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Protective effect of secondary plant metabolites from *Ipomoea aquatica* Forsk. against carbofuran induced damages

Sanjukta Datta[a], Mahuya Sinha[b], Dipesh Das[b], Santinath Ghosh[a] & Pubali Dhar[c*]

Department of [a]Chemical Technology and [b]Physiology, University College of Science and Technology, University of Calcutta, 92, A.P.C. Road, Kolkata 700 009, India
[c]Laboratory of Food Science and Tecnology, Food and Nutrition Division, Department of Home Science, University of Calcutta, 20 B Judges Court Road, Kolkata 700 027, India

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Plausible interactions between food contaminants and natural constituents *in vivo* and protective effect of polyphenols present in *I. aquatica* against carbofuran toxicity in Charles Foster rats were evaluated. Determinations based on antioxidant enzyme activities showed significant alterations in glutathione, glutathione peroxidase, superoxide dismutase and catalase in tissues (liver and brain) and plasma of pesticide treated group while polyphenolic extracts from *I. aquatica* (IAE) attenuated their activities when given alongwith carbofuran. IAE decreased enhanced lipid peroxidation levels in plasma and erythrocyte membrane and cholesterol levels in brain and plasma. IAE also minimized histopathological degenerative changes produced by carbofuran. While single cell gel electrophoresis showed that secondary metabolites in leafy vegetables produced a combinatorial effect with pesticide at cellular level, DNA fragmentation level in bone marrow cells showed a decline in the IAE treated rats. Food safety adversely affected by various chemical contaminants can be retained by plant polyphenols and secondary plant constituents that can be found together in bolus. Therefore, the present study gives an insight into the protective role of naturally found polyphenols against pesticide toxicity.

Keywords: Antioxidant enzymes, Carbofuran, Flavonoids, Lipid peroxidation, Oxidative stress

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from daily metabolism of food and often generated as byproducts of biological reactions or exogenous environmental factors like xenobiotics[1]. Being a part of normal oxidative metabolism when produced in excess, however, cause tissue injury, cell membrane lipid peroxidation (LPO), decreased membrane fluidity and other DNA mutations leading to cancer, degenerative and other diseases[2].

Carbamates are the most widely used pesticides in substitution to organochlorates and organophosphates due to their low residual persistence[3,4,5] However, their indiscriminate use has led to toxicity, both accidentally and occupationally. Their role as a neurotoxicant, showing behavioural and psychological consequences, has been well established[6,7]. Correlation between accumulation of acetylcholine and extent of lipid peroxidation has been reported[8]. Milatovic *et al.*[9] showed that AchE inhibitors such as carbofuran cause myopathy by excessive formation of ROS leading to LPO through mitochondrial dysfunction and dyshomeostasis. Sub-acute intraperitoneal administration of carbofuran induced has been reported[10,11]. The lipophilic nature of carbofuran has been reported to cause oxidative injury resulting in perturbations in the membrane structure and functions[12,13]. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR), are the main enzymatic defenses and act in concert with panoply of non-enzymatic antioxidants.

Besides the body's normal levels of such antioxidant substances, natural food components affect the inbuilt antioxidative defense of the body. Natural food items like fruits, vegetables including the green leafy ones, beverages etc., have long been proved to be great radical scavengers and inhibitors of lipid peroxidation both *in vivo* and *in vitro*. Green leafy vegetables are rich sources of many nutrients and their beneficial role has partly been attributed to the antioxidant components present in them of which the major

* Correspondent author
Telephone: +91 9433122560
E-mail: pubalighoshdhar@yahoo.co.in
SD: sanj_9231@yahoo.co.in, MS: mahuyasinha@gmail.com, DD: dkdas1983@gmail.com, SG: santinathghosh@yahoo.com.hk

portion is formed by the flavonoids, isoflavones, lignans, catechins and isocatechins[14]. Sulfur containing phytochemicals glucosinolates and S-methylcystein sulfoxide in cruciferous vegetables are effective against carcinogens[15]. Orally administered butanolic extract of *Paronychia argentea* protects against chlorpyrifos ethyl induced toxicity, possibly through the inhibition of increased LPO in addition to inhibition of triglyceride accumulation, plasma membrane destruction and neutrophil infiltration in the liver tissue[16]. Leafy vegetable extracts of *Enydra fluctuans*, prevent lipid peroxidation by inhibiting the production of free radicals and also by protecting the rats from the deleterious effects of acephate by altering the antioxidant levels in the body to a great extent[17].

The presence of 8-oxo-dG in urine or its accumulation in kidney cells has been linked to DNA damage in body cells[18]. Also, studies of damaged cells by methods like comet assay, chromosomal aberrations, micronuclei tests have provided much understanding in the phenomenal changes that take place in the body cells due to the deleterious effects of the commonly used carbamates due to their relatively lower toxicity levels[19].

There is an urgent need to improve our knowledge on interaction of food contaminants and food components both *in vitro* and *in vivo*. *I. aquatica* Forsk., a locally available green leafy vegetable has been selected in the present study and polyphenolic food constituents were extracted. In the present study the efficacy of polyphenolic extract against carbofuran toxicity, based on disturbance of oxidative-antioxidative balance has been investigated.

