CHAPTER 3
INTRODUCTION

There are a plethora of reports which suggest that diet rich in antioxidants has the potency to reduce the risk of development of various chronic diseases\(^2\)\(^3\)\(^7\). A common denominator of most chronic diseases is the involvement of oxidative stress, related to the production of reactive oxygen and nitrogen species and free radicals\(^2\)\(^3\)\(^8\). Intervention trials with single isolated compounds such as vitamin E and C have shown lesser antioxidant ability compared to the use of crude whole extracts\(^2\)\(^4\). The most appropriate reason behind such outcomes may be because of the wide range of polyphenols present in the crude extracts. Polyphenols have different ionic strength, osmolality, electric charges and chemical concentration which altogether provide a summated effect to protect against harmful agents\(^2\)\(^5\).

The positive outcome of our study revealing the protective role of PFE against arsenic-induced hepatotoxicity led us to further investigate and compare the efficacy of two more dietary products in reducing arsenic-induced hepatotoxicity. The next phase of the study was designed to compare the efficacy of green tea / pomegranate fruit extract versus red wine polyphenols in ameliorating arsenic-induced hepatotoxicity.

We selected green tea and resveratrol as our next choice for investigation because these two phytochemicals are widely consumed around the world and popularly reported in scientific world as anti-cancer agents. The polyphenols present in green tea provide a broad spectrum of health support\(^2\)\(^3\)\(^9\). Green tea enhances body’s antioxidant defense system promoting cellular function. Similarly resveratrol is a natural polyphenolic compound present in grapes, peanuts and other plants and food products. Resveratrol is an active compound of red wine. Red wines contain 2.8-5.8 mg/L of resveratrol depending upon the variety of grapes used for fermentation.
The reason behind popularity of green tea (GT) as a popular beverage worldwide is its aroma, flavor and health benefits. Green tea polyphenols are subjects of considerable amount of research and are reported to have beneficial role against oxidative stress\textsuperscript{240}. Green tea catechins are also reported to reduce hepatic fibrosis\textsuperscript{187}. The principal catechins found in tea are epicatechingallate (EG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)\textsuperscript{241}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{popular_diets.png}
\caption{Popular dietary phytochemicals}
\end{figure}

Literature study indicates that GT has potent anti-oxidative, anti-inflammatory and even chemopreventive properties\textsuperscript{242}. Epidemiological studies suggest that tea consumption may have protective effects against human cancers\textsuperscript{243}.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{catechins.png}
\caption{Catechins present in green Tea}
\end{figure}
Besides green tea, resveratrol (3,5,4’-trihydroxy-trans-stilbene), a plant derived polyphenolic compound belonging to the family of stilbenes\(^{244}\) is found abundantly in certain grapes and is noted for its high anti-oxidative properties\(^ {245}\). Having anti-inflammatory, anti-atherosclerosis and anti-oxidative properties, resveratrol is also reported to act as a chemopreventive agent\(^ {246}\). Resveratrol is a phytoalexin produced by the enzyme stilbene synthase in response to environmental stresses, such as vicissitudes in climate, exposure to ozone, sunlight and heavy metals, and infection by pathogenic microorganisms\(^ {245}\). Resveratrol exists in both cis- and trans-stereoisomeric forms. Exposure to heat and ultraviolet radiation can cause trans-resveratrol to isomerize to the cis-resveratrol.

![Figure C3.3: structure of cis- and trans- resveratrol](image)

It is primarily found in the skin of grapes as well as in other fruits and plants, such as raspberries, blueberries, mulberries, Scots pine, Eastern white pine, and knotweed\(^ {245}\). Resveratrol has been shown to exhibit a wide range of health-promoting benefits for the coronary, neurological, hepatic and cardiovascular systems\(^ {246,247}\). It has been shown to inhibit inflammation, viral infection, oxidative stress and platelet aggregation.
Our investigation till now showed that overproduction of reactive oxygen species and development of immune suppressive milieu is one of the main reasons of arsenic-induced toxicity. Pomegranate supplement was observed to scavenge ROS, reduce arsenic accumulation and restrict activation of inflammatory pathways to protect the cells from death. These findings prompted us to conduct a comparative study using two more phytochemicals which could be used as protective supplements against arsenic toxicity.

