CHAPTER V

EVALUATION OF MEMEORY ENHANCING AND NEUROPROTECTIVE EFFECTS OF *MORINGA OLEIFERA*, *ANACARDIUM OCCIDENTALE* AND *NELUMBO NUCIFERA* IN STRESS-EXPOSED RATS

5.1 Introduction

To date, the daily lifestyles are full of pressures, demands and frustrations leading to the increased stress. Repetitive stress exposure has been reported to produce the detrimental effects on the damage and dysfunctions of various organs including brain damage. A substantial evidence obtained from both animals and human has demonstrated that stress can alter brain cell properties, disturb cognitive processes, such as, learning and memory, and consequently limit the quality of human life (Kim and Diamond, 2002; Mizoguchi *et al.*, 2000). In addition, stress is also regarded to precipitate or exacerbate many neuropsychiatric disorders such as depression, Alzheimer's disease, Parkinson's disease, schizophrenia, and others (Johansson *et al.*, 2010; Mazure, 1995; Schwab and Zieper, 1965).

Stress response, a complex reaction in the organisms, occurs via 2 main systems including sympathetic nervous system and hypothalmo-pituitary-adrenal (HPA) axis. The stimulation of sympathetic nervous system gives rise to the secretion and elevation of catecholamine leading to the enhanced oxidative load (Ahmad *et al.*, 2012) resulting in memory deficit (Kumar *et al.*, 2009). In addition, the stimulation of HPA-axis which results in the increased secretion and elevation of glucocorticoids (cortisol in humans; corticosterone in a variety of animals including rodents) is also reported to induce hippocampal damage and results in memory deficit (McEwen, 2007; Wang *et al.*, 2006). Recent study has revealed that the memory deficit induced by stress can be attenuated by substance possessing antioxidant effect such as Ascorbic acid (Kumar *et al.*, 2009). Therefore, the neuroprotective and cognitive enhancing effect of substances possessing antioxidant effects has gained much attention.

Moringa oleifera, a plant in the family of Moringaceae, has been widely used as food and medicine in many countries including Thailand for a long time. Its leaves have been consumed as indigenous vegetable by Asian village people. Recently, it has been demonstrated that *M.oleifera* leaves extract protects against oxidative related brain damage such as stroke and dementia (Kirisattayakul *et al.,* 2013; Sutalangka *et al.,* 2013). It also enhances memory performance by suppressing acetylcholinesterase and improved oxidative stress in hippocampus (Sutalangka *et al.,* 2013). The extract also improved stress response by decreasing serum corticosterone level and suppress MAO-B activity in stress exposed rats (PRABSATTROO *et al.*, 2015). In addition, it has been reported that *M.oleifera* leaves extract is safe up to 2 g/kg BW (Awodele *et al.,* 2012).

Anacadium accidentale, a plant in the family of Anacardiaceae, native plant in dry areas of the Central America and northern part of South America. Now, it is widely distributed in Thailand. *A.occidentale* leaves have been used as the traditional medicine. It has been reported that *A.occidental* leaves extract showed antioxidant, antidiabetic (Kamtchouing *et al.*, 1998), antiulcerogenic effect (Konan *et al.*, 2007). Acute toxicity study, crude hydro-ethanolic extract did not produce toxic symptoms in rats in doses up to 2 g/kg (Konan *et al.*, 2007).

Nelumbo nucifera, a plant in the family of Nymphaeaceae, has been used as a medical plant in Chinese medicine and Ayurvedic medicine (Mukherjee *et al.*, 2009). It has been reported that *N.nucifera* flowers extract showed antioxidant effect (DPPH, FRAP), antibacterial activity (Durairaj and Dorai, 2010), antitumor activity (Durairaj and Dorai, 2010), inhibition of Acetylcholinesterase (AChE) (Durairaj and Dorai, 2010).

Based on the safety consumption, the anti-stress, antioxidant, neuroprotective and cognitive enhancing effect of *M.oleifera* leaves, *A.accidentale* leaves and *N.nucifera* flower extract mentioned earlier, the protective effect against brain damage and memory impairment induced by stress of selected Thai medicinal plants has been considered. Therefore, we aimed to determine the effect of *M.oleifera* leaves, *A.accidentale* leaves and *N.nucifera* flower extract on spatial memory and hippocampal damage in restraint stress-exposure rats. In addition, the possible underlying mechanism was also investigated.

5.2 Materials and Methods

5.2.1 Plant Materials and Preparation

5.2.1.1 Preparation of *Moringa oleifera* leaves extract

Fresh leaves of *M. oleifera* were harvested during November-December, 2010 from Khon Kaen province, Thailand. After the authentication, the herbarium specimen was deposited at Integrative Complementary Alternative Medicine Research Center, Khon Kaen University (voucher specimen 2010002). The fresh leaves were cleaned, cut in to small pieces and dried in oven at 40^{o}C. The dried plant material was ground into powder and extracted with 50% hydro-ethanolic by maceration technique. The extract was filtered through Whatman filter paper number 1 and concentrated with rotator evaporator at 45°C. Then, the yielded extract was kept at 4°C till used. The percentage yield of the extract was 17.49%. The concentration of total phenolic compounds was 62.333±0.008 mg GAE·g^{-1} extract. In addition, the contents of ferulic acid and quercetin were 0.003±0.0001 mg FAE·mg^{-1} extract and 0.444±0.0001 mg QE·mg^{-1} extract respectively.

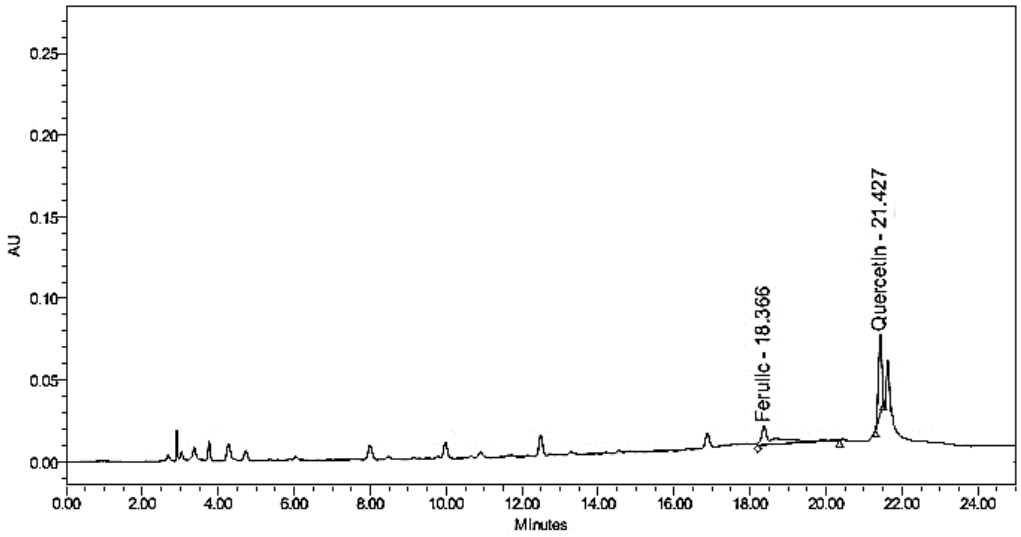

Figure 5-1 The fingerprint chromatogram of 50%hydroalcoholic extract of *Moringa oleifera* leaves used in this study

5.2.1.2 Preparation of *Anancardium occidentale* leaves extract

A. occidentale leaves were collected from Phuket province and authenticated by Associate Professor Panee Sirisa-ard, Faculty of Pharmacy, Chiang Mai University. After the authentication, the herbarium specimen was deposited there. The fresh leaves were cleaned, cut in to small pieces and dried in oven at 40°C. The dried plant material was ground into powder and extracted with 95% hydro-ethanolic by maceration technique. The extract was filtered through Whatman filter paper number 1 and concentrated with rotator evaporator at 45°C. Then, the yielded extract was kept at 4°C till used. The percentage yield of the extract was 17.32%. The concentration of total phenolic compounds was 102.963±0.006 mg GAE·g^{-1} extract. In addition, the contents of gallic acid and quercetin were 7.771±0.003 mg GAE·mg^{-1} extract and 0.617±0.0001 mg QE·mg^{-1} extract respectively.

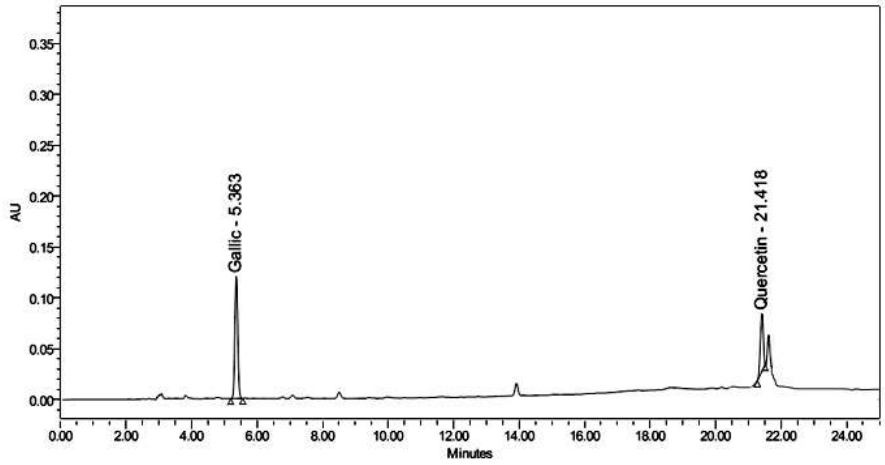

Figure 5-2 The fingerprint chromatogram of 50%hydroalcoholic extract of *Anacardium occidentale* leaves used in this study

5.2.1.3 Preparation of *Nelumbo nucifera* flower extract

Fresh flower of *N.nucifera* were harvested during November-December, 2012 from Khon Kaen province, Thailand. *N.nucifera* were authenticated by Dr. Nopachai Chansilp, Rajamangala University of *Technology* Tawan-ok. After the authentication, the herbarium specimen was deposited at Integrative Complementary

Alternative Medicine Research Center, Khon Kaen University. The fresh flowers were cleaned, cut in to small pieces and dried in oven at 40°C. The dried plant material was ground into powder and extracted with 50% hydro-ethanolic by maceration technique. The extract was filtered through Whatman filter paper number 1 and concentrated with rotator evaporator at 45°C. Then, the yielded extract was kept at 4°C untill used. The percentage yield of the extract was 10.23%. The concentration of total phenolic compounds was 152.963±0.009 mg GAE·g^{-1} extract. In addition, the contents of quercetin were 0.456±0.0001 mg QE·mg^{-1} extract.

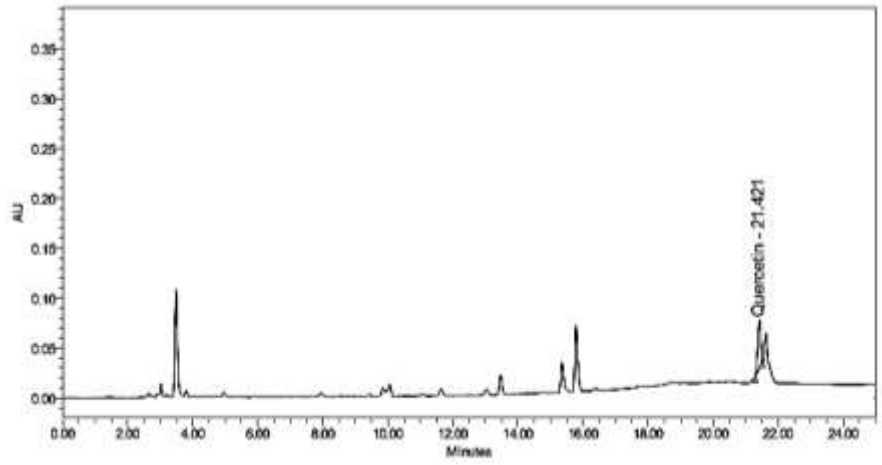

Figure 5-3 The fingerprint chromatogram of 50%hydroalcoholic extract of *Nelumbo nucifera* flowers used in this study

5.2.2 Animal treatment

Adult male Wistar rats, weight 250–350 g were obtained from National Laboratory Animal Center, Salya, Nakorn Pathom province. They were housed, six per cage, under standard conditions and maintained on a 12:12 dark-light cycle. Temperature was controlled at 24±1°C. Food and water were available *ad libitum* throughout the experiments.

5.2.3 Experimental protocol

This study was divided into 3 parts. The first part was designed to evaluate the cognitive enhancing effect of *Moringa oleifera* Lam leaves in restraint rats whereas the second and the third parts were set up to determine the cognitive

enhancing effect of *Anacardium occidentale* Linn leaves and *Nulumbo nucifera* Gerth flower in restraint rats.

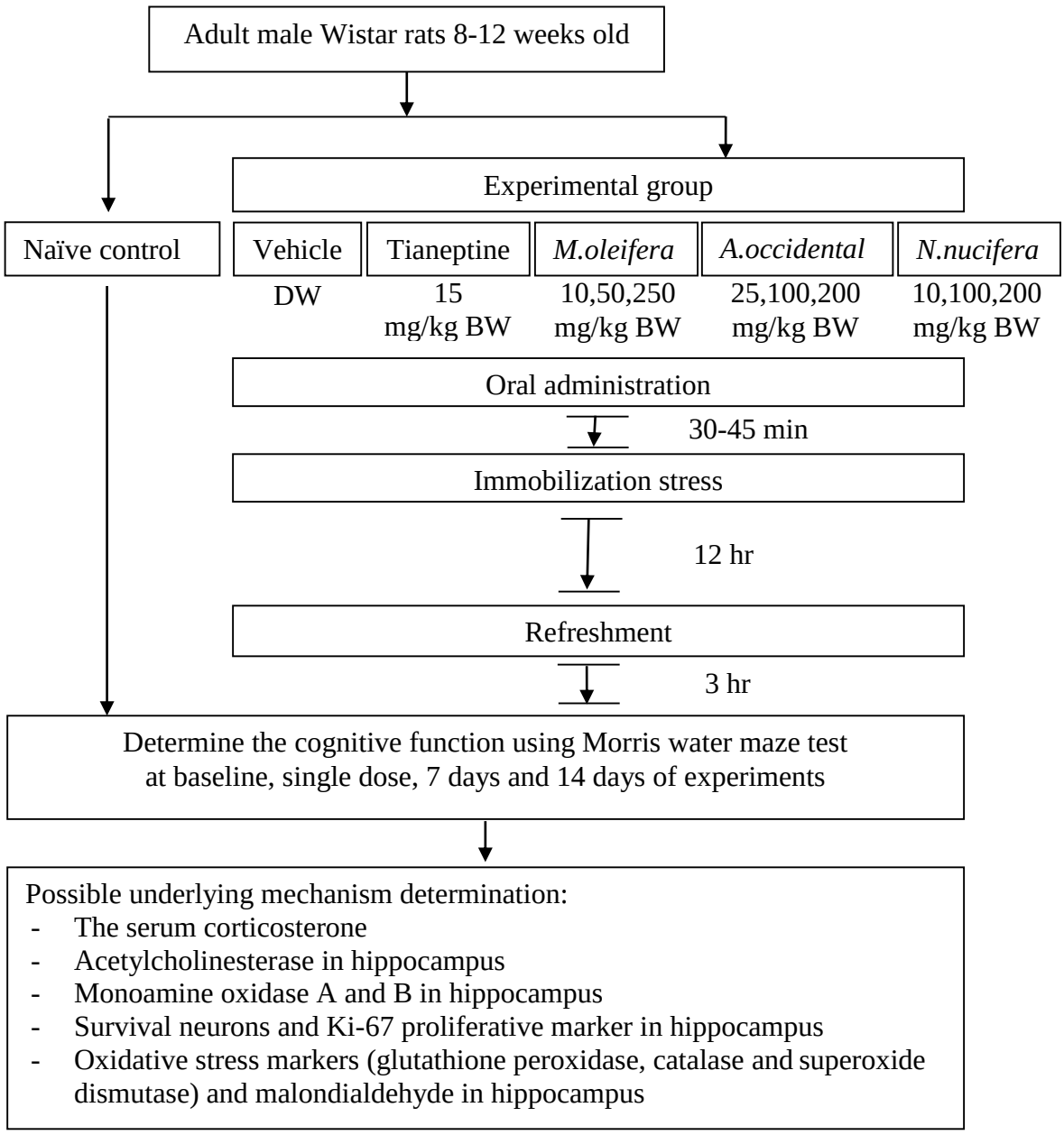

Figure 5-4 The schematic diagram represents the experimental protocol of this study

Adult male Wistar rats, weighing 250-350 g (8-12 weeks of ages) (n = 6/group) were trained for learning and memory since 7 days. The trained animals were divided into various groups as following; naïve control, vehicle treated group,

Tianeptine treated group and 3 various doses of plant extract (*M.oleifera;* at dose of 10,50 and 250 mg/kg BW, *A.occidentale;* 25,100 and 200 mg/kg BW and *N.nucifera*; 10, 100 and 200 mg/kg BW) treated groups. Rats in all groups except naïve control were orally administrated the assigned substances before exposed to 12 hours immobilization stress.

5.2.4 Morris water maze test

The water maze is a circular pool with 160 cm in diameter, 60 cm in height filled with water and 42 cm in depth. The water pool was filled with tap water at temperature of 23 – 2 4º C. The pool was divided into 4 quadrants and the water surface was covered with nontoxic milk powder. The removable platform was submerged in the center of one quadrant. Visual cues were placed at fixed places in order to facilitate spatial orientation of the animal and the positions of the cues throughout the study period. The animals must be trained to memorize the location of the hidden platform by forming the association between its location and the visual cues. During training session, animals were allowed to find the platform within 60 seconds. If the animal failed to find the platform within 60 sec, it was gently guided to the platform and stayed there for 10 sec, and its escape latency was recorded as 60 sec. If an animal found the platform within 60 sec, it was allowed to remain there for 10 sec and was then placed into a cage until next trial. Each animal was subjected to four acquisition trials per day for four consecutive days. After the last trial in each session, the rat was towel-wiped and placed in a drying chamber for 5 to 15 min and then returned to the home cage. The time which the animals spent to climb on the hidden platform was recorded as escape latency. 24 hour later, the platform was removed and the rat was released into the quadrant diagonally opposite to the quadrant which previously contained the platform and the time which each animal spent swimming in this quadrant was recorded as retention time.

5.2.5 Corticosterone assay

At 7 and 14 days of study period, the blood of each animal was collected and kept on ice. Then, it was centrifuged immediately at 2000 ×g at 4 °C for 15 min. The obtained serum was kept at −80 °C until use. Corticosterone levels were measured using Corticosterone Double Antibody Radioimmunoassay Kit (MP

Biomedicals) for the quantitative determination of corticosterone in rat and mice serum. The results were showed as ng/ml.

5.2.6 Determination of acetylcholine esterase (AChE) activity

Hippocampus was isolated and prepared as hippocampal homogenate and the determination of acetylcholinesterase (AChE) activity in hippocampus was performed. AChE activity was determined using Ellman method (Ellman *et al.*, 1961). Protein concentrations were determined in brain tissue supernatants by the method of Lowry and colleagues (Classics Lowry *et al.*, 1951) using bovine serum albumin as standard.

5.2.7 Determination of monoamine oxidase A and B activity

Monoamine oxidase type A and B (MAO-A and MAO-B) were determined by the continuous peroxidase-linked photometric assay which was carried out in the 96-well plate modified from Holt *et al.* (Holt *et al.*, 1997). The hippocampus of rats were collected and prepared as a homogenate with RIPA buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxylcholate, 0.1% SDS and 1% TritonX-100). Brain homogenate was centrifuged at 14, 000 g, at 4 ^{o}C for 20 min. After the centrifugation, the supernatant was harvested to determine MAO-B activity. Briefly, the well contained 120 µl amino substrate (2.5 mM tyramine (Sigma-Aldrich) in potassium phosphate buffer), 40 µl chromogenic solution (1 mM vanillic acid (Sigma), 0.5 mM 4-aminoantipyrine, 4 U/ml peroxidase in potassium phosphate buffer, 40 µl of brain homogenate and then incubated for 30 min at 37 °C. In order to determine the specific MAO-A activity the brain homogenate was pre-incubated with clorgyline (selective MAO-A-I) for 30 min at 37 °C. MAO-B activity was determined by pre-incubating the reaction mixture with pargyline (irreversible MAO-B-I). The reactions were read with microplate reader at 490 nm using a microplate reader.