Materials and Methods

Chemicals—Methanol, Hematoxylin and H_2O_2 were purchased from Sisco Research Laboratory (SRL), Mumbai, India; glutathione (GSH), GR, NADPH, DTNB, flavonoid standards of rutin hydrate, rutin and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO); BSA standard were purchased from E. Merck India Pvt. Ltd., Mumbai, India. Carbofuran pesticide was supplied by Anu Products Limited, Haryana, India.

Preparation of leafy vegetable extract—The leafy vegetable, *Ipomoea aquatica*, was collected from the local field and was authenticated by the Central National Herbarium, Shibpur, India vide letter no CNH/120/2011/TechII/607. The polyphenolic compounds were extracted by following the methods developed in the laboratory[14]. Briefly, the whole plants (stem and leaves) of *Ipomoea* were washed thoroughly, oven dried and ground to powder. It was then extracted using 80:20 methanol:water and concentrated in a rotary evaporator. The concentrates were pooled and the final concentrate was lyophilized to obtain the dry matter. The required amount for the dose (20 mg polyphenolic compounds expressed as gallic acid equivalents/kg body wt) was dissolved in water to obtain the water extract. It was then stored at -40 °C for further use.

Animal diet and treatment—Male albino rats of Charles foster strain, weighing 100-130 g, were caged singly and provided with balanced diet and water *ad libitum*. The diets composed of fat free casein, 18%; fat, 20% (sunflower oil); starch, 55%; salt-mixture 4% (composition of salt mixture No.12 (in g) : NaCl 292.5, KH_2PO_4 816.6; $MgSO_4$ 120.3; $CaCO_3$ 800.8; $FeSO_4.7H_2O$ 56.6; KI 1.66; $MnSO_4.2H_2O$, 9.35; $ZnCl_2$ 0.5452; $CuSO_4.5H_2O$, 0.9988, $CoCl_2.6H_2O$ 0.0476) cellulose 3%; one multivitamin capsule (vitamin A I.P. 10,000 units, thiamine mononitrate I.P. 5mg , vit B I.P. 5 mg, calcium pantothenate USP 5mg, niacinamide I.P. 50 mg, ascorbic acid I.P. 400 units, cholecalciferol USP 15 units, menadione I.P. 9.1 mg, folic acid I.P. 1 mg, vitamin E USP 0.1 mg) per kg of diet. The diets were adequate in all nutrients. The animals were maintained at 12 h light/dark conditions. The animal experiment was carried under the supervision of the Animal Ethical Committee of the Department of Chemical Technology, University of Calcutta.

The animals were divided into the following 4 groups of 8 rats each: Gr. I served as the control (C) and was provided with normal diet. The animals in Gr. II (F) were gavaged with the pesticide carbofuran only at a dose of 0.1 mg/kg body wt, Gr. III animals (IAE) were administered by gavaging, the leafy vegetable extract at a dose of 20 mg/kg body wt and Gr. IV animals (IAEF) were given the pesticide, at a dose of 0.1 mg/kg body wt along with the vegetable extract (20 mg/kg body wt). The dose used for the pesticide was calculated as $1/100^{th}$ of the LD_{50}, so that it showed no adverse effects or mortality in the animals.

All the treated rats were gavaged for 14 days and were sacrificed under mild anesthesia; blood was collected, and tissues were immediately excised, blotted, and stored frozen (-40 °C) for further analysis.

Determination of total polyphenols and flavonoid—Total polyphenol content of the leafy vegetable was determined by the Folin-ciocalteau method as described by Matthaus[20]. The total flavonoid was also determined using the methods of Chang *et al.*[21] after slight modification.

HPLC analysis of the leafy vegetable extract—The hydrolyzed sample for HPLC detection of the polyphenols was prepared according to the method of Hertog *et al*[22]. In brief, 0.5 g of the dried material was taken in a flask along with BHA (2g/L) and sonicated for 5 min in 40 mL of 65% methanol. 10 mL of 6 *N* HCl was added and nitrogen purged for 60 sec. The mixture was then heated on a water bath at 90 ^{o}C for 2 h with constant stirring. It was then cooled, filtered and sonicated for 5 min and then used for HPLC analysis according to the method of Siddhuraju and Becker[23]. In brief, a gradient elution was employed for flavonoids other than catechins with a mobile phase consisting of 50 m*M* H_3PO_4, *p*H 2.5 (solution A), and acetonitrile (solution B) which is as follows: isocratic elution 95% A/5% B, 0-5 min; linear gradient from 95% A/5% B to 50% A/50% B, 5-55 min; isocratic elution 50% A/50% B, 55-65 min; linear gradient from 50% A/50% B to 95% A/5% B, 65-67 min; post-time 6 min before the next injection. The system, WATERS 2487 is equipped with a C-18 column (Nova-Pak C_{18}, 3.9 × 150 mm). 280 nm, 340 nm and 370 nm wavelengths were selected for peak detection. The flow rate of the mobile phase was 0.8 mL/min, and the injection volume was 20 µL. The peaks were identified using authentic standards.

Plasma and erythrocyte membrane lipid peroxidation—Lipid peroxidation of the plasma and erythrocyte membrane was estimated based on thiobarbituric acid reactive acid substances (TBARS) followed by the method of Draper and Hadley[24]. Briefly, in 0.2 mL of plasma and erythrocyte membrane samples, 1 mL of 0.67% TBA and 2 mL 20% TCA were added, and heated at 90 °C for 10 min. The absorbance was then noted at 532 nm against reagent blank.

