Journal of Enzyme Inhibition and Medicinal Chemistry, April 2009; 24(2): 320–327

Antioxidant and hepatoprotective potential of *Aegle marmelos Correa.* against CCl_4-induced oxidative stress and early tumor events

TAJDAR HUSSAIN KHAN & SARWAT SULTANA

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

(*Received 6 December 2007; accepted 13 March 2008*)

Abstract

The antioxidant properties and inhibitory effect on early tumor promoter markers of *A. marmelos* (25 and 50 mg/Kg b. wt. orally) have been evaluated. Male Wistar rats were pre-treated for seven consecutive days with *A. marmelos* prior to CCl_4 (1 mL Kg^{-1} body weight p. o., in corn oil [1:1 v/v]) treatment. Pre-treatment with *A. marmelos* suppressed lipid peroxidation (LPO), xanthine oxidase (XO) and release of serum toxicity marker enzymes viz, SGOT, LDH, SGPT dose-dependently and significantly ($p < 0.001$). Hepatic antioxidant status viz, reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), quinone reductase (QR), catalase (CAT) were concomitantly restored in *A. marmelos*-treated groups ($p < 0.001$). In addition, *A. marmelos* pretreatment also prevented the CCl_4-enhanced ornithine decarboxylase (ODC) and hepatic DNA synthesis significantly ($p < 0.001$). In conclusion, carbon tetrachloride-induced liver toxicity was strikingly attenuated by *A. marmelos* treatment and the study gives some insight into the mechanisms involved in diminution of free radical generating toxicants and enhancement of the antioxidant armory, hence preventing further tissue damage, injury and hyperproliferation.

Thus, these findings indicate that *A. marmelos* attenuates CCl_4-mediated hepatic oxidative stress, toxicity, tumor promotion and subsequent cell proliferation response in Wistar rats.

Keywords: Aegle marmelos Correa.*; antioxidants; CCl_4, tumor promotion, ornithine decarboxylase, carbon tetrachloride, protection*

Introduction

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) formation and scavenging by antioxidants. Excess generation of ROS can cause oxidative damage to biomolecules resulting in lipid peroxidation, mutagenesis and carcinogenesis. Oxidative damage plays an important role in carcinogenesis [1,2]. 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 [3–5]. The role of ROS has been implicated in many human degenerative diseases, including aging and cancer [6,7]. CCl_4 is an hepatotoxic halomethane capable of causing hepatocellular fatty degeneration and centrilobular necrosis in both animal and human. [8–11]. It has several uses: as a degreaser, solvent, a fire extinguisher and a substrate for the synthesis of fluorocarbon and other industrial and laboratory reagents [12]. A number of studies have revealed that the toxicity is initiated by its metabolic products, the highly reactive tri-chloromethyl free radical and its peroxy radical [13]. CCl_4 is metabolically activated by cytochrome P450 to form CCl_3 free radicals, which initiate lipid peroxidation in the cell. CCl_4 induces

Correspondence: S. Sultana, Department of Medical Elementology and Toxicology, Faculty of Science, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi 110062, India. Tel: 0091 11 26089688. Ext. 5565/5566. Fax: 0091 11 26059663. E-mail: sarwat786@rediffmail.com

ISSN 1475-6366 print/ISSN 1475-6374 online © 2009 Informa UK Ltd.
DOI: 10.1080/14756360802167754

liver necrosis and Kupffer cells may possibly phagocytose the necrotic cell remnants. CCl_4 metabolites react with polyunsaturated fatty acids to propagate a chain reaction leading either to lipid peroxidation or binding covalently to lipids and proteins, resulting in destruction of cell membranes and liver damage [14,15]. It is well documented that antioxidants play a significant role in protecting living organisms from the toxic effects of chemical substances such as carbon tetrachloride and carcinogens. Therefore, the effects of antioxidants prior to the administration of CCl_4 on antioxidant enzyme and drug-metabolizing activities were assessed. The importance of nutrition in protecting living organisms from the toxic effects of environmental carcinogens has been realized [16].

