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EFFICACY OF *EUPHORBIA THYMIFOLIA* LINN PHYTOSTEROL ON ROTENONE INDUCED PARKINSON'S DISEASE: BEHAVIOURAL AND NEUROCHEMICAL STUDY

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ABSTRACT Previous works showed antianxiety, antidepressant and nootropic activity of *E. thymifolia* total sterol (ETTS) with facilitatory effect on retention of acquired learning and memory. This has driven to explore neurorejuvenating potential of ETTS against rotenone induced parkinson's disease (PD) by assessing behavioral and neurochemical alterations on mice. The present experiment was designed to assess effect of ETTS on behavioural dysfunction, neurochemical abnormality and oxidative stress in mice brain on rotenone induced PD model. ETTS 75 mg/kg ($P < 0.01$-0.001) treated group showed significant reversal of body weight loss, locomotor score, rota rod muscle grip and rearing behavior induced by rotenone. ETTS has significantly ($P < 0.001$) reversed the increased lipid peroxidation and declined glutathione and nitric oxide scavenging ability. Significant increase ($P < 0.05$-0.001) in gama amino butyric acid and dopamine level was observed in ETTS treated group whereas acetylcholine esterage level has not been changed significantly. The results substantiate protective effect of ETTS on dopaminergic neurons against degenerative effects of rotenone may be by virtue of free radical scavenging ability. Significant improvement in the neurobehavioral, oxidative and neurochemical parameters was observed ETTS administration along with rotenone exposure.

KEYWORDS : *Euphorbia thymifolia* Linn, phytosterol, rotenone, parkinsonism, behavior, neurotransmitter, antioxidant

INTRODUCTION

Parkinson's disease (PD) is a major progressive neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain (Calne, 1992). PD affects about 1% of the population over the age of 65 years as the second most common chronic and progressive neurodegenerative disorder after Alzheimer's disease (AD) Zhang and Román, 1993). The prominent clinical features are motor impairment such as tremors at rest, bradykinesia, rigidity and postural instability (Berardelli et al., 2001). Dopamine depletion leads to an increased cholinergic activity in the striatum resulting in an imbalance between dopaminergic and cholinergic effects on the striatal control of the motor function. Evidence showed that in addition to loss of dopaminergic neurons in PD there is also early disturbances in cholinergic neurotransmission which is mediate as symptoms like dementia and cognitive impairments (Caviness et al., 2007).

Despite the advances in modern medicine the neuurodegenerative diseases like AD and PD have been a big challenge to the medical scientists as they are difficult to diagnose and cure at initial stages. The neurodegeneration is not just genetic problem but can also occur in response to several environmental factors (Van Den Eeden et al., 2003). Several pesticides and herbicides via water or food enters in to the cells and affect the mitochondrial succinate dehydrogenase functioning, and they are capable of crossing the blood brain barrier to inhibit growth and development of nervous system by causing oxidative stress (Zeliger, 2013). Oxidative stress has been strongly implicated in the pathophysiology of PD (Miller et al., 2009). Under physiological conditions, human body is continuously exposed to reactive oxygen metabolites (eg, the superoxide radical, hydrogen peroxide and hydroxyl radical) and nitrogen species. These can be counterbalanced by number of enzymatic (catalase, glutathione peroxidase and superoxide dismutase) and nonenzymatic (glutathione, ascorbic acid, carotenoids, and vitamin E) antioxidant (Halliwell, 2001). Several studies have indicated the existence of elevated levels of oxidative stress in postmortem brain tissue in PD patients (Zhang et al., 1999).

Herbal antioxidants can prevent brain damage by enhancing oxygen utilization and increasing the uptake of glucose, in turn promoting nerve transmission, improving the synthesis of neurotransmitters in the area of the brain most affected by PD and AD. Vinpocetine, ginkgo and curcuma has antioxidant effect on the brain protecting the brain form free radical damage. *Euphorbia thymifolia* Linn (Family-Euphorbiaceae) is commonly known as Dudhi. It is an annual herb found in waste lands, along roadsides and wall sides under humid condition. *E. thymifolia* has slender, smooth and profusely branched reddish coloured stems (Kirtikar and Basu, 2006). *E. thymifolia* contain kaempferol, cosmosiin, quercetin, β-amyrine, β-sitosterol, campesterol, stigmasterol, epitaraxerol, n-hexacosanol, euphorbol, 24-methylene cycloartenol and cholesterol (Nadkarni, 2007), and are reported to have antioxidant activity (Kunanusorn et al., 2009).

