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# DOSE DEPENDENT EFFICACY OF QUERCETIN IN PREVENTING ARSENIC INDUCED OXIDATIVE STRESS IN RAT BLOOD AND LIVER

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***Abstract: The aim of this study was to evaluate the protective efficacy of Quercetin, one of the most widely used flavonoid, against acute arsenic exposure on biochemical variables suggestive of changes in blood and hepatic oxidative stress in rats. Exposure to arsenic caused a significant decrease in blood d-aminolevulinic acid dehydratase (ALAD) activity and accompanied by an increase in urinary ALA excretion. These changes were accompanied by a decrease in blood glutathione (GSH),an increase in the level of blood Reactive oxygen species and arsenic levels. An increase in the level of liver thiobarbituric reactive species along with a concomitant decrease in the activities of glutathione peroxidase and reduced glutathione content were also observed in arsenic administered rats. The toxicity induced by arsenic was significantly reversed by the simultaneous administration of 50 mg/kg quercetin. Quercetin administration at two lower doses (10 and 25 mg/kg) provided less pronounced recovery in the altered biochemical variables compared to the higher dose. The results suggest adequate intake of quercetin during arsenic exposure may prevent arsenic absorption and oxidative stress.***

**Key words:** Arsenic, Oxidative stress, Quercetin, Prevention

## INTRODUCTION

Arsenic, a major constituent of earth's crust, is present in various concentrations and in different oxidation states in the atmosphere, soil and water. Arsenic is carcinogenic, teratogenic, genotoxic, and can arrest mitosis, alter DNA synthesis and repair, presumably by binding to thiols of DNA polymerase and produce chromosome aberrations in exposed individuals [1]. The reported mechanism of arsenic induced toxicity is the disruption of prooxidant/ antioxidant balance in the biological system which is responsible for causing skin and liver cancer [2]. Thus in order to redeem the balance, antioxidants are administered to reduce the reactive oxygen species (ROS) generated in the process.

Flavonoids are phenolic phytochemicals that represent substantial constituents of the non energetic part of the human diet and are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against reactive oxygen species (ROS). Flavonoids are ubiquitous in nature and are categorized, according to their chemical structure, like flavonols (Quercetin, kaempferol, Myricetin), flavones (Apigenin), flavanones (Naringenin), isoflavones (Genistein), flavan-3-ol (catechins), anthocyanidins and chalcones [3]. Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. The high biological activity of flavonoids is due to their capability for redox reactions and to complexation with metal ions. Flavonoids scavenge OH radicals and superoxide ($O_2^-$) anions; they also reduce some metal cations to lower oxidation states [4,5].

Flavonoids have been reported to bring benefits in lowering inflammation and oxidative stress and exert positive effects in cancer, cardiovascular and chronic inflammatory diseases. Flavonoids are known to inhibit aldoreductase [6] and thereby block the accumulation of dulcitol, which is responsible for osmotic stress. They are also known to inhibit lipid peroxidation [7, 8], which markedly increases in patients suffering from diabetes mellitus [9, 10] and various liver diseases [11].The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Figure 1). Quercetin, (3, 5, 7, 3, 4-pentahydroxy flavon), the most abundant dietary flavonol [12], is a potent antioxidant because it has all the right structural features for free radical scavenging activity (Figure 1).. Previous studies have shown that quercetin and other flavonoids have a broad range of pharmacological properties, including carcinostatic and antiviral activities, suppression of cell proliferation, modification of eicosanoid synthesis, protection of LDL from oxidation, prevention of platelet aggregation, stabilization of immune cells, and relaxation of cardiovascular smooth muscle [13, 14]. It is found to be the most active of the flavonoids in studies, and many medicinal plants owe much of their activity to their high quercetin content. Quercetin has demonstrated significant anti-inflammatory activity because of direct inhibition of several initial processes of inflammation. Quercetin was found to scavenge free radicals and reduce the oxidability and cytotoxic effects of low density lipoproteins [15,16]. A diet rich in quercetin was reported to inhibit the development of carcinogen-induced rat mammary cancer [17], colonic neoplasia [18] and oral carcinogenesis [19]. The present study was designed to investigate the dose dependent preventive efficacy of quercetin to prevent Arsenic-induced oxidative stress and liver injury in rats.

