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ROLE OF AQUEOUS EXTRACT OF *Cynodon dactylon* IN PREVENTION OF CARBOFURAN- INDUCED OXIDATIVE STRESS AND ACETYLCHOLINESTERASE INHIBITION IN RAT BRAIN

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Abstract

The present study was designed to investigate the ameliorating effect of aqueous extract of C. *dactylon* on carbofuran induced oxidative stress (OS) and alterations in the activity of acetylcholinesterase (AChE) in the brain of rats. Vitamin C was used as a positive control. Wistar rats were administered with single sub-acute oral dose (1.6 $mgkg^{-1}$ b.wt.) of carbofuran for 24 h. The OS parameters such as lipid peroxidation (LPO) and the activities of antioxidant enzymes including super oxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), and that of AChE were studied in brain. Carbofuran treatment significantly increased the activities of SOD and CAT by 75 and 60%, respectively. It also induced the level of LPO by 113%. In contrast, the activities of GST and AChE were recorded to be diminished by 25 and 33%, respectively. Pretreatment of the rats with aqueous extract of *C. dactylon* (oral; 500$mgkg^{-1}$) restored SOD activity completely but CAT activity only partially (7%). Carbofuran induced LPO was moderated by 95% in the brain of *C. dactylon* treated rats. The observed changes in OS parameters in *C. dactylon* treated group were comparable to that observed in vitamin C (200 $mg\text{-}kg^{-1}$ b. wt.) treated group. Surprisingly, *C. dactylo*n treatment significantly recovered the activity of AChE to a smilar level as observed in the brain of control group. In contrast vitamin C treatment did not cause significant change in the activity of AChE in carbofuran treated group. There were no noticeable changes in the aforementioned study parameters in the brain of rats receiving C. *dactylon* and vitamin C, only. The results suggest that the study is extremely important in the context of development of new anticholinestesterase and antioxidant antidotes against carbofuran from *C. dactylon.*

Key words: Carbofuran, *C. dactylon*, Antidote, AChE, Oxidative stress, brain.

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Abbreviations: OS: Oxidative stress ; **AChE**: acetylcholin-esterase ; ***C. dactylon***: *Cynodon dactylon.*

INTRODUCTION

Carbofuran causes reversible inhibition of the activity of acetylcholinesterase (AChE). As a result an exceedingly high concentration of acetylcholine is built up at synaptic junctions in CNS (the major sites of enzyme activity) which leads to hypercholinergic excitatory processes (1). During carbofuran induced hypercholinergic excitotoxicity, the flow of oxygen through brain and muscle is greatly increased and use of ATP is greater than the rate of its generation (7). This metabolic stress invokes the rate of production of reactive oxygen species (ROS) and byproducts of their reaction with cellular biomacromolecules (8). ROS are kept in check in body by antioxidant molecules such as vitamins (A, C and E,);

glutathione, and enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed), glutathione-S-transferase (GST) and mitochondrial monooxygenases. An imbalance between ROS production and antioxidant defense towards former exposes the body/cells to oxidative stress (OS) (29).

Relatively greater rate of O_2 utilization and poor antioxidant defense of brain render it more vulnerable to OS in comparison to other organs of the body (18). Presence of redox-active metal ions such as iron, copper, manganese and zinc as well as high concentrations of polyunsaturated lipids (biomolecules with high OS susceptibility) may aggravate the damaging effect of ROS to the brain. In an earlier study we observed that brain possesses considerably lower ROS scavenging activity than that of liver (28). Carbofuran has been reported to cause elevated lipid peroxidation, generation of ROS and diminished activity of ROS quenching enzymes in a number of studies (14, 29).

The emergence of new mechanisms pertaining to oxidative stress in addition to existing paradigm of anticholinesterase toxicity of carbamates and irresolute effectiveness and toxicity of currently existing cholinesterase reactivating (oxime) and anticonvulsant (benzodiazepine) based treatment regimens (2, 31) warrant for the search of new treatments for carbamate neurotoxicity.

