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ORIGINAL ARTICLE

Protective Effect of *Terminalia chebula* against B[α]p Induced Memory Impairment and Neurodegenerative Changes in Mice

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ABSTRACT

Accumulating evidences from pre-clinical studies have demonstrated that the Benzo[α]pyrene (Bαp) induces memory and cognitive impairments. This study designed to test the neuroprotective effects of Terminalia Chebula on Benzo[α]pyrene mediated memory deficits and neurodegenerative changes in mice. The objective of this study is to evaluate Terminalia Chebula in Bαp induced cognitive and memory impairment model in mice. Mice injected with Bαp (5 mg/kg., i.p) alternately for 28 days and Morris water maze were performed. On 23 to 26th day Morris water maze training were perfomed and on 27th day the prob trial were performed. Oral dose of Terminalia Chebula 20 mg /kg and 40 mg/kg administered daily from 14th to 28th days. On 28th day, the mice sacrificed and biochemical, neurochemical, neuroinflammatory parameter and histological parameter analyzed. Further the oxidative and inflammatory biomarkers level, caspase-3 activity, mitochondrial dysfunction were assessed in the brain homogenates Bαp administration resulted in alterations in behavioral parameters with increased escape latencies; reduced time spent in the target quadrant, decreased number of crossing the platform in MWM test. Notably, Terminalia Chebula treatment exhibited dose dependent improvements on behavioral, biochemical and histological findings in Bαp intoxicated mice. However, high dose Terminalia chebula treatment significantly ameliorated behavioral, biochemical changes and rescued from the various histological abnormalities in Bαp injected mice. Study concludes that high dose Terminalia Chebula treatment provides neuroprotective effects against Bαp induced memory deficits and neurodegenerative changes by anti-oxidative, inflammation suppressant, correction of mitochondrial dysfunction, inhibitory effects on necrosis and apoptosis.

Keywords: *Benzo[α]pyrene (Bαp), Terminalia Chebula (TC), Morris Water Maze (MWM), Cognitive, memory impairement, Neurodegenerative.*

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INTRODUCTION

Benzo[α]pyrene (Bαp) is one of the polycyclic aromatic hydrocarbons (PAHs) and environmental pollutant present in various sources such as coal processing waste products, petroleum, tobacco and smoke. Bαp considered as the Group 1 carcinogenic [1].Exposure of Bαp occur through different routes such as ingestion of contaminated food and water and through inhalation of particulate in air, and smoking [2]. Bαp is involved in diverse range of diseases including cytotoxicity, mutagenic, and carcinogenetic property also include immunosupression and hormonal disorder in body [3] The high lipophilicity of Bαp allows crossing blood brain barrier and induces neurological abnormalities like memory impairment and neurodegenerative changes [4]. Administration of Bap causes impairment motor activity, neurotransmitter imbalance and neurobehavioral toxicity by increasing oxidative stress [5].Further, administration of Bαp at a single dose leads to the alteration dopamine and serotonin neurotransmission in rat brain [6]. Exposure of Bαp causes the disruption of glutaminergic neurotransmitter in brain and also reduced the expression of GluR1 and GluR2 mediated by increasing SNAP -25 protein level [8].Terminalia chebula is one of the best known for medicinal plant belongs to the family Combretaceae and it is a medium sized tree distributed in India, Nepal, Sri-Lanka and Southeast Asia. Commonly known as chebulinic myrobalan black myrobalan (English), *harahra* (Hindi).It is a deciduous tree about 25 cm long. . The bark is usually dark brown in color. It shows yellow to orange -brown when ripped. [9].Experimental study reported that extract of

Terminalia Chebula showed its protective effect on acetic acid induced colitis on rats and 70% of methanolic extract of *Terminalia chebula* showed its protective effect on iron induce liver toxicity in mice [10].The major consistutent of Terminalia chebula fruit are gallic acid, ellagic acid, chebulic acid ,chebulinic acid and chebulagic acid [11]. It reported that the *Terminalia chebula* shows neurological effect on PC12 cells, also inhibits ROS production and influx of calcium [12]. However, the objective of this study is to evaluate *Terminalia Chebula* in Bαp induced cognitive and memory impairment model in mice.