5.2.8 Determination of oxidative stress markers

In order to determine the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), the brains tissues were weighed and homogenized with a phosphate buffer. Then, the brain homogenates were centrifuged at 3000 g for 15 min at 4 °C. The supernatant was used for bioassays. The activity of SOD was determined using a xanthine/

xanthine oxidase reaction which was used as a superoxide ($O_2^{\cdot}$) generator. Based on the previous findings that oxidized cytochrome c was reduced by the superoxide radical while SOD competed with superoxide radical to reduce cytochrome c. The rate of reduction of cytochrome c was recorded spectrophotometrically at 550 nm. (McCord and Fridovich, 1969). SOD activity was presented as unit/mg·protein. CAT was measured by modified methods of Chance and Maehly (Chance and Maehly, 1955). The activity of CAT was determined based on the chemical titration on the conversion over time of hydrogen peroxide to water and oxygen gas by using enzyme catalase. According to this method, the enzyme solution was allowed to react with hydrogen peroxide for varying periods of time and measuring the excess peroxide remaining by titration with potassium permanganate. CAT activity was expressed as unit/mg·protein. Glutathione peroxidase was determined by the procedure of Rotruck et al (Rotruck *et al.*, 1973) with some modifications. Hydrogen peroxide was used as the substrate and incubated with glutathione. The remaining GSH was detected by 5,5'-dithiobis-(2-nitrobenzoic acid) reagent and measured with spectrophotometer at 410 nm. GSH-Px activity was expressed as unit/mg·protein. Level of malondialdehyde (MDA) was indirectly estimated by thiobarbituric acid reaction assay (TBARS). The TBARS assay measures oxidative stress by quantifying the peroxidative damage to lipids that occurs with free radical generation. Free radical damage to lipids results in the production of MDA, which reacts with TBA under conditions of high temperature and acidity generating a chromogen that can be measured either spectrophotometrically (Uchiyama and Mihara, 1978).

5.2.9 Histological study

5.2.9.1 Tissue preparation

The brains were removed after the transcardial perfusion with NSS and postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C overnight. Then, the brains were immersed sequentially in a cryoprotectant containing 30% sucrose for 48-72 h. Serial sections of tissues were cut at 10 μm thick. Sections were stored in phosphate buffer and they were picked up on slides coated with a 0.01% aqueous solution of a high molecular weight poly–L-lysine. The serial cut sections were either stored at 4°C or processed immediately.

5.2.9.2 Determination of the neurons density in hippocampus

Adjacent series of sections throughout cerebral cortex, striatum and hippocampus from all groups were stained with 0.5% cresyl violet. All sections of cerebral cortex and hippocampus were investigated with the aid Olympus light microscope model BH-2 (made in Japan). To determine the density of neuron, fine representative non-adjacent sections which contained cerebral cortex, striatum and hippocampus were selected for analysis. The observer was blind to the treatment at time of analysis. The density of neuron was determined at 40X magnification.

5.2.9.3 Morphological Analysis

Six coronal sections from each rat in each group were studied quantitatively. Neuronal counts were performed by eye using a ×40 objective with final field 255 μm^2 and bregma coordination according to the following stereotaxic coordinates: AP -4.8 mm, lateral ± 2.4-6 mm, depth 3-8 mm. The observer was blinded to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained 50 soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm^2. All data are represented as number of neurons per 255 μm^2.

5.2.10 Western blot analysis

Western blot analysis was used to determine the expression of Ki-67 in hippocampus (Titheradge, 1998). Briefly, hippocampus of each rat was isolated and prepared and homogenized in ice cold RIPA buffer with protease inhibitors. The solution was then centrifuged at 14,000 g at 4 °C for 20 minutes. The supernatant was collected and measured the level of protein by using NANO drop Spectrophotometers. Equal amounts of protein (50 µg) were fractionated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). Each step was preceded by rinses (three times for 5 min) in 0.05% Tris-buffer saline with Tween-20. Membranes were blocked with 5% skim milk in Tris-buffer saline with 0.05% Tween-20) and incubated overnight in primary antibody against Ki-67, a proliferative marker (1:500). The membranes were then incubated with horseradish peroxidase-linked secondary antibody (1:4,000) for 1 hr at room temperature. Then the signal was enhanced with a Thermo Scientific™ Pierce™ ECL Substrate chemiluminescence kit (Pierce™ ECL

Western Blotting). Images were acquired by ImageQuant LAS 4000, GE Healthcare. Band densities were quantified with NIH-ImageJ (Version 1.48V; National Institutes of Health,USA). The PVDF was reprobed with the beta actin antibody (1:2,000) as a loading control.

5.2.11 Statistical analysis

The data were expressed as mean ± SEM. The significance of differences among the groups were assessed using one way analysis of variance (ANOVA) test followed by LSD multiple comparison test using SPSS, version 13. P-value < 0.05 was considered as significance.

5.3 Results

5.3.1 Effect and Possible Underlying Mechanism of *M.oleifera* leaves Extract on Memory Impairment and Neurodegeration in Stress-Exposed Rats

5.3.1.1 Effect of *M.oleifera* leaves extract on spatial memory

Figure 5-5 showed that rats which subjected to immobilization stress and received vehicle or water significantly increased escape latency after a single dose of administration and at both 7 and 14 days of treatment (P-value<.01, .001 and .01 respectively; compared with control group). Tianeptine could attenuate the increased escape latency in restraint stress rats throughout the study period (P-value<.05, .001 and.01 respectively; compared with vehicle+stress treated group). It was found that *M.oleifera* leaves at dose of 250 $mg.kg^{-1}$ BW could attenuate the elevation of escape latency at 7 and 14 days after treatment (P-value<.01 all; compared with vehicle+stress treated group). No significant changes were found in other groups.

Figure 5-6 also showed that rats which were exposed to 12-h restraint stress and received vehicle decreased the retention time after a single dose of administration and at both 7 and 14 days of treatment (P-value<.05, .01 and .01 respectively; compared with control group). Tianaptine could mitigate the reduction of retention time in restraint rats (P-value<.001, .001 and.01 respectively; compared with vehicle+stress treated group). Interestingly, *M.oleifera* leaves extract at doses of 50 and 250 $mg.kg^{-1}$ BW could attenuate the reduction of retention time induced by restraint stress in male rats (P-value<.05 all; compared with vehicle+stress treated group).

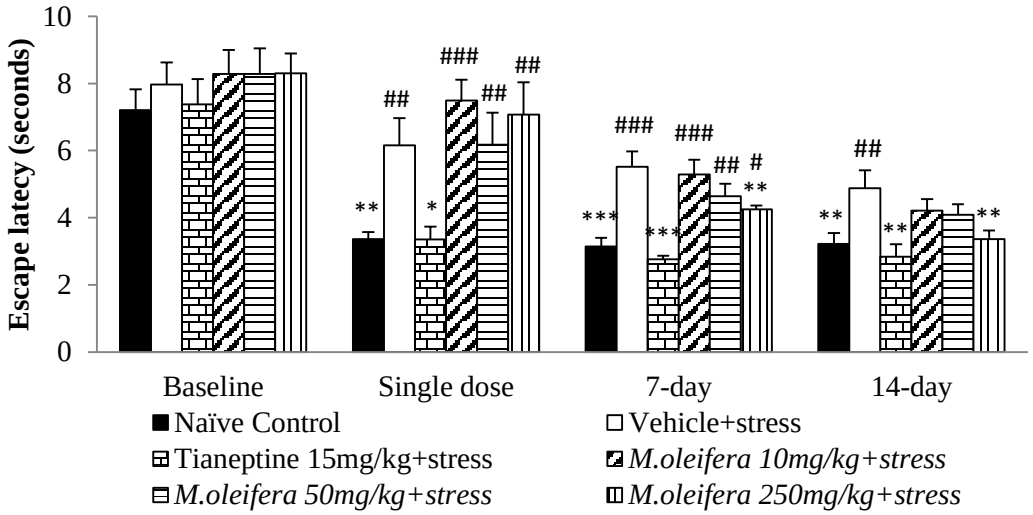

Figure 5-5 The effect of hydro-alcoholic extracts of *M. oleifera* leaves extract on escape latency of stress-exposed rats at baseline, after a single dose, 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). *,**,****P*-value <0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#,\#\#\#}$ *P*-value < 0.05, 0.01 and 0.001 respectively; compared with control group

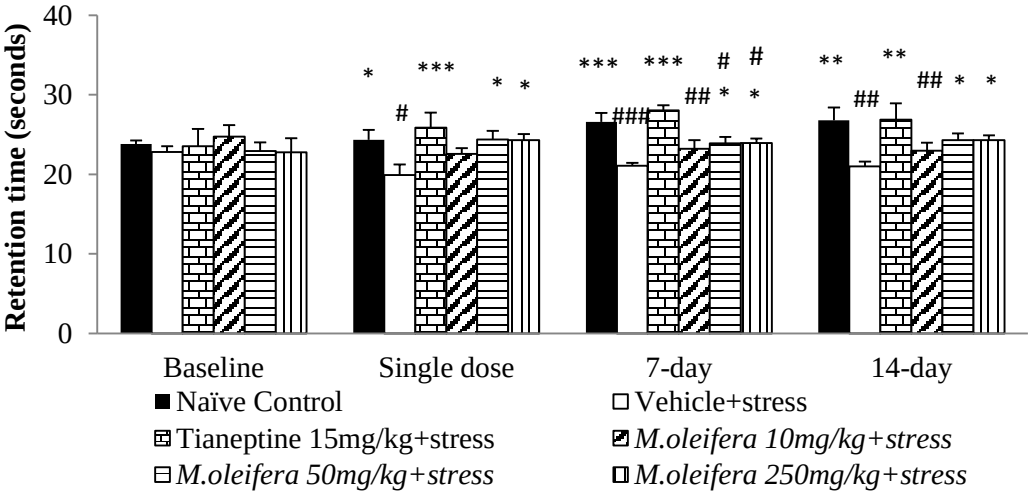

Figure 5-6 The effect of hydro-alcoholic extracts of *M. oleifera* leaves extract on retention time of stress-exposed rats at baseline, after a single dose, 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). *,**,****P*-value <0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#}$ *P*-value < 0.05 and 0.01 respectively; compared with control group

5.3.1.2 Effect of *M.oleifera* leaves extract on serum corticosterone level

Rats which received vehicle and repetitive stress exposure significantly increased serum corcosterone levels (p-value<.05 all; compared to naïve control) both at 7 and 14 days of exposure time (Figure 5-7). Tianeptine failed to modulate the elevation of serum corticosterone in stress exposed rats. *M.oleifera* leaves extract at doses used in this study mitigated the elevation of serum corticosterone in stress exposed rats (p-value<.01, .001 and .05 respectively; compared to vehicle+stress) at 7 days of exposure time. However, only stress exposed rats which received low and high doses significantly mitigated the elevation of this parameter when the exposure time was prolonged to 14 days (p-value<.05 and .01 respectively; compared to vehicle+stress).

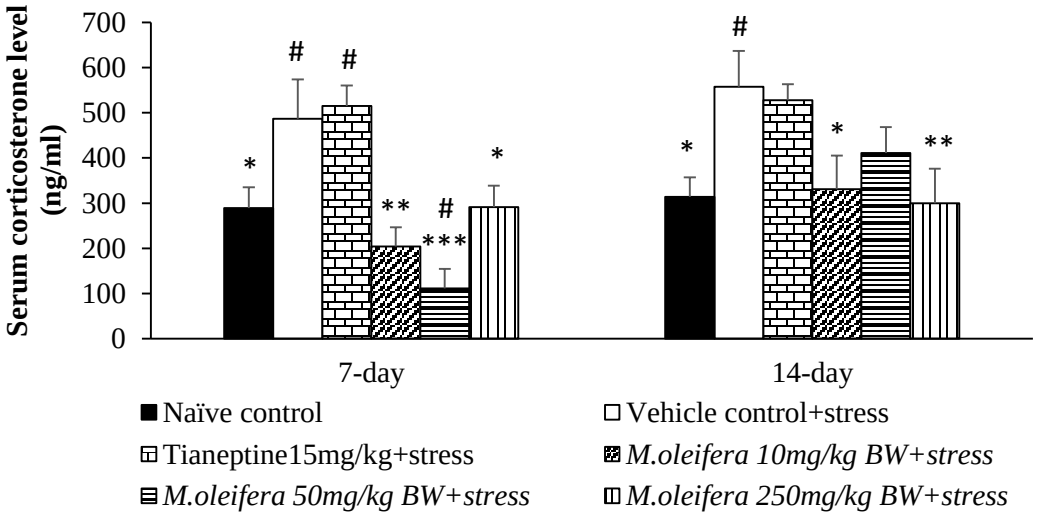

Figure 5-7 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on serum corticosterone level of stress-exposed rats at 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). $^{*,**,***}P$-value <0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#}P$-value < 0.05 compared with control group

5.3.1.3 Effect of *M.oleifera* leaves extract on acetylcholine esterase

Figure 5-8 showed the effect of *M.oleifera* leaves extract on AChE activity in hippocampus. Repetitive exposure to restraint stress significantly increased AChE activity in hippocampus (*P*-value<.01; compared to naïve control). However, this elevation was attenuated by Tianeptine and *M.oleifera* leaves extract both at medium and high doses (*P*-value<.05, .01 and .01 respectively; compared to vehicle+stress).

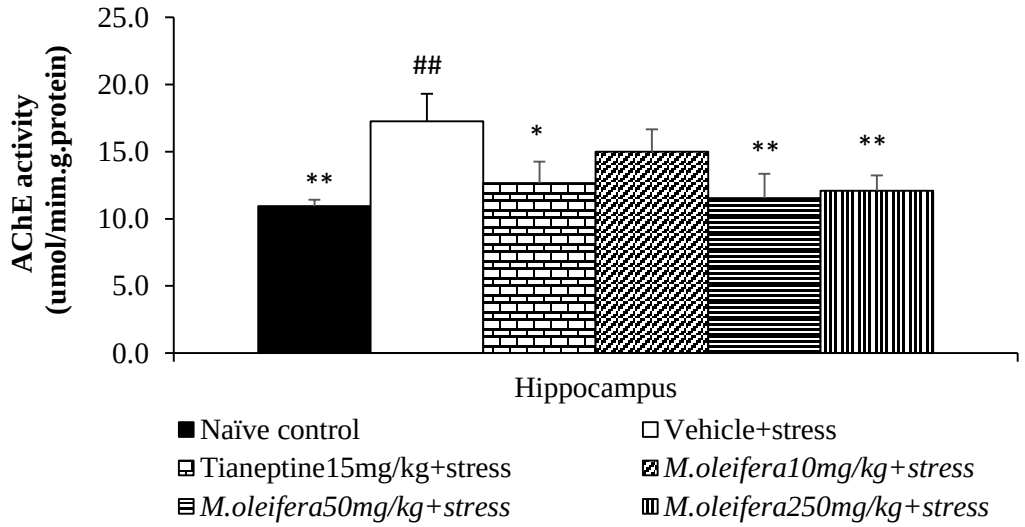

Figure 5-8 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on acetylcholine esterase of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05 compared with control group

5.3.1.4 Effect of *M.oleifera* leaves extract on monoamine oxidase-A and B

Rats which received vehicle and subjected to immobilization stress significantly increased the activity of both MAO-A and MAO-B in hippocampus (*P*-value <.001 all; compared to naïve control). Tianeptine mitigated the elevation of both MAO-A and MAO-B (*P*-value<.001 all; compared to vehicle+stress). In addition, rats which received *M.oleifera* leaves extract at doses of 50 and 250

mg/kg plus immobilization stress for 14 days also decreased MAO-B activity in hippocampus (*P*-value<.01 all; compared to vehicle +stress). No significant change of MAO-B in rats which received low dose of extract plus stress was observed. In addition, all doses of the extract used in this study also failed to produce significant changes of MAO-A in hippocampus as shown in Figure 5-9).

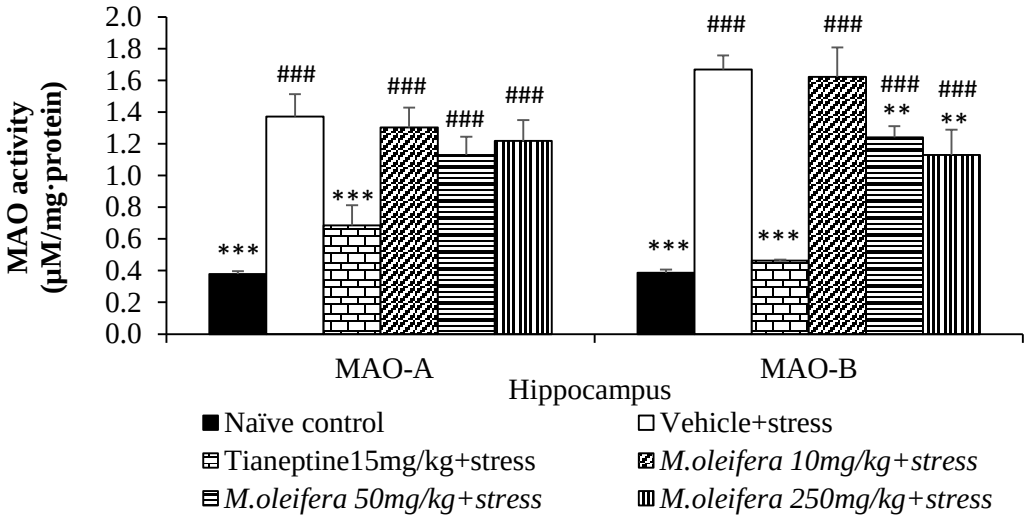

Figure 5-9 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on monoamine oxidase type A and B of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). **,*** *P*-value < 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{###}$ *P*-value < 0.001 compared with control group.

5.3.1.5 Effect of *M.oleifera* leaves extract on oxidative stress markers

Figure 5-10- 5-13 and table 5.1 showed that repetitive restraint stress significantly decreased SOD, CAT and GSH-Px activities but increased MDA level in hippocampus (*P*-value<.01, .001, .05 and .001 respectively; compared to naïve control group). Rats which received Tianeptine plus stress significantly increased SOD and CAT activities but decreased MDA level in hippocampus (*P*-value< .01, .001 and .001 respectively; compared to vehicle +stress). All doses of *M.oleifera* decreased MDA level (*P*-value<.05 all; compared to vehicle +stress) but increased SOD (*P*-value< .001,.001 and .01 respectively; compared to

vehicle +stress) in hippocampus of stress exposed rats. The increased CAT in hippocampus was observed in rats which received the extract at doses of 10 and 50 mg/kg (*P*-value<.05 and .001respectively; compared to vehicle +stress). No changes in GSH-Px activity in any groups were observed.