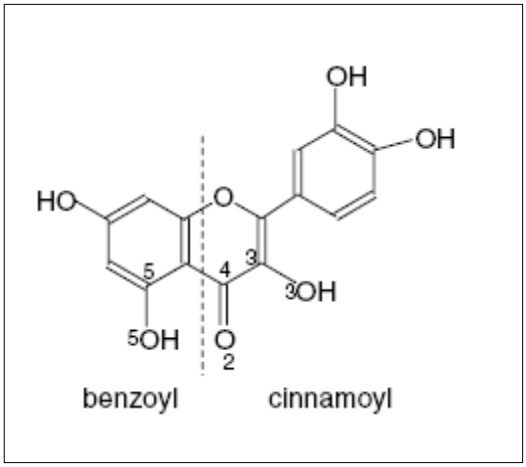



**Fig. 1**: Chemical structure of Quercetin

## MATERIALS AND METHODS

**Chemicals:** Sodium m- arsenite ($NaAsO_2$, molecular weight 129.9) and Quercetin (molecular weight 338.26) were procured from Sigma Chemical (USA). All other analytical laboratory chemicals and reagents were purchased from Merck (Germany), Sigma (USA) or BDH chemicals (Mumbai, India). Ultra pure water prepared by Millipore (New Delhi, India) was used through out the experiment to avoid metal contamination and for the preparation of reagents and buffers used for various biochemical assays in our study.

**Animals and treatments:** Adult male Wistar rats (120–130 g), procured from animal house facility of Defence Research and Development Establishment (DRDE), Gwalior were kept in clean plastic cages and allowed to acclimatize in the laboratory environment for 7 days to light from 06:00 to 18:00 h alternating with 12 h darkness in an air-conditioned room with temperature maintained at 25±2°c. Balanced food (Amrit feeds, Pranav Agra, New Delhi, India; metal contents of diet, in ppm dry weight, Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) and drinking water were given to the animals *ad libitum*. Animals were randomly divided into five groups of 5 animals and treated for 4 weeks as follows:

Group I: Normal animals
Group II: Arsenic (1mg/kg, intraperitoneal)
Group III: Arsenic + Quercetin (1mg/kg i.p. + 10mg/kg, orally, once, daily)
Group IV: Arsenic + Quercetin (1mg/kg i.p. + 25mg/kg, orally, once, daily)
Group V: Arsenic + Quercetin (1mg/kg i.p. + 50mg/kg, orally, once, daily)

After 4 weeks, 48 h after the last dose of arsenic and quercetin, animals were sacrificed under light ether anesthesia by decapitation. Blood was collected in heparinized vials. The liver was

removed by transverse abdominal incision, washed with normal saline and all the extraneous materials were removed before studying various parameters.

**Biochemical assays**

**Clinical haematological variables:** White blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), haematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and platelet (PLT) counts were measured on Sysmex Hematology Analyzer (model K4500).

**Urinary d-aminolevulinic acid:** Urinary d-aminolevulinic acid was estimated as described by the method Davis et al. [20]. 24 hr samples were collected in disposable polypropylene tubes (acidified with a drop of acetic acid) and stored at 0°C until analyzed. The separation and isolation of d-ALA was performed by employing a dual chromatography column set up in tandem position with a convenient support rack. The top chromatography column was filled with Dowex-2W×8, 200-400 mesh $Cl^-$ form resin and the bottom column with Dowex-50W×8, 200-400 mesh, $H^+$ form resin. The top column was washed with 10 ml of water and allowed to drain through both the columns. Urine sample was applied to the top column. The top sample applied was passed through the resin followed by lower column resin and then eluted out. The top column was now washed three times with water and was allowed to drain through both columns like the urine sample. The top anionic columns were removed and bottom cationic columns are placed in empty, numbered test tubes, δ-ALA is eluted individually from each column with sodium acetate trihydrate (1.0 M) and collected in a test tube. 0.2 ml of acetyl acetone was added to test tubes and mixed. Test tubes were then placed in boiling water bath for 10 min and cooled to room temp. Added equal amount of freshly prepared Ehrlich reagent mixed. Optical density was read at 555 nm after 15 min. The development of pink color indicates the presence of δ-ALA.

