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Protective role of *Ipomoea aquatica* Forsk. crude extract on rat tissues in the presence of acephate and carbofuran by histopathology and cytometric determination

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Ipomoea aquatica Forsk., commonly called water spinach ('*kalmi*' in Bengali) is an underexploited local green leafy vegetable having enormous scope as a cheap antioxidant source. The study explores the ameliorative effect of aqueous *I. aquatica* extract (IAE) in acephate and carbofuran treated Wistar male rats. Aqueous IAE (@ 20 mg/kg body wt.), administered to rats treated with organophosphate acephate (@ 30 mg/kg body wt.) and carbamate carbofuran (@ 0.1 mg /kg body wt.), attenuated the cholinesterase activity in brain, liver and cellular blood and reformed the histological perturbations in the brain cortex as well as the kidney anomalies, to a good extent. The IAE also upregulated the NF-E2-related factor-2 (Nrf-2) and MnSOD gene expression against pesticide toxicity. Hence, results of the present study intervenes into a new approach of justifying the deleterious side effects of pesticides that are commonly used, and how green leafy vegetables can help ameliorate those harmful effects.

Keywords: Flowcytometry, MnSOD, NF-E2-related factor-2, Nrf-2-ARE pathway, Pesticide toxicity, Water spinach

Carbofuran ($C_{12}H_{15}NO_3$; 2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), commonly known as furadan and acephate (O,S-dimethyl acetyl-phosphoramidothioate) are broad spectrum pesticides used against a wide range of insects, sometimes also as nematicides and acaricides for their short half-lives and lower persistence in the environment[1]. However, as alternatives to persistent organochlorates, the rampant use of these pesticides has caused detrimental outbreaks amongst the non-target species[2]. The presence of carbofuran has been reported to affect non-target mammalian systems and new born babies[3]. Its higher affinity towards adipose depots in the body exerts adverse effects on vital organs like brain, liver[4], skeletal muscles, and heart[5]. Oral administration of carbamates, has been shown to produce neuronal injury by excessive generation of ROS and nitrogen species[6], leading to lipid peroxidation (LPO), mitochondrial dyshomoeostasis, reduction of neuronal energy level[7], increased cytochrome c oxidase (COX) activity[8] and altering neurotransmitter concentration[9].

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Biotransformation of acephate to methamidophos, a more potent inhibitor of acetylcholine esterase (AChE), is a critical determinant of its toxicity. In mammals, methamidophos inhibits the enzyme carboxyamidase that converts acephate to methamidophos, thereby, ceasing further biotransformation of acephate[10]. A study on mammalian cell lines showed how the residual effects of such pesticides can cause toxicity at molecular levels[11].

Potential sources of antioxidants have been reported in several types of plant materials like fruits, vegetables, leaves, oilseeds, cereals, barks, roots, spices and herbs[12]. The ethno botanical reports suggest that green leafy vegetables can be antidiabetic[13], antihistaminic, anticarcinogenic[14], and antibacterial[15]. Recent studies have shown that leafy vegetables are rich in flavonols like kaempferol, quercetin and their derivatives[16]. Quercetin has been reported to decrease lipid peroxidation, upregulate the expression of serum high density lipoprotein (HDL) – associated paraoxonase1 (PON-1) in the HuH7 human hepatoma cell line[17], inhibit oxidized low density lipoprotein (LDL) triggered apoptosis and increase intracellular glutathione (GSH) down regulation in COS-1 cells[18]. Even, topical application of quercetin has been reported to effectively

modulate growth factors and cytokines, helping in wound healing[19]. It has been reported that administration of carbofuran orally, caused neuronal injury, which was mitigated by the administration of aqueous extract of *Cynodon dactylon* and Vitamin C[20]. The protective properties of ginger[21] and curcumin[22] against pesticide neurotoxicity has also been documented. Previous studies in the author's laboratory, have proved the efficacy of aqueous *Ipomoea aquatica* and *Enydra fluctuans* extracts, against the deleterious effects of acephate[23] and carbofuran[24,25], respectively.

