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Protective effects of syringic acid on neurobehavioral deficits and hippocampal tissue damages induced by sub-chronic deltamethrin exposure

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ABSTRACT

Recent developments in the field of insecticide exposure have led to a renewed interest in alternative antioxidant therapy. The present study was to investigate the neuroprotective role of syringic acid (SA, 25 mg/kg/day) on the neurotoxicity and oxidative damage induced by deltamethrin (DTM, 1.28 mg/kg/day during two months) in CA1/3 pyramidal neurons. Animals were divided into 4 groups (n = 16/group) (250–270 g) for control, DTM, SA and DTM + SA. DTM and SA were administered by oral gavage daily. Rats that were given sub-chronic DTM had revealed a significant increase in caspase-3 levels, impaired recognition memory, reduced antioxidant activity and enhanced free radicals in the hippocampus. The results showed that SA ameliorated neurobehavioral alterations, reduced reactive oxygen/nitrogen species, pyknosis in the CA1/3 and increased antioxidant enzyme activity. In conclusion, SA (25 mg/kg/day) had potential neuroprotective and therapeutic impacts against sub-chronic DTM exposure via its antioxidant and antiapoptotic efficacy. Therefore, it can be used as a neuroprotective natural plant-derived agent against DTM-induced neurotoxicity.

1. Introduction

Low doses of insecticide toxicity have serious damaging impacts on public health and the environment. Direct or indirect consumption of insecticide, especially contaminated food and water leads to health risk mainly causes neurodegenerative maladies. Deltamethrin (DTM) is one of the alpha-cyano type II synthetic neuroactive pyrethroid and considered to be the most reliable insecticide in most countries due to its low mammalian toxicity and high eradication role, but DTM induced oxidative damage has been reported in several experimental animal models (Chandra et al., 2013; Köprücü et al., 2008; Sharma et al., 2013). DTM is a highly lipophilic compound that can easily pass through the blood-brain barrier and act on the hippocampus (Gasmi et al., 2017). The main target site of DTM is the axonal sodium channel and results in several neurobehavioral sub-lethal consequences. DTM is acting directly on the axons in the hippocampus by delaying the closure of the voltage-gated sodium channels. This effect extended the permeability of the nerve to Na^+ and generates a sequence of recurrent nerve signals (Narahashi, 1992, 1996; Silver et al., 2014; Soderlund and Bloomquist, 1989). The increase of ROS destroys the neurons, which leads to subsequent activation of voltage-gated receptors, allowing calcium influx and trigger the paths of cell death low doses of DTM exposure leads to the simple neurobehavioral and motor deficits to complicated neurodegenerative diseases. Some of them are spontaneous aggressive uncontrolled behavior, immobility, loss of coordination, paralysis, tremor, hyperactivity, hyperexcitability, convulsions, hypersalivation, choreoathetosis and contraction (Gray, 1985; Vijverberg and van den Bercken, 1990). In addition, administering DTM

Abbreviations: C, control; CA, cornu ammonis; Co, corn oil; Cu, copper; DCF, 7′-dichlorodihydrofluorescein; DCFH, dichlorodihydrofluorescein; DCFH-DiOxyQ, dichlorodihydrofluorescein dioxyq; DTM, deltamethrin; ESI, positive electrospray ionization; H-E, haematoxylin and eosin; HPLC, high-performance liquid chromatography; LD, lethal dose; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; OF, open field; RAM, radial arm maze; RME, reference memory errors; RNS, reactive nitrogen species; ROS, reactive oxidative species; SA, syringic acid; TAC, total antioxidant capacity; UFLC, ultra-fast liquid chromatography; WME, working memory errors; WHO, World Health Organization

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to Wistar rats results in increased reactive oxygen/nitrogen species (ROS/RNS), neurodegeneration and alterations neuronal morphology of hippocampus (Adibhatla and Hatcher, 2008; Banerjee et al., 2001; Dubey et al., 2012; Halliwell and Chirico, 1993; Hossain et al., 2008; Husain et al., 1996; Abdollahi et al., 2004; Rehman et al., 2006; Van Den Bosch et al., 2002). Thus, studies that have evaluated the effects of DTM exposure on learning and memory focused on the CA1/3 pyramidal neurons (Chen et al., 2006; Hossain et al., 2015; Hossain et al., 2008; Huang et al., 2010; Husain et al., 1996; Li et al., 2005; Li et al., 2006; Martinez-Larranaga et al., 2003; Meyer et al., 2008; Sun et al., 2007; Zhao et al., 1995). Therefore, there is a need to find alternative strategies to synthetic pyrethroid, this has thus led to an increasing interest in the development of plant-derived natural products. The natural antioxidant compounds of plants, especially polyphenols, are the subject of numerous studies for their utilize in the treatment of many diseases. Research to date has tended to focus on syringic acid (SA) which is a product of microbial metabolism of anthocyanins and polyphenols that are available especially in fruits and alcoholic beverages (Cotoras et al., 2014; Pacheco-Palencia et al., 2008). SA occurs in several plants, such as *Isatis indigotica* (Radix isatidis) (Cikman et al., 2015), *Capparis spinosa* (Turgut et al., 2015), *Herba dendrobii* (Wei et al., 2012), *Medicago rigidula* (Farag et al., 2007), *Ardisia elliptica* and *Euterpe oleracea* (Pacheco-Palencia et al., 2008). SA exhibits multiple pharmacological properties, such as an antioxidative (Memon et al., 2010) anticarcinogenic, chemoprotective (Hudson et al., 2000) antiproliferative (Orabi et al., 2013; Wei et al., 2012), anti-endotoxic, antimicrobial, anti-inflammatory (Abaza et al., 2011; Bogert and Jacob, 2013; Cho et al., 1998; Wei et al., 2012), anti-angiogenic, antidiabetic (Nadeem et al., 2011) and antihyperglycemic activities (Guimaraes et al., 2007; Kumar et al., 2012; Thipparaboina et al., 2016). Some studies have brought SA into the forefront by describing neuroprotective (Rekha et al., 2014) and antidepressant effects (Cao et al., 2016; Dalmagro et al., 2017). Numerous studies have reported a dose-dependent reduction of oxidative stress in hippocampal cells by SA; however, few studies have investigated SA as a neuroprotective agent (Guven et al., 2015; Kim et al., 2013; Tokmak et al., 2015). Therefore, the present study aimed the neurotoxicity effect of DTM in CA1/3 pyramidal neurons and its effects on learning and memory via oxidative stress and apoptotic mediators, also the neuroprotective role of SA against DTM toxicity as an antioxidant agent.

