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Differential cholinesterase inhibition in the rat brain regions by dichlorvos and protective effect of *Decalepis hamiltonii* roots

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ABSTRACT

Dichlorvos (DDVP) causes neurotoxicity primarily by inhibiting cholinesterase (ChE) which is the characteristic feature of organophosphate pesticides. In this study, we found for the first time that DDVP shows differential inhibition of ChE (acetylcholinesterase + butyrylcholinesterase) in various rat brain regions. A single dose of DDVP (1/3 LD_{50}) after 16 h of treatment elicited ChE inhibition in the brain regions which was highest in striatum and lowest in cerebellum. The inhibition of ChE by DDVP has been shown to be accompanied with induction of oxidative stress. Further, we investigated the protective potential of the aqueous extract of the roots of *Decalepis hamiltonii* (DHA), having potent antioxidant constituents, against DDVP-induced ChE inhibition in various rat brain regions. Pretreatment of rats with multiple doses of DHA, 50 and 100 mg/kg b.w., for 7 consecutive days did not produce any significant change in ChE activity. Pretreatment of rats with DHA, at high dose, significantly protected against DDVP-induced ChE inhibition in all the brain regions except cerebellum. Pretreatment of rats with DHA, at low dose, showed significant protection in striatum, cortex, and pons against DDVP-induced ChE inhibition. The protective activity of DHA can be attributed to the characterized potent antioxidant constituents which could have an important role in preventing ChE inhibition by inducing the DDVP detoxifying enzymes. We strongly believe that these antioxidant constituents are prospective novel nutriceuticals.

1. Introduction

Dichlorvos (2,2′-dichlorovinyl dimethyl phosphate – DDVP), an organophosphorus compound (OPC), is a contact and stomach – acting insecticide with fumigant action (Kovacic, 2003). Non-target species including humans are frequently affected by OPCs throughout the world. OPCs inhibit ChE by either phosphorylating, phosphonylating, phosphoramidation, or phosphinylation of its active site, causing accumulation of acetylcholine (ACh) at synapses, which results in excessive stimulation of cholinergic receptors on postsynaptic cells, leading to cholinergic toxicity (Bajgar et al., 2007). Inhibition of brain ChE is generally regarded as a standard biochemical marker and most sensitive measure of organophosphate (OP) toxicity (Worek et al., 2005). It has been demonstrated that there is differential pattern of ChE inhibition in the brain regions of rat by bromophos and ethylbromophos (Santhoshkumar et al., 1996). It has been reported that OPCs may induce oxidative stress on acute exposure in humans and animals (Milatovic et al., 2006; Kaur et al., 2007). Lately it has been clearly shown that DDVP toxicity involves induction of oxidative stress apart from the characteristic inhibition of ChE (Soltaninejad and Abdollahi, 2009). Further, the treatment with antioxidants has been shown to reduce the toxicity of DDVP either by prevention of ChE inhibition and/or by speedy reactivation of the inhibited ChE (Pena-Llopis et al., 2003; Buyukokuroglu et al., 2008).

Tuberous roots of *Decalepis hamiltonii* (Wight and Arn.) (family: Asclepiadaceae) are traditionally consumed as pickles and juice for its alleged health promoting properties in Southern India. We have earlier shown that the roots of *D. hamiltonii* possess potent antioxidant properties and isolated/characterized the antioxidant constituents which could be associated with their alleged health benefits (Srivastava et al., 2006a,b, 2007). We have also shown that DHA protects against ethanol-induced neurotoxicity by ameliorating the oxidative stress (Srivastava and Shivanandappa, 2009a). In this study we have examined how different brain regions of the rat respond to DDVP toxicity in terms of ChE inhibition. Further, as inhibition of ChE by DDVP is linked with oxidative stress, we tested the hypothesis that DHA, having potent antioxidants, can reduce/prevent DDVP-induced ChE inhibition.

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2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (ATCI), 5.5′, -dithio-bis 2-nitrobenzoic acid (DTNB) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis MO, USA). Technical grade DDVP (dichlorvos; 2,2′-dichlorovinyl dimethyl phosphate) was obtained from India Medical Corporation (Mumbai, India). All other chemicals were purchased from Sisco Research Laboratories, Mumbai, India and were of highest purity grade available.