Significant work has been carried out for identifying naturally occurring agents that can provide a defence against oxidative stress. Natural antioxidants have a broad range of biochemical activities, including inhibition of ROS generation, scavenging of free radicals; alteration of intracellular redox potential and they also have anticancer or anticarcinogenic properties [17–19]. The plant, tree *A. marmelos*, commonly known as Bael (in Hindi), Golden apple, Stone apple (in English) is found widely throughout the Indian peninsula and Ceylon, Burma, Thailand and Indo-China. The unripe dried fruit of *A. marmelos* is used as astringent, diarrhoea. It is prescribed for diarrhoea dyspepsia and dysentery. The leaves are used in the treatment of ophthalmia, febrile delirium and various types of inflammation including acute bronchitis. The roots are used in the treatment of fever, stop pain in the abdomen, the cardiac palpitation and urinary disorders. *A. marmelos* is also useful in disorders of bile, cough and various dysfunctions [19–21]. The present study was designed to investigate whether *A. marmelos* inhibited CCl_4-induced oxidative stress in rat liver and to characterize the mechanism of its anti-oxidative effects.

Materials and methods

Chemicals

Whole plant methanolic extract of *A. marmelos* was purchased from Saiba Industries Pvt. Ltd, Mumbai, India, 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), 2, 6-dichlorophenolindophenol (DCPIP) nitroblue tetrazolium (NBT), 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°C under a 12-h light/dark cycle. The animals were acclimatized for one week immediately prior to the study. They had free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water ad-libitum.

All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Chennai, India.

Treatment

For oxidative stress and liver toxicity markers. Animals of all the groups were sacrificed under light anaesthesia after 24 h of CCl_4 treatment and blood was collected by cardiac puncture for serum separation and stored at 4°C for the estimation of GOT, GPT and LDH. Tissue was processed for post-mitochondrial fraction and cytosol preparation as described by Athar and Iqbal, [22].

For the [^{3}H] thymidine incorporation study:. The same treatment regimen was followed except that all the animals were given intraperitoneal [^{3}H] thymidine (25 μCi per animal) 2 h before sacrifice. 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.

For estimation of ODC activity. All the animals were sacrificed under ether anaesthesia after 24 h of CCl_4 treatment and were processed for post-mitochondrial fraction and cytosol preparation as described by Athar and Iqbal, [22].

Estimation of reduced glutathione

Reduced glutathione was determined by the method of Jollow et al. [23]. 1.0 mL samples of Post-mitochondrial supernatant (PMS) were precipitated with 1.0 mL of sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h 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 (0.1 M, 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, [24]. The reaction mixture consisted of 2 mL phosphate buffer (0.1 M, pH 7.4), 0.95 mL hydrogen peroxide (0.019 M) and 0.05 mL PMS in a final volume of 3 mL. Changes in absorbance were recorded at 240 nm. 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. [25]. The reaction mixture consisted of 1.425 mL sodium phosphate buffer (0.1 M, pH 7.4), 0.2 mL reduced glutathione (1 mM), 0.025 mL CDNB (1 mM) 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 enzyme activity was calculated as nmol CDNB conjugate formed $min^{-1} mg^{-1}$ protein using a molar coefficient of $9.6 \times 10^3 M^{-1} cm^{-1}$.

Glutathione reductase activity

Glutathione reductase activity was assayed by the method of Carlberg and Mannervick [26] as modified by Mohandas et al. [27]. 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 0.2 mL (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 6.22×10^3 M/cm.

Glutathione peroxidase activity

Glutathione peroxidase activity was assayed by the method of Mohandas et al. 1984 [27] as described by Athar and Iqbal [22]. 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 0.2 mL (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 $6.22 \times 103 M^{-1} cm^{-1}$.

Glucose-6-phosphate dehydrogenase activity

The activity of glucose-6-phosphate dehydrogenase was assayed by the method of Zaheer et al. [28]. The reaction mixture consisted of 300 μL tris-HCl buffer (0.05 M, pH 7.6), 0.1 mL NADP (0.1 mM), 0.1 mL glucose-6-phosphate (0.8 mM), 0.1 mL $MgCl_2$ (8 mM), 0.3 mL PMS and 2.4 mL distilled water in a total volume of 3 mL. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADPH oxidized $min^{-1} mg^{-1}$ protein using a molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Quinone reductase activity

Quinone reductase activity was measured by the method of Benson et al. [29]. The assay mixture consisted of 0.1 mL cytosolic fraction (10%), 0.7 mL 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, DCPIP (0.29%) and 2 mL of tris-HCl buffer (25 mM, pH 7.4) with a final volume of 3 mL and the absorbance 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, [30]. The reaction mixture consisted of 0.2 mL PMS which was incubated for 5 min at room temperature with 0.8 mL phosphate buffer (0.1 M, pH 7.4). Then 0.1 mL xanthine (9 mM) was added to the reaction mixture and kept at 37°C for 20 min, which was followed by the addition of 0.5 mL of 10% perchloric acid and 2.4 mL of double distilled water in a total volume of 4 mL. 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 290 nm.

