Neuroprotective evaluation of *Tribulus terrestris* L. in aluminum chloride induced Alzheimer's disease

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Abstract: *Tribulus terrestris* (*T.T*) is enriched with steroidal saponins and flavonoids which have neuroprotective effect. The study was aimed to explore the potential of *T.T* methanol extract (*T.T* ME) for anti-Alzheirmer activity along with its safety evaluation. Plant was characterized by physicochemical, phytochemical and GCMS analyses whereas acute oral toxicity (OECD 425) was performed for safety evaluation. $AlCl_3$ induced Alzheimer's disease rat model was used for anti-alzheirmer activity. *T.T* ME was given orally at 100, 300 and 1000 mg/kg doses for 21 days and behavioral parameters were observed on 22nd study day. Physicochemical parameters were in permissible limits. GCMS analysis showed eight different compounds and benzene dicarboxylic acid showed maximum % peak area (64.19). No mortality was noted in acute toxicity study. Behavioral studies showed highly significant ($p<0.001$) improvement in *T.T* ME treated groups. Antioxidant enzymes and acetylcholinesterase levels were significantly ($p<0.05$) improved on treatment with *T.T* ME. Histopathological analysis indicated that neurofibrillary tangles were significantly improved in *T.T* ME treated groups. Biochemical and behavioral results suggested that *T.T* contained lead compounds which are effective in the treatment of Alzheimer disease.

Keywords: Aluminum chloride, anti-Alzheimer activity, *Tribulus terrestris.*

INTRODUCTION

In the developed countries neurodegenerative disorders are becoming more prevalent among people over 65 years due to genetic and environmental factors. It is predicted that 1 among 85 individuals will be affected by Alzheimer's disease (AD) in 2050 (Uddin *et al*., 2016). AD is a progressive neurodegenerative disorder which is mainly characterized by neuronal loss in brain which results in short term memory loss and cognitive impairments. The initial clinical sign is loss of short term memory and with prognosis of disease worsening of cognitive and learning disabilities signs as forgetting names and words during speech, mood swings, inability to calculate, inability to use daily living objects and tools appeared (Lakshmi *et al*., 2015). Pathological hallmarks include apoptotic neuronal death, highly phosphorylated tau proteins over expression (Nampoothiri *et al*., 2015), neurofibrillary tangles, amyloid plaques (due to degeneration of neuronal processes beta- amyloid proteins are accumulated), oxidative stress and cholinergic dysfunction (Bais *et al*., 2017). It is reported that excessive oxygen species and free radicals are the causative factors in pathogenesis of AD (Kimura & Ohno, 2009). Aluminum is a well-known neurotoxin ubiquitous at earth crust after oxygen and silicone has a valuable role in AD pathology due to its reported neurotoxic and cholinotoxic effects in animal experimentation and clinical studies (Walton, 2012). It has ability to enter and accumulate in central nervous system and alter the level of acetylcholine which have main role in neurochemistry of AD. Pharmaceuticals available for treatment of AD such as acetyl cholinesterase inhibitors drugs i.e. rivastigmine, donepezil, memantine, glantamine and secretase inhibitors have many side effects (Müller, 2007). Now a day in therapeutic research there is a need of novel, effective and safe natural medicines to treat neurodegenerative disorders to avoid side effects associated with synthetic medicines. Phytomedicines being natural remedy served humanity since ancient time. It is reported in animal experiments and clinical studies that *Tribulus terrestris* L. (*T.T*) family Zygophyllaceae have immense medicinal importance in neurodegenerative disorders due to its constituents such as steroidal saponins (Sun *et al*., 2015), flavonoids (Kumar & Khanum, 2012), spirostanol and furanostol steroids, alkaloids and cinammic acid. *T. T* is traditionally used in developing countries for infertility treatment in males due to its popular aphrodisiac properties. This plant is effective in treatment of asthma, urinary dysfunctions, vasoconstriction, hypertension, opthalmia (Qureshi *et al*., 2010). It has antioxidant, anticancer, anthelmintic, antimicrobial and antifungal properties (Al-Bayati & Al-Mola, 2008). Up till now scientific data on

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neuroprotective effect of *T.T* ME on aluminum chloride induced AD model is not reported. The current study was therefore aimed to evaluate anti-neurodegenerative effect of *T.T* ME in aluminum chloride induced AD model.

MATERIALS AND METHODS

Chemicals and drugs

Ethanol, chloroform, Folin-Ciocalteau reagent, gallic acid piperine, bovine serum albumin, sodium hydroxide, aluminum nitrite, quercetin, aluminum chloride and methanol of analytical grades were purchased from sigma-Aldrich (USA), rivastigmine was obtained from Novartis Pharma (Pvt.) Ltd Pakistan.

Animals

Fifteen weeks old healthy Wistar rats (male) with weight range of 200-250 g (for neuroprotective study), and nine weeks old nulliparous and non-pregnant female rats (for acute oral toxicity study) were purchased from animal house of University of Agriculture, Faisalabad, Pakistan and placed in Government College University Faisalabad animal house one week prior to the start of experimentation in order to acclimatize the animals. Animal house was maintained at standard conditions (25±3^{o}C room temperature, 12-hour light and dark cycles and 30-60% humidity). Animals were caged separately in groups. They had free excess to food and water.

Plant collection and extraction

T.T fruits were collected from West canal offices Faisalabad in the month of June 2017. This plant was identified by Botany Department of University of Agriculture Faisalabad Pakistan and a voucher specimen was submitted in herbarium after authentication No: 625-1-2018. It was dried under shade for 2 months. It was grounded into a fine powder. *T.T* methanol extract (*T.T* ME) was prepared by macerating 2 kg powder in 5 L of methanol.

Quantitative phytochemical analysis of T.T ME

Saleem *et al*. (2014) method was adopted for quantification of primary metabolites (total proteins, total carbohydrates, total lipids, total glycosaponins) and secondary metabolites (total pholyphenolics, total flavonoids and total alkaloids).