2.2. Preparation of the root powder and extraction

The roots of *D. hamiltonii* were collected from B.R. Hills, Karnataka, India and identity confirmed by a botany Professor at Mysore University. A voucher specimen was maintained in Botany department of Mysore University, Mysore, Karnataka, India.

Tuberous roots of *D. hamiltonii* were washed with water, followed by crushing with a roller to separate the inner woody core from the outer fleshy layer. The fleshy portion was collected, dried at 40 °C in a hot air oven and fine powdered. The powder was used for extraction. We have earlier reported that aqueous extract of *D. hamiltonii* shows high antioxidant activity among the different solvent extracts (Srivastava et al., 2006a,b). The aqueous extract was prepared by homogenizing the root powder in warm water (50 °C) and allowed to stand for 24 h, filtered with Whatman paper No. 1 and the filtrate was lyophilized and weighed (17% of the root powder). To quantify/characterize the extract composition total polyphenolic content was measured (13.8 mg/g extract) by the method of Singleton and Rossi (1965). Aqueous extract of *D. hamiltonii* was chosen for this study as it shows highest antioxidant activity among the different solvent extracts.

2.3. Animals and treatments

Sixty day old adult male Wistar rats (180–200 g) were divided into different groups, of eight each. The Institute Animal Ethics Committee guidelines were followed for the animal experiments. In a 90 days dietary study on rats it was established that the root extract of *D. hamiltonii* is safe to the mammalian system at the highest dose used in this study (unpublished data). Based on the preliminary experiments the dose of DHA was decided. Experiments on protective activity of the aqueous extract of the roots of *D. hamiltonii* were done with multiple dose (7 consecutive days) oral pretreatment by gavage at the doses of 50 and 100 mg/kg b.w. The toxicant was administered orally by gavage on the 7th day 1 h after the *D. hamiltonii* aqueous extract (DHA) administration. The dose of the toxicant was 1/3 LD_{50} of DDVP based on the MSDS sheet provided by the supplier. Animals were sacrificed by deep ether anesthesia and respiratory arrest under humane conditions after 16 h of toxicant administration. No visual signs of toxicity were observed. Exposure time was chosen based on earlier report demonstrating maximum ChE inhibition at 16 h after which it starts recovering (Santhoshkumar et al., 1996).

2.4. Experimental design and groupings

2.4.1. Groups

Group I – Control (Sunflower oil); Group II – DHA (100 mg/kg b.w. in saline) (7 days); Group III – DHA (50 mg/kg b.w.) (7 days) + DDVP (47 mg/kg b.w.); Group IV – DHA (100 mg/kg b.w.) (7 days) + DDVP (47 mg/kg b.w.); Group V – DDVP (47 mg/kg b.w.) in Sunflower oil.

2.5. Sample preparation

Rat brain was quickly removed, washed in ice-cold saline and blotted dry. Brain was placed on ice and different regions viz. cortex, striatum, cerebellum, pons, thalamus, hippocampus, and medulla were dissected out as described by Santhoshkumar et al. (1996). A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) using Potter-Elvehjem type homogenizer followed by centrifugation at 10,000 × *g* at 4 °C for 10 min in a Sorvall high speed refrigerated centrifuge and the supernatant was used for assaying ChE.

2.6. Cholinesterase assay

ChE activity was assayed spectrophotometrically by measuring the rate of hydrolysis of the substrate ATCI, according to the method of Ellman et al. (1961). The reaction mixture (3.0 ml) in 0.1 M phosphate buffer (pH 7.4) contained 25 μl of 10% (w/v) tissue homogenate, 0.33 mM DTNB and 0.5 mM ATCI at 37 °C; the reaction was started by the addition of substrate and change of absorbance at every 30 s for 5 min was followed at 412 nm in a UV-Vis spectrophotometer. Appropriate blanks were run in parallel to eliminate the effect of confounding DTNB-reactive substances in the tissue. The enzyme activity was calculated by the extinction coefficient ($E_{412} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$) and was expressed as nmol of ATCI hydrolyzed $min^{-1}\ mg^{-1}$ of protein.