Green tea was prepared by brewing 1.5 g of green tea leaves in 100 ml (1.5%) of freshly boiled milli Q water (100°C) for 5 minutes. The liquor was then fed to mice by oral gavage at a volume of 200 µl for an average weight of 28-30g mice. Ho et al.\textsuperscript{248} reported that single dose of 1.5% GT extract could initiate DNA repair and increase heme oxygenase-1 expression and provide protection against genotoxicity. The dose selected in this study has been reported to delay the onset of cancer in a population consuming an average of 6 cups of green tea for 12 months\textsuperscript{249}. Resveratrol was solubilized in 1.5% carboxymethyl cellulose (CM cellulose) because of its low solubility in water. A vehicle control was maintained to eliminate the indulgences of CM-cellulose in experimental findings. In the earlier two chapters we have mentioned that arsenic causes dose-dependent damage and greatest toxicity was observed at the highest dose. Therefore, to restrict increase in experimental groups, in the set of resveratrol supplementation we selected only five core groups: control, vehicle control (CM cellulose), Res, As3 and As3+Resveratrol. 20 mg/kg body weight of resveratrol was provided to the animals by oral gavage once daily.

In vitro MTT study revealed that 94% of hepatic cells were metabolically active even at the highest dose of arsenic with resveratrol co-treatment (at the selected dose). Moreover Juan et al.\textsuperscript{250} reported that administration of resveratrol at 20 mg/kg body weight for 28 days did not show any adverse health effect on animals. All the experimental conditions were similar as the first phase of study and were conducted for 30 days.
SPECIFIC OBJECTIVES OF THIS CHAPTER

- To examine the protective effect of green tea and resveratrol against arsenic-induced hepatotoxicity.
- To compare the effectiveness of pomegranate, green tea and resveratrol in protection from arsenic-induced hepatotoxicity.
DESIGN OF THE COMPARATIVE STUDY

CONTROL

GT/ Res

As1

As2

As3

As1 +GT

As2+GT

As3+ GT/Res

30 days study was done and liver was isolated

HISTOPATHOLOGICAL STUDY

IMAGING &IHC

HEPATIC TOXICITY TEST

FLOWCYTOMETRIC ANALYSIS

ANTIOXIDANT ENZYME STUDY

WESTERN BLOT

H&E STAINING OF LIVER SECTION

SEM & NUCLEAR LOCALIZATION

ALT, ALP, LDH, AST

ROS ESTIMATION

SOD, CAT, GSH, LPO

APOPTOTIC PROTEIN TRANSCRIPTION FACTOR
Results and Discussion

Comparative histopathology and structural changes in liver tissue sections

We first analyzed the histopathology of the liver sections obtained from the experimental groups. The period of study for the experimental groups was similar to the time period used for assessing PFE efficacy against arsenic toxicity. According to our hypothesis, it was observed that both GT and Res showed recovery of liver damage due to arsenic exposure (Figure 3.1). Hematoxylin & eosin stain showed that both GT and resveratrol prevented infiltrating cells, prevented vacuole formation and ballooning of cells and maintained the central vein structure (Figure 3.1; panel 1; indicated by arrows). Picrosirius study showed the extent of fibrosis in the liver (Figure 3.1; panel 2). The red-orange stains and the reddish tinge which were prominent in the arsenic group were reduced in the phytochemical-supplemented groups. The change in color was mostly localized around the central vein of the liver section in the polyphenols-supplemented groups (Figure 3.1; panel 2).

Electron microscopic analysis of the structural differences of liver tissues among the arsenic and polyphenols treated groups show that the abnormalities noticed in the arsenic group was remarkably ameliorated in the GT and Res co-administered group (Figure 3.1; panel 3; indicated by arrows). Data obtained from histological staining and scanning electron microscopy provided us with a qualitative analysis which indicates that recovery was observed to be more in the PFE group.
Figure 3.1: Panel-1 represents the H&E staining of liver section. Panel-2 represents picrosirius staining of liver section. Panel-3 represents SEM of liver. First column represents the liver sections from control. Second column represents liver section from As3. Third column represents the sections from As3+P groups. Fourth and Fifth represents the liver sections of As3+GT group and As3+R group. The images are representative of 10 random fields.
Biochemical analysis of rescue from arsenic-induced hepatotoxicity upon Green Tea (GT) and Resveratrol (Res) treatment