Nootropics are reported to increase stress tolerance and alleviate depression by replenishing or increasing the brain's supply of specific neurotransmitters. Lemon balm (*Melissa officinalis*) having antidepressant effect and Ginseng an anti-anxiety adaptogen normalizes physical stress and mental consequences to improve cognitive function in people with mild to moderate AD and PD (Akhondzadeh et al., 2003; Wang et al., 20016; Khadrawy et al., 2016). Previous studies substantiate antianxiety and moderate depressant activity with modulating antidepressant property of *E. thymifolia.* Neurorejuvenating activity of *E. thymifolia* phytosterol has also been reported reinforcing its protective role against progression of dementia (Bigoniya et al., 2013). Nootropic effect of *E. thymifolia* had been associated with anti-cholinesterase activity, serotonin inhibitory and absence dopamine induced reinforcing effect (Bigoniya and Shukla, 2015). The present study has been attempted to evaluate neuroprotective effect of *E. thymifolia* phytosterol against rotenone induced PD on mice. Rotenone is a pesticide and toxin that is used to induce PD in an animal model as it can reproduce many key pathological features of PD such as oxidative damage, aggregation, oxidative stress-induced striatal dopaminergic terminal degeneration, selective nigrostriatal loss, cognitive deficits and depression-like behavior (Zaitone et al., 2012). As PD is a multifactorial disease characterized by oxidative stress, energy crisis, motor dysfunction, excitotoxicity, neurotransmitter imbalance and protein aggregation the effect of *E. thymifolia* phytosterol on PD was assessed through measurement of motor function, behavioral aspects, and brain level of antioxidant and neurotransmitters.

MATERIALS AND METHODS

Drugs and chemicals

Rotenone, acetylthiocholine iodide, acetylthiocholine, 1, 1, 3, 3-tetramethoxypropen, griess reagent and 5,5′-dithiobis-2-nitrobenzoic acid (Sigma Aldrich, USA), vinpocetine (Micro Labs Ltd, Bangalore), sodium dodecyl sulfate, thiobarbituric acid, glutathione disulphide, nicotinamide adenine dinucleotide phosphate, sodium nitroprusside, ninhydrin and phthalaldehyde (Merck Millipore, India) were purchased. All other solvents and reagents used in the study were of analytical grade.

Plant material and separation of total phytosterol

The plant was collected in the month of August- September 2013 from Bhopal (M.P.) India. It was made completely clean, dust free and allowed to get dried under shade. Plant was identified and

authenticated by Dr. Tariq Husain, Scientist and Head Herbarium and Angiosperm Taxonomy, National Botanical Research Institute, Lucknow (India) and a specimen voucher no. 97313 was assigned. *E. thymifolia* total sterol (ETTS) fraction was processed and separated as per the previously described method (Bigoniya and Shukla, 2015).

Experimental animals

Male and female breed Swiss Albino mice weighing between 18-22 gm were used in the experiments. All the experiments were performed between 9:30 to 16:30 hr to avoid diurnal and circadian variations. All the animals were housed at a temperature of 22 ± 2C and in a relative humidity of 65 ± 5% following a 12:12 hr L: D cycle. The animals were housed in polypropylene cages with paddy husk bedding, free access to water ad libitum and fed with standard commercial pelleted chow (Hindustan Lever). All the experimental procedures and protocols were approved by Institutional Animal Ethics Committee of the institution (Proposal number IAEC/RCP/Dec-2013/12) and were performed in accordance with the guidelines of the IAEC.

Group division

Albino mice of both sexes were randomly divided into nine groups containing 6 animals per group. Group I: Represented vehicle control treated with 0.1 ml/20 gm (dimethyl sulfoxide + polyethylene glycol-300, 1:1 v/v), i.p., Group II: Represented negative control treated with PD inducing agent Rotenone 2 mg/kg, s.c, Group III: vinpocetine 5 mg/kg, i.p. (Shibota et al., 1982), Group IV: ETTS 25 mg/kg, i.p., Group V: ETTS 50 mg/kg, i.p., Group VI: ETTS 75 mg/kg, i.p., Group III to VI were treated with vinpocetine or different doses of ETTS 30 min after PD inducing agent rotenone for a period of 35 days. Evaluation of various test parameters like catalepsy, locomotor activity, rota rod fall off time and rearing behaviour was done on 0 day, and periodically on every 7th day and on 35th day.

Dosing

ETTS, vinpocetine and rotenone was emulsified in vehicle (dimethyl sulfoxide + polyethylene glycol-300, 1:1, v/v) and prepared freshly before administration by injection. Acute toxicity study reported earlier showed LD_{50} of 283.00 mg/kg (i.p) and the dose range 25, 50 and 75 mg/kg was selected for evaluation of pharmacological activities (Bigoniya et al., 2013).

Body weight

Body weight of all the animals were recorded on 0, 7th, 14th, 21st, 28th and on 35th day of drug treatment. Group average body weight was calculated and percentage change in body weight gain was also calculated in respect to zero day weight.

Cataleptic behavior

Catalepsy is defined as reduced ability to initiate movement and a failure to achieve correct posture. The duration of catalepsy was noted at 0, 7, 14, 21, 28 and 35th day 30 min after respective drug administration following Bar test. The phenomenon was measured as the duration, when the mouse maintained an imposed position with both front limbs extended and resting on 8 cm high wooden bar (8 × 8 cm) and was scored accordingly to following scoring system. To better measure the catalepsy (avoiding false results) the animals were tested twice at each time interval and only the greater duration of time was recorded. In between the measurements animals were returned to their home cages. The scoring system is as follows; time 0-10 sec grade 0, 11-30 sec grade 1, 31-60 sec grade 2, 61-120 sec grade 3 and > 120 sec grade 4. Mean score of each group was calculated (Narasapur and Dashputra, 1995).