Enzymatic and non-enzymatic antioxidant activities—The tissues were first homogenized in phosphate buffer (50 m*M* potassium phosphate buffer at *p*H 7.0 for catalase and 1 *M* potassium phosphate buffer at *p*H 7.0 for SOD, GSH and GPx) and centrifuged. The supernatant was then used for measuring the following antioxidant enzymes. Catalase was measured according to the method of Aebi[25]. CAT activity was measured spectrophotometrically and expressed as U/mg protein by the rate of decrease of hydrogen peroxide at 240 nm. SOD activity was assayed by measuring the auto oxidation of haematoxylin[26]. GSH was determined as per Ellman[27]. Total activity of GPx (GPx EC.1.11.1.9.) was determined in tissue homogenates and plasma[28]. The total protein was determined by the method of Lowry *et al*[29].

Extraction of tissue lipids and determination of total cholesterol—Tissue lipid was extracted by the method of Folch *et al*[30]. One gram of tissue (brain and plasma) was homogenized with 1 mL of 0.74% KCl and 2 mL of different proportions of chloroform and methanol for 2 min and then centrifuged. The mixture was left overnight and the chloroform layer was filtered through a Whatman filter paper (No. 1). The chloroform layer was dried and the tissue lipid contents were measured. The total cholesterol from the extracted lipid was determined by the standard kit method.

DNA fragmentation assay—DNA fragmentation of bone marrow cells was determined by the diphenylamine assay of Taylor[31]. Cell suspension (1 mL, not less than 5×10^5 and no more than 5×10^6, in order to obtain an OD 600 for DNA > 0.04 and < 1.200) was delivered in tubes labeled B (bottom) and centrifuged at 200 g at 4 °C for 10 min. Supernatants were carefully transferred in new tubes labeled S (supernatant). To the pellet in tubes B, 1.0 mL TTE solution was added, vortexed vigorously and then centrifuged at 20,000 g for 10 min at 4 °C. Supernatants were carefully transferred in new tubes labeled T. 1.0 mL TTE solution was added to tubes B and 1.0 mL of 25% TCA were added to tubes T, B and S and vortexed vigorously. They were then allowed to precipitate overnight at 4 °C. After incubation, precipitated DNA was recovered by pelleting for 10 min at 20,000 g at 4 °C. Supernatants were discarded by aspiration. The DNA was hydrolyzed by adding 160 mL of 5 % TCA to each pellet and heating them for 15 min at 90 °C in a heating block. To each tube was added 320 mL of freshly prepared DPA solution, then vortexed and color development was allowed for about 4 h at 37 °C or overnight at room temperature. Aliquots (200 mL) of coloured solution (ignoring dark particles) were transferred from each tube to a well of a 96-well microtiter plate and measured at 600 nm in an ELISA reader. The percentage of fragmented DNA was calculated using the formula:

Fragmented DNA(%) = (S + T) / (S+T+B) × 100

where S, T and B are the OD 600 of fragmented DNA in the S, T and B fractions, respectively.

Histopathological study—The liver were excised and after fixing in 10% formalin saline for 3-4 days, the tissues were embedded in paraffin wax. Uniform sections of 5 m thickness were cut and stained with haematoxylin and eosin by routine procedures. The stained sections were examined for pathological changes[32].

Single cell gel electrophoresis (Comet assay)— Lymphocytes were analyzed by the comet assay according to the method of Singh *et al*[33]. The cells were suspended in low melting point agarose (0.65%) and 75 L of suspension was quickly layered over slides which were precoated with normal melting point agarose (0.65%), immediately covered with a cover slip and the slides were placed on + 4 ºC for 10-15 min. After solidification, the coverslip was gently removed and immersed in cold lysing solution (2.5 *M* NaCl, 100 m*M* EDTA, 10 m*M* Tris *p*H 10 in which 10 % DMSO and 1 % Triton X-100 were added) at 4 ºC for 1 h. The slides were removed and placed on a horizontal gel electrophoresis platform covered with electrophoresis buffer (300 m*M* NaOH, 1 m*M* EDTA *p*H>13). The slides were left in the solution for 20 min to allow the unwinding of the DNA. The DNA was electrophoresed (25 V, 300 mA) for 20 min. After electrophoresis, the slides were removed and rinsed with neutralization buffer (0.4 *M* Tris, *p*H 7.5). Each slide was stained with 50 L of 20 g/mL ethidium bromide. The slides were then observed under a Leica DM IL HC Inverted Research Microscope (Wetzlar, Germany).

Statistical analysis- Statistical analysis of the result was performed using Origin 7.0 software. Data were expressed as mean±SD. Differences among the experimental groups were analyzed using one-way ANOVA and the comparisons between the means were carried out using the Tukey test; $P<0.05$ was considered as statistically significant in the experiments.

Results

Total polyphenol and flavonoid contents and identification of components—In the present study, the crude extract of *I. aquatica* was used and the active components present in it were analyzed by HPLC (Fig. 1) which showed the presence of flavonoids like rutin, apigenin and quercetin as major components along with some unidentified materials. The total polyphenol and flavonoid contents were recorded to be 55.95 ± 0.002 (mg /g dry wt basis expressed as GAE) and 13.49 ± 0.001 (mg QE/ g of dry wt of leafy vegetable), respectively.