GC-MS analysis to determine chemical composition of T.T ME

GCMS analysis was conducted using Clarus system 500 perkin Elmer. Gas chromatograph was equipped with turbo mass gold mass detector Perkin Elmer with elite -1 spectrometer (100% dimethy poly siloxane), 30 m × 0.25 mm ID × 1 µm capillary column. Flow rate of helium gas was maintained at the rate of 1 mL/min. Initial temperature was set at 110 ºC and raised upto 280 ºC (with an increase of 5 ºC per minute) and maintained for 9 min. Injection volume (2 µL) at the temperature of 250 ºC at an ionization voltage of 70 eV was used in split ratio. Mass spectral range was set at 45-450 (m/z) with 0.5 second of scan interval. Total time of GCMS running was 36 min with solvent delay 0-2 min. The percentage amount of each component was calculated by comparing its average peak area to total peak area. Software of turbo mass 5.2 was used to analyze mass spectra and chromatogram.

The unknown compounds present in methanol plant extract was compared with known compounds which are stored in database of national institute and technology library (NIST). The name, molecular weight and structure were confirmed from NIST library.

Approval from animal ethics committee

Animal studies were conducted after getting approval from Institutional Review Board (IRB)/Animal Ethical Committee GC University with an authorized number; GCUF/ERC/1980. Experimental procedures were according to rules of Institute of Laboratory Animal Resources, Commission on Life Sciences University, National Research Council (1996).

Acute oral toxicity study

OECD test guidelines 425 were adopted to study acute oral toxicity of *T.T* ME Limit dose (2000 mg/kg) was administered orally to a single female rat which kept under strict observation for initial 30 min and then for 4 hr consecutively. After survival of this female rat four more animals were given same dose. Five animals was kept in control group and given 1% CMC. All the treated and control group animals were handled in an identical manner. Both groups were closely monitored after every 30 min for 6 hr and then after regular intervals for 14 days. Changes in body weight and behavioural parameter were recorded at regular time intervals. After fourteen days, weights of all animals were recorded and blood samples were collected under anaesthesia by cardiac puncture. Serum was separated for biochemical and haematological examinations. Vital organs (heart, liver and kidney) of each animal were separated after cervical dislocation and preserved in 10% formalin solution for histopathological examination (Guideline, 2001)

Experimental study design

Healthy male Wistar rats were selected for this study and divided into six groups (n=6).

Group I: Served as control group and treated with vehicle (distilled water) 1 mL/kg.

Group II: Served as disease control and treated with aluminum chloride 100 mg/kg, p.o.

Group III: Served as standard group and treated with aluminum chloride 100 mg/kg, p.o. and rivastigmine 100 mg/kg, p.o.

Group IV: Treated with aluminum chloride 100 mg/kg, p.o. and *T.T* ME 100 mg/kg p.o.
Group V: Treated with aluminum chloride 100 mg/kg, p.o. and *T.T* ME 300 mg/kg p.o.
Group VI: Treated with aluminum chloride 100 mg/kg, p.o. and *T.T* ME 1000 mg/kg p.o.

Animals in each group were treated daily for 21 days. Behavior and weight were recorded at the start and at the end of study period. After 24 hr of administration of last dose, animals were sacrificed under light anesthesia by cervical dislocation and brains were isolated, washed with phosphate buffer and preserved at −80 ºC for biochemical and histopathological studies.

Behavioral analyses

Water maze was designed to investigate spatial learning, memory and task strategy. Morris water maze test was performed according to method by Morris (Morris, 1984).The open field test was used for simultaneous investigation of exploration, anxiety and locomotion in animals (Brown *et al*., 1999). Time spent in central area, stretch attend postures, grooming, defecation, rearing and freezing were observed according to Brown *et al*. (1999).Y- maze task is used in behavioral neurosciences to determine the spatial memory, short term memory and cognitive defect in rodents. Y- Maze task was performed according to method adopted by Aydin *et al*. (2006) with little modification. Anxiety and exploratory behavior of rats were observed by head dipping in hole board apparatus. This test was performed according to method adopted by Tillerson & Miller. (2003). Wire hanging test was performed to investigate the neuromuscular strength of animal according to method of Chitra *et al*. (2017). Apparatus used for this test was constructed of horizontal stainless steel grids placed on three inches wide and 50 cm high wooden walls. The hanging time was recorder from 30 seconds to 1 minute. Elevated plus-maze task was used to evaluate the memory and exploration in exteroceptive behavioral model. This test was performed and results were interpreted according to method adopted by Parle *et al*. (2005). At day first (at 20th day of study) animals were gently placed at an open arm facing away from platform and transfer latency was recorded. After 24 hours (at 21st day of study) same procedure was repeated and transfer latency was recorded to analyze cognition, learning and memory deficit (Parle *et al*., 2005).

Estimation of biochemical parameters

Each animal was sacrificed under light anesthesia of isoflurane and brain was removed by cervical dislocation. Isolated brains were washed with ice cold normal saline to remove blood and stored at –80 °C. Brain homogenates were prepared in a tissue homogenizer by using 0.1 M phosphate buffer (pH 7.4) which was composed of 1mmol ethylene diamine tetra-acetic acid (EDTA), 10 mM potassium chloride, 0.25 M sucrose and 1 mM phenylmethyl sulfonylfluoride. It was centrifuged at 800 rpm at 4°C for thirty minutes to obtain supernatant (Lakshmi *et al*., 2015)

MDA level indicates the level of lipid peroxidation which was estimated by the method of Ohkawa *et al*., (1979). Catalases level was estimated by method of Aebi. (1984) with some modifications. Superoxide dismutase (SOD) activity was estimated by adopting method of Kakkar *et al*. (1995). For estimation of glutathione peroxidase a reaction mixture was formulated by method adopted by Chirta *et al*. (2017).

Estimation of acetylcholinesterase activity

Phosphate buffer (2.6 mL, pH 8.00, 0.1M) was added to 100 µL of 2,4 dithiobis nitrobenzoic acid and 20 µL of acetylthiocholine iodide. An aliquot of tissue homogenate (0.4 mL) was added to this mixture. In this test yellow color was appeared due to reaction of 2,4 dithiobis nitrobenzoic acid with thiocholine and absorbance was recorded at 412 nm. Acetyl cholinesterase activity was calculated by using following formula

$$R = 5.74 \times 10^{-4} \times \frac{A}{CO}$$

Where CO is the original concentration of tissue (mg/mL), A is the change in absorbance /min and R is the rate in moles of substrate hydrolyzed /min/gram of tissue (Lakshmi *et al*., 2015).