**Blood δ- aminolevulinic acid dehydratase (ALAD):** The activity of blood δ-aminolevulinic acid dehydratase (ALAD) was assayed by Berlin and Schaller [21]. Total volume of 0.2 ml of heparinized blood was mixed with 1.3 ml of distilled water and incubated for 10 min at 37°C for complete hemolysis. After adding 1 ml of standard δ aminolevulinic acid (ALA), the tubes were incubated for 60 min at 37°C. Enzyme activity was stopped after 1 h by adding 1 ml of 10% trichloroacetic acid (TCA). After centrifugation (1500 x g) of reaction mixture, equal volume of Ehrlich reagent was added to the supernatant and the absorbance was recorded at 555 nm after 5 min.

**Blood Glutathione (GSH):** Blood GSH concentration was determined following the procedure of Ellman [22], and modified by Jollow et al. [23]. 0.2 ml of whole blood was added to 1.8 ml of distilled water and incubated for 10 minutes at 37. °C for complete hemolysis. After adding 3ml of 4% sulphosalicylic acid, tubes were centrifuged at 2500-x g for 15 minutes. 0.2 ml of supernatant was mixed with 0.4 ml of 10 mM solution of 5,5' dithiobis-(2 nitro benzoic acid) (DTNB) in the presence of 1 ml phosphate buffer (0.1M pH 7.4). The absorbance was recorded at 412 nm.

**Reactive oxygen species (ROS) level in blood***:* Amount of ROS in blood was measured using 2', 7'-dichlorofluorescin diacetate (DCFDA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci et al. [24]. Briefly, 5% RBC hemolysate was prepared and diluted to 1.5% with ice-cold 40 mM tris –HCl buffer (pH 7.4) and further diluted to 0.25% with the same buffer and placed on ice. The samples were divided into two equal fractions. In one fraction 40-µl of 1.25 mM DCFDA in methanol was added for ROS estimation. Another fraction in which 40-µl of methanol was added, served as a control for tissue/hemolysate auto fluorescence. All samples were incubated for 15 min in a water bath at 37°C. Fluorescence was determined at 488 nm excitation and 525 nm emission wavelength using a fluorescence plate reader (Tecan Spectra Fluor Plus).

**Thiobarbituric acid reactive substances (TBARS) level in liver:** Tissue lipid per oxidation was measured by method of Ohkawa et al. [25]. 1 ml of tissue homogenate, prepared in 0.15M KCl (5% w/v), was incubated for 1 hr at 37°C followed by addition of 10% TCA, mixed thoroughly and centrifuge at 3000 rpm for 10 min. 1 ml TBA was added to 1 ml supernatant and the tube were kept

in boiling water bath for 10 minutes till the pink color appeared. 1 ml of double distilled water was added to after cooling the tubes and absorbance was measured at 532 nm.

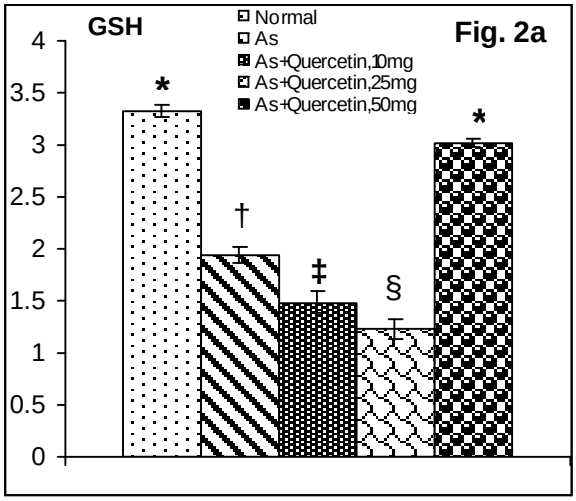
**Glutathione peroxidase (GPx) activity in liver:** Glutathione peroxidase activity was measured by the procedure of Flohe and Gunzler [26]. Reaction mixture contained 0.3 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of GSH (2mM), 0.1 ml of sodium azide (10mM), 0.1 ml of $H_2O_2$ (1mM) and 0.3 ml of supernatant (as prepare for measuring SOD activity). Complete mixture was incubated at 37 °C for 15 min, and reaction was terminated by adding 0.5 ml of 5 % TCA. Tubes were centrifuged at 1500xg for 5 min and supernatant was collected. 0.2 ml of phosphate buffer (0.1M pH 7.4) and 0.7ml of DTNB (0.4 mg/ml) was added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm.