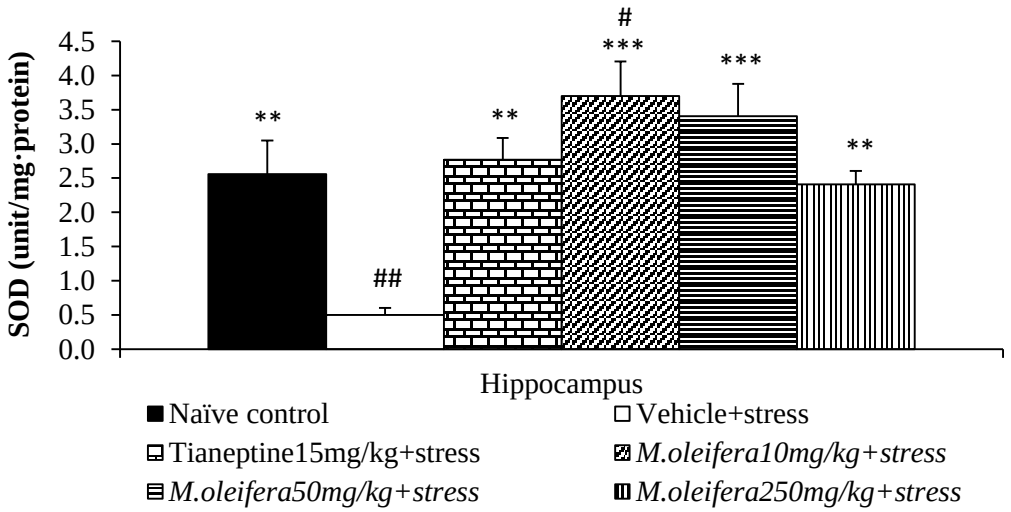

Figure 5-10 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on superoxide dismutase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). **,****P*-value < 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#}$*P*-value < 0.05 and 0.01 respectively; compared with control group

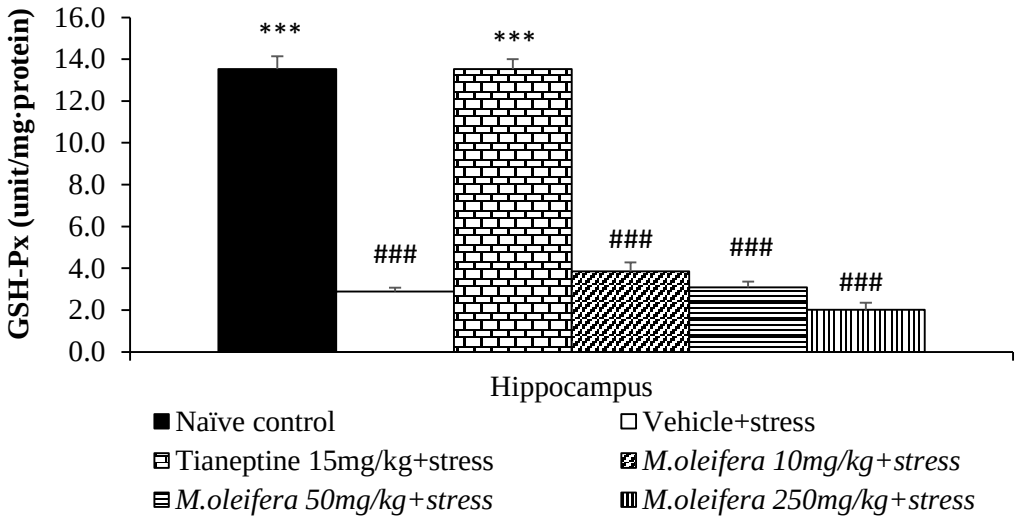

Figure 5-11 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on glutathione peroxidase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). $^{***}P$-value < 0.001; compared with vehicle plus stress. $^{###}P$-value < 0.001 respectively; compared with control group

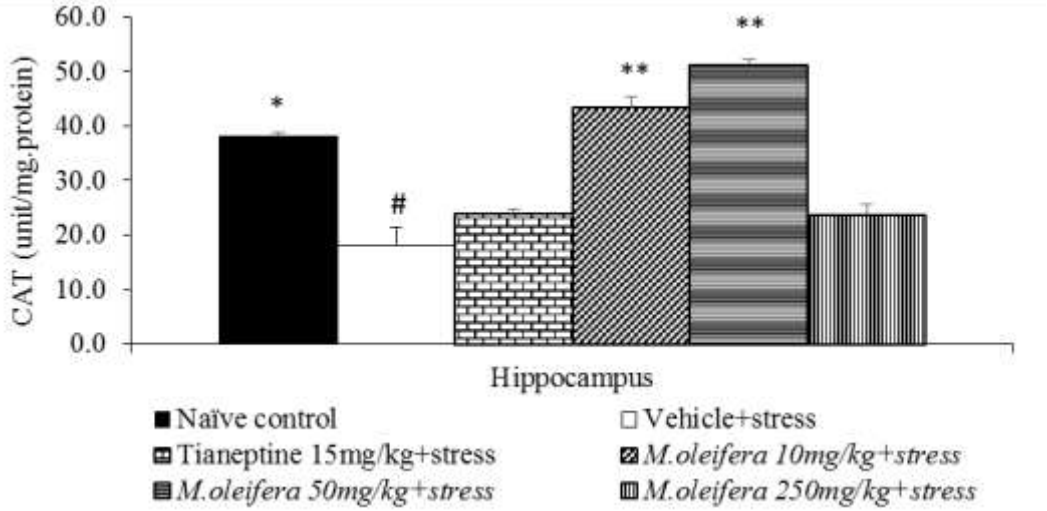

Figure 5-12 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on catalase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). $^{**,***}P$-value < 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#}P$-value < 0.05 and 0.01 respectively; compared with control group

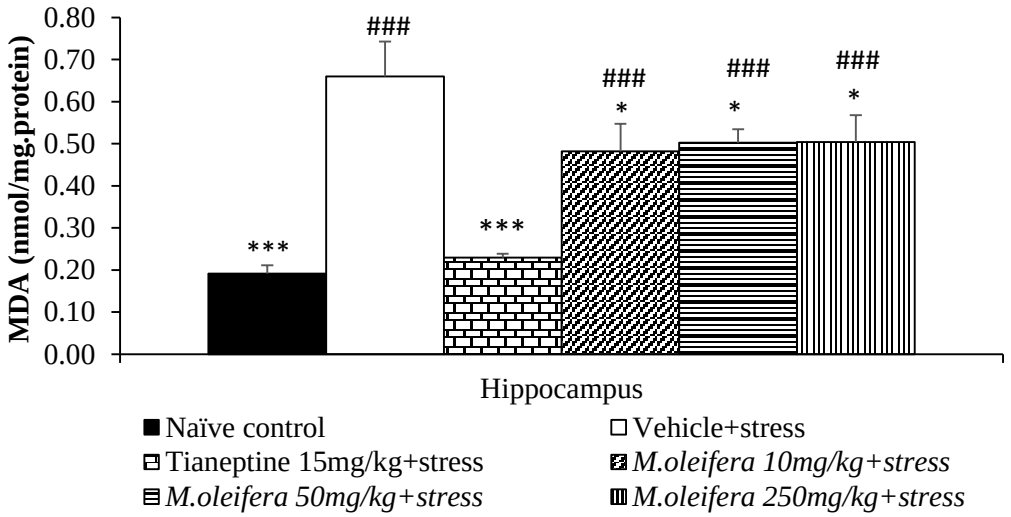

Figure 5-13 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on malondialdehyde level in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). $^{*,***}P$-value < 0.05 and 0.001 respectively; compared with vehicle plus stress. $^{###}P$-value < 0.001; compared with control group

Table 5.1 Effect of *M.oleifera* leaves extract on oxidative stress markers (SOD,GSH-Px, CAT, MDA)

Parameters	**SOD**	**GSH-Px**	**CAT**	**MDA**
Group	**unit/mg·protein**	**unit/mg·protein**	**unit/mg·protein**	**nmol/mg·protein**
Naïve control	2.6±0.5**	13.5±0.6***	38.1±5.4*	0.19±0.02***
Vehicle+stress	0.5±0.1$^{\#\#}$	2.9±0.2$^{\#\#\#}$	18.2±5.5$^{\#}$	0.66±0.08$^{\#\#\#}$
Tianeptine 15mg/kg +stress	2.8±0.3**	13.5±0.5***	23.9±5.3	0.23±0.01***
M.oleifera 10 mg/kg +stress	3.7±0.5$^{\#,***}$	3.8±0.4$^{\#\#\#}$	43.3±6.0**	0.48±0.07$^{\#\#\#,*}$
M.oleifera 50 mg/kg +stress	3.4±0.5***	3.1±0.3$^{\#\#\#}$	51.2±4.6**	0.50±0.03$^{\#\#\#,*}$
M.oleifera 250mg/kg +stress	2.4±0.2**	2.0±0.3$^{\#\#\#}$	23.6±5.4	0.50±0.06$^{\#\#\#,*}$

*,**,*** P < .05, .01 and .001 respectively; compared to vehicle plus stress.

$^{\#,\#\#,\#\#\#}$ P < .05, .01 and .001 respectively; compared to Naïve control.

5.3.1.6 Effect of *M.oleifera* leaves extract on survival neurons

It was found that rats which obtained vehicle plus stress showed the decreased neurons density in CA1, CA2, CA3 and dentate gyrus (P-value<.01 all; compared to naïve control). Tianeptine increased neuron density in

all areas mentioned earlier in stress exposed rats (*P*-value<.05, .05, .001 and .05 respectively; compared to vehicle +stress). Interestingly, *M.oleifera* leaves extract at doses of 10, 50 and 250 mg/kg produced the significant increase in neurons density in CA1 (*P*-value<.01, .05 and .01 respectively; compared to vehicle +stress), CA2 (*P*-value<.01, .05 and .01 respectively; compared to vehicle +stress), CA3 (*P*-value<.001, .05 and .01 respectively; compared to vehicle +stress) and dentate gyrus (*P*-value<.01, .05 and .01 respectively; compared to vehicle +stress) as shown in Figure 5-14-Figure 5-17.

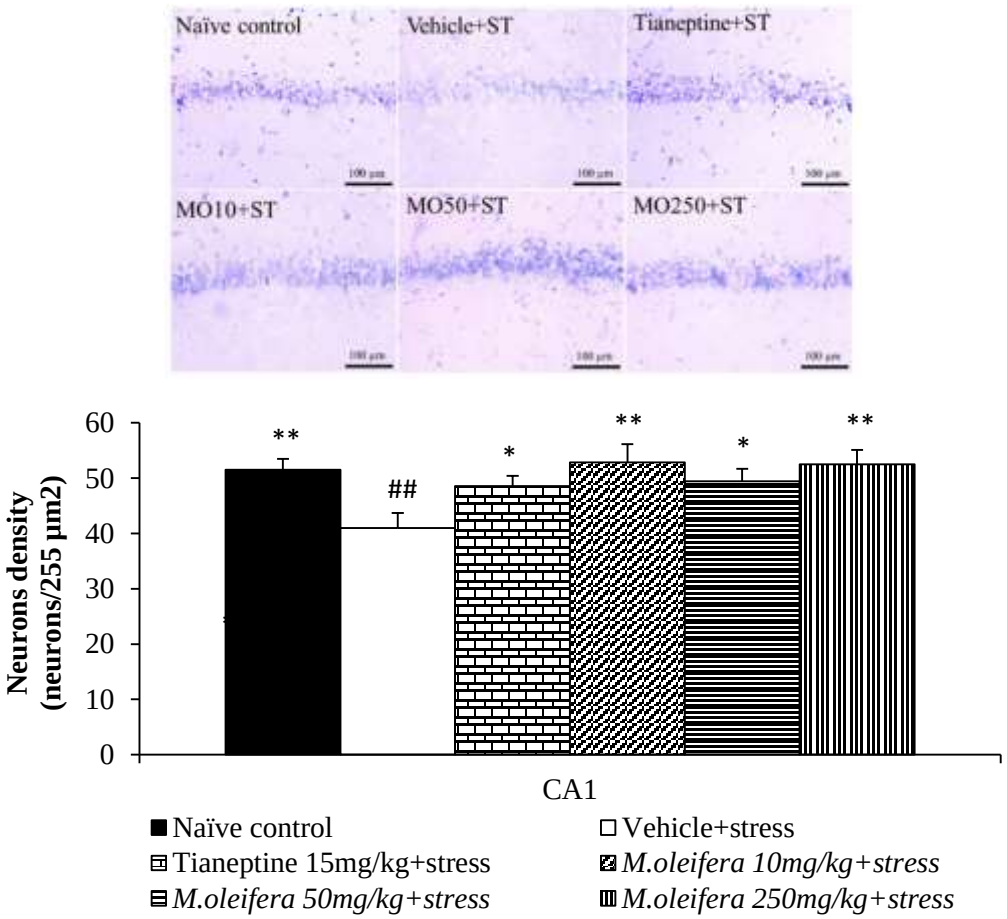

Figure 5-14 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on the density of survival neurons in CA1 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,***P*-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#}$*P*-value < 0.01; compared with control group

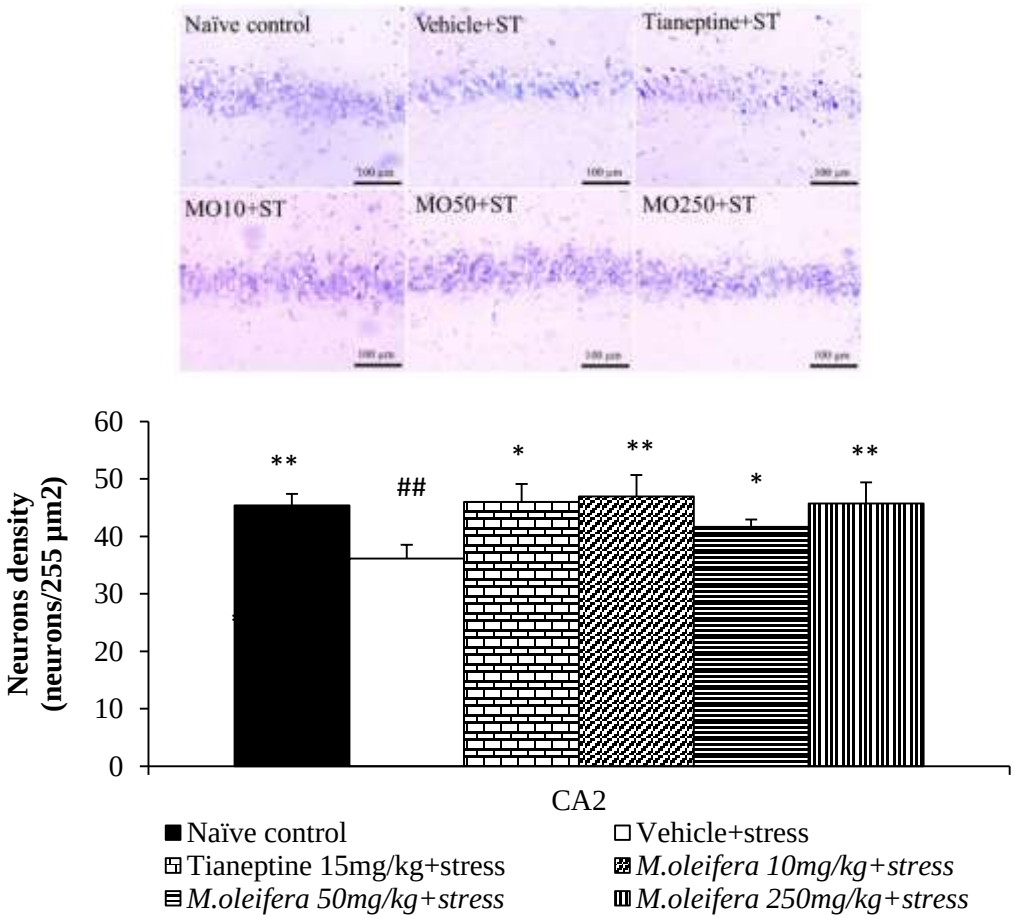

Figure 5-15 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on the density of survival neurons in CA2 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,***P*-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#}$*P*-value < 0.01; compared with control group

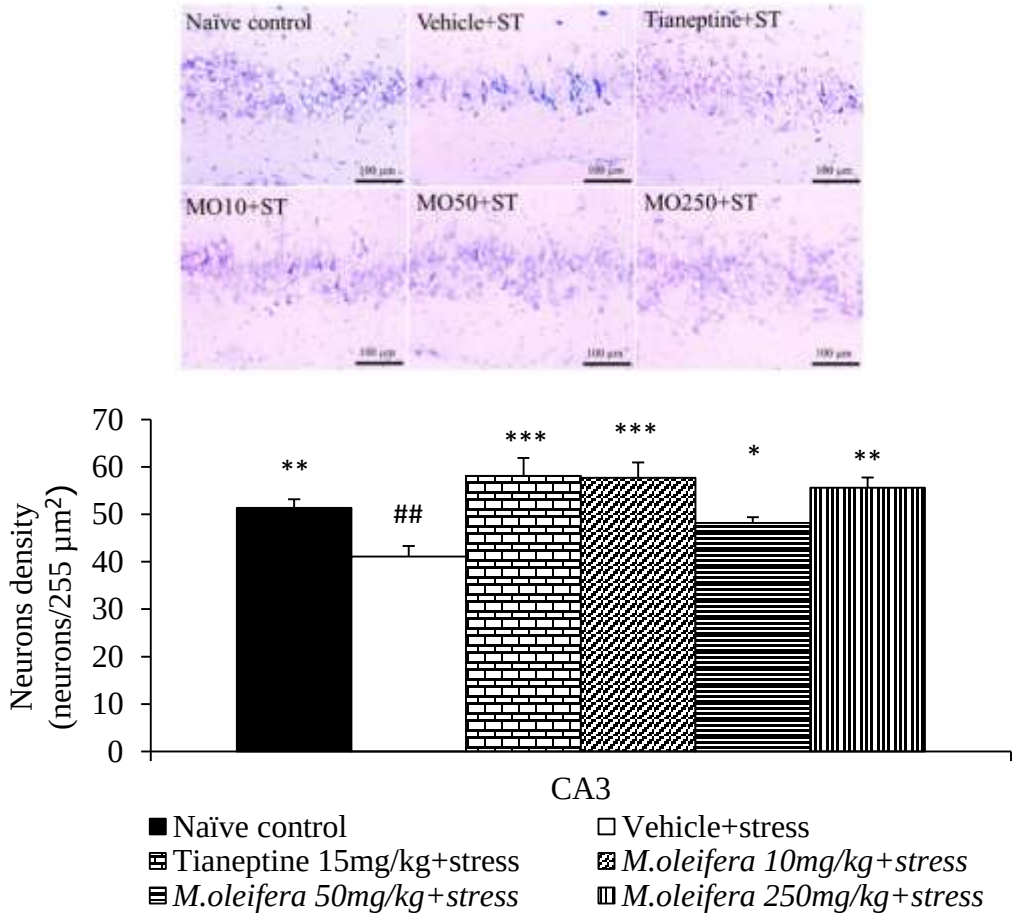

Figure 5-16 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on the density of survival neurons in CA3 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). $^{*,**,***}P$-value < 0.05 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#\#}P$-value < 0.01; compared with control group

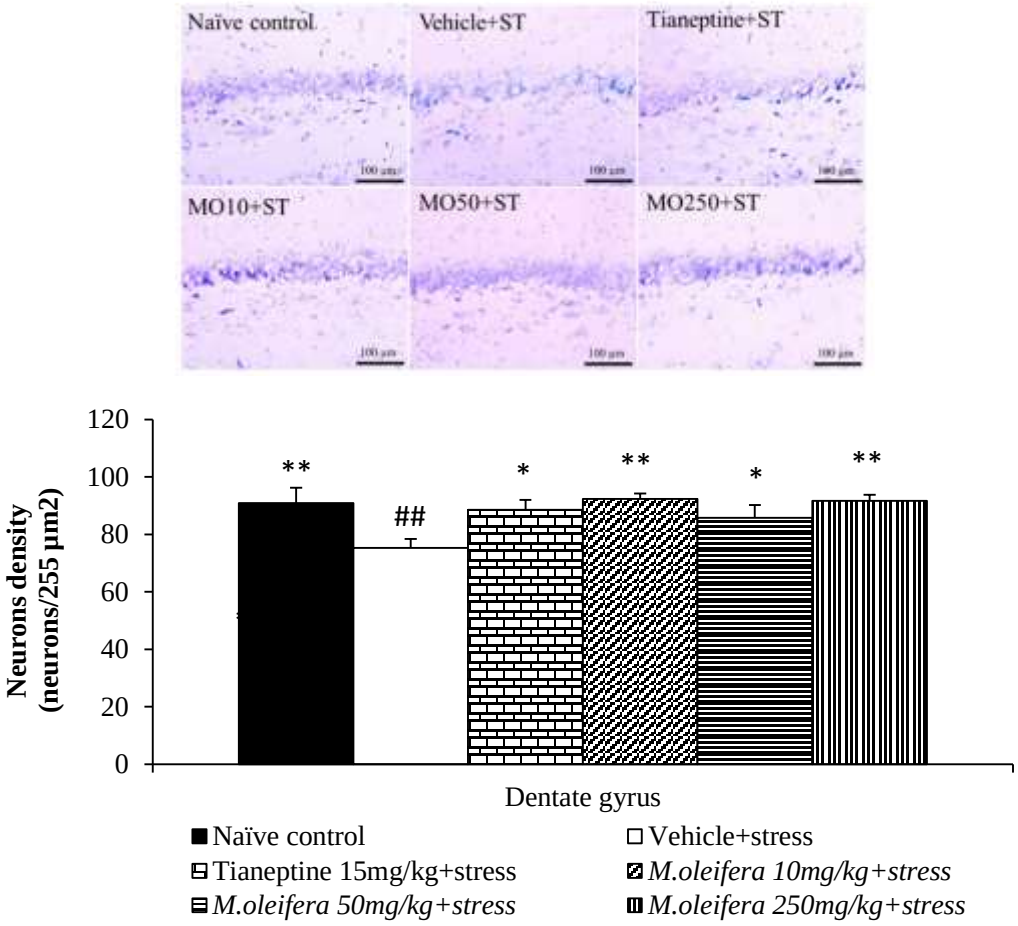

Figure 5-17 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on the density of survival neurons in dentate gyrus of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). $^{*,**}P$-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#}P$-value < 0.01; compared with control group

