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Antioxidant and Hepatoprotective Potential of Soy Isoflavones Against CCl_4 Induced Oxidative Stress and Early Tumor Events

Tajdar Husain Khan [*a,b] and Sarawat Sultana[b]

[a]*Department of Pharmacology, College of Pharmacy, Alkharj University, Alkharj 11942, Kingdom of Saudi Arabia*

[b]*Section of Chemoprevention and Nutrition Toxicology, Department of Medical Elementology and Toxicology, Jamia Hamdard(Hamdard University), Hamdard Nagar, New Delhi-110062, India*

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Address for Correspondance: Tajdar_hamdard@rediffmail.com

Abstract— In the present study we have evaluated the hepatoprotective efficacy of soy isoflavones (SIF), against carbon tetrachloride (CCl_4) induced oxidative damage and early tumor response in male Wistar rats. Methods: Single treatment of CCl_4 (1:1 v/v in corn oil was administered orally at dose of 1ml kg^{-1} body weight) induced oxidative stress and enhanced early tumor markers. Results: CCl_4 treatment resulted in marked reduction of hepatic glutathione content and activities of hepatic anti-oxidant enzymes, viz., glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PD) and phase-II metabolising enzymes such as glutathione-*S*-transferase (GST) and quinone reductase (QR) significantly as compared to control group ($p<0.001$). Concomitant enhancement in xanthine oxidase (XO) and malanodialdehyde (MDA) formation was observed with simultaneous induction in serum toxicity markers and tumor promotion markers, viz., SGPT, SGOT, LDH, ornithine decarboxylase (ODC) activity and thymidine [^{3}H] incorporation into hepatic DNA were also found significant($p<0.001$). Pretreatment of rats with SIF (20 and 40 mg kg^{-1}body weight, orally) resulted in a significant decrease in XO ($p<0.05$) level and MDA formation. There was decrease in GPT, GOT and LDH release in serum ($p<0.001$) and concomitant and dose dependent decrease in hepatic ODC activity and DNA synthesis significantly ($p<0.001$). There was also significant restoration of hepatic glutathione content, anti-oxidant enzymes and other phase-II metabolizing enzymes ($p <0.001$). Conclusions: Thus, these findings show that SIF attenuates CCl_4-mediated hepatic oxidative stress, toxicity, tumor promotion and subsequent cell proliferation response in Wistar rats.

Keywords : Soy Isoflavones, Antioxidants, CCl4, Tumor Promotion, ODC.

INTRODUCTION

Herbal products are gaining progressively attention due to less toxicity and high efficacy against free radical mediated diseases [1,2]. Soybean decreases the risk of various diseases and pathological conditions, including various types of cancers, osteoporosis, menopausal symptoms, and coronary heart disease [3-6]. The chemopreventive effects of soybean and soy containing food may be related to their isoflavone content. Main isoflavones are genistein, daidzein and glycitein [5-8]. These molecules may be conjugated with a 7-O-b-glucoside, a 6''-O-malonylglucoside or a 6''-O-acetylglucoside group play a crucial role in the prevention of oxidation-related diseases including atherosclerosis, hypertension [9], breast cancer [10], or inflammatory bowel syndrome [11]. Soy saponins are another class of natural glycosides found in soybean seeds and soy-derived products. Flavonoids have gained importance as scavengers of free radicals and a potent inhibitor of lipidperoxidation [12, 13]. In spite of the estrogenic activity, isoflavones show DNA topoisomerase activity, synthesis and release of TGF^{β}, modulation of apoptosis, and potent inhibitory activity of tyrosine specific protein kinases [5,6,14].

It has been reported that population having high intake of isoflavones show lower incidence of cardiovascular diseases, osteoporosis, kidney diseases and cancer risk [6, 15]. Genistein and daidzein have also been shown to prevent 8-hydroxy-2'-deoxyguanosine formation in toxicant exposed cells [16-18].

The marked vulnerability of the liver to chemical damage has been shown as a function of several contributing factors including its anatomical proximity to blood supply, role in metabolism and excretion of xenobiotics. Hence prevention of hepatotoxic damage is of great concern [19]. CCl_4 is an established hepatotoxicant for both animal and human. CCl_4 is known as a degreaser, solvent and is used as a fire extinguisher and also as a substrate for the synthesis of fluorocarbon and other industrial and laboratory reagents [20-22]. Its toxic nature is due to production of trichlorocarbon ($CCl3^{*}$) radical, which can initiate the oxidation of macromolecules and lipids leading to oxidative stress [22, 23]. Oxidative damage plays an important role in carcinogenesis [24,25]. ROS generated by mitochondria or from other intracellular or extracellular sites can cause cell damage and initiate various degradation processes. ROS-induced lipid peroxidation has been implicated in neoplastic transformation. The role of ROS has been implicated in many human degenerative diseases, including aging and cancer [26-28].

Soybeans and soyfoods potentially have multifaceted health-promoting effects. Keeping in view, the potential health promoting effects of soybeans and soyfoods we have hypothesized that the soy isoflavones may inhibit oxidative stress and toxicity induced by CCl_4 in animals.

MATERIALS AND METHODS

Chemicals

Novasoy® 40% soy isoflavone concentrate procured from Archer Daniels Midland Co. Decatur, Illinois, EDTA, Tris, Reduced glutathione (GSH), oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate reduced (NADPH), bovine serum albumin (BSA), 1,2-dithio-bisnitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitro benzene (CDNB), phenylmethyl sulfonylfluoride (PMSF), nitroblue tetrazolium (NBT), phenylmethyl sulfonylfluoride (PMSF), Brij-35, Pyridoxal-phosphate, 2-mercaptoethanol, dithiothreitol, Tween 80 were obtained from Sigma chemicals Co (St Louis, MO). DL [^{14}C] ornithine and [^{3}H] thymidine were purchased from Amersham Corporation (Little Chalfort, UK). All other chemicals were of the highest purity and commercially available.

Animals

Eight week old adult male Wistar rats (150-200 g) were obtained from the Central Animal House Facility of Hamdard University, New Delhi and were housed in a ventilated room at 25 ± 2 ^{0}C under a 12-h light / dark cycle. The animals were acclimatized for one week before the study and had free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water ad libitum.