To compare the percentage reversal of hepatotoxic markers by the polyphenols we repeated the enzymatic assay for measuring the hepatotoxic marker levels. A significant elevation of ALP (Alkaline phosphate), ALT (Alanine transferase), AST (Aspartate transferase) and LDH (Lactate dehydrogenase) levels with respect to control was observed in the As-1, As-2 and As-3 groups (Figure 3.2). We separately estimated the toxicity levels after Green Tea (GT) supplementation. After GT supplementation a decrease in AST, ALT, ALP and LDH levels was observed. In the GT-supplemented group, AST, ALT, LDH and ALP levels showed significant drops, tending towards normal values.

Figure 3.2: Estimation of serum hepatotoxic markers. (A) Represents LDH level in serum of experimental animals (B) Represents serum ALP level (C) Represents serum AST level (D) Represents ALT level. ‘*’ represents significant difference (p<0.05) between control and arsenic exposed groups, ‘#’ represents significant difference between only arsenic and Arsenic +GT co-administered groups. Data are represented as mean ±SEM.
We did the similar serum hepatotoxic marker tests from the experimental animals after
treatment with resveratrol. In this phase of study we selected only the highest dose of arsenic
and the efficiency of resveratrol in reverting arsenic-induced toxicity was tested by co-
administering resveratrol with the highest dose of arsenic. Similar to our earlier findings, a
significant increase in the ALP, LDH, ALT levels were observed (Figure 3.3). It was very
interesting to observe that in all the three sets, the highest increase was observed in the ALT
level. A 3.4 fold increase in ALT was observed in the As3 group which was reduced to only
1.3 fold increase with resveratrol supplementation. ALT is localized in the cytoplasm of the
hepatocytes, thus elevated levels in all the experimental sets indicates acute hepatic injury\textsuperscript{197}. The other hepatotoxic markers were also reduced by resveratrol co-administration. These
observations were similar to our previous findings which clearly indicated that dietary intake
of natural extract can protect against arsenic-induced toxicity.
Figure 3.3: Estimation of serum hepatotoxic markers. (A) Represents LDH level in serum of experimental animals (B) Represents serum ALP level (C) Represents serum AST level (D) Represents ALT level. ‘*’ represent significant difference (p<0.05) between control and arsenic exposed groups, ‘#’ represents significant difference between only arsenic and Arsenic + Res co-administered groups. Data are represented as mean ±SEM

Comparative analysis of the percentage reversal of arsenic-induced hepatotoxicity by PFE, Green Tea and Resveratrol

Next we compared the percentage reversal of liver enzymes towards normal after arsenic exposure upon supplementation with PFE, GT and Res. It was observed that, when considering AST and ALT levels (Figure 3.4), PFE was most effective in reversal towards control levels. Comparing LDH levels, it was observed that both PFE and resveratrol showed equal percentage of reversal. But considering ALP level, resveratrol showed highest percentage of reversal. Based on this comparative study we find that among the four
enzymes PFE showed better result of normalizing three of them. Next in the queue is the efficacy of resveratrol. Green tea was positioned third in reversing the toxic markers toward normal. This might be because of its high clearance rate. Based on the results obtained from this part of the study, it was evident that all the dietary polyphenols could significantly reverse arsenic-induced liver toxicity.

Figure 3.4: Comparative Estimation of serum hepatotoxic markers. (A) Represents percentage reversal of LDH level selected at the highest dose of arsenic (B) Represents percentage reversal serum AST level at the highest dose of arsenic (C) Represents percentage reversal of serum ALT level at the highest dose of arsenic (D) Represents percentage recovery of serum ALP level at the highest dose of arsenic.
ROS scavenging by green tea and resveratrol

Previously we have confirmed that arsenic mediates toxicity by generating excessive reactive oxygen species and PFE could scavenge the free radicals (Figure No. 2.9). To find if green tea could do the same we estimated intracellular ROS generation by flow cytometry. We stained the cells with DHE and measured the relative fluorescence intensity by a flow cytometer. DHE is a cell permeable blue fluorescence dye which upon entering cell reacts with superoxide ion to form oxyethidium\(^{252}\). In accordance to our previous findings, a significant increase in ROS was observed in the arsenic-exposed groups but interestingly green tea-supplemented groups showed decreased DHE emission which signifies a protective role of green tea. Ability of scavenging ROS fits the criteria for being a savior against arsenic toxicity. Thus this provides us an important clue of green tea being a potent candidate after PFE.

