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International Journal of Current Research
Vol. 6, Issue, 10, pp.9006--9013, October, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

AMELIORATIVE POTENTIAL OF CERTAIN ANTIOXIDANTS ON ENODSULFAN INDUCED NEUROTOXICITY ON CHOLINESTERASES IN THE OLFACTORY LOBE OF MICE

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ARTICLE INFO

Article History:
Received 14th July, 2014
Received in revised form
09th August, 2014
Accepted 24th September, 2014
Published online 25th October, 2014

Key words:

Endosulfan,
Neurotoxicity,
Antioxidants,
Olfactory lobe,
Neurotransmitter enzymes.

ABSTRACT

Endosulfan, an organochlorine pesticide had been in global use as a part of pest management strategy. Its exposure has resulted in multifaceted damage to various organs including brain. Hence, there is an urgent need to seek out certain herbal, biochemical and pharmaceutical formulations with an innate potential to curb toxicities induced by endosulfan. In the present study, an effort has been made to elucidate the protective effects of antioxidants viz., trans- resveratrol, alpha- lipoic acid and vitamin E against endosulfan induced neurotoxicity in olfactory lobe of the brain of Swiss albino mice on the basis of altered histochemical localization of acetylcholinesterase (AChE), butyrylcholinesterase (BChE). The experimental protocol constituted of ten batches, consisting of six mice each. First group served as the control where mice were administered only vehicle (olive oil). The second group were administered only endosulfan (2.45 mg/kg body weight/day for 15 days) while the third, fifth, seventh and ninth experimental groups were administered only resveratrol (5 mg/kg body weight/day for 15 days), alpha- lipoic acid (20 mg/kg body weight day for 15 days), only vitamin E (50 mg/kg body weight/day for 15 days) solitary and in combination. The fourth, sixth, eighth and tenth experimental groups were administered antioxidants in solitary and combination form one hour prior to endosulfan administration. As compared to the control group, it was observed that exposure of endosulfan at a dose of 2.45 mg/kg body weight/day distinctly altered the distribution pattern of acetylcholinesterase and butyrylcholinesterase neurotransmitter enzymes, thereby delineating its toxic effects on the mice brain. The only antioxidants treated groups i.e. group III, V, VII and VIII, showed an enzymatic distribution profile similar to that of control group. In other antioxidant plus endosulfan treated groups i.e. group IV,VI,VII,X it was observed that endosulfan induced AChE, BChE inhibition in different layers of olfactory lobe involved in mapping of odour and receiving information from axons of olfactory receptor nucleus were ameliorated and brought back to normal. Hence, this study clearly delineates toxic effects of endosulfan on the olfactory lobe of mice which were ameliorated on administration of antioxidants.

INTRODUCTION

Endosulfan, a chlorinated cyclodiene pesticide has been reported to influence the distribution pattern of neurotransmitter enzymes present in the brain. Central nervous system affectations have been the hallmark of endosulfan exposure in experimental animals. Its exposure and associated accumulation in brain has been co-related with its ability to cross the blood brain barrier where it further causes functional mutilations (Chan *et al*., 2006). Acetylcholinesterase, the major excitatory neurotransmitter enzyme in the peripheral nervous system plays an important physiological role in neurotransmission (Nachmansohn and Neumann, 1975 and Lu *et al.*, 2012). Butyrylcholinesterases on the other hand, possess unique enzymatic properties and is widely distributed in the nervous system, pointing to its possible involvement in neural function (Çokugras, 2003 and Darvesh *et al*., 2003). Hence, in the present study effect of endosulfan and antioxidants on the cholinesterases has been investigated. To sustain the normal functioning of brain different antioxidants as dietary supplements might prove beneficial. Resveratrol, the active ingredient in red grapes, peanuts, berries, and several other food plants has been reported to provide neuroprotection against ischemia, seizure, and neurodegenerative disorders (Karuppagounder *et al*., 2009; Cuzzola *et al*., 2011 and Li *et al*., 2012).

Alpha-lipoic acid, found in a variety of foods, notably kidney, heart and liver meats as well as spinach, yeast, broccoli and potatoes have beneficial effect against the severity of CNS disorders (Packer *et al.*, 1995; Lynch, 2001; Munch *et al*., 2011).