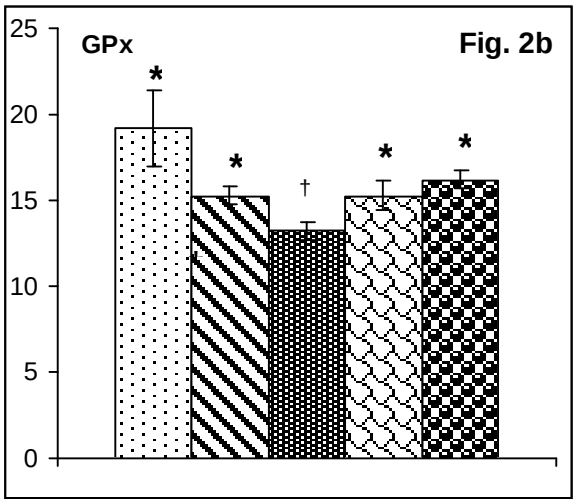
**Reduced glutathione (GSH) level in liver:**GSH level was measured as described by Hissin and Hilf [27]. Briefly, 0.25 g of tissue sample was homogenized on ice with 3.75 ml of phosphate–EDTA buffer and 1ml of 25% $HPO_3$ which was used as a protein precipitant. The total homogenate was centrifuged at 10,000×g for 30 min at 4 °C. For the tissue GSH assay, 0.5 ml supernatant and 4.5 ml phosphate buffer (pH 8.0) were mixed. The final assay mixture (2.0 ml) contained 100µl supernatant, 1.8ml phosphates–EDTA buffer and 100µl o-pthaldehyde (OPT; 1000µg/ml in absolute methanol, prepared fresh). After mixing, fluorescence was determined at 420nm with an excitation wavelength of 350 nm using a spectrofluorometer (Perkin Elmer, LS-55, UK).

**Statistical Analysis**: Data are expressed as means ± SEM. Data comparisons were carried out using ANOVA followed by Bonferroni test. A significance of < 0.001 and < 0.05 was considered significant.

## RESULTS

**Effect on clinical haematological variables:** Table 1 indicates the toxic effects of arsenic exposure and the preventive efficacy of Quercetin on some clinical hematological variables. Except for a significant decrease in WBC and HCT counts following arsenic exposure, most of the other hematological variables remained significantly



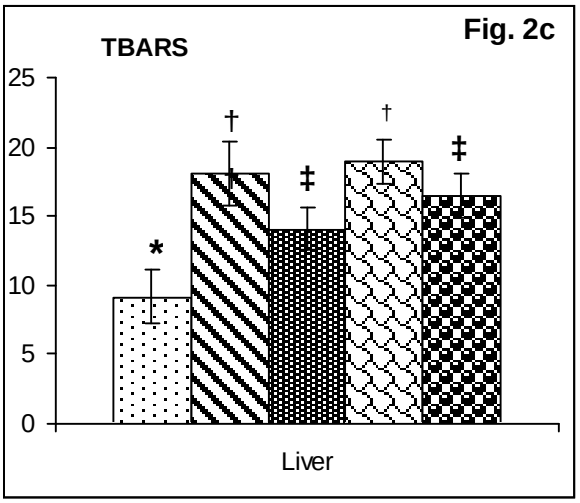




**Fig. 2:** Abbreviation used and units: Fig. 2a- GSH- Reduced Glutathione as mg/gm tissue; Fig. 2b - GPx- Glutathione peroxidase as µg/min/mg protein;Fig. 2c- TBARS- Thiobarbituric acid reactive species as mg/gm tissue.
Values are mean ± SE; n=5.

