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EFFECT OF *Cynodon dactylon*, ON METHYL MERCURY-an ENVIRONMENTAL POLLUTANT INDUCED NEUROTOXICITY

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Abstract

Methylmercury (MeHg) is highly toxic, and its principal target tissue in human is the nervous system, which has made MeHg intoxication a public health concern for many decades. *Cynodon dactylon* (Poacae) (CD) is a well-known traditional plant used as a folk remedy in treatment of many symptoms and diseases. The present study was designed to evaluate the effect of CD ethanolic seed extract (EECD) on MeHg-induced neurotoxicity in cerebellum of rats. Phytochemical screening was done to identify the presence of alkaloids and Flavanoids. The phyto chemical screening which had a high level of phenols and amino acids. This is further confirmed by the FT-IR profile which shows the peak at the specific functional groups. Male Wistar rats were administered with MeHg orally at a dose of 5 mg/kg b.w. for 21 days. Experimental rats were given MeHg and also administered with EECD (750 mg/kg, orally) 1 hr prior to the administration of MeHg for 21 days. After MeHg exposure, we observed that activities of catalase, superoxide dismutase, glutathione (GSH) peroxidase, and the level of GSH were reduced. Behavioral changes such as, decreased motor activity, and spatial short-term memory were noted down during MeHg exposure. EECD pre-treatment offered protection from these behavioral changes. These observations highlighted that EECD has a protective effect against MeHg-induced neurotoxicity.

Key words: Methylmercury, *Cynodon dactylon*, Cerebellum, Behavioural.

I. Introduction

Methyl mercury (MeHg) is a pervasive environmental contaminant that causes marked neurobehavioral effects, including memory deficits and anxiety-like effects [1]. Because MeHg is more toxic than other forms of mercury, and mercury is mostly deposited in the environment in its ionic form (Hg [II]), the biogeochemical cycling of mercury in the environment plays a key role in modulating mercury toxicity [2]. In vivo exposure to MeHg causes its accumulation inside mitochondria followed by a series of biochemical changes, including reduced cellular respiration and decreases in the activity of mitochondrial enzymes such as cytochrome C oxidase, superoxide dismutase, monoamine oxidase (MAO) and succinate dehydrogenase [3,4]. *Cynodon dactylon (*L.) Pers. Family: Poaceae, also known as Bermuda grass, Devil's grass, Couch grass, Triticum repens, and Indian Doab, is a resilient and perennial grass native to the warm temperate and tropical regions [5]. C. dactylon is claimed to have anti-diabetic and anti-emetic [6] hypolipidemic [7] anti-inflammatory, antimicrobial [8] properties. The root and rhizomes are also used in the treatment for depression, vomiting, cough, epilepsy, and haemorrhage[9]. According to the pharmacological profile of *Cynodon dactylon* it is reasonable to assume that these extracts may have some other neuroactivities. Therefore, the present study was designed to investigate the neuroprotective effect of these extracts by using various experiments in rats.

II. MATERIALS AND METHODS

Chemicals Required

All the chemicals used were of analytical grade. Methylmercuric chloride was purchased from Sigma Chemicals where as Glutathione reductase (GR), glutathione (GSH)-reduced form, glutathione oxidized form (GSSG), tert-butyl hydro peroxide, 5, 50-dithiobis-(2-nitrobenzoicacid (DTNB) b-Nicotinamide adenine dinucleotide phosphate reduced (NADPH) were purchased from SRL(PVT.LTD) of India.

Plant collection identification and preparation of plant extract

The fresh leaves of *Cynodon dactylon.*L. Were collected from local market of Tirichirapalli. The plants was identified and authenticated by Dr.Sussai Raj Department of Botany St.Joseph College,(Autonomous) with a voucher number **SJCBOT2168**.

The dried powder of plant leaves of *Cynodon dactylon* L. (25g) were successively extracted with 100ml ethanol in a soxhlet apparatus at 60-70ºC each for 10-12h consecutively. Solvents used were of analytical grade. Ethanol was removed from the extract under vacuum and a semisolid mass was obtained. The yield of ethanolic extracts was 11.75% w/w for EECD. The extracts were stored in sterile amber colored storage vials in refrigerator until used for further experimentation.

Phytochemical Screening of C*ynodon dactylon.*L

Phytochemical screening was done to identify the presence of alkaloids and Flavanoids.