Plasma and erythrocyte membrane lipid peroxidation—Effect of *in vivo* administration of carbofuran and IAE on the plasma and erythrocyte membrane lipid peroxidation (Fig. 2) was measured. Carbofuran treatment enhanced the plasma lipid peroxidation significantly ($P<0.05$) in comparison to the control group of rats fed with normal diet as well as the group treated with both extract and carbofuran (Fig. 2A). Polyphenolic treatment significantly reduced the peroxidation from control group. On the other hand, *I. aquatica* extract along with carbofuran treatment resulted in efficient recovery and the levels of TBARS formation were significantly lowered ($P<0.05$) than the carbofuran treated group. Erythrocyte membrane lipid peroxidation in the carbofuran treated group showed a significant increase ($P<0.05$) compared to the normal diet fed rats (Fig. 2B). A significant decrease ($P<0.05$) in the erythrocyte membrane lipid peroxidation was observed both in the rats given only polyphenol and the rats treated with carbofuran along with

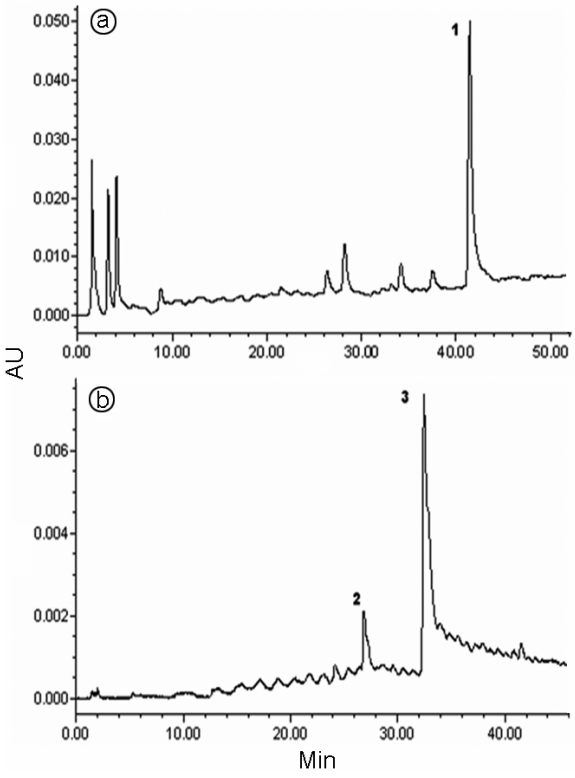

Fig. 1—HPLC chromatograms of *I. aquatica* extract at (A) 280 nm (B) 370 nm. The peaks identified are 1.Apigenin 2. Rutin 3.Quercetin

polyphenol. Plasma and erythrocyte membrane LPO levels in IAEF group were comparable to the control counter parts.

Enzymatic and non-enzymatic antioxidant activities—A decrease in the activity of catalase enzyme was observed in the liver of the carbofuran treated group and a significant increase was observed in plasma and brain. However, the level of the enzyme seemed to have been restored significantly to a normal state in the IAEF group (Fig. 3A). The level of SOD activity showed a significant decrease in the carbofuran treated group in all the tissues which seemed to have gained a significant increase in the group treated with the *I. aquatica* extract and carbofuran (Fig. 3B). A significant increase in GSH level in both liver and brain and a decrease in plasma was observed in the carbofuran treated group. However, a significant increase in the GSH level was observed in both liver and plasma in the IAE group. The GSH level reached a near normal status in case of plasma in the group treated with the *I. aquatica* extract along with carbofuran (Fig. 3C). The GPx activity in both liver and brain in the carbofuran treated group had shown a significant increase, whereas, a decrease had been noticed in case of plasma. However, restoration of the enzyme level to the normal status had been observed in the group treated with the *I. aquatica* extract along with carbofuran (Fig. 3D).

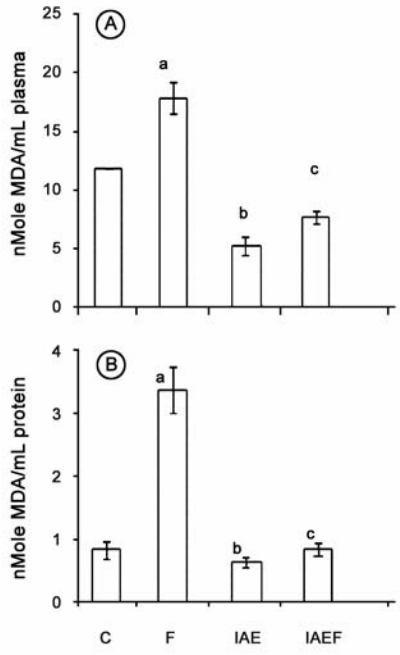

Fig. 2—Effect of *I. aquatica* extract on MDA level in plasma (A) and erythrocyte membrane (B) of rats treated with carbofuran. [Values are mean ± SD from 8 animals in each group. C=Control; F=Group treated with 0.1 mg/kg body wt. of carbofuran only; IAE=Group treated with 20 mg/kg body wt. of *I. aquatica* extract only; IAEF=Group treated with both *I. aquatica* extract and carbofuran. [a] F *vs* C, IAE, IAEF (P<0.05); [b] IAE *vs* F, IAEF (P<0.05); [c] IAEF *vs* F, IAE (P<0.05)]

Total cholesterol—The total cholesterol levels in plasma and brain significantly increased in carbofuran treated rats. However, treatment with the *I. aquatica* extract did have a protective effect by minimizing the level of cholesterol in plasma and brain (Fig. 4).