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Vitamin E is a potent lipid soluble antioxidant, found in green leafy vegetables, lean meats, poultry, fishes, beans, eggs, nuts, vegetable oils, whole grains and fortified cereals has vital role against Parkinson's and Alzheimer's disease (Etminan *et al*., 2005 and Shen and Ji, 2012). The study pertaining to histochemical localization of all these neurotransmitter enzymes is of prime importance as different studies related to their biochemical, haematological and other aspects have been conducted earlier however, there is lack of substantial data regarding the qualitative distributional pattern of these enzymes. The findings of present investigation clearly demarcate the distribution of these enzymes in olfactory region of brain under the impact of endosulfan and antioxidants, administered in combination and solitarily along with and without endosulfan respectively. Hence, in the present investigation resveratrol, alpha lipoic acid and vitamin E have been considered to check and control the hazardous potential of endosulfan.

MATERIALS AND METHODS

Test Chemical and Antioxidants

The neurotoxicity was induced by using technical grade endosulfan of 99% purity (CAS No 115-29-7) obtained from Shree Pesticides Pvt. Ltd., Udaipur (Rajasthan, India). The antioxidants vitamin E and alpha- lipoic acid were purchased from Hi Media, India. Stilbene trans- resveratrol was procured from Cayman, USA. Acetyl thiocholine iodide, butyryl thiocholine iodide was obtained from Hi Media, India. All the other chemicals used in the present investigation were of analytical grade.

Animal models

Healthy, male, adult Swiss albino mice, *7*- 8 weeks old and weighing 28±7 gm were maintained in plastic cages, bedded with sterilized rice husk in a well ventilated room at 27± 2°C temperatures with relative humidity of 50- 55% and 12±1 hour's dark and light phase. They were fed with a standard diet and were given *ad libitum* access to water. This experimental study was approved by the Institutional Animal Ethics Committee (approval no. 1098/AC/07/CPCSEA). All the doses of antioxidants and endosulfan were prepared by dissolving in olive oil and were given orally by gastric gavage method. All the doses of antioxidants were administered 1 hour prior to endosulfan administration.

Experimental design

The mice were divided into ten groups, minimum of 6 mice per group, and their dose protocol was as follows:

a) Group I: Control group mice which were administered olive oil as a vehicle per day for 15 days
b) Group II: Experimental group mice which were administered endosulfan (2.45 mg/kg body weight/day for 15 days)
c) Group III: Experimental group mice which were administered resveratrol (5 mg/kg body weight/day for 15 days)
d) Group IV: Experimental group mice which were administered endosulfan (2.45 mg/kg body weight/day for 15 days) and resveratrol (5 kg/kg body weight /day for 15 days)
e) Group V: Experimental group mice which were administered alpha- lipoic acid (20 mg/kg body weight day for 15 days)
f) Group VI: Experimental group mice which were administered endosulfan (2.45 mg/kg body weight/day for 15 days) and Alpha- lipoic acid (20 mg/kg body weight/day for 15 days)
Group VII: Experimental group mice which were administered Vitamin E (50 mg/kg body weight/day for 15 days)
h) Group VIII: Experimental group mice which were administered Endosulfan (2.45 mg/kg body weight/day for 15 days) and Vitamin E (50 mg/kg body weight for 15 days)
i) Group IX: Experimental group mice which were administered Resveratrol (5 mg/kg body weight/day for 15 days), Alpha- lipoic acid (20 mg/kg body weight/day) and Vitamin E (50 mg/kg body weight/day for 15 days)
j) Group X: Experimental group mice which were administered Endosulfan along with Resveratrol (5 mg/kg body weight/day for 15 days), Alpha- lipoic acid (20 mg/kg weight/day for 15 days) and Vitamin E (50 mg/kg body weight/day for 15 days)

On completion of dose administration, the animals were killed by cervical dislocation. Cranium was opened to expose the brain.

Histochemical Localization of AChE and BChE

After 24 hours of administration of last dose, the animals were sacrificed by cervical dislocation, and olfactory lobe was immediately removed and fixed in chilled calcium formol (40 C) for 18-20 h in a refrigerator. Sections (15 µ) were cut on the cryostat and were processed for histoenzymological localization as per the method of Karnovsky and Roots (1964).