2. Materials and methods

2.1. Animals

A total of sixty-four male *Rattus norvegicus* Wistar albino rats, weighing 250–270 g, were used in the present study. They were obtained from the Laboratory Animals Center of the Akdeniz University School of Medicine and also this study was conducted on Akdeniz University School of Medicine. The rats were housed in polypropylene cages (40 × 50 × 20 cm) and they were kept under a 12/12 h light/dark cycle at 21 ± 2 °C and relative humidity of 60 ± 10%. Food restriction was applied to the animals for learning and memory tests. Overall the parameters of this diet per under NRC guidelines for Wistar rats and all processes were approved in advance by the Animal Care and Use Committee (IACUC) of Akdeniz University (protocol number 615-2017.01.05). The rats were acclimatized for one week before starting the treatment and all efforts were made to minimize animal suffering under standard conditions in accordance with the Ethical Principles for the Care and Use of Laboratory Animals.

2.2. Chemical preparation and administration protocol

Deltamethrin ($C_{22}H_{19}Br_2NO_3$, (*S*)-a-cyano-d-phenoxybenzyl-(1*R*,3*R*)-e-(2,2-dibromovinyl)-2,2-dimethylcyclo-propane-1-carboxylate, Decis®; Bayer AG, Leverkusen, Germany) and Syringic Acid ($C_9H_{10}O_5$, 4-hydroxy-3,5-dimethoxybenzoic acid, Cat: 530-57-4, SA ≥ 98%; Santa Cruz Biotechnology, Santa Cruz, CA, USA) solutions were dissolved in corn oil. The dose that was selected is 1.28 mg/kg/day for DTM (purity 98%) and 25 mg/kg/day for SA (5.78 mg/ml at 25 °C) during two months respectively. The SA was vortexes and diluted with corn oil to a final volume of 5.78 mg/ml for 5 min. 1.28 mg/ml concentrations of DTM was used at room temperature. 1.28 mg/kg (1/100 LD_{50}) was selected as the DTM dose to elicit oxidative stress in Wistar rats without having morbidity (Tayebati et al., 2009).

2.3. Sub-chronic toxicological study

The rats were randomly divided into four groups of sixteen males (n = 16) each group for Control, DTM, DTM + SA, and SA. Each rat was administered by oral gavage once daily for two months. The effects of treatment on body weight changes in the rats were also evaluated by weighing each rat once every week. Behavioral test was performed using the Noldus Ethovision XT system (Noldus Information Technology, Wageningen, The Netherlands).

Group I was referred to as the Control (C) and treated with corn oil (0.5 ml/rat, Schneider, Istanbul, Turkey).

Group II was received DTM (1.28 mg/kg/day ~1/100th of its LD50).

Groups III was co-administered with DTM and SA (at 1.28 mg/kg/day and 25 mg/kg/day); the rats in the DTM + SA group were given SA and DTM together in the same time. The SA and DTM were vortexes and diluted with corn oil for 5 min.

Group IV was treated with SA alone (at 25 mg/kg/day).

2.4. Behavioral testing

All rats were tested for behavioral tests (n = 64) and then used for histology (n = 24) and biochemistry (n = 40) analysis. Behavioral tests were performed during the experiments after 6 h later from exposure to DTM and SA. Before starting the behavioral tests, we restricted the rats' food intake, gradually reducing their body weight to 80% of their ad libitum weights and we handled animals for 5 min/day (7 days) before starting the test. Four rats were housed per cage before food restriction began. Radial Arm Maze (RAM) was applied to all groups 15 days before sacrifice and Open Field was applied to all groups one-day before sacrifice (Fig. 1).

2.4.1. Open field (OF)

General locomotor activity and behavioral activity level of Wistar rats were evaluated in the OF test. We based our protocol on that

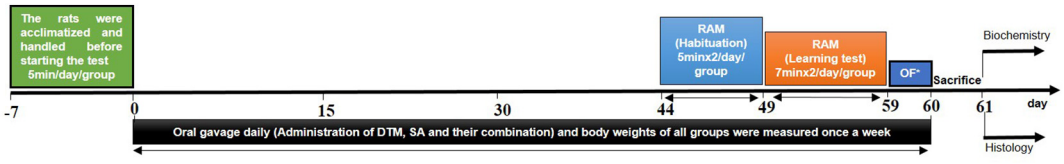

Fig. 1. Flow chart of the experimental protocol (day). RAM: radial arm maze test; OF: open field test; DTM: deltamethrin; SA: syringic acid. RAM test takes 15 consecutive days for each group. The first 5 days were the habituation phase and the next 10 days were the learning test. The first 5 consecutive days (2 trial/day) are made to prevent the maze stress. In learning test each trial was 7 min (10 consecutive day) according to the RAM protocol. Asterisks (*) signify 5 min/day/group.

previously described by Denenberg (1969) and Berry et al. (2008). OF maze was used for this analysis, 40 cm (length) × 80 cm (width) × 80 cm (height), was made from black high density and non-porous plastic. The field was divided into 16 equal 20 cm^2 squares using the Noldus Ethovision XT system. At the beginning of the experiment, the rats were placed in the central area of this field and their movements were recorded digitally for 5 min according to the (Denenberg, 1969). Above all, it is used not only to measure their motor functions but also to investigate anxiety, frequency, total distance and mean velocity (Berry et al., 2008).

2.4.2. Radial arm maze (RAM)

RAM was designed to assess the short/long term learning and memory in Wistar rats. We based our protocol on that previously described by Jakubowska-Dogru et al. (2003) and Noorafshan et al. (2013). In the RAM test, data on reference memory error (RME), working memory error (WME) and time spent to complete a trial were evaluated. RME was evaluated as the number of entries into an arm without feed; WME was evaluated as the number of re-entries into an arm after the food was already consumed. Rats were trained in the task until they performed at or above 80% accuracy for 3 out of 4 consecutive training days. Re-entry of the animal, which was expected at the correct arm where the target pots were located, to the arm with food that it had already taken was recorded as a WME and entry to an arm that was never baited was recorded as a RME (Jakubowska-Dogru et al., 2003; Noorafshan et al., 2013). After SA and DTM administration, rats were placed to home cages for 6 h to allow for absorption of the substance, then rats were moved to the behavior room for testing on the RAM.

2.4.2.1. Apparatus. We used an eight-arm radial maze (arm length 42 cm, arm width 12 cm, hub diameter 40 and height 40 cm, raised 50 cm above the floor) made of black Perspex (*Perspex*® Acrylic, Darwen, UK) with manually operating guillotine doors separating the central area from each of the eight arms.

2.4.2.2. Habituation. Rats were placed to the RAM apparatus on 5 consecutive days (x2 trial) and the intertrial interval was 30 min–1 h. At the beginning of each habituation trial, the rat was placed in the center of the maze and all eight doors were opened and the rat was allowed to explore the maze for a maximum of 5 min. All eight target pots were baited, rats were allowed to move freely and obtain the food rewards. The habituation phase was completed when either all eight targets had been retrieved or 5 min had elapsed.