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.7. Statistical analysis

The data are expressed as means ± S.E. of eight observations ($n = 8$). Statistical significance was determined using ANOVA and Newman–Keuls test as post hoc test. $p < 0.05$ was taken as significantly different.

3. Results

Results show differential ChE activity in the rat brain regions. The ChE activity in the brain regions was in the following order:

Table 1
Protective effect of *D. hamiltonii* root extract (aqueous) against DDVP-induced ChE inhibition in the brain regions of rat.

Group	ChE* activity in brain region						
	Hippocampus	Thalamus	Pons	Cortex	Medulla	Striatum	Cerebellum
I	$21.35^{d} \pm 1.98$	$41.23^{c} \pm 3.25$	$52.87^{d} \pm 4.28$	$21.42^{d} \pm 1.88$	$39.61^{c} \pm 2.93$	$92.33^{d} \pm 7.18$	$15.38^{b} \pm 1.33$
II	$21.22^{d} \pm 1.29$	$42.34^{c} \pm 4.12$	$51.67^{d} \pm 4.36$	$21.84^{d} \pm 1.74$	$39.24^{c} \pm 2.62$	$93.15^{d} \pm 8.24$	$15.22^{b} \pm 1.26$
III	$11.28^{c} \pm 1.02$	$33.17^{a} \pm 1.47$	$32.78^{b} \pm 2.91$	$14.18^{b} \pm 1.24$	$20.45^{a} \pm 1.76$	$51.24^{b} \pm 4.83$	$12.35^{a} \pm 1.09$
IV	$14.62^{b} \pm 1.33$	$35.34^{b} \pm 1.22$	$36.94^{c} \pm 3.41$	$16.09^{c} \pm 1.32$	$26.39^{b} \pm 2.18$	$64.33^{c} \pm 4.11$	$12.72^{a} \pm 1.07$
V	$9.39^{a} \pm 0.74$	$31.75^{a} \pm 2.54$	$28.54^{a} \pm 2.16$	$12.42^{a} \pm 1.03$	$18.65^{a} \pm 1.43$	$38.77^{a} \pm 3.29$	$11.99^{a} \pm 1.07$

Group: I – Control; II – DHA (100 mg/kg bw); III – DHA (50 mg/kg bw) + DDVP (47 mg/kg bw); IV – DHA (100 mg/kg bw) + DDVP (47 mg/kg bw); V – DDVP (47 mg/kg bw). Different superscript letters indicate that the means differ significantly ($p < 0.05$).

* nmol ATCI hydrolysed $min^{-1}\ mg^{-1}$ protein.

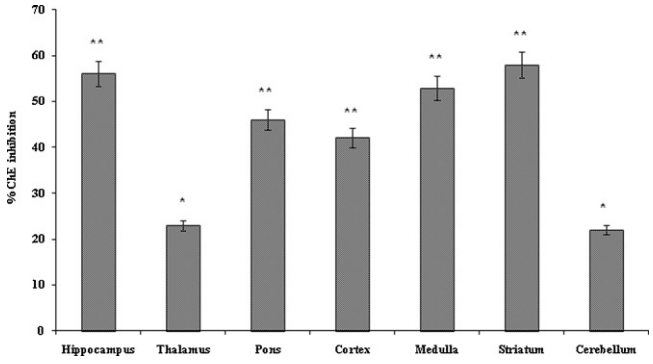

Fig. 1. Differential inhibition of ChE in rat brain regions by acute dose of DDVP (47 mg/kg). * and ** indicate significance at $p < 0.05$ and $p < 0.01$ level between control and DDVP treated groups.

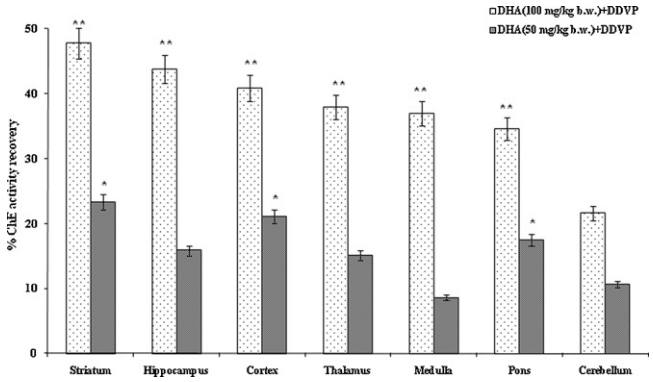

Fig. 2. Protective effect of aqueous extract of *Decalepis hamiltonii* roots (pretreatment) against DDVP-induced ChE inhibition in the rat brain regions. % ChE activity recovery was calculated by comparing with DDVP induced inhibition. * and ** indicate significance at $p < 0.05$ and $p < 0.01$ level between DDVP and DHA + DDVP treated groups.