MATERIAL AND METHODS

Animal

Thirty- eight Swiss Albino mice weighing (30- 35g) were obtain from Lala Lajpat Rai University of Venitary and animal sciences, Hisar and kept in Central Animal House of ISF College of Pharmacy, Moga Punjab (India).The animals were kept in cages in well controlled atmosphere with 12 hr light /dark cycle. Animals maintained on a diet in the form of dry pellets and water *ad libium* and behavioiural parameter were performed. The Protocol of study approved by the Institutional Animal Ethics Committee (IAEC), and follow up under the Committee for the purpose of control and supervision of Experiments on animals (CPCSEA) guidelines for the use and care of experimental animals.

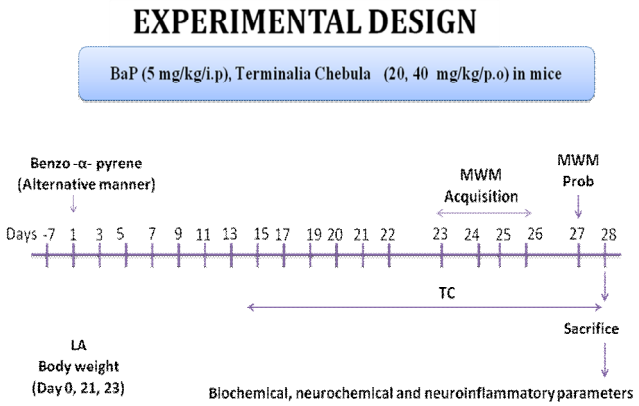
Chemicals

Benzo[α]pyrene purchased from Sigma- Aldrich, USA and Terminalia Chebula purchased by Natural RemediedsPvt.Ltd Bangalore. Interleukin -1 beta (IL-1β), interleukin-6(IL-6) and tumor necrosis factor – alpha (TNF-α) kits were purchased from Krishgen Biosystem, India .All chemicals and other biochemical reagents were used of good quality .All drugs were freshly prepared before use.

Experimental Design

S.No	Group	Animal species	Number of Animals used
1	Normal	Mice	8
2	Vehicle	Mice	6
3	Bαp	Mice	8
4	Bαp +tc(20 mg/kg)	Mice	8
5	Bαp +tc (40mg/kg)	Mice	8
6	Total Animal	Mice	38

Plan of work



Establishment of Benzo[α] pyrene induced cognitive and memory impairment model

Bαp stock solution was prepared and dissolved in the1ml/kg olive oil. Animal given a 5mg/kg of Bαp alternatively for a period of 28 days while *Terminalia Chebula* (20 mg/kg and 40 mg/kg) were orally disperse in 1 % of CMC for a period of 14 days regularly Behavioiural parameter were performed. On 23,24,th 25 thand 26th day Morris water maze were perfomed and on 27th day hidden platform prob trial were performed .On 28th day mice were sacrificed and biochemical ,neurochemical, neuroinflammatory parameter were analyzed.

The Morris Water maze test

Morris water maze test were perfomed in mice according to R.Morris method [13].In Morris water maze test a submerged platform and video tracking software (Any maze 6.2) were used. The Morris consists of a circular pool of diameter 6 ft and 2 ft depth of open field maze. Twenty-four hours after pre training mice trained over a period of four trials using the hidden platform placed at fixed in the centre of one of the four quadrants. Location of the platform remains constant in all trials. Place the mice gently on the walls of the pool and began the trial. Mice were allowed to swim for 120 s or until it found the platform. Each mice was performed four consecutive trials within a period of 60 s. If mice is unable to locate the platform it was gently guided towards platform and allowed to stay for 20 sec and measure the escape latency. Escape latency time (ELT) is a measure to find a hidden platform in targeted quadrant as a note of acquisition or learning. On last day, the platform removed and mice memory analyzed. The mean time spent in targeted quadrant to find hidden platform as an index of memory and number of times the targeted platform crossed during the prob trial measured for each mice.