Experimental design

To study the biochemical, histopathological and serological changes 25 male Wistar rats were divided into five groups. Group I served as saline treated control. Group II served as negative control and was administered CCl_4 (1 ml kg^{-1} body weight p. o., in corn oil [1:1]). Group III served as positive control and was given higher dose (D_2) of SIF for seven consecutive days. Groups IV and V were pretreated with SIF at doses 20 and 40 mg kg^{-1} body weight for seven consecutive days followed by CCl_4 intoxication on the 7th day. All animals were sacrificed 24 h after CCl_4 intoxication. Serum was separated and stored at 4 ^{0}C for the estimation of GOT, GPT and LDH. Tissue was processed for the estimation of hepatic ornithine decarboxylase (ODC) activity and other biochemical parameters.

For histopathological study livers were immediately rinsed with cold saline excised and quickly fixed in ice-cold 10% buffered formalin. Formalin-fixed material was processed for hematoxylin and eosin staining. The histopathological studies and their interpretation were carried out in the Maulana Azad Medical College, New Delhi, India by Professor, R.K. Bhatnagar, Department of Pathology.

For [^{3}H] thymidine incorporation study, same treatment regimen was followed except all the animals were given intraperitoneal [^{3}H] thymidine (25 μCi per animal) 2 h before killing. Time of sacrifice was after 48 h of CCl_4 intoxication; liver sections were quickly

excised, rinsed with ice-cold saline, freed of extraneous material and processed for the quantification of [^{3}H] thymidine incorporation into the hepatic DNA.

Biochemical estimations:

Tissue processing and preparation of post mitochondrial supernatant (PMS) were done as described by Athar and Iqbal [29]. All the biochemical estimations were completed within 24 h of animal sacrifice.

Estimation of reduced glutathione

Reduced glutathione was determined by the method of Jollow et al., [30]. One ml PMS was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for one hour and then centrifuged at 1200 g for 20 min at 4°C. The assay mixture contained 0.4 ml supernatant, 2.6 ml sodium phosphate buffer (0.1M, pH 7.4) and 0.2 ml DTNB (100 mM) in a total volume of 3.0 ml. The yellow colour developed, was read immediately at 412 nm on a spectrophotometer.

Catalase activity

Catalase activity was measured by the method of Claiborne, [31]. The reaction mixture consisted of 2ml phosphate buffer (0.1M, pH 7.4), 0.95 ml hydrogen peroxide (0.019M) and 0.05 ml PMS in a final volume of 3ml. Changes in absorbance were recorded at 240nm. Catalase activity was calculated as nmol H_2O_2 consumed min^{-1} mg^{-1} protein.

Glutathione-S-transferase activity

Glutathione-S-transferase activity was estimated by the method of Habig et al, [32]. The reaction mixture consisted of 1.425 ml sodium phosphate buffer (0.1M, pH 7.4), 0.2 ml reduced glutathione (1mM), 0.025 ml CDNB (1mM) and 0.3 ml PMS (10% w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nmol CDNB conjugate formed $min^{-1}mg^{-1}$ protein using a molar coefficient of 9.6 x $10^3M^{-1}cm^{-1}$.

Glutathione reductase activity

Glutathione reductase activity was assayed by the method of Carlberg and Mannervick [33] as modified by Mohandas et al, [34]. The assay system consisted of 0.1 M, pH 7.4 sodium phosphate buffer, 0.5 mM EDTA, 1 mM GSSG, 0.1 mM NADPH and PMS (10% w/v) in a total mixture of 2.0 ml. The enzyme activity was measured at 340 nm and calculated using a molar extinction coefficient of 6.22 x 10^3M/cm.

Glutathione peroxidase activity

Glutathione peroxidase activity was assayed by the method of Mohandas et al, [34]; as described by Athar and Iqbal [29]. The assay mixture consisted of 0.1 M, pH 7.4 sodium phosphate buffer, 1 mM EDTA, 0.2 mM NADPH, 1 mM sodium azide, 1 IU/ml glutathione reductase, 0.25 mM H_2O_2, and PMS (10% w/v) in a total volume of 2.0 ml. The activity was recorded at 340 nm and calculated using a molar extinction coefficient of 6.22 x 103$M^{-1}cm^{-1}$.

Glucose-6-phosphate dehydrogenase activity

The activity of glucose-6-phosphate dehydrogenase was assayed by the method of Zaheer et al, [35]. The reaction mixture consisted of 0.3ml tris -HCl buffer (0.05M, pH 7.6), 0.1ml NADP (0.1mM), 0.1ml glucose-6-phosphate (0.8mM), 0.1ml $MgCl_2$ (8mM), 0.3ml PMS and 2.4ml distilled water in a total volume of 3ml. The changes in absorbance were recorded at 340nm and enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

Quinone reductase activity

Quinone reductase activity was measured by the method of Benson et al, [36]. The assay mixture consisted of 0.1 ml cytosolic fraction (10%), 0.7ml of BSA (0.1 %), 0.02 ml tween-20 (1 %), 0.1 ml of FAD (150 M), 0.02 ml of NADPH (0.2 mM), 0.05 ml of 2,6,DCIP (0.29 %) and 2 ml of tris-HCl buffer (25 mM, pH 7.4) with a final volume of 3 ml and the optical density was read at 600 nm for 3 min. The enzyme activity was calculated as nmol 2,6, DCIP reduced $min^{-1}mg^{-1}$ protein.

Xanthine oxidase activity

The activity of xanthine oxidase was assayed by the method of Stripe and Della Corte, [37]. The reaction mixture consisted of 0.2 ml PMS which was incubated for five minutes at room temperature with 0.8ml phosphate buffer (0.1M, pH 7.4). Then 0.1ml xanthine (9mM) was added to the reaction mixture and kept at 37°C for 20 min, which was followed by the addition of 0.5ml of 10% perchloric acid and 2.4ml of double distilled water in a total volume of 4ml. After 10 min, the mixture so obtained was centrifuged at 4000-rev min^{-1} for 10 min and μg uric acid formed min^{-1} mg^{-1} protein was recorded at 290nm.