5.3.1.7 Effect of *M.oleifera* leaves extract on Ki-67 proliferative marker

Figure 5-18 showed that repetitive exposure to restraint stress significantly decreased the level of Ki67, an adult neurogenesis marker, in hippocampus (P-value<.05; compared with naïve control). Tianeptine and the extract at doses of 5 and 10 mg/kg failed to produce the change of Ki67 level in hippocampus of stress exposed rats. However, stress exposed rats which received the extract at dose

of 250 mg/kg could increase Ki67 level in the mentioned area (*P*-value<.01; compared to vehicle +stress)

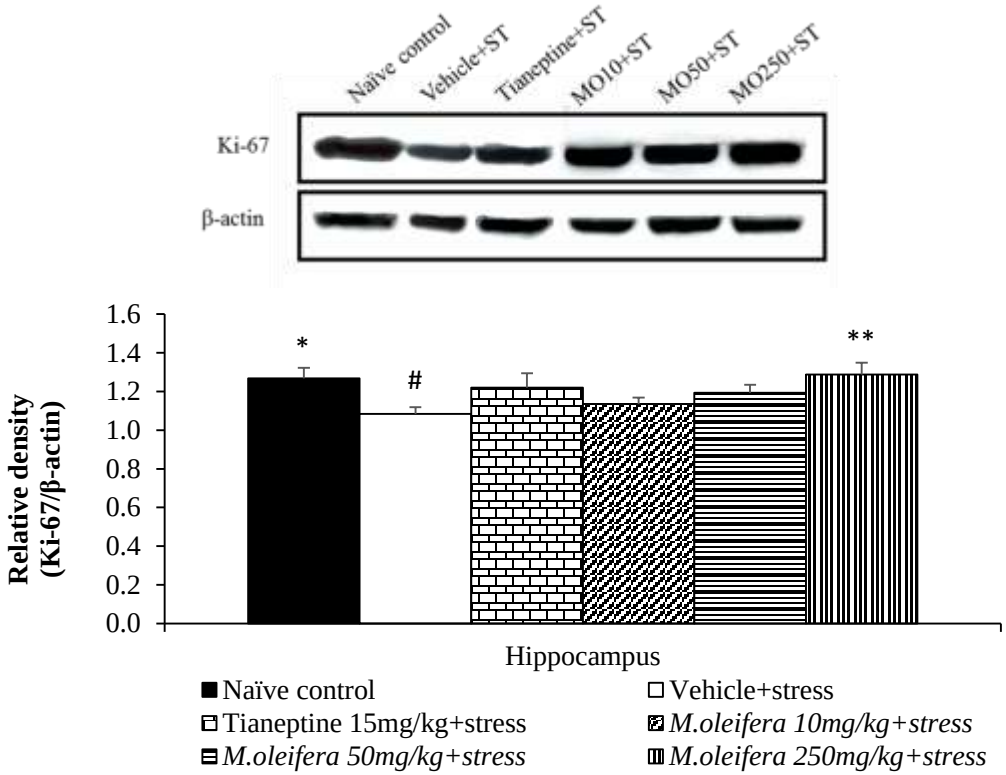

Figure 5-18 The effect of hydro-alcoholic extracts of *M.oleifera* leaves extract on Ki-67 proliferative marker in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05, and 0.01 respectively; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05; compared with control group

5.3.2 Effect and Possible Underlying Mechanism of *A.occidentale* leaves Extract on Memory Impairment and Neurodegeration in Stress-Exposed Rats

5.3.2.1 Effect of *A.occidentale* leaves extract on spatial memory

Figure 5-19 showed that rats which subjected to restraint stress and received vehicle or water significantly increased escape latency after a single dose of administration and at both 7 and 14 days of treatment (*P*-value<.01, .001 and .01 respectively; compared with naïve control group). Tianeptine attenuated the increased escape latency in restraint stress rats throughout the study period (P-value<.01, .001 and.001 respectively; compared with vehicle+stress treated group).

It was found that *A.occidentale* at all doses significantly decreased escape latency in restraint stress rats at both 7 and 14 days of treatment (*P*-value <.001 all; compared to vehicle+stress)

Figure 5-20 also showed that rats which were exposed to 12-h restraint stress and received vehicle decreased the retention time after a single dose of administration and at both 7 and 14 days of treatment (*P*-value<.05, .01 and .05 respectively; compared with naïve control group). Tianptine could mitigate the reduction of retention time in restraint rats (*P*-value<.001, .001 and .01 respectively; compared with vehicle+stress treated group). Interestingly, *A.occidentale* leaves extract at all doses could attenuate the reduction of retention time induced by restraint stress in male rats at single dose of administration (*P*-value <.01,.01 and .05 respectively; compared to vehicle+stress) and 7 days of treatment (*P*-value <.001, .001 and .01 respectively; compared to vehicle+stress).The high dose of *A.occidentale* leaves extract increased retention time in restraint stress rats at 14 days of experimental period (*P*-value <.001; compared to vehicle+stress).

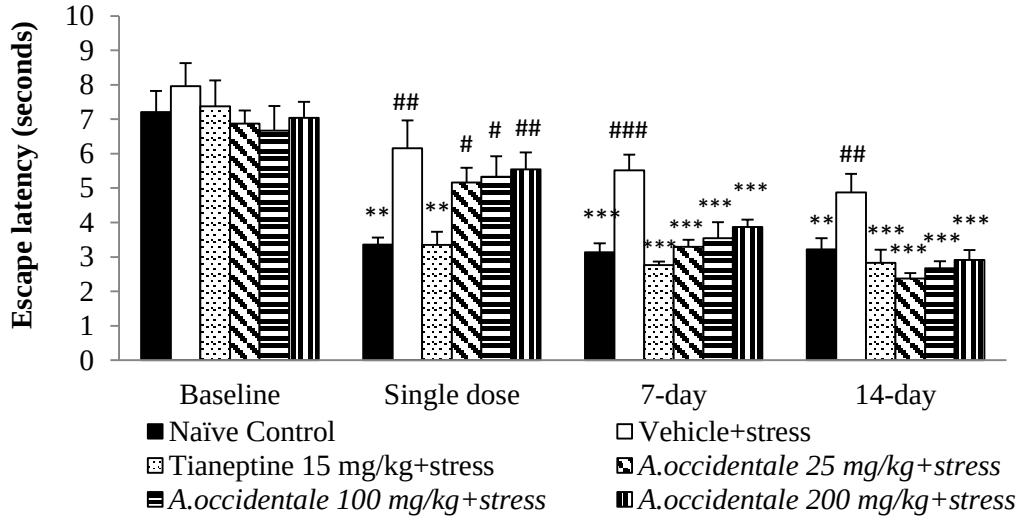

Figure 5-19 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on escape latency of stress-exposed rats at baseline, after a single dose, 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). **,****P*-value <0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#,\#\#\#}$ *P*-value < 0.05, 0.01 and 0.001 respectively; compared with control group

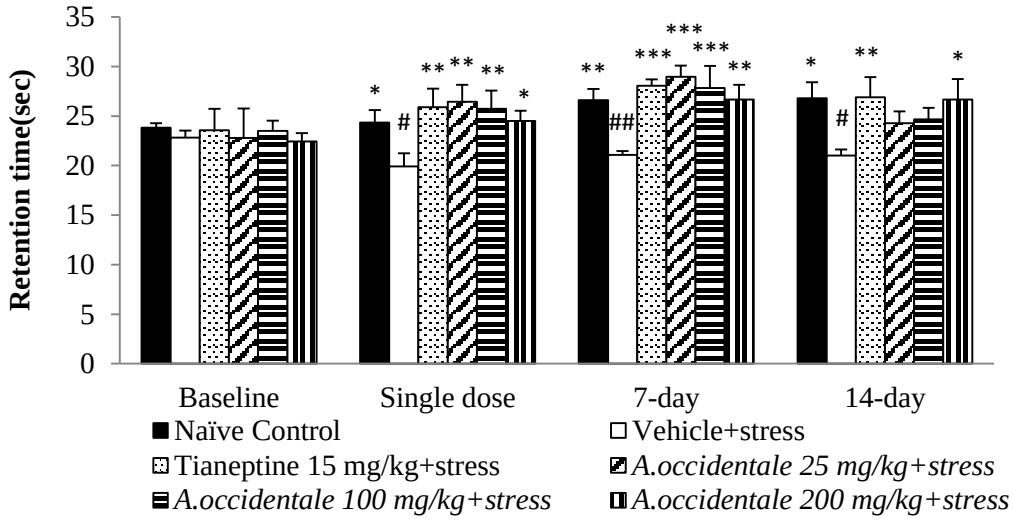

Figure 5-20 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on retention time of stress-exposed rats at baseline, after a single dose, 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). *,**,****P*-value <0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#}$ *P*-value < 0.05 and 0.01 respectively; compared with control group

5.3.2.2 Effect of *A.occidentale* leaves extract on serum corticosterone level

Rats which received vehicle and repetitive stress exposure significantly increased serum corcosterone levels (*P*-value<.01 all; compared to naïve control) both at 7 and 14 days of exposure time (Figure 5-21). Tianeptine failed to modulate the elevation of serum corticosterone in stress exposed rats. *A.occidental* leaves extract failed to show the decrease of serum corticosterone in stress exposed rats at 7 days of exposure time. However, only stress exposed rats which received medium dose of extract significantly mitigated the elevation of this parameter when the exposure time was prolonged to 14 days (*P*-value<.05; compared to vehicle+stress).

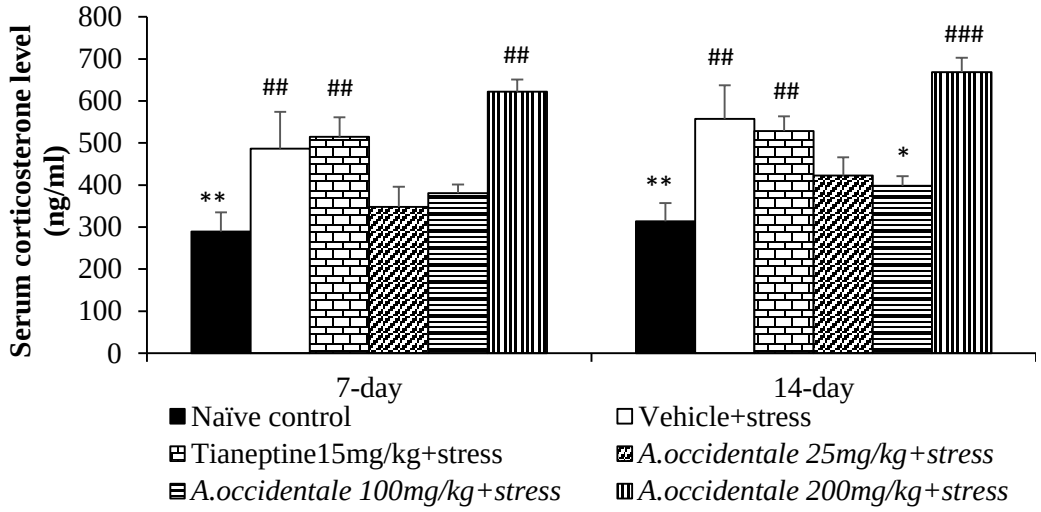

Figure 5-21 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on serum corticosterone level of stress-exposed rats at 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). *,***P*-value <0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#,\#\#\#}$ *P*-value < 0.01 and 0.001 respectively; compared with control group

5.3.2.3 Effect of *A.occidentale* leaves extract on acetylcholineesterase activity

Figure 5.22 showed that rats which subjected to restraint stress and received vehicle significantly increased AChE activity in hippocampus (*P*-value<.01; compared to naïve control). Tianeptine attenuated the increased AChE activity in restraint stress rats (*P*-value<.05 ; compared to vehicle+stress). It was found that *A.occidentale* leaves extract at all doses significantly decreased AChE activity in restraint stress rats (*P*-value<.05, .05, and .001 respectively; compared to vehicle+stress).

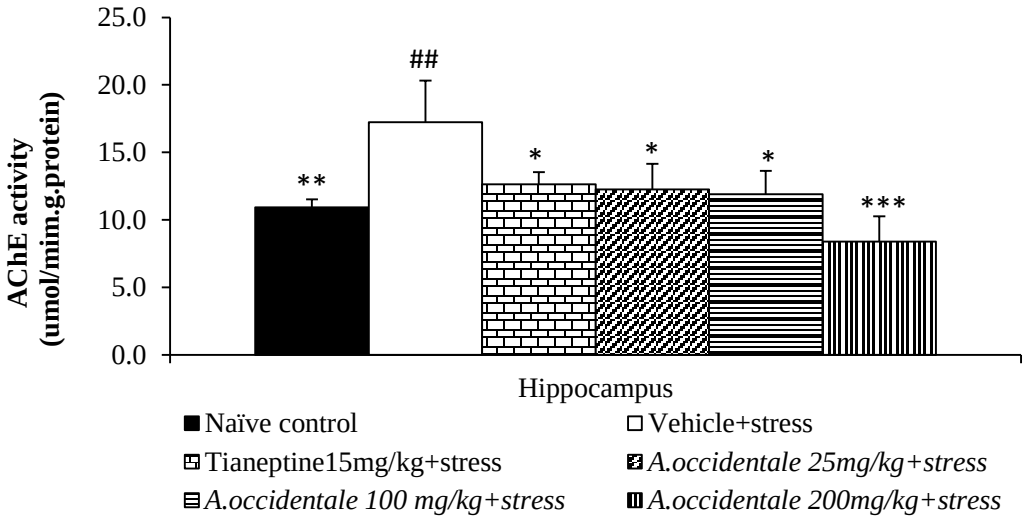

Figure 5-22 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on acetylcholineesterase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,**,*** *P*-value < 0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01 compared with control group.

5.3.2.4 Effect of *A.occidentale* leaves extract on monoamine oxidase-A and B

Rats which received vehicle and subjected to restraint stress significantly increased the activities of both MAO-A and MAO-B in hippocampus (*P*-value <.001 all; compared to naïve control). Tianeptine mitigated the elevation of both MAO-A and MAO-B in restraint stress rats (*P*-value<.001 all; compared to vehicle+stress). All doses of *A.occidentale* leaves extract significantly decreased MAO-A and B activities in restraint stress rats (*P*-value<.001 all; compared to vehicle +stress) in hippocampus (figure 5-23).

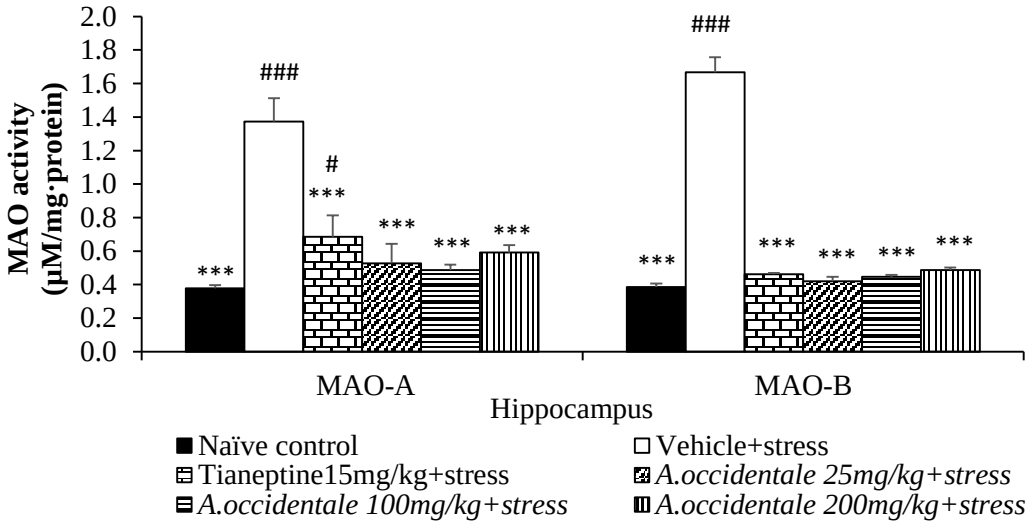

Figure 5-23 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on monoamine oxidase type A and B of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *** *P*-value < 0.001 respectively; compared with vehicle plus stress. #,### *P*-value < 0.05 and 0.001 compared with control group.

5.3.2.5 Effect of *A.occidentale* leaves extract on oxidative stress markers

Figure 5-24-5-27 and table 5.2 showed that repetitive restraint stress significantly decreased SOD, CAT and GSH-Px activities but increased MDA level in hippocampus (*P*-value<.001, .05, .001 and .001 respectively; compared to naïve control group). Rats which received Tianeptine plus stress significantly increased SOD and GSH-Px activities but decreased MDA level in hippocampus (*P*-value< .001 all respectively; compared to vehicle +stress). Rats were received *A.occidentale* and repetitive restraint stress significantly increased SOD and GSH-Px activities but decreased MDA level in hippocampus. All doses of *A.occidentale* increased SOD (*P*-value<.001, .001 and .01 respectively; compared to vehicle +stress) and GSH-Px (*P*-value<.001 all; compared to vehicle +stress). MDA level in hippocampus of stress –exposed rats which received all treatments significantly decreased (*P*-value < .001 all; compared to vehicle +stress). No significant changes were observed in CAT activity in any group of *A.occidentale*.

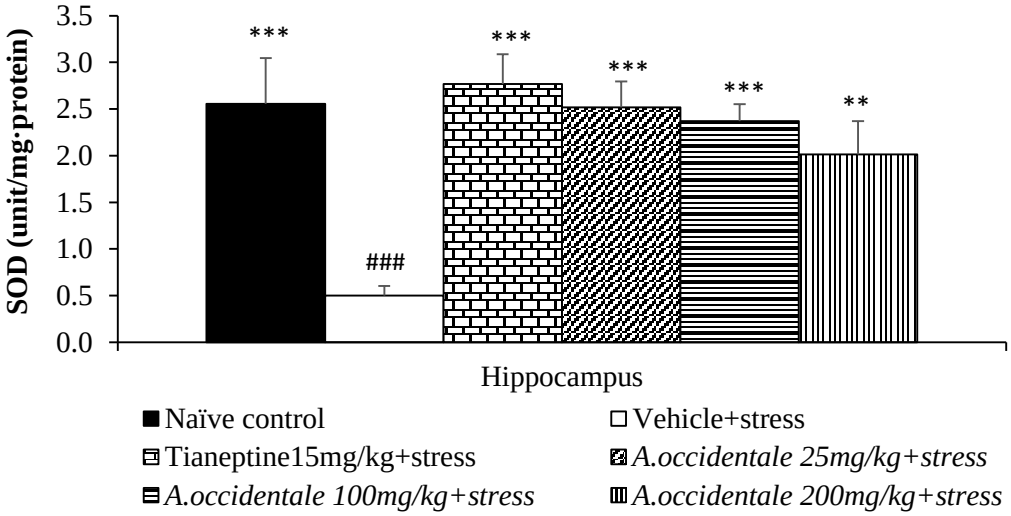

Figure 5-24 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on superoxide dismutase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). **,*** *P*-value < 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{###}$ *P*-value < 0.001; compared with control group.