Biochemical Assessment

Determination of Acetylcholinesterase Assay (AchE)

The whole brain AchE activity was measured and described by the method Ellman *et* al[14]The brain sample was read spectrophotometrically at 420 nm using DU 640 B Spectrophotometer ,Beckman Coulter Inc,CA,USA.

Determination of Myeloperoxidase Activity (MPO)

The myeloperoxidase activity in whole brain were measured and described by the method Grishman *et al* [15]The brain sample was read spectrophotometrically at 420 nm using DU 640 B Spectrophotometer ,Beckman Coulter Inc,CA,USA.

Determination of Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS activity in brain were described by the method Ohkawa *et al*[16].Absorbance were measured spectrophotometrically at 420 nm using DU 640 B Spectrophotometer, Beckman Coulter Inc, CA, USA.A standard curve was plotted by using 1,1,3.3 tertramethoxyopropane (1-10 nM) to calculate the estimation of TBARS.

Estimation of Reduced Glutathione (GSH)

The GSH activity in brain were described by the method Boyne and Ellman[17] Absorbance were measured spectrophotometrically at 412 nm using DU 640 B Spectrophotometer, Beckman Coulter Inc, CA, USA.Standard curve was plotted by using reduced form of glutathione (10 -100 uM).

Estimation of Catalase activity in brain

The catalase activity in brain were described by the method Aebi .H[18]. The rate of decomposition of H2O2 measured spectrophotometrically from changes in absorbance at 240nm for 2 minutes at 30 seconds interval. Activity of catalase expressed as units (k)/mg protein.

Estimation of pro-inflammatory Cytokines (TNF-α, IL-1β, and IL-17) levels

Quantification of TNF –α, IL-1β and IL-17 was examined by using ELISA kit. It is a solid phase sandwich enzyme linked immunosorbent assay (ELISA).Pro inflammatory cytokines measured by using immunosorbent assay kit in mice brain.

Estimation of TBARS activity in brain

TBARS measured as lipid peroxidation in brain. The TBARS performed by method of Ohkawa. The absorbance measured spectrophotometrically at 532 nm using DU 640B spectrophotometer, Beckman Coulter Inc., CA, USA. A standard calibration curve using 1, 1, 3, 3- tertramethoxyopropane (1–10 nM) was plotted to calculate the concentration of TBARS.

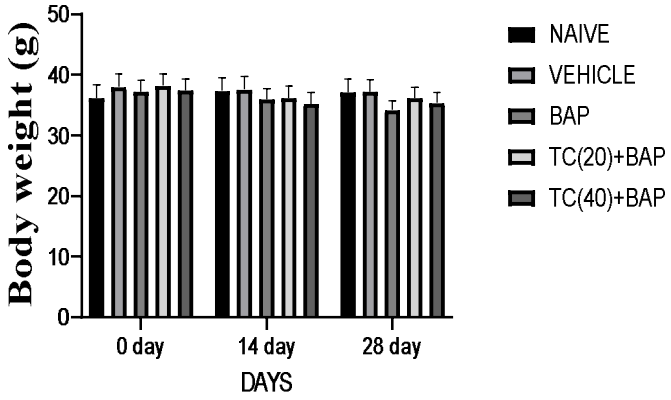
Statistical Analysis

Analysis of the data was performed using Graph Pad PRISM statistical software (v. 5.01) by using 1way ANOVA and with Tukey's multiple comparisons test as post hoc. Data for ELT, path length, average swim speed and TSTQ in the MWM were analyzed using two-way analysis of variance (ANOVA), followed by post-hoc Bonferroni's tests and one-way ANOVA, followed by post-hoc Tukey's multiple-range test for all other measures. All the data are presented as mean ± SEM. Significance was accepted at $P<0.05$.