Fourier transform IR spectroscopy (FT-IR)

FT-IR spectroscopy of liquid sample of Ethanolic extract of *Cynodon dactylon* L. it was done on a bio-red FTIS-46 model USA. The sample was mixed with dried potassium bromide (kbr) pellet and compressed on a salt disc. For reading the spectrum that analyses the functional groups of the plants

Experimental animals

Six to eight week old Wistar male rats (n=24 grouped into 4) were used for the study after obtaining approval of the Institutional Animal Ethics Committee **(IAEC NO 011712).** The animal were maintained under standard conditions and administrated with drugs as depicted in table 4.1. Three groups of the rats were induced with oxidative stress by giving methylmercury (5mg/kg) and one group served as control. Rats were divided into four experimental groups of six animals each. Group I: control; group II: MeHg (5 mg/kg, b.w.) (Yamashita et al. 2004) orally for 14 days; group III: MeHg + EECD (750 mg/kg, b.w.) orally 1 h prior to the administration of MeHg for 14 days; group IV: EECD alone (750mg/kg, b.w.) orally for 14 days.

Behavioral Parameters

All the behavioral parameters were performed at room temperature in a calm room without any outside interference. All of the behavioral parameters were performed between 10AM and 5PM.

Open Field Test

Open field tests were performed in a separated room with no interference noise or human activity. The locomotor activity was assessed during the treatment in sessions of 6 minutes using an open-field box measuring 56cm(long)x42cm(wide)x40cm(high) with the floor divided into 12 squares. The duration of the trials (6min) was based on well-standardized protocols currently used in the literature [53, 44] as a measure of locomotor activity. The number of squares crossed with the four paws was used as a measure of locmotor activity.

Forced Swim Test

The forced swim test was performed according to the method of Porsolt et al [10]. A vertical glass cylinder (25cm high, 14cm in diameter) was filled with water (30ºC) to a depth of 20 cm. The water depth was adjusted so that the animals must swim or float without their hind limbs or tail touching the bottom. For testing, each animal was placed in the cylinder for 6 min, and the latency to float, and the duration of floating (i.e. the time during which rat made only the small movements necessary to keep their heads above water) was scored. As suggested by Porsolt, only the data scored during the last 4 min were analysed and presented.

In the forced swim procedure, rats were forced to swim in un-escapable situation. After a period of vigorous struggling, the animal becomes immobile, or makes only those movements necessary to keep its head above the water. The immobility observed in this test is considered to reflect a state of despair. The forced swim test has a high degree of pharmacological validity as reflected by its sensitivity to major classes of antidepressants, including tricyclics antidepressant and Selective Serotonin Reuptake Inhibitors (SSRIs)[11].

Tissue Preparation

After treatment period, experimental animals and control animals were killed by cervical dislocation. Brains were immediately taken out and washed with ice cold saline to remove blood and kept at -80_C. The cerebellum was rapidly dissected from the intact brain carefully on ice plate according to the stereotaxic atlas of Paxinos and Watson [12]. The cerebellum was homogenized individually in Tris buffer (pH 7.4). The tissue homogenate (10%) was made (w/v), which was centrifuged at 3,0009g for 10 min. The resulting pellet (P1) consisting of nuclear and cellular material was discarded. The supernatant (S1) containing mitochondria, synaptosomes, microsomes, and cytosol was further ultracentrifuged at 25,0009g for 1 h. Pellet had membrane fraction, while the supernatant had cytosol fraction. In this study, all biochemical estimations were performed in the cytosol fraction. Homogenates were kept at -80_C and thawed just before the start of biochemical estimation. All processes were carried out in cold conditions.

Biochemical determination

Thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation, were estimated by the method of Okhawa et al. [13]. The amount of TBARS was determined spectrophotometrically at 532 nm and expressed as lmoles of TBARS/mg protein. Protein carbonyl levels were measured by the method of Levine et al. [14] and expressed as nmoles/ mg protein. The level of reduced glutathione (GSH) was measured by the method of Moron et al. [15] on the basis of the reaction of 5, 50-dithiobis-2-nitrobenzoic acid which is readily reduced by sulfhydryls forming a yellow substance which was measured at 412 nm and expressed as l moles/mg protein. The enzyme

glutathione peroxidase (GPx) was assayed according to the method of Rotruck et al. [16]. The assay takes advantage of concomitant oxidation of NADPH by GR, which was measured at 340 nm. Enzyme activity was expressed as lg/min/mg protein. GR activity was assayed by the method of Carlberg and Mannervik[17]. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein. Superoxide dismutase (SOD) activity, expressed as units/mg of protein, was based on the inhibition of superoxide radical reaction with pyrogallol (Marklund and Marklund [18]. Catalase (CAT) activity was determined by following the decrease in 240 nm absorption of hydrogen peroxide (H2O2). It was expressed as nanomoles of H2O2 reduced/min/mg of protein (Aebi 1984) [19]. The protein content was measured by Lowry et al. [20].

