in dose dependent manner thereby affects immune function and neurotransmitter release. P2RX7 is a purinergic ATP-binding calcium channel expressed in microglial cells, various brain regions and considered a candidate gene in type I diabetes (Elliott and Higgins, 2004). Genistein also induced the expression of FGF17, a secreted growth factor, can both enhance cellular proliferation and inhibit apoptosis and, play an important role in normal and pathological growth in many organ systems (Polnaszek et al., 2004; Guddo et al., 2006). Genistein regulated the expression of AKT3 that is the key intermediate of signaling pathways has been shown to regulate various cellular processes and controlling cell growth, proliferation, survival. Expression of AKT3 mRNA has been

shown to be upregulated in estrogen receptor-negative breast carcinomas and androgen-insensitive prostate cancer cell lines suggesting that AKT3 may contribute to the aggressiveness of steroid hormone-insensitive carcinomas (Nakatani et al., 1999). Enzymes involved in ETS and glycolysis were differentially expressed by Genistein treatment. Cyp4a14 was induced more than 4.4-fold by the higher dose of Genistein (500 mg/kg). Cyp4a14 belongs to the cytochrome family CYP 450 that are heme containing mixed-function oxidases that play a key role in the metabolism of hydrophobic endogenic substances (sterols, prostaglandins, and fatty acids) and ingested foreign compounds such as drugs, pesticides and pollutants (Hodek et al., 2002). These proteins are involved in interactions with flavonoid compounds in three ways: flavonoids can induce biosynthesis of certain CYPs, they can modulate enzymatic activity of CYPs and, finally, flavonoids can be metabolized by several CYPs. During metabolization cytochrome P450 gives rise to metabolites with associated biological activities distinctly different from those of the parent compound (Nielsen et al., 1998; Breinholt et al., 1999, Breinholt et al., 2000b). It could thus be speculated that some of the flavonoid metabolites rather than parent compound might mediate the biological response. Interestingly, the most recognized gene, Succinate dehydrogenase complex, subunit A (SDH A), flavoprotein (Fp) was downregulated more than two fold in the high dose group 500 mg/kg of Genistein. The main function of SDH A is to accept the electron from succinate during the conversion of succinate to fumarate in citric acid cycle. Its downregulation clearly indicated the impairment in metabolic regulation at high dose of Genistein. Differential regulation of other genes (*Ndufs7, Cyc1, Cyb5*) of electron transport chains by Genistein may induce the premature electron leakage to oxygen, generating superoxide and potentially resulting in increased oxidative stress. Apoptosis-inducing factor (AIF) is a caspase-independent apoptosis effector. In vivo, AIF directly involve in the protection against neuronal apoptosis induced by oxidative stress (van Empel et al., 2005). Down regulation of this gene in present study clearly indicated the generation of oxidative stress following apoptosis induction in liver of mice. Insulin I and II were differentially expressed during Genistein exposure. These different insulins are encoded by two nonallelic genes by beta-cells with more than 90% homology in rodents (Lomedico et al., 1979). Previously Ling et al., 1998 found that prolonged exposure of Islet beta-cells to high glucose levels differentially regulates the rat insulin I and II mRNA level. However, the