Estimation of lipid peroxidation

The assay of microsomal lipid peroxidation was done according to the method of Wright et al. [31]. The reaction mixture consisted of 580 μL phosphate buffer (0.1 M, pH 7.4), 200 μL microsome, 200 μL ascorbic acid (1 mM) and 20 μL ferric chloride (100 mM) in a total volume of 1 mL. The mixture was incubated at 37°C in a shaking water bath for 1 h. Then 1 mL 10% trichloroacetic acid and 1 mL 0.67% TBA were added. All the tubes were placed in a boiling water bath for 20 min. The tubes were placed in an ice bath and then centrifuged at 2500 × g 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 535 nm.The results were expressed as nmol MDA formed $h^{-1} g^{-1}$ tissue at 37°C using a molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

Lactate dehydrogenase in serum (LDH) activity

Lactate dehydrogenase activity was estimated in serum by the method of Kornberg, [32]. 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 (AST & ALT)

Serum AST and ALT were determined by the method of Reitman and Frankel, [33]. Substrate (0.5 mL) (2 mM 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 ALT and AST, respectively. Then to the reaction mixture, 0.5 mL of DNPH (1 mM) 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 the product read at 505 nm.

Estimation of incorporation of [3H] thymidine in hepatic DNA

The isolation of hepatic DNA and incorporation of [^{3}H] thymidine in DNA was done by the method of Smart et al. [34] as described by Athar and Iqbal [22]. Liver was quickly removed cleaned free of extraneous material and homogenate (10% w/v) was prepared in ice-cold water containing an equal volume of ice-cold TCA (10%). The precipitate thus obtained was washed with cold trichloroacetic acid (TCA) (5%) and incubated with cold perchloric acid (PCA) (10%) at 4°C overnight. After 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 a 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). The amount of DNA in the filtrate was estimated by the diphenylamine method of Giles and Myers, [35]. The amount of [^{3}H] thymidine incorporated was expressed as DPM μg^{-1} DNA.

Estimation of the activity ornithine decarboxylase

ODC activity was determined using 0.4 mL hepatic 105,000 × g supernatant fraction per assay tube by measuring the release of CO_2 from DL- [^{14}C] ornithine by the method of O'Brien et al. [36]. 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 400 μl 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 other 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 hr of incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of glass tubes and the solution was continued for 1 h to ensure complete absorption of CO_2. 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 CO_2 released/h/mg protein.

Results

Effect on antioxidant system and LFT

Antioxidant potential of *A. marmelos* against CCl_4-induced hepatic oxidative stress in Wistar rats was assessed. CCl_4 administration result in depletion of hepatic glutathione, its metabolizing enzymes GST and GR and antioxidant enzymes CAT, GPx, QR and G6PD by 51, 59, 39, 88, 51, 77 and 69% respectively as compared with the saline treated control group (as shown in Tables I & II). CCl_4 also caused elevation in the activity of XO to 80% and increase in the levels of lipid peroxidation level i.e. MDA formation to 141% and liver toxicity markers ALT, AST and LDH by 154–456% (as shown in Tables III & IV).

Pre-treatment with *A. marmelos* (25 mg and 50 mg^{-1} Kg body weight) restored hepatic glutathione level to 20–54%, glutathione-metabolizing enzymes GST and GR by 25–70%, and is shown in Table I. Antioxidant and glutathione dependent enzymes like CAT, GPx and G_6PD were restored by to 25–525% and are shown in Table II. A marked depletion in levels of XO to 51–54%, and lipid peroxidation level by 25–34% and restoration of QR by 187–260% is shown in Table III. Concomitant down regulation of release of ALT, AST and LDH to 37–62% in serum was observed and is shown in Table IV.

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. *A. marmelos* showed a significant ($p < 0.05$) protection of GSH level in a dose-dependent manner. Prophylactic administration of *A. marmelos* significantly ($p < 0.001$), decreased the MDA levels as compared to only CCl_4 treated

The treatment schedule is presented below.