RESULTS

In the present study, distribution profile of AChE and BChE activities in the different layers of olfactory lobe was observed. In the control group, AChE activity was lacking in the non medullated nerve fibers i.e. PFL (peripheral fibrillar layer). On the other hand, GL (glomerular layer) rich in concentrated synaptic contacts revealed strong positive activity. Deep staining was also observed in interglomerular fibers. The tufted cells of EP (ependymal) layer were strongly stained while EP itself showed mild AChE activity. Intense activity was seen in the ML (mitral cell layer). Moderate activity was observed in the fibrous IP layer. The GRL (granular cell layer) which is made up of gra nule cells revealed mild to moderate activity (Plate 1; Fig.1; Table 1).

Each layer of olfactory lobe treated with endosulfan showed considerable reduction in the AChE enzyme reaction (Plate 1; Fig.2; Table 1). It was found that the mice pre -treated with antioxidants in endosulfan treated groups prevented inhibition of AChE activity quite similar to that of control group (Plate 1; Fig.4,6,8, and 10; Table 1).

Table 1. Effect of endosulfan and antioxidants on the distribution of ache in different layers of the olfactory lobe

Olfactory Lobe Layers	Groups									
	C	E	R	R+E	L	L+E	V	V+E	R+L+V	R+L+V+E
Peripheral Fibrillar Layer (PFL)	- -	- -	+ -	±	+ -	+ + -	±	- -	+ -	±/- -
Glomerular Layer (GL)	+ + + +	+ -	+ + +	+ + + +	+ + -	+ + -	+ + +	+ + -	+ + +	+ + -
Ependymal Layer (EPL)	+ + -	+ -	+ + -	+ -	+ + +	+ + -	+ + -	+ -	+ + -	+ + -
Mitral Cell Layer (ML)	+ + +	+ -	+ + +	+ + +	+ + -	+ + +	+ + + +	+ + -	+ + + +	+ + +
Internal Plexiform Layer (IPL)	+ + +	+ -/±	+ + +	+ + -	+ + -	+ + +	+ + -	+ + -	+ + +	+ + -
Granular Cell Layer (GRL)	+ + -	+ -	+ + +	+ -	+ + -		+ + +	+ -	+ + +	+ + -

Histoenzymological index taken for enzymatic activity was as follows:
+ + + + (very strong) > + + + (strong) > + + - (moderate) > + - (mild) > ± (negligible) > - - (no activity)

