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RESEARCH ARTICLE

ANTIOXIDANTS AND ANTI-INFLAMMATION POTENTIALOF AMARANTHUS VIRIDIS LINN AGAINST 3-NP–INDUCED HUNTINGTON'S DISEASE IN RATS

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Abstract

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease. Accordingly, 3-nitropropionic acid (3-NP) has been found to effectively produce HD-like symptoms. Amaranthus viridis Linn (HAEAV), popularly known in Chauraiya in Hindi," may act as a neuroprotective agent in vitro andin vivo. We evaluated the hypothesis that the hydro-ethanolic extract of Amaranthus viridis Linn could prevent behavioral and oxidative alterations induced by 3-NP in rats. 30 albino Wistar female rats were divided into 5 groups: (1) control, (2) HAEAV(400 mg/kg), (3) 3-NP, (4) HAEAV(200 mg/kg) + 3-NP, and (5) HAEAV(400 mg/kg) + 3-NP.Animals in groups 3, 4, and 5 received 15 mg/kg 3-NP daily from days 8–14. At day 14, parameters of locomotor activity and biochemical evaluations were performed. Indeed, rats treated with 3-NP showed decreased locomotor activity compared to controls. Additionally, 3-NP increased levels of reactive oxygen species and lipid peroxidation and decreased ratio of CAT, TT, and GSHactivityinbrain tissues. Our results suggest that rats pretreated with HAEAVprior to 3-NP treatment showed neuroprotective effects when compared to 3-NP treated controls, which may be due to its antioxidant properties.

Introduction:-

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor impairment, cognitive decline and psychiatric symptoms that worsen as the disease progress [1]. Although the exact cause of neuronal death in HD remains unknown, it has been postulated that the abnormal aggregation of the mutant huntingtin protein may cause toxic effects in neurons, leading to a cascade of pathogenic mechanisms associated with transcriptional dysfunction, oxidative stress, mitochondrial alterations, apoptosis, bioenergetic defects and subsequent excitotoxicity [2].HD patients often exhibit deficits in executive tasks requiringplanning, cognitive flexibility and problem solving. HD poses challenges for health and social care professionals due to its complexity and unpredictability. With an incidence of 2–10 per 100,000, HD afflicts 30,000 people in USA and another 250,000 persons are genetically at risk [3]. Several animal models exist for HD such as stereotactic injection of kainic, quinolinic and ibotenic acids into specific region of the brain, but systemic administration of 3-nitropropionic acid (3-NP) is the recent and popularly used one [4]. 3-NP crosses the blood–brain barrier and at cellular level it is an irreversible inhibitor of the electron transport enzyme succinate dehydrogenase (SDH), a mitochondrial complex II enzyme, responsible for the oxidation of succinate to fumarate in Kreb's cycle. Subsequently it blocks the transport of electrons in oxidative

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phosphorylation, causing decreased ATP levels in brain. It affects normal brain electrical activity and oxidative stress has been suggested to play a role in 3-NP toxicity. Neurons are metabolically highly active hence, processes that affect the mitochondrial function invariably leads to neuronal death [5-6]. Accumulating data indicates that 3-NP produces free radicals and consequent disturbance of glutathione redox cycle and the inflammation associated with 3-NP, also acts as a contributing factor for neuronal damage and further free radical generation [6]. 3-NP also causes severe damage to the neurons present in other regions of brain like hippocampus, cortex and hypothalamus [7].In this scenario, anti-oxidant therapy has been suggested as a potential method to prevent neurodegenerative disorder, such as HD. In this way, natural products from plants would be an important tool for treating these pathologies.Amaranthus viridis L. (Amaranthus) (HAEAV) commonly called as Chauraiya in Hindi is an erect much branched glabrous herb, 30-60 cm high, distributed in all tropical countries. The traditional uses are in diuretic, analgesic, anti-pyretic, vermifuge, anti-ulcer, hypoglycemic, hypolipidemic, laxative, asthma and veneral diseases. Antioxidant activity and phenolic content of raw and branched Amaranthus viridis L were reported. Amaranthus viridis L also possesses antiviral activity [8].

Study Aims

A wide variety of pharmacological actions of HAEAV were reported— antioxidant,anti-cancer, anti-inflammatory, antinociceptive and neuro-endocrineactivities. However, no research has been carried out to investigate the efficacy of hydro-ethanolic extract of HAEAV in 3-nitropropionic acid in rats. The present study has been designed to Neuroprotective Effect of Amaranthus viridis linn Against 3-NP–Induced Huntington's Disease in rats and attempts to understand the mechanismof its therapeutic effect with reference to biochemical analysis.