Groups	Treatment Regimen: Treatment on 1st to 6th day	Treatment on 7th Day
1. Control	Water 10 mL/Kg Orally	Corn oil 1 mL/Kg b. wt. Orally
2. Only D2	D2 (mg/Kg b. wt.) in 10 mL water, Orally	D2 (mg/Kg b. wt.) in 10 mL water, Orally
3. D1 + Toxicant	D1 (mg/Kg b. wt.) in 10 mL water, Orally	D1 in 10 mL water + CCl_4 1 mL/Kg (1:1 in corn oil) Orally
4. D2 + Toxicant	D2 (mg/Kg b. wt.) in 10 mL water, Orally	D2 in 10 mL water + CCl_4 1 mL/Kg (1:1 in corn oil) Orally
5.Only Toxicant	Water 10 mL/Kg Orally	CCl_4, 1 mL/Kg b. wt. (1:1 in corn oil) Orally

D1 = *A. marmelos* 25 mg/Kg (methanolic extract was dissolved in water), D2 = *A. marmelos* 50 mg/Kg (methanolic extract was dissolved in water).

group. However *A. marmelos* alone group produced no significant changes in GSH content and MDA formation. CCl_4 administration resulted in a significant ($p < 0.001$) rise in the levels of AST, ALT and LDH in serum by 154–456% as compared to saline treated control, respectively. Pre-treatment with *A. marmelos* showed a marked dose dependent inhibition of AST, ALT and LDH by 46–62%, 38–58% and 37–47% respectively.

Effect on early markers of tumor promotion

Figures 1 and 2 shows the significant modulation of early markers of tumor promotion like ODC activity and hepatic DNA synthesis in rat by 162–414%. CCl_4 treatment resulted in increase in the rate of [^{3}H] thymidine incorporation into hepatic DNA and increase in ODC activity significantly ($p < 0.001$), which is a marker of tumor promotion. The pre-treatment of rats with *A. marmelos* showed a marked ($p < 0.001$) suppressing effect on the rate of [3H] thymidine incorporation into hepatic DNA by 35–60% of treated control, and inhibited ODC activity by 14–45% of treated control, in a dose-dependent manner.

Discussion

Plants are part of the diet and a number of them exhibit medicinal properties. A number of Indian plants are also being used in regular medicinal practice. The therapeutic properties of numerous herbal plants have been documented in ancient Indian literature and in more recent time the preparations have been found to be effective in the treatment of diseases [37]. There are numerous reports, which indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases [38,39]. Use of plant food/products has become an important area of research, to prevent xenobiotic induced damage and oxidative stress due to their non-toxic effects [40]. We are constantly exposed to an array of environmental pollutants, industrial chemicals that acts as free radical generators. There are several reports, which suggested that the total extract might be more effective than its single constituents [41,42].

The present study provides a substantial amount of scientific data to show the chemopreventive potential of *A. marmelos* by using CCl_4, a known hepatotoxicant and free radical generator, associated biochemical end points in a rat model. We studied the effect

Table I. Effect of pretreatment of *A. marmelos* on the CCl_4-mediated depletion in the activity of glutathione content and its metabolizing enzymes in the liver of Wistar rats.

Treatment Groups	Reduced glutathione (n moles GSH/gm tissue)	Glutathione reductase (n moles NADPH oxidized/ min/mg protein)	Glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein)
Group I Normal pellet diet	0.856 ± 0.014	483.77 ± 25.94	1269.47 ± 29.44
Group II Only *A. marmelos* 50 mg/Kg b. wt.	0.789 ± 0.006	451.08 ± 33.96	1252.23 ± 51.04
Group III *A. marmelos* 25 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	0.502 ± 0.016[c]	396.52 ± 20.56[c]	752.23 ± 47.28[b]
Group IV *A. marmelos* 50 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	0.644 ± 0.002[b]	366.95 ± 10.44[c]	895.60 ± 24.56[b]
Group V Only CCl_4 (1 mL/Kg b wt)	0.419 ± 0.022[a]	294.60 ± 15.37[a]	525.56 ± 31.63[a]

D1 = *A. marmelos* 25 mg/Kg; D2 = *A. marmelos* 50 mg/Kg; [a] $p \leq 0.001$ on comparison of normal control with CCl_4 treated group and only *A. marmelos* group; [b] $p \leq 0.001$ and; [c] $p \leq 0.005$ on comparison of *A. marmelos* treated groups with only CCl_4 treated group.