The Nrf2-ARE pathway has recently been exploited as a valuable therapeutic agent for neurodegenerative diseases[26]. Overexpression of Nrf-2 causes an increase in the endogenous antioxidant status of the body cells, especially the brain, rendering protective effect to the body cells[27]. The objective of this study is to elucidate the protective side of green leafy vegetables in the presence of commonly used pesticides,even at the molecular level. This approach in the present study is a first of its kind, where protein expression has been recorded, besides, highlighting the histopathological and chemical assays, determining cellular damage.

Materials and Methods

Chemicals

Methanol and acetylthiocholine iodide were purchased from Sisco Research Laboratory (SRL), Mumbai, India; DTNB was purchased from Sigma Chemical Co. (St. Louis, MO); BSA standard was purchased from E. Merck India Pvt. Ltd., Mumbai, India. Carbofuran pesticide was supplied by Anu Products Limited, Haryana, India. Acephate (Technical Grade 85% purity) was obtained from M/S Hindustan Insecticides Limited, Delhi, India.

Preparation of Ipomoea aquatica aqueous extract

The leafy vegetable, *Ipomoea aquatica* Forsk, was collected from the local field and was authenticated by the Central National Herbarium, Shibpur, India vide letter no CNH/ 120/2011/Tech II/607. It is a locally available green leafy vegetable belonging to the family Convolvulaceae.

The polyphenolic compounds were extracted by following the methods developed in the laboratory[28]. 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 weight) was dissolved in water to obtain the water extract. It was then stored at −40°C for further use.

Preparation of pesticide solution

The pesticide solutions were prepared by dissolving them in distilled water to obtain a clear solution. They were prepared on a daily basis so as to obtain the required dose of 30 mg of acephate/kg body wt. and 0.1 mg of carbofuran/ kg body wt. The dose used for the pesticide was calculated as $1/100^{th}$ of the LD_{50}, so that it showed no mortality in the animals.

Animal diet and treatment

Male albino Wistar rats, 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. 5 mg, vitamin B I.P. 5 mg, calcium pantothenate USP 5 mg, 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. 1.0 mg, vitamin E USP 0.1 mg) per kg of diet. The diets were adequate in all nutrients. They were maintained at 12 h light/dark conditions at normal room temperature. 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 6 groups comprising 8 rats (n=6) in each group. Group I (C) served as the control and was provided with normal diet. Group II animals (IAE) were fed (by gavaging) with the leafy vegetable extract @ 20 mg/kg body wt. The animals in Group III (A) were gavaged with the pesticide acephate only @ 30 mg/kg body wt. and Group IV (F) with carbofuran only @ 0.1 mg/kg body wt. Group V animals (IAEA) were given acephate @ 30 mg/kg body wt, along with the leafy vegetable extract (20 mg/kg body wt.). Group VI animals (IAEF) were given carbofuran @ 0.1 mg/kg body wt. along with the leafy vegetable extract (20 mg/ kg body wt.).

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

Determination of acetylcholine esterase (AChE) activity in erythrocytes and tissue homogenates

The AChE activity was determined according to the procedure of Ellman's colorimetric method[29]. Briefly, a 30 µL aliquot of tissue homogenate or diluted cellular blood was added to 3 mL of phosphate buffer containing 5,5'-dithio-bis-nitrobenzoic acid (DTNB) and acetylthiocholine iodide (AChI) and incubated at 37ºC water bath for 6 min. The activity was then determined using Shimadzu spectrophotometer at 412 nm. The AChE content was expressed as µmoles of ACh hydrolyzed/mg protein/min. The specific activity of enzyme was calculated using molar absorbance $13.6 \times 103M^{-1}cm^{-1}$. The reaction mixture without the enzyme protein served as a control in this assay. The protein was calculated according to the Lowry method[30].

Histopathological study

Tissues from the kidney and brain were excised and after fixing in 10% formalin saline and picric acid respectively for 3-4 days, the tissues were embedded in paraffin wax. Uniform sections of 5 µM thickness were cut and stained with hematoxylin and eosin by routine procedures. The stained sections were examined for pathological changes[31]. The microscopic slides were observed at 40X magnification.

Hepatocyte isolation

A fresh hepatocyte suspension was obtained by *in situ* liver perfusion with collagenase (0.05%, p/v) through a two-step perfusion technique of Seglen[32].