III.RESULTS AND DISCUSSION

Preliminary phtochemical screening listing the presence of compounds.

S.no	Phytocompounds	Presence	Absence	Adapted method
1	Alkaloids	+		Wagner's reagent
2	Carbohydrates	++		Molisch's test
3	Cardiac glycosides	++		Keller Kelliani's test
4	Flavonoids	+++		Alkaline reagent test
5	Phenols	++		Ferric chloride test
6	Phlobatannins		–	Precipitate test
7	Amino acid	++		1% ninhydrin solution in acetone
8	Saponins	+		Foam test
9	Sterols		–	Liebermann-Burchard test
10	Tannins		–	Braymer's test
11	Terpenoids		–	Salkowki's test
12	Quinones		–	
13	Oxalate	+		
14	Diterpenes		–	
15	Glycosides		–	
16	Anthraquinones		–	

***+++ denotes-High intensity, *++ denotes- mild intensity, *+ = denotes presence, *- = denotes- absence**

The *Cynodon Dactylon.*L is a potential plant with numerous phytocompounds that are initial screened by a preliminary phytochemical screened using various biochemical methods. The table furnished the details of the presence and absence of the phytocompounds (represented in the figure).Where flavonoids occupy the highest intensity persued by carbohydrates, cardiac glycoside, phenol and aminoacids. The result significant the rich source of all biochemical constituents such as carbohydrates and proteins.

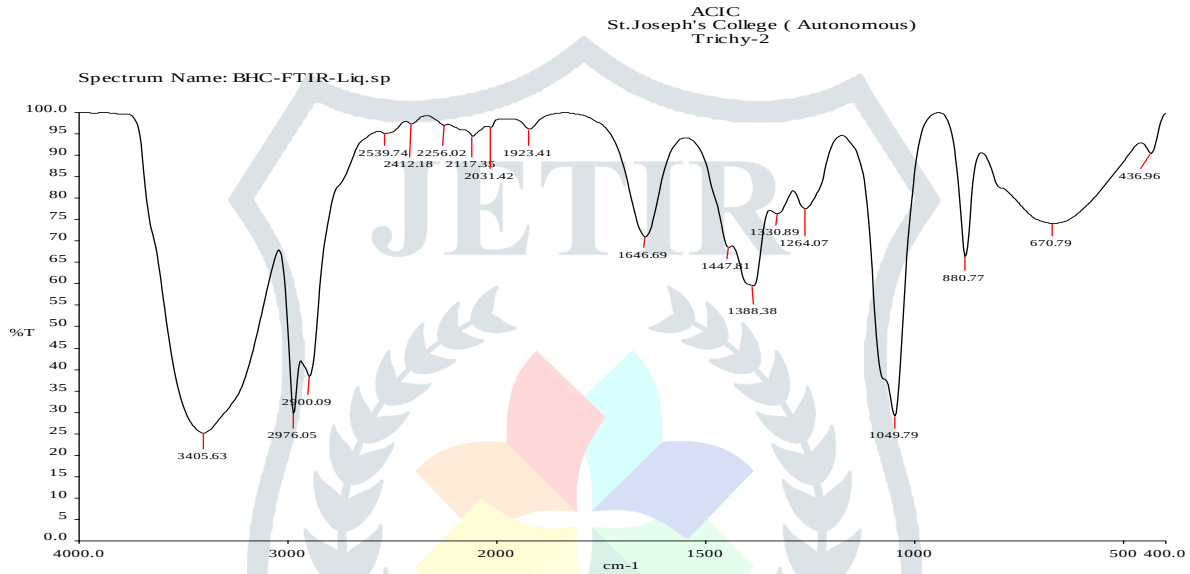
Interpretation of functional groups by FT-IR analysis- Wave length and functional groups of spectrum peaks