striatum > pons > thalamus > medulla > cortex > hippocampus > cerebellum (Table 1). The regional ChE activity levels are similar to those reported earlier for a different rat strain (Hammond et al., 1994). A single dose of DDVP (1/3 LD_{50}) after 16 h elicited differential ChE inhibition in the brain regions which ranged from 58% (striatum) to 22% (cerebellum) and the inhibition was in the following order: striatum > hippocampus > medulla > pons > cortex > thalamus > cerebellum (Fig. 1 and Table 1). In this study we are showing for the first time the differential ChE inhibition pattern by acute dose of DDVP in major brain regions of rat. Further, the ChE inhibition by DDVP could not be correlated to its activity levels in the respective brain region. The ChE inhibition pattern was distinctly different from that of bromophos and ethylbromophos as reported earlier (Santhoshkumar et al., 1996).

Administration of DHA alone did not produce any significant change in ChE activity (Table 1). Pretreatment of rats with DHA, at high dose, provided significant protection against DDVP-induced ChE inhibition in all the brain regions except cerebellum which was in the following order: striatum (48%) > hippocampus (44%) > cortex (41%) > thalamus (38%) > medulla (37%) > pons (35%). Pretreatment of rats with DHA, at low dose, showed significant protection in striatum (23%), cortex (21%), and pons (17%) only (Fig. 2 and Table 1). The data clearly demonstrates role of DHA in preventing ChE inhibition and/or speedy rate of its reactivation.

4. Discussion

OP compounds (OPCs) are recognized for their ability to induce toxicity in mammals through inhibition of ChE, leading to accumulation of acetylcholine and subsequent activation of cholinergic, muscarinic and nicotinic receptors (Kobayashi et al., 2007). In this study, we found that DDVP shows differential inhibition of ChE in various brain regions. This is the first report on the ChE inhibition pattern by acute dose of DDVP in different brain regions of rat. Santhoshkumar et al. (1996) reported a regional variation in ChE inhibition with bromophos and ethylbromophos. Differential inhibition of ChE in the brain regions may have implications in the pattern of neurotoxic effects produced by OPCs. The degree of enzyme inhibition and the rate of recovery in the brain regions could be important in the time course of neurotoxic symptoms. In this study ChE activity was assayed after 16 h DDVP exposure as this time point has been demonstrated to elicit peak inhibitory effect after which ChE starts to recover (Shivanandappa et al., 1988; Santhoshkumar and Shivanandappa, 1994).

However, it is difficult to explain the neurotoxicity based on ChE inhibition alone. Many reports indicate that OPCs cause inhibition of ChE as well as induce oxidative stress marked by increased lipid peroxidation, altered antioxidant enzyme activities, and reduced glutathione (GSH) (Ranjbar et al., 2002; Shadnia et al., 2005; Lopez et al., 2007). The role of oxidative stress is underlined by the fact that antioxidants have been shown to prevent/ameliorate DDVP toxicity (Suke et al., 2008). In this study we observed amelioration of DDVP induced ChE inhibition by DHA, which could be due to the high antioxidant activity of its constituents. Green tea extract, which is rich in antioxidants, has also been shown to protect OPC toxicity (Khan and Koura, 2007). It is reported that antioxidants like phenyl-N-tert-butylnitrone (PBN), vitamin E, and N-acetyl-L-cysteine (NAC) abolish both ChE inhibition and oxidative stress induced by DDVP (Gupta, 2004). Pena-Llopis et al. (2003) reported that NAC increased the tolerance levels towards DDVP toxicity and increased the survival time in European eels. Further, it has been shown that NAC increased mice survival rates against fenthion, an OPC, induced acute toxicity.