Recently there has been a surge in the application of antioxidant compounds such as vitamins C, vitamin E, N-acetyl cysteine and melatonin in mitigating carbamate toxicity especially in experimental animals (15, 29, 32). Plants have been a rich source of medicinally active compounds and play an important role in drug discovery since times immemorial. A number of these compounds such as phenolics and flavonoids, terpenoids etc. are highly effective in mitigating OS and OS associated disorders (19). Our previous study reporting efficient antioxidant action in conjunction with couple of recent studies showing prevention of brain hypercholinergic excitotoxicity by turf grass, *Cynodon dactylon* (*C. dactylon*) (21, 25, 30) led us to select this plant as antioxidant source for studying the possible mitigation of carbofuran induced toxicity. Keeping the aforementioned facts in view, in the present study we envisaged to explore the impact of aqueous extract of *C. dactylon* on the carbofuran mediated changes in the activity of target enzyme acetylcholinesterase as well as enzymatic and non-enzymatic oxidative stress parameters in the brain of rats.

MATERIALS AND METHODS

Chemicals

Technically pure (99.6%) carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl N-methylcarbamate) in powder form was supplied by Rallis India Limited (Bangalore, India) as a gift. Pyrogallol, reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from HIMEDIA Laboratories, India. Bovine serum albumin (BSA), acetylthiocholine-iodide (ATI), 3,5-dithionitrobenzoic acid (DTNB) were purchased from Loba Chemie Pvt. Ltd., India. All other chemicals used in the study, were purchased from E. Merk, Darmstad, Germany.

Collection of plant material and preparation of its aqueous extract

C. dactylon (2 kg) was collected from the campus of University of Allahabad, Allahabad, India. It was identified and authenticated by Prof. B.D Singh Taxonomist Botanical Survey of India. A voucher specimen (AA 518) has been submitted. The whole green plant was washed thoroughly with water and air-dried (25^0 C for seven days in absence of sunlight). The whole plant material was extracted with boiling water for 48 h. The resulting dark brown extract was cooled and filtered through Whatmann no. 1 filter paper, evaporated to dryness and residue was dissolved in water. The concentrated extracts were lyophilized to get powder for the experiment (w/w 1.3%).

Animals and their maintenance

Male albino rats (Wistar strain) weighing between 100 and 150g purchased from CDRI- Lucknow, India were used throughout the study. The animals were housed at 22 ± 2^0C in polypropylene cages (6 rats per cage), fed with standard pellet diet obtained from Pashu Ahar Kendra, Varanasi, India and had free access to drinking water. These animals were acclimated for one week under laboratory conditions each time before starting the experiment. The protocols used in the study were according to the guidelines for use and care of laboratory animals and were approved by the Institutional Ethics Committee in the University.

Treatment regimen

Animals were divided into six groups, each containing 6 animals. Animals in control group (C) received 0.5 mL of groundnut oil, orally (placebo). Animals in carbofuran treated group (CF) received single oral dose of 1.6 mg carbofuran kg^{-1} body weight equivalent to 20% LD_{50} dissolved in 0.5mL of groundnut oil. Animals of group (Vit C) received vitamin C at 200 mg kg^{-1} body weight in 0.5 ml distilled water. Animals placed in groups (Vit C+CF) received vitamin C at 200 mg kg^{-1} body weight 30 minutes prior to carbofuran 1.6 mg carbofuran kg^{-1} body weight treatment. Animals in group (Cd) received *C. Dactylon* extract, 500 mg kg^{-1} body weight dissolved in distilled water. Animals in group (Cd+CF) received *C. Dactylon* extract, 500 mg kg^{-1} body weight 30 minutes prior to carbofuran 1.6 mg carbofuran kg^{-1} body weight treatment. All the doses were given once and animals were watched for

24 h and the experiments were performed after the stipulated period. Groundnut oil has been used as a vehicle for being safe.