Methods:-

Animals

The experiments were carried out in Wistar rats (200–250 g) obtained from central animal house of Sree Siddaganga College of Pharmacy, tumakuru, Karnataka (India). They were kept in polyacrylic cages and maintained under standard husbandry conditions (room temperature 22± 2 °C and relative humidity of 45–64%) with 12 h light/dark reverse cycle (lights turned on at 7:30 A.M.). The food in the form of dry pellets and water were made available ad libitum. All behavioral experiments were carried out between 9:30A.M. and 3 P.M. The protocol was reviewed and approved by the Institutional Animal Ethics Committee and the animal experiments were carried out in accordance with the Institutional Ethical Committee of Sree Siddaganga College of Pharmacy guidelines for use and care of animals.

Plant material and extraction

The aerial part of plant Amaranthus viridis Linn. was collected from Namadha chilume, Tumakuru, Karnataka. The plant was identifiedand authenticated. The Amaranthus viridis Linn. Plant will be collected and then washed, air-dried. The Hydroalcoholic extraction of the leaves and stems of the plant were carried out separately, by suspending 100 g of the powder of each part in 2000 ml of 80% ethanol: water (80:20 v/v). The extraction was done by cold maceration for 3days at room temperature. The extracts were filtered through wattman filter paper. After filtration extract is subjectedto evaporation. Finally, product is collected. However, the dose 200 and 400 mg/kg extract does will be prepared from concentrated extract which will be dissolved in normalsaline.

Preliminary phytochemical screening

The hydro-ethanol extract of Amaranthus viridislinn was screened for the presence of various phytoconstituents like steroids, alkaloids, glycosides, flavonoids, carbohydrates, amino acids, proteins and phenolic compounds [9].

Drugs and treatment schedule

The following drugs were used in the present study: 3-NP (sigma Aldrich, Karnataka, India) was diluted with distilled water (adjust pH 7.4) and administered oral rout to animals. HEAV extract was suspended in distilled water solution and administered by per oral route in a constant volume of 0.5 ml per 100 g of body weight. The albino Wistar rats were randomly divided into five groups of 6 animals in each. Group 1, vehicle treated control group, Group 2, Received vehicle HEAV extract (400 mg/kg, p.o.); Group 3,3-NP (15 mg/kg, i.p); Group 4, received HAEAV (200 mg/kg, p.o.) + 3-NP (15 mg/kg, i.p) for 14 days; Group 5, received HAEAV (400 mg/kg, p.o.) +3-NP (15 mg/kg, i.p).

In groups 4 and 5, extract was administered 1 hour prior to 3-NP administration. On 15^{th} day all the rats were anesthetized and then sacrificed by cervical decapitation. Brain tissue was excised immediately and rinsed in ice-chilled normal saline. A known weight of the brain tissue was homogenized in 5.0 ml of 0.1 M Tris– HCl buffer (pH

7.4) solution. The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameter Histopathological study.

Behavioral assessment

Beam Walking Test

Beam walking test was used to evaluate gross vestibulomotor function. The apparatus consisted of a rod 120 cm in length and with a diameter of 2.3 cm. A wooden box (20 cm 9 20 cm 9 10 cm) was set at one end of the rod as a nest for motivating the animal to cross the beam. The apparatus was suspended 50 cm above a cushion, which protected the animals against fall injury. Rats were trained twice daily for 2 days for motor coordination. The time taken to traverse the beam was recorded. The cut-off time was taken as 120sec [11].

Hanging wire test.

The rats were allowed to hold a steel wire with the forepaws (2 mm in diameter and 35 cm in length), placed at a height of 50 cm over a cushion support. The length of time the rat was able to hold the wire was recorded. The latency to the grip loss is considered as an indirect measure of grip strength [12].

Actophotometer test

Locomotor activity was evaluated by using actophotometer. Animals from all the groups in MSG induced neurotoxicity model were placed in actophotometer for 10 minutes and score was recorded. Difference in the change in locomotor activity in different groups of animals in the model was recorded [13].

Measurement of oxidative stress parameters

All the biochemical parameters were measured in the brain homogenate on day 15.

Catalase estimation

Catalase activity was assayed by the method of Luck (1971), wherein the breakdown of hydrogen peroxides was measured at 240 nm. Mixture consisted of 3 ml of hydrogen peroxides phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and change in absorbance was recorded at 240 nm with UV-1700 Spectrophotometer, Shimadzu, Japan. The results were expressed as micromole H2O2 decomposed per mg of protein/min [14].