Table II. Effect of pretreatment of *A. marmelos* on the CCl_4-mediated depletion in the activity of antioxidant enzymes in the liver of Wistar rats.

Treatment Groups	Catalase (n moles H_2O_2 consumed /min/mg protein)	Glutathione peroxidase (n moles NADPH oxidized /min/mg protein)	Glucose-6-phosphate Dehydrogenase (nmoles NADP reduced /min/mg protein)
Group I Normal pellet diet	105.79 ± 6.29	428.14 ± 6.48	93.87 ± 13.54
Group II Only *A. marmelos* 50 mg/Kg b. wt.	99.91 ± 2.54	433.86 ± 19.9	91.62 ± 12.46
Group III *A. marmelos* 25 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	45.67 ± 2.7[a]	274.54 ± 31.48[a]	62.00 ± 5.71[c]
Group IV *A. marmelos* 50 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	78.32 ± 3.28[b]	360.43 ± 72.62[b]	88.92 ± 11.7[b]
Group V Only CCl_4 (1 mL/Kg b wt)	12.21 ± 1.39	208.80 ± 8.98[b]	29.54 ± 3.61[a]

D1 = *A. marmelos* 25 mg/Kg; D2 = *A. marmelos* 50 mg/Kg; [a] $p \leq 0.001$ on comparison of normal control with CCl_4 treated group and only *A. marmelos* group; [b] $p \leq 0.001$ and; [c] $p \leq 0.005$ on comparison of *A. marmelos* treated groups with only CCl_4 treated group.

of *A. marmelos* on the biochemical parameters and early markers of tumor promotion induced by CCl_4. Reduction in GSH levels, an endogenous cellular antioxidant, is indicative of an insult by a toxic foreign agent, such as CCl_4 in present case. GSH, the major intracellular non-protein thiol, is mainly known as a nucleophilic scavenger and an enzyme-catalyzed antioxidant in electrophilic/oxidative tissue injury [43]. GSH plays an important role in the maintenance of the intracellular redox state. The intracellular level of GSH, which differs from one cell type to another, may be crucial for ROS-induced NF-κB response. It is widely recognized that the ROS is involved in the activation of NF-κB [44]. They may play a role as second messengers, when they are produced in physiological concentrations [45]. Extracellular GSH catabolism may be involved in the modulation of cell signalling and activation of transcription factors [46]. Restoration of the depleted GSH and GST on pre-treatment of animals with *A. marmelos* point out its role as a chemopreventive measure. It has been suggested that *A. marmelos* protects by inhibiting CCl_4 mediated toxicity through decrease in the level of lipid peroxidation and reducing hepatic XO levels.

GR retains reduced GSH whereas GP_X utilizes it for decomposition of lipid peroxides/hydroperoxides and other ROS. A substantial decreases in hepatic GSH with related decrease in GR, GPx, QR, GST and G6PD levels on CCl_4 administration was observed in the present study and a dose-dependent distinct restoration was observed with prophylactic treatment of *A. marmelos*. Quinone reductase is a vital line of protection in conjunction with superoxide generation by redox cycling of quinones [47]. Fruits and green

Table III. Effect of pretreatment of *A. marmelos* on the CCl_4-mediated alteration in the activities of xanthine oxidase, quinine reductase and enhancement of lipid peroxidation in the liver of Wistar rats.

Treatment Groups	Xanthine Oxidase (μg uric acid formed/ min/mg protein)	Lipid Peroxidation (n moles MDA formed/ hr/gm tissue)	Quinone Reductase (nmoles dichloroindophenol reduced/ min/mg protein)
Group I Normal pellet diet	0.509 ± 0.049	4.15 ± 0.05	587.66 ± 17.7
Group II Only *A. marmelos* 50 mg/Kg b. wt.	0.440 ± 0.005	3.91 ± 0.22	532.45 ± 21.63
Group III *A. marmelos* 25 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	0.58 ± 0.023[b]	7.45 ± 0.33[c]	395.74 ± 45.84[b]
Group IV *A. marmelos* 50 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	0.51 ± 0.028[b]	6.56 ± 0.047[b]	495.78 ± 28.60[b]
Group V Only CCl_4 (1 mL/Kg b wt)	0.917 ± 0.33[a]	9.99 ± 0.36[a]	137.70 ± 16.53[a]

D1 = *A. marmelos* 25 mg/Kg; D2 = *A. marmelos* 50 mg/Kg; [a] $p \leq 0.001$ on comparison of normal control with CCl_4 treated group and only *A. marmelos* group; [b] $p \leq 0.001$ on comparison of *A. marmelos* treated groups with only CCl_4 treated group;cp < 0.005.