DNA fragmentation in bone marrow cells—DNA fragmentations had been observed in the bone marrow cells of the rats treated with carbofuran which seemed to have been prevented to a large extent in case of the group treated with both the pesticide, carbofuran, and the extract of *I. aquatica* (Table 1).

Single cell gel electrophoresis (Comet assay)—The protective effect of IAE was evident in the cells treated with the extract in the presence of the pesticide, where minimal comet formation was observed. Damaged cells with comet formations were visible in the carbofuran treated rat liver cells (Fig. 5).

Histopathological study- Highly damaged liver lobules with central vein distortions and localized necrosis were found in the carbofuran treated rat liver cells (Fig. 6 d), whereas, partially normal cells were observed in those that were treated with the extract along with carbofuran. Normal liver cells were observed in the group treated with only the extract (Fig. 6).

Discussion

Peroxidative damages caused by residual food contaminants like pesticides have been reported but there is a paucity of information on the *in vivo* interaction of food components like polyphenols with pesticides. Antioxidative enzyme activities of liver which acts as the prime organ for phase I and phase II reaction in the removal of food contaminants need to be evaluated in presence of both food constituent polyphenols, and food contaminant, carbofuran. Numerous natural food compounds have been reported to modulate the Cytochrome P450 1A and Cytochrome P450 3A isoform expression through aryl hydrocarbon receptor in rat liver microsomes[34]. Furthermore, Foster *et al.*[35] demonstrated that garlic components exhibit an inhibitory effect on Cytochrome P450 3A4 mediated metabolism. On the other hand carbamates are metabolized by liver

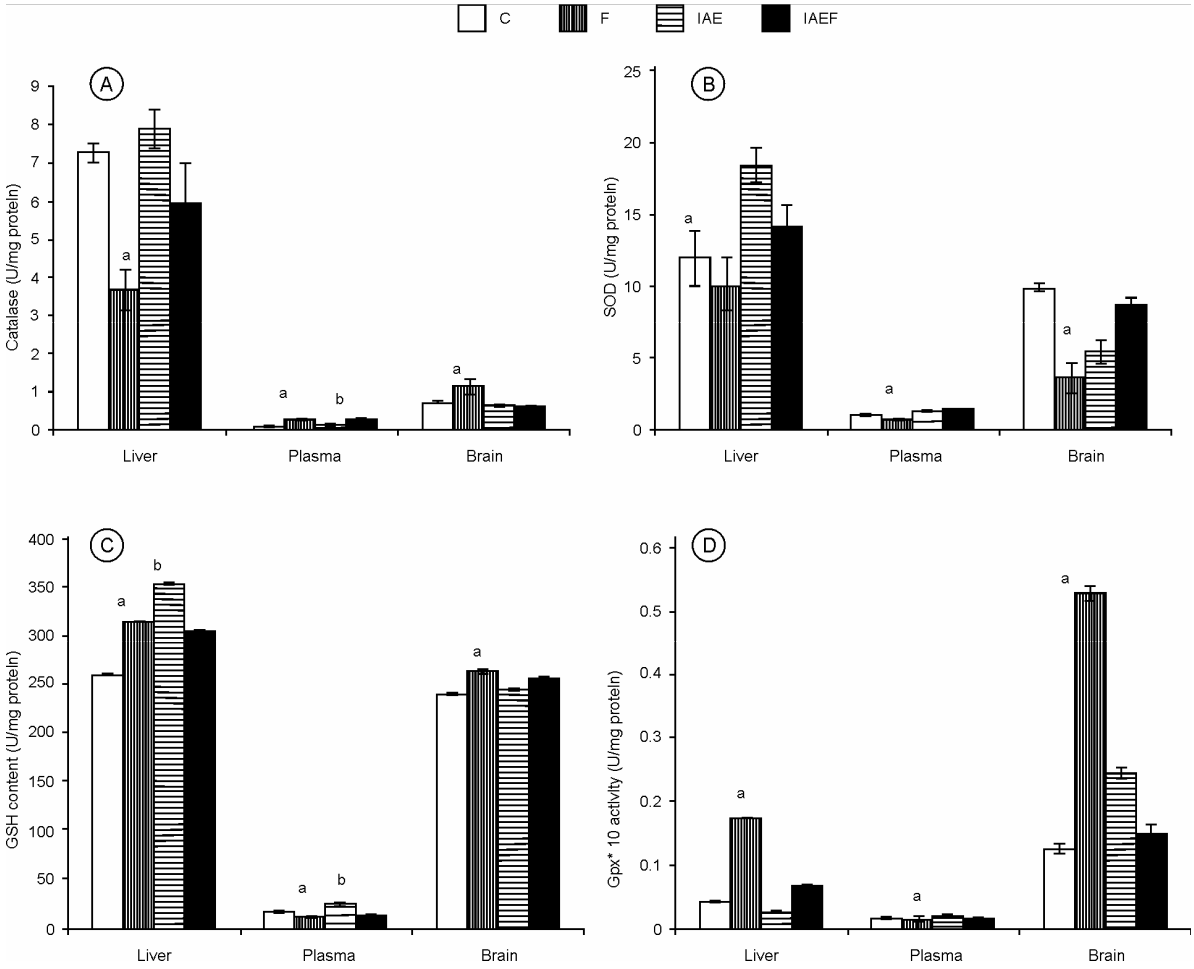

Fig. 3—Effect of *I. aquatica* extract on the liver, brain and plasma catalase (A), SOD (B), glutathione (C) and GPx (D) activity in carbofuran treated rats. [Values are mean ± SD from 8 animals in each group. C=Control; F=Group treated with 0.1 mg/kg body wt. of carbofuran only; IAE=Group treated with 20 mg/kg body wt. of *I. aquatica* extract only; IAEF=Group treated with both *I. aquatica* extract and carbofuran. [a] F *vs* C, IAE, IAEF (P<0.05); [b] IAE *vs*F, IAEF (P<0.05)]