Measurement of lipid peroxidation

The quantitative measurement of lipid peroxidation in the brain striatum was performed according to the method of Wills (1966). The amount of malondialdehyde (MDA), a measure of lipid peroxidation, was measured by reaction with thiobarbituric acid at 532 nm using a Perkin Elmer lambda 20 spectrophotometer. The values were calculated using the molar extinction coefficient of the chromophore (1.56 ×105 M-1 cm-1) and expressed as a percentage of the vehicle-treated group (the protein concentration was 0.876 mg·mL-1) [15]

Estimation of Reduced glutathione (GSH)

GSH in brain tissue was quantified by the method described shortly here in, 5% tissue homogenate was prepared in 20 mM EDTA, pH 4.7, and 100 mL of homogenate or pure GSH was added to 0.2 M Tris-EDTA solution (1.0 mL, pH 8.2) as well as 20 mM EDTA, pH 4.7 (0.9 mL) followed by 20 mL of Ellman's reagent (10 mmol/L DTNB in methanol). After 30 min of incubation at room temperature, samples were centrifuged, and the absorbance was recorded at 412 nm[16].

Total thiols

This assay is based on the principle of formation of relatively stable yellow color by sulfhydryl groups with DTNB.37 Briefly, 0.2 ml of brain homogenate was mixed with phosphate buffer (pH 8), 40 ml of 10mM DTNB, and 3.16 ml of methanol. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blanks [16].

Superoxide dismutase (SOD)

SOD to inhibit the autooxidation of adrenaine to adrenochrome at basic pH.33 Briefly, 25 ml of the supernatant obtained from brain homogenization was centrifuged was added to a mixture of epinephrine (0.1 mM) in carbonate solution (pH 10.2) for a total volume of 1 ml and subsequently measured at 295 nm to find the composition adrenochrome. The SOD(U/mg of protein) activity was calculated [16].

Statistical analysis

The data were analyzed by using analysis of variance (ANOVA) followed by Tukey's test. All the values are expressed as mean ± SEM. In all tests, the criterion for statistical significance was $P < 0.05$.

Results:-

Extraction and percentage of yield of Hydroalcoholic extraction of HAEAV.

Sl.no	Extract	Colour of extract	Consistency	% of yield
1	Hydroalcoholic	Greenish brown	Semisolid	7.9603%.

Preliminary phytochemicals investigation ofHAEAV

SI. No.	Type of test	HAEAV
1	Test for Flavonoids	+
2	Test for Alkaloids	-
3	Test for Tannins	+
4	Test for Protein and Amino acids	+
5	Test Cardiac glycosides	+
6	Test Saponin	+
7	Test for Anthraquinone	+
8	Test for Reducing sugar	-

Indication- Positive +, Negative -

The Preliminary phytochemicals investigation of HAEAV were carried out as per the standard procedure given in reference book (Khandelwal and kokate). The result relieved the presence of various phytoconstituents like flavonoids, tannins, proteins and amino acids, carbohydrates, Saponin, Anthraquinone and alkaloids, reducing sugars were absent in the HAEAV. The results are summarized in Ta

Behavioral assessment

Balance beam

The body balance and motor coordination were significantly ($p<0.001$ affected by the 3- NP alone when compared to normal control group. The normal control group crossed the beam in (5.1±0.57s). In 3-NP alone treated animals crossed in (16.50±1.07s). However, HAEAV treated group animals crossed the beam in (5.24±0.47s) which was comparable with the normal group. In contrast, the HAEAV group pre-treated with various doses shows a significant ($p<0.001$) effect against 3-NP induced damage in muscle coordination. The HAEAV low and high dose crossed the beam at (9.46±0.98 &4.41±0.35s) respectively. The results are compiled in Table-1 and graphically depicted inFig-A

Hangingwire

There was a significantly ($p<0.001$) reduction in the grip strength of the animals treated with the 3-NP alone (5.1±0.42 s) when compared to the normal group (11.82±1.23s).However, the HAEAV pre-treated groups showed a significant ($p<0.001$) increases in the muscle grip strength. The HAEAV low dose &high dose showed the increases in the muscle grip strength (9.12±0.33 & 13.13±0.75) when compared to inducer control. The results are compiled in Table-1 and graphically depicted in Fig-A