Table IV. Effect of pretreatment of *A. marmelos* on the CCl_4-mediated enhancement of liver toxicity markers in Wistar rats.

Treatment Groups	ALT (IU/L)	AST (IU/L)	LDH (nmol NADH oxidized/min/mg protein)
Group I Normal pellet diet	24.23 ± 0.59	16.96 ± 0.28	392.66 ± 5.72
Group II *A. marmelos* 50 mg/Kg b. wt.	22.07 ± 1.26	16.67 ± 1.42	367.38 ± 8.18
Group III *A. marmelos* 25 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	46.71 ± 0.87[b]	50.78 ± 6.59[b]	626.79 ± 6.14[b]
Group IV *A. marmelos* 50 mg/Kg b. wt. + CCl_4 (1 mL/Kg b wt)	31.19 ± 1.21[b]	36.15 ± 3.49[b]	533.49 ± 8.35[b]
Group V Only CCl_4 (1 mL/Kg b wt)	74.88 ± 4.39[a]	94.28 ± 9.17[a]	997.68 ± 11.05[a]

D1 = *A. marmelos* 25 mg/Kg; D2 = *A. marmelos*s 50 mg/Kg; [a] $p \leq 0.001$ on comparison of normal control with CCl_4 treated group and only *A. marmelos* group; [b] $p \leq 0.001$ on comparison of *A. marmelos* treated groups with only CCl_4 treated group.

vegetables have been shown to act as chemopreventive agents by means of quinone reductase induction [48]. Since perturbations of cell proliferation and death are considered essential events, studies were conducted on the deregulation of two specific parameters, viz. the induction of ornithine decarboxylase (ODC, an enzyme related to cell proliferation) and DNA synthesis. This could be the consequence of the activation of various mechanisms by exposure to toxicant. *A. marmelos* treatment prior to dosing with CCl_4 restrain the levels of serum toxicity markers as compared to only CCl_4 group. CCl_4 administration also resulted in significant induction in hepatic ODC and [^{3}H] thymidine uptake into hepatic DNA. These changes were significantly inhibited by prophylaxis with *A. marmelos*. Findings in the present study are that *A. marmelos* suppressed CCl_4 induced cellular oxidative stress and reversed the induction in hepatic ODC activity and thymidine incorporation.

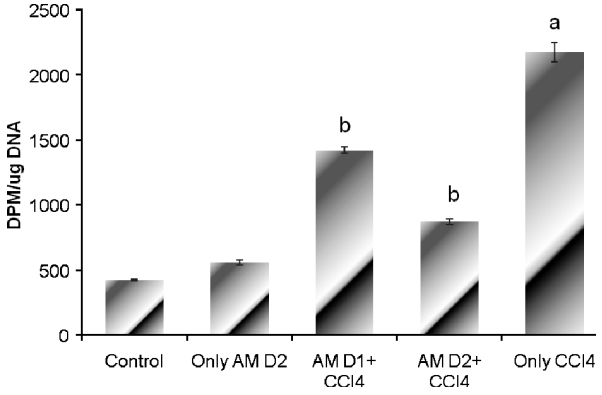

Figure 1. Effect of pre-treatment of *A. marmelos* on CCl_4-induced cell proliferation response. [b]$p < 0.001$, compared with the corresponding value for treatment with only CCl_4 treated group. [a]$p < 0.001$ compared to corresponding value for saline treated control group. D1 = *A. marmelos* 25 mg/Kg; D2 = *A. marmelos* 50 mg/Kg.

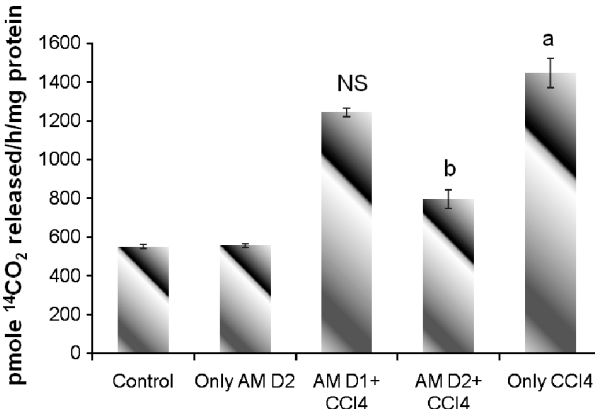

Figure 2. Effect of pre-treatment of *A. marmelos* on CCl_4-induced ODC activity. [b]$p < 0.001$, compared with the corresponding value for treatment with only CCl_4 treated group. [a]$p < 0.001$ compared to corresponding value for saline treated control group. D1 = *A. marmelos* 25 mg/Kg; D2 = *A. marmelos* 50 mg/Kg.