Observation of histopathological alteration in rat brain

To investigate the neurodegeneration animals were sacrificed in each group (control, disease control, standard, 100 mg/kg, 300 mg/kg and 1000 mg/kg) by cervical dislocation and brains were isolated and fixed in 4% paraformaldehyde. Transverse sections of 5 µm thickness of paraffin embedded brain tissues were sliced with microtome, stained with hematoxylin and eosin dye and observed under microscope.

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM. One and Two way ANOVA followed by bonferroni post-test were applied for statistical analysis by using graphpad prism version 5. $P<0.05$ was set as statistically significant value.

RESULTS

Quantitative phytochemical analysis of T.T ME

Primary metabolites (total-proteins, -carbohydrates, -glycosaponins and -lipid contents) were quantified and their percentage in the extract in decending order is as follows: Total carbohydrates (60%) > total glycosaponins (48.5%) > total lipid contents (33.3%) > total proteins (11.37%). Linear regression equation "y = 0.024x and R^2 = 0.9867" constructed from various concentrations of bovine serum albumin was used to calculate total proteins. Among secondary metabolites, total polyphenolics were found in highest concentration i.e. 23% whereas total alkaloids and total flavonoids were 18% and 4% respectively (fig. 1). Gallic acid standard

curve with linear regression equation y = 0.0006x and R^2 = 0.9957 was used quantifying alkaloids, whereas quercetin standard curve with linear regression equation "y = 0.001x and R^2 = 0.9748" and piperine standard curve with linear regression equation "y = 0.00396x and R^2 = 0.9954" were employed in quantification of flavonoids and polyphenolics respectively.

GCMS analysis of T.T ME

GC-MS analysis of *T.T* ME clearly indicated the presence of eight different compounds (fig. 2). Results revealed that pthalic acid (1,2-benzenedicarboxylic acid) (64.19%) was found as major component in *T.T* ME and seven minor components such as pregnane-11,20-dione, 3,21bis[(trimethylsilyl)oxy]-,20-[O(phenylmethyl)oxime] (7.06%), Cortol, penta-trimethylsilyl ether (3.31%), Silane,trimethyl(phenethylthio)(14.95%),3Acetoxydodecane (2.24%), Phenyl acetic acid (Benzeneacetic acid) (0.71%), Tetradecanoic acid (2.74%) and Anthiaergostan-5,7,9-trien,15-[2,4-dinitrophenylazo] (4.83%) (table 1).

Acute oral toxicity study

Minor changes were noted in behavioral parameters of treatment group as elevation of fur was observed throughout the study immediately after dosing for next two hours then it became normal. Respiration rate increased which got normal after 30 min of dosing. Somatomotor activity was also slightly increased during the study period. Other behavioral parameters were quite normal when compared to control group. Body weight of treatment and control groups animals raised in identical manner. No mortality was recorded during the study so LD_{50} was found greater than 200 mg/kg.

Effects of T.T ME on hematological, biochemical parameters in acute toxicity study

Table 2 showed the effect of *T.T* ME on hematological parameters in which no significant change was noted in hemoglobin, TLC, RBC, HCT, MCV, MCH, MCHC, neutrophils, lymphocytes, monocytes and eosinophil's as compared to control group. Platelet count increased significantly ($p<0.001$) as compared to control group. Cholesterol level decreased significantly ($p<0.001$) whereas triglycerides increased significantly ($p<0.001$) when compared with control group values. HDL, LDL, VLDL levels of treatment group showed non-significant change with reference to control group values (table 3). Blood urea and creatinine values ($p > 0.05$) exhibited non-significant change with reference to control group values (Table 4). ALT, AST, protein, albumin, globulin, A/G ratio of treatment group were changed non-significantly as compared to control group values whereas alkaline phosphatase level increased significantly ($p< 0.001$) as compared to that of control group which is indicative sign of biliary obstruction (table 4).

Histopathological examination in in acute toxicity study

Histological examination indicated preserved architecture of cardiac tissues, hepatocytes and kidney tissues in *T.T* ME treatment group identical to control group organ architecture. *T.T* ME did not produce unfavorable effects at physiological parameters. Although some changes in biochemical parameters (increased level of alkaline phosphatases) were observed in treatment group due to biliary tract obstruction (fig. 3).

Neuroprotective study

Effect of T.T ME on behavioral parameters in aluminum chloride induced AD model

Morris water maze task indicated that after training session the escape latency (in seconds) was increased significantly ($p<0.001$) in disease control group as compared to control and treatment groups. The escape latency was improved in *T.T* ME treatment groups and in standard group. Visual behavioral assessment indicated that animals in aluminum chloride induced model group were slowed in speed toward platform and thigmotaxis behavior (time spent at periphery of pool as an index of anxiety and platform searching strategy) was greater as compared to control and *T.T* ME treatment groups (table 5).

Neurobehavioral observation of open field test indicated that time spent at periphery was greater significantly ($p < 0.001$) as compared to time spent at center of apparatus among all groups. Total distance travelled in apparatus, rearing and duration at center were decreased in disease control group. Defecation, freezing and stretch attend postures were observed more frequently in disease control group. Control, *T.T* ME treatment groups and standard groups travelled more distance and crossed a more number of lines at periphery of chamber as compared to disease control group as depicted in (table 6).

The stepdown latency was decreased significantly ($p< 0.05$) in disease control as compared to control group and is significantly improved in *T.T* ME treatment group and in standard group. Retention time at platform was greater in control, *T.T* ME treatment and in standard group (Fig. 4)

In Y-maze task it was investigated that there was a significant difference in the percentage of spontaneous alteration among all groups. Percentage of spontaneous alteration was decreased significantly ($p<0.001$) in disease control group as compared to control and other *T.T* ME treatment and standard group (table 7)

It was investigated that the number of head dipping, locomotion and exploratory behavior was decreased significantly ($p<0.05$) in disease control group as compared to control group. The exploratory behavior and head dipping was significantly improved in standard and *T.T* ME treatment group in such manner 100 mg/kg <300 mg/kg <1000 mg/kg (fig. 5).