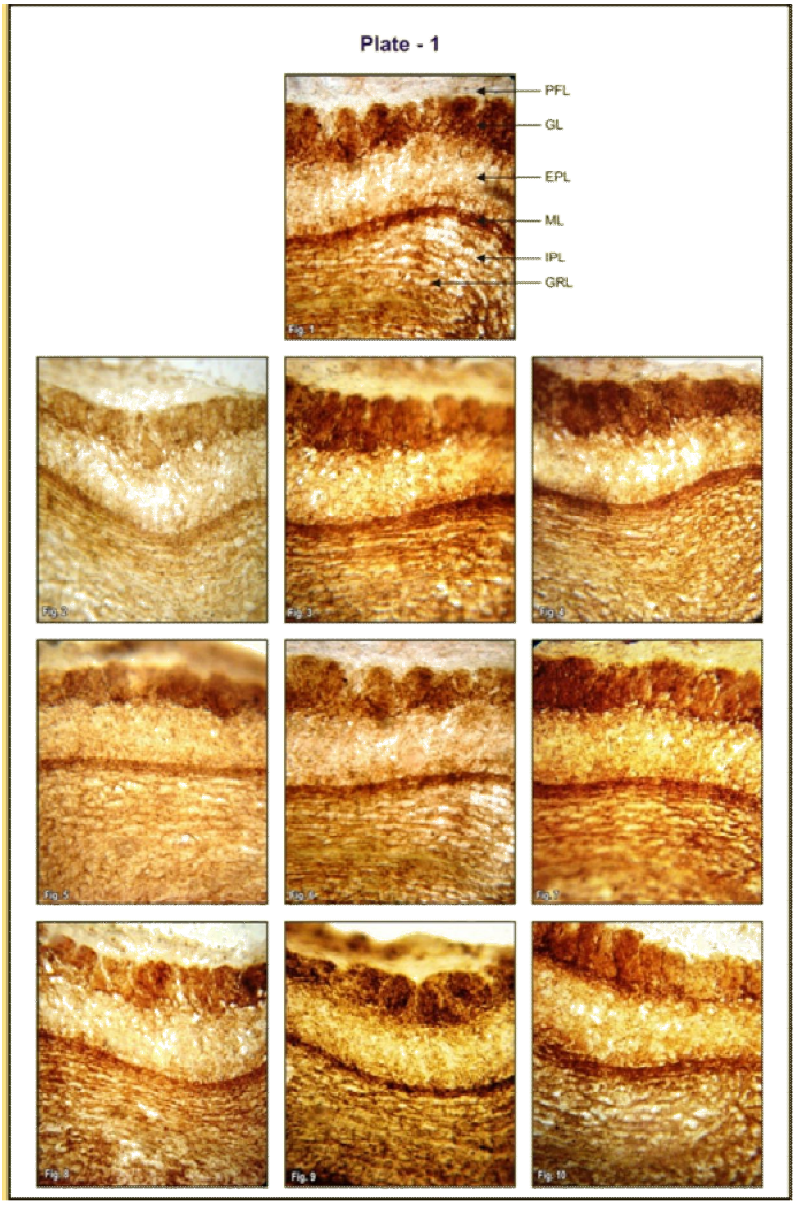

Plate 1. Histochemical distribution of ache activity in different layers of olfactory lobe (100 x)

Fig: 1. Control group showing absence of AChE activity in the non medullated nerve fibers i.e. PFL (Peripheral Fibrillar Layer). Very strong activity was observed in GL (Glomerular Layer). Moderate activity was observed in GRL (Granular Cell Layer), IPL (Internal Plexiform Layer) and EL (Ependymal Layer). Intense activity was observed in ML (Mitral Cell Layer).

Fig: 2. Endosulfan treated experimental group showing markedly reduced AChE activity in each layer with maximum reduction in EPL and GRL.

Fig: 3, 5, 7 and 9. Resveratrol, alpha- lipoic acid and vitamin E solitary and combined treated experimental groups showing mild AChE activity in PFL and strong activity in GL and ML.

Fig: 4, 6, 8 and 10. Resveratrol plus endosulfan, alpha- lipoic acid plus endosulfan, vitamin E plus endosulfan and combined antioxidants plus endosulfan treated experimental groups showing amelioration in AChE activity in EPL and GRL.

The purely antioxidants treated groups administered solitary and in combination revealed elevated AChE activity in PFL, which was unstained in other groups. Maximum elevation in AChE activity in all the layers was seen in Group 9 collectively treated with resveratrol, alpha-lipoic acid and vitamin E (Plate 1; Fig. 3, 5, 7, 9; Table 1). BChE activity was negligible in the non medullated nerve fibres i.e. PFL layer of control group. On the other hand, GL rich in concentrated synaptic contacts revealed very strong positive activity. Deep staining was also observed in interglomerular fibres.

The EP layer was moderately stained. Intense activity was seen in the ML. Moderate activity was observed in the fibrous IP layer. The GRL which is made up of granule cells revealed strong activity (Plate 2; Fig.1; Table 2). In the present study, each layer of olfactory lobe treated with endosulfan showed considerable reduction in the BChE enzyme reaction (Plate 2; Fig.2; Table 2). It was found that the mice pre -treated with antioxidants in endosulfan treated groups prevented inhibition of BChE activity quite similar to that of control group (Plate 2; Fig.4,6,8,and 10; Table 2).