RESULTS

Effect of *Terminalia chebula* in body weight of Bαp intoxicated mice

There is no significant difference found in body weight among Bαp treated group and control group before 28 days treatment. On 28thday Bαp administered significantly, decreases the body weight when compared to normal Furthermore on 28th day both TC treatment (20 mg/kg and 40 mg/kg) normalize the body weight when compare to control as shown in figure 1.



Effect of Terminalia Chebula in MWM of Bαp in toxicated mice

MWM is a test performed for spatial memory and learning in rodents. During the four days of training performed in consecutive days, we conclude that mice in control group immediately find the hidden platform than Bαp injected group. The Bαp injected group mice significantly decreases the ELT time when compared with control from days 2 to 4 shown in figure 2.The group treated with TC (20mg/kg and 40 mg/kg) mice significantly improved the ELT from days 2 to 4 when compare to control group in hidden platform MWM.

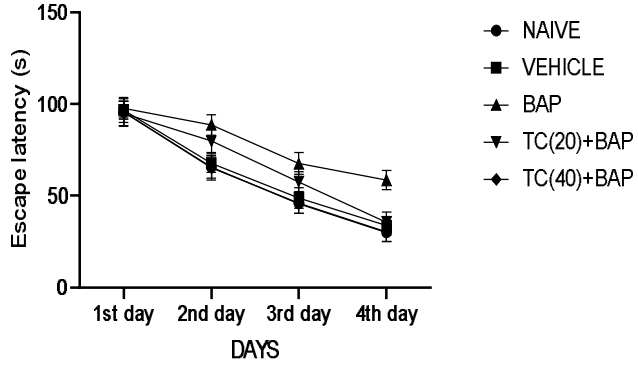


Figure 2: Effects of Terminalia Chebula doses on mean escape latency in BaP (benzo-α-pyrene) administered mice analyzed by Morris water maze task in hidden platform MWM. Significant differences between the group's $*p < 0.05$ BaP and TC (20) +BaP groups vs. vehicle and naive groups. @p<0.05 TC (20) +BaP and TC (40) +BaP vs. BaP group alone. #p<0.05 ChA (40) +BaP vs. TC (20) +BaP group.

In prob trial, the Bαp injected mice decreases the time in (Q4) targeted quadrant. While TC treated group mice (20 mg/kg and 40 mg/kg), also increase in TSEQ as shown in figure 3(a).Thus it concludes that Cha enhanced the spatial Recognization in the MWM test.

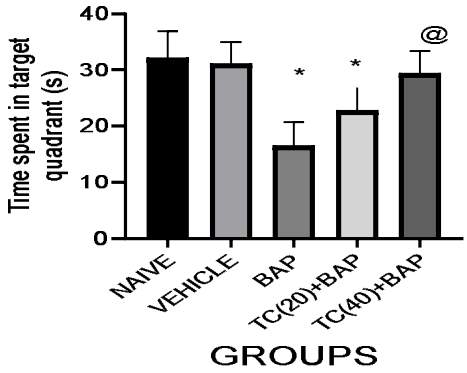


Figure 3 (a) Effects of *Terminalia Chebula* doses on time spent in target quadrant in BaP (benzo-α-pyrene) administered mice analyzed by MWM task in prob trial .Significant differences between the groups $*p <$ 0.05 vs. vehicle and naïve groups.@p<0.05 v/s BaP group alone.

The swim path of each mice were recorded the number of times the targeted annulus were recorded for each group of mice as shown in figure 3(b).