Locomotor activity

Locomotor activity was recorded with a digital actophotometer (Dolphin, India) at 0, 7, 14, 21, 28 and 35th day 30 min after respective drug administration. Mouse was individually placed in digital actophotometer for 10 min to score locomotor activity. Mean change in the locomotor activity was calculated for each group. Percentage changes in locomotion score were calculated for each group (Kulkarni, 2005).

Muscle grip performance

Digital rotarod apparatus (Jyoti Scientific, India) was used to evaluate the muscle relaxing effect. The animals were placed individually on the rotarod, rotating at a speed of 25 rpm to score the fall off time. Respective groups of animals were assessed for their performance on the rotarod after 30 min of drug treatment at 0, 7, 14, 21, 28 and 35th days of drug administration. Percentage changes in fall off time were calculated for each group (Kulkarni, 2005).

Rearing behavior

Mouse was placed in a clear cylinder are normally engaged in exploratory behavior including rearing. Rearing behavior is characterized as lifting of forelimbs above shoulder level to make contact the wall of the cylinder with either one or both forelimbs. Mouse was placed in clear plexiglass cylinder (height 30 cm, diameter 20 cm) for 5 minutes under low red-light conditions (10 lux) and the number of rears was quantified. Removal of both forelimbs from the cylinder wall and contact with the table surface is required before another rear was counted. Respective groups of animals were assessed for their rearing performance 30 min after respective drug treatment at 0, 7, 14, 21, 28 and 35th days of drug administration. Percentage changes in rearing counts were calculated for each group (Fleming et al., 2004).

Relative brain weight

All animals were euthanized under ether anaesthesia on 35th day 2 hrs after last drug dose administration. The brain was quickly dissected out on an ice-cold plate, washed with ice-cold phosphate-buffered saline (pH 7.4), weighed, frozen on powdered dry ice before transferring to −80°C freezer until the biochemical analyses. Relative weight of brain per 100 gm body weight was calculated for each animal and group average weight was represented.

Preparation of brain tissue homogenate

The frozen brain samples were taken out and thawed on the day of experiment. The brain tissues were homogenized with 0.1 M phosphate-buffered saline (pH 7.4) using a Teflon-glass tissue homogenizer (Remi, India) and centrifuged (Remi, India) at 3000 rpm (4C) for 10 min. The supernatant was collected to give a final concentration of 5% weight/volume (w/v), kept on ice and used for the biochemical assays. For dopamine estimation brain tissues were homogenized with acidified (0.2 N acetic acid) n-butanol to give 10% weight/volume (w/v) homogenate.

Estimation of lipid peroxidation

The thiobarbituric acid (TBA) test was followed for measuring the peroxidation of lipids. Lipid peroxidation was assayed by measuring the level of malondialdehyde in brain tissues based on the principle that lipid peroxidation initiated in the tissue microsomes by ferric ions leads to the formation of small amounts of malondialdehyde (MDA). TBA reacts with MDA forming a diadduct, pink chromogen that have peak absorbance at 532 nm detected spectrophotometrically according to the method devised by Reddy and Lokesh (1992) using ultraviolet (UV-VI8) spectrophotometer (Shimadzu, Japan) at a 532 nm wavelength. As external standard, 1, 1, 3, 3-tetramethoxypropen was used, and the level of lipid peroxidases was expressed as nM of MDA/ mg of brain protein.

Estimation of reduced glutathione

Glutathione (GSH) react directly with O_2^*, OH^*, O^* free radicals by radical transfer process, which yields thiol radicals. This thiol radical or the sulfhydryl group present in GSH forms a colored complex with 5-5 Dithiobis-2 nitrobenzoic acid (DTNB) measured colorimetrically at 412 nm following method of Tietze (1969), and Beutler and Kelly (1963). The amount of GSH was determined using molar extinction coefficient of 13.600/min and the results expressed as g/gm of brain tissue.

Estimation of nitric oxide

Nitric oxide (NO) generated from sodium nitroprusside interacts with oxygen to produce nitrite ions, which on addition of Griess reagent converts into a deep purple azo compound that gives absorbance at 546 nm (Green et al., 1982). Measurement of endogenous nitrite concentration was used as an indicator of NO production and the scavengers of NO compete with oxygen leading to reduced products of nitric oxide (Marcocci et al., 1994). The percent NO scavenging activity was measured in comparison to standards known concentrations of sodium nitrite.

Estimation of acetylcholinesterase activity

Acetylcholinesterase (AchE) activity was determined following modified method of Ellman et al. (1961) as described by Gorun et al. (1978). The principle of the method involves measurement of the yellow colour nitrobenzoate (TNB) ion produced as acetylthiocholine

is hydrolysed to thiocholine which reacts with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The optical density of the yellow color compound formed at 412 nm was measured spectrophotometrically at 412 nm, every minute for a period of 3 minutes. Glutathione 2.5 mM was used as the standard.