Table 1: GC- MS analysis of *T.T* ME

No	RT	Name of compound	M. Wt	Molecular formula	Peak area %
1	4.437	Pregnane-11,20-dione (Neuroactive steroid)	597	$C_{34}H_{55}NO_4Si_2$	7.0
2	4.832	Cortol, penta trimethylsilyl ether	728	$C_{36}H_{76}O_5Si_5$	3.31
3	10.759	Silane	210	$C_{11}H_{18}SSi$	14.95
4	14.628	Acetoxydodecane	228	$C_{14}H_{28}O_2$	2.24
5	15.026	Phenyl acetic acid	218	$C_{14}H_{18}O_2$	0.71
6	15.382	Myristic acid	256	$C_{16}H_{32}O_2$	2.74
7	16.587	Anthiaergostan-trien	574	$C_{34}H_{46}N_4O_4$	4.83
8	19.198	Pthalic acid	390	$C_{24}H_{38}O_4$	64.19

Table 2: Effect of *T.T* ME on hematological and biochemical parameters (acute toxicity study)

Parameter	Unit	Control	Treatment
Haemoglobin	g/Dl	13.38 ± 0.33	13.65 ± 0.44 ^{ns}
TLC	$\times10^9$/L	11.83 ± 0.44	10.60 ± 0.26 ^{ns}
RBC	$\times10^{12}$/L	6.43 ± 0.23	6.26 ± 0.31 ^{ns}
HCT	%	42.46 ±1.34	37.70 ± 0.40 ^{ns}
MCV	Fl	62.13 ± 2.43	56.10 ± 1.03 ^{ns}
MCH	Pg	22.10 ± 1.64	18.43 ± 0.29 ^{ns}
MCHC	%	33.06 ± 0.48	33.69 ± 0.64 ^{ns}
Platelets	$\times10^9$/L	583.33 ± 9.82	796.33 ± 19.5 ***
Neutrophils	%	9.00 ± 2.08	2.66 ± 0.88 ^{ns}
Lymphocytes	%	84.66 ± 3.71	94.66 ± 1.20 ^{ns}
Monocytes	%	1.66 ± 0.39	1.50 ± 0.28 ^{ns}

Values are expressed as mean ± SEM, ****P<0.001* and ns: non - significant as compared to control.

Table 3: Effect of *T.T* ME on lipid profile (acute toxicity study)

Parameter	Unit	Control	Treatment
Cholesterol	mg/dL	140.6 ± 2.33	126.6 ± 1.45 ***
Triglycerides	mg/dL	113.3 ± 0.88	110 ± 0.57 ^{ns}
HDL	mg/dL	41.6 ± 1.76	44.0 ± 1.154 ^{ns}
LDL	mg/dL	123.0 ± 1.52	120.3 ± 0.88 ^{ns}
VLDL	mg/dL	20.0 ± 1.54	25.33 ± 2.9 ^{ns}

Values are expressed as mean ± SEM, ****P<0.001* and ns: non - significant as compared to control.

Table 4: Effect of *T.T* ME on liver and kidney function test (Acute toxicity study)

Parameter	Unit	Control	Treatment
Bilirubin	mg/dL	0.70 ± 0.11	0.70 ± 0.05 ^{ns}
ALT	µ/L	29.33 ± 1.76	23 ± 0.57 ^{ns}
AST	µ/L	30.66 ± 1.76	32 ± 1.15 ^{ns}
Alkaline Phosphatase	µ/L	180.00 ± 17.32	355 ± 2.88 ***
Protein	g/dL	6.69 ± 0.11	6.63 ± 0.18 ^{ns}
Albumins	g/dL	4.43 ± 0.29	3.96 ± 0.12 ^{ns}
Globulin	g/dL	2.53 ± 0.27	2.80 ± 0.11 ^{ns}
A/G		1.90 ± 0.11	1.43 ± 0.88 ^{ns}
Blood urea	mg/dL	38.00 ± 1.52	33 ± 1.52 ^{ns}
Serum creatnine	mg/dL	0.80 ± 0.05	0.76 ± 0.02 ^{ns}

Values are expressed as mean ± SEM, ****P<0.001* and ns: non - significant as compared to control.

Table 5: Effect of *T.T* ME on Morris water maze in $AlCl_3$ induced AD model

Group	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 4
	Escape latency in seconds			
Control	19.33±1.2	23.67±1.2	22.00 ± 1.5	19.3 ± 1.2
Disease control	43.33±1.2***	40.00 ± 2.0***	42.66 ±1.4***	45.33 ±1.7***
Standard	21.00±0.5 ns	22.33 ± 0.8 ns	20.00 ± 0.5ns	19.00 ± 0.5ns
100 mg/kg	36.00 ± 1.5***	32.66 ± 0.8***	38.00 ± 0.5***	32 ± 0.5***
300 mg/kg	32.00 ± 1.2***	28.3 ± 0.3 ns	30.3 ± 0.8***	34.00 ± 0.5***
1000 mg/kg	19.00 ± 0.5 ns	23.61 ± 0.3 ns	23.31 ± 0.3 ns	20.00 ± 0.5 ns

Values are expressed as mean ± SEM, ****P<0.001* and ns: non - significant as compared to control.

Table 6: Effect of *T.T* ME on open field test in $AlCl_3$ induced AD model

Group	Total No. of lines crossed	Freezing (second)	Rearing /10 min	Grooming /10 min
Control	27.00 ± 0.5	0.00 ±0.0	5.00 ± 0.2	3.00 ± 0.5
Disease control	9.00 ± 0.0***	80.0± 0.5***	0.00 ± 0.0***	0.00 ±0.0***
Standard	25.66 ±0.6 ns	10.33 ± 0.8***	5.00 ± 1.5 ns	3.66 ± 0.8 ns
100 mg/kg	19.30± 1.8***	30.00 ± 1.15***	4.66 ± 1.2 ns	5.00 ± 0.5 ns
300 mg/kg	21.00 ± 0.5**	19.00 ± 0.5***	3.66 ± 0.3 ns	2.66 ± 0.3 ns
1000 mg/kg	24.00± 0.5 ns	0.33 ± 0.0 ns	5.00 ± 0.5 ns	4.00 ± 0.5 ns

Values are expressed as mean ± SEM, ****P<0.001* and ns: non - significant as compared to control.