Cytochrome P450 3A4 which shows an interaction between food contaminants and food constituents in hepatic targets. Thus, the present study reports about the oxidative-antioxidative balance in *in vivo* condition, in presence of food constituents like polyphenols and food contaminant, carbofuran.

The inhibiting action of carbamate pesticides on cholinesterase activity may lead to oxidative stress by producing free radicals in the tissue[36,37] Carbamates like carbofuran, exposure was found to increase the oxidative damage in rat tissues[10,12,38,]. The increase in lipid peroxides in rat tissues on exposure to carbamates like carbofuran, benomyl and thiram has been demonstrated earlier[39,40]. Extensive data are still lacking on the oxidative damages induced by these carbamates on body cells and tissues[39,41-44]. End products of LPO are believed to be largely responsible for the cytotoxic effects observed in various neurodegenerative conditions[45,46]. IAE treatment to carbofuran exposed animals was effective in bringing the LPO levels to that of control. The decrease in LPO may be partly due to the direct scavenging of ROS by highly active components of *I. aquatica* which showed high *in vitro* radical scavenging activity or could be due to retention of intracellular GSH. Phytochemicals in the form of phenolic compounds are a crucial part of the nutrition provided by vegetables and fruits, including the green leafy ones[47]. Among the various polyphenolic compounds, the flavonoids form the major class including the flavones, isoflavones, flavonols, flavanols, etc. and leafy vegetables are a good store house for these components[48]. Flavonoids in plants are generally present in their glycosylated and sulphated derivative forms, which are readily absorbed by the body cells[49]. Quercetin (3, 3′, 4′, 5,

7-pentahydroxyflavone), is a commonly and widely distributed flavonoid group present in plants, mainly in the glycosidic forms such as rutin (5, 7, 3, 4 -OH, 3-rutinose). Quercetin and rutin are the flavonoids that are most frequently consumed in the diets[50]. In the present study, among the various plant phenolics, rutin, quercetin, and apigenin have been identified in the methanolic extract of the leafy vegetable, *I. aquatica*. They have been best described as free radical scavengers and mediators of peroxidation reactions in the body. Hence, the protective role of this leafy vegetable extract comes to the forefront through this study.

Liver is the main organ where most of the metabolisms and also the detoxification of various toxic materials or their metabolites take place. Conjugation of enzymatic moieties to harmful molecules is a well known pathway of removal of toxic materials from the system. In the present study, the antioxidant enzyme profiles of the liver including SOD, catalase, GPx and the non-enzymatic GSH, and also lipid peroxidation levels have shown much alteration in the presence of carbofuran as also in the rats treated with IAE (P<0.05).

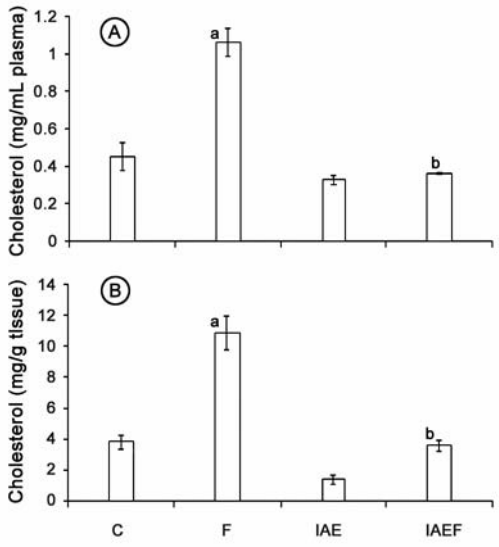

Fig. 4—Effect of *I. aquatica* extract on the total cholesterol level in plasma (A) and brain tissues (B) in rats treated with carbofuran. [Values are mean ± SD from 8 animals in each group. C=Control; F=Group treated with 0.1 mg/kg body wt. of carbofuran only; IAE=Group treated with 20 mg/kg body wt. of *I. aquatica* extract only; IAEF=Group treated with both *I. aquatica* extract and carbofuran. [a] F *vs* C, IAE, IAEF (P<0.05); [b] IAEF *vs*F, IAE (P<0.05)]

Table 1—DNA fragmentation in bone marrow cells of rats treated with carbofuran

[Values are mean± SD for 3 animals in each group]

Groups	Fragmented DNA (%)
C	0.73±0.071
F	6.98±0.110[a]
IAE	1.22±0.002
IAEF	3.73±0.012

C=Control, F=Group treated with 0.1 mg/kg body weight of carbofuran only, IAE=Group treated with 20 mg/kg body weight of *I. aquatica* extract only, IAEF=Group treated with both *I. aquatica* extract and carbofuran [a] F *v*s C, IAE, IAEF (P<0.05)

SOD plays an important role in dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen, and acts as the first line of defense[51]. Catalase in turn coverts the peroxide to molecular oxygen and water, thus, nullifying the prooxidative effect of the free radicals[52]. However, the presence of superoxide radicals inhibits the activity of catalase. Singlet oxygen and peroxyl radicals also affect SOD and catalase activities[53]. The free radical production as an outcome of oxidative stress induced by carbofuran, thus, may have reduced the activities of these enzymes. On the other hand, a dose of the antioxidant in the form of the *I. aquatica* extract might have restored their functions through redox cycling, thus replenishing the enzymes normal pool.