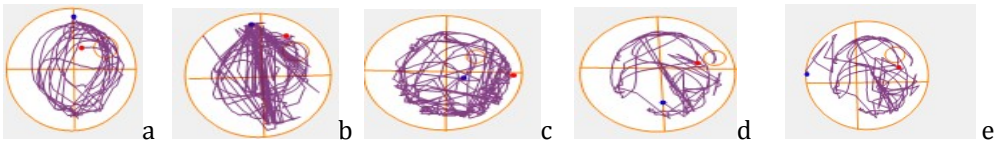


Figure 3(b): Diagrammatic representation of mice swimming path in prob trial when exposed to Bαp. A represents the naïve group .B represents the vehicle group .C represents the Bαp toxin induced group and D and E represents *Terminalia Chebula* treatment group 20 and 40 mg /kg respectively.

Effect of Terminalia Chebula in on AchE content in hippocampus of Bαp intoxicated mice.

The ACh content measured in mice hippocampus after MWM test. Administration of Bαp significantly increased the activity of AchE when compared with control (P<0.05) (Figure 4). Oral administration of TC (20 mg/kg) showed significantly increased in the activity of AchE. However TC (40 mg/kg) improved the activity of AchE in Bαp treated mice (p<0.05).

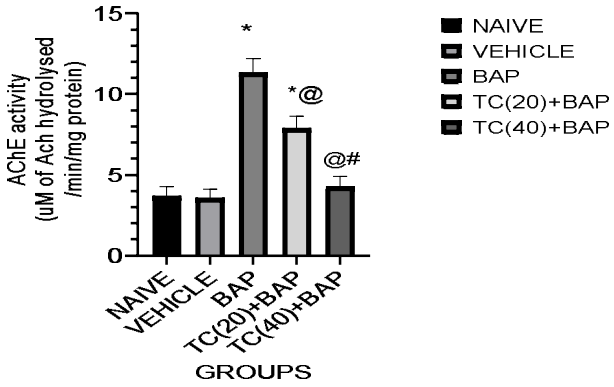


Figure 4: Effect of TC on brain (hippocampus) Acetylcholinesterase activity) in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vs BαP, #p < 0.05 vs TC (20) + BaP and TC (40) +BaP vs. BaP group alone.

Effect of Terminalia Chebula on MPO content in hippocampus of Bαp intoxicated mice

The analysis of MPO has been observed .The activity of MPO was significantly increases in the Bαp administrative mice as compared to control (p<0.05) as shown in (Figure 5). Oral administration of TC (20 mg /kg) showed significantly increased in the activity of MPO. However Terminalia Chebula (40 mg/kg) improved the activity of MPO in Bαp treated mice (p<0.05).

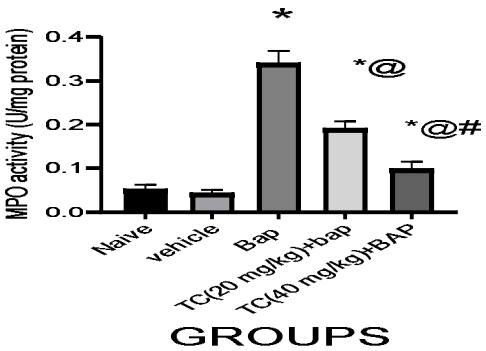


Figure 5: Effect of ChA on brain (hippocampus) MPO activity) in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vs BαP, #p < 0.05 vs TC (20) + BaP and TC (40) +BaP vs. BaP group alone.

Effect of chebulinic acid on GSH content in hippocampus of Bαp intoxicated mice

The activity of GSH was significantly depleted in the Bαp administrative mice as compared to control group (p<0.05) as shown in (figure 6.) Oral administration of TC (20 mg /kg) showed significantly decreases in the activity of GSH. However TC (40 mg/kg) increases the activity of GSH in Bαp treated mice (p<0.05).