S.no	Frequency range	Bond	Functional group
1	3405.63	O–H stretch, H–bonded	alcohols, phenols
2	2976.05	C–H stretch	Alkanes
3	2900.09	C–H stretch	Alkanes
4	2539.74	H–C=O: C–H stretch	Aldehydes
5	2412.18	H–C=O: C–H stretch	Aldehydes
6	2256.02	–C≡C– stretch	Alkynes
7	2117.35	–C≡C– stretch	Alkynes
8	1646.69	C=O stretch	carbonyls (general)
9	1447.81	C–C stretch (in–ring)	Aromatics
10	1388.38	C–H rock	Alkanes
11	1330.89	C–H rock	Alkanes
12	1264.07	N–O symmetric stretch	nitro compounds

13	1049.79	C–N stretch	aliphatic amines
14	880.77	=C–H bend	Alkenes
15	670.79	C–H “oop”	Aromatics
16	436.96	C–Br stretch	alkyl halides

The functional groups of retrieved from the spectra specifies the various constituents present in the phtocompounds of the plants. This result was found on par with the phyto chemical screening which had a high level of phenols and amino acids. This is further confirmed by the FT-IR profiles which shows the peak at the specific functional groups. Hence all the essential groups of the plant is revealed through this significant studies. The intensity of flavonoids was high and generally these group of phytocompounds act as an anti-oxidative elements, this was further proved by means bio chemical oxidative study. The treated plant extract showed on optimal intensity in the oxidative level.

Diagrammatic representation of the FT-IR profile



The plant has the potential to react against Methylmercury (MeHg) which is well known causative agent of minamata disease that is been proved by animal studies. The essential phyto compounds of the plant were analyzed by a preliminary phyto compound screening and FTIR studies which showed an elevated level of flavonoids. Interestingly, flavonoids are the fore most compounds that act as an antioxidative agent. Hence the plant can combat neurodegenerative changes that take place with an increased oxidative stress. Oxidative stress has been known to contribute to MeHg induced central nervous system (CNS) damage [21]. In this current study, we have shown for the first time that EECD possesses protective effects against MeHg-induced neurotoxicity in rats. From a toxicological point of view, it is important to state that no visible signs of toxicity in the EECD treated rats were detected.

Effect of EECD on MeHg induced Forced swim test and Open Field test in control and experimental rats

Response to inescapable aversive situation interpreted as a measure of depression like behaviour. MeHg exposed rats showed (p<0.01) longer immobility time than control animals. EECD treatment was significantly (p<0.05) reduced the immobility time when compared to MeHg induced group. EECD alone treated resembled to that of control group. The locomotor activity was found to be significantly decreased (p<0.01) in MeHg induced animals compared to control groups. EECD treated rats showed significantly (p<0.05) increased locomotor activity. EECD alone treated group did not show any effect on the locomotor activity.

Group	Treatment	Immobility Time (Sec)	Locomotor Activity (No.of.crossing)
Group I	Control	5.83 ± 2.85	15.16 ± 3.06
Group II	MeHg	9.16 ± 2.63**	6.16 ± 2.85**
Group III	MeHg + EECD	6.5 ± 2.42*	11.33 ± 3.14*
Group IV	EECD	6.5 ± 2.42	19 ± 3.08

Data represents mean ± SD of 6 rats in each group. Values represents the time duration of Immobility. Values presented as crossed squares in the open field area. $^{**}p<0.01$ significantly different from control group; $^{*}p<0.05$ significantly different from MeHg induced group, using one way ANOVA with Tukey's post hoc test

In this present study open field test a reduced locomotor activity was noted in MeHg intoxicated rats. Previous studies also reported that that MeHg inhibits the locomotor in rodents [22] and in humans [23]. EECD treatment increased the locomotor activity. In forced swim test methylmercury intoxicated rats showed longer immobility. It represented as depression like behaviour. The result obtained by this study are in agreement with Nabi et al [24] who reported that alterations in depression like behaviour induced by MeHg. EECD treatment decrease the immobility.

Effect of EECD on MeHg induced level of TBARS in cerebellum in control and experimental rats

Level of LPO was observed in cerebellum of control and experimental rats. Level of LPO were found to be significantly ($p<0.01$) increased in MeHg-induced rats compared with control. Administration of EECD significantly decreased the levels of the TBARS ($p<0.05$).