Glutathione mediated detoxification in the body, forms the most important antioxidant defense lines in cells. Formation of less toxic intermediates, by conjugation reactions, protects the body cells by reducing the injury levels[54,55]. The importance of such detoxification in the body cells becomes evident, when GSH level, together with its coupled enzyme system gets disrupted due to excessive consumption[41,56]. Depletion in glutathione level due to carbamates has been reported earlier[42,57]. However, in the present study, an increase in GSH and GPx levels has been noticed in presence of carbamates, which may be an outcome of the reduced activities of both SOD and catalase that has enhanced the activities of both GSH and GPx. Also, the high levels of hydroperoxides in the tissues of the carbofuran treated animals might have triggered this action. To reduce these peroxides to stable non-radical lipid alcohols, GPx utilizes GSH, thus, oxidizing them to GSSG which is regenerated by glutathione reductase[58] thus bringing GSH to normal levels. Supplementation of the extract has shown an alteration in their levels, so as to help the body regain their normal or near normal activity. Thus, the role of the flavonoid rich extract, as an important source of antioxidant, has been highlighted, which helps in replenishing the enzyme pools in the body when met with a toxic insult as in the present study.

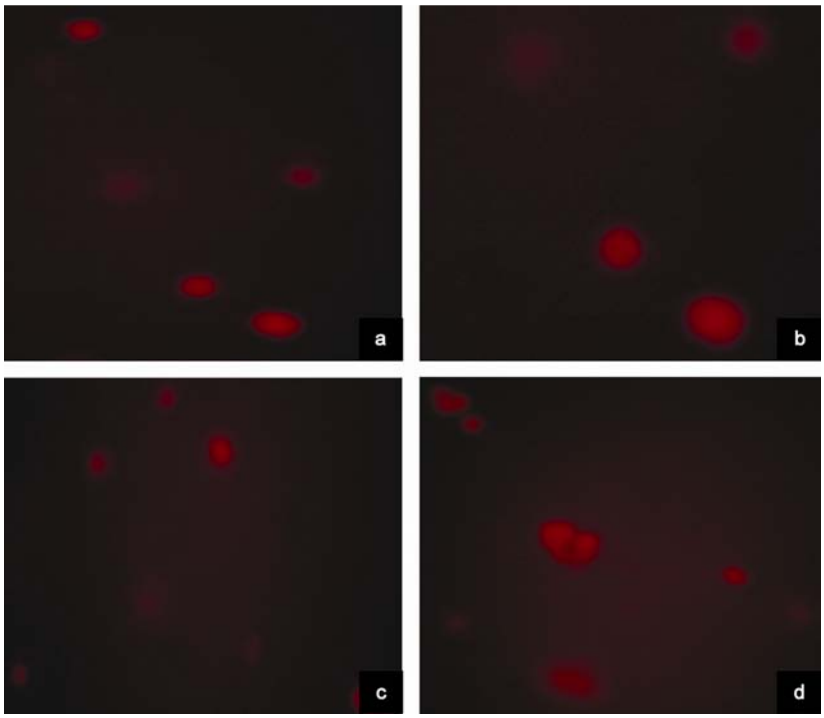

Fig. 5—Single cell gel electrophoresis of liver cells (a) normal cells showing no comet formation, (b) *I. aquatica* extract treated cells showing no comet formation, (c) carbofuran treated cells protected from comet formation to a large extent in presence of the extract of *I. aquatica* , (d) damaged cells showing comet formation in the carbofuran treated cells [100X]