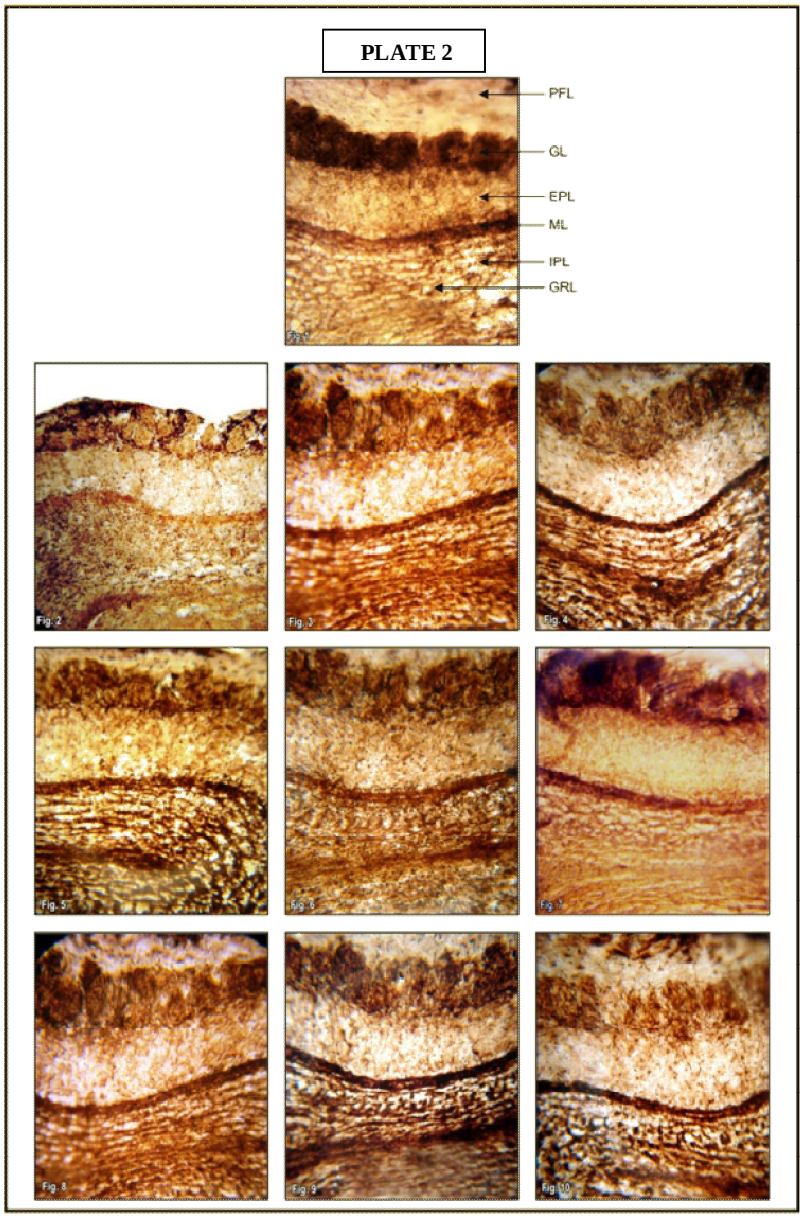

Plate 2. Histochemical distribution of BChE activity in different layers of olfactory lobe (100 x)

Fig: 1. Control group showing mild to negligible of BChE activity in the non medullated nerve fibers i.e. PFL (Peripheral Fibrillar Layer). Very strong activity was observed in GL (Glomerular Layer). Moderate activity was observed in GRL (Granular Cell Layer), IPL (Internal Plexiform Layer) and EL (Ependymal Layer). Strong activity was observed in ML (Mitral Cell Layer).

Fig: 2. Endosulfan treated experimental group showing markedly reduced BChE activity in each layer with maximum reduction in EPL and GRL.

Fig: 3, 5, 7 and 9. Resveratrol showed moderate activity in PFL. Alpha- lipoic acid showed strong. Vitamin E and combined treated experimental groups showed mild BChE activity in PFL. Strong activity in GL, ML was observed in all the groups. Strong to moderate activity was observed in EPL.

Fig: 4, 6, 8 and 10. Resveratrol plus endosulfan, alpha- lipoic acid plus endosulfan, vitamin E plus endosulfan and combined antioxidants plus endosulfan treated experimental groups showing amelioration in BChE activity in EPL and GRL.

The purely antioxidants treated groups administered solitary and in combination revealed elevated BChE activity in PFL, which was unstained in other groups. Maximum elevation in BChE activity in all the layers was seen in Group 9 collectively treated with resveratrol, alpha-lipoic acid and vitamin E (Plate 2; Fig. 3, 5, 7, 9; Table 2).