Actophotometer

3-NP alone treated decreases the locomotor count when compared with normal control group.as the normal group shows (90.70±13.32) locomotor count per 5min which is greater than the 3-NP alone treated (62.51±1.72). Due to this reduction was statistically insignificant. but on the other hand, the HAEAV pre-treatment showed significantly ($p<0.001$) increases in the locomotor count. As the doses of HAEAV (200 mg/kg & 400 mg/kg) showed the increased locomotor count (105.6±4.38 & 127.3±2.61) respectively. The results are compiled in Table-1 and graphically depicted in Fig-A

Fig. A. Effect of HAEAV on Behavioral assessment graphical representation

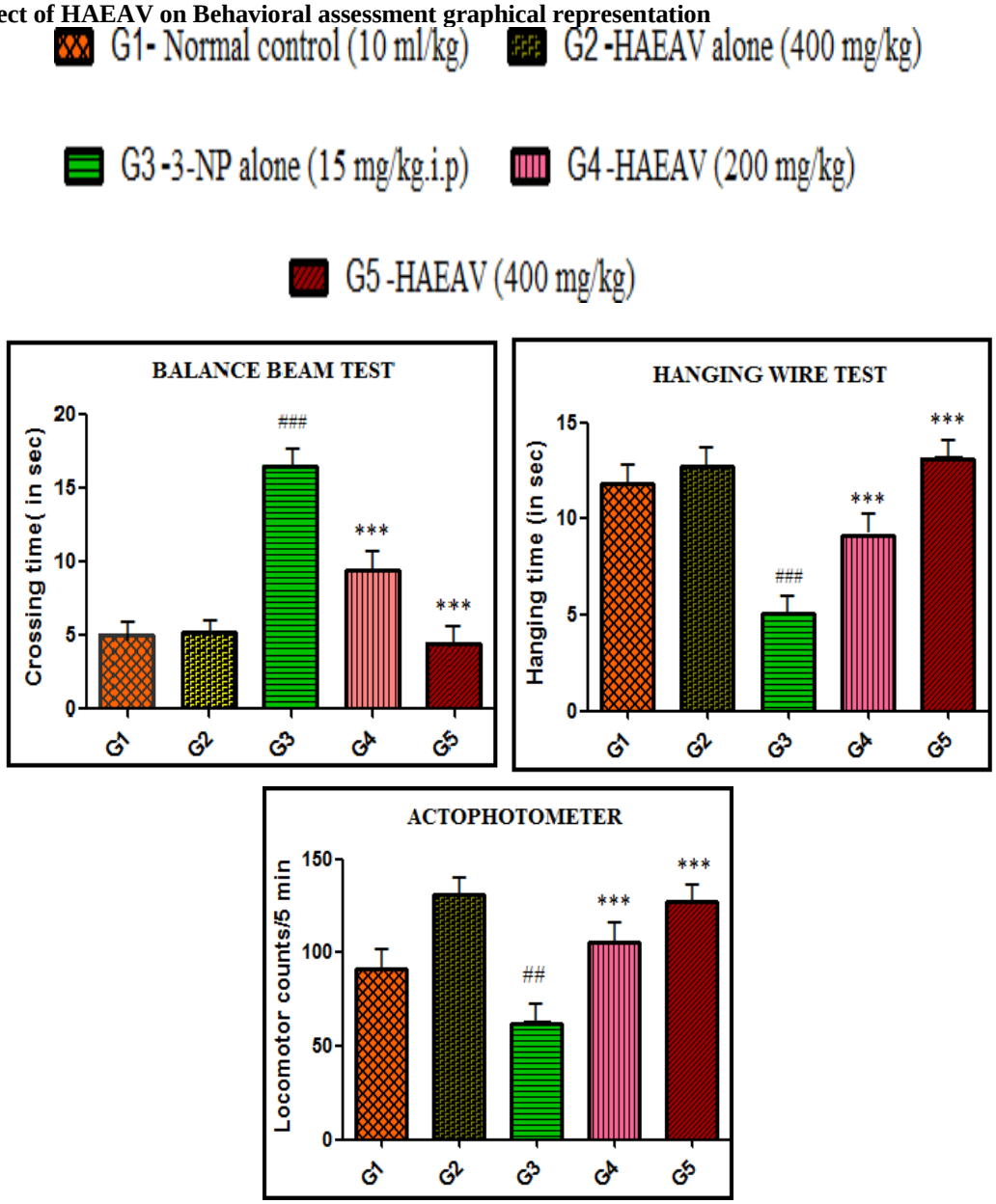


Fig. A:- Effect of HAEAV on Beam walk, hanging wire, Actophotometer in 3-NP induce neurotoxicity in rats. Each bar represents mean± SEM (n=6), ###p<0.001, ##p<0.01, #p<0.05 compared to normal control; ***p<0.001, **p<0.01, *p<0.05 compared to MSG alone group (One-way ANOVA followed by Tukey's multiple comparison tests at the significance level p<0.05.