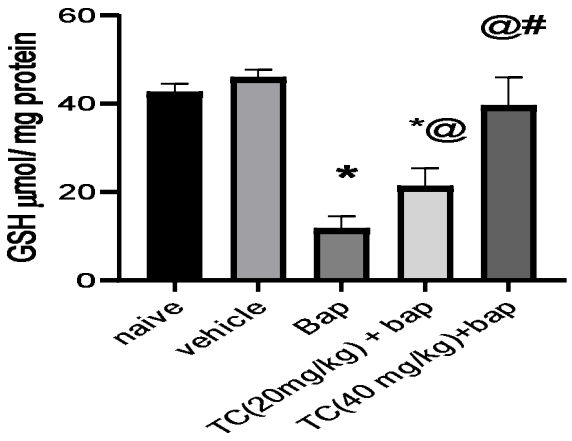


Figure 5: Effect of ChA on brain (hippocampus) GSH activity) in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vsBaP, #p < 0.05 vs TC (20) + BaP

Effect of Terminalia Chebula on Catalase content in hippocampus of Bαp intoxicated mice.

The activity of Catalase was significantly depleted in the Bαp administrative mice as compared to control group ($p<0.05$) as shown in (figure 7).Oral administration of TC 20 mg/kg and 40 mg/kg significantly increases the activity of catalase when compare to Bαp administrative mice.

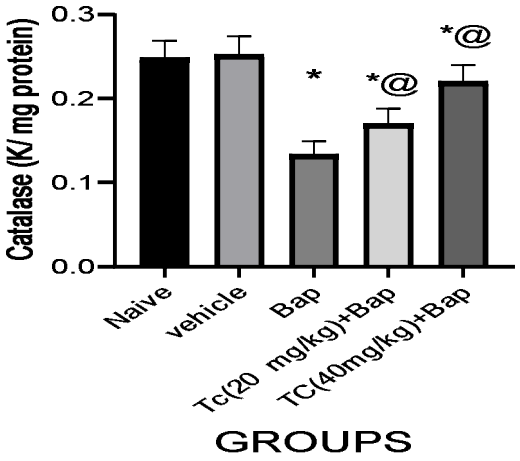


Figure 7: Effect of TC on brain (hippocampus) Catalase activity in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vs BαP, #p < 0.05 vs TC(20) + BaP and TC(40) +BaP vs. BaP group alone

Effect of Terminalia Chebula on MDA content in hippocampus of Bαp intoxicated mice

The activity of TBARS was significantly increases in the Bαp administrative mice as compared to control group ($p<0.05$) as shown in (figure 8).Oral administration of TC 20 mg/kg and 40 mg/kg significantly decreases the activity of MDA when compared to the Bαp administrative mice ($p<0.05$).

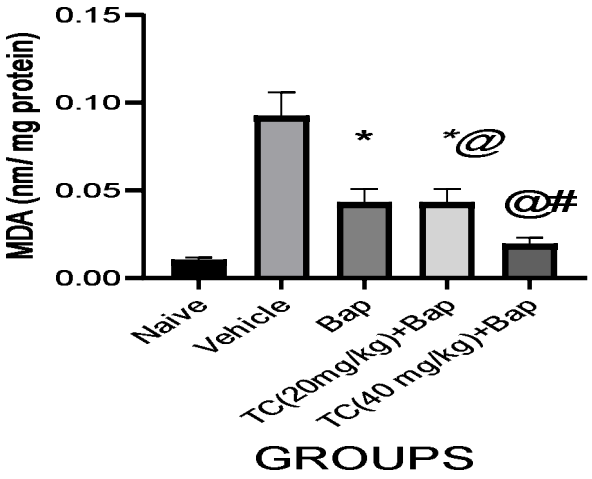


Figure 8: Effect of TC on brain (hippocampus) MDA activity in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vs BαP, #p < 0.05 vs ChA (20) + BaP and TC (40) +BaP vs. BaP group alone.

Effect of Terminalia Chebula on TNF-α (anti-inflammatory) in hippocampus of Bαp intoxicated mice.