Flow cytometric analysis of protein expression

Isolated Hepatocyte were fixed in 4% paraformaldehyde in PBS (pH-7.4) for 20 min at room temperature and permeabilized in 0.1% Triton X-100 in PBS with 0.1% FBS for 5 min. After washing twice in PBS with 3% FBS, permeabilized cells were incubated with primary antibody (p-Akt, Nrf2 and Mn-SOD) on ice for 2 h and washed twice in PBS. Cells were then incubated with a FITC conjugated goat anti rabbit IgG, as a secondary antibody for 30 min on ice and washed twice in PBS. Stained cells were acquired and analyzed using BD FACSAria III (Becton Dickinson, Franklin Lakes, NJ) equipped Flow Jo software[33].

Statistical analysis

The data was 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 all the experiments[34].

Results

Determination of acetylcholine esterase activity

AChE catalyzes the hydrolysis of acetylcholine, facilitating nerve impulse transmission. Both carbofuran and acephate treatment showed a decrease in the activity, whereas, pre-treatment with IAE, ameliorated the condition to a good extent in the tissues, as well as blood, enhancing the activity to a near normal status (Fig. 1).

Histological examinations

Histopathological determinations showed severe morphological changes in both the cortical region of the brain and kidney, in both acephate and carbofuran treated rats. The pyramidal cells showed distortion and lesser in number than the normal state, in the brain cortex and the glomerular structure in the kidney showed morphological damage, with widening of parietal and visceral leaves of Bowman's capsules in the pesticide treated groups. Treatment with the polyphenolic extract showed effective decrease in the damaged cellular structures (Fig. 2).

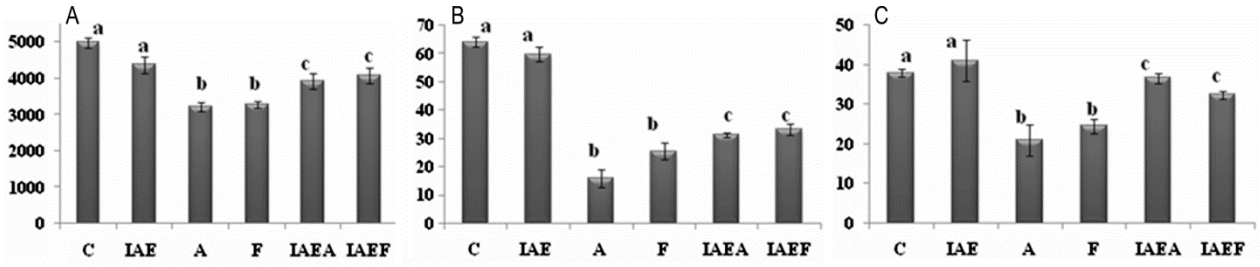

Fig. 1 — Cholinesterase activity: Cholinesterase activity in rats (A) erythrocytes; (B) liver; and (C) brain, treated with IAE alone and in the presence of acephate and carbofuran. [Values are expressed as Mean ± SD. n=6. C= Control; IAE= *Ipomoea aquatic* extract treated cells; A= Acephate; F= Carbofuran; IAEA= IAE treated cells in presence of acephate; IAEF = IAE treated cells in presence of carbofuran.[a] Significantly different from A,F, IAEA and IAEF; $P < 0.05$; [b] Significantly different from C, IAE, IAEA and IAEF; $P < 0.05$; and [c] Significantly different from C, IAE,A and F; $P < 0.05$]