Table 2. Effect of endosulfan and antioxidants on the distribution of BChE in different layers of the olfactory lobe

Olfactory Lobe Layers	Groups									
	C	E	R	R+E	L	L+E	V	V+E	R+L+V	R+L+V+E
Peripheral Fibrillar Layer (PFL)	±/ + -	- -	++ -	+ + -	+++	+ + -	+ -	+ + +	+ -	+ + -
Glomerular Layer (GL)	++++	++ -	+++	+++	+ ++	++ +	++++	+ ++	+++	++-
Ependymal Layer (EPL)	++ -	+ -/±	+++	++ -	++ -	+ -	++ -	++ -	+ + -	+ -
Mitral Cell Layer (ML)	+++	+-/±	+++	+++	++++	+++	+++	+++	++++	+++
Internal Plexiform Layer (IPL)	++-	+-	+++	++-	++++	+++	++-	+++	++++	+++
Granular Cell Layer (GRL)	+++	++-/±	+++	++-	++++		++-	+++	++++	+++

Histoenzymological index taken for enzymatic activity was as follows:
++++ (very strong activity) > +++ (strong) > + + - (moderate) > + - (mild) > ± (negligible) > - (no activity

DISCUSSION

In the present investigation, an attempt was made to evaluate the protective role of antioxidants viz., trans-resveratrol, alpha –lipoic acid and vitamin E against neurotoxic effects induced by endosulfan in the olfactory lobe of Swiss albino mice. The results and observations of the present study significantly depicted the adverse effects of endosulfan as manifested by alterations in the histoenzymological distribution of cholinesterases. Endosulfan, an organochlorine pesticide has been reported to influence the distribution pattern of neurotransmitter enzymes present in the brain. There are several reports establishing the role of endosulfan in inhibiting the release of neurotransmitters like cholines, adenosine, GABA and serotonin present in the brain (Gupta, 1976; Cole and Casida, 1986; Ansari *et al*., 1987; Lakshmana and Raju, 1994; Paul *et al.* 1994; Pomes *et al.*, 1994; Paul *et al.*, 1995; Rosa *et al.*, 1996; Markey *et al.*, 2007 and Yu, 2008). The neuroprotection rendered by the antioxidants resveratrol, alpha-lipoic acid and vitamin E did not allowed the pesticide to cause disturbances and alterations in the histochemical and biochemical profile of these enzymes, which was rather similar to that of control group.

As cholinesterase's (ChE's) are also found to be localized in blood brain barrier, any alterations in the permeability of blood brain barrier can manipulate the activity of cholinesterase's (Sinha and Shukla, 2003). Olfactory system has been associated with choice of mate and food localization (Brennan and Zufall, 2006; Pifferi and Menini, 2011). In the present study, it was observed that endosulfan exposure led to significant variations in the distributional pattern of AChE activity. Substantial data suggest a correlation between AChE and these functions, therefore, any disruption in the cholinergic system results into mutilation of various functions associated with olfactory lobe. A reduction in cholinergic enzyme activity (both AChE and BChE) was observed in the present study on endosulfan intoxication in different layers of mice olfactory lobe, which suggests that organochlorine pesticides deteriorate the normal functioning of CNS. These observations are in concurrence with the findings of earlier reports which indicate the possible role of AChE in neurotransmitter system and its involvement in olfaction, learning, memory and mood processes etc (Nair *et al*., 2004; Hasselmo, 2006 and Miwa *et al*., 2011). There has been paucity of related information pertaining to the endosulfan induced variations in the distributional pattern of AChE and BChE activity in olfactory lobe of brain. However, numerous other biochemical studies have been conducted on endosulfan induced alterations in the levels of neurotransmitter enzymes which are in concurrence with the results of present investigation. These studies significantly show a decline in AChE levels (Gupta 1976; Anand *et al*., 1980a; Kushwah and Dikshith, 1981; Banerjee *et al*., 1999). Kiran and Varma, (1988) and Naqvi and Vaishnavi (1993) reported endosulfan linked decline in acetylcholinesterase which adversely affect central nervous system. Assis *et al.* (2011) also investigated the *in vivo* and *in vitro* effects of the pesticide endosulfan on the cholinesterase (ChE) activity in the rats. Besides all these, Dutta and Arends, (2003); Sarma *et al*. (2009) and Pereira *et al.* (2012) have also observed similar results in fishes. The results of all the above mentioned research studies are in concurrence with present investigation which clearly delineates the hazardous potential of endosulfan with an ability to inhibit the AChE activity.