Estimation of Gama amino butyric acid

Gama amino butyric acid (GABA) level was determined in mice brain spectrofluorometrically following the method of Lowe et al. (1958) as described by Uchida and O'Brein (1964). GABA formed was estimated at excitation/emission wavelength of 377 /451 nm in a spectrofluorometer (Jasco FP-777, with a source of xenon arc lamp 150 watt, JASCO Ltd., Tokyo, Japan). Samples were read against a GABA standard and expressed as µg GABA/mg of brain protein.

Estimation of dopamine

The quantitative determination of dopamine levels was carried out fluorometrically according to the method of Chang (1964) modified by Ciarlone (1978) using spectrofluorometer. The method is based on estimation of dopamine fluorophors formed after oxidation by iodine at excitation/emission wavelengths of 320/375 nm.

Estimation total protein

The brain total protein was determined by the Folin-Ciocalteau reagent method of Lowry et al. (1951) with slight modification with Autoanalyzer (Star 21 Plus, India) using commercial kit of Aspen Diagnostics Pvt. Ltd., India.

Statistical analysis

The values were expressed as Mean ± SEM. Statistical comparison was performed using one way analysis of variance ANOVA to assess the Statistical significance, followed by Dunnett multiple comparison test. P value of less than 0.05 was considered as statistically significant.

RESULTS

Body weight

Body weight of all groups were recorded on 0, 7th, 14th, 21st, 28th and on 35th day of treatment which showed 39.01% weight gain in vehicle control group. Rotenone treated group showed 43.88% decrease in weight gain whereas vinpocetine and ETTS at 75 mg/kg had 25.44% and 16.77% decrease respectively. Relative brain weight of all the groups were reported and non-significant changes were observed compared to vehicle control group (Table 1).

Table 1: Effect of *E. thymifolia* total sterol on body weight and relative brain weight of mice on rotenone induced Parkinson disease

Treatment (mg/kg, i.p)	Body weight (gm)						Brain Wt. (gm/100 gm body Wt.)
	0 Day	7th Day	14th Day	21st Day	28th Day	35th Day	
Vehicle (0.1 ml WFI)	18.30 ± 1.24	19.66 ± 1.56 (+7.43)	20.60 ± 2.01 (+12.56)	21.32 ± 1.24 (+14.16)	23.38 ± 1.33 (+27.55)	25.44 ± 1.28 (+39.01)	1.75 ± 0.20
Rotenone (2, s.c)	32.79 ± 2.11	31.78 ± 1.86 (3.08)	30.15 ± 1.22 (8.05)	24.37 ± 1.45 (25.67)	20.05 ± 1.48 (38.85)	18.40 ± 1.25 (43.88)	1.54 ± 0.19^{ns}
Rotenone + Vinpocetine (5)	23.38 ± 1.98	24.51 ± 1.45 (+4.83)	22.80 ± 1.05 (2.48)	20.35 ± 1.04 (12.95)	19.37± 1.07 (17.15)	17.43 ± 1.06 (25.44)	1.92 ± 0.15^{ns}
Rotenone + ETTS (25)	24.22 ± 2.04	23.41 ± 1.22 (3.46)	22.64 ± 1.07 (6.52)	20.57 ± 1.23 (15.10)	19.39 ± 1.40 (19.94)	16.22 ± 1.10 (29.44)	1.46 ± 0.09^{ns}
Rotenone + ETTS (50)	22.99 ± 1.87	22.40 ± 1.45 (2.56)	21.90 ± 1.32 (4.74)	21.38 ± 1.87 (7.00)	18.33 ± 1.32 (20.26)	18.50 ± 1.64 (23.61)	1.64 ± 0.12^{ns}
Rotenone + ETTS (75)	36.19 ± 2.36	35.88 ± 2.93 (0.85)	35.44 ± 2.57 (2.07)	34.16 ± 2.48 (5.60)	30.44 ± 2.09 (15.88)	30.12 ± 2.05 (16.77)	1.90 ± 0.14^{ns}

All the values are M ± SEM per group containing 6 animals per group. Ns = non-significant compared to vehicle control group. The values in parenthesis donates to percentage increase or decrease in per group body weight of the animals compared to zero day value.

Cataleptic behavior

Catalepsy was noted at 0, 7, 14, 21, 28 and 35th days after respective drug administration following Bar test. All the treated groups showed zero score upto 7th day of treatment. Rotenone gradually increased cataleptic score following 14 days treatment and showed maximum score 3.45 on 35th day. Vinpocetine treated group showed catalepsy on 21st day and peak catalepsy score was 3.02 on 35th day. ETTS at 75 mg/kg dose showed cataleptic score of 3.28 on 35th day. Vinpocetine and ETTS at all tested doses had non significant effect on rotenone induced catalepsy (Table 2).