Figure 3.5: Intracellular ROS estimation (A) Represents the flow cytometric analysis of ROS after DHE staining (B) Graphical representation of generated ROS after arsenic exposure and its reversal in the GT supplemented groups. ‘*’ Represents significant difference between control and arsenic groups. ‘#’ represents significant difference between only arsenic and Gt supplemented groups at p<0.05.
Next we examined the scavenging capacity of resveratrol. The ROS scavenging ability of resveratrol was measured by fluorimetry. The protective role of resveratrol against inflammation and carcinogenesis has been largely attributed to its anti-oxidative properties\textsuperscript{253}. The structural determinants of the diverse property of resveratrol are obscure but the number and position of hydroxyl groups have been suggested for contributing toward anti-oxidative property of resveratrol\textsuperscript{254}. It was observed that at the highest dose of arsenic a 6 fold increase in the reactive oxygen species was observed with respect to control (Figure 3.6). In the resveratrol co-administered group, ROS generation was significantly reduced (3.5 fold increase over control). This observation confirms that resveratrol efficiently protects against excess ROS generation by arsenic.

![Fluorimetric analysis of ROS](image)

**Figure 3.6:** Fluorimetric analysis of ROS. ‘**’ represents the significant difference between control and arsenic group. ‘#’ represents difference between arsenic and resveratrol-supplemented group.
Comparative analysis of reducing power and DPPH scavenging activities of the three dietary polyphenols (PFE, GT and Res)

Ou et al., reports that intake of dietary antioxidants help in maintaining an optimal antioxidant status and therefore maintain normal physiological homeostasis. We performed two separate assays - reducing power and DPPH scavenging - to compare the antioxidant efficacies of PFE, GT and Res in the presence of arsenic (Figure 3.7).

The chemical diversities of antioxidants makes it difficult to separate and quantify individual antioxidants from the extract so it was deemed advisable to measure the total reducing power and DPPH scavenging power of PFE, GT and resveratrol. Reducing power and DPPH scavenging depends on the hydrogen atom transfer reaction between the antioxidant molecule and oxidant. The comparative data revealed that among the selected doses for PFE, GT and resveratrol, the selected dose of PFE had higher reducing power. To illustrate the fact that PFE has a higher capability than the other two polyphenols we performed the DPPH assay. The assay is based on the theory that the hydrogen donor is the antioxidant. DPPH accepts hydrogen from the antioxidant and the scavenging capacity of the antioxidants is measured proportional to the decrease in color of the test samples. PFE showed higher DPPH scavenging ability with respect to control and in comparison with both resveratrol and green tea. Even with the highest dose of arsenic, PFE showed the highest reducing power compared to the other two dietary polyphenols (Figure 3.7). This data clearly denotes that PFE is more efficient in reducing hepatotoxicity.
Figure 3.7: (A) Comparative representation of percentage change of reducing assay of PFE, GT and Res over control at the selected dose in this study. (B) Comparative DPPH scavenging assay of three selected dose of polyphenols. (C) Comparative estimation of Percentage changes over control in reducing power of three polyphenols. (D) Comparative presentation of DPPH scavenging at highest dose of arsenic.

Molecular pathways
The data presented so far involving histopathological studies, hepatic enzyme levels and intracellular ROS generation, made it amply clear that both green tea and resveratrol were able to impart a degree of protection against arsenic-induced hepatotoxicity. In the second phase of the study we did a comprehensive study investigating the role of the chosen polyphenols in activating redox-sensitive transcription factors and their role in preventing oxidative stress-induced cell death. In this phase of the study to get a closer look of the molecular pathway governed by GT and Res. We evaluated the efficacy of both green tea and resveratrol in maintaining cellular redox balance. To adapt to and survive oxidative stress situations, cells up-regulate an elaborate network of cytoprotective proteins facilitated by the activation of redox-sensitive transcription factors like Nrf2 and NF-κB. Western blot analysis was done to investigate the involvement and activation of Nrf2 and NF-κB and the results are presented in the following sections.
Immunohistochemical determination of Nrf2 nuclear localization after supplementation with green tea