Table 7: Effect of *T.T* ME on Y-maze test in $AlCl_3$ induced AD model

Groups	Total No. of arm entries	Total No. of triads	% Spontaneous alteration	Laterality index
Control	11.00 ± 0.5	3.00 ± 0.0	38.00 ± 0.5	0.14
Disease control	2.33 ± 0.33***	0.00 ± 0.0***	0.00 ± 0.0***	-0.33
Standard	11.00 ± 0.5 ns	3.33 ± 0.3 ns	37.00 ± 0.5 ns	0.14
100mg/kg	8.00 ± 0.5***	1.00 ± 0.0**	21.33 ± 0.8***	-0.2
300mg/kg	7.66 ± 0.33***	2.00 ± 0.0 ns	34.14 ± 0.4***	0
1000mg/kg	15.66 ± 1.7 ns	4.00 ± 0.0 ns	37.1 ±0.49 ns	0.1

Table 8: Effect of *T.T* ME on antioxidant parameters in $AlCl_3$ induced AD model

Groups	CAT (IU/µL)	SOD (IU/µL)	MDA (TBA mg/mL)	GSH µ/mg protein	GPx µ/mg protein
Control	0.717±0.006	0.06±0.002	73.33±0.47	0.73±0.012	9.50±0.25
Disease control	0.54±0.001***	0.024±0.001***	98.07±0.36***	0.24±0.009***	3.20±0.05***
Standard	0.711±0.005 ns	0.059±0.002 ns	70.49±0.36 ns	0.68±0.009 ns	8.46±0.27 ns
100 mg/kg	0.67±0.001 ns	0.034±0.002***	75.70±0.27 ns	0.37±0.015***	4.36±0.27***
300 mg/kg	0.693±0.009 ns	0.036±0.009***	61.58±3.34***	0.55±0.02***	10.46±0.08***
1000 mg/kg	0.703±0.012 ns	0.062±0.002 ns	46.23±1.92***	0.71±0.009 ns	10.10±0.49 ns

Values are expressed as mean ± SEM, (n = 6), ****P<0.001* and ns: non - significant as compared to control.

We observed that wire hanging time was decreased significantly ($p<0.05$) and falling down was significantly increased in disease control group as compared to control group, standard and *T.T* ME treatment groups. There was non-significant ($p>0.05$) difference in wire hanging time and falling down among control group standard and *T.T* ME treatment groups (fig. 6).

We found that in elevated plus maze experiment the transfer latency in control group was decreased significantly ($p<0.001$) as compared to disease control group at 20th and 21st day. The transfer latency was significantly improved in *T.T* ME treatment group and standard group as compared to disease control group (fig. 7).

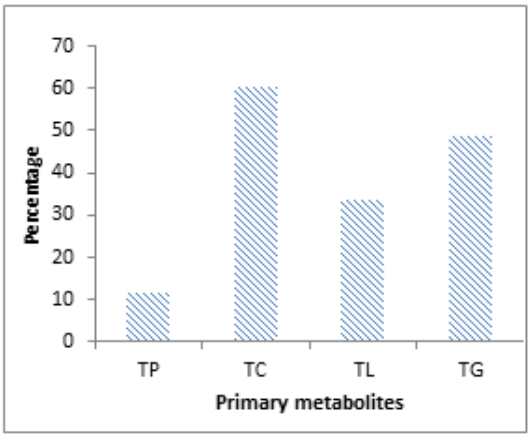
Effect of T.T ME on biochemical parameters in aluminum chloride induced AD model

It was evaluated in biochemical parameters estimation that $AlCl_3$ exposure produced the significant decrease (p

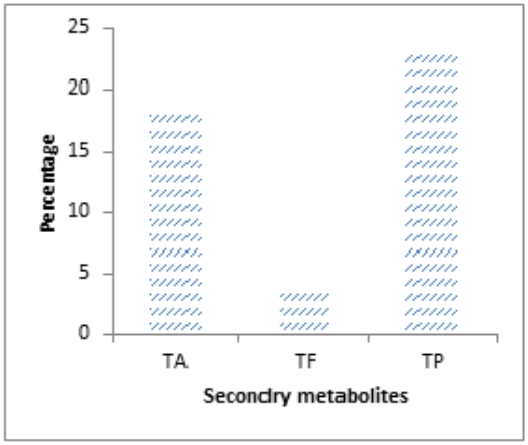
>0.05) in the level of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) level in disease control group. *T.T* ME and rivastigmine significantly improved the level of CAT, SOD, GPx and GSH *T.T* ME treatment group (1000 mg/kg) and in standard group. Melondialdehyde (MDA) was significantly increased (p>0.05) in disease control group and significantly recovered in *T.T* ME 1000 mg/kg treatment group (table 8).

Effect of T.T ME on acetyl cholinesterase activity in aluminum chloride induced AD model

We observed that acetyl cholinesterase (AchE) activity was significantly decreased (p>0.05) in disease control group as compared to control group. AchE activity was recovered significantly in *T.T* ME treatment groups (100 mg/kg < 300 mg/kg <1000 mg/kg) (fig. 8).



TP: Total proteins, TC: Total carbohydrates, TL: Total lipids, TG: Total glycosaponins



TA: Total alkaloids, TF: Total flavonoids, TP: Total Polyphenolics

Fig. 1: Estimation of *T.T* ME primary and secondary metabolites.