Table 2: Effect of *E. thymifolia* total sterol on cataleptic score of mice on rotenone induced Parkinson disease

Treatment (mg/kg, i.p)	Catalepsy score					
	0 Day	7th Day	14th Day	21st Day	28th Day	35th Day
Vehicle (0.1 ml WFI)	00	00	00	00	00	00
Rotenone (2, s.c)	00	00	1.63 ± 0.02	1.82 ± 0.05	2.83 ± 0.03	3.45 ± 0.31
Rotenone + Vinpocetine (5)	00	00	00	1.58 ± 0.02	2.06 ± 0.02	3.02 ± 0.12^{ns}
Rotenone + ETTS (25)	00	00	1.81 ± 0.04	2.20 ± 0.04	2.60 ± 0.05	3.51 ± 0.22^{ns}
Rotenone + ETTS (50)	00	00	1.45 ± 0.02	2.01 ± 0.03	2.55 ± 0.07	3.46 ± 0.51^{ns}
Rotenone + ETTS (75)	00	00	1.07 ± 0.01	1.84 ± 0.05	2.59 ± 0.06	3.28 ± 0.80^{ns}

All the values are M ± SEM per group containing 6 animals per group. Ns = non-significant compared to rotenone treated group.

Table 3: Effect of *E. thymifolia* total sterol on locomotor activity of mice on rotenone induced Parkinson disease

Treatment (mg/kg, i.p)	Locomotor score on actophotometer						% decrease in locomotion on 35th day
	0 Day	7th Day	14th Day	21st Day	28th Day	35th Day	
Vehicle (0.1 ml WFI)	257.1 ± 10.44	272.56 ± 11.22	262.51 ± 11.41	291.68 ± 10.14	284.77 ± 12.36	286.53 ± 13.62	-
Rotenone (2, s.c)	255.44 ± 11.65	218.16 ± 10.43	172.50 ± 12.40	141.71 ± 9.33	81.75 ± 3.58	51.88 ± 2.15	79.68
Rotenone + Vinpocetine (5)	263.62 ± 12.41	207.53 ± 9.68	182.49 ± 12.51	148.80 ± 8.66	118.92 ± 6.42	107.3 ± 7.38***	59.28
Rotenone + ETTS (25)	195.84 ± 11.50	183.61 ± 11.72	175.34 ± 13.00	142.46 ± 11.11	108.92 ± 8.90	62.48 ± 3.15^{ns}	68.09
Rotenone + ETTS (50)	204.87 ± 12.33	187.35 ± 11.35	172.80 ± 12.42	148.76 ± 10.02	112.66 ± 8.44	76.69 ± 3.62^{ns}	62.51
Rotenone + ETTS (75)	239.18 ± 13.10	202.91 ± 10.22	182.41 ± 12.70	161.98 ± 9.45	140.33 ± 7.93	91.88 ± 4.84**	61.58

All the values are M ± SEM per group containing 6 animals per group. $^{***}P < 0.001$, $^{**}P < 0.01$ and ns = non-significant compared to rotenone treated group.

Locomotor activity

Rotenone caused 79.68% decrease in locomotor activity following 35 days treatment compared to zero day value. Vinpocetine and ETTS at 75 mg/kg dose was recorded respectively with 59.28% and 61.58% decrease in locomotor score. Compared to rotenone treated group significant reversal of decreased locomotion was reported by vinpocetine ($P < 0.001$) and ETTS ($P < 0.01$) as induced by rotenone treatment on 35th day (Table 3).

Muscle grip performance

Rotenone showed 79.18% decrease in rotarod falloff time on 35th day of treatment. Vinpocetine and ETTS at 75 mg/kg dose reverted the muscle relaxant activity induced by rotenone with respectively 55.84% and 41.13% decrease in fall off time compared to zero day value. Compared to rotenone treated group significant reversal of muscle relaxant activity was observed in vinpocetine ($P < 0.001$) and ETTS all doses treated groups ($P < 0.01$-0.001) on 35th day (Table 4).

Table 4: Effect of *E. thymifolia* total sterol on muscle relaxant activity of mice on rotenone induced Parkinson disease

Treatment (mg/kg, i.p)	Rotarod fall off time (sec)						% decrease in fall off time on 35th day
	0 Day	7th Day	14th Day	21st Day	28th Day	35th Day	
Vehicle (0.1 ml WFI)	275.13 ± 11.22	288.26 ± 13.37	282.42 ± 12.80	276.64 ± 11.91	281.37 ± 11.71	290.29 ± 13.62	-
Rotenone (2, s.c)	256.24 ± 11.36	249.00 ± 12.73	124.48 ± 9.00	119.51 ± 8.75	79.14 ± 4.32	53.33 ± 2.85	79.18
Rotenone + Vinpocetine (5)	284.11 ± 12.12	271.78 ± 12.92	242.16 ± 10.00	156.50 ± 9.62	148.28 ± 9.23	124.44 ± 8.91***	55.84
Rotenone + ETTS (25)	256.85 ± 12.41	268.22 ± 11.85	238.98 ± 10.00	170.31 ± 9.38	138.74 ± 8.44	101.34 ± 7.76**	60.54
Rotenone + ETTS (50)	274.72 ± 13.56	280.14 ± 11.76	212.46 ± 10.00	184.11 ± 9.14	156.73 ± 9.25	141.26 ± 8.44***	48.58
Rotenone + ETTS (75)	268.46 ± 10.14	271.83 ± 10.23	212.15 ± 10.00	184.26 ± 9.43	170.34 ± 9.19	158.32 ± 8.74***	41.13

All the values are M ± SEM per group containing 6 animals per group. ***$P < 0.001$ and **$P < 0.01$ compared to rotenone treated group.