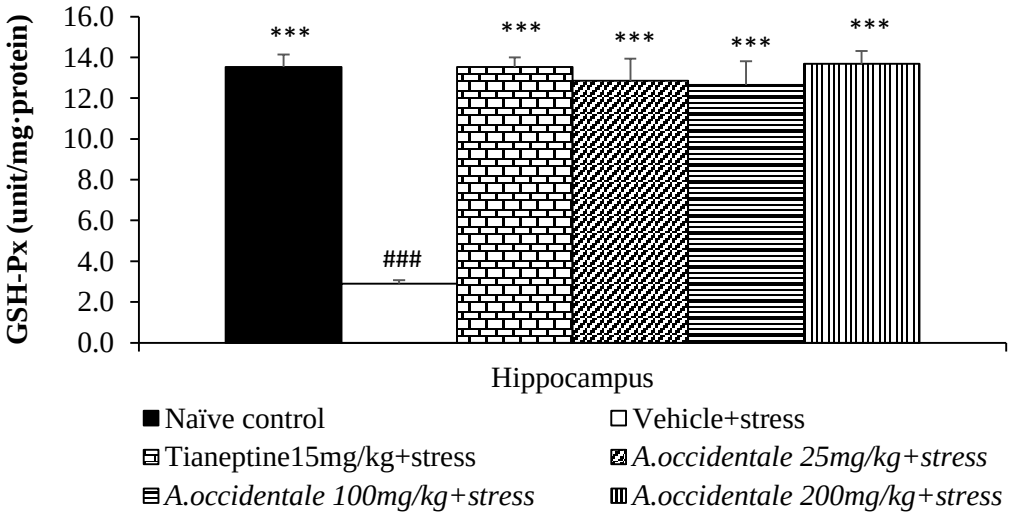

Figure 5-25 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on glutathione peroxidase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *** *P*-value < 0.001; compared with vehicle plus stress. $^{###}$ *P*-value < 0.001 respectively; compared with control group

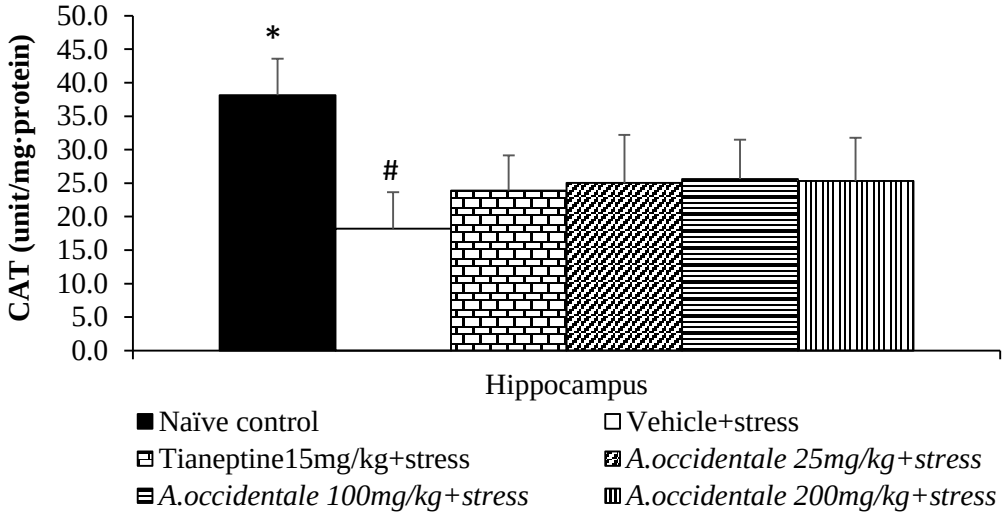

Figure 5-26 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on catalase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). * *P*-value < 0.05; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05; compared with control group

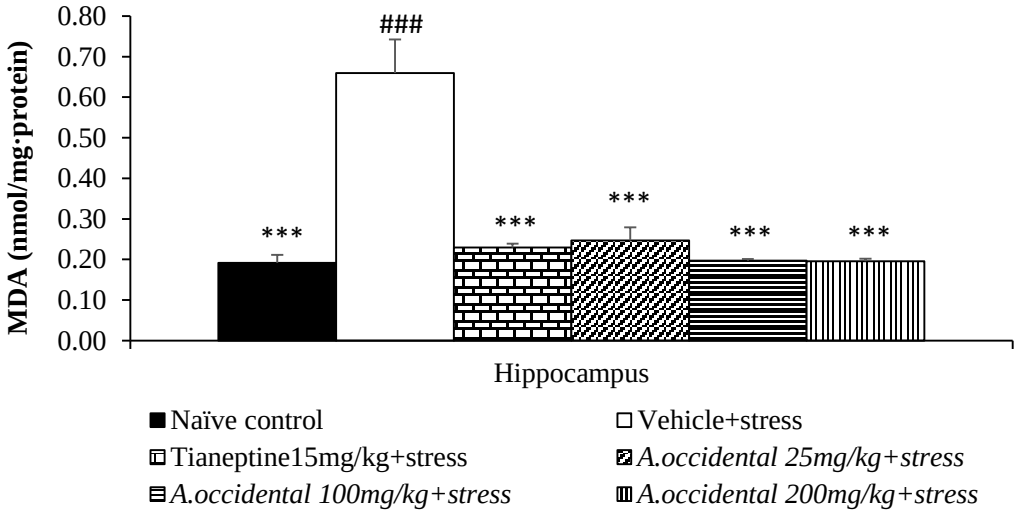

Figure 5-27 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on malondialdehyde level in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). ****P*-value < 0.001; compared with vehicle plus stress. $^{\#\#\#}$ *P*-value < 0.001; compared with control group

Table 5.2 Effect of *A.occidentale* leaves extract on oxidative stress markers (SOD, GSH-Px, CAT, MDA) in hippocampus of stress-exposed rats

Parameters	SOD	GSH-Px	CAT	MDA
Group	unit/mg·protein	unit/mg·protein	unit/mg·protein	nmol/mg·protein
Naïve control	2.6±0.5***	13.5±0.6***	38.1±5.4*	0.19±0.02***
Vehicle+stress	0.5±0.1$^{\#\#\#}$	2.9±0.2$^{\#\#\#}$	18.2±5.5$^{\#}$	0.66±0.08$^{\#\#\#}$
Tianeptine 15 mg/kg+stress	2.8±0.3***	13.5±0.5***	23.9±5.3	0.23±0.01***
A.occidentale 25mg/kg+stress	2.5±0.3***	12.9±1.1***	25.1±7.2	0.25±0.03***
A.occidentale 100mg/kg+stress	2.4±0.2***	12.7±1.2***	25.6±5.9	0.20±0.00***
A.occidentale 200mg/kg+stress	2.0±0.4**	13.7±0.6***	25.3±6.5	0.20±0.01***

*,**,*** $P < .05, .01$ and $.001$ respectively; compared to vehicle plus stress.

$^{\#,\#\#,\#\#\#}$ $P < .05, .01$ and $.001$ respectively; compared to Naïve control.

5.3.2.6 Effect of *A.occidentale* leaves extract on survival neurons

It was found that rats which obtained vehicle plus stress showed the decreased neurons density in CA1, CA2, CA3 and dentate gyrus (*P*-value<.01 all; compared to naïve control). Tianeptine increased neuron density in all areas mentioned earlier in stress exposed rats (*P*-value<.05, .01, .001 and .05 respectively; compared to vehicle +stress). Interestingly, *A.oocidentale* leaves extract at doses of 25, 100 and 200 mg/kg produced the significant increase in neurons density in CA1 (*P*-value<.01, .01 and .05 respectively; compared to vehicle +stress), CA2 (*P*-value<.001 all; compared to vehicle +stress), CA3 (*P*-value<.001, .05 and .01 respectively; compared to vehicle +stress) and dentate gyrus (*P*-value<.05, .01 and .01 respectively; compared to vehicle +stress) as shown in figure 5-28 – Figure 5-31.

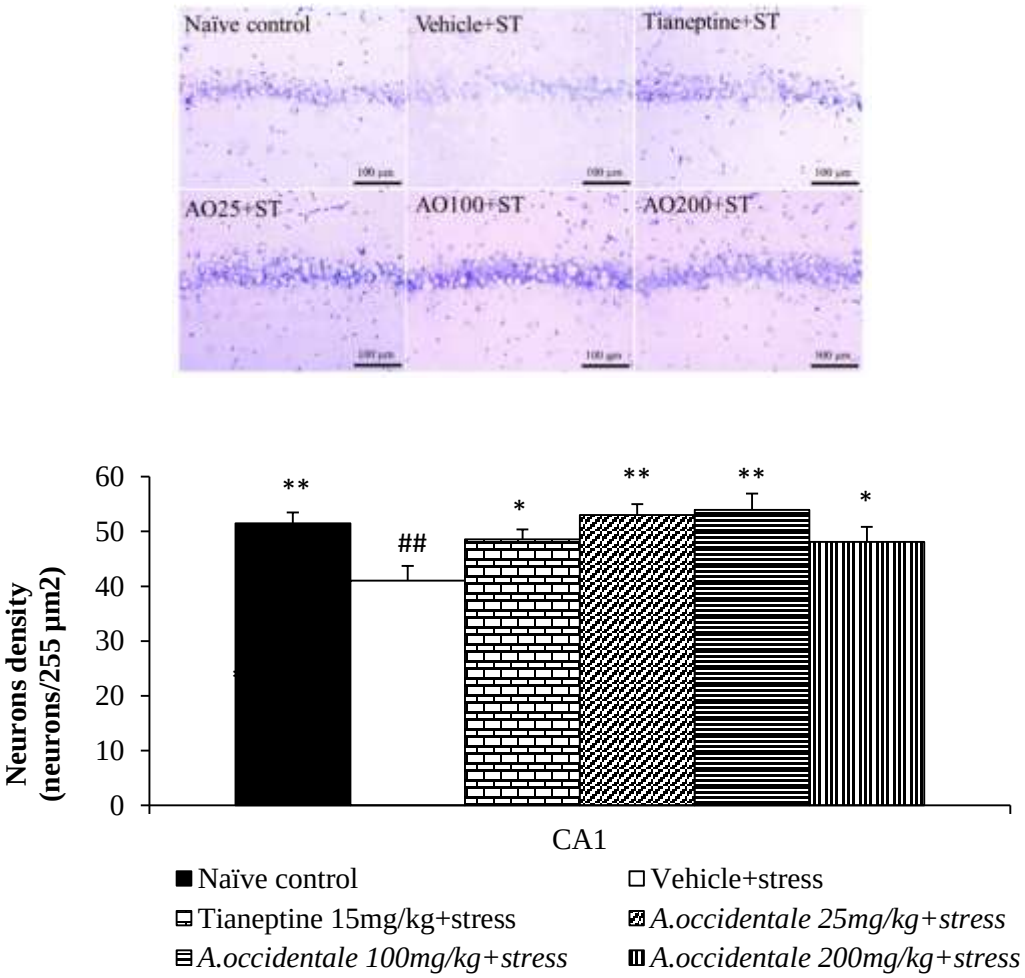

Figure 5-28 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on the density of survival neurons in CA1 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01; compared with control group

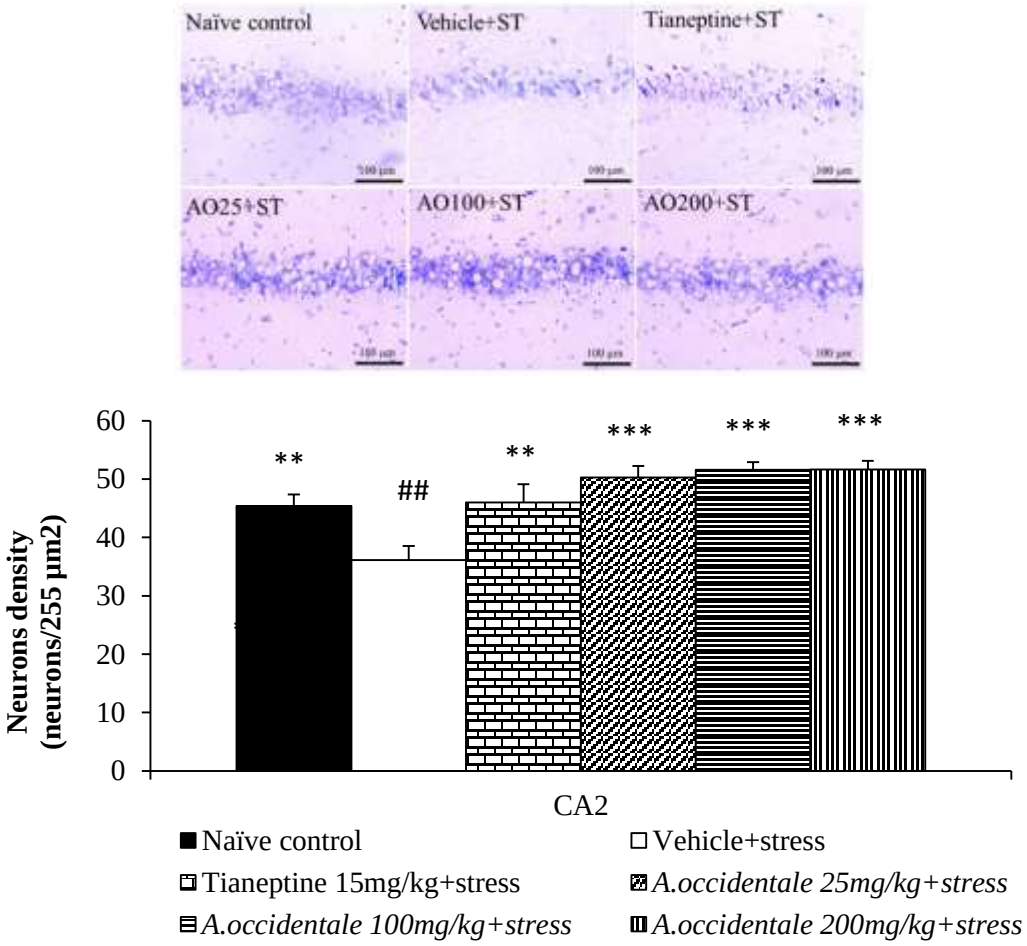

Figure 5-29 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on the density of survival neurons in CA2 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). **,*** *P*-value < 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01; compared with control group

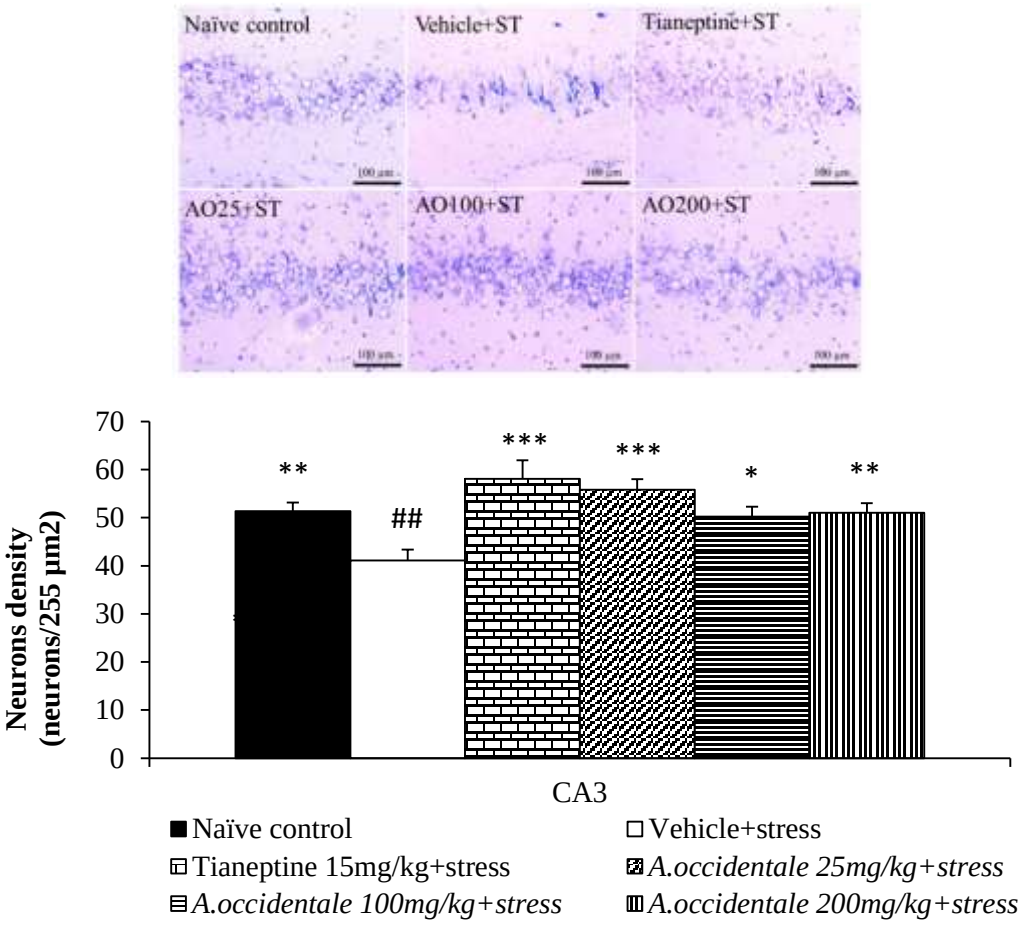

Figure 5-30 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on the density of survival neurons in CA3 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,**,*** *P*-value < 0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01; compared with control group

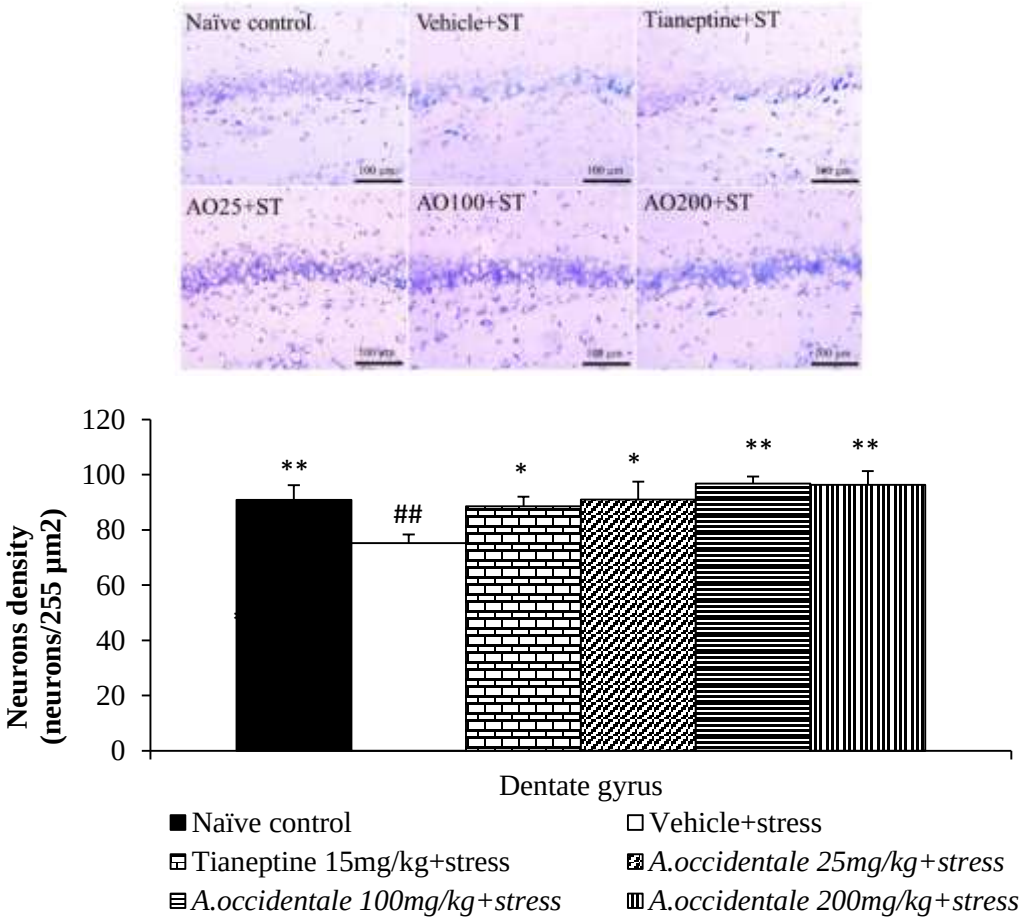

Figure 5-31 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on the density of survival neurons in dentate gyrus of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01; compared with control group

5.3.2.7 Effect of *A.occidentale* leaves extract on Ki-67 proliferative marker

Figure 5.32 showed that repetitive exposure to restraint stress significantly decreased the level of Ki67, an adult neurogenesis marker, in hippocampus (*P*-value<.05; compared with naïve control). Tianeptine failed to produce the change of Ki67 level in hippocampus of stress exposed rats. However, stress exposed rats which received all doses of *A.occidentale* leaves extract could

increase Ki67 level in the mentioned area (*P*-value<.01, .05 and .01 respectively; compared to vehicle +stress)

