

5. SUMMARY

Flavonoids are polyphenolic compounds widely distributed in plants kingdom; therefore they are the part of our daily diet. They are classified into flavonols, flavonones, flavones and isoflavones. They have been divided on the basis of their structure; having an identical backbone but different substituent groups. Due to various beneficial effects flavonoids are being recommended as food supplements. Nevertheless, they are identified as environmental contaminants since two decades. Most of the studies have pointed out their mutagenic, genotoxic, cytotoxic, pro-oxidant and moreover carcinogenic behaviour but none of study has clarified their hepatotoxic and nephrotoxic nature. Additionally, reports are inadequate on the effects of flavonoids on gene expression. In the present work three different flavonoids, each representing a separate class has been included so that the basic toxicity profile for a representative flavonoid of respective class could be evaluated. Hence, Apigenin from flavone, Genistein from isoflavone and Quercetin from flavonol class of flavonoids were selected based on the available literature about their toxicity and preliminary studies.

Swiss albino mice of 8 to 10 weeks of age weighing 25-30 g were employed in this study. They were dosed with varying concentration of different flavonoids (Apigenin, Genistein and Quercetin) to observe the maximum effect on the level of markers of hepatotoxicity. Similar doses of flavonoids were used to study their effect on kidney. Lower doses of apigenin (25 and 50 mg/kg), genistein (125 and 250 mg/kg) and quercetin (500 and 1000 mg/kg) did not cause any toxicity neither in liver nor in kidney. However, level of alkaline phosphatase in 1000 mg/kg group of quercetin was increased that may be due to its release from other tissues like bone, intestinal or kidney. Higher doses of apigenin (100 and 200 mg/kg), genistein (500 and 1000 mg/kg) and quercetin (1500 and 2000 mg/kg) did cause damage in liver tissue. Kidney biomarkers (CRT and BUN) were unaltered in animals treated with apigenin, significantly increased in highest dose of genistein (100 mg/kg) while non-significantly increased in higher dose groups of quercetin. With the effect of increased biomarkers level in higher dose groups of flavonoids a damaged histoarchitectures of liver tissue in same groups were found. Histology of liver in lower treatment groups and kidney in all treatment groups were normal as compared to controls.

The reason for this dose specific flavonoid toxicity in mice was further investigated and found that flavonoids at higher doses cause generation of reactive oxygen species resulting in oxidative stress. A five to six fold increase in ROS level in PBMCs of higher treatment groups of apigenin indicated the damage in liver may be due to ROS which can also damage essential biological molecules like proteins, DNA and lipids and alter the activity of antioxidant enzyme of tissue. As a result of ROS generation, an increase in Lipid peroxidation level and decrease in total Glutathione content in higher treatment group of flavonoids were found. Decrease in the protein content of SOD in liver of higher treatment groups of apigenin and genistein results a decrease in the activity subsequently lack of combating capacity against oxidative stress. In contrast, an increase in the protein content of SOD in liver of higher treatment groups of quercetin results the generation of peroxides. Furthermore, alteration in the activities and mRNA level of other major antioxidant enzymes i.e. CAT, GPX, GR and GST were observed in liver and kidneys of higher treatment group of flavonoids. As oxidative stress being a major part in toxicity it was imperative to study the most important protein regulated during stress condition. Therefore, the expression of Hsp70 was measured. Protein level of Hsp70 was significantly decreased in higher treatment group of flavonoids. Decrease in the level of Hsp70 indicated that the cell may undergo towards apoptotic phase and further substantiated the previous results as well as imparted to study the effect of flavonoids at genomic level to reveal mechanistic details.

Microarray based gene expression profiling has attained an important role in toxicological investigations of important therapeutic agents. It is believed that high throughput expression profiling facilitates prediction of toxicity and interpretation of mechanism of toxicity based on distinct gene expression changes. In the present study differential expression of genes in mice liver at statistical criteria (i.e. $p < 0.05$ and fold change > 2) were measured. This increase the statistical confidence in the detection of important genes and cellular processes with a probable role in the initiation and propagation of toxicity as well as those possibly involved in regeneration during the early phase of tissue response. 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. mRNA expression in mice liver was assessed with 22,827 unique probes after apigenin treatment whereas 60,000 unique probes were assessed after genistein and quercetin treatment. In mice liver exposed to apigenin results in differential regulation of 48 genes consisting 36 were up regulated and 12 were down regulated. Few genes (*Bnip3l*, *Neol*, *Clca1*, *Idh3a*, *Pank2*, *Prpsap1*, *Eif5B*, *Polr2h*, *Zfp110*) were engaged in the regulation of apoptosis, oxidative stress and cell growth. Following genistein exposure when less statistically stringent criterion ($p < 0.05$ and 1.2 fold change) was applied, 1082 differentially expressed genes were identified consisting of 381 up-regulated and 701 down-regulated genes while high stringency ($p < 0.05$ and 2 fold change) identified only 40 differentially expressed genes consisting 20 up-regulated and 20 down-regulated genes. The most striking finding of genistein treatment is the massive down regulation of oxidative stress and glutathione metabolism related genes. Following quercetin exposure a less statistically stringent criterion ($p < 0.05$ and 1.2 fold change) filtered 202 differentially expressed genes consisting of 142 up-regulated and 60 down-regulated genes while high stringency ($p < 0.05$ and 2 fold change) identified only 155 differentially expressed genes consisting 36 up-regulated and 119 down-regulated genes. The quantitative modelling of quercetin microarray data suggested a highly significant relationship between MAPK expression and other genes in the stress-signalling subnetwork. About 60% of total genes in the network were down-regulated in which heat shock proteins were contributed more.

This study is a first comprehensive study at biochemical, histological and genomic level collectively to assess the toxicity of certain flavonoids (Apigenin, Genistein and Quercetin) and their gene expression signatures in mouse model. Small differences in chemical structure of flavonoids led to changes in their biological effects, and were gene specific. The study further establishes the hepatotoxicity is generated by these flavonoids at higher doses that may be mediated through oxidative stress.