Rearing behavior

Table 5: Effect of *E. thymifolia* total sterol on rearing score of mice on rotenone induced Parkinson disease

Treatment (mg/kg, i.p)	Rearing counts						% decrease in rearing counts on 35th day
	0 Day	7th Day	14th Day	21st Day	28th Day	35th Day	
Vehicle (0.1 ml WFI)	28.30 ± 1.52	29.14 ± 1.70	30.66 ± 1.63	28.31 ± 1.11	28.55 ± 1.20	26.34 ± 1.41	-
Rotenone (2, s.c)	27.25 ± 1.13	24.13 ± 1.32	18.50 ± 0.92	5.80 ± 0.48	5.35 ± 0.31	4.15 ± 0.42	84.77
Rotenone + Vinpocetine (5)	31.17 ± 1.48	28.66 ± 1.41	26.25 ± 1.04	18.14 ± 0.82	18.01 ± 0.90	14.54 ± 0.79***	53.35
Rotenone + ETTS (25)	21.33 ± 1.25	18.84 ± 1.25	15.16 ± 0.78	12.40 ± 0.35	11.35 ± 0.88	5.85 ± 0.15ns	72.57
Rotenone + ETTS (50)	24.13 ± 1.60	19.44 ± 1.24	16.58 ± 0.86	13.37 ± 0.66	10.10 ± 0.71	7.54 ± 0.36*	68.75
Rotenone + ETTS (75)	21.17 ± 1.22	19.05 ± 1.02	17.96 ± 0.97	12.11 ± 0.78	9.15 ± 0.58	8.72 ± 0.48**	64.94

All the values are M ± SEM per group containing 6 animals per group. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and ns = non-significant compared to rotenone treated group.

Rotenone treatment for 35 days induced 84.77% decrease in rearing score whereas vinpocetine and ETTS at 75 mg/kg dose showed 53.35% and 64.94% decrease in respect to zero day value. Significant increase in rearing score was observed in vinpocetine ($P < 0.001$) and ETTS at 75 mg/kg ($P < 0.01$) group compared to rotenone treated group (Table 5).

Antioxidant and neurotransmitter level of brain

MDA brain content in rotenone treated group was significantly higher ($P < 0.01$) than vehicle control group. Vinpocetine and ETTS at 75 mg/kg dose has extreme significantly ($P < 0.001$) decreased MDA level of brain. Brain glutathione level and NO scavenging ability was significantly ($P < 0.05$-0.001) reduced by rotenone, and ETTS at 50 and 75 mg/kg dose has significantly ($P < 0.001$) reversed the decline. Rotenone treatment has significantly ($P < 0.01$-0.001) decreased brain tissue AchE, GABA and dopamine content compared to vehicle treated group. Significant increase ($P < 0.05$-0.001) in the level of GABA and dopamine was observed in ETTS 75 mg/kg treated group (Table 6), whereas AchE level has not been changed significantly by ETTS. Similar effect was observed with vinpocetine.

Table 6: Effect of *E. thymifolia* total sterol on antioxidant parameters and neurotransmitter level of mice brain on rotenone induced Parkinson disease

Treatment (mg/kg, i.p)	MDA (nM/mg of protein)	GSH (µg/gm of tissue)	NO (% Scavenging)	AchE (µmol/ SH/gm brain tissue/min)	GABA (µg/mg of brain protein)	Dopamine (µg/mg of brain protein)
Vehicle (0.1 ml WFI)	24.03 ± 1.25	12.86 ± 1.06	30.02 ± 1.40	5.46 ± 0.33	3.38 ± 0.12	0.65 ± 0.03
Rotenone (2, s.c)	35.03 ± 1.33c	5.91 ± 0.43a	3.78 ± 0.25c	2.32 ± 0.12c	1.25 ± 0.08b	0.23 ± 0.01c
Rotenone + Vinpocetine (5)	10.73 ± 1.05***	70.86 ± 2.12***	78.92 ± 2.04***	2.20 ± 0.60ns	4.08 ± 0.44***	0.35 ± 0.02*
Rotenone + ETTS (25)	28.23 ± 1.03ns	13.43 ± 1.02**	40.54 ± 1.63***	2.47 ± 0.41ns	2.58 ± 0.24ns	0.24 ± 0.02ns
Rotenone + ETTS (50)	18.81 ± 1.07*	19.19 ± 1.35***	47.92 ± 1.58***	2.68 ± 0.15ns	3.65 ± 0.50***	0.30 ± 0.03ns
Rotenone + ETTS (75)	15.73 ± 1.22***	32.25 ± 1.86***	54.59 ± 1.96***	2.82 ± 0.21ns	3.74 ± 0.41***	0.37 ± 0.04*

All the values are M ± SEM per group containing 6 animals per group. c$P < 0.001$, b$P < 0.01$ and a$P < 0.05$ compared to vehicle control group. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and ns = non-significant compared to rotenone treated group.