Immunohistochemical analysis of TNF-α observed .The activity of TNF-α was significantly increases in the Bαp administrative mice as compared to control group as shown in (figure 9). This effect is significantly reversed by the TC 20 mg/kg treatment in hippocampus as compare to Bαp group. TC 40 mg/kg attenuates level of TNF-α.

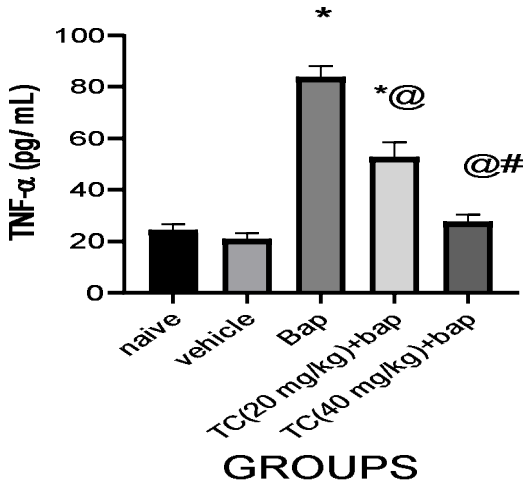


Figure 9: Effect of TC on anti-inflammatory activity of TNF-α in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vs BαP, #p < 0.05 vs TC (20) + BaP and TC (40) +BaP vs. BaP group alone.

Effect of Terminalia Chebula on IL-1β (anti-inflammatory) in hippocampus of Bαp intoxicated mice.

Immunohistochemical analysis of IL-1β observed .The activity of IL- 1β was significantly increases in the Bαp administrative mice as compared to control group as shown in (figure 10).This effect is significantly reversed by the TC 20 mg/kg treatment in hippocampus as compare to Bαp group.TC 40 mg/kg attenuates the level of IL-1β.

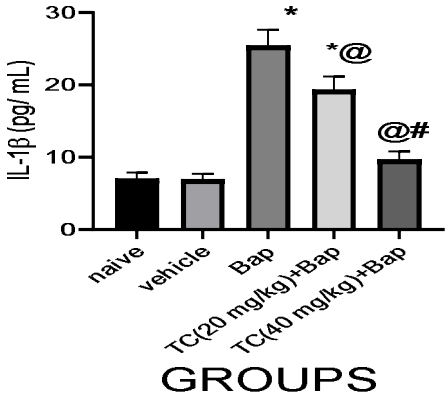


Figure 10: Effect of TC on anti-inflammatory activity of IL-1β in Benzo[α] treated mice .Significant differences between the groups *P < 0.05 VS NAÏVE AND VEH, @P < 0.05 VS BAP, #P < 0.05 VS TC (20) + BAP AND TC (40) +BAP VS. BAP GROUP ALONE.

Effect of Terminalia Chebula on IL-17 (anti-inflammatory) in hippocampus of Bαp intoxicated mice.
Immunohistochemical analysis of IL-17 observed .The activity of IL- 17 was significantly increases in the Bαp administrative mice as compared to control group as shown in (figure 11).This effect is significantly reversed by the TC 20 mg/kg treatment in hippocampus as compare to Bαp group.TC 40 mg/kg attenuates the level of IL-17.

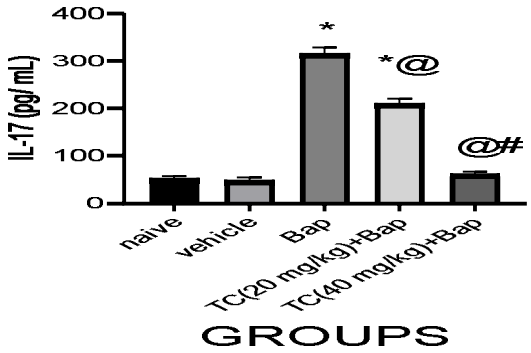


Figure 11: Effect of TC on anti-inflammatory activity of IL-17 in Benzo[α] treated mice .Significant differences between the groups *p < 0.05 vs naïve and veh, @p < 0.05 vs BαP, #p < 0.05 vs TC (20) + BaP and TC (40) +BaP vs. BaP group alone.