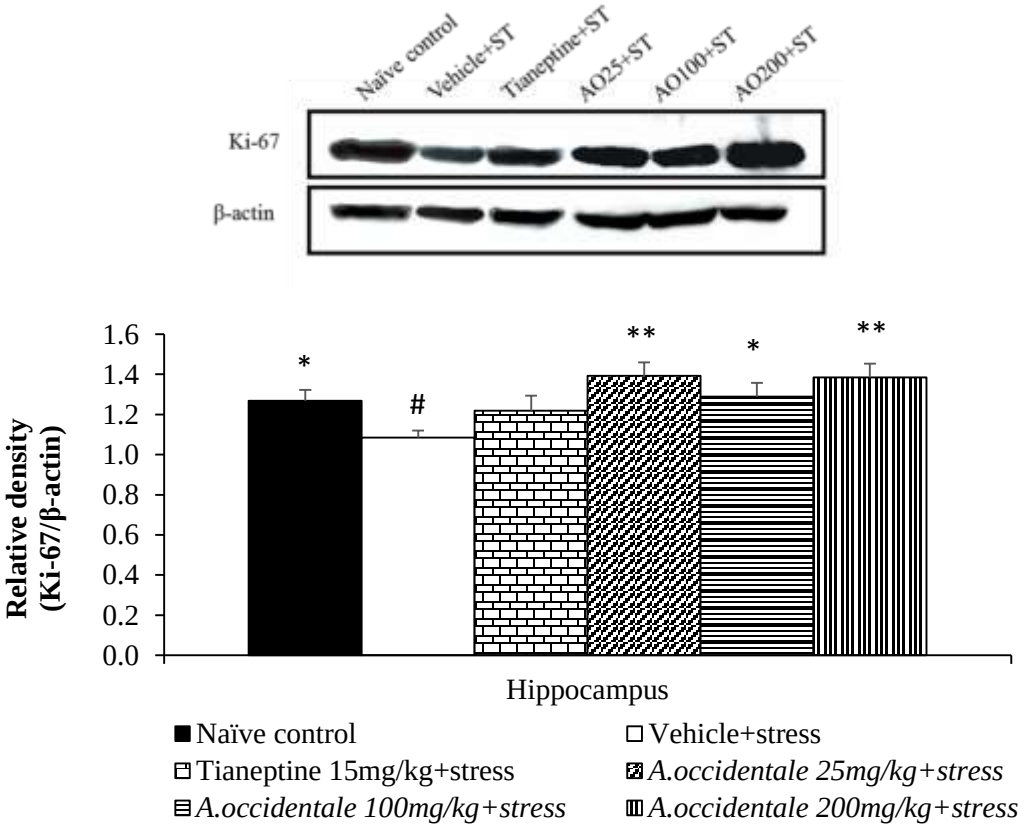

Figure 5-32 The effect of hydro-alcoholic extracts of *A.occidentale* leaves extract on Ki-67 proliferative marker of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05, and 0.01 respectively; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05; compared with control group

5.3.3 Effect and Possible Underlying Mechanism of *N.nucifera* Flowers Extract on Memory Impairment and Neurodegeneration in Stress-Exposed rats

5.3.3.1 Effect of *N.nucifera* flowers extract on spatial memory

Figure 5-33 showed that rats which subjected to restraint stress and received vehicle or water significantly increased escape latency after a single dose of administration and at both 7 and 14 days of treatment (P-value<.001 all; compared with naïve control group). Tianeptine attenuated the increased escape latency in restraint stress rats throughout the study period (*P*-value<.001 all;

compared with vehicle+stress treated group). It was found that rats which received *N.nucifera* at all doses and exposed to 12 h-restraint stress significantly decreased escape latency (*P*-value <.001; compared to vehicle+stress) at a single dose, 7 and 14 days of treatment.

Figure 5-34 also showed that rats which were exposed to 12-h restraint stress and received vehicle decreased the retention time after a single dose of administration and at both 7 and 14 days of treatment (*P*-value<.05, .001 and .01 respectively; compared with naïve control group). Tianaptine could mitigate the reduction of retention time in restraint rats (*P*-value<.01 all; compared with vehicle+stress treated group). Interestingly, all doses of *N.nucifera* at all doses used in this study could mitigate the reduction of retention time in restraint rats (*P*-value <.05 all; compared to vehicle+stress) at 14 days of experimental period.

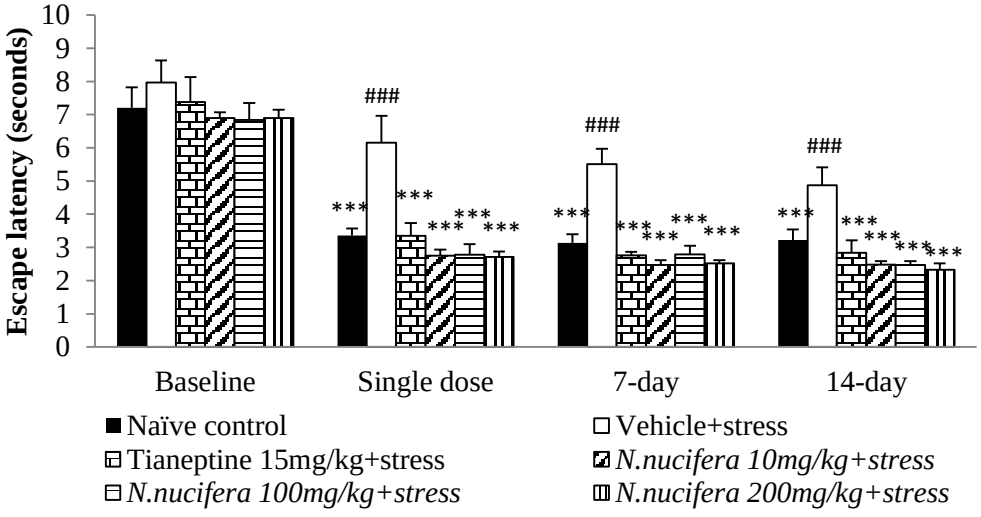

Figure 5-33 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on escape latency of stress-exposed rats at baseline, after a single dose, 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). ****P*-value <0.001; compared with vehicle plus stress. $^{###}$ *P*-value < 0.001 respectively; compared with control group

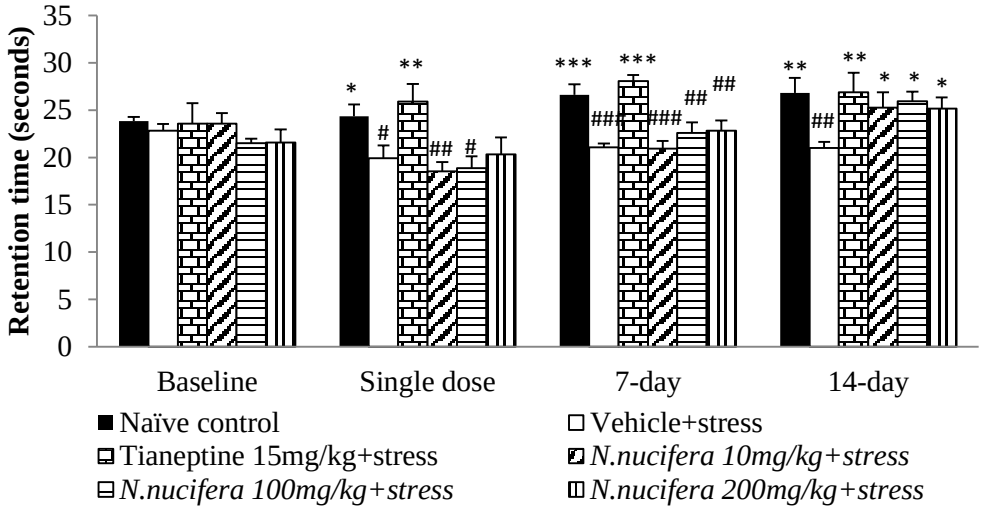

Figure 5-34 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on retention time of stress-exposed rats at baseline, after a single dose, 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). *,**,****P*-value <0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#,\#\#\#}$ *P*-value < 0.05,0.01 and 0.001 respectively; compared with control group

5.3.3.2 Effect of *N.nucifera* flowers extract on serum corticosterone level

Rats which received vehicle and repetitive stress exposure significantly increased serum corticosterone levels (*P*-value<.05 all; compared to naïve control) both at 7 and 14 days of exposure time (Figure 5-35). Tianeptine failed to modulate the elevation of serum corticosterone in stress exposed rats. *N.nucifera* flowers extract at low doses mitigated the elevation of serum corticosterone in stress exposed rats at 7 and 14 days of exposure time (*P*-value<.05 and .001 respectively; compared to vehicle+stress). Stress-exposed rats which received the extract at doses of 100 and 200 mg.kg^{-1} BW significantly mitigated the elevation of this parameter when the exposure time was prolonged to 14 days (p-value<.05 all; compared to vehicle+stress).

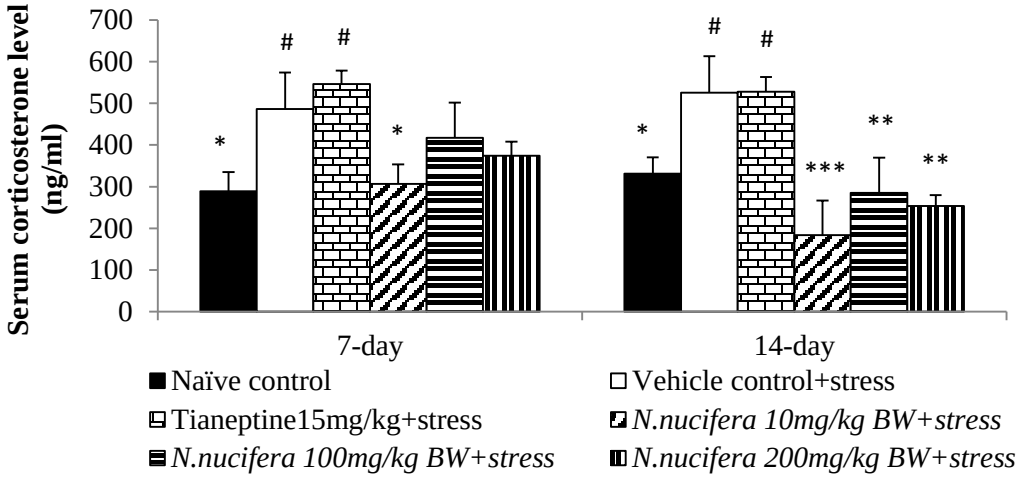

Figure 5-35 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on serum corticosterone levels of stress-exposed rats at 7 days and 14 days of treatment. Data were expressed as mean±S.E.M. (n=6/group). *,**,****P*-value <0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05; compared with control group

5.3.3.3 Effect of *N.nucifera* flowers extract on acetylcholineesterase activity

Figure 5-36 showed that rats which subjected to restraint stress significantly increased AChE activity in hippocampus (*P*-value<.001; compared to naïve control). Tianeptine attenuated the increased AChE activity in restraint stress rats (*P*-value<.01; compared to vehicle+stress). It was found that *N.nucifera* flowers extract at all doses significantly decreased AChE activity in restraint stress rats (*P*-value<.001 all; compared to vehicle+stress).

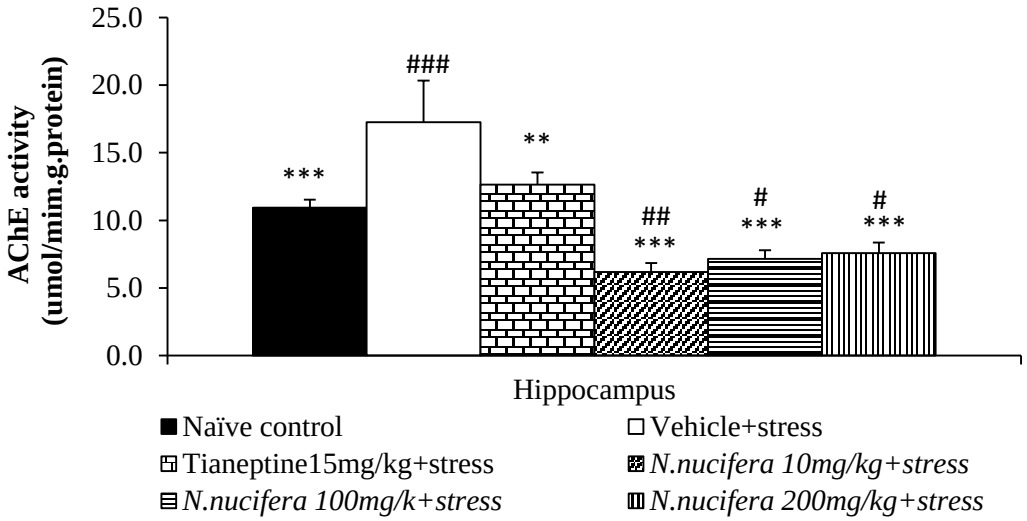

Figure 5-36 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on acetylcholineesterase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). **,*** *P*-value < 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#,\#\#,\#\#\#}$ *P*-value < 0.05, 0.01 and 0.001 compared with control group

5.3.3.4 Effect of *N.nucifera* flowers extract on monoamine oxidase-A and B

Rats which received vehicle and subjected to restraint stress significantly increased the activity of both MAO-A and MAO-B in hippocampus (*P*-value <.001 all; compared to naïve control). Tianeptine mitigated the elevation of both MAO-A and MAO-B (*P*-value<.001 all; compared to vehicle+stress). Rats subjected to restraint stress and received *N.nucifera* flowers extract at all doses significantly decreased MAO-A and B activities in hippocampus (*P*-value<.001 all; compared to vehicle +stress) as shown in Figure 5-37).

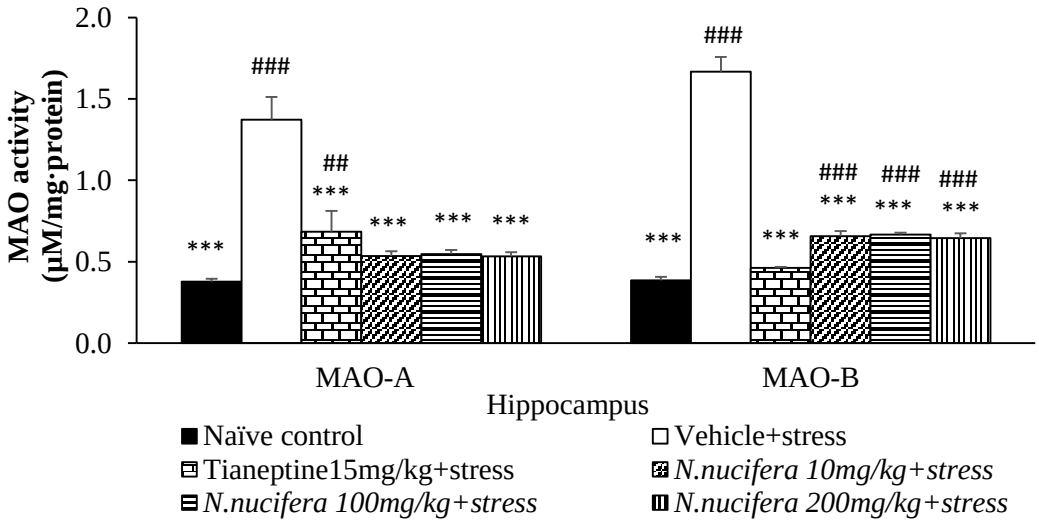

Figure 5-37 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on monoamine oxidase type A and B of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *** *P*-value < 0.001 respectively; compared with vehicle plus stress. ##,### *P*-value < 0.01 and 0.001 compared with control group

5.3.3.5 Effect of *N.nucifera* flowers extract on oxidative stress markers

Figure 5.38 – Figure 5-41 and table 5.3 showed that repetitive restraint stress which received vehicle significantly decreased SOD, CAT and GSH-Px activities but increased MDA level in hippocampus (*P*-value<.001, .05, .001 and .001 respectively; compared to naïve control group). Rats which received Tianeptine plus stress significantly increased SOD GSH-Px activities but decreased MDA level in hippocampus (*P*-value < .001 all; compared to vehicle +stress). All doses of *N.nucifera* significantly decreased MDA level in hippocampus (*P*-value<.001 all; compared to vehicle +stress) but they failed to produce the significant changes on SOD and GSH-Px activities in hippocampus of stress-exposed rats. However, all doses of *N.nucifera* increased CAT activity (*P*-value<.05 all; compared to vehicle +stress) in hippocampus of stress exposed rats.

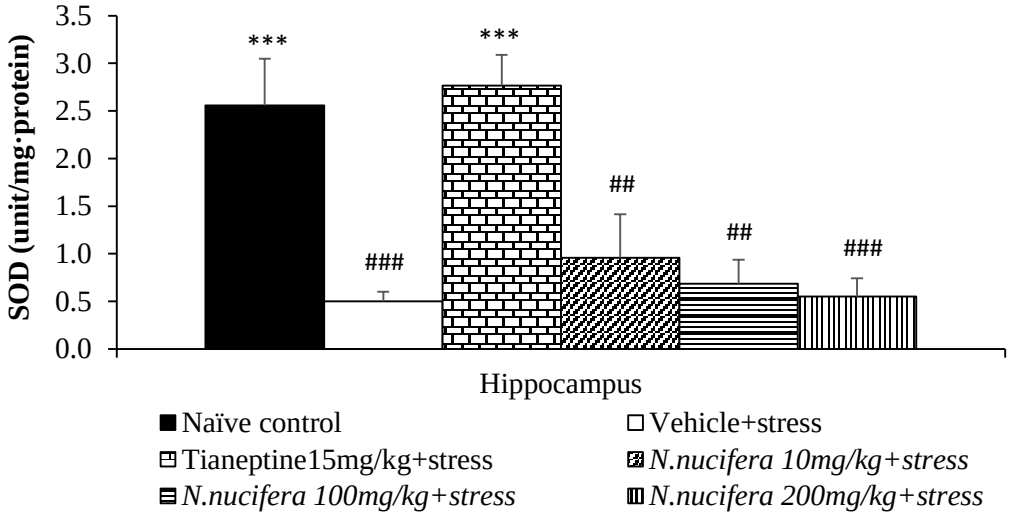

Figure 5-38 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on superoxide dismutase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *** *P*-value < 0.001; compared with vehicle plus stress. ##,### *P*-value < 0.01 and 0.001 respectively; compared with control group

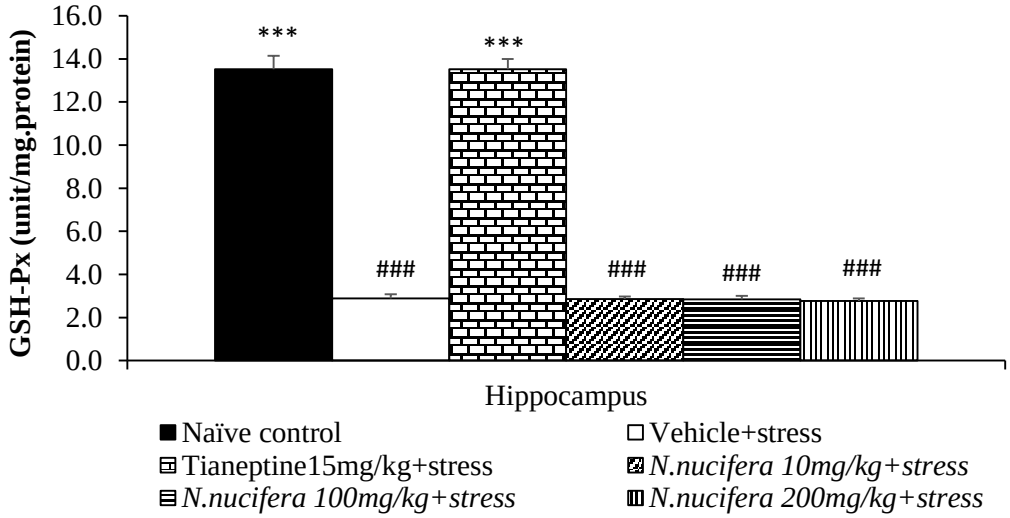

Figure 5-39 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on glutathione peroxidase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *** *P*-value < 0.001; compared with vehicle plus stress. ### *P*-value < 0.001; compared with control group