DISCUSSION

PD, a neurodegenerative disease results mainly from the death of dopaminergic neurons in the substantia nigra. Rotenone is extensively studied in animal models of PD recreation. Chronic rotenone treatment causes inhibition of complex I throughout the brain, cell death in the striatum, toxicity to dopaminergic neurons and glutamatergic dysfunction leading to mitochondrial impairment (Alam and Schmidt 2002; Hoglinger et al., 2003; Greenamyre et al., 1999; Bertabet et al., 2000). Rotenone administered in rodent causes highly selective dopaminergic degeneration and alpha-synuclein aggregation in dopaminergic neurons and on chronic subcutaneous exposure reproduce characteristic features of PD via free radicals mediated oxidative stress (Sherer et al., 2007). Previous studies indicated antianxiety, depressant, neurorejuvenating activity of *E. thymifolia* phytosterol. All these profile is suggestive of presence of neuroprotective effect of ETTS that can slow down the progression of PD. Vinpocetine is a synthetic derivative of the Vinca alkaloid vincamine was used as standard drug. Vinpocetine is reported to have cerebral blood-flow enhancing and neuroprotective effects, and is used for treatment of cerebrovascular disorders and age-related memory impairment (Dézsi et al., 2002; Szilágyi et al., 2005). Chronic treatment with vinpocetine exhibit neuroprotective effect in rotenone-induced Parkinsonian rat due to antioxidant action (Szilágyi et al., 2012).

Animals developed symptoms of PD such as body weight loss, motor function impairment, behavioral and brain biochemical changes following 35 days of rotenone (2 mg/kg, s.c) treatment. Rotenone exposure for 35 days was tolerated by animals with a mortality rate of about 12%. The body weight of rotenone treated animals decreased gradually till 2nd week and after 3rd week the decline in the body weight was drastic till the end of 5th week. The observations are slightly different to that reported by Nehru et al. (2008) and Sharma and Nehru (2013). Rotenone exposure related decline in body weight of animals may be related to delay in gastric emptying that occurred during rotenone intoxication (Edwards et al., 1992). Gastrointestinal dysfunction like bloating, dysphagia, constipation and weight loss are the most common symptom of PD (Natale et al., 2008). *E. thymifolia* phytosterol treatment has moderately checked the extent of body

weight loss induced by rotenone. Rotenone treatment caused increase in cataleptic score of the animals 2nd week onward. ETTS and vinpocetine both was ineffective in decreasing catalepsy in all the tested doses. Vinpocetine do not antagonize reserpine induced catalepsy and is reported to reduce efflux of dopamine and acetylcholine evoked by glutamate and NMDA (Kiss et al., 1991). Severe reduction in locomotion, rearing score and rotarod fall off time was observed after 3rd week of rotenone exposure. Co-administration of ETTS showed significant improvement in locomoter activity, rearing score and rotarod fall off time at the end of 3rd week and much evident further at the end of 5th week.

Dopaminergic neurons exert greater oxidative stress than other neurons due to the generation of hydrogen peroxide during monoamine oxidase-driven dopamine metabolism (Kweon et al., 2004). Rotenone causes oxidative stress exhausting the intrinsic antioxidant system that results in cell death due to ATP depletion and complex I inhibition in mitochondria (Naoi et al., 2005). Rotenone exposure significantly decreases GSH level and NO scavenging ability at the end of 5th week. MDA as an indicator of lipid peroxidation, was significantly elevated by rotenone treatment at 5th wee. All these changes signify alteration in the antioxidant defense system concerned with manifestation of the PD symptoms following chronic rotenone treatment. These observations of remarkable changes in biochemical oxidative stress markers following rotenone exposure reinforce the previous findings that free radical generation and oxidative damage is involved in the neuronal abnormalities related to PD (Adams and Odunze 1991; Singh et al., 2007; Kaur et al., 2011). Depletion in the levels of the thiol reducing agents, GSH and GSH disulfide, is the earliest reported biochemical event occurring in the Parkinsonian substantia nigra following selective loss of complex I activity that contribute to subsequent dopaminergic cell death. Subsequent excessive levels of NO in may play an important role in the behavioral deficits and loss of nigral tyrosine hydroxylase-positive neurons observed in the subacute rotenone-induced rat model of PD (Xiong et al., 2015).

Several studies revealed that NO evoked striatal dopamine release and the significant increase in the NO scavenging ability of ETTS treated animals signifies that ETTS could revert the effect of rotenone, stimulating the release of dopamine ameliorating pathological condition of PD (Lorrain and Hull, 1993; Kiss et al., 1999). ETTS treatment reduced lipid peroxidation and simultaneous increase in GSH level. Control over PD can be achieved by regulating the mitochondrial oxidative damage related activity by employing neuroprotective agents. Decreased lipid peroxidation may result in lesser utilization of glutathione which is the first line of defense against free radical insult and thus decrease GSH content found in rotenone along with ETTS treated group. Plant derived antioxidants have been shown to ameliorate the loss of cognitive function by protecting susceptible neurons, maintain motor control to reduce motor complications, and sustain nigrostriatal integrity and functionality reducing PD risk (Vauzour et al., 2008; Gao et al., 2012; Gao et al., 2015). *E. thymifolia* contain phytosterol like kaempferol, cosmosiin, quercetin, β-amyrine, β-sitosterol, campesterol, stigmasterol, epitaraxerol, n-hexacosanol, euphorbol, 24-methylene cycloartenol and cholesterol that are reported to have antioxidant activity (Mosango, 2008). Plants possessing strong antioxidant activity can inhibit generation or causes neutralization of free radicals responsible for lipid peroxidation of neuronal cell membrane. Vinpocetine, ginkgo and curcuma has antioxidant effect on the brain protecting the brain form free radical damage (Yan et al., 2007; Dua et al., 2009).