Table 1:- Behavioral assessment using actophotometer, balance beam and hanging wire.

Treatment	Balance beam(in sec)	Hanging wire(in sec)	Actophotometer(scorecount/5min)
Normal control	5.1±0.57	11.82±1.23	90.70±13.32
HAEAV	5.24±0.47***	12.73±0.61***	131.3±12.4***
3-NP	16.50±1.07###	5.1±0.42###	62.51±1.72##
HAEAV (200mg/kg) +3-NP	9.46±0.98***	9.12±0.33**	105.6±4.38***
HAEAV(400mg/kg) +3-NP	4.41±0.35***	13.13±0.75***	127.3±2.61***

Table.1. Effect of HAEAV on Beam walk, Hanging, Actophotometer in 3-NP induce neurotoxicity in rats. Each bar represents mean± SEM (n=6), $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ compared to normal control; *******p<0.001, ******p<0.01, *****p<0.05 compared to MSG alone group (One-way ANOVA followed by Tukey's multiple comparison tests at the significance level p<0.05.

Measurement of oxidative stress parameters

Catalase

In the present study, the 3-NP alone group shows (0.12±0.1) reduction in levels of catalase when compared to normal control group. 3-NP alone treated group showed significant (p<0.01) reduction in the tissue catalase levels when compare with normal control group. Furthermore, when pre-treatment with HAEAV, it shows significantly (p<0.001) increase in the catalase levels when compared with the inducer group. When treated with HAEAV high dose 400 mg/kg (0.24±0.11) correspondingly shows increase in the catalase levels when compared with inducer group. When rats are administered with low dose 200 mg/kg (0.21±0.02) shows statistically non-significantly. The results are compiled in Table 2 and graphically depicted in Fig.B.

LPO

In the study, when 3-NP alone treated shows (26.64±4.65) increase in tissue levels of LPO when compared with normal group (26.64±4.65). In the 3-NP alone treated group showed show significantly (p<0.01) increase in the tissue LPO levels, when compared with normal group. When HAEAV is pre-treatment, it shows (p<0.001) significantly reduction in the tissue LPO levels when compared with the 3-NP alone treated group. Whereas the HAEAV lower dose (200 mg/kg &400mg/kg) decreases the tissue LPO levels (15.62±1.10 and 12.65±0.75 respectively) when compared to the 3-NP alone treated animals and low dose 200 mg/kg is statistically insignificant. The results are compiled in Table.2 and graphically depicted in Fig.B.

TT

In the estimation of TT, 3-Np treated group shows (0.64±0.02) reduced in tissue levels when compared with the control group (0.86±0.03) 3-Np treated group significantly diminished the tissue TT level compared a normal control.Pre-treatment of HAEAV shows significantly increases when compared with the 3-Np group. However, the low dose 200 mg/kg & high dose 400mg/kg HAEAV (0.88±0.03& 0.87±0.02 respectively) shows significantly increases in the TT tissues levels. The results are compiled in Table.2 and graphically depicted in Fig.B.

GSH

In the estimation of GSH, 3-NP alone treated group shows (20.58±0.15) importantly decrease in the tissue GSH levels when compared with the normal control group (51.92±5.23). The 3-NP alone treated group significantly (P<0.001) diminution the tissue GSH level when compared a normal control. The pre-treatment of HAEAV shows significantly (P<0.001) increase when compared to 3-NP group. However, the low dose 200 mg/kg & high dose 400mg/kg HAEAV (34.11±2.74 & 43.26±0.40 respectively) shows (p<0.001) significantly increases in the tissue GSH levels. But low dose is statically insignificantly. The results are compiled in Table.2 and graphically depicted in Fig.B.

SOD

In the treatment of 3-NP alone treated group showed (9.07±0.61) reduced tissue SOD levels when compared with the normal control (22.27±1.25). The 3-NP alone treatment shows (p<0.001) significant decreases in the tissue SOD levels. When HAEAV low doge 200 mg/kg increases in tissue SOD levels (15.76±1.85) when compared to the 3- NP alone group. But, statistically insignificant shows (p<0.01). Whereas in thehigh dose HAEAV 400 mg/kg increase the tissue SOD levels when compared to the 3-NP alone group. Which shows statistically (p<0.001). The results are compiled in The results are compiled in Table.2 and graphically depicted in Fig.B.