Butyrylcholinesterase, is primarily localized in the white matter of the central nervous system. It is a non-specific cholinesterase enzyme involved in neural function. It is has also been reported to serve as an important tool to monitor the toxic effects of pesticides (Stefanidou *et al*., 2009). As expected, in endosulfan treated experimental group, lower BChE activity was observed in the regions of white matter viz., PFL, GL, EPL and ML of olfactory lobe. Although detailed histoenzymological studies on BChE activity has been lacking in the literature but the result of present study correlates with those of Bano and Bhatt (2007) and Bist and Bhatt (2009 and 2010) in which reduced BChE was observed due to lindane induced neurotoxic effects. Reduction in plasma BChE has also been reported in many studies on exposure of farm workers to endosulfan and other pesticides (Khan *et al.*, 2008; Rastogi *et al*., 2008 and Pathak *et al.*, 2011). Attademo *et al.* (2011) also reported intoxication of endosulfan in form of reduced BChE levels in the blood plasma of frog *Leptodactylus chaquensis.* Antioxidants have been reported to safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. These are intimately involved in the prevention of cellular damage. They have been reported to protect neurons against damage and many neurological disorders including Alzheimer's, Parkinson's and neuropathy etc. Therefore, antioxidants such as trans-resveratrol, alpha-lipoic acid and vitamin E were used in the present study to counterbalance the hazardous effects of endosulfan. It was

observed that all these antioxidants were able to render protection against endosulfan induced alterations in the distributional profile of neurotransmitter enzymes. The altered activities of AChE and BChE on endosulfan administration were modulated and brought back to normal profile quite similar to that of control on administration of antioxidants. It is thus assumed that application of these antioxidants as probable preventive agents could be targeted in therapeutic amelioration of endosulfan induced neurological abnormalities.

Many scientists have reported the neuroprotective potential of resveratrol, among which the reports of Raval *et al.* (2008), Johnston *et al*. (2006); Yáñez *et al*. (2006); Zhang *et al.* (2010), Marambaud *et al*. (2005); Gupta *et al*. (2012); Kennedy *et al*. (2012) and Miranda *et al*. (2012) substantiate the beneficial properties of resveratrol in rendering protection against various neurological disorders including brain ischemia, seizures, and Parkinson's disease, Huntington's disease, Alzheimer's disease and strokes etc. Similarly, several studies of Packer *et al.* (1997); Hagen *et al.* (1999); Packer *et al*. (2001); Yilmaz *et al.* (2002) Wollin and Jones, (2003); Akkas *et al*. (2007) and Hegazy and Ali, (2011); have revealed protective effects of lipoic acid in aging, diabetes mellitus and vascular and neurodegenerative diseases, all in which free radicals are involved. This study also showed that vitamin E moderately attenuated the toxic manifestations of endosulfan. Vitamin E has been proven to encompass antioxidant properties and has an important role in protecting biological systems (Azman *et al*., 2001). Butterfield *et al.* (2002); Evans *et al*. (2002) Fariss and Zhang, (2003) Berman and Brodaty, 2004 and Amara *et al.* (2011) has reported it as one of the best therapeutic strategies in neurodegenerative disorders associated with oxidative stress. Ambali *et al.* (2012) and Yavuz *et al.* (2005) demonstrated AChE restoration properties of vitamin E following organophsphorus pesticide exposure like that of chlorpyrifos. It cannot be denied that the best protection against oxidative stress comes from a wide assortment of interrelated antioxidants and antioxidant cofactors (Jacob *et al*., 1995). In the present study, best results were obtained where combination of antioxidants was administered against endosulfan. The results from the studies of Bano and Bhatt, (2010). Bano and Bhatt, (2007); Pal *et al*. (2009); El-Hossary *et al*. (2009); Bist and Bhatt, (2009); Srilatha *et al*. (2010); Das *et al*. (2010) support the findings of present study. Hence, it can safely be concluded that antioxidants such as trans resveratrol, alpha- lipoic acid and vitamin E are most effective neuroprotective agents. Nevertheless, combination of all the three antioxidants rendered maximum protection as when administered in combination than given solitarily along with endosulfan. This may be due to the intrinsic properties that these antioxidants possess. Thus, it can be hypothesized that the diet rich in multiple antioxidants can act as an effective neuroprotective source minimizing the various neurological anomalies associated with pesticide toxicity.

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