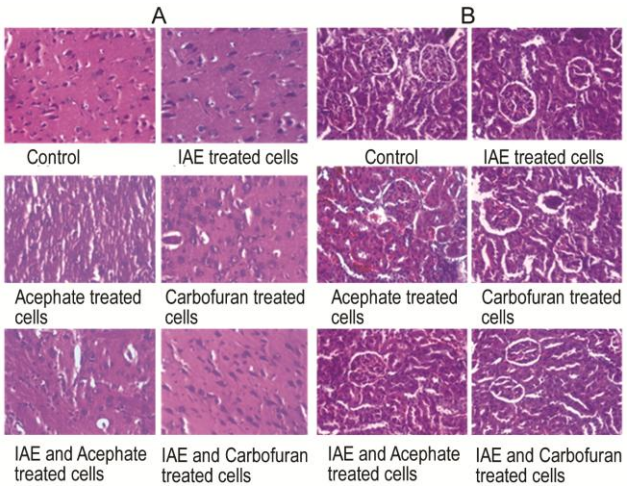

Fig. 2 — Histopathological study: Histopathological determinations of (A) Cortical region of brain; and (B) kidney cells showing distortions in cellular structures [lesser number of normal pyramidal cells in brain and distorted glomerular structure in kidney] in pesticide treated cells and the near normal cellular arrangements in the IAE pretreated cells.

Up-regulation of Mn-SOD expression via Nrf2 activation

It was hypothesized that the protective effect of IAE, against acephate and carbofuran induced oxidative stress, resulted from the induction of antioxidant genes, such as MnSOD and its transcription factor Nrf-2. The results showed that the extract significantly increased Nrf2 and MnSOD expression over control group as observed from relative FITC fluorescence intensity. The results obtained from flowcytometry also showed that the expression level of Nrf-2 and its transcriptionally regulated protein MnSOD, to be significantly down-regulated in the pesticide treated groups as compared to the control group. Furthermore, the group treated with both the plant extract (IAE) and the pesticides, showed significant up-regulation of Nrf-2 and MnSOD expression, as compared to the groups treated only with the pesticides, indicating the protective role of the extract through the activation of Nrf-2 (Fig. 3).