Estimation of lipid peroxidation

The assay of microsomal lipid peroxidation was done according to the method of Wright et al. [38]. The reaction mixture consisted of 0.58ml phosphate buffer (0.1M, pH 7.4), 0.2ml microsome, 0.2ml ascorbic acid (1mM) and 0.02ml ferric chloride (100mM) in a total volume of 1ml. The mixture was incubated at 37°C in a shaking water bath for 1h. Then 1ml 10% trichloroacetic acid and 1ml 0.67% TBA was added. All the tubes were placed in a boiling water bath for 20min. The tubes were placed in an ice bath and then centrifuged at 2500g for 10 min. The amount of malanodialdehyde (MDA) formed in each of the samples was assayed by measuring the optical density of the supernatant at 535nm.The results were expressed as nmol MDA formed h^{-1} g^{-1} tissue at 37°C using a molar extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1}.

Lactate dehydrogenase (LDH) activity

Lactate dehydrogenase activity was estimated in serum by the method of Kornberg, [39]. The reaction mixture consisted of serum, NADH (0.02 M), Sodium pyruvate (0.01 M), sodium phosphate buffer (0.1 M, pH 7.4) and distilled water in a total volume of 3.0 ml. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADH oxidized $min^{-1}mg^{-1}$ protein.

Determination of serum oxaloacetate and pyruvate transaminases (GOT & GPT)

Serum GOT and GPT were determined by the method of Reitman and Frankel, [40]. Each substrate (0.5 ml) (2mM α-ketoglutarate and either 200 mM α L-Alanine or L-Aspartate was incubated for 5 min at 37°C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with sodium phosphate buffer. The reaction mixture was incubated for exactly 30 min and 60 min for GPT and GOT, respectively. Then to the reaction mixture, 0.5 ml of DNPH (1mM) was added and left for another 30 min at room temperature. Finally, the colour was developed by addition of 5.0 ml of NaOH (0.4 N) and product read at 505 nm.

Estimation for tumor markers

Ornithine decarboxylase activity

ODC activity was determined using 0.4 ml hepatic 105,000g supernatant fraction per assay tube by measuring the release of CO2 from DL- [^{14}C] ornithine by the method of O'Brien et al, [41]. The liver was homogenized in Tris–HCl buffer (pH 7.5, 50 mM) containing EDTA (0.4 mM), pyridoxal phosphate (0.32 mM), PMSF (0.1 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (4.0 mM) and Tween 80 (0.1%) at 4°C using a Teflon-glass homogenizer. In brief, the reaction mixture contained 400µl enzymes and 0.095 ml co-factor mixture containing Pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brij 35 (0.02%) and DL-[^{14}C] ornithine (0.05 µCi) in total volume of 0.495 ml. After adding buffer and cofactor mixture to blank and others tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanolamine and methoxyethanol mixture (2:1) in the central well and kept in water-bath at 37°C. After 1 h of incubation, injecting 1.0 ml citric arrested the enzyme activity acid solution (2.0 M) along the sides of glass tubes and the solution was continued for 1 h to ensure complete absorption of CO2. Finally, the central well was transferred to a vial containing 2 ml ethanol and 10 ml toluene based scintillation fluid. Radioactivity was counted in liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as pmol CO2 released $h^{-1}mg^{-1}$ protein.

Hepatic DNA synthesis

The isolation of hepatic DNA and incorporation of [^{3}H] thymidine in DNA was done by the method of Smart et al, [42] as described by Athar and Iqbal [29]. Livers were quickly removed cleaned free of extraneous material and homogenate (10% w/v) was prepared in ice-cold water. The precipitate thus obtained was washed with cold trichloroacetic acid (TCA) (5%) and incubated with cold perchloric acid (PCA) (10%) at 4°C for over night. After the incubation it was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%) followed by incubation in boiling water bath for 30 min, and filtered through Whatman 50. The filtrate was used for [^{3}H] thymidine counting in liquid scintillation counter (LKB-Wallace-1410) by adding the scintillation fluid. The amount of DNA in the filtrate was estimated by diphenylamine method of Giles and Myers, [43].The amount of [^{3}H] thymidine incorporated was expressed as DPM μg^{-1} DNA.

Protein estimation

Protein content in all samples was estimated by the method of Lowry et al, [44] using bovine serum albumin as standard.

Statistical analysis

The level of significance between different groups is based on ANOVA test followed by Dunnett's t test.

Histopathology

Fixation, dehydration, infiltration and block preparation

The tissues were then dehydrated by passing through graded series of ethyl alcohol (50%, 70%, 90% and 100%) for one hour in each giving two changes. These were then cleared in xylene (two changes of one hour each). The cleared tissues were placed for 5 minutes in xylene containing molten paraffin wax at 50-60°C for infiltration. After giving two changes of molten paraffin (1 hour each) the tissues were embedded in fresh paraffin wax. Embedded tissue was sectioned (5-6 µ thickness) on a microtome and spread on albumin coated glass slides. These were then dried at 35-40°C.

Staining Procedure

Sections were deparaffinized by dipping in xylene (2 changes). These slides were then passed through graded concentration of ethyl alcohol (100%, 90%, 70%, 50% and 30%) with two changes of 2 minutes each, and then kept stained with hematoxylin for 1 minute and again washed in running water thoroughly. Slides were then passed through 50% and 70% ethyl alcohol and subsequently put into eosin stain (prepared in 70% ethyl alcohol). These were then passed through graded series of ethyl alcohol [70%, 90%, 100%, 100% + xylene (1:1)] and finally given two changes of xylene. The slides were mounted in D.P.X. covered with glass cover slips and kept for drying at room temperature.

RESULTS

Here we have evaluated the antioxidant and anti-proliferative potential of SIF against CCl_4 induced hepatic oxidative stress in wistar rats. CCl_4 intoxication leads to depletion of hepatic glutathione, its metabolizing enzymes GST and GR and antioxidant enzymes CAT, GPx, QR and G6PD by 38 %, 70 %, 41 %, 50 %, 30 %, 44 % and 71 % respectively as compared with the saline treated control group. CCl_4 also caused elevation in the activity of XO to 99 % and increase in the levels of MDA formation to 290 % and liver toxicity markers SGPT, SGOT and LDH by 56-304 %.