2.4.2.3. Testing. As seen in Fig. 1, rats received 2 acquisition trials per day for 10 consecutive days during the learning test. In the learning phase, 4 randomly selected arms were baited. Six hours after dosing, each rat was placed in the central area of the RAM and the rats were allowed to enter the open arms and retrieve the foods in 7 min described by (Noorafshan et al., 2013). The learning test started with opening the doors, allowing the rat to enter the arms, and ended with the rat retrieving the last food or reaching 7 min. After each test, the labyrinth was cleaned with 70% ethanol to prevent the rats from acting based on their sense of smell. Behavioral tests were performed by a video and an observer, blinded to the rat's treatment. During the testing, food restriction was applied to trigger explorative seeking behavior in maze and body weight is measured once a week.

2.5. Animal sacrifice

After 2 months of treatment, the body weights of all rats were measured again and 200 mg/kg ketamine (Cas: 6740-88-1, ketamine hydrochloride; Pfizer, Lake Forest, IL, USA) with 10 mg/kg xylazine (Xylazine Bio 2%; Bioveta, Komenského, Czech Republic) were perfused by cardiac puncture under anesthesia. Perfusion was fixed with SF for biochemistry and with 10% formalin for histology. The rats were decapitated 24 h after the last day of exposure. Total brain tissues ($n = 64$) were removed and allocated for histopathological and immunohistochemical assays ($n = 24$) and hippocampal tissues were removed ($n = 40$) for biochemical analyses.

2.6. Biochemical analyses

2.6.1. Determination of total antioxidant activity

Total antioxidant activity in the hippocampus was evaluated using the Total Antioxidant Capacity (TAC) Assay kit (STA-360, OxiSelect™; Cell Biolabs Inc., San Diego, CA, USA). Hippocampus tissues were homogenized tissue on cold PBS and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was recovered and diluted with PBS for protein determination and TAC value. The TAC test is based on the reduction of Cu^{+2} into Cu^{+1} by antioxidants. Following reduction, Cu^{+1} reacts with a chromogenic reagent with maximum absorbance at 490 nm. The net absorbance values of the antioxidants were compared with a known uric acid standard curve. All results were corrected according to the total protein levels of the hippocampal tissue.

2.6.2. Determination of reactive oxygen/nitrogen species

ROS levels were measured with OxiSelect™ in the Vitro ROS/RNS Assay Kit (STA-347, OxiSelect™; Cell Biolabs Inc. San Diego, CA, USA). Hippocampus tissues at 10–50 mg/ml in PBS were homogenized and centrifuged at 10,000 × *g* for 5 min. DCF standards were prepared a 1:10 dilution series in the concentration range of 0–10 μM by diluting the 1 mM DCF stock in 1× PBS. The oxidants in the tissue provide to accelerate the oxidative reaction after adding a catalytic converter. Dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ), which is a specific ROS/RNS fluorogenic, with chemical properties similar to 2′.7′-dichlorodihydrofluorescein diacetate, was used in the test. The DCFH probe was prepared and the samples were measured fluorometrically against hydrogen peroxide and 7′-dichlorodihydrofluorescein (DCF) standard. Free radical content of the existing oxidant molecules was determined by comparison to a pre-determined DCF or hydrogen peroxide standard curve. Fluorescence intensities of the samples were normalized according to hippocampal tissue protein levels.

2.6.3. Determination of glutamic acid/glutamine levels

Standard for glutamine and glutamic acid were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO USA) Glutamine and Glutamic acid standard solution was prepared by weighing 0.01 g of each compound into a 10 ml glass tube. Then, 1 ml of 98–100% formic acid (Sigma-Aldrich, St. Louis, MO USA) and 9 ml LC-grade water were added. An optimized multiple reaction monitoring (MRM) method was developed using ultra-fast liquid chromatography (UFLC) coupled with tandem mass spectrometry (MS/MS). A UFLC system (LC-20 AD UFLC XR, Shimadzu Corporation, Japan) was coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). Chromatographic separations were carried out using an HPLC column (Inertsil ODS-4, 3 × 100 mm, 2 μm, GL Sciences Inc. Tokyo, Japan) maintained at 25 °C. Glutamine and Glutamic acid was detected using a gradient elution with a flow rate of 0.4 ml/min. Mobile phase solvent A was water with 0.1% formic acid and 1% acetonitrile and solvent B was acetonitrile with 0.1% formic acid. Gradient program was solvent B, 5–50% (0–1 min), 50–95% (1–2 min), 95% (2–3 min) and 5% (3–4 min). Injection volume was 5 μl. MRM transitions and responses were automatically optimized for Glutamine and Glutamic acid in positive electrospray ionization (ESI). In the positive ESI-MS mode the precursor and product *m/z* values were as follows: Glutamine precursor ion 146.9 and product ions 84.1/130.2; Glutamine precursor ion 147.9 and product ions 84.1/129.7. Retention time of Glutamine and Glutamic acid was 1.05 and 1.07 min, respectively. Response to Glutamine and Glutamic acid were optimized to a linear calibration range from 50 to 1000 ng/ml and a sample analysis time of 4 min. All

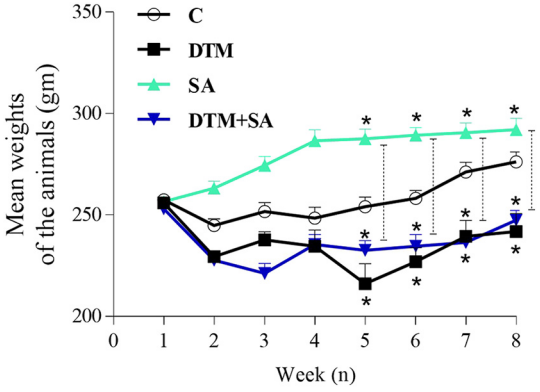

Fig. 2. BWG (gm/week). Mean weights of the animals. SA and control (C) groups revealed that a significantly increased weights of the animals at the end of 8th week and DTM showed a decline on total body weight. Asterisks (*) signify a statistically significant effect compared with control; *$p < 0.05$, according to significant difference (Tukey) post-hoc.

results were corrected according to the total protein levels of the hippocampal tissue.

2.6.4. Total protein assay

After homogenization of the hippocampus in cold distillated water (2 ml), tissue protein levels were measured in 50 µl of the homogenate according to the procedure of Lowry et al. (1961). The quantity of protein was calculated in milligrams in the 10% tissue homogenate after centrifugation at 10,000 ×*g*. All results were corrected according to the total protein levels of the tissues.

2.7. Histologic evaluations

2.7.1. Histopathological analysis

Animals were perfused with 10% formalin and after decapitation, the brain tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature, tissues were then treated with a graded ethanol series (70–100%) and chelated with xylol after dehydration. It was followed by routinely processed for paraffin embedding to obtain 5 µm sections. 5-µm thick serial sections were stained with haematoxylin and eosin and cresyl violet and examined under an optical microscope (Leica DM750 Microsystems, Bannockburn, IL, USA).