Figure 3.8 A: Immunohistochemistry of liver sections. 1\textsuperscript{st} column represents the DAPI staining of the hepatic nuclei. 2\textsuperscript{nd} column represents the Nrf2 staining with PE-tagged fluorophore. 3\textsuperscript{rd} column represents the overlap of two images. Panel-1 represents control sections Panel-2: As1, Panel-3: As2 and Panel-4: As3 sections. The images are representative of 10 random fields at magnification 40X.
Figure 3.8 B: Immunohistochemistry of liver sections. 1\textsuperscript{st} column represents the DAPI staining of the hepatic nuclei. 2\textsuperscript{nd} column represents the Nrf2 staining with PE-tagged fluorophore. 3\textsuperscript{rd} column represents the overlap of two images. Panel-5 represent GT sections, Panel-6: As1+GT, Panel-7: As2+GT and Panel-8: As3+GT sections. The images are representative of 10 random fields at magnification 40X.
Figure 3.8 A & B shows the activation of Nrf2 after arsenic intoxication. Nrf2 was PE-tagged and for nuclear staining DAPI was used. The Nrf2-Keap1 pathway is the main cellular defense pathway against oxidative and electrophilic stress\textsuperscript{207}. Arsenic affects a multitude of biological systems which leads to activation of Nrf2 and up-regulates its downstream genes which help to adapt to the cellular stress situation\textsuperscript{208}. In our study, in the control group, the merged image showed distinct localization of PE tagged Nrf2 outside the nucleus but with increasing doses of arsenic, fused emission of PE and DAPI was observed which indicates nuclear localization of Nrf2 (Figure 3.8 A; panel 2,3,4; column 3\textsuperscript{rd}). IHC of the green tea only group showed similar localization pattern of Nrf2 as that of control (Figure 3.8 B panel 1; Column 3rd). In the arsenic + GT-supplemented groups, Nrf2 nuclear localization was observed to be restricted (Figure3.8B panel 6,7,8; column 3rd).

We also analyzed the protein expression of Keap1 in all the experimental groups (Figure3.9). Keap1 act as regulatory sensors and are modified upon exposure to oxidative or electrophilic stress thereby releasing Nrf2 for nuclear localization\textsuperscript{213}.

![Figure 3.9: (A) Representative Western blot analysis of Nrf2 and Keap1 (B) Densitometric analysis of the corresponding blots of Nrf2 and Keap1](image-url)
It was observed that there was a dose-dependent activation of Nrf2 in the arsenic-exposed groups. But in the arsenic plus green tea-supplemented groups, the activation of Nrf2 was restricted. In parallel, with increase in Nrf2 activation, Keap1 expression was also observed to decrease in the arsenic-intoxicated group (Figure 3.9). This provides evidence that change in the oxidative stress situation of the cell augment release of Nrf2 from its inhibitory adaptor protein.

We also assessed the protective role of resveratrol by analyzing the activation of both the transcription factor Nrf2 and NF-κB. Since Nrf2-Keap1 signaling pathway clearly determines the change in the electrophilic condition within the cell, we examined co-localization of Nrf2 and Keap1 in all the experimental groups after treatment with resveratrol using confocal microscopy. From our previous experiments with PFE and GT (Figure 2.11 & 3.8), it was clear that arsenic exposure at the three doses increased Nrf2 activation in a dose-dependent way. Co-localization study of Keap1 and Nrf2 revealed that with increase in dose of arsenic a decrease in co-localization of the two molecules was evident while Western blotting of Keap1 showed an increase in expression on exposure to arsenic. The reverse phenomenon was observed in the PFE and GT supplemented groups. Similar to our earlier findings, immune staining of liver sections after resveratrol treatment also revealed the same fact. Consistent findings from our study endorsed the fact that arsenic potentiates Nrf2-Keap1 signaling pathway. Optimal function of Keap1/Nrf2/ARE pathway may help to show or prevent chronic disease progression but aberrant increased activation has deadly outcomes. Our investigation so far strongly indicates that dietary supplementation of PFE, GT and Res can rescue hepatocytes from excessive ROS generation due to arsenic, reduce hepatotoxicity and prevent aberrant activation of Nrf2. Figure 3.10 shows the confocal imaging done to study co-localization of Nrf2 and Keap1. The liver sections were stained with FITC tagged Nrf2 (3rd column) and PE tagged Keap1 (4th column). The 5th column
represents the pixel intensity analyzed by calculating emission from two fluorochrome tagged to two different molecules. Analysis of merged image by confocal FluoView software showed that co-localization was observed in the control, Res and As3+R group but lesser co-localization was evident in the arsenic group.