Acknowledgements

Authors are thankful to Indian Council of Medical Research, New Delhi, India for providing fellowship and funds to carry out this study.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Olinski R, Siomek A, Rozalski R, Gackowski D, Foksinski M, Guz J, Dziaman T, Szpila A, Tudek B. Oxidative damage to DNA and antioxidant status in aging and age-related diseases. Acta Biochim Pol 2007;54(1):11–26.
[2] Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nature Rev Cancer 2003;3:276–285.
[3] Orrenius S. Reactive oxygen species in mitochondria-mediated cell death. Drug Metab Rev 2007;39(2–3):443–455.
[4] Hristozov D, Gadjeva V, Vlaykova T, Dimitrov G. Evaluation of oxidative stress in patients with cancer. Arch Physiol Biochem 2001;109:331–336.

[5] Davies KJ, Hochstein P. Ubisemiquinone radicals in liver implications for a mitochondrial Q cycle *in vivo*. Biochem Biophys Res Commun 1982;107:1292–1299.
[6] Harman D. Free radical theory of aging, increasing the functional life span. Ann N Y Acad Sci 1994;717:1–15.
[7] Simonian NY, Coyle JT. Oxidative stress in neurodegenerative disease. Annu Rev Pharmacol Toxicol 1996;36:83–106.
[8] Slater RF. Free radicals and tissue injury: Fact and fiction. Br J Cancer 1987;8:5–10.
[9] Recknagel RO, Glende EA, Dolak JA, Waller RL. Mechanism of CCl_4 toxicity. Pharmacol Ther 1989;43:139–154.
[10] Soni MG, Mehendale HM. Evidence for hepatic failure in the interactive toxicity of chlordecone and carbon tetrachloride. Fundam Appl Toxicol 1993;21:442–450.
[11] Luster MI, Simeonova PP, Gallucci RM, Bruccoleri A, Blazka ME, Yucesoy B. Role of inflammation in chemical-induced hepatotoxicity. Toxicol Lett 2001 2001;120(1–3): 317–321.
[12] NAS (National Academy of Science). Chloroform, carbon tetrachloride and other halomethanes: An Environmental Assessment. Washington, DC: NAS; 1978.
[13] Stoyanovsky DA, Cederbaum AI. Metabolism of carbon tetrachloride to trichloromethyl radical: An ESR and HPLC-EC study. Chem Res Toxicol 1999;12:730–736.
[14] Recknagel RO, Glende Jr. EA, Dolak JA, Waller RL. Mechanisms of carbon tetrachloride toxicity. Pharmacol Ther 1989;43:139–154.
[15] Jorquera F, Culebras JM. Gonz,/~Lez-Gallego J. Influence of Nutrition on Liver Oxidative Metabolism. Nutrit 1996;12(6): 442–447.
[16] Finkel T, Holbrook NJ. Oxidants, oxidative stress and biology of ageing. Nature 2000;408:239–247.
[17] Johnson IT, Williamson G, Musk SR. Anticarcinogenic factors in plant foods: A new class of nutrients. Nutr Rev 1994;7: 175–204.
[18] Dragsted LO, Strube M, Larsen JC. Cancer-protective factors in fruits and vegetables: Biochemical and biological background. Pharmacol Toxicol 1993;72:116–135.
[19] Kirtikar KR, Basu BD, an ICS In: second, editor. Indian Medicinal Plants. Dehradun, India: B/S International Book Distributors; 1935. p 448–502.
[20] Nadkarni AK. Dr. KM Nadkarni's Indian Materia Medica., Vol.1 Bombay, India: Popular Prakashan Company; 1986. p 45.
[21] Parmar C, Kaushal MK. Aegle marmelos. Wild Fruits. New Delhi, India: Kalyani Publishers; 1982. p 1–5.
[22] Athar M, Iqbal M. Ferric nitrilotriacetate promotes N-diethylnitrosamine-induced renal tumorigenesis in the rat: Implications for the involvement of oxidative stress. Carcinogenesis 1998;19:1133–1139.
[23] Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. Bromobenzene induced liver necrosis: Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic intermediate. Pharmacol 1974;11:151–169.
[24] Claiborne A. Catalase activity. In: Greenwald RA, editor. CRC Handbook of Methods in Oxygen Radical Research. Boca Raton: CRC Press; 1975. p 283–284.