*, †, ‡, § Differences between values with matching symbol notations within each column is not statistically significant at 5% level of probability

**Table 1:** Effect of quercetin on arsenic induced hematological variables in rats

| | Normal animal | As | As + Quercetin, 10 mg | As + Quercetin, 25mg | As + Quercetin, 50mg |
|---|---|---|---|---|---|
| WBC | 9.2±1.2* | 6.5±0.54† | 6.4±0.34† | 8.2±0.61* | 9.3±0.26* |
| RBC | 6.1±0.47 | 6.0±0.43 | 6.9±0.20 | 8.2±0.29 | 7.9±0.31 |
| HGB | 11.9±0.9 | 10.6±0.7 | 10.7±0.9 | 12.1±0.9 | 13.3±0.7 |
| HCT | 45.7±2.6* | 36.9±2.6† | 34.4±2.6† | 41.0±2.3* | 43.1±1.5* |
| MCV | 46.9±3.0 | 50.8±1.7 | 49.4±2.7 | 50.2±1.8 | 54.3±0.4 |
| MCH | 14.2±1.4 | 15.9±0.7 | 15.3±1.13 | 14.7±0.7 | 16.6±0.3 |
| MCHC | 30.1±1.2 | 31.2±0.7 | 30.97±0.8 | 29.3±0.7 | 30.6±0.7 |

**Abbreviation used and units-** RBC- Red blood cells as $10^3/\mu l$; WBC– White blood cells as $X10^6/\mu l$; Hb- Hemoglobin as g/dl; HCT-Hematocrit as %; MCV- Mean cell Volume as fL; MCH- Mean Cell Hemoglobin as pg; MCHC- Mean Cell Hemoglobin Concentration as g/dL; PLT- Platelet as $X10^3/\mu l$. Values are mean±SE; n=5. *, †, æ% Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability.

**Table 2:** Effect of quercetin on changes in biochemical variables suggestive of heme synthesis pathway and blood oxidative stress in rats

| | U-ALA | ALAD | GSH | ROS | Blood Arsenic |
|---|---|---|---|---|---|
| Normal animal | 0.08±0.01* | 6.9±0.9* | 3.6±0.2* | 0.36±0.05 | 0.02±0.05* |
| Arsenic | 0.94±0.03† | 2.7±0.2† | 2.4±0.3† | 1.4±0.02† | 9.4±0.12† |
| As + Quercetin,10mg | 1.02±0.04† | 2.2±0.5† | 2.5±0.3† | 1.3±0.00† | 7.9±0.20† |
| As+ Quercetin, 25 mg | 0.65±0.03‡ | 4.8±0.2‡ | 2.5±0.1† | 1.1±0.00† | 8.1±0.23‡ |
| As+ Quercetin, 50 mg | 0.12±0.04* | 6.3±0.1* | 2.8±0.1† | 0.51±0.00 | 1.71±0.00 |

**Abbreviation used** and units- ALAD- δ-aminolevulinic acid dehydratase as nmol/min/ml erythrocytes; U-ALA Urinary δ-aminolevulinic acid as mg/dl; GSH- Glutathione as mg/ml; ROS- ηmoles $min^{-1}ml^{-1}$ of RBC. Blood arsenic - µg/dl
Values are mean±SE; n=5. *, †, ‡Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability

unchanged. Supplementation of quercetin (50 mg/kg dose) led to a pronounced recovery in HCT and WBC count close to the normal group compared to other doses of quercetin. Interestingly, HGB and RBC counts were higher after quercetin administration and were comparable to the normal value.

**Effects on biochemical variables in blood:** Table 2 shows the effect of arsenic exposure and co-administration of Quercetin with arsenic at three different doses on some blood biochemical variables indicative of alterations in heme biosynthesis and oxidative stress. The blood ALAD activity inhibited significantly following As exposure. Co-administration of Quercetin (25 and 50 mg/kg) was able to increase ALAD activity towards normal level compared to the lower dose. U-ALA activity showed significant increase in arsenic exposed groups (Table 2) which again showed significant recovery on quercetin administration (25 and 50 mg/kg). Blood GSH decreased significantly on arsenic exposure which remained unchanged on Quercetin administration except for some marginal recovery on 50 mg/kg, Quercetin administration. Blood ROS level increased significantly on arsenic exposure and co-administration of Quercetin particularly at the level of 25 and 50 mg/kg significant reduced the level towards normal. Blood arsenic which increased significantly on exposure showed significant reduction on Quercetin administration.