cellular contents of insulin I over insulin II fluctuate at different physiological states of the body (Wang et al., 1988). Several genes and their isoforms of glycolysis pathway were down-regulated however few genes and their isoforms were upregulated. For example Glucose-6-phosphatase, catalytic subunit and Enolase 2, gamma neuronal shows an increase at higher dose. In anaerobic section of glycolysis, lactate dehydrogenase-A level decreased up to two fold by the higher doses of Genistein. Lactate is formed from pyruvate in a variety of microorganisms in a process called lactic acid fermentation. The reaction also takes place in cells of higher organisms when the amount of oxygen is limiting, as in muscle during intense activity. The reduction of pyruvate by NADH to form lactate is catalyzed by lactate dehydrogenase. Inductions of gene expression for many enzymes involved in cholesterol, steroid, triglyceride and fatty acid metabolism were analyzed. It is well recognized that genes of CYP 450 family not only participate in xenobiotic metabolism but also extensively involve in Fatty acid, cholesterol, steroid and triglyceride metabolism. Differential regulation of various genes of CYP 450 family like Cyp4a29, Cyp7b1, Cyp4a14, Cyp2d10, Cyp2d26, Cyp7b1, Cyp3a25, Cyp2d9, Cyp3a41b by different doses of Genistein further suggested that there are comparable regulation mechanisms and that CYP enzymes may be involved in related oxidative stress metabolic pathways. Genistein also induced solute carrier family genes; Slc27a5, Slc10a2, and Slc37a4 that may involve in vacuole formation in hepatocytes through transport of fatty acids (Hirsch et al., 1998). Therefore, It has been indicated the lower doses of Genistein were less effective while higher doses were potentially induced the hepatocellular vacuolization. Increased expression of one of the most significant gene of phase 2 xenobiotic metabolism, Sul1e1 in the higher treatment group could increase their capacity for Genistein sulfation and likely contributes to their enhanced resistance against this compound and would also ameliorate glutathione depletion by enhancing this alternative route of Genistein detoxification. Additionally Genistein affects the glucose homoeostasis by regulating Insig1 and Insig2 genes. An important molecular receptor, *retinoid X receptor alpha*, was downregulated in 125, 250 and 500 mg/kg dose groups. It has been found that *RXR* dimerises with other nuclear receptors (e.g. *PPAR*) and regulates the expression of different genes involved in fatty acid and triglyceride metabolism (Amoutzias et al., 2007). Genistein regulates the different

isoforms of hydroxysteroid dehydrogenase isomerases (Hsd3b1, Hsd3b7, Hsd17b4, Hsd3b5, and Hsd17b11). These enzymes have major role in steroidogenesis.

Following Quercetin exposure mRNA expression in mice liver was assessed with 60,000 unique probes. A less statistically stringent criterion ($p < 0.05$ and 1.2 fold change) filtered 1082 differentially expressed probes consisting of 381 up-regulated and 701 down-regulated probes while high stringency ($p < 0.05$ and 2 fold change) identified only 155 differentially expressed probes consisting of 36 up-regulated and 119 down-regulated probes. For analyzing the Quercetin induced stress at molecular level the microarray data were quantified on the interrelated behavior of genes within gene interaction networks. The quantitative modeling suggested a highly significant relationship between MAPK expression and other genes in the stress-signaling subnetwork. About 60% of total genes in the network were downregulated in which Heat shock proteins have contributed more. Most of the heat shock proteins were down regulated. Recent studies have shown that Quercetin inhibits specifically the synthesis of heat shock proteins at the level of mRNA accumulation and transcription without affecting the other protein synthesis (Hosokawa et al., 1990). In fact, these findings also indicated that Quercetin inhibits the synthesis of Hsp70 at transcriptional and translational level consequently may lead towards apoptosis of liver cells. Quercetin upregulated the expression of *Uaca* (Uveal autoantigen with coiled-coil domains and ankyrin repeats). *Uaca* plays an important role in regulation of stress-induced apoptosis and promotes apoptosis by via three pathways, apoptosome up-regulation, LGALS3/galectin-3 down-regulation and NF-kappa-B inactivation (Sakai et al., 2003 and 2004). Another upregulated gene was a transcription factor; Jun proto-oncogene related gene d belongs to jun proto-oncogene family. It has been proposed that Jun protect the cells from p53-dependent senescence and apoptosis (Aggarwal et al., 1999). TAO (Thousand and one amino acid) kinase 3 was another upregulated gene by Quercetin. TAO kinase activates p38 MAPK in response to various toxic stimuli. TAO regulates p38 via their ability to phosphorylate and activate the MAPK kinases MEK3 and 6 (Hutchison et al, 1998; Chen et al, 2003). TAO kinases were found to be activated acutely by ionizing radiation, ultraviolet radiation, and hydroxyurea. Upregulation of this gene in present study indicated the presence of any toxic stimuli in cell. Quercetin induced the expression of different family members of Fibroblast growth factors (Fgf 13, 14 and 21). Fibroblast growth factors are a family of growth