Effect of T.T ME on histopathology of brain tissues in aluminum chloride induced AD model

Histopathological examination of transverse section of control group indicated the normal architecture. Gross neurodegenerative pathological signs such as neurofibrillary tangles and vacuolated cytoplasm and pigmentation were observed in disease control group. In standard group and *T.T* ME treatment groups (100 mg/kg and 300 mg/kg) neuronal loss and neurofibrillary tangles were slightly improved, whereas in *T.T* ME treatment group (1000 mg/kg) neurofibrillary tangles and neuronal loss were significantly improved (fig. 9).

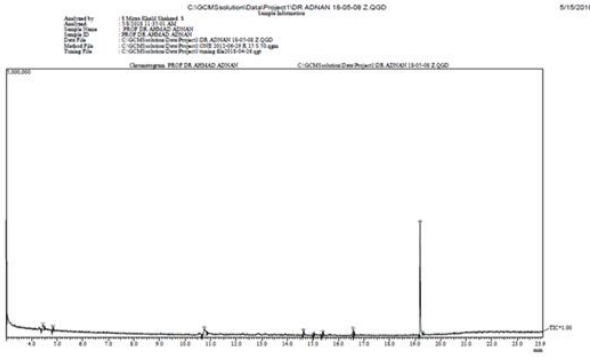


Fig. 2: GC-MS chromatogram of *T.T* ME

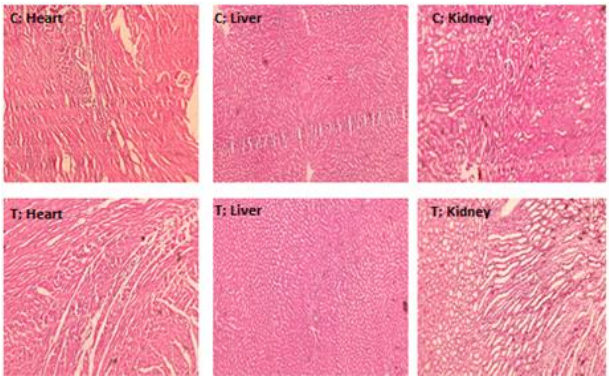
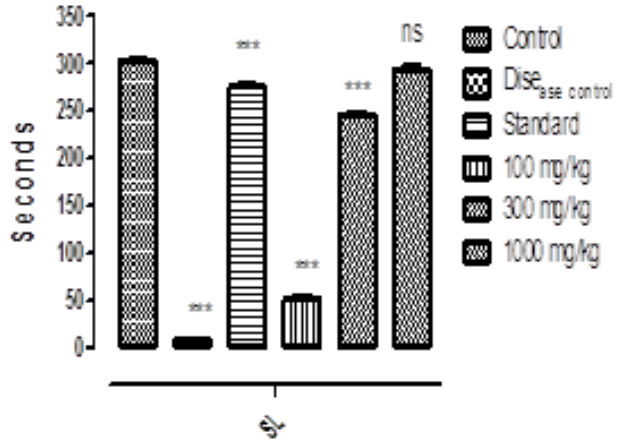


Fig. 3: Histopathological examination of heart, liver and kidney of control group (C) and treatment group (T).

DISCUSSION

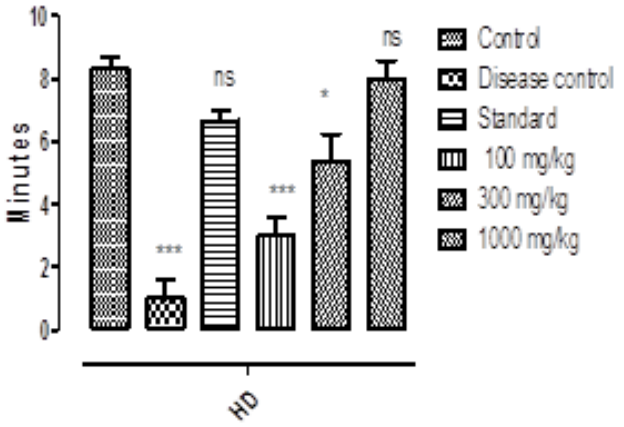
In modern health care system neurodegenerative disorders particularly AD are gathering momentum. Deficit of particular treatment, inflated morbidity and expensive medications of neurodegenerative disorders elucidate the significance of research on revolutionary herbal neuroprotective agents. Phytochemical analyses of *T.T* confessed its medicinal importance due to existence of saponins, alkaloids, flavonoids and polyphenolics. Food supplements and plant extracts enriched with flavonoids are responsible for holding the potential to restrain neurodegeneration and improve memory, cognitive functions and learning. Flavonoids rich plant extracts are effective in neurodegenerative disorders by their modulatory action on intracellular signaling pathways which promote cell survival and by modulating age-related neuronal functions. *T.T* has a favorable amount of flavonoids and polyphenolics which are strong antioxidant and has anti- ischemic activity can be considered as precursor molecule for development of

novel neuroprotective agents. Flavonoids actuate neuroprotective potential mostly by stimulating neuronal regeneration and by curtailing oxidative stress (Luo *et al*., 2002).



SL: Step down latency, Values are expressed as mean ± SEM, (n = 6), ****P<0.001* and ns: non - significant as compared to control.

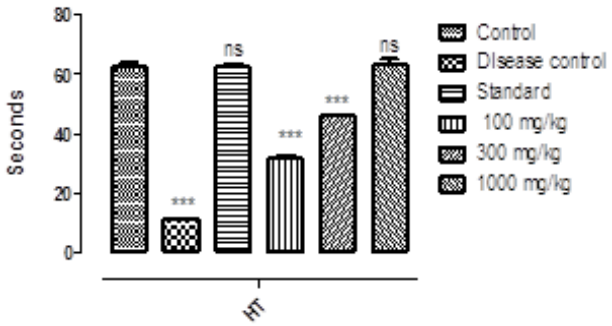
Fig. 4: Effect of *T.T* ME on passive avoidance test in $AlCl_3$ induced AD model



HD: Head dipping, Values are expressed as mean ± SEM, (n = 6), ****P<0.001* and ns: non - significant as compared to control.