Biochemical studies

Preparation of brain homogenate for determination of OS parameters

After 24 hr of the treatment animals were sacrificed using mild chloroform anesthesia and cervical dislocation causing minimal pain. The whole brain was excised, washed in isotonic ice-cold NaCl (0.9%) solution, blotted to dryness and weighed. 10% (w/v) brain tissue homogenate was made in 100 mM sodium-phosphate buffer (pH 7.4) containing 150mM potassium chloride (KCl) using Potter-Elvehjam homogenizer fitted with a Teflon-coated pestle under ice cold condition (4-6°C). The homogenate was centrifuged at 9,000×g for 30 min. The pellet was discarded and the supernatant was used for estimation of different OS parameters.

Determination of oxidative stress parameters in rat brain: Lipid peroxidation (LPO) was determined in supernatants of brain homogenates by colorimetric estimation of malondialdehyde (MDA) / thiobarbituric acid reactive substances (TBARS) formed. The results were expressed as nmol MDA/mg protein using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (24). The activity of SOD (E.C.1.15.1.1) was assayed by monitoring pyrogallol autooxidation at 412 nm for 3 min in presence/absence of the enzyme (supernatant). One unit of the enzyme activity was expressed as fifty percent inhibition of autooxidation of pyrogallol per minute (22). The activity of CAT (E.C. 1.11.1.6.) was determined by monitoring decrease in the absorbance at 240 nM for 3 min as a function of H_2O_2 consumption Beers and Sizer (3). One unit of CAT activity was defined as micromoles of H_2O_2 decomposed per min using molar extinction coefficient of H_2O_2 (43.6 $M^{-1}cm^{-1}$). GST (EC: 2.5.1.18) activity was estimated as described earlier (9). The change in absorbance was recorded spectrophotometrically at 340 nm for 3 min. One international unit (IU) of GST activity was defined as nanomoles of GSH–CDNB conjugate formed per minute using molar extinction coefficient (6.22×10^3 M^{-1} cm^{-1}). The aforementioned enzyme activities were determined at room temperature using UV-visible double beam spectrophotometer (ELICO, model: SL-160) with quartz cuvettes (1 cm light path) against distilled water as blank.

Preparation of tissue homogenates for determination of activity of acetylcholinesterase (AChE, EC: 3.1.1.7)

For estimation of AChE activity, a homogenate (10%, w/v) was prepared from brain tissue in 50mM sodium phosphate buffer (pH 8.0) containing 0.1% Triton X-100 using Potter-Elvehjam homogenizer fitted with a Teflon-coated pestle under ice cold condition (4-6°C). The homogenates were kept for 30 min in cold with intermittent stirring and centrifuged at 4°C for 30 min at 10,000 g. Supernatant was either used for determination of protein contents or for assay of AChE activity.

Determination of AChE activity in rat brain

The activity of AChE was determined as described earlier (6). The reaction mixture (3 ml) contained 1.5 ml of 100mM sodium phosphate buffer (pH 8.0), 0.3 ml of 5mM DTNB [5, 5'-dithiobis-(nitro benzoic acid) prepared in 10 mM sodium phosphate buffer, pH 7.5 containing 15 mg sodium bicarbonate added per 10 ml of solution, 0.3 ml of 5mM acetylthiocholine iodide (ATI), 0.1 ml of 10% homogenate and 0.8 ml of distilled water. The increase in absorbance was monitored at 412 nm for 3 min in a UV-visible double beam spectrophotometer (ELICO, model: SL-160) with quartz cuvettes (1 cm light path) against distilled water as blank. One unit of AChE activity was expressed as nanomoles of substrate hydrolyzed/min/mg protein under experimental conditions.

Estimation of total protein content

Wherever needed, total protein content in the homogenates was determined spectrophotometrically as described earlier (20) using BSA as a standard.

Statistical Analysis

All values were expressed as mean ± SD. Each experiment was performed in triplicate and data were averaged. Each group represents determinations on six animals and represents the average of individual determination. Statistical significance of the observed difference in various determinations was tested using one way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple pair wise comparisons between the various treated groups. Values with $P<0.05$ were considered as statistically significant.