**Effect on hepatic oxidative stress and lipid per oxidation:** Figure 2 shows the preventive efficacy of Quercetin on arsenic induced changes in some biochemical variables suggestive of oxidative stress. Liver GSH level decreased significantly in arsenic exposed animals. Except for 50 mg/kg administration of Quercetin, none of other two doses had any marked effect on GSH level. GPx activity decreased marginally except for a significant decrease in animals co-administered with 10 mg/kg Quercetin compared to all other treatments. Hepatic TBARS content showed significant increase in arsenic exposed animals (Fig 2) and co-administration of Quercetin with arsenic marginally decreased TBARS level towards the normal value except for the animals co-administered Quercetin at 25 mg/kg dose.

## DISCUSSION

In the present study, we aimed at determining the beneficial effects of Quercetin using three different doses against arsenic induced oxidative stress in blood and hepatic tissues based on some selected biochemical variables. There are enough evidence in the literature which support that natural flavonoid like Quercetin have the greater ability than other potent antioxidant like vitamin C in preventing arsenic toxicity [28]. The antioxidant efficacy of Quercetin could be attributed due to, (i) its higher diffusion rate into the membranes [29] allowing it to scavenge free radicals at various sites; (ii) its pentahydroxyflavone structure, allowing it to chelate metal ions [30]; (iii) regeneration of endogenous and exogenous antioxidants like vitamin C and E and glutathione and (iv) presence of sulfhydryl group in the structure justified its selection in this study against arsenic toxicity. The generation of free radicals under various physiological and pathological conditions has contributed to the imbalance between pro-oxidant and antioxidants resulting oxidative stress which is recognized as one of the important biochemical mechanism of arsenic [31].

In our results, significant inhibition of delta-aminolevulinic acid dehydratase (δ-ALAD) activity was observed in arsenic exposed group. δ-ALAD, a zinc dependent metalloenzyme plays a key role in heme biosynthesis pathway and its inhibition by arsenic results in the accumulation of its substrate delta-aminolevulinic acid (δ-ALA) that rapidly oxidized to generate free-radicals such as superoxide ion, hydroxyl radical, and hydrogen peroxides responsible for producing various toxic effects in arsenic toxicity [32]. In the present study, decrease δ-ALAD activity and increase in urinary-ALA excretion after arsenic exposure support this hypothesis. Quercetin at 10 mg/kg showed beneficial dose in recuperating the δ-ALAD activity near to normal group as compared to other doses whereas U-ALA activity showed marked significant restoration in all the three doses of Quercetin.

Glutathione is an important component of the antioxidant defense mechanism and it functions as a direct scavenger of free radicals [33]. Mishra and Flora [34] reported the protective efficacy of quercetin against arsenic induced GSH depletion which supports our results. Quercetin at all three doses gradually showed significant decrease conflicting that it acts as prooxidant or has some GSH inhibitory activity [35] which is in agreement with our results. The depleted GSH level following arsenic exposure may be due to the consumption of GSH during quercetin metabolism by utilizing one GSH molecule to convert P-quinone methide 1 metabolite into 6-glutathionyl quercetin [36].

In the present study, increased lipid peroxidation associated with decreased antioxidant status in arsenic treated rats can therefore related to insufficient antioxidant potential. Quercetin at 25 mg/kg dose showed marked increase in TBARS level as compared to arsenic exposed group. While, the other two doses of quercetin decreased TBARS level near to the normal group. GPx acts as preventive antioxidants responsible for detoxification of free radicals. The GPx activity decreased in arsenic exposed group may be due to the decreased synthesis of enzymes or oxidative inactivation of enzymes protein. Quercetin at 10 mg/kg decreases the GPx activity significantly as compared to other doses of quercetin.

Thus, the above study provides a probable insight on the beneficial effects of Quercetin intake at three different doses tried in the present study; co-administration of Quercetin (50 mg/kg) with arsenic was most effective in preventing arsenic poisoning by reducing oxidative stress. Besides, providing beneficial effects in reversing the altered biochemical variables quercetin intake could also be useful in enhancing endogenous antioxidant levels.

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