Fig. 5: Effect of *T.T* ME on hole board test in $AlCl_3$ induced Alzheimer ś disease model

Therefore flavonoids existing in *T.T* extract can be considered as lead compounds for development of neuroprotective agents. *T.T* antioxidant activity and inhibition of lipid peroxidation was demonstrated by Zheleva-Dimitrova *et al*., (2012).The neuroprotective and antioxidant potential of *T.T* are evident by our findings of GCMS analysis which revealed the presence of eight different compounds. In present study GCMS analysis of *T.T* ME revealed the presence of benzene dicarboxylic acid with maximum peak area. Our findings of GCMS analysis are harmonious with investigations of Jung Choi *et al*. (2009). They evaluated the neuroprotective effect of benzenedicarboxylic acid dinonyl ester by decreasing oxidative stress. They isolated benzenedicarboxylic acid dinonyl ester from ethanol extract of *Rosa laevigata*. They evaluated that infusion of benzenedicarboxylic acid dinonyl ester into mice improved the learning and memory disabilities as well as improved catalase activity and acetylcholinesterase level (Jung Choi *et al*., 2009). Pregnane-11, 20-dione a neuroactive phytosteroid have antioxidant activity (Mooradian, 1993). Cortol, penta-trimethylsilyl ether and its derivatives reported in previous studies have antioxidant potential (Girao *et al*., 1999). It was reported by Gold & Grieb that phenyl acetic acid reinforce endogenous antioxidant activity (Glód & Grieb, 2004). Henry *et al*., (2014) have reported in his study that fatty acids present in food have strong antioxidant activity with longer chain length as mysistic acid (Henry *et al*., 2002). Benzoic acid derivative "benzene dicarboxylic acid" also has strong antioxidant potential (Velika & Kron, 2012). Therefore *T.T* may have effective role in oxidative stress induced neurodegenerative disorders like AD. Aluminum is ubiquitous at our earth crust and implicated in etiopathogenesis of neurodegenerative disorders characterize by neuropsychiatric, behavioral changes and cognitive impairments like semantic memory, altered visuoperceptions, impairment of working memory, lack of learning in new information.

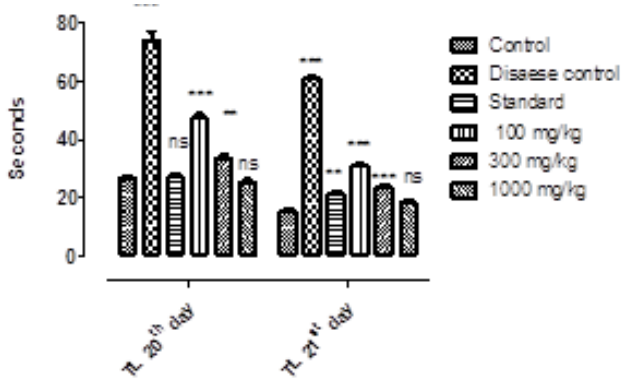


HT: Hanging time, values are expressed as mean ± SEM, (n = 6), Values are expressed as mean ± SEM, (n = 6), ****P<0.001* and ns: non - significant as compared to control.

Fig. 6: Effect of *T.T* ME on wire hanging test in $AlCl_3$ induced AD model

Our study explicated neuroprotective activity of *T.T* in aluminum chloride induced behavioral and biochemical changes attributed by oral administration of aluminum chloride for twenty one days. Aluminum is ubiquitous at our earth crust and implicated in etiopathogenesis of neurodegenerative disorders characterize by neuropsychiatric, behavioral changes and cognitive impairments like semantic memory, altered visuoperceptions, impairment of working memory, lack of learning in new information. Aluminum has ability to amass in all brain regions typically in hippocampus and cortex (Deloncle & Guillard, 1990). Hippocampus and cortex are principally concerned with memory and

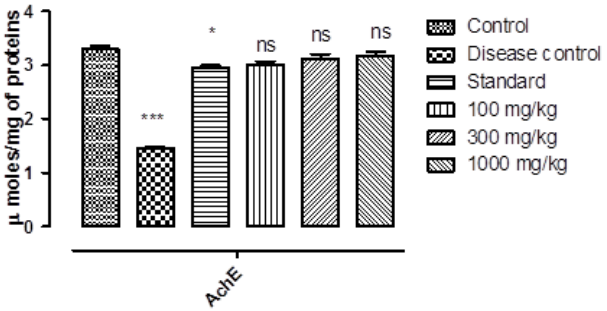
perception, which are critically influenced in AD (Buckner *et al*., 1999). Exposure to aluminum triggered locomotor and cognitive deterioration indicating its CNS depressant activity (Bhalla *et al*., 2010). Cognitive behavioral alterations are conjugated with locomotor disabilities due to CNS depressant effects of aluminum chloride. In current study impairment in spatial memory, task strategy and locomotion were obvious from reduced execution in Morris water maze test, open field test, Y maze task and elevated plus maze task. Morris water maze test findings of increased escape latency in aluminum chloride treated groups congruous with previous studies of Petrasek *et al.* (2018) and *T.T* ME treated groups displayed improved latency indicating neuroprotective effect of *T.T*. In open field test decline in exploration, learning and locomotion on treatment with aluminum chloride is compatible with previous study (Thenmozhi *et al*., 2015) was significantly improved by administration of *T.T* ME. In Y- maze task it was investigated that sustained cognition, short term and innate ability of rodents to alternate the arms or neophilia was impaired by chronic administration of aluminum chloride. *T.T* ME treated groups and rivastigmine (standard drug) treated groups showed the improved cognitive behavior and locomotion in Y-maze task, these findings were in corroboration with study of Hrnkova *et al*. (2007). Our findings of wire hanging test suggested that *T.T* consumption enhance neuromuscular strength which was declined by chronic administration of aluminum chloride.



TL 20th day: transfer latency at 20th day, TL 21st day: transfer latency at 21st day, Values are expressed as mean ± SEM, (n = 6), ****P<0.001* and ns: non - significant as compared to control.