2.7.2. Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded 5-µm-thick serial sections were first deparaffinized in xylene and rehydrated in graded alcohol. The sections were subjected to antigen retrieval by boiling in a microwave for 7 min in sodium citrate buffer (0.01 M, pH 6.0), followed by a 20 min cool-down. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (7 ml; Cas: 108600, Merck, Kenilworth, USA) in methanol (63 ml) for 15 min. The sections were then incubated overnight (12 h) at 4 °C in a humified chamber with anti-cleaved-caspase-3 antibody (dilution 1:200, Biovision activated Caspase-3, Cell Signaling, California, USA). The tissue samples were marked on the object slide, washed in phosphate buffer solution (PBS) for 15 min and then left in room temperature for 60 min. Then the primary antibody was applied and washed with PBS and incubated for 1 h at room temperature with a secondary biotinylated antibody (Cas: BA-1000, Vector, Burlingame, USA) at a 1:400 dilution. And then washed with PBS for 15 min before incubating with the streptavidin peroxidase (Cas: TS-125-HR, Thermo Scientific™ Lab Vision™, Waltham, USA) for 30 min and 3,3′-Diaminobenzidine tablets (DAB) (D4168-50SET, Sigma Aldrich, St. Louis, USA). After the sections were washed with PBS, the slides were developed with DAB and counterstained with Haematoxylin and Eosin.

2.7.2.1. Determination of apoptotic indices. The number of apoptotic neurons in a section expressed as a fraction of the total number of cells, apoptotic index, measures an apoptotic state. An activated caspase-3 labeling apoptotic index was calculated in CA1/3 pyramidal neurons and the number of apoptotic neurons in immunostained sections was counted (cells/mm^2) under an optical microscope (Leica DM750 Microsystems, Bannockburn, IL, USA). In each subfield, the number (cells/mm^2) and percentage (%) of immunopositive stained cells was calculated. For all the methods, the slides were examined by two independent and blinded observers comparing the results to evaluate the interobserver reproducibility. In cases of discordance among the investigators, the slides were reviewed and discussed until a unanimous diagnosis was obtained.

2.8. Statistical analysis

The data were analyzed with SPSS 21.0 software (IBM Corp., Armonk, NY, USA) and was expressed as the mean ± SEM (standard error of the mean) in all cases. Data were analyzed by mix ANOVA and Tukey's Cramer test was used for multiple comparisons. Results are presented as the mean ± standard error. A p-value < 0.05 was considered significant.

3. Results

3.1. Effect of treatments on bodyweight change

As seen in Fig. 2, there was a progressive gain in body weight after SA administration when compared with Control, DTM + SA and DTM from weeks 1 to 8 of the study. The weight changes were detected as 18.76 ± 8.52 g, 35.38 ± 1.8 g, −5.71 ± 9.8 g and −8.06 ± 5.7 g in C, SA, DTM + SA and DTM group, respectively. Bodyweight change significantly decreased in the DTM group and DTM + SA group compared with the SA group between the 5th–8th week of the study ($p < 0.05$). No deaths were observed after exposure to DTM.

3.2. Learning and memory test

3.2.1. Open field

3.2.1.1. Total distance. As seen in Fig. 3A, SA caused significant increase in total distance (3066.11 ± 150.63 cm, $p < 0.001$) compared with the DTM (1869.66 ± 168.54 cm, $p < 0.001$) and DTM + SA (1965.94 ± 97.43 cm) groups, however DTM decreased the total distance compared with the other groups ($p < 0.01$). After treatment, a significant increase was observed in the SA group (3066.11 ± 150.63 cm, $p < 0.001$) and this effect was significantly higher when compared with the C group (2621.03 ± 128.43 cm).

3.2.1.2. Mean velocity. DTM-induced rats caused a significant decline (400.06 ± 35.74 cm/s, $p < 0.001$) in mean velocity compared with C (538.6 ± 22.75 cm/s, $p < 0.001$). As seen in Fig. 3B, a significant increase was more frequent in the SA group (618.46 ± 20.42 cm, $p < 0.001$) compared with the DTM group. DTM + SA (491.76 ± 25.09 cm/s, $p < 0.05$) also increased in altering the mean velocity as for DTM. Mean velocity (cm/s) results from the SA was also detected to be positively correlated with the total distance of the SA; highest increase in SA treated group was determined as (618.46 ± 20.42 cm, $p < 0.001$) compared with C (538.6 ± 22.75 cm/s, $p < 0.05$).

3.2.1.3. Number of squares entered. According to the frequency data, a significant reduction was detected in DTM (10.42 ± 1.17 n, $p < 0.01$) compared with C group (15.72 ± 1.14 n, $p < 0.01$). And the number of squares entered in DTM + SA group as follows 11.07 ± 0.75. As seen in Fig. 3C, SA treated rats (23.45 ± 1.83 n, $p < 0.001$) caused a significant increase for the number of squares compared with the DTM