DISCUSSION
In vitro study stated that salidroside protects the PC12 against the hypoglycemia and serum limitation model that induce cytotoxicity and inhibit the production of ROS. One of experimental study demonstrated that neuroprotective effect of *Terminalia chebula* extract against OGD-R and hydrogen peroxide (H_20_2) induces injury in cell on rat pheochromocytoma cell line (PC12).Thus; study concludes that extract of *Terminalia chebula* exhibits the protective effect against OGD-R, H202 and LPS induced cell death [20]. *Terminalia chebula* showed its protective effect on microglia cells and further inflammatory markers such as TNF-α, IL-1β, IL-6, PGE-2 and COX-2 were measured also concluded that these markers were reduced after the treatment of *Terminalia chebula* extract in microglia cells [21].
Administration of Bαp results impairment in motor activity, causes neurobehavioral toxicity by increasing oxidative stress [22] Chronic exposure of Bαp disrupts the neurotransmitter level ,causes alteration in cholinergic system, monoaminergic system and oxidative stress induces learning and memory deficits[23].
Sub acute administration of Bαp (5mg/kg) induce neurotoxic effects over a period of 45 days resulted in memory impairment (escape latency increased, time spent in target quadrant was decreased while oral dose Terminalia Chebula treatment 20 mg /kg and 40 mg /kg attenuates the learning and memory deficits in mice[24]. Our findings are in line with previous studies, which have also demonstrated that Terminalia Chebula exerts beneficial effects on memory dysfunction in other neurotoxicity models such as in Scopolamine memory impairment model [25] and anti – epileptic model.

Bap administration resulted in oxidative and inflammatory stress, which observed in brain homogenates of mice after 28th day study period. Earlier studies have also shown the elevation of oxidative stress markers MDA, NO and SOD and were up regulation in Bap intoxicated rat over a period of 90 days. Administration of Bαp 0.02 and 0.2 mg/kg sub acute oral exposure over a period of 3 weeks improve the sexual behavior in mice and alters the monoaminergic neurotransmission system (26) .Our findings are in line with these previous studies, which have reported anti-oxidant and anti-inflammatory effects of *Terminalia Chebula.*
Apart from this, the Bap intoxicated mice in our study showed increased infiltration of immune cells in brain, necrosis, degenerative changes and up- regulation of Capase- 3 activities. These immune cells are visible in toxicity group may be due to damage of BBB and activation of cytokines which recruit immune cells at target sites that aggravate ROS formation and induce cytotoxicity Previous studies have reported that TC attenuates inflammation, pro-apoptotic factors activation and other pathological changes in neurotoxicity or neurodegenerative model. With linked to this extract of *Terminalia chebula* showed the protective effect on ischemia stroke model in rats. Administration of *Terminalia chebula* for 7 days through intragastricly after the middle cerebral artery occlusion (MCAO) and further neuroprotective effects, antioxidant, anti inflammatory activity ,energy and amino acid metabolism and others glycerophospholoipid metabolism were identified. Thus, our study concluded that *Terminalia chebula* improves MCAO rats through different energy metabolism, amino acid metabolism and glycerophospholoipid metabolism [12]. We observed reduced inflammatory stress, oxidative damage, immune cell infiltration and caspase-3 inhibition in CA treated mice challenged with Bap toxin.

CONCLUSION

We conclude that Bap intoxication resulted in memory impairments due to increased oxidative-inflammatory stress in brain. Moreover, the elevated of caspase-3 activity, MPO activity and histological changes including necrosis, degenerative changes in hippocampal layers and immune cell infiltration was present in Bap administered mice. TC treatment dose dependently ameliorated memory impairment and histological damage by anti-oxidative and inflammatory property, inhibition of immune cells infiltration.

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