Pretreatment with soy isoflavones (20 mg and 40mg^{-1} kg body weight) restored hepatic glutathione content to 18-37 %, glutathione-metabolizing enzymes GST and GR by 43-146 %, and is shown in Table1. Antioxidant and glutathione dependent enzymes like CAT, GPx and G6PD to 21-113 % and are shown in Table 2. There was a marked depletion in levels of XO to 24-31 %, and MDA formation by

22-35 % and restoration of QR by 22-63 % is shown in Table3. Down regulation of release of GPT, GOT and LDH to 24-59 % in serum was observed and is shown in Table 4.

Treatment Groups	**Reduced glutathione (μ M GSH reduced h^{-1} g^{-1} m tissue)**	**Glutathione reductase (nM NADPH oxidized min^{-1} mg^{-1} protein)**	**Glutathione-S-transferase (nM CDNB conjugate formed min^{-1} mg^{-1} protein)**
Group I Control	0.910 ± 0.0469	372.37 ± 11.17	167.73 ± 4.77
Group II Only CCl_4 (1ml kg^{-1} body weight)	0.560 ± 0.006*	220.27 ± 14.76*	50.86 ± 2.37*
Group III Only SIF 40 mg kg^{-1} body weight)	0.860 ± 0.027	403.36 ± 9.6	161.58 ± 11.25
Group IV SIF 20 kg^{-1} body weight. + CCl_4 (1ml kg^{-1} body weight)	0.659 ± 0.012#	314.58 ± 3.14#	111.33 ± 10.28#
Group V SIF 40 kg^{-1} body weight. + CCl_4 (1ml kg^{-1} body weight)	0.765± 0.026#	335.30 ± 7.50#	124.92 ± 4.83#

Table 1 Effect of pretreatment of soy isoflavones on the CCl4 mediated depletion in glutathione content and its metabolizing enzymes in the liver of Wistar rats

***p≤ 0.001 on comparison of normal control with CCl_4 treated group and only soy isoflavone group.**

#p≤ 0.001 on comparison of soy isoflavones treated groups with only CCl_4 treated group.

Treatment Groups	**Catalase (nM H_2O_2 consumed min^{-1} mg^{-1} protein)**	**Glutathione peroxidase (n M NADPH oxidizedmin^{-1} mg^{-1} protein)**	**Glucose-6-phosphate Dehydrogenase (nM NADP reduced min^{-1} mg^{-1} protein)**
Group I Control	191.91 ± 2.96	470.93 ± 12.33	66.62 ± 2.17
Group II Only CCl_4 (1ml kg^{-1} body weight)	95.18 ± 1.77*	328.03 ± 7.48*	19.51 ± 0.53*
Group III Only SIF40 mg kg^{-1} body weight)	181.24 ± 3.95	469.91 ± 8.64	65.55 ± 2.57
Group IV SIF20 mg/kg body weight. + CCl_4 (1ml kg^{-1} body weight)	115.15 ± 3.65#	396.75 ± 5.47#	25.71 ± 1.09#
Group V SIF40 mg/kg body weight. + CCl_4 (1ml kg^{-1} body weight)	162.66 ± 3.84#	433.81 ± 6.92 #	41.65 ± 2.36#

Table 2 Effect of pretreatment of soy isoflavones on the CCl4 mediated alteration in the activity of antioxidant enzymes in the liver of Wistar rats

***p≤ 0.001 on comparison of normal control with CCl_4 treated group and only soy isoflavone group.**
#p≤ 0.001 on comparison of soy isoflavones treated groups with only CCl_4 treated group.

Treatment Groups	**Quinone Reductase (nM dichloroindophenol reduced min^{-1} mg^{-1} protein)**	**Lipid Peroxidation (n M MDA formed min^{-1} mg^{-1} protein)**	**Xanthine Oxidase (μg uric acid formed min^{-1} mg^{-1} protein)**
Group I Control	304.86 ± 6.22	3.86 ± 0.153	0.273 ± 0.029
Group II Only CCl_4 (1ml kg^{-1} body weight)	172.10 ± 2.8*	15.05 ± 0.078*	0.543 ± 0.001*
Group III Only SIF40 mg kg^{-1} body weight)	298.84 ± 4.52	4.30 ± 0.194	0.270 ± 0.006
Group IV SIF20 mg/kg body weight. + CCl_4 (1ml kg^{-1} body weight)	210.80 ± 5.59 #	11.74 ± 0.113#	0.415 ± 0.003#
Group V SIF40 mg/kg body weight. + CCl_4 (1ml kg^{-1} body weight)	280.98 ± 8.73#	9.83 ± 0.109#	0.373 ± 0.003#

Table 3 Effect of pretreatment of soy isoflavones on the CCl4 mediated alteration in the activity of QR, LPO and XO in the liver of Wistar rats

*p≤ *P≤ 0.001 on comparison of normal control with CCl_4 treated group and only soy isoflavone group.

#p≤ 0.001 on comparison of soy isoflavones treated groups with only CCl_4 treated group.

Treatment Groups	**SGPT (IU l^{-1})**	**SGOT (IU l^{-1})**	**LDH (nmol NADPH oxidized min^{-1} mg^{-1} protein)**
Group I Control	17.56 ± 0.345	24.56 ±0.345	359.05 ± 5.79
Group II Only CCl_4 (1ml kg^{-1} body weight)	70.90 ± 0.412*	79.60± 2.234*	587.29 ± 6.39*
Group III Only SIF40 mg kg^{-1} body weight)	17.02 ± 0.490	24.80 ± 0.459	357.14 ± 3.85
Group IV SIF20 mg/kg body weight + CCl_4 (1ml kg^{-1} body weight)	35.76 ± 0.178#	60.83 ± 0.447#	397.96 ± 2.65 #
Group V SIF40 mg/kg body weight + CCl_4 (1ml kg^{-1} body weight)	29.34 ± 0.219#	47.92 ± 0.675#	389.62 ± 2.87#

Table 4 Effect of pretreatment of soy isoflavones on the CCl4 mediated alteration in the liver toxicity markers in the serum

***p≤ 0.001 on comparison of normal control with CCl_4 treated group and only soy isoflavone group**
#p≤ 0.001 on comparison of soy isoflavones treated groups with only CCl_4 treated group.