RESULTS

Effect of *C. dactylon* aqueous extract on the activity of super oxide dismutase (SOD) in brain of rat treated with carbofuran: The activity of SOD, which removes super oxide anion radical (O_2^{-0}) by catalyzing its dismutation, to reduce one molecule to H_2O_2 and oxidize another to O_2 (10); was recorded to be elevated by 75% $p \leq 0.001$ in brain of rats after subacute oral treatment with carbofuran. Pretreatment of rats with *C. dactylon* aqueous extract held SOD activity to its value corresponding to that observed in control rats receiving vehicle i.e. groundnut oil, only (Fig 1). The protection offered by *C. dactylon* was in agreement with that of pretreatment of rats with vitamin C.

Effect of *C. dactylon* aqueous extract on the activity of catalase (CAT) in brain of rat treated with carbofuran: The activity of another antioxidant enzyme CAT which neutralizes peroxide radicals into H_2O and O_2, registered increase of 60% in brain ($p \leq 0.01$) of rats treated with carbofuran (Fig 2). The aqueous extract of *C. dactylon* could offer only a partial preservation of the enzyme activity (7%) in carbofuran treated rats; however, vitamin C treatment retained the activity near its level observed in the brain of vehicle only, treated rats.

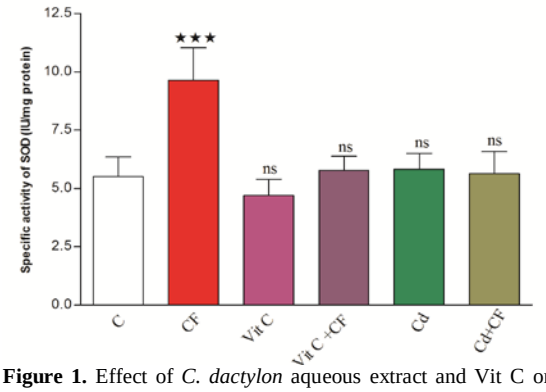

Figure 1. Effect of *C. dactylon* aqueous extract and Vit C on the activity of SOD in brain of rat treated with carbofuran. C refers to control group treated with 0.5 ml groundnut oil; CF refers to single sub acute oral dose (1.6 mg-kg^{-1} bwt equivalent to 20% LD_{50}) of carbofuran. Vit C and Cd refer to the rats treated with 200 and 500 mg-kg^{-1} bwt Vitamin C and *C. dactylon* aqueous extract, respectively. However, Vit C+ CF and Cd+ CF group received the aforementioned doses of Vit C and *C. dactylon* 30 min prior to oral treatment of rats with 1.6 mg-kg^{-1} bwt carbofuran. All the treatments were given as single oral dose and experiments were carried out after 24 h of treatment. SOD activity was determined as described in Materials and Methods and expressed as international units. One international unit (IU) of enzyme activity has been defined as fifty percent inhibition of pyrogallol auto oxidation per min. Specific activity of the enzyme was expressed in terms of enzyme activity (IU/mg protein), values were expressed as Mean ± SD; n=6, where n=number of determinations. The sign (***) indicates values significantly different from control at $p \leq 0.001$.