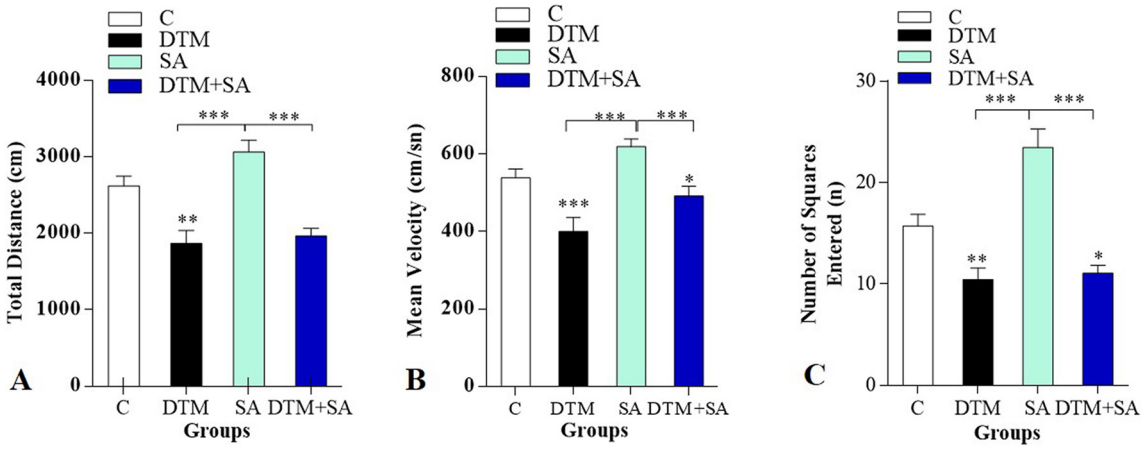

Fig. 3. Effects of SA and DTM on Open Field (OF). The total distance (cm), mean velocity (cm/sn) and number of squares entered (n) were evaluated as parameters of locomotor activity. A. DTM significantly decreased the total distance (cm) ($p < 0.01$). SA (3066.11 ± 15 cm) significantly increased total distance (cm) compared with the DTM (1869.66 ± 16 cm) and DTM + SA (1965.94 ± 9 cm) ($p < 0.001$). B. DTM significantly reduced mean velocity (cm/sn) ($p < 0.001$). SA (618.46 ± 20.42 cm/sn, $p < 0.001$) and DTM + SA (491.76 ± 25.01 cm/sn, $p < 0.05$) significantly increased mean velocity (cm/sn) compared with DTM (400.06 ± 35.74 cm/sn). C. A significant reduction was detected in DTM. SA (23.45 ± 1.8 n) significantly increased number of squares entered (n) as a parameter of locomotor activity compared with the DTM (10.42 ± 1.1 n) ($p < 0.001$). Asterisks (*) signify a statistically significant effect; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to Tukey post-hoc for multiple comparisons.

(10.42 ± 1.17 n, $p < 0.001$).

3.2.2. Radial arm maze (RAM)

3.2.2.1. Time spent to complete a trial. As seen in Fig. 4A, a significant decline in the time spent to complete a trial in Control (C) (2.19 ± 0.31 min, $p < 0.05$), SA (1.19 ± 0.16 min, $p < 0.05$) and DTM + SA (1.66 ± 0.24 min, $p < 0.05$) groups compared with the DTM group (6.02 ± 0.26 min, $p < 0.05$) on days 1–10. The DTM + SA (1.26 ± 0.19 min, $p = 0.009$, $p < 0.05$) group significantly difference than the DTM (6.22 ± 0.22 min, $p < 0.05$)

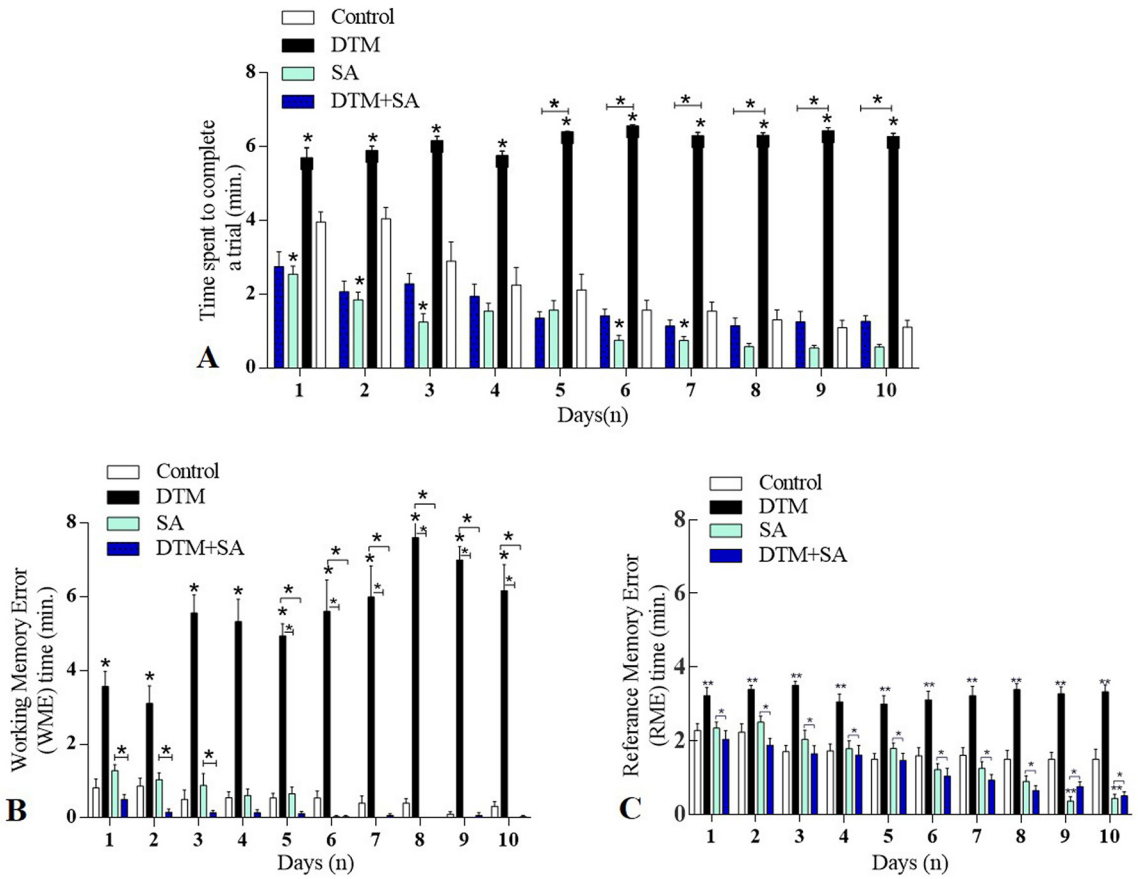

Fig. 4. RAM results. Values are shown as the mean and standard deviation. A. Time spent to complete a trial (min/day). DTM revealed a significant increase in time spent to complete a trial (min/day) compared with the C, SA and DTM + SA groups ($p = 0.009$, $p < 0.05$). B. WME (min/day) results indicated that DTM had a significant decline in working memory errors compared with the SA and DTM + SA groups ($p < 0.05$). C. DTM revealed a significant increase in the reference memory errors, RME compared with the SA ($p < 0.01$). The results reveal that reference memory, which was corrupted by DTM, was improved by SA.

group especially after day 5 and time spent significantly decreased over trials for SA treated group compared with C on days 1, 2, 3, 6 and 7 respectively (SA, (1st day) 2.55 ± 0.22 min; (2nd day) 1.85 ± 0.2 min; (3rd day) 1.25 ± 0.23 min; (6th day) 0.75 ± 0.13 min, (7th day) 0.74 ± 0.11 min $p < 0.05$; C, (1st day) 3.96 ± 0.27 min; (2nd day) 4.05 ± 0.3 min; (3rd day) 2.9 ± 0.52 min; (6th day) 1.57 ± 0.26 min, (7th day) 1.54 ± 0.24 min $p < 0.05$).