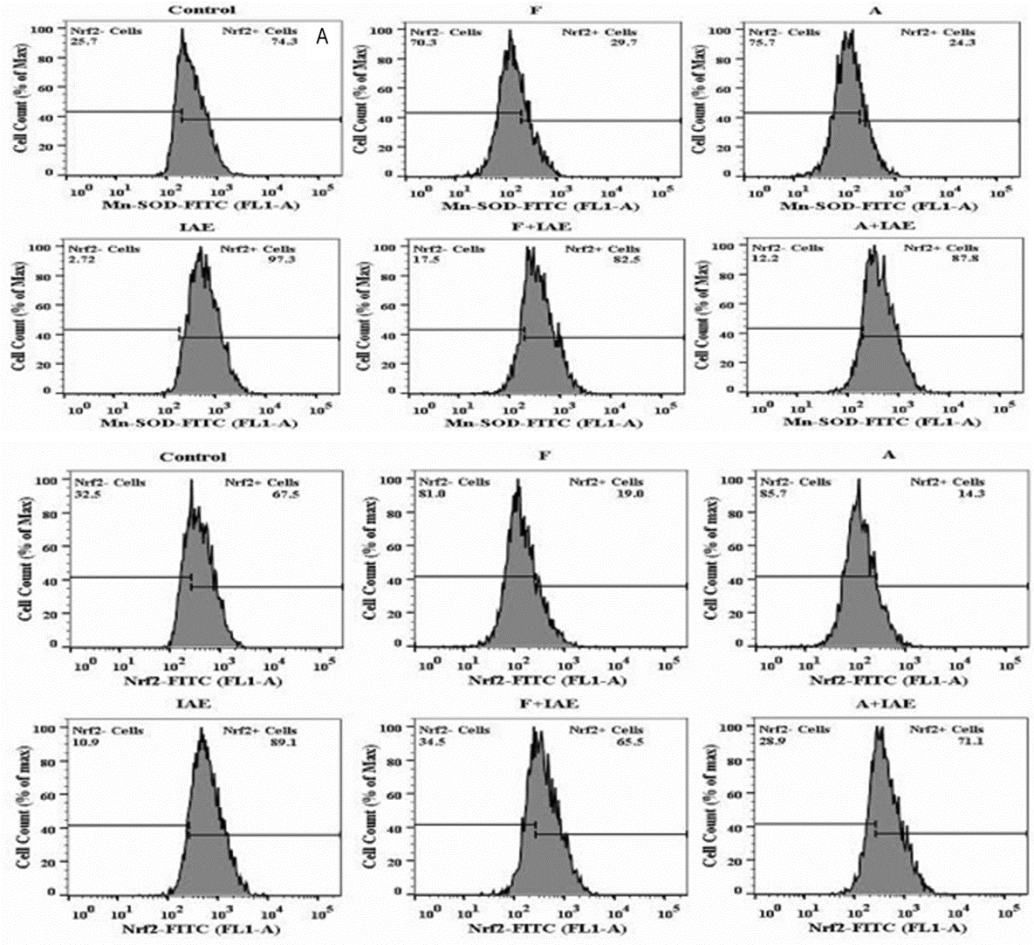

Fig. 3 — Gene regulation**:** Upregulation of MnSOD expression via Nrf-2 activation in acephate and carbofuran treated rats (IAE= *Ipomoea aquatica* extract; F= Carbofuran; A= Acephate)

Discussion

The primary target of most pesticides (mostly organophosphates and carbamates) is acetylcholinesterase (AChE) that hydrolyzes acetylcholine-a major neurotransmitter, resulting in the accumulation of acetylcholine and activation of muscarinic and nicotinic receptors[35]. The absorption of these pesticides can, thus, be assessed by measuring the decrease in AChE, the rapidity of accumulation being dependant on the dose[36]. The present study showed a decrease in the enzyme activity, in the cellular part of blood (erythrocytes), liver and brain cells in the pesticide treated groups over which the extract seemed to have a maximum normalizing effect. Both acephate and carbofuran administration induced weakness, tremors and facial movements[37]. Structural alterations of the brain tissues due to changed lipid structures, hampers the functional integrity of the cholinergic neurons, thereby decreasing the enzyme activity[38].

Generation of reactive oxygen species, as a byproduct of normal oxidative mechanisms, especially mitochondrial functions, for maintaining intracellular homeostasis and signal transduction, is a well established data[39]. In the present study, degenerative changes of brain tissues and kidney morphologies showed good recovery in the presence of the leafy vegetable extract. Microscopic evaluation of the kidney showed necrotic areas, plasmic debris and damaged glomerular morphologies with distorted Bowmen's capsules in both acephate as well as carbofuran treated cells. Renal injuries can be attributed to the high elimination rates of the pesticides and their metabolites that might alter the morphological status of the kidney cells, besides the oxidative insults that are borne by the cells in addition. Lipophilic nature of the pesticides causes their accumulation in the high adipose contents of the brain, proving detrimental to brain health and its degeneration[40]. Antioxidative properties of the green leafy vegetable extract seemed to ameliorate these side effects.

Endogenous antioxidant capacity of body cells is regulated through the activation of the transcription factor NF-E2-related factor-2 (Nrf-2) which regulates MnSOD gene expression by binding to the antioxidant responsive element (ARE), thus, activating or inhibiting a cascade of proteins that determines the protection of cells in the presence of any toxin in the body[41]. The phytoextract upregulated the antioxidant genes MnSOD and Nrf-2 in rat liver cells. IAE mainly protected the liver cells from acephate and carbofuran induced oxidative stress by elevating the intracellular antioxidant enzymes via the enhanced accumulation of a transcription factor, Nrf-2, and dramatically upregulated the expression of antioxidant gene MnSOD. The data confirmed that IAE mediated upregulation of MnSOD and Nrf-2, is critically governed by the pesticide induced oxidative stress. The presence of pesticide molecules in the body activates the detoxification procedures in the body that enhances the production of several antioxidant enzymes like SOD, Catalase, several reductases and peroxidases, which, in turn might be switched on by the transcription of a multitude of genes by the Nrf-2 ARE pathway[42]. These genes play an important role in cytoprotection from several oxidative insults and injuries in various tissues, most important being the brain[43].

Conclusion

The present study elaborates a first of its kind finding on the regulation of protein expression by hepatocytes in presence of both the pesticides and their protection to a large extent by the leafy vegetable extract, which was justified by the up-regulation of protein expression of the cells. Histopathological determinations and cholinesterase activities also proved the protective side of the extract. Thus, it can be concluded, that such green leafy vegetables can affect the body cells at a molecular level, and further studies are necessary to understand the complex mechanisms by which they act and provide protection to the body cells against toxic insults.

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Conflict of interest

The authors declare no conflict of interest.

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