Group	Treatment	Units/mg of protein
Group I	Control	4.43 ± 0.33
Group II	MeHg	8.11 ± 0.3**
Group III	MeHg+EECD	6.43 ± 0.28*
Group IV	EECD	5.05 ± 0.46

Data represents mean±SD of six rats in each group. TBARS units expressed as µmoles of TBARS/mg protein. $^{**}p<0.01$ significantly different from control group; $^{*}p<0.05$significantly different from MeHg induced group, using one-way ANOVA with Tukey's post hoc test.

Effect of EECD on MeHg induced level of GSH and GPx in cerebellum in control and experimental rats.

Level of GSH and activity of GPx was observed in cerebellum of control and experimental rats. The level of GSH and GPX activity was significantly decreased ($p<0.01$) in MeHg-induced rats compared with control. Administration of EECD significantly increased the GSH level and GPx activity ($p<0.05$). EECD alone treated groups resembles control.

Group	**Treatment**	**GSH (Units/mg of protein)**	**GPx (Units/mg of protein)**
Group I	Control	10.21 ± 0.24	30.46 ± 0.3
Group II	MeHg	7.31 ± 0.28**	23.33± 0.28**
Group III	MeHg+EECD	8.9 ± 0.26*	28.66 ± 0.6*
Group IV	EECD	9.18 ± 0.34	29.45± 0.32

Data represents mean±SD of six rats in each group. GSH units expressed as µM/mg protein. GPx units expressed as µg/min/mg protein.$^{**}p<0.01$ significantly different from control group; $^{*}p<0.05$significantly different from MeHg induced group, using one-way ANOVA with Tukey's post hoc test.

Effect of EECD on MeHg induced activity of SOD and CAT in cerebellum in control and experimental rats.

Activity of SOD and CAT was observed in cerebellum of control and experimental rats. The activity of SOD and CAT was found to be significantly ($p<0.01$) reduced in MeHg intoxicated groups when compared to control rats. EECD treatment significantly ($p<0.05$) increased the activity of SOD and CAT. EECD alone treated rats resembled control rats.

Group	**Treatment**	**SOD (Units/mg of protein)**	**CAT (Units/mg of protein)**
Group I	Control	12.51 ± 0.23	7.75 ± 0.18
Group II	MeHg	9.51 ± 0.28**	5.26 ± 0.21**

Group III	MeHg+EECD	11.51 ± 0.3*	6.26 ± 0.21*
Group IV	EECD	12.18 ± 0.24	7.26 ± 0.21

Data represent mean ± SD of 6 rats in each group. SOD units expressed as enzyme activity of inhibit 50% of pyrogallol auto-oxidation. CAT units expressed as nmol of H_2O_2 reduced/min/mg protein. $^{**}p<0.01$ significantly different from control group; $^{*}p<0.05$significantly different from MeHg induced group, using one-way ANOVA with Tukey's post hoc test.

Mechanistically, it is well known that MeHg-induced neurotoxicity is related to oxidative stress, which is closely associated with glutamate and calcium dyshomeostasis[25]. Notably, the glutathione antioxidant system has been reported to represent a molecular target for the deleterious effects of MeHg in the central nervous system[26]. Gpx activity was significantly reduced in MeHg exposed rats when compared to control. EECD significantly increased the activity. Glutathione reductase is an important enzyme involved in the reduction of glutathione disulfide (GSSG also known as oxidized glutathione) to glutathione (GSH), using NADPH as a reducing cofactor (Glu et al. 2000). TBARS level was elevated in MeHg induced rats in our study. This indicates lipid peroxidation. EECD decrease the level of TBARS. Previous study also reported that *cynadon dactylon* decreases the oxidation levels in the brain [27]. GSH system is an important target in mediating MeHg neurotoxicity. In accordance with mori et al[28], we found from our result that MeHg induction caused reduction of GSH. It interestingly EECD increased the GSH level. SOD and CAT are the antioxidants play a vital role in preventing both free radical damage and generating oxidative stress like conditions. From our studies SOD and CAT activity was decreased in MeHg intoxicated rats. These results obtained by this study are in agreement with Sumathi et al [29] Due to protein degradation DNA and RNA content were decreased in MeHg induced group. EECD increased the DNA and RNA content.

IV.CONCLUSION

In conclusion, the results provide evidence for the first time that POEE exerts significant in vivo protective effects on cerebellum and cortex against MeHg-induced neurotoxicity. Further studies at the compound level are yet to be explored.

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