3.2.2.2. Working memory error (WME). The working memory errors significantly increase on 1th–10th day in DTM (5.49 ± 0.56 min/day, $p < 0.05$) *group* compared with the C (0.5 ± 0.16 min/day, $p < 0.05$), SA (0.45 ± 0.1 min/day, $p < 0.05$) and DTM + SA group (0.12 ± 0.06 min/day, $p < 0.05$) (Fig. 3B). A significantly main effect on days 1–3 was demonstrated for WME, $F_{27, 600} = 110.3$, $p < 0.05$, between the SA and the DTM + SA group, indicating that the percentage of memory errors increased by DTM. As seen in Fig. 4B, after day 5 a significant decreases were observed in WME, especially in the SA (0.06 ± 0.02 min/day, $p < 0.05$) and DTM + SA (0.08 ± 0.04 min/day, $p < 0.05$) groups compared with the DTM (6.11 ± 0.5 min/day, $p < 0.05$). Therefore, DTM + SA treated rats decreased working memory errors more rapidly than the DTM group.

3.2.2.3. Reference memory error (RME). As seen in Fig. 4C the RME results indicated that C (1.71 ± 0.2 min/day, $p < 0.01$), SA (1.46 ± 0.16 min/day, $p < 0.01$) and DTM + SA (1.24 ± 0.18 min/day, $p < 0.01$) groups were a significant decrease on 1th–10th days for utilizing reference memories in the retrieval of food compared with the DTM group (3.25 ± 0.19 min/day, $p < 0.01$). A significant main effect of SA treatment was detected for reference memory type, $F_{27, 600} = 56.52$, $p < 0.001$, therefore the percentage of correct arm entries was significantly different from the control particularly on days 9 (SA, 0.35 ± 0.12 min/day, $p < 0.01$; C, 1.5 ± 0.18 min/day, $p < 0.01$) and 10 (SA, 0.42 ± 0.11 min/day, $p < 0.01$; C, 1.5 ± 0.26 min/day, $p < 0.01$). Compared with DTM, SA showed a significant effect on reference memory on 1–10 day test (see Fig. 4C) ($p < 0.05$). In contrast to the effects of SA, DTM impaired learning.

3.3. Biochemical analyses

3.3.1. Total antioxidant activity (nM UAE/mg·ml^{-1})

Total antioxidant activity, the quantitative indicator for the total antioxidant capacity against free radical overproduction, was significantly reduced after DTM compared with the control group. The SA (61.33 ± 15.07 UAE/mg, $p < 0.01$) and DTM + SA (61.78 ± 9.78 UAE/mg, $p < 0.01$) groups were significantly higher antioxidant activity than the DTM (33.14 ± 4.09 UAE/mg, $p < 0.01$). As seen in Fig. 5A, DTM significantly decreased total antioxidant activity compared with the control group ($p < 0.01$). The antioxidant levels of the DTM + SA and SA were increased while TAC levels decreased under DTM toxicity compared with the DTM (33.14 ± 4.09 UAE/mg, $p < 0.01$). Additionally, SA treatment was demonstrated the neuroprotective effect by reducing DTM-induced oxidative stress and rising antioxidative effect, compared with the control (60.93 ± 10.95 UAE/mg, $p < 0.01$).

3.3.2. ROS/RNS activities (nM/mg·ml^{-1})

The oral administration of DTM leads to a significant increase in the reactive oxygen/nitrogen levels of the hippocampus compared with the control group ($p < 0.05$). Analysis of ROS depicts the significantly reduced ROS/RNS levels upon SA treatment (117.97 ± 4.02 nM/mg·ml^{-1}, $p < 0.01$) and DTM + SA (212.23 ± 6.07, nM/mg·ml^{-1}, $p < 0.01$) groups. As seen in Fig. 5B, DTM stimulated neurotoxicity recorded significantly increased levels of ROS (288.63 ± 19.13, $p < 0.05$) compared with the control (169.15 ± 19.31, $p < 0.05$) group.

3.3.3. Glutamine and glutamic acid level (μg/ml)

As seen in Fig. 5C, glutamine levels (μg/ml) in the DTM (9.92 ± 0.56 μg/ml, $p < 0.05$) groups differed significantly from those in the SA group (7.52 ± 1.34 μg/ml, $p < 0.05$). SA significantly reduced glutamine levels compared with control (9.68 ± 1.79 μg/ml), whereas DTM + SA (8.77 ± 0.77, μg/ml) had no effect. These results indicated that DTM impaired short and long term memory through glutamine pathways. As seen in Fig. 5D, glutamic acid levels (μg/ml) tended to be higher in the DTM (15.06 ± 0.99 μg/ml, $p > 0.05$) group than in the C, SA, DTM + SA (13.68 ± 1.8 μg/ml; 13.89 ± 1.66 μg/ml; 13.02 ± 1.23 μg/ml, $p > 0.05$) groups. DTM group showed no significant effects ($p > 0.05$).

3.4. Histological analysis

3.4.1. Active caspase-3 immunohistochemistry of the CA1/CA3

The total number of cells stained with active caspase-3 was proportioned to the total cell count at the site, and the percentage of apoptotic neurons was determined (Fig. 6A–D). As seen in Fig. 6E significantly higher percentage of apoptotic neurons was stained for active caspase-3 in the DTM (36.52 ± 2.38, $p < 0.01$) compared with the control (12.82 ± 0.35, $p < 0.001$), SA (10.96 ± 0.31, $p < 0.001$) and DTM + SA (15.38 ± 0.67, $p < 0.05$) groups in the CA1 pyramidal neurons; there was a significant difference was also between the DTM and the DTM + SA groups ($p < 0.05$). As seen in Fig. 6F, the percentage of apoptotic neurons was significantly higher in DTM (40.81 ± 3.2, $p < 0.01$) compared with the control (13.05 ± 0.41, $p < 0.01$), SA (11.77 ± 0.50, $p < 0.001$) and DTM + SA groups (14.35 ± 0.60, $p < 0.001$); this difference was also between the SA and DTM + SA groups ($p < 0.01$) in CA3 pyramidal neurons.

3.4.2. Histopathological analysis

3.4.2.1. Haematoxylin and eosin staining. As seen in Fig. 7A, histopathological analyses of the hippocampus tissue of the control group showed a normal histological view except for a few numbers of apoptotic neurons. Fig. 7B indicated that the most neuronal damage was detected in the CA1/3 pyramidal neurons of the DTM group. DTM showed obvious apoptotic and neurodegenerative characteristics in the form of central chromatolysis, neuronal swelling, disorganization and lysis of cell membranes. The CA1/3 pyramidal neurons characterized by pyknotic or lysed nuclei in the DTM group. As seen in Fig. 7C–D, the pyramidal neurons of the hippocampus in the DTM + SA group and SA treated group revealed small numbers of apoptotic, degenerated, necrosed neurons and gliosis.