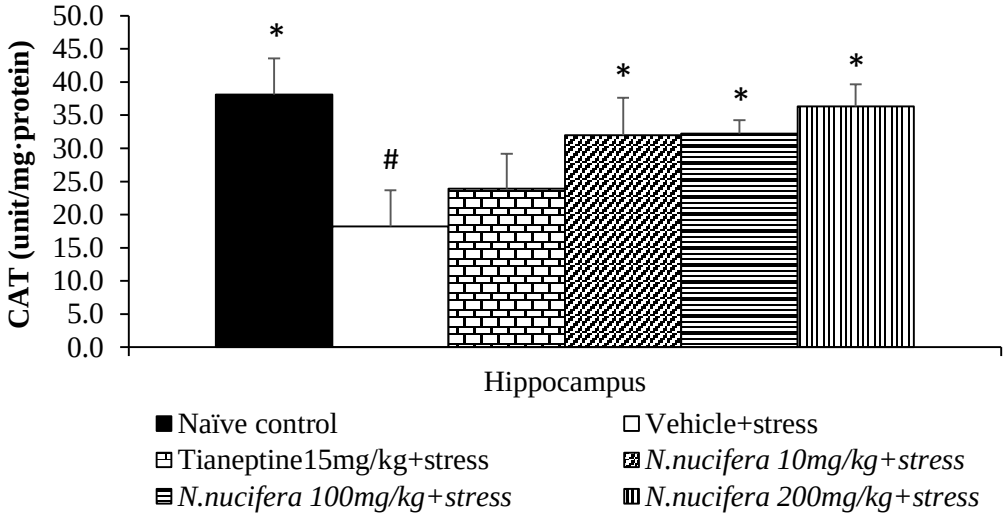

Figure 5-40 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on catalase activity in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). * *P*-value < 0.05; compared with vehicle plus stress. # *P*-value < 0.05; compared with control group

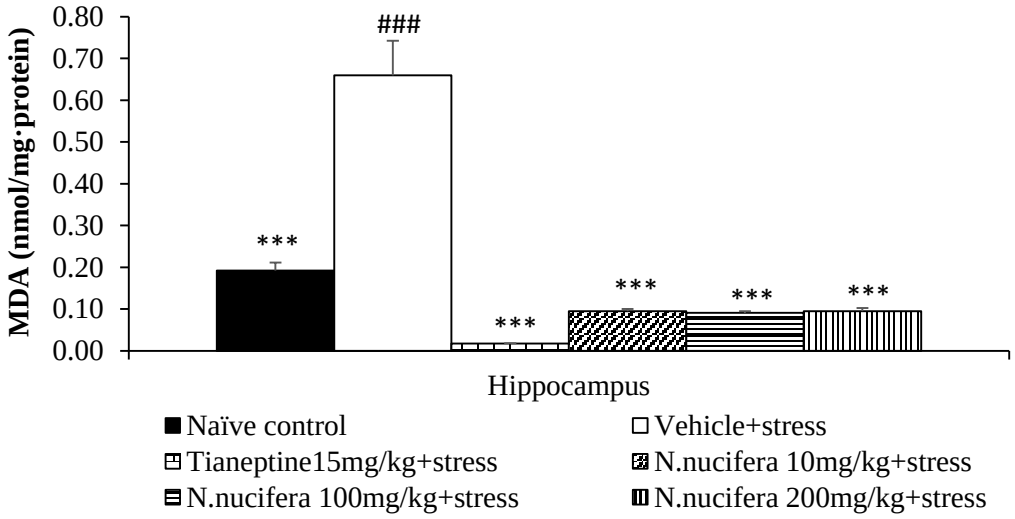

Figure 5-41 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on malondialdehyde level in hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *** *P*-value < 0.001; compared with vehicle plus stress. ### *P*-value < 0.001; compared with control group

Table 5.3 Effect of *N.nucifera* leaves extract on oxidative stress markers (SOD, GSH-Px, CAT, MDA)

Parameters	**SOD**	**GSH-Px**	**CAT**	**MDA**
Group	**unit/mg·protein**	**unit/mg·protein**	**unit/mg·protein**	**nmol/mg·protein**
Naïve control	2.6±0.5***	13.5±0.6***	38.1±5.4*	0.19±0.02***
Vehicle+stress	0.5±0.1$^{\#\#\#}$	2.9±0.2$^{\#\#\#}$	18.2±5.5$^{\#}$	0.66±0.08$^{\#\#\#}$
Tianeptine 15mg/kg +stress	2.8±0.3***	13.5±0.5***	23.9±5.3	0.23±0.01***
N.nucifera 10mg/kg +stress	1.0±0.5$^{\#\#}$	2.8±0.1$^{\#\#\#}$	32.0±5.6*	0.09±0.00***
N.nucifera 100mg/kg +stress	0.7±0.2$^{\#\#}$	2.8±0.2$^{\#\#\#}$	32.2±2.1*	0.09±0.00***
N.nucifera 200mg/kg +stress	0.5±0.2$^{\#\#\#}$	2.8±0.1$^{\#\#\#}$	36.3±3.3*	0.09±0.01***

*,**,*** P < .05, .01 and .001 respectively; compared to vehicle plus stress.

$^{\#,\#\#,\#\#\#}$ P < .05, .01 and .001 respectively; compared to Naïve control.

5.3.3.6 Effect of *N.nucifera* flowers extract on survival neurons

It was found that rats which obtained vehicle plus stress showed the decreased neurons density in CA1, CA2, CA3 and dentate gyrus (*P*-value<.01, .01, .05 and .01 respectively; compared to naïve control). Tianeptine increased neuron density in all areas mentioned earlier in stress exposed rats (*P*-value<.05, .01, .01 and .05 respectively; compared to vehicle +stress). Interestingly, *N.nucifera* flowers extract at doses of 10, 100 and 200 mg/kg produced the significant increase in neurons density in CA2 (*P*-value<.05, .05 and .001 respectively; compared to vehicle +stress) and CA3 (*P*-value<.01, .01 and .001 respectively; compared to vehicle +stress) of stressed rats. Low dose of *N.nucifera* produced the significant increase of neurons density in dentate gyrus (*P*-value<.05; compared to vehicle +stress) as shown in Figure 5-42 – Figure 5.45. No significant changes were observed in CA1 at all doses of *N.nucifera*.

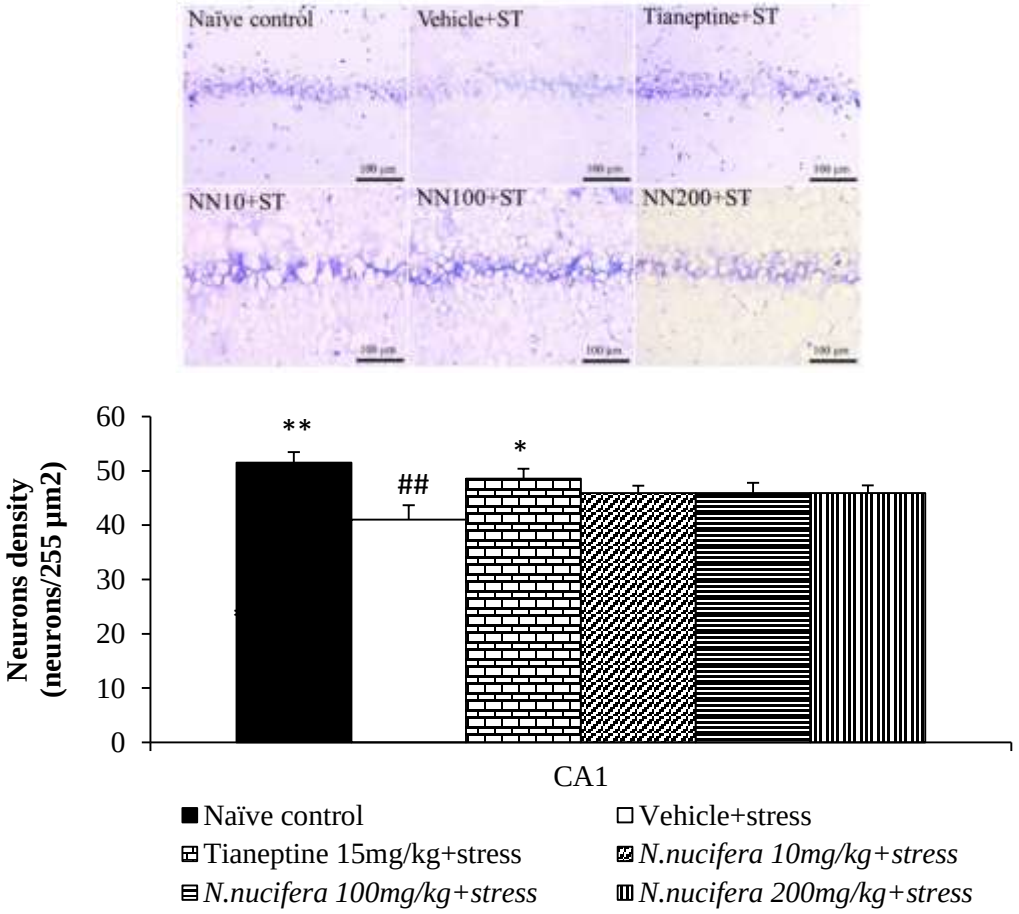

Figure 5-42 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on the density of survival neurons in CA1 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05 and 0.01 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01; compared with control group

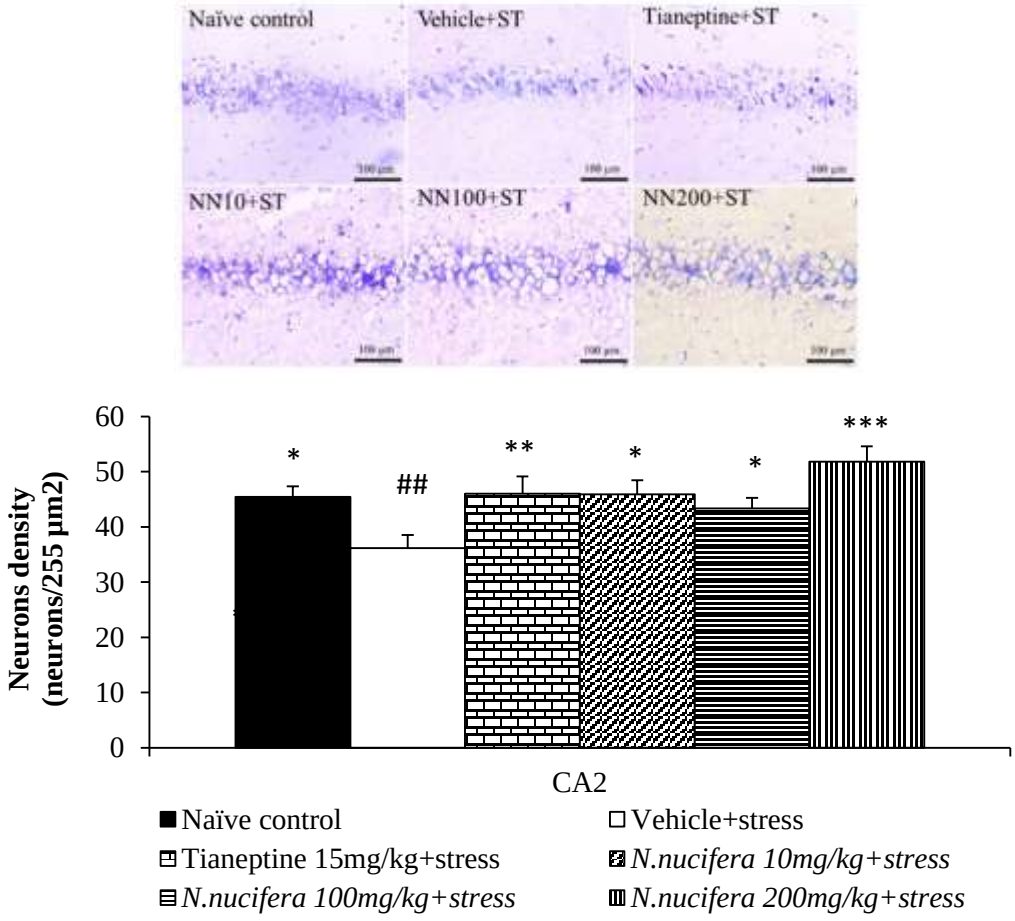

Figure 5-43 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on the density of survival neurons in CA2 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,**,*** *P*-value < 0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#\#}$ *P*-value < 0.01; compared with control group

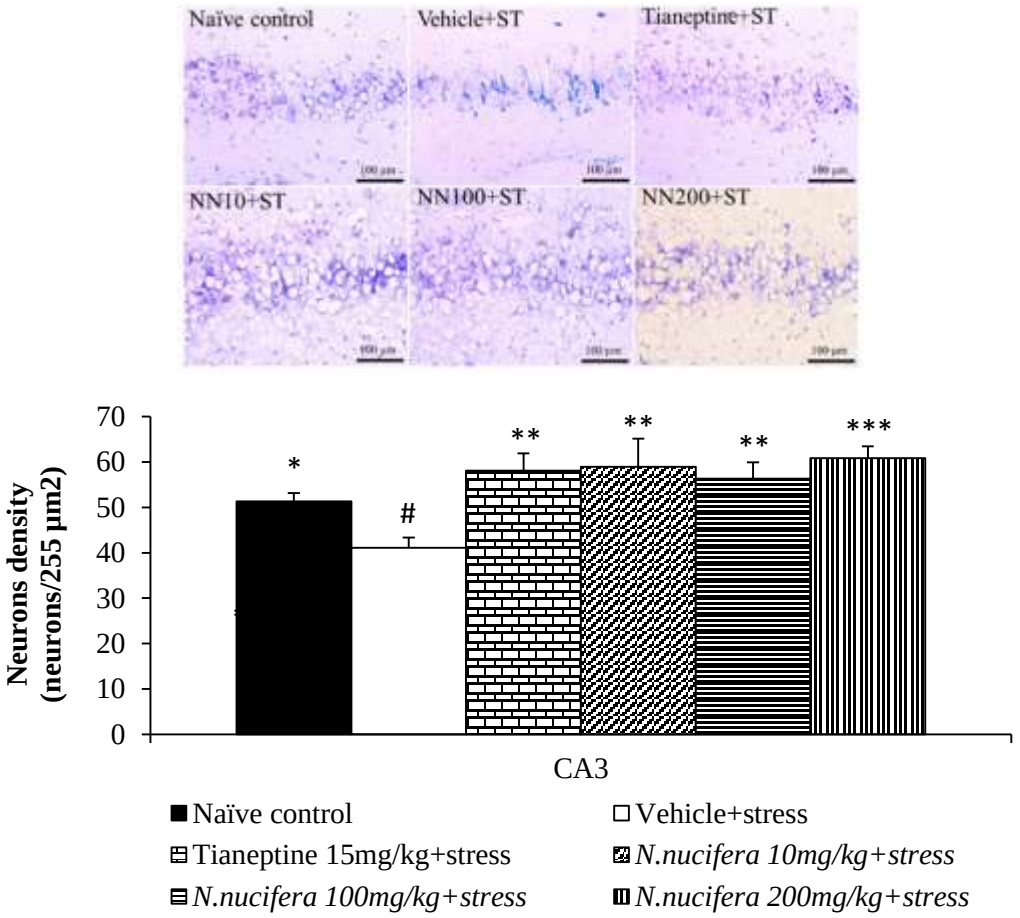

Figure 5-44 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on the density of survival neurons in CA3 of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,**,*** *P*-value < 0.05, 0.01 and 0.001 respectively; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05; compared with control group

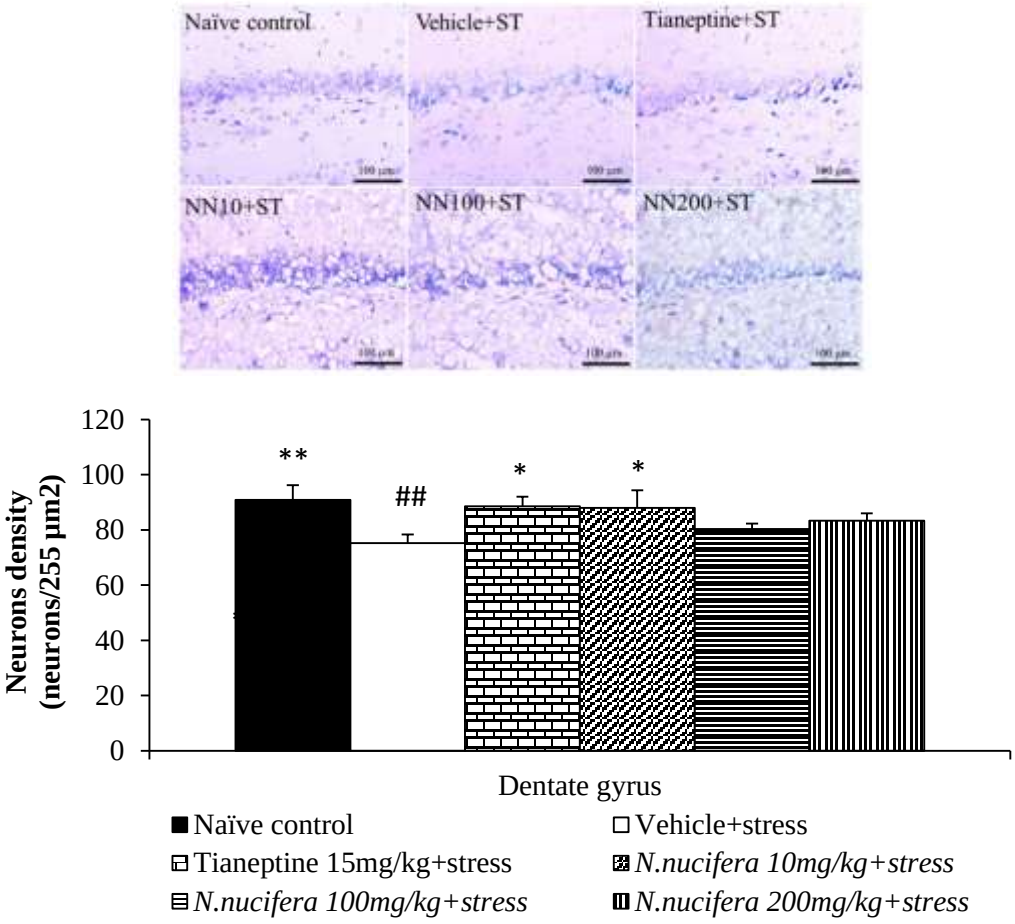

Figure 5-45 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on the density of survival neurons in dentate gyrus of hippocampus of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). *,** *P*-value < 0.05, and 0.01 respectively; compared with vehicle plus stress. $^{\#}$ *P*-value < 0.05; compared with control group

5.3.3.7 Effect of *N.nucifera* flowers extract on Ki-67 proliferative marker

Figure 5-46 showed that repetitive exposure to restraint stress significantly decreased the level of Ki67, an adult neurogenesis marker, in hippocampus (*P*-value<.05; compared with naïve control). Tianeptine and *N.nucifera* extract at doses of 100 and 200 mg/kg failed to produce the change of Ki67 level in hippocampus of stress exposed rats. However, stress exposed rats which received *N.nucifera* extract

at dose of 10 mg/kg could increase Ki67 level in the mentioned area (*P*-value<.05; compared to vehicle +stress)

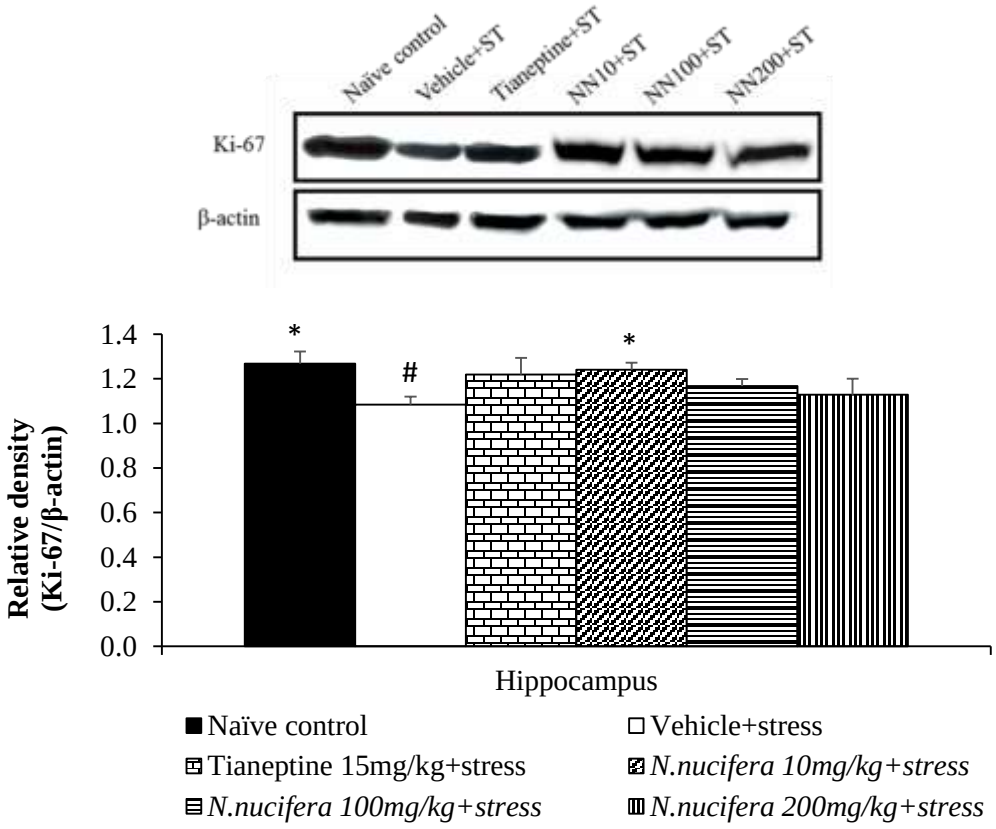

Figure 5-46 The effect of hydro-alcoholic extracts of *N.nucifera* flowers extract on Ki-67 proliferative marker of stress-exposed rats. Data were expressed as mean±S.E.M. (n=6/group). * *P*-value < 0.05; compared with vehicle plus stress. # *P*-value < 0.05; compared with control group

5.4 Discussion

5.4.1 Effect of *M.oleifera* leaves extract on memory impairment and neurodegeneration in restraint rats

The current study has clearly demonstrated that *M.oleifera* leaves extract improved spatial memory, neurons density, oxidative stress status (SOD and CAT) and the level of Ki67 but decreased AChE, MAO-B activities in hippocampus and MDA level. In addition, the extract also suppressed serum corticosterone level.