CCl_4 administration resulted in significant ($p<0.001$) depletion in hepatic GSH and its dependent enzymes with simultaneous decrease in antioxidant enzymes level and enhancement of liver toxicity markers. Soy isoflavones showed a significant protection of GSH content in a dose dependent manner ($p<0.001$). Prophylactic administration of SIF prior to CCl_4 treatment significantly decreased the MDA levels, with soy isoflavones showing inhibition of 35 %, at significance ($p<0.001$), respectively in a dose dependent manner. However SIF alone group produced no significant changes in GSH content and MDA formation. The effect of prophylactic administration of SIF on CCl_4 mediated leakage of liver marker enzymes in serum is shown in Table 4. CCl_4 administration resulted in a significant ($p<0.001$) rise in the levels of SGOT, SGPTand LDH in serum by 224 %. 304 % and 64 % of saline treated control, respectively. Pretreatment with SIF showed a marked dose dependent alleviation of SGOT, SGPT and LDH by 40 %, 59 % and 34 % respectively.

Figure 3 and 4 shows the significant modulation of early markers of tumor promotion like ODC activity and hepatic DNA synthesis in rat. The effect of prophylactic treatment of rats with SIF on CCl_4 induced rate of [^{3}H] thymidine incorporation into hepatic DNA. Administration of CCl_4 resulted in significant ($p<0.001$) increase in the rate of [^{3}H] thymidine incorporation into hepatic DNA and 179 % increase in ODC activity significantly ($p<0.001$) which is a marker of tumor promotion. The pretreatment of rats with SIF showed a

marked ($p<0.001$) suppressing effect on the rate [^{3}H] thymidine incorporation into hepatic DNA by 53 % of treated control, and inhibited ODC activity by 51 % of treated control, in a dose dependent manner.

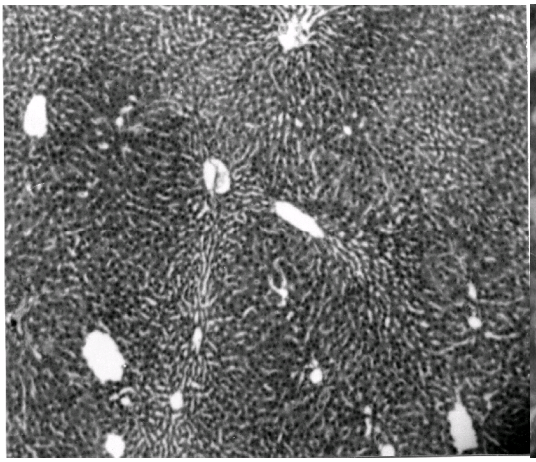

Fig (2B): CONTROL (H&E: X100)

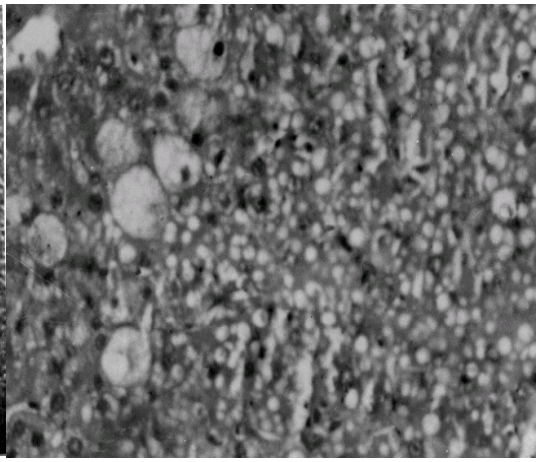

Fig (2A): CCl_4 (H&E: X500)

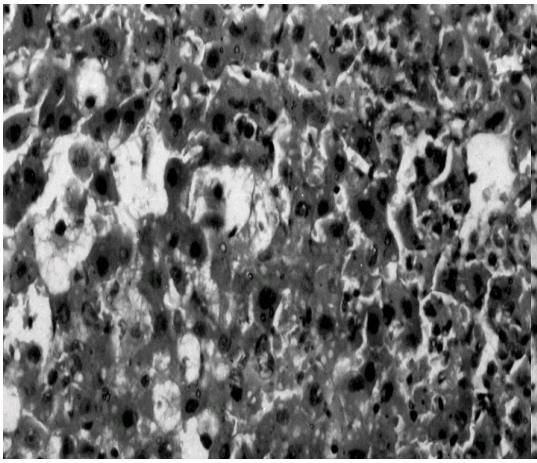

Fig (2C): CCl_4 +SIF (D_1) (H&E: X 500)

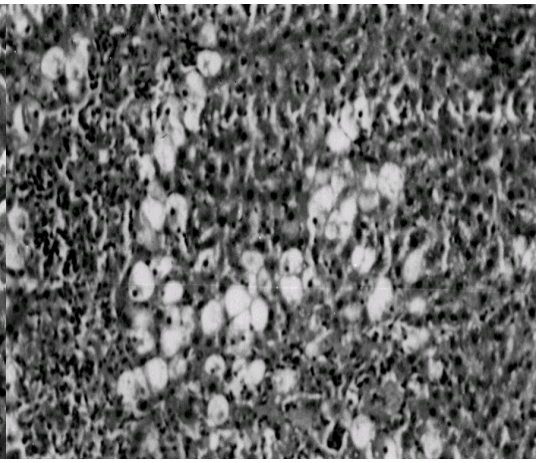

Fig (2D): CCl_4 +SIF (D2) (H&E: X 500)

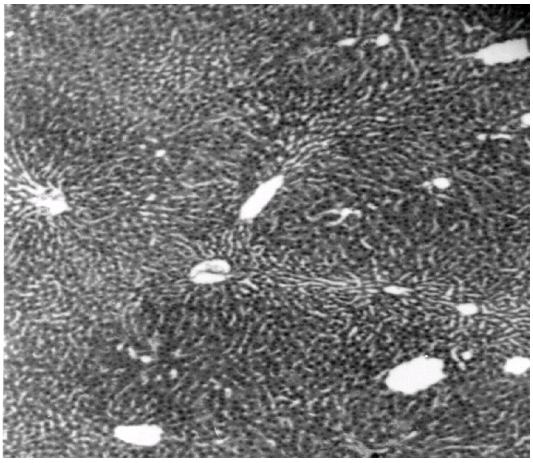

Fig (2E): ONLY SIF (D2) (H&E: X100)

However, on the basis of the results obtained from our studies, we conclude that SFI acts as a modulator of CCl4 induced by adversely affecting free radical generation and hyperproliferative response in Swiss albino mice.