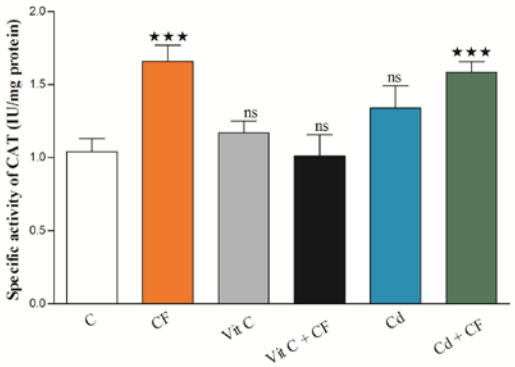

Figure 2. Effect of *C. dactylon* aqueous extract and Vit C on the activity of CAT in brain of rat treated with carbofuran C refers to control group treated with 0.5 ml groundnut oil; CF refers to single sub acute oral dose (1.6 mg-kg^{-1} bwt equivalent to 20% LD_{50}) of carbofuran. Vit C and Cd refer to the rats treated with 200 and 500 mg-kg^{-1} bwt Vit C and *C. dactylon* aqueous extract, respectively. However, Vit C+ CF and Cd+ CF group received the aforementioned doses of Vit C and *C. dactylon* 30 min prior to oral treatment of rats with 1.6 mg-kg^{-1} bwt carbofuran. All the treatments were given as single oral dose and experiments were carried out after 24 h of treatment. CAT activity was determined as described in Materials and Methods section and expressed as international units. One international unit (IU) of CAT activity was defined as micromoles of H_2O_2 decomposed per min**.** Specific activity of the enzyme was expressed in terms of enzyme activity (IU/mg protein), values were expressed as Mean ± SD; n=6, where n=number of determinations. The sign (***) indicates values significantly different from control at $p \leq 0.001$. ns denotes value not statistically different from group C.

Effect of *C. dactylon* aqueous extract on the activity of glutathione S transferase (GST) in brain of rat treated with carbofuran: GST functions as a major detoxification enzyme either by conjugating the toxic molecules to glutathione for their removal from the cell through mercapturic acid pathway (16) or by conjugation of glutathione to lipid peroxides which are by products of reaction of ROS with membrane lipids and could be equally devastating as ROS in catalyzing oxidative damage of membrane lipids in chain reactions (26, 33). Its activity was observed to decline by 25%, $p \leq 0.05$; in brain of rats upon treatment with carbofuran (Fig 3). *C. dactylon* and Vit C treatments could restore enzyme activity to its near normal levels observed in control group of rats treated with vehicle, only.

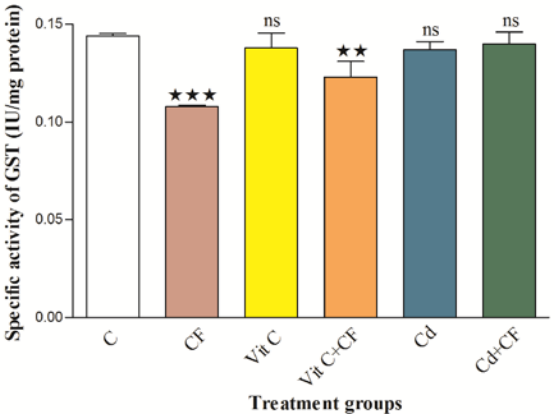

Figure 3. Effect of *C. dactylon* aqueous extract and Vit C on the activity of GST in brain of rat treated with carbofuran C refers to control group treated with 0.5 ml groundnut oil; CF refers to single sub acute oral dose (1.6 mg-kg^{-1} bwt equivalent to 20% LD_{50}) of carbofuran. Vit C and Cd refer to the rats treated with 200 and 500 mg-kg^{-1} bwt Vit C and *C. dactylon* aqueous extract, respectively. However, Vit C+ CF and Cd+ CF group received the aforementioned doses of Vit C and *C. dactylon* 30 minutes prior to oral treatment of rats with 1.6 mg-kg^{-1} bwt carbofuran. All the treatments were given as single oral dose and experiments were carried out after 24 h of treatment. GST activity was determined as described in Materials and Methods section and expressed in terms of international units. One international unit (IU) of GST activity was expressed as nanomoles of GSH–CDNB conjugate formed per minute. Specific activity of the enzyme was expressed in terms of enzyme activity (IU/mg protein), values were expressed as Mean ± SD; n=6, where n=number of determinations. The sign (***) indicates values significantly different from control at $p \leq 0.001$. the sign (**), denotes that the value is significantly different from control group at $p \leq 0.01$. However, ns denotes value not statistically significant from group C.