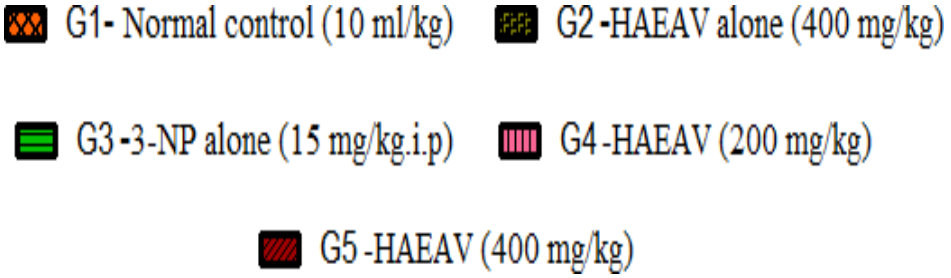
Table 2:- Estimation of anti-oxidant enzymes levels.

Treatment	Catalase (U/mg of tissue)	LPO (nm/g of tissue)	Total thiols(nm/g of tissue)	GSH (nm/g of tissue)	SOD (U/ml of tissue)
Normal control (10 ml/kg,p.o)	0.27±0.01	12.33±1.17	0.86±0.03	51.92±5.23	22.27±1.25
HAEAV alone	0.23±0.01***	11.72±0.33**	0.82±0.01**	37.57±2.70*	19.32±1.83**

(400mg/kg)					
3NP(15mg/kg,i.p)	0.12±0.1##	26.64±4.65##	0.64±0.02##	20.58±0.15###	9.07±0.61###
HAEAV(200mg/kg)+ 3NP(15mg/kg,i.p)	0.21±0.02***	15.62±1.10**	0.88±0.03***	34.11±2.74**	15.76±1.85**
HAEAV(400mg/kg)+ 3- NP(15 mg/kg,i.p)	0.24±0.11***	12.65±0.75***	0.87±0.02***	43.26±0.40***	17.50±1.52***

Table.2. Effect of HAEAV on GSH, LPO, CAT, TT, SOD in 3-NP induce neurotoxicity in rats. Each bar represents mean± SEM (n=6), $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ compared to normal control; ***p<0.001, **p<0.01, *p<0.05 compared to MSG alone group (One-way ANOVA followed by Tukey's multiple comparison tests at the significance levelp<0.05.

Fig.B:- Effect of HAEAV on brain tissue antioxidant levels in 3-NP induced neurotoxicity in rats.



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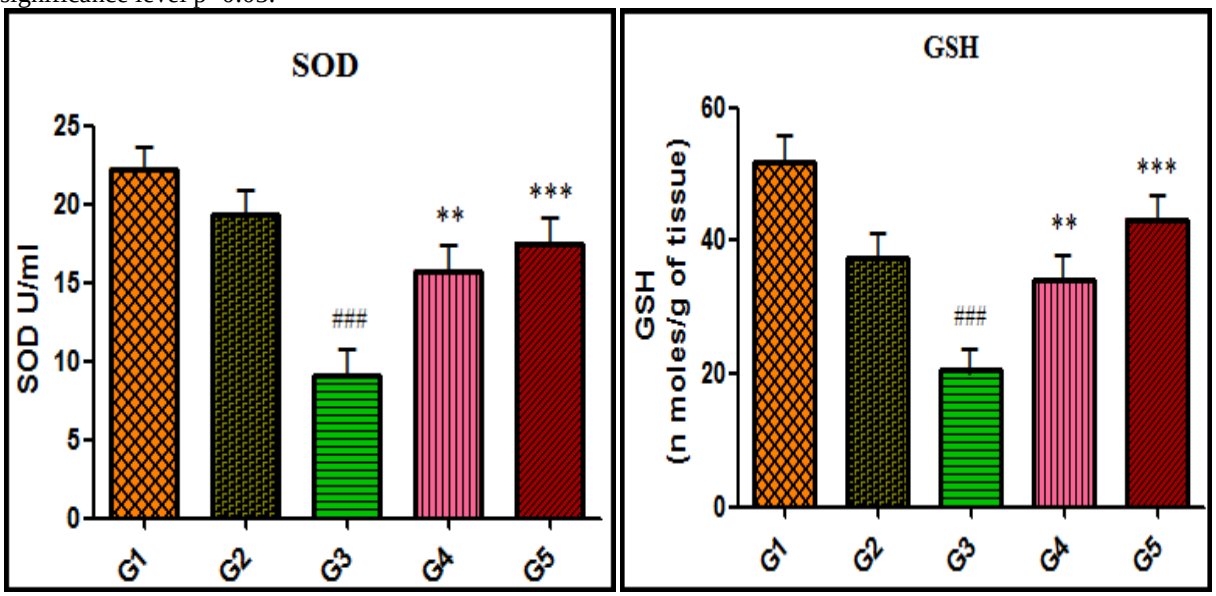