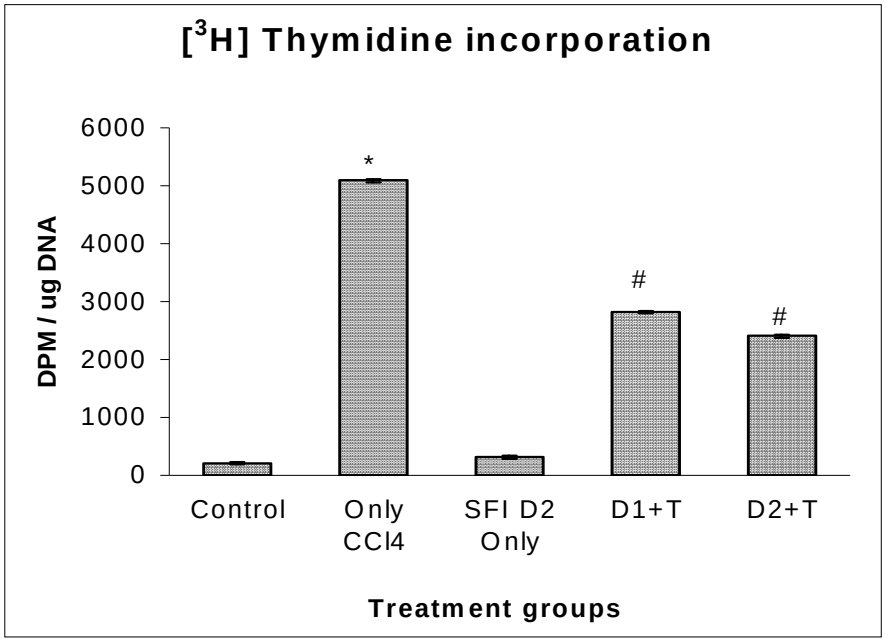

Figure 3

***p≤ 0.001 on comparison of normal control with CCl_4 treated group and only soy isoflavones group.**
#p≤ 0.001 on comparison of soy isoflavones treated groups with only CCl_4 treated group.

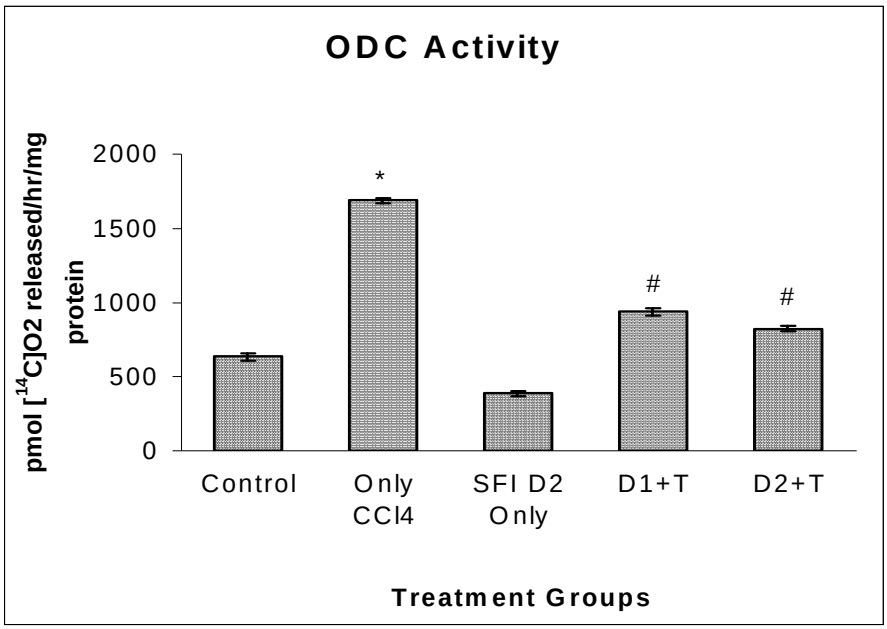

Figure 4

DISCUSSION

There has been a considerable emphasis laid down on the use of dietary constituents to prevent xenobiotic induced damage and oxidative stress due to their non-toxic effects [45, 46]. The carcinogenesis is multi-step process and free radicals are believed to be

involved in each of these steps initial step being oxidative stress. In our day-to-day life, we are constantly exposed to an array of chemicals that acts as free radical generators. So, emphasis has been laid on the study of prevention of oxidative stress and early tumor-promoting stages where prevention is practically possible. Studies have also suggested that the whole mixture may be more effective than its specific constituents [11, 47, 48]. These observations are congruent with the use of the soy isoflavones in our study. In fact, favorable effects have been suggested even with the consumption of a mixture of isoflavones [18]. We studied the effect of SIF on the biochemical parameters and early markers of tumor promotion induced by CCl_4. Single oral CCl_4 dose induced significant depletion in the hepatic GSH content and its metabolizing enzymes. Depletion in GSH levels, a natural cellular antioxidant, is suggestive of the insult by toxic foreign agent, which is CCl_4 in present case [49]. Recovery of the depleted GSH and GST on pretreatment of animals with SIF indicate its role as chemopreventive measure [50]. CCl_4 causes increased formation of pro-oxidants and a concomitant decrease in the antioxidant status of the cells [51]. CCl_4 hepatotoxicity depends on the major unstable radical trichloromethyl radical (CCl_3*) which rapidly reacts with oxygen molecule and form trichloromethyl peroxy radical, which is reported as highly reactive species. These free radicals attack microsomal lipids, proteins and leads to peroxidation products [52,53]. They also covalently bind to macromolecules and proteins and thus initiate a site for secondary biochemical process, which is the ultimate cause for unfolding of the panorama of several pathological sequences of CCl_4 metabolism. It has been suggested that SIF protects by inhibiting CCl_4 mediated toxicity through decreasing levels of MDA formation and reducing hepatic XO levels. The mechanisms by which the dietary agents inhibit hepatic damage induced by CCl_4 involve modulation of intracellular and extracellular metabolic pathway.