3.4.2.2. Cresyl violet staining. As seen in Fig. 8A, histopathological analyses of CA1/3 pyramidal neurons of the control group showed normal histological appearance except for a few numbers of apoptotic neurons. Fig. 8B the DTM group exhibited damaged neurons with condensed chromatin and shrinkage of Nissl bodies and basophils. Fig. 8C showing normal pyramidal neurons and less apoptotic neurons in the SA group. CA1/3 pyramidal neurons were stained strongly with cresyl violet following Nissl shrinkage of some neural cells in the DTM, whereas the pyramidal neurons of DTM + SA group showed small neuromorphological changes compared with DTM group (Fig. 8D).

4. Discussion

The present study indicated that DTM significantly increased immobility time and time spent in the central zone in the RAM, but reduced locomotor activity in the OF and time spent in closed arms, without inducing significant effects on total distance, mean velocity, frequency, spatial and working memory. DTM-induced male Wistar rats have shown significantly decreased locomotor activity and this effect may be due to alteration in the antioxidant and oxidant balance system (Husain et al., 1994). The reason for the change in spontaneous

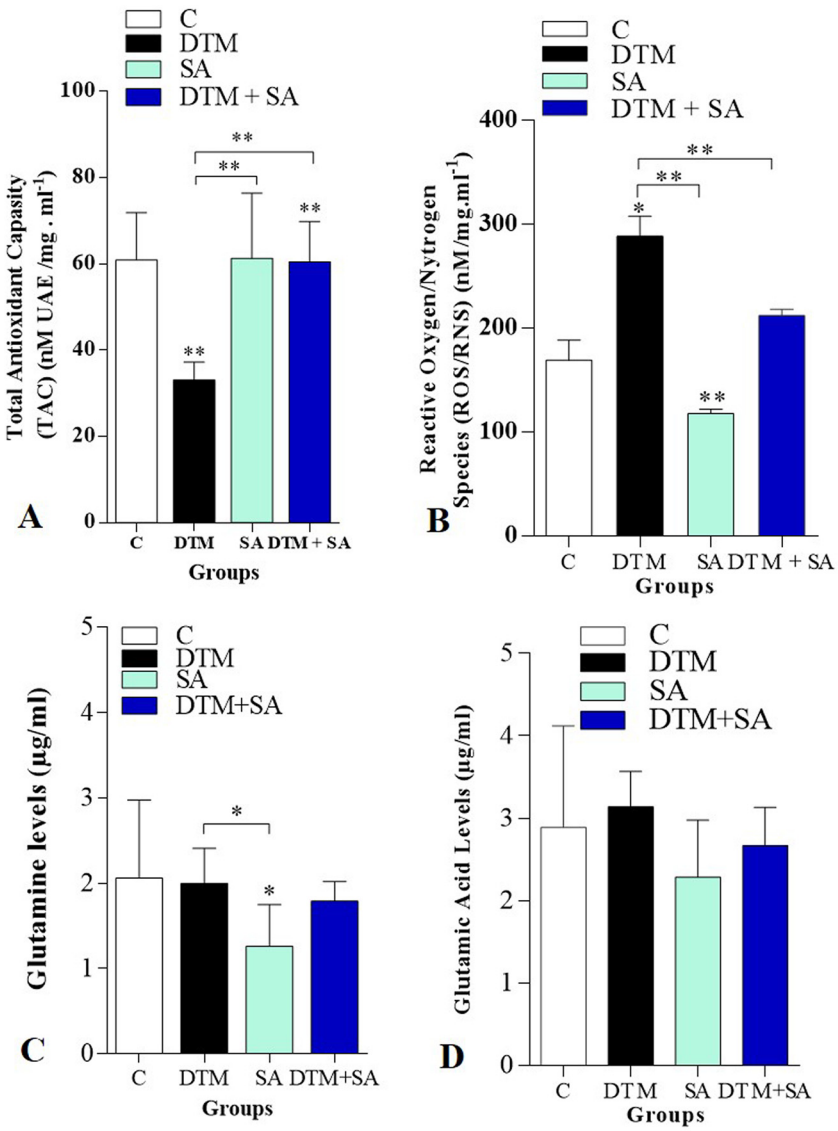

Fig. 5. Results of TAC (nM UAE/mg/ml protein), RO/NS (nM/mg/ml protein), glutamine and glutamic acid (µg/ml) levels. A. SA significantly increased the total antioxidant activity compared with the DTM and DTM + SA groups ($p < 0.01$). DTM significantly decreased the antioxidant activity compared with the control ($p < 0.01$). As seen in Fig. 4A, DTM + SA and SA groups indicated higher antioxidant levels ($p < 0.01$). B. DTM group ($p < 0.05$) revealed that reactive oxygen and nitrogen species were significantly increased compared with DTM + SA ($p < 0.01$). SA treatment was showed significantly lower RO/NS activities than the DTM group ($p < 0.01$). C. DTM had significantly increased glutamine levels (µg/mL) than the SA group because of the impaired levels of glutamine ($p < 0.05$). D. The glutamic acid levels were not statistically significant between the groups ($p > 0.05$).

locomotor activity or increased time of inactivity is the accumulation of DTM and its metabolites in the hippocampus after increased administration time or may be the result of increased anxiety (Redei et al., 2001). Thus, SA demonstrated reinforcing learning and memory, antioxidant activity, neuroprotectivity, affecting multiple aspects of locomotion, with stimulating total distance, mean velocity, and frequency. The administration time and concentration of SA facilitating, antioxidative, antiapoptotic and neuroprotective effects were accompanied by cognitive effects on neuronal activity in the CA1/3 pyramidal neurons. The increased locomotor activity of SA induced treatment may be explained by the fact that its antioxidative and neuroprotective effects for decreasing anxiety. After administrated orally by 1.28 mg/kg/day of DTM for two months, increased neurodegeneration results in the hippocampus are caused a physiological and behavioral disturbance in DTM induced rats and produced a varying degrees of neurotoxicological and neurobehavioral symptoms. A clear behavioral disturbance was more frequent in DTM induced rats on locomotor activity, exploratory, learning and memory test with significant behavioral changes such as an increased stress, short-term memory loss and impairs learning as a result of the continuous increment in hippocampal ROS (Gasmi et al., 2017; Husain et al., 1996; Husain et al., 1994). Loss of learning and memory cause an increased risk of disorders with the elevation of inflammatory activity may be an important mediator of DTM exposure (Gasmi et al., 2017). Learning and memory are greatly affected by chronic stress and it can be more sensitive to memory deficits compared with non-stressful situations. The hippocampus seems involved in several types of learning and memory processes (Redei et al., 2001). The results of the RAM test verified that the DTM caused a defect in the reference memory, raised latency and decreased the number of entries and time spent in the target quadrant (Kemble et al., 1997). On the other hand, SA treatment improves learning dysfunction and memory retention while reducing oxidative stress and apoptosis (Turgut et al., 2015). Learning, spatial and working memory, which were impaired by DTM, was improved by SA after 10-day-test, especially after day 5 ($p < 0.05$) as an antioxidant agent.