Figure 3.10: Co-localization study of Nrf2 and Keap1. 1st column represents the DAPI staining of the hepatic nuclei. 2nd column represents the Nrf2 staining with FITC-tagged fluorophore. 3rd column represents the Keap1 staining with PE-tagged fluorophore. 4th column represents merged pictures of 2nd and 3rd columns. Images are representative of 10 random fields at 40X magnification.
Besides Nrf2, the ubiquitous transcription factor NF-κB also responds to intrinsic and extrinsic cellular stress situations\(^{257}\). NF-κB is activated in the cytoplasm by disruption of the association of NF-κB with Inhibitor of κB protein (IkB). The phosphorylation of IkB by the IkB kinase (IKK) complex results in the degradation of IkB, leading to the nuclear translocation of NF-κB where it can transactivate NF-κB target genes involved in cell proliferation, anti-apoptosis, survival, etc\(^{257}\).

In our study, Western blot analysis led to the observation that cells exposed to arsenic exhibited an enhancement of NF-κB expression in the whole cell lysate (Figure 3.11). GT treatment to arsenic-administered animals markedly abrogated arsenic-induced activation of NF-κB compared with the only arsenic-exposed groups. Activation of NF-κB was further confirmed by investigating the phosphorylation status of IkB. It was seen that arsenic treatment caused an increase in the levels of phosphorylated IkB.
Activation of both Nrf2 and NF-κB were restricted by supplementation with resveratrol

Figure 3.12: Western blot analysis of Keap1, Nrf2, NFκB, IκB from whole cell lysate of liver fraction isolated after resveratrol treatment. (A) Represents the densitometric analysis of Keap1 (B) Represents the densitometric analysis of Nrf2 (C) represents the densitometric analysis of IκB (D) Represents the densitometric analysis of NFκB. Blots are representative blot of three separate

Consistent to our previous studies, Western blot analysis of both Nrf2 and NF-κB in nuclear lysate showed increased expression in the arsenic groups. In resveratrol-supplemented groups the activation of both the transcription factors were less (Figure 3.12).
Activation of Nrf2 target genes

**Figure 3.13 A:** Analysis of HO-1 and NQO1 activities (A) Represents enzymatic activity of HO-1 after GT supplementation. (B) Represents NQO1 enzymatic activity after GT supplementation.

**Figure 3.13 B:** HO-1 and NQO1 activities (A) Represents enzymatic activity of HO-1 after Res supplementation. (B) Represents NQO1 enzymatic activity after Res supplementation. '*' Represents significant difference between control and arsenic and '#' represents significant difference between only arsenic and both Res and GT groups.
The NQO1 and HO-1 enzymes are implicated in protection against oxidative stress\textsuperscript{258} and carcinogenesis, and their functions include stabilization of the p53 tumor suppressor\textsuperscript{259}. Literature study reports that NQO1-deficient mice show reduced p53 induction and apoptosis, increased susceptibility to chemically induced tumors\textsuperscript{260} and impaired NF-κB function\textsuperscript{261}. Figure (3.13 A & B) shows the enzymatic activity of HO-1 and NQO1 in the different experimental groups. A significant increase in the activity was observed in the arsenic doses and supplementation of both GT and Res showed less activity compared to the only arsenic-exposed group. Induction of cytoprotective enzyme at gene level was confirmed by analysis of the PCR expression pattern of HO-1. Evidence of up-regulation of adaptive strategy by the cell compelled us to examine the endogenous defense activity of the cell.