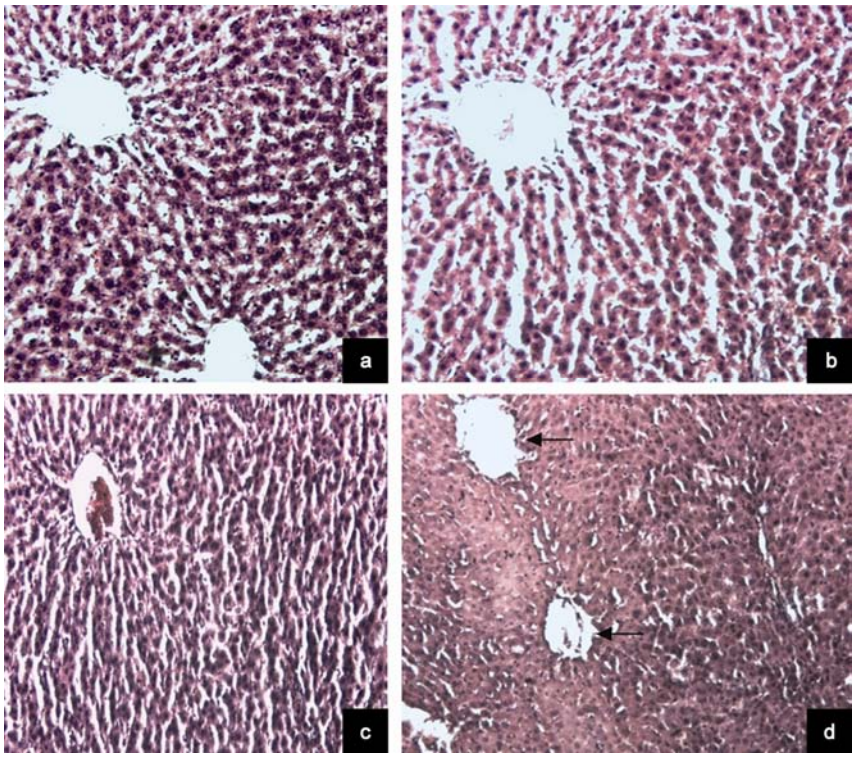

Fig. 6—Pictomicrographs of liver cells (a) showing normal liver cells in control rat, (b) liver cells of rats treated with *I. aquatica* extract alone, (c) liver cells protected by the extract of *I. aquatica* from the damage incurred by pesticide treatment, (d) totally damaged liver cells showing disoriented liver lobules due to pesticide treatment (the arrows showing distorted central veins) [100X].

The disruption of formation of lipoprotein has been reported by Hassan ***et al.***[59] as one of the factors leading to accumulation of cholesterol in carbofuran treated mice. Data presented in the present investigation showed that carbofuran caused a general hypercholesterolemia. Cholesterol is usually obtained in the diet but, if necessary, can be synthesized quantitatively in liver, beside intestine and other tissues under requirement[60]. It is reasonable to suggest that carbofuran had increased tissue lipogenesis, probably, through acceleration of acetyl CoA, which was supposed to be the precursor of cholesterol biosynthesis[60]. On the other hand, no significant changes were noticed with groups treated with IAE.

Plasma cholesterol levels are considered valuable indicator of drug induced disruption of lipid metabolism and development of fatty liver and altered cholesterol levels are implicated in impaired biliary excretion[61]. In the present study, cholesterol increase in the tissues might be due to the inhibition in the activity of enzymes involved in cholesterol break up that resulted in deposition of cholesterol into the cell. Similar results were also reported in rats treated with dimethoate[62].

DNA fragmentation is one of the earliest events in apoptosis. In the late apoptotic process, the cells break into a number of membrane-bound apoptotic bodies containing one or more fragments of nucleus. Case studies on human subjects working as floriculturists have been reported recently where pesticides have caused DNA fragmentations[63]. Several studies on Indian farmers have confirmed the fact that pesticides have a huge impact on DNA constitution of the body cells and tissues[64,65]. Chromosomal aberrations, sister chromatid exchanges and micronuclei formations in cells are common aspects of pesticide toxicity[66]. However, it is noteworthy to mention that data confirming DNA fragmentations in the bone marrow cells by the method presently used is almost lacking. Hence, from the present study, it can also be concluded that this assay is a simple method of assessing DNA damage in bone marrow cells.

DNA damage has been identified as a marker of cellular damage in many studies. Pesticide related oxidative stress in human subjects have been well studied where oxidative stress and DNA damage have been proposed as the major mechanisms that affect health, sometimes leading primarily to cancerous developments in the body cells and tissues, besides various neurological disorders[67]. Since the major metabolic procedures are taken up by the liver cells, most of the damage is incurred upon them. Hepatocellular damage in rats focuses on the severity of the pesticide even at minimal doses. Extract of *I. aquatica* clearly reduced the DNA damage possibly by quenching the free radicals produced by carbofuran.

Histomorphological alterations like centrilobular necrosis and extensive hemorrhage in the carbofuran treated rats have been well demarcated from the normal liver cells of the leafy vegetable extract treated rats. Thus, the severity of hepatic damage caused by such pesticides was confirmed by the histopathological examination of liver sections under light microscopy and also how green leafy vegetables can avert such perturbations in body tissues to a good extent.

In conclusion, the dietary polyphenolic compounds like rutin, quercetin, apigenin, along with some other components present in green leafy vegetables like *I. aquatica*, definitely enhances body's antioxidative status and their interaction with food contaminants like carbofuran reduces oxidative stress generated by pesticides that are consumed occasionally by us through various foodstuffs. Therefore, recommendations for inclusion of green leafy vegetables in our daily diet, as a natural source of antioxidant, are highlighted through the present study.

Practical application

The present study portrays the effectiveness of phytochemicals in the form of polyphenolic compounds in combating the deleterious side-effects of harmful chemicals like pesticides. Green leafy vegetables found ubiquitously are natural and cheap sources of a huge range of antioxidants besides being store houses of healthy nutrients. Their antioxidative nature has ushered them the capability of fighting oxidative stress situations rendered by several xenobiotic components inside the body systems. The results of the present study can thus be extrapolated to human cases giving us the opportunity to understand how these cheap plant sources can affect our systems *in vivo* in the real scenario. They are indeed good sources of natural antioxidants that can be had either in the raw or processed form. Encapsulation of the extracted polyphenolic components will be another endeavour in the present case.

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Conflict of interest

The authors declared that there is no conflict of interest.

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