The data obtained from this study clearly showed that repetitive restraint stress significantly enhanced both MAO-A and MAO-B in hippocampus. It

has been reported that MAO-A plays an important role on the inactivation of serotonin, norepinephrine and dopamine whereas MAO-B plays an important role on dopamine (Bortolato *et al.*, 2008). Therefore, the available norepinephrine and dopamine in hippocampus were decreased following the repetitive exposure to restraint stress. The elevation of monoamine oxidase can increase oxidative stress status via the oxidative deamination of neurotransmitters which in turn generate hydrogen peroxide (Youdim *et al.*, 2006) giving rise to the excess free radicals leading to the neurodegeneration and finally results in the decreased neuron density in hippocampus. These changes were in agreement with our data which showed the increased MAO-A and MAO-B together with the increased MDA, a lipid peroxidation product occurring via the attack of free radicals and polyunsaturated fatty acid of membrane, and the decreased neurons density in hippocampus. Repetitive restraint stress also stimulated HPA-axis and the serum corticosterone level leading to the neurodegeneration (McEwen, 2007). The neurodegeneration both via the increased both types of MAO and the increased corticosterone can sequentially induce memory deficit. In addition, the current result also showed that repetitive restraint stress also increased AChE activity which in turn decreased the cholinergic function in hippocampus which was in agreement with the previous study (Das *et al.*, 2005; Youdim *et al.*, 2006). The enhanced cholinergic function can improve stress-related-memory deficit and increased the level of Ki67 which in turn increased adult neurogenesis in hippocampus (Kee *et al.*, 2002) and increased neuron density in hippocampus which in turn increased memory.

It was found that *M.oleifera* leaves extract at all doses used in this study could improve oxidative stress status and neurons density in hippocampus but only the medium and high doses could improve memory deficit in stress exposed rats. The decreased oxidative stress status in rats treated with *M.oleifera* leaves extract might occur via the decreased MAO-B, enhanced SOD and CAT activities and decreased MDA level in hippocampus. In addition, the extract also suppressed serum corticosterone level which in turn might decrease neurodegeneration in hippocampus. Since the enhanced neuron density played a role on the improve memory, we did suggest that the improved neurons density via the decreased free radicals and serum corticosteone might partly contribute a role on the improved memory but might not be

a principal factor. Interestingly, high dose of extract also increased Ki67 giving rise to the increased adult neurogenesis in hippocampus leading to the increased neuron density. This phenomenon could also increase memory performance. *M.oleifera* leaves extract also suppressed AChE activity in hippocampus resulted in the enhanced cholinergic function which in turn improved memory (Ikarashi *et al.*, 2004). The increased cholinergic function could also increase hippocampal neurogenesis (Kotani *et al.*, 2006) giving rise to the enhanced neuron density and finally leading to the improved memory. The data obtained from this study has demonstrated that *M.oleifera* leaves extract showed the multi-target action. The action appeared to depend on the administration dose. Only the high dose could show the enhanced neurogenesis in hippocampus. Based on the finding that quercetin enhanced neurogenesis (Tchantchou *et al.*, 2009) together with the improved neurodegeneration and spatial memory via the decreased oxidative stress and the suppression of AChE, the beneficial effect of *M.oleifera* leaves extract observed in this study might be associated with quercetin content in the extract. However, further research concerning the determination of precise active ingredient is still essential.

In conclusion, *M.oleifera* leaves extract are the potential neuroprotective and cognitive enhancer regimen against stress related brain damage and memory deficit. It exerts the effect via multi-target including the decreased neurodegeneration via the decreased free radicals and the decreased corticosterone, the increased neurogenesis and the increased cholinergic function. The beneficial effect of the extract might be associated with quercetin content in the extract. Therefore, *M.oleifera* leaves extract might be served as the anti-stress food supplement. However, further researches to confirm the precise active ingredient and clinical trial are necessary.

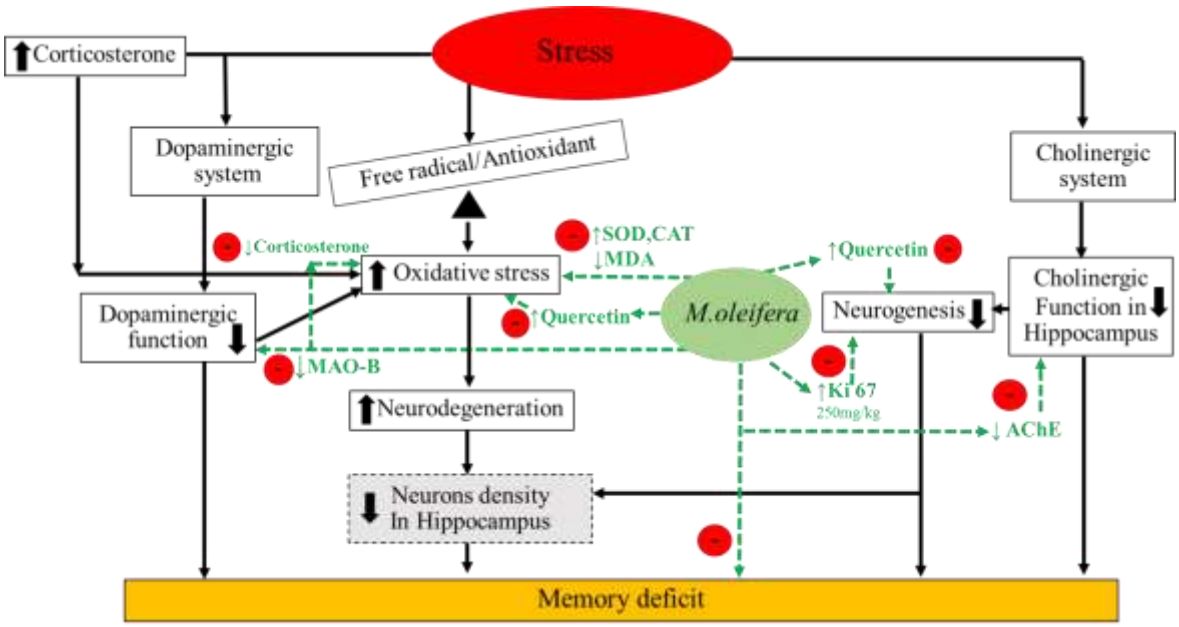

Figure 5-47 Schematic diagram showed the possible underlying mechanism of *M.oleifera* on neuroprotective and memory enhancing effects in stressed rats

5.4.2 Effect of *A.occidentale* leaves extract on memory impairment and neurodegeneration in restraint rats

Accumulative lines of evidence showed that chronic restraint stress could enhance oxidative stress (Abidin *et al.*, 2004), memory impairment (Abidin *et al.*, 2004; McLaughlin *et al.*, 2007) and decreased hippocampus neurogenesis (Yun *et al.*, 2010). Our data also revealed the memory impairment and the increased MDA level in hippocampus. Therefore, the current findings also showed the enhanced of oxidative status in hippocampus of stress-exposed rats as similar as that findings of Abindin nad coworkers (Abidin *et al.*, 2004). The decreased Ki67, a neuroproliferation marker, in hippocampus was also observed. Thus, the current study also demonstrated the decreased hippocampal neurogenesis which was consistent with the previous study (Yun *et al.*, 2010). In addition, the elevation of corticosterone induced by stress could also induce neurodegeneration (Touyarot and Sandi, 2002). Therefore, the decreased neuron density observed in the rats exposed to 12 h-immobilization stress which received vehicle may possibly due to the decreased neurogenesis and the

elevation of corticosterone and oxidative stress (Abidin *et al.*, 2004; Touyarot and Sandi, 2002; Yun *et al.*, 2010).

Memory performance is a complex phenomenon which depends on many factors. It has been well established that the severity of memory impairment is associated with the neurodegeneratiion in hippocampus (Devi *et al.*, 2003; Oswald and Good, 2000). Based on the finding mentioned earlier, the decreased neuron density in hippocampus in this study might play a pivotal role on the memory impairment induced by stress. Interestingly, *A.occidentales* leaves extract at all doses used in this study could produce the significant elevations of SOD and GSH-Px activities which in turn decreased oxidative stress status in hippocampus and finally gave rise to the increased neuron densities in all subregions of hippocampus. In addition, all doses of *A.occidentale* leaves extract also increased the expression of Ki67 in hippocampus. Therefore, it has been suggested that the memory enhancing effect of *A.occidentale* may partly due to the decreased neurodegeneration and the increased neuroproliferation in hippocampus which in turn lead to the increase of neuron density and memory improvement in stress –exposed rats.

Although the elevation of corticosterone could also mitigate neurodegeneration in hippocampus and memory impairment, no closed association between serum corticosterone and the neuron density in hippocampus was observed. Only the medium dose of extract could mitigate the serum corticosterone. Therefore the neuroprotective effect of the medium dose of extract might occur as the result of the decreased oxidative stress and the increased neurodgeneration in hippocampus together with the decreased of corticosterone, a stress hormone.

In conclusion, this study is the first study to demonstrate that *A.occidentale* leaves extract possesses the neuroprotective and memory enhancing effect in stress-exposed rats. Therefore, it may possibly be served as food supplement against neurodegeneration and memory impairment induced by stress. However, further researches to confirm the precise active ingredient and clinical trial are necessary.

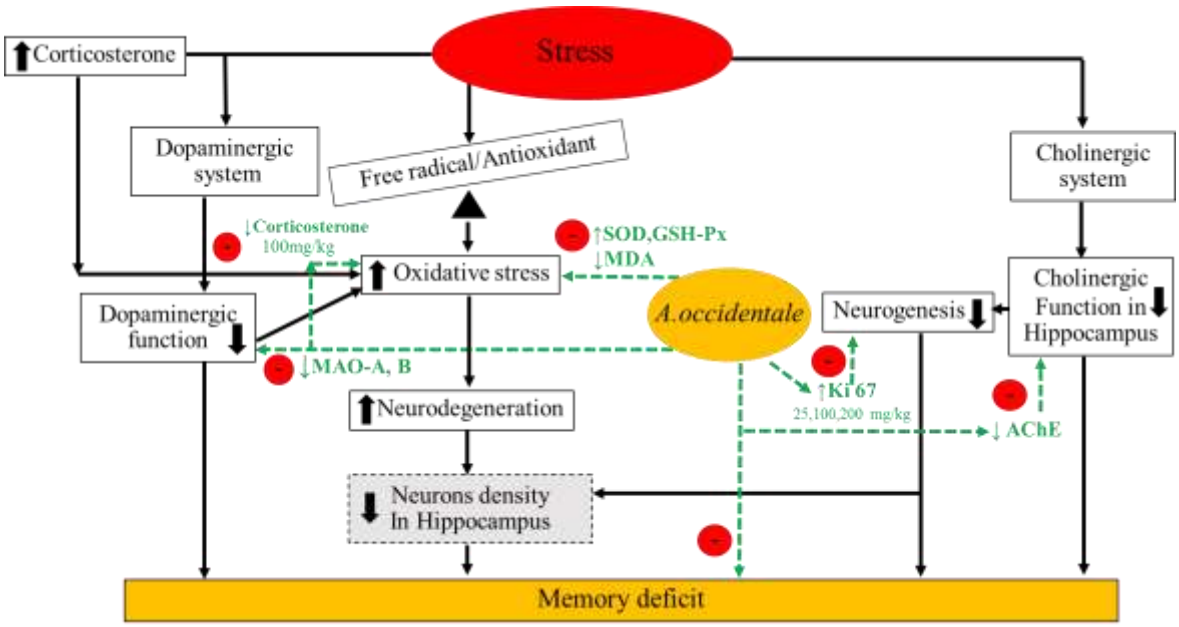

Figure 5-48 Schematic diagram showed the possible underlying mechanism of *A.occidentale* on neuroprotective and memory enhancing effects in stressed rats

5.4.3 Effect of *N.nucifera* flowers extract on memory impairment and neurodegeneration in restraint rats

It has been well known that hippocampus is a heterogeneous structure. Various subregions of hippocampus perform different functions. CA3 is preferentially involved in acquisition, consolidation and retention phase of memory (Lee and Kesner, 2004; Lee and Kesner, 2004b; Steffenach *et al.*, 2002) whereas CA1 is involved in retention and retrieval of memory (Lee and Kesner, 2004; Lee and Kesner, 2004b) and dentate gyrus (DG) is important for encoding (Lee and Kesner, 2004b). It has been recently shown that CA2 plays minor role on spatial memory but it plays an important role on social memory and social recognition (Caruana *et al.*, 2012). In addition, hippocampus is also recognized as the special area which neurogenesis can occur throughout adult lives (Kempermann *et al.*, 1997). Recent findings have shown that adult neurogenesis play the role on learning and memory (Kim *et al.*, 2009).

Various processes of learning and memory depend not only on area specificity of hippocampus but also depend on various types of neurotransmitters.

It has been shown that acetylcholine is essential for the encoding process, the first phase of memory and dopamine plays the crucial role on both acquisition and retention of memory (da Silva *et al.*, 2012). Norepinehrine is also implicated in hippocampus-based learning and memory especially acquisistion process via adrenergic receptor (Gertner and Thomas, 2006).

The data obtained from this study demonstrated that the stress-exposed rats which received *N.nucifera* flowers extract at all doses used in this study showed the enhanced neuron density in CA2 and CA3 in hippocampus together with the improved spatial memory and the suppression effects of AChE, MAO-A and MAO-B. On the basis of the information mentioned earlier, it has been suggested that the memory enhancing effect of *N.nucifera* flowers extract may occur partly via the increased neuron density in CA3 which in turn increase the acquisition, consolidation and memory retention of memory leading to the improved spatial memory. In addition, *N.nucifera* flowers extract may also suppress AChE and both types of MAO resulting in the increased available ACh, NE and DA in hippocampus which in turn increase encoding process and retention of memory and finally resulting in the improved memory.

In this study, the decreased serum corticosterone levels and the decreased oxidative stress status were also observed in rats which subjected to 12 h-immobilization stress and received *N.nucifera* flowers extract at the dosage range used in this study. The decreased serum corticosterone might increase the neurons density in hippocampus via the decreased excitotoxicity induced by corticosterone in hippocampus (Abraham *et al.*, 2001). Besides the decreased serum corticosterone, the decreased oxidative stress in hippocampus also contributes the important role on the increased density of survival neurons in the mentioned area. Although the oxidative stress status was very much improved reflecting by the decreased MDA level, only the elevation of CAT activity was observed. Therefore, other factors such as the increased non-enzymatic antioxidant activity and the decreased oxidative stress formation might also contribute the role on the reduction of MDA level. *N.nucifera* flowers extract also increased Ki67 expression which in turn indicated the increased hippocampal neurogenesis in stress-exposed rats which received low dose (10 $mg.kg^{-1}$ BW) of extract. Therefore, besides the mechanisms just mentioned, the increased

neurons density in hippocampus in stress- exposed rats which received low dose of *N.nucifera* might be attributed by the increased adult neurogenesis in hippocampus.

In conclusion, *N.nucifera* flowers extract was the potential neuroprotective and cognitive enhancer agent against stress related brain damage and memory deficit. The possible underlying mechanisms are associated with the improved oxidative stress status, the increased adult neurogenesis and the increased neurotransmitters which play the role on learning and memory such as acetylcholine, dopamine and norepinephrine. However, further investigation about the precise active ingredient, kinetic and clinical trial studies are still required.

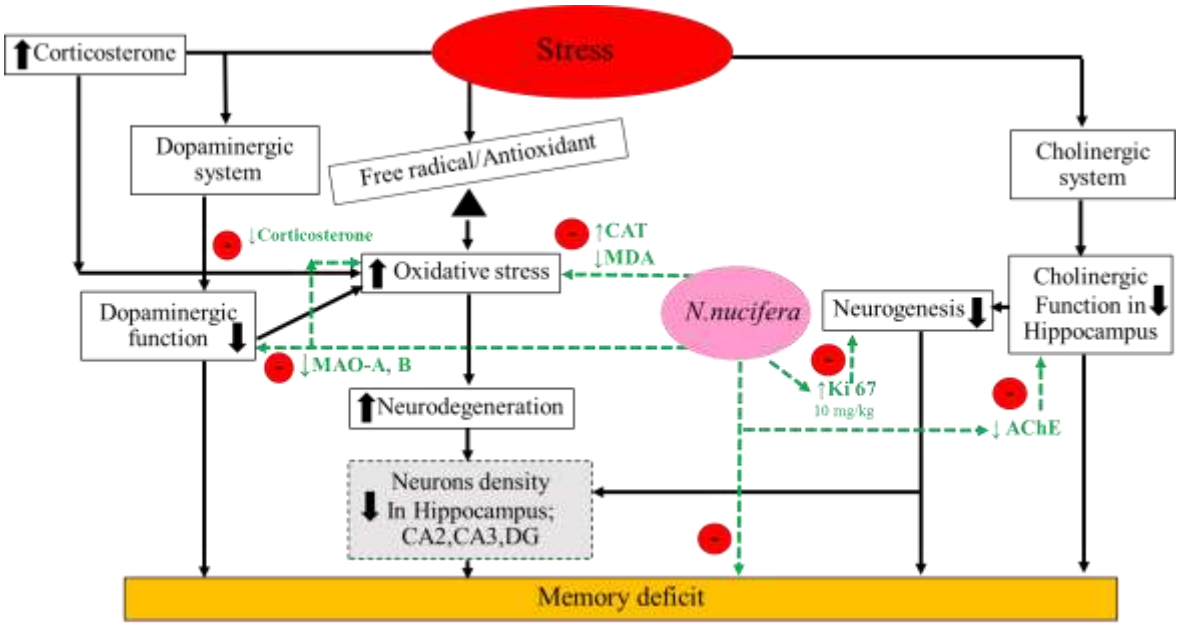

Figure 5-49 Schematic diagram showed the possible underlying mechanism of *N.nucifera* on neuroprotective and memory enhancing effects in stressed rats

5.5 Conclusion

This study is the first study to demonstrate the scientific evidence concerning the neuroprotective and memory enhancing effect of the *M.oleifera*, *A.occidentale* and *N.nucifera*, the medicinal plants reputed for neuroprotective and memory enhancing in traditional folklore and their underlying mechanisms. Interestingly, these plants can

exert memory enhancing effect via the central effect by enhancing the cholinergic and dopaminergic function in the key areas which control memory function and increase neurons density by decreasing oxidative stress and increasing proliferation of neurons in hippocampus. They can also provide benefits as neuroprotectant and memory enhancer against stress-induced memory deficit and stress-induced sexual dysfunction. In addition, most of them are not expensive and easy to approach. Therefore, they may be served as the potential neuroprotective and memory enhancing agent. However, further researches concerning the active ingredient, pharmacokinetic and drug interaction are essential before moving to the clinical trial.