The protective effect of antioxidants can be understood by considering the metabolism of OPCs. Organophosphates are detoxified principally by glutathione-S-transferases (GST), esterases, and Cytochrome P450s (Fujioka and Casida, 2007). Glutathione dependent demethylation of DDVP by GST is the main metabolic pathway of DDVP in the rat liver (Dicowsky and Morello, 1971). It has been shown that plant extracts enhance the antioxidant profile by interacting with the antioxidant response elements (AREs) that transcriptionally regulate the genes related to antioxidant enzymes, including GST (Ferguson, 2001). It has also been shown that the γ-glutamylcystein synthetase (γ-GCS), a key enzyme in the glutathione synthesis, is also transcriptionally regulated by AREs (Moskaug et al., 2005). We have recently reported that DHA enhances the antioxidant profile of rat liver and brain (Srivastava and Shivanandappa, 2009b). DHA raises the *in vivo* levels of GST and GSH which could be instrumental in the metabolic detoxication of DDVP or its metabolites.

The OP-induced oxidative damage can be attenuated through the use of antioxidants which can be used complimentarily with current methods of treatments for OP toxicity (Cankayali et al., 2005). The aqueous extract of *D. hamiltonii* roots contains potent antioxidants (activity guided purification) namely; 4-hydroxy isophthalic acid, 4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6,2′,6′-dilactone, 14-aminotetradecanoic acid, 4-(1-hydroxy-1-methylethyl)-1-methyl-1,2-cyclohexane diol, 2-hydroxymethyl-3-methoxybenzaldehyde, 2,4,8 trihydroxybicyclo [3.2.1]octan-3-one (Srivastava et al., 2006b, 2007). This study provides evidence that antioxidant rich plant extract offers protection against OP-induced neurotoxicity as evident from reduced ChE inhibition. The exact mechanism of prevention is not clear but could involve mitigation of oxidative stress as well as the boosting of the antioxidant defenses including GSH. The antioxidant compounds isolated are

potential novel nutriceuticals and need further experimental investigation for their use as therapeutic agents for treating neurodegenerative diseases involving oxidative stress.

Conflict of interest

None.

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References

Bajgar J, Fusek J, Kuca K, Bartosova L, Jun D. Treatment of organophosphate. Intoxication using cholinestrase reactivators: facts and fiction. Mini Rev Med Chem 2007;7:461–6.

Buyukokuroglu ME, Cemek M, Yurumez Y, Yavuz Y, Aslan A. Antioxidative role of melatonin in organophosphate toxicity in rats. Cell Biol Toxicol 2008;24:151–8.

Cankayali I, Demirag K, Eris O, Ersoz B, Moral AR. The effects of N-acetylcysteine on oxidative stress in organophosphate poisoning model. Adv Ther 2005;22:107–16.

Dicowsky L, Morello A. Glutathione-dependent degradation of 2,2 dichlorovinyl dimethyl phosphate (DDVP) by the rat. Life Sci 1971;10:1031–7.

Ellman GL, Courtney KD, Andres V, Feather-Stone RM. A new and rapid colorimetric determination of cholinestrase activity. Biochem Pharmacol 1961;7:88–95.

Ferguson LR. Role of plant polyphenols in genomic stability. Mutat Res 2001;475: 89–111.

Fujioka K, Casida JE. Glutathione S-transferase conjugation of organophosphorus pesticides yields S-phospho-S-aryl-, and S-alkylglutathione derivatives. Chem Res Toxicol 2007;20:1211–7.

Gupta RC. Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. Toxicol Mech Methods 2004;14:103–43.

Hammond P, Rao R, Koenigsberger C, Brimijoin S. Regional variation in expression of acetylchlinestrase mRNA in adult rat brain analyzed by *in situ* hybridization. Proc Natl Acad Sci U S A 1994;91:10933–7.

Kaur P, Radotra B, Minz RW, Gill KD. Impaired mitochondrial energy metabolism and neuronal apoptotic cell death after chronic dichlorvos (OP) exposure in rat brain. Neurotoxicology 2007;28:1208–19.

Khan SM, Koura G. Subacute oral toxicity of chlorpyriphos and protective effect of green tea extract. Pest Biochem Physiol 2007;89:118–23.