GR maintains GSH reduced where as GPx utilizes it for decomposition of lipid peroxides/hydro peroxides and other ROS. Substantial decrease in hepatic GSH with concomitant decrease in GR, GPx, QR, GST and G6PD levels on CCl_4 administration, a dose dependent marked recovery was observed with prophylactic treatment of SIF. Earlier studies have shown that active principles of SIF, genistein and diadzein, have potential to increase cellular antioxidants like GSH and other phase II metabolizing enzymes [54, 55] SIF treatment prior to CCl_4 intoxication restored the levels of serum toxicity markers as compared to negative control only CCl_4 group. CCl_4 administration resulted in significant induction in hepatic ODC and [^{3}H] thymidine uptake into hepatic DNA, prophylactic treatment with SIF prior to CCl_4 intoxication significantly inhibited ODC activity and rate of [^{3}H] thymidine incorporation. Protective action of SIF is observed by several mechanisms viz, by counteracting free radicals, inducing several antioxidant enzymes and restoring GSH levels. They all contribute to its potential as a modulator of oxidative damage produced by CCl_4. It reduces the risk of acute hepatic injury induced by CCl_4 and thus preventing the extent of post-necrotic hepatic injury.

CONCLUSION

Our data suggest that the efficacy of soy isoflavones is due to its antioxidant nature, antiproliferative response and ability to inhibit the activity of ODC and hepatic DNA synthesis this protective nature of soy isoflavones may be attributed to all three constituents'

genistein, daidzein and glycetin. Present study suggests that SIF as antioxidant is an effective chemopreventive tool against carcinogenic agents in which of free radicals are involved.

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REFERENCES

[1] T.H. Khan, T. Jahangir, L. Prasad, S. Sultana. Inhibitory effect of apigenin on benzo(a)pyrene-mediated genotoxicity in Swiss albino mice. J. Pharm Pharmacol. 2006, 58(12):1655-60.

[2] A. Hudecova, E. Ginter., The influence of ascorbic acid on lipidperoxidation in guinea pigs intoxicated with cadmium. Food Chem. Toxicol. 1992, 30:1011-1031.

[3] V.E. Archer. Cooking methods, carcinogens and diet-cancer studies. Nutr Cancer, 1988, 11: 75-79.

[4] K.A. Steinmetz, J.D. Potter. Vegetables, fruits and cancer: Epidemiology. Cancer Causes Control. 1991,2: 325-357.

[5] S. Barnes. The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. Lymphat. Res. Biol. 2010,8(1):89-98.

[6] C. Nagata. Factors to consider in the association between soy isoflavone intake and breast cancer risk. J. Epidemiol. 2010,20(2):83-9.

[7] Wei H, Bowen R, Cai Q, Barners S, Wang Y. 1995. Antioxidant and antipromotional effect of the soybean isoflavone genistein. Proc Exp Biol Med. 1:124-130.

[8] M. Messina, S. Barnes., The role of soy products in reducing risk of cancer. J. Nat. Cancer Instit. 1991,83:541–6.

[9] E. Park, J.I. Shin, Park et al., Soy isoflavones supplementation alleviates oxidative stress and improves systolic blood pressure in male spontaneously hypertensive rats. J Nutrit. Sci. Vitaminol. 2005,51(4): 254–259.

[10] R. A. DiSilvestro, J. Goodman, E. Dy, & G. Lavalle,. Soy isoflavone supplementation elevates erythrocyte superoxide dismutase, but not plasma ceruloplasmin in postmenopausal breast cancer survivors. Breast Cancer Res Treat. 2005, 89(3): 251–255.

[11] A. Wiseman, Crohn's disease leading to bowel cancer may be avoided by consumption of soya isoflavones: Adjunct-chemotherapy with oxaliplatin. Medical Hypotheses. 2006,66(5): 934–935.

[12] W. Bors, W. Heller, C. Michel, M. Saran. Flavonoids as antioxidants: Determination of radical scavenging efficiencies. Methods Enzymol., 1990,186: 343-355.

[13] T.H. Khan, S. Sultana., Apigenin induces apoptosis in Hep G2 cells: possible role of TNF-alpha and IFN-gamma. Toxicology. 2006,217(2-3):206-12.

[14] D.F. Birt, S. Hendrich, W. Wang. Dietary agents in cancer prevention: flavonoids and isoflavonoids. Ann N Y Acad Sci., 2001, 90: 157-177.

[15] J.M. Hodgson, K.D. Croft, I.B. Puddey, T.A. Mori, L.J. Beilin. Soybean isoflavonoids and their metabolic products inhibit in vitro lipoprotein oxidation in serum. J. Nutrit. 1996,7: 664-669.

[17] A. Arora, M.G. Nair, G.M. Strasburg. Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. Arch Biochem Biophys., 1998,356: 133-141.

[18] J. Kang, T.M. Badger, M.J. Ronis, X. Wu. Non-isoflavone phytochemicals in soy and their health effects. J. Agric. Food Chem. 2010,58(14):8119-33.

[19] T. Wang, K. Shankar, M.J. Ronis, H.M. Mehendale. Mechanisms and outcomes of drug and toxicant-induced liver toxicity in diabetes. Crit Rev Toxicol. 2007,37(5):413-59.

[20] National Academy of Science (NAS). Chloroform, carbon tetrachloride and other halomethanes: an Environmental Assessment, NAS, Washington, DC,1978.

[21] J.V. Bruckner, R. Luthra, G.M. Kyle, et al. Influence of time of exposure to carbon tetrachloride on toxic liver injury. Ann Rev Chronopharmacol., 1984,1: 373-376.

[22] L.W.D. Weber, M. Boll, A. Stampfl., Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit. Rev. Toxicol. 2003,33: 105–136.

[23] O. Faroon, C.T. De Rose, L. Smith. ATSDR evaluation of health effects of chemicals, I. Carbon tetrachloride: health effects, toxicokinetics, human exposure and environmental fate. Toxicol Indust Health. 1994, 10: 1-118.