Effect of *C. dactylon* aqueous extract on the extent of lipid peroxidation (LPO) in brain of rat treated with carbofuran: The malonyldialdehyde (MDA), the secondary product of lipid peroxidation, is frequently used as a biomarker of oxidative stress in various pathological conditions as well as in pesticide exposure induced stress. Table 1 exhibits a sharp rise in the

Table 1. Effect of carbofuran oral treatment for 24h on the levels of membrane lipid peroxidaton (LPO) in terms malonyldialdehyde released/mg protein in rat brain and liver.

Treatment group	LPO (MDA released/mg protein) in brain	Prooxidant/antioxidant (P/A) ratio
C	0.87±0.03	0.129
CF	1.85±0.2***	0.162
Vit C	0.94±0.049	0.156
C. dactylon extract	1.04±0.06	0.150
Vit C+ CF	0.92±0.05	0.125
C. dactylon extract + CF	1.02±0.02	0.138

The sign (***) shows that the value is significantly different from group C at p≤ 0.001.

levels of MDA (113%, P>0.001) in brain of carbofuran treated rats, which was accentuated significantly by 95% in the brain of rats receiving treatment with aqueous extract of *C. dactylon*. The protection offered by *C. dactylon* was comparable to that offered by pretreatment of rats with Vit C.

Effect of *C. dactylon* aqueous extract on the activity of activity of acetylcholinesterase (AChE) in brain of rat treated with carbofuran: AChE hydrolyzes acetylcholine at synapses to terminate synaptic transmission and is also target for inhibitory effect of carbamate. Figure 4 shows the activity of AChE in the brain of different treatment groups which shows that upon oral treatment with carbofuran for 24 h its activity was inhibited by 33%; P>0.001 than that in control group of rats. *C. dactylon* treatment exhibited considerable recovery (higher than Vit C) and brought its activity to near the value observed in control group of rats. This startling observation is extremely important in the context of exploration of *C. dactylon* in mitigating the hypercholinergic toxicity of carbamates as well as other compounds. This would be discussed in detail in the discussion.

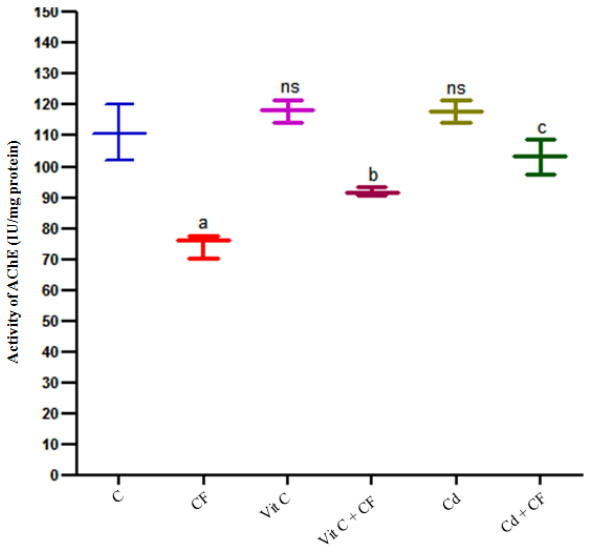

Figure 4. Effect of *C. dactylon* aqueous extract and Vit C on the activity of activity of AChE in brain of rat treated with carbofuran. C refers to normal control group treated with 0.5 ml groundnut oil; CF refers to single sub acute oral dose (1.6 mg-kg^{-1} bwt equivalent to 20% LD_{50}) of carbofuran. Vit C and Cd refer to the rats treated with 200 and 500 mg-kg^{-1} bwt Vit C and *C. dactylon* aqueous extract, respectively. However, Vit C+ CF and Cd+ CF group received the aforementioned doses of Vit C and *C. dactylon* 30 min prior to oral treatment of rats with 1.6 mg-kg^{-1} bwt carbofuran. All the treatments were given as single oral dose and experiments were carried out after 24 h of treatment. AChE activity was determined as described in Materials and Methods and expressed as international units. One international unit (IU) of activity was expressed as nanomoles of ATI hydrolyzed per min. Specific activity of the enzyme was expressed in terms of enzyme activity (IU/mg protein), values were expressed as Mean ± SD; n=6, where n=number of determinations. The signs a, b and c indicates that the value is significantly different from normal at p≤0.001, from CF treated group at p≤0.01 and from CF treated group at p≤0.05 respectively. ns means that value is not significantly different from normal control group