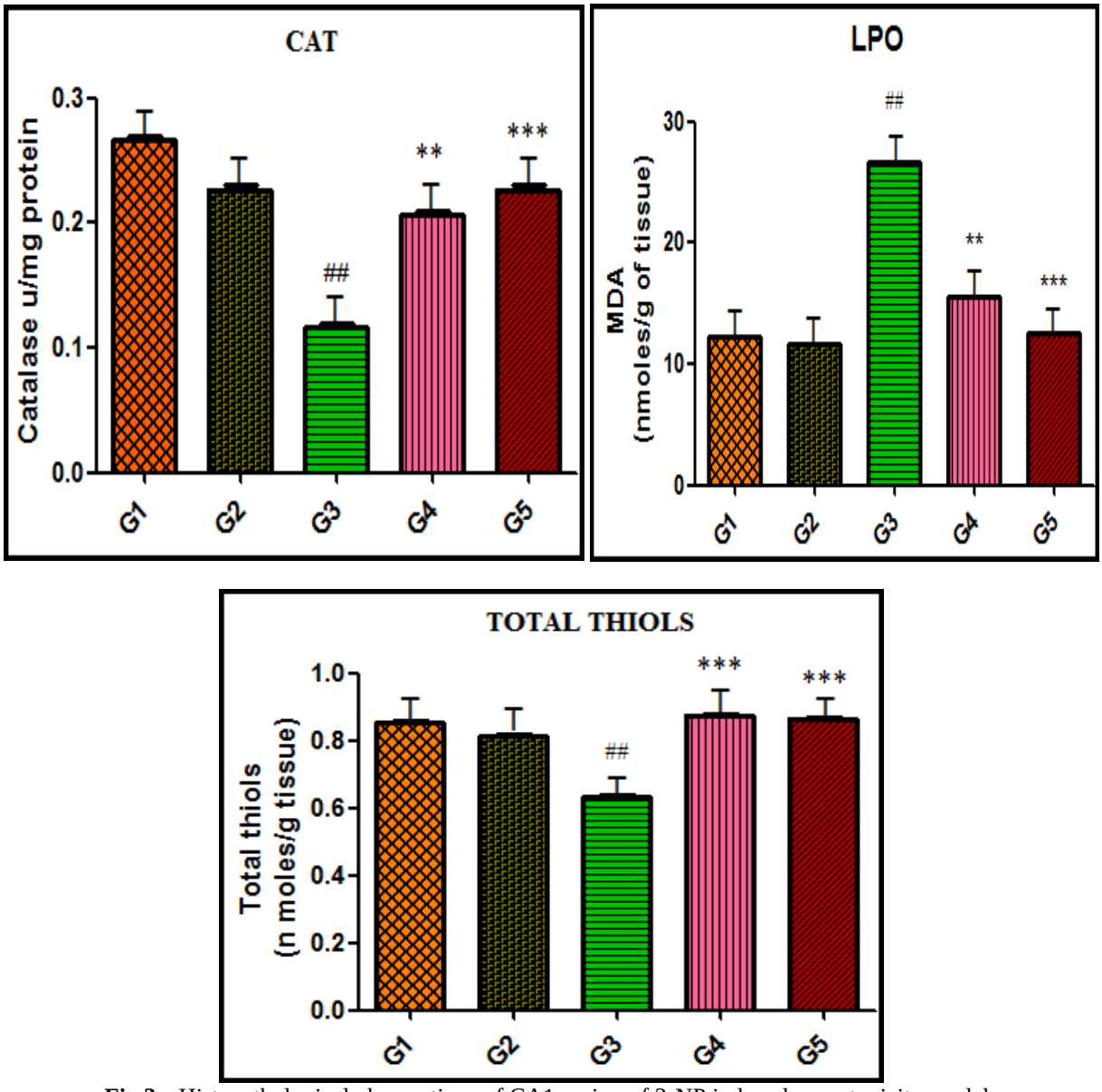

Fig.3:- Histopathological observations of CA1 region of 3-NP induced neurotoxicity model.