[24] S.P. Hussain, L. J. Hofseth, C. C. Harris., Radical causes of cancer. Nature Rev Cancer. 2003,3: 276-285.

[25] R. Olinski, A. Siomek, R. Rozalski et al., Oxidative damage to DNA and antioxidant status in aging and age-related diseases. Acta Biochim. Pol. 2007,54(1):11-26.

[26]S. Orrenius. Reactive oxygen species in mitochondria-mediated cell death. Drug Metab Rev. 2007, 39(2-3):443-55.

[27] D. Hristozov, V. Gadjeva, T. Vlaykova, G. Dimitrov., Evaluation of oxidative stress in patients with cancer. Arch Physiol Biochem. 2001,109:331–6.

[28] D. Harman., Free radical theory of aging, increasing the functional life span. Ann N Y Acad Sci. 1994, 717, 1–15.

[29] M. Athar, M, Iqbal. Ferric nitrilotriacetate promotes N-diethylnitrosamine-induced renal tumorigenesis in the rat: implications for the involvement of oxidative stress. Carcinogenesis, 1998, 19: 1133–1139

[30] D.J. Jollow, J.R. Mitchell, N. Zampaglione, J.R. Gillette. Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic intermediate. Pharmacol. 1974, 11: 151-169.

[31] A. Claiborne. Catalase activity. In: Greenwald RA (ed). CRC Handbook of Methods in Oxygen Radical Research. CRC Press, Boca Raton., 1985,pp. 283-284.

[32] W.H, Habig, M.J. Pubst, W.B. Jokoby., GST the first enzymatic step in mercapturic acid formation. J. Biol Chem. 1974, 249:7130-7139.

[33] I. Carlberg, B. Mannervik. Glutathione reductase levels in rat brain. J Biol Chem., 1975, 250: 5475-5480.

[34] J. Mohandas, J.J. Marshall, G.G. Duggin, J.S. Horvath, D. Tiller., Differential distribution of glutathione and glutathione related enzymes in rabbit kidney. Cancer Res. 1984, 44: 5086 –5091.

[35] N. Zaheer, K.K. Tiwari, P.S. Krishnan. Exposure and solubilization of hepatic mitochondrial shunt dehydrogrnases. Arch Biochem Biophys. 1965,109:646 – 648.

[36] A.M. Benson, M.J. Hunkeler, P, Talalay. Increase of NADPH; Quinone reductase activity by dietary antioxidant. Possible role in protection against carcinogenesis and toxicity. Proc Nat Acad Sci. USA, 1980, 77: 5216-5220.

[37] F. Stripe, E. Della Corte., The regulation of rat liver Xanthine oxidase. J Biol Chem. 1969, 244: 3855-3863

[38] J.R. Wright, H.D. Colby, P.R. Miles. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Arch Biochem Biophys. 1981,206: 296 –304.

[39] A. Kornberg. Lactic dehydrogenase of muscle. In Methods in Enzymology. Colowick S P, Kaplan NO. (eds.) Academic Press, New York, 1955,1:pp. 441-443.

[40] S. Reitman, S.A. Frankel. Colorimetric method for the determination of serum oxaloacetic and glutamic pyruvic transaminases. Ame. J. Clinical. Pathol. 1957,28: 56-63.

[41] T.G. O`Brien, R.C. Simsiman, R.K. Boutwell. Induction of the polyamine biosynthesis enzymes in mouse epidermis by tumor promoting agents. Cancer Res. 1975,35: 1662-1670.

[42] R.C. Smart, M.T. Huang, A.H. Conney. Sn1, 1,2;diacylglycerols mimic the effects of TPA in vivo by inducing biochemical changes associated with tumor promotion in mouse epidermis. Carcinogenesis. 1986,7: 1865-1870.

[43] K.W. Giles, A. Myers., An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature. 1965,206: 93.

[44] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall. Protein measurement with the phenol reagent. J Biol Chem. 1964,193: 2370-2378.

[45] L.R. Ferguson., Antimutagens as cancer chemopreventive agents in diet. Mutat Res. 1994, 307:395-410.

[46]T.H. Khan, S. Sultana., Antioxidant and hepatoprotective potential of Aegle marmelos Correa. against CCl4-induced oxidative stress and early tumor events. J. Enzyme Inhib. Med. Chem. 2009,24(2):320-7.

[47]R.F. Anderson, L.J. Fisher, Y. Hara, et al. Green tea catechins partially protect DNA from .OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. Carcinogenesis, 2001, 22: 1189–1193.

[48] G. Santana-Rios, G.A. Orner, A. Amantana et al., Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. Mutat Res. 2001,495: 61–74.

[49] A. Alam, N. Khan, S. Sharma, M. Saleem, S. Sultana. Chemopreventive effect of Vitis vinifera extract on 12-O-tetradecanoyl phorbol 13-acetate-induced cutaneous oxidative stress and tumor promotion in murine skin. Pharmacol. Res., 2002, 46: 557–564.

[50] A. Singh, S.S. Handa. Hepatoprotective activity of Apium graveolens and Hygrophila auriculata against paracetamol and thioacetamide intoxication in rats. J. Ethnopharmacol. 1995,49: 119-126.

[51] M.T. Anon, A. Ubeda, M.J. Alcaraz.. Protective effects of phenolic compounds on CCl4-induced toxicity in isolated rat hepatocytes. Z Naturforsch [C], 1992,47: 275-279.

[52] M. Comporti.,. Biology of diseases: lipid peroxidation and cellular damage in toxic liver injury. Laboratory Investigator., 1985,53: 599-623.

[53] S.V. Rana, S.R. Singh, S. Verma., Mercury induced lipid peroxidation in liver, kidney, brain and gills of a fresh water fish Channa punctatus. Jap. J. Ichthyol. 1995, 42: 255-259.

[54] Q. Guo, G. Rimbach, H. Moini, S. Weber, L. Packer., ESR and cell culture studies on free radical-scavenging and antioxidant activities of isoflavonoids. Toxicology. 2002,179: 171–180.

[55] L.C. Appelt, M.M. Reicks. Soy induces phase II enzymes but does not inhibit dimethylbenz[a]anthracene-induced carcinogenesis in female rats. J Nutr., 1999, 129:1820-1826.