DISCUSSION

Accumulating evidences are interesting but still poorly understood biochemical pathway for generation of oxidative stress, excitotoxicity and AChE inhibition caused by carbofuran suggest a need for detailed investigation of these processes in mammalian systems (13, 29, 32). Thus the present study was designed to study the possible association between carbofuran induced anticholinesterase toxicity and oxidative stress in the brain of the rats exposed to sub-acute concentration of carbofuran.

The results indicate that subacute oral treatment of carbofuran lead to significantly enhanced activities of SOD and CAT ($P < 0.05$). The elevated activities of SOD and CAT in rat brain in response to carbofuran exposure suggests

the activation of compensatory mechanism evolved by the animal system to protect it from the damage due to free radical-mediated oxidative stress after pesticide exposure, leading to a reduction in the accumulation of excess ROS in the brain. However, the threshold level of induction and quantum of its influence depends on many defined and yet to be understood factors including the magnitude of the oxidative stress generated as well as on the dose of stressor (27).

In contrast to the responses of SOD and CAT, the activity of GST, a multifaceted detoxifying and antioxidant enzyme was recorded to be inhibited in the brain of carbofuran treated rats; this could be either due to suicidal binding of GST to carbofuran, a mechanism employed by GST to remove toxic compounds from body (17) or feedback inhibition of the enzyme by the products of membrane lipid peroxidation. However, both hypotheses need further investigation.

The pretreatment of rats with *C. dactylon* or Vit C conserved the level of LPO to its level corresponding to that observed in control group as opposed to significant upsurge observed in the brain of carbofuran treated rats. It is important to note that, products of LPO exert cytotoxic effects observed in various neurodegenerative conditions (11). Presence of large amount of polyunsaturated fatty acids (PUFA), consumption of large amounts of oxygen per unit weight (about 20% of the total amount used in humans) and a relatively weak antioxidant defense system substantiate the aforementioned observation of the levels of LPO in the different treatment groups (8). The observed preventive effect of *C. dactylon* or Vit C in terms of LPO suggest a possible regenerative effect of these preparation

The most striking observation in the present study was *C dactylon* treatment driven amelioration of AChE activity impairment caused by carbofuran treatment in the of brain of rats treated with carbofuran. This suggests that *C dactylon* may directly confer protection against carbofuran excitotoxicity mediated oxidative stress in the brain. It is important to mention that similar observations have been made in a separate study in which the ethanol extracts of *C. dactylon* was found to cause significant rats from status epileptics (25); the author explains the presence of rich concentrations of brain biogenic amines in the plant to be responsible for the observed effect. The aforementioned observation is extremely pertinent in the context of present study as status epileptics happen to by far most obvious symptom of anticholinesterase toxicity (12). In another closely related study, butanolic extract of *P. argentea* (L.) protected rat fetuses form the toxicity of chloropyriphos-ethyl, another pesticide with similar mechanism of action (34).

In agreement with a large number of studies showing association between carbofuran exposure, AChE inhibition, oxidative stress, DNA damage and many other pathological implications etc. (5, 12, 23) as well as epidemiological data a very recent case report has presented similar observations in the aforementioned alterations in occupational applicators exposed to carbofuran (35). Therefore, in the light of experimental findings and earlier evidences, the present study clearly highlights the importance of exploitation of *C. dactylon* in developing nontoxic natural antidotes against carbofuran toxicity, which is mediated via cholinesterase inhibition and/or oxidative stress. The isolation, characterization and clinical validation of different fractions obtained from this extract may lead to identification of active principle ingredient of this plant extract against carbofuran intoxication.

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Other articles in this theme issue include references (36-51).

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