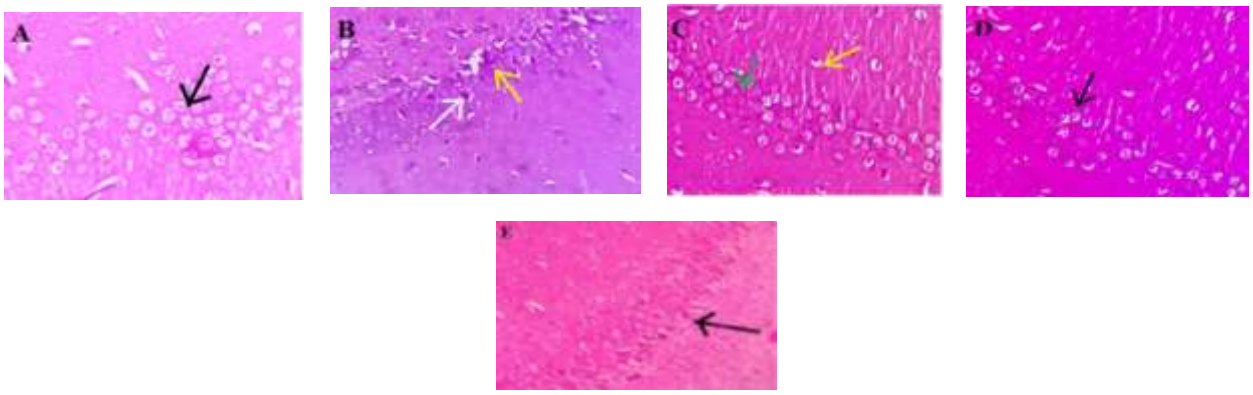

Fig.C:- Histopathological changes of hippocampal region of brain in 3-NP induced neurotoxicity model exhibited by (a) Normal control, (b) 3-NP alone (15 mg/kg .p.o.), (c) Low dose HAEAV (200 mg/kg),(d) High dose HAEAV (400 mg/kg), (e) HAEAV alone.

Black- normal cells of CA1 cells, Yellow- Thinning of CA1 region, White- shrunken and clumped CA1 layer cells. Green- Nuclear pyknosis.

Discussion:-

The present study highlights the protective role of HAEAV on 3-NP induced neurotoxicity by assessing its morphological, behavioral, biochemical and histopathological parameters. The Phytochemical constituent from natural sources is found to have antioxidant property and may be used in prevention and treatment of the neurodegenerative diseases. So, we have been done experiment aimed to study the effect of HAEAV on3-nitropropionic acid induced neurotoxicity inalbinoWisterrat. Theintraperitonially administrations of 3-nitropropionic acid for 14 days significantly produce behavioral alterations (reduced locomotor, grip strength performance, increases time in beam) and oxidative damage. 3-NP administration can cause both hypoactivity and hyperactivity depends on frequency and time of dosing. Animals which received 3-NP for 14 days exhibit marked neuronal loss and movement disorders are related to basal ganglia lesions.The HAEAV treatment improved the hypoactivity, as evident by several behavioral investigations such as movement analysis and locomotor activity. It was also observed that there is significant increase in motor deficits, cognitive function, grip strength, body coordination and restored the body weight and anti-oxidant levels in brain. Further, histopathological observation of hippocampal region of rat brain section indicates the protection of neuron in 3-NP. The 3-NP administration in early reports alsoshowed cognitive deficit, motor deficit and hypokinetic activity which resembles clinical symptom related to Huntington disease. Pre-treatment and treatment with both the doses of HAEAV significantly protected the 3-NP induced increase in behavior and motordeficit.The administration of 3-NP for 14 days, produced oxidative stress (increased levels of LPO (as evident by increased MDA levels) and depleted levels of endogenous antioxidant enzyme (catalase, reduced glutathione, TT, SOD levels). These results support the oxidative stress based on the theory of neurotoxicity caused by 3- NP. On the other hand,treatment with HAEAV has significantly reversed the 3-NP induced changes. However, treatment with the high dose of HAEAV has produced more significant reversal of catalase, GSH, TT, SOD than low dose. The reason could not establish this study however; the LPO produces similar changes suggesting the possible involvement of antioxidant action of HAEAV in preventing 3- NPneurotoxicity. Subsequently, the above dissection has revealed that HAEAV protective action against 3-NP induced neurotoxicity in rat. This protective effect might be partway due to its antioxidants and anti-inflammation properties. Our discovery also showed the significant neuroprotective and antioxidant action against chemically induced neurotoxicity in rats by showing well preserved levels of endogenous antioxidation and inhibition of lipid peroxidation and the neuroprotective effects of HAEAV may be to its antioxidants and anti- inflammation potential.

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