Kobayashi H, Suzuki T, Sakamoto M, Hashimoto W, Kashiwada K, Sato I, et al. Brain regional cholinestrase activity and muscarinic acetylcholine receptors in rats after repeated administration of cholinestrase inhibitors and its withdrawal. Toxicol Appl Pharmacol 2007;219:151–61.

Kovacic P. Mechanism of organophosphates (nerve gases and pesticides) and antidotes: electron transfer and oxidative stress. Curr Med Chem 2003;10:2705–9.

Lopez O, Hernandez AF, Rodrigo L, Gil F, Pena G, Serrano JL, et al. Changes in antioxidant enzymes in humans with long-term exposure to pesticides. Toxicol Lett 2007;171:146–53.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;13:265–75.

Milatovic D, Gupta RC, Aschner M. Anticholinestrase toxicity oxidative stress. Sci World J 2006;6:295–310.

Moskaug JO, Carlsen H, Myhrstad MC, Blomhoff R. Polyphenols and glutathione synthesis regulation. Am J Clin Nutr 2005;81:277S–83S.

Pena-Llopis S, Ferrando MD, Pena JB. Increased recovery of brain cholinestrase activity in dichlorvos-intoxicated European eels *Anguilla anguilla* by bath treatment with N-acetylcysteine. Dis Aquat Organ 2003;55:237–45.

Ranjbar A, Pasalar P, Abdollahi M. Induction of oxidative stress and acetylcholinestrase inhibition in organophosphorous pesticide manufacturing workers. Hum Exp Toxicol 2002;21:179–82.

Santhoshkumar P, Shivanandappa T. Differential in vivo inhibition of the foetal and maternal brain acetylcholinestrase by bromophos in the rat. Neurotoxicol Teratol 1994;16:227–32.

Santhoshkumar P, Karanth S, Shivanandappa T. Neurotoxicity and pattern of cholinestrase inhibition in the brain regions of rat by bromophos and ethylbromophos. Fundam Appl Toxicol 1996;32:23–30.

Shadnia S, Azizi E, Hosseini R, Khoei S, Fouladdel S, Pajoumand A, et al. Evaluation of oxidative stress and genotoxicity in organophosphorus insecticide formulators. Hum Exp Toxicol 2005;24:439–45.

Shivanandappa T, Joseph P, Krishnakumari MK. Response of blood and brain cholinesterase to dermal exposure of bromophos in the rat. Toxicology 1988;48:199–208.

Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. Am J Enol Viticult 1965;16:144–58.

Soltaninejad K, Abdollahi M. Current opinion on the science of organophosphate pesticides and toxic stress: a systematic review. Med Sci Monit 2009;15:RA75–90.

Srivastava A, Shivanandappa T. Neuroprotective effect of *Decalepis hamiltonii* roots against ethanol-induced oxidative stress. Food Chem 2009a;119:626–9.

Srivastava A, Shivanandappa T. *Decalepis hamiltonii* roots boost antioxidant status of rat liver and brain. J Sci Food Chem 2009b;14:2461–6.

Srivastava A, Shereen. Harish R, Shivanandappa T. Antioxidant activity of the roots of *Decalepis hamiltonii* (Wight & Arn.). LWT-Food Sci Technol 2006a;39:1059–65.

Srivastava A, Harish R, Shivanandappa T. Novel antioxidant compounds from the aqueous extract of the roots of *Decalepis hamiltonii* (Wight and Arn.) and their inhibitory effect on low-density lipoprotein oxidation. J Agric Food Chem 2006b;54:790–5.

Srivastava A, Rao LJM, Shivanandappa T. Isolation of ellagic acid from the aqueous extract of the roots of *Decalepis hamiltonii*: antioxidant activity and cytoprotective effect. Food Chem 2007;103:224–33.

Suke SG, Ahmed RS, Pathak R, Tripathi AK, Banerjee BD. Attenuation of phosphamidon-induced oxidative stress and immune dysfunction in rats treated with N-acetylcysteine. Braz J Med Biol Res 2008;41:765–8.

Worek F, Koller M, Thiermann H, Szinicz L. Diagnostic aspects of organophosphate poisoning. Toxicology 2005;214:182–9.