factors involved in angiogenesis, wound healing, and embryonic development. FGFs are key players in the processes of proliferation and differentiation of wide variety of cells and tissues. Quercetin was found to downregulate the expression of major antioxidant enzymes GR, GST, CAT. Expression of GPX was unaltered whilst SOD was upregulated by Quercetin treatment. These antioxidant enzymes neutralize the free radicals generated in the cell during any disease condition or metabolism of xenobiotics. Superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2) and this H_2O_2 is further converted to water and oxygen by the help of catalase and glutathione peroxidase. GPX eliminates H_2O_2 and other lipid peroxides by utilizing reduced glutathione and converts reduced glutathione (GSH) to oxidized glutathione (GSSG). Further GSSG is reduced to GSH by the action Glutathione reductase (GR). Glutathione S-transferase (GSTs) catalyzes the conjugation of reduced glutathione-via a sulfhydryl group- to electrophilic centers on a wide variety of substrates including endogenous compounds such as peroxidised lipids as well as breakdown of xenobiotics (Douglas, 1987). Altered expression of antioxidant enzymes clearly indicated the oxidative stress in liver of mice following higher amount of Quercetin. Other genes of oxidative stress and glutathione metabolism related pathways were also downregulated. One of the most important gene nuclear factor, erythroid derived 2, like 2 (Nrf2) was under expressed. Nrf2 is able to induce genes important in combating oxidative stress, thereby activating the body's own protective response. Peroxiredoxins (Prdx), a thioredoxin-dependent peroxidase, expression was also reduced. Two forms of peroxiredoxins; Prdx3 and Prdx1 were downregulated by the Quercetin treatment. They play a multifunctional role in the elimination of damaging reactive oxygen species (ROS). PRDX3 is located in the mitochondria and over-expressed in different cancers, possibly guarding of emergent tumor cells against apoptosis (Noh et al., 2001; Kinnula et al., 2002). The downregulation of PRDX3 may decline the ability of mitochondria to neutralize the ROS and potentiate the early apoptosis (Liu et al., 2010). PRDX1 involves in the reduction of H_2O_2 and alkyl hydroperoxides and plays a significant role in protection of cell against stress. Simultaneous downregulation of PrnP (Cellular prion protein) and Stip1 (Stress induced phosphoprotein 1) genes by Quercetin treatment augments the stress inside the cell. Stip1 provides potential to germ cells to survive in stress conditions (Mizrak et al., 2006) and PrnP cooperates with Stip1 to regulate SOD activity in PrnP-deficient

neuronal cell line (Sakudo et al., 2005). Differential regulation of epidermal growth factor receptor (EGFR; ErbB-1; HER1 in humans) and AKT3 has been observed by Quercetin treatment. EGFR occupies the cell-surface and activated by binding to its specific ligands and found to be associated with a number of cancers, including lung and anal cancers (Walker et al., 2009). AKT3 is the key intermediate of signaling pathways and has been shown to regulate various cellular processes and controlling cell growth, proliferation, survival (Nakatani et al., 1999). Higher dose of Quercetin decreased the expression of PKD3. Protein kinase D3 (PKD3) belongs to the PKC (a serine/threonine-protein kinase) family and phosphorylates a wide variety of protein targets and is known to be involved in diverse cellular signaling pathways. PKD3 was significantly higher in human prostate tumors and its cell lines (PC3 and DU145 cells) and contributes to prostate cancer cell growth and survival. Over-expression of wild-type PKD promoted S phase entry, whereas depletion of endogenous PKD3 resulted in G0-G1 phase cell cycle arrest and inhibits cell proliferation (Chen et al., 2008).

Thus, the high throughput gene expression profiling in the case of mice treated with Apigenin, Genistein and Quercetin was successful in distinctly characterizing the gene expression signatures specific to tissues and the type of flavonoids administered. Furthermore, present differential gene expression profiling suggests the involvement of multiple pathways ranging from oxidative stress pathways to changes in apoptosis and immune surveillance in determining the toxicological or other changes observed in liver and kidney tissues of Swiss mice exposed to flavonoids. Moreover, identification of robust gene expression alterations in the absence of biochemical and histological markers at lower doses of flavonoids, encourage microarray technology as a useful tool to understand molecular events at an earlier stage. However, assigning precise molecular functions and understanding complete functional significance of each of the differentially regulated genes is difficult at present and requires further studies at different doses both at gene and protein level. Moreover, many of the genes identified in this study have no biological function assigned as yet and may be important in initiation and progression of disease or may merely represent an initial protective signature which might otherwise have led to more serious effects under chronic exposure of flavonoid.