[25] Habig WH, Pubst MJ, Jokoby WB. GST the first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249: 7130–7713.
[26] Carlberg I, Mannervik B. Glutathione reductase levels in rat brain. J Biol Chem 1975;250:5475–5480.
[27] Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller D. Differential distribution of glutathione and glutathione related enzymes in rabbit kidney. Cancer Res 1984;44:5086–5091.
[28] Zaheer N, Tiwari KK, Krishnan PS. Exposure and solubilization of hepatic mitochondrial shunt dehydrogrnases. Arch Biochem Biophys 1965;109:646–648.
[29] Benson AM, Hunkeler MJ, Talalay P. Increase of NADPH; Quinone reductase activity by dietary antioxidant. Possible role in protection against carcinogenesis and toxicity. Proc Natl Acad Sci USA 1980;77:5216–5220.
[30] Stripe F, Della Corte E. The regulation of rat liver Xanthine oxidase. J Biol Chem 1969;244:3855–3863.
[31] O'Brien TG, Simsiman RC, Boutwell RK. Induction of polyamine biosynthesis enzymes in mouse epidermis by tumor promoting agents. Cancer Res 1975;35:1662–1670.
[32] Wright JR, Colby HD, Miles PR. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Arch Biochem Biophys 1981;206:296–304.
[33] Kornberg A In: Colowick SP, Kaplan NO, editors. Lactic dehydrogenase of muscle. In Methods in Enzymology. New York: Academic Press; 1955. p 441–443.
[34] Reitman S, Frankel SA. Colorimetric method for the determination of serum oxaloacetic and glutamic pyruvic transaminases. Amer J Clin Pathol 1957;28:56–63.
[35] Giles KW, Myers A. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature 1965;206:93.
[36] Smart RC, Huang MT, Conney AH. 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.
[37] Jain SK. Ethnobotany and research on medicinal plants in India. Ciba Found Symp 1994;185:153–164, discussion 164-8.
[38] Sies H, editor. Antioxidants in Disease, Mechanisms and Therapy New York: Academic Press; 1996.
[39] Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 1992;119:598–620.
[40] Ferguson LR. Antimutagens as cancer chemopreventive agents in diet. Mutat Res 1994;307:395–410.
[41] Anderson RF, Fisher LJ, Hara Y, Harris T, Mak WB, 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.
[42] Santana-Rios G, Orner GA, Amantana A, Provost C, Wu SY, Dashwood RH. Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. Mutat Res 2001;495:61–74.
[43] Lieberman R, Crowell JA, Hawk ET, Boone CW, Sigman CC, Kelloff GJ. Development of new cancer chemoprevention agents: Role of pharmacokinetic/pharmacodynamic and intermediate endpoint biomarker monitoring. Clin Chem 1998;44:420–427.
[44] Nanxin L, Michael K. Is NF-κB the sensor of oxidative stress? FASEB 1999;13:1137–1143.
[45] Baeuerle PA, Rupec RA, Pahl HL. Reactive oxygen intermediates as second messengers of a general pathogen response. Pathologie Biologie 1996;44:29–35.
[46] Marie-Jose A, Milica E, Mojgan M, Christine M, Sylvia D, et al. Gamma-Glutamyl transpeptidase -dependent glutathione catabolism results in activation of NF-κB. Biochem Biophys Res Commun 2000;276:1062–1067.
[47] Perchellet JP, Perchellet EM, Gali HU, Gao XM. Oxidant Stress and Multistage carcinogenesis. In: Mukhtar H, editor. Skin Cancer: Mechanism & Human Relerence. Boca Raton, FL: CRC Press, Inc. 1995. p 145–180.
[48] Tawfiq N, Heaney RK, Plumb JA, Fenwick GR, Musk SR, Williamson G. Dietary glucosinolates as blocking agents against carcinogenesis: Glucosinolate breakdown products assessed by induction of quinone reductase activity in murine hepa1c1c7 cells. Carcinogenesis 1995;16(5):1191–1194.