Fig. 7: Effect of *T.T* ME on elevated plus-maze in $AlCl_3$ induced AD model

Our investigations of passive avoidance test indicated that exposure to aluminum chloride produced worse effects on retention memory with decreased step through latency and insensitivity to electric shock in shock apparatus alike to previous studies (Lakshmi *et al*., 2015). Current study manifested that aluminum chloride exposure induce alteration in neophilia, curiosity and exploratory behavior in rodents corresponding to previous literature (Zerrouki *et al*., 2016), *T.T* ME and rivastigmine improved exploratory behavior and curiosity in hole board apparatus. These behavioral alterations and locomotor impairments are compounding consequences of lipid peroxidation, oxidative stress and compromised cholinergic neurotransmission induced by administration of aluminum chloride. *T.T* ME treatment ameliorated cognitive disabilities and locomotion abnormalities in this study indicating its neuroprotective potential in AD.



Values are expressed as mean ± SEM, (n = 6), Values are expressed as mean ± SEM, (n = 6), ****P<0.001* and ns: non - significant as compared to control.

Fig. 8: Effect of *T.T* ME on acetylcholinesterase activity (µmole/mg of proteins) in brain tissue

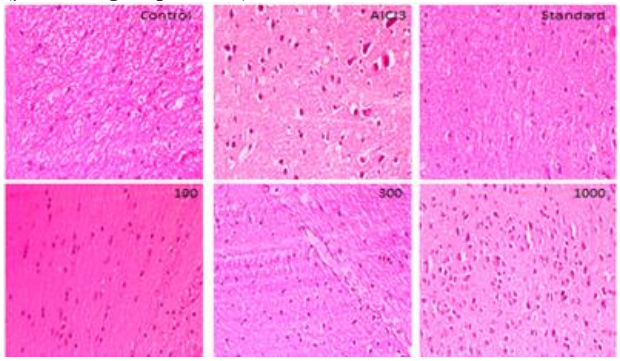


Fig. 9: Histopathological analysis of brain tissues in aluminum chloride induced AD model

Vitiated cholinergic neurotransmission is principal factor elaborated in etiopathogenesis of neuro-psychiatric and biochemical alterations in AD. Impaired cholinergic neurotransmission in cortex and hippocampus transmitted by two mechanisms, firstly due to reduction in acetylcholine release and diminished activity of choline acetyltransferase (Rodriguez-Puertas *et al*., 1994). Secondly elevated level of AchE originate decline in acetylcholine level by humiliating available acetylcholine at synapse. Contradictory to some previous studies who reported that the level of AchE significantly decreased in AD brain as compared to normal (Arendt *et al*., 1992). This study explored that the level of AchE was decreased on exposure to aluminum chloride. Aluminum is a well-known neurotoxic metal instigates oxidative stress, which has excessive propensity to impair activity of this enzyme. Perhaps because of this potential level of AchE was significantly decreased upon treatment with

aluminum chloride in disease control group. Our findings corroborated with preceding studies in which they noticed biphasic response of AchE, elevated level of AchE in short phase and decreased level of AchE in second phase after chronic administration of aluminum chloride (Kaizer *et al*., 2008). Our findings are consistent with second phase indicating reduced activity of AchE in disease control group. We suppose that the level of AchE decreased due to sedate deposition of aluminum in brain and it's binding to anionic sites of enzyme which contributed oxidative stress. However undermine cholinergic neurotransmission due to altered acetylcholine synthesis and release are more pronounced effects than partial influence on AchE, altered by aluminum exposure which was significantly improved upon treatment with *T.T* ME and standard drug rivastigmine (acetyl cholinesterase inhibitor).

Literature reported that oxidative stress has primary role in etiopathogenesis of neurodegenerative disorders like AD (Nampoothiri *et al*., 2014). Aluminum cause oxidative stress by generation of reactive oxygen species and by peroxidation damage to lipids and proteins in hippocampus and cortex. The level of reactive oxygen species and free radicals was elevated because of decreased level of first line of defense antioxidant enzymes in aluminum chloride induced cognitive deficit animal model. Oxidative damage caused by aluminum resulted in reduced level of first line agent of defense antioxidant enzymes like catalase, superoxide dismutase, glutathione reductase and peroxidase. The level of antioxidants is decreased due to Golgi disruption, decreased axonal mitochondria turnover and reduced level of synaptic vesicles which cause the release of damaging species such as melondialdehyde, carbonyls, peroxynitrites and superoxide dismutase. Oxidative damage and cognitive disabilities are strongly linked with each other so that it is evaluated that an agent which have antioxidant activity will be effective in treatment of neurodegenerative disorders like AD (Mook-Jung *et al*., 1999). Our results were in agreement with previous studies as treatment with aluminum chloride in disease control group cause the significant decrease in the level of first line agent of defense antioxidant enzymes and increased the level of melondialdehyde. The levels of antioxidant enzymes as CAT, SOD, GPx and GSH were recovered with *T.T* ME treated groups and level of MDA was significantly decreased due to antioxidant potential of this plant. It was reported that neurodegeneration and myocardial injury was associated with decreased level of SOD (Lebovitz *et al*., 1996). In a previous study it was reported that many mental disorders were linked with deficiency of CAT (Khan *et al*., 2010). GPx is an important antioxidant enzyme present in mitochondria and cytosol has inhibitory effect on oxidative stress and lipid peroxidation and its deficiency is linked with neurotoxic effects (Chabory *et al*., 2009). GSH is another antioxidant enzyme act as scavenger of singlet oxygen and hydroxyl radicals and as reservoir of reduced glutathione. Neurodegeneration and aging process are linked with decreased level of GSH (Santambrogio *et al*., 2015). First line defense antioxidant enzymes have indispensable role in protection against reactive oxygen species, singlet oxygen and superoxide radicals. *T.T* phytochemical analysis indicated that it has a favorable amount of flavonoids which have metal chelation ability with cadmium, beryllium, aluminum, zinc and iron. Therefore its flavonoids form chelate with aluminum and decrease the accumulation of aluminum and increase its excretion. Histopathological analysis explored that *T.T* ME recovered the neuronal loss, neurofibrillary tangles, pigmentation and neuroinflammation induced by aluminum chloride. This study indicated that *T. T* ME have neuroprotective effects in Alzheimer ś disease model by improving biochemical and behavioral parameters.

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