![Figure 3.14: (A) PCR analysis of HO-1 genes in the eight experimental groups (B) Densitometric analysis of the PCR bands.](image)

Figure 3.14: (A) PCR analysis of HO-1 genes in the eight experimental groups (B) Densitometric analysis of the PCR bands.
Evaluation of augmentation of endogenous cellular anti-oxidative effects by green tea and resveratrol

**Figure 3.15 and 3.16** shows the activities of SOD, catalase and glutathione reductase; and levels of lipid peroxidation and GSH after supplementation with GT and resveratrol. Arsenic exposure caused a significant depletion (p<0.05) of superoxide dismutase activity with respect to control (**Figure 3.15**). A significant (p<0.05) change in activity was observed at the three doses of arsenic with respect to control, which was reverted in the GT and Res supplemented groups. It is noteworthy to mention that resveratrol was more efficient in doing the job than GT (**Figure 3.15**).

A highly significant (p<0.05) dose-dependent elevation of LPO levels was observed in the groups administered with arsenic (**Figure 3.15**). The LPO levels show significant decline with the treatment of GT and resveratrol. The change was observed to be significant at three doses both with control and also in the supplemented groups.

The major non-enzymatic regulator γ-glutamyl-cysteinyll-glycine (GSH) level also showed a significant decrease with respect to control in the arsenic-exposed groups. GSH is present in cells either in the reduced (GSH) or oxidized (GSSG) form and participates in the redox reaction by reversible oxidation of its thiol group. Glutathione reductase (GR) is a ubiquitous flavoenzyme that catalyzes NADP-(H)-dependent reduction of GSSG to GSH. This enzyme is responsible for maintaining adequate level of GSH pool for maintaining the redox balance.

To justify the decrease of GSH we assessed the enzymatic activity of GR. It was observed that at arsenic dose 1 (0.01mg/l) the GR activity was more than that in dose 2 (0.05 mg/l) and dose 3 (0.1 mg/l) but the activity was restored by polyphenol suppletionation. The rise in GR activity was reflected by slight increase in the GSH level in the arsenic+GT groups.
Data obtained in our study indicates that arsenic inhibits GR activity which causes decrease in GSH level and renders the hepatocytes more susceptible to oxidative insult.

It was surprising that in all the three different sets of study it was observed that in spite of Nrf2 activation, the antioxidant enzymes were less active in the arsenic-exposed groups. Experimental data shows that the equilibrium between these enzymatic and non-enzymatic antioxidants got disturbed which possibly led to the accumulation of reactive species. Thus from these data we can infer that arsenic interferes with the enzymatic activities, whereas the supplementation of polyphenols can boost the antioxidant defense mechanism even when the Nrf2 is restricted. This provides an insight that polyphenols can exert Nrf2-independent protection.
Figure 3.15: SOD activity, CAT activity, LPO level, GSH level, and GR activity in eight experimental groups. All data are represented as mean±SEM and significant at p<0.05. * represents significant difference between arsenic-exposed groups and control group; # represents significant difference between only arsenic and arsenic+GT group. Data are represented as mean±SEM.
Figure 3.16: SOD activity, CAT activity, LPO level, GSH level, and GR activity in eight experimental groups. All data are represented as mean ± SEM and significant at p<0.05. ‘*’ represents significant difference between arsenic-exposed groups and control group. ‘#’ represents significant difference between arsenic and arsenic+Res groups. Data are represented as mean ± SEM.
Green Tea and Resveratrol protects against arsenic-induced apoptosis by maintaining mitochondrial membrane potential

A variety of physiological death signals, as well as pathological cellular insults, trigger the genetically programmed pathway of apoptosis\textsuperscript{262}. Apoptosis manifests in two major execution programs downstream of the death signal: the caspase pathway and organelle dysfunction, of which mitochondrial dysfunction is the best characterized\textsuperscript{263}.

Decrease in antioxidant enzyme activity and a dose-dependent increase in the intracellular ROS generation in the arsenic-exposed groups give a clear indication towards induction of apoptosis. A loss of mitochondrial membrane potential (MMP) may be critical mediator of apoptosis. The outward pumping of protons across the inner mitochondrial membrane produces a proton gradient that drives the conversion of ADP to ATP\textsuperscript{264}. Decrease in MMP induces opening of mitochondrial permeability transition pores which may lead to the release of mitochondrial apoptosis initiation factors\textsuperscript{265}.