Dopamine and acetylcholine are two primary factors directly involved in PD. Other CNS neurotransmitters such as acetylcholine, glutamate and GABA also play a role in the pathophysiology of the PD. In fact, neurotransmitter imbalance exists in the extrapyramidal system with DA and GABA deficiency with acetylcholine and glutamate surplus. The significant loss of DA in the striatum plays a critical role in the pathogenesis of PD, resulting in low dopamine and high acetylcholine levels. Earlier study reports showed decline in the dopamine and AchE level following rotenone exposure correlates to significant and progressive decrease in total locomotor activity and increased rigidity (Costall and Naylor, 1974). The loss of dopaminergic inhibition favors an increased level of acetylcholine which causes hyperactivity and due to continuous stimulation without inhibition, the characteristic symptoms of tremor, rigidity and muscle fatigue develop leading to postural instability (Swathi et al., 2013). AchE is the enzyme responsible for termination of acetylcholine action at receptor sites by hydrolysis. The very common approach to treat PD is to increase the levels of dopamine while simultaneously decreasing the levels of acetylcholine by increasing level of AchE enzyme activity. At the end of 5th week, rotenone exposure caused a significant decline in the level of neurotransmitter dopamine and GABA. ETTS has significantly increased dopamine and GABA level but effect on AchE was insignificant indicating its ability of increasing dopamine and GABA level while simultaneously not decreasing acetylcholine availability. Vinpocetine also showed similar profile. Vinpocetine supplementation is reported to prevent fall of mitochondrial membrane potential, rise in ROS generation and acetylcholine esterase activity associated with the hypoxia-reoxygenation injury establishes neuroprotection in primary hippocampal cell culture (Solanki et al., 2011).

Previous study reported potential anti-cholinesterase role of *E. thymifolia* steroid (17). Inhibition or reduced activity of AchE enzyme can curtail the progress of neurodegeneration in Parkinson's and Alzehimer's disease (Bohnen et al., 2012). ETTS corrects motor function abnormality may be by reserving dopaminergic system but not catalepsy due to noninfluence on cholinergic transmission. The PD induced motor dysfunction clearly correlates with the nigrostriatal dopaminergic cell loss and dopamine deficiency within basal ganglia which are essential in regulating the various body movements (Selby, 1990). Thus, supplementation with ETTS showed significant improvement in locomoter activity but not catalepsy at the end of 5th week.

PD devastates the input to striatal GABAergic neurones, so GABA is unable to exert presynaptic inhibitory function and thus glutamate exerts enhanced excitotoxic effect in the extrapyramidal system. ETTS treatment significantly reverted the decline in brain GABA level induced by 5 week rotenone administration. Dopaminergic and muscarinic cholinergic neurons have antagonistic interaction between each other, which is enabled through presynaptic GABAergic neurons via $GABA_A$ receptors and glutaminergic neurons via NMDA receptors (Bosch et al., 2011). Drugs enhancing GABAergic neurotransmission with an agonistic effect at $GABA_A$ receptors could be of therapeutic value in the treatment of PD (Werner and Covenas, 2014). The results of previous study indicated anxiolytic and anti-stress effect of *E. thymifolia* phytosterol implicating involvement with central GABAergic transmission.

E. thymifolia phytosterol showed facilitatory effect on associative learning and memory, reversed acetylcholine deficit induced amnesia acting as a potential anti-cholinesterase, corrects serotonin induced Behavioural performance but devoid of any effect on nigrastratial dopaminergic pathway indicating absence of reinforcing effect. The study demonstrates protective effect of ETTS on dopaminergic neurons against degenerative effects induced by rotenone may be by virtue of free radical scavenging ability causing efficient reduction of rotenone induced lipid peroxidation and oxidative stress. ETTS may provide protection to the antioxidant defense system in dopaminergic neurons hence, lesser dopaminergic neurodegeneration. Present study demonstrated significant improvement in the neurobehavioral, oxidative as well as neurochemical parameters following chronic administration of ETTS as caused by rotenone exposure. ETTS given with rotenone attenuated the rotenone-induced oxidative stress and exhibited dose dependent protective effect in PD induced animals. The levels of glutathione and scavenging ability of MDA and NO has increased in the brain of animals with PD as a compensatory mechanism to deal with oxidative stress. ETTS possess neuroprotective effects with regard to positive changes in body weight, rearing behavior, grip strength, locomotion, neurotransmitter and oxidative imbalance in PD induced animals.

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