In our study, in the arsenic-exposed groups the change of mitochondrial membrane potential is significantly higher (Figure 3.17). However, in the dietary polyphenols-supplemented groups, the membrane potential shows a trend towards recovery (Figure 3.17). This finding stands in support of the fact that low chronic doses of arsenic exposure can disrupt MMP and initiate cell death by apoptosis.

After green tea supplementation, it was observed that the percentage of cells emitting red florescence was increased in comparison to the only-arsenic-exposed groups. Similar to this finding it was also observed that resveratrol also showed a potential efficacy in maintaining transmembrane potential.
A major site of activity of the Bcl2 proteins is the mitochondrial membrane (Hockenbery et al. 1990). In our study it was observed that exposure to arsenic caused a decrease in the expression of the anti-apoptotic protein Bcl2 and an increase in the expression of the pro-apoptotic protein Bax (Figure 3.19). This increase in the Bax/Bcl2 ratio was prevented by PFE, GT and resveratrol co-administration. Confirmation of arsenic-induced apoptosis was done by assessing the activation status of caspase-3 by Western blot.
Figure 3.18: Flow cytometric analysis of JC-1 staining of hepatocytes for determining the mitochondrial transmembrane potential in the four experimental groups after resveratrol treatment.

Similar patterns of activation were observed in the expression of Bax, Bcl2 and caspase-3 in all the experimental groups after polyphenols supplementation. The results obtained from all the sets document the fact that antioxidant-rich dietary intake may boost the endogenous defense systems and impart a protective mechanism against arsenic-induced hepatotoxicity by modulating the various signaling pathways.
Figure 3.19: Western blot analysis of Bcl2, Bax, Caspase-3 in the eight experimental group after GT supplementation. (B) Represents the densitometric change in all the experimental groups with respect to control.

Figure 3.20: Western Blot analysis of Bcl2, Bax, Caspase-3 in the five experimental groups after Res supplementation. (B) Densitometric analysis of bands with respect to control.
Analysis of the comparative study

Histopathological data provides a vivid picture of protection against arsenic intoxication by supplementation of dietary polyphenols. A clear improvement in the liver architecture was observed in the PFE, GT and Res co-administered groups. Supplementation of dietary polyphenols in the arsenic-intoxicated groups could efficiently reduce arsenic-induced excess ROS generation and could prevent cellular lipid peroxidation. Even the elevated levels of hepatotoxic markers were decreased after supplementation of dietary polyphenols.

By doing a comparative analysis of the three sets of experimental data, it was observed that PFE efficiently reduced ROS by more than 2 fold and GT could diminish ROS generation by 1.7 fold at the highest dose of arsenic. On the other hand, resveratrol could reduce ROS by 1.4 fold. Interestingly, 2.2 fold decrease, 2 fold decrease and 1.5 fold decrease in LPO levels was observed after PFE, GT, Res supplementation. The reversal of hepatotoxic markers was more efficiently done by PFE in comparison to GT and Res.

Due to ROS generation the SOD activity was found to be compromised in all the three doses of arsenic and the decrease in activity was more pronounced at the highest dose of arsenic but with the supplementation of PFE, the activity was increased by 4 fold. Similar to this observation, GT and Res supplementation could also reverse the SOD activity by 1.4 and 1.7 folds respectively. The GSH level depletion at the arsenic highest dose was prevented by polyphenolic extract supplementation. PFE most efficiently restored the GSH level to near control level at the highest dose, then GT and Res.

Considering the data obtained from the reducing power assay and the DPPH assay it was observed that at the highest selected dose of arsenic PFE contributed the highest protection among the three dietary polyphenols.
To summarize, it must be mentioned that all the polyphenols showed improvement against arsenic intoxication. Both qualitative and quantitative data puts an emphasis that among the three polyphenols PFE could more efficiently fight back the oxidative stress situation by imparting highest free radical scavenging potency among the three. The signaling pathway undertaken by the three polyphenols was similar which reflects that the mode of imparting protection by the dietary polyphenols is similar.
SCHEMATIC REPRESENTATION OF THE FINDINGS