The neuronal damage mechanism of DTM-induced toxicity can result from the triggering impacts caused by impaired voltage gates of sodium channel gating mechanism, glutamine excitotoxicity, chronic oxidative stress and increased levels of apoptosis (Hossain et al., 2015; Hossain et al., 2008; Huang et al., 2010; Silver et al., 2014). And a significant increase was recorded in the rate of apoptosis in the DTM-induced CA1/3 pyramidal neurons; this status is the result of free radicals induced neurodegeneration or disruption of neurotransmitter transport mechanism (Wu and Liu, 2000b). The reason for neuronal apoptosis is the toxicity of the neurodegenerative mechanism, which is pivotal to evade the compromise followed by invading of reactive

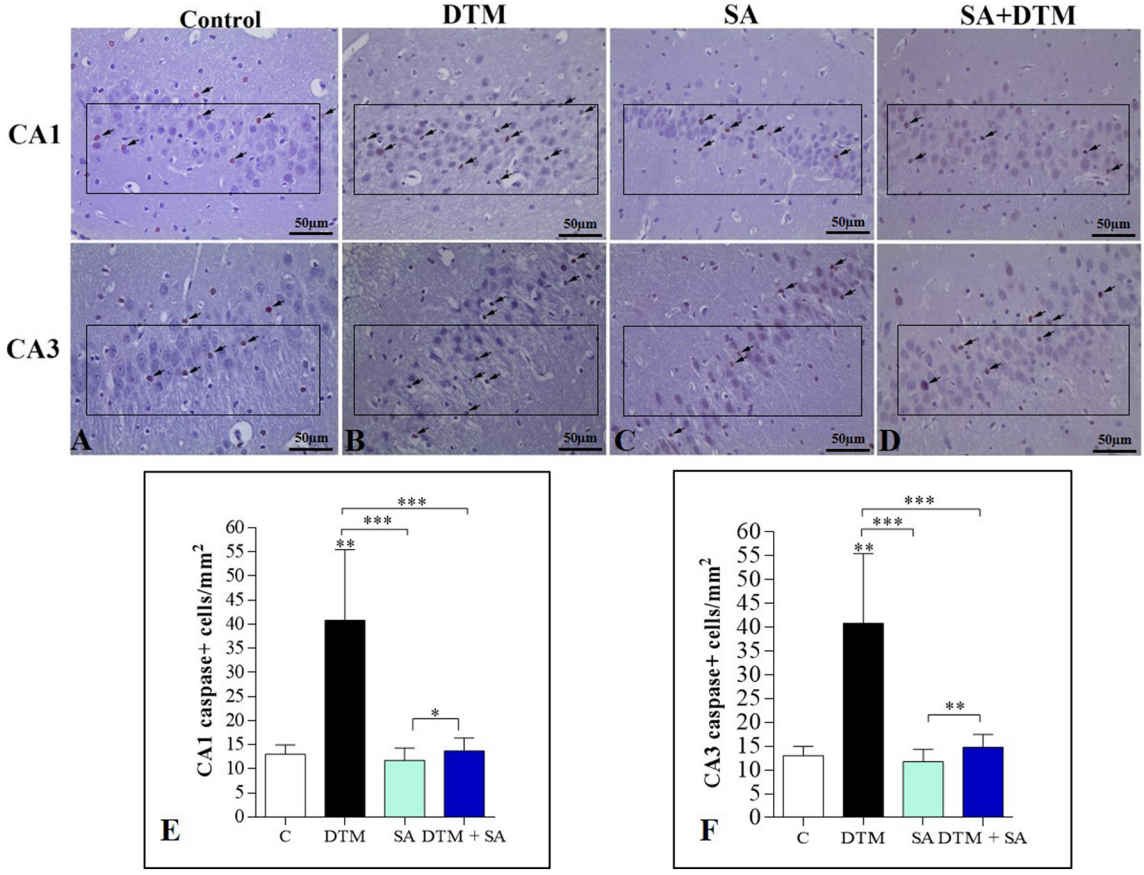

Fig. 6. Representative active caspase 3 immunohistochemistry in the CA1/3 pyramidal neurons of different groups. A. Control group showing a few numbers of cas-3 immunostaining reaction and apoptotic cells are indicated by arrows in the CA1/3 pyramidal neurons. B. DTM group showing strong nuclear immunopositive reaction in numerous cells (arrows). C. SA group showing slightly immunostaining reaction (arrows). D. SA + DTM group showing reduction in the number of cas-3 immunostained cells (arrows). E–F. Number of CA1/3 caspase + cells/mm^2. E. DTM had a significantly higher percentage of apoptotic cells than the control ($p < 0.01$), SA ($p < 0.001$) and DTM + SA ($p < 0.001$) groups in CA1 pyramidal neurons. This significance was also observed between the SA and SA + DTM groups ($p < 0.05$) in CA1. F. DTM revealed that significantly increased percentage of apoptotic cells compared with the control ($p < 0.01$), SA ($p < 0.001$) and DTM + SA ($p < 0.001$); this difference was also observed between the SA and DTM + SA groups ($p < 0.01$) in CA3. Asterisks (*) signify a statistically significant effect; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to Tukey post-hoc for multiple comparisons.

oxygen/nitrogen species. DTM induced neurodegeneration in the hippocampus detected by IHC-staining which occurred in CA1/3-pyramidal neurons (Wu and Liu, 2000a).Our results demonstrate cas 3-involved neurodegeneration, reactive gliosis, and neurogenesis in DTM-induced excitotoxicity. After DTM administration is liable for increased ROS activation and promotes necrosis and apoptosis by activating caspases. DTM influences the pyramidal neuron density of the CA1/3 by incited of morphological changes, vacuolization, necrosis, glial degeneration, disorganization (Sharma et al., 2015; Tayebati et al., 2009) and a reduction in weight/thickness of hippocampus (Husain et al., 1994). Additionally, there was a significant increase in degenerative neurons such as; nuclear pyknosis, degeneration of eosinophilic cytoplasm, neuronal Nissl shrinkage and increased oxidative stress in the hippocampus (Wu and Liu, 2000a). This increasing is the result of inhibition of the antioxidant system and accumulation of the metabolites of DTM in the hippocampus; this accumulation triggers the rate of cell apoptosis. And as a result of the instability between the raising generation of reactive oxygen/nitrogen radicals and the decreasing antioxidative activity.

Kim and Tokmak et al., revealed that SA significantly reduced oxidative damage, apoptosis, and neurodegeneration on CA1/3 pyramidal neurons (Kim et al., 2013; Tokmak et al., 2015). In an in vitro study on reperfusion following the withdrawal of oxygen-glucose in hippocampal neural cells, SA considerably decreased apoptosis in the hippocampal neurons (Cao et al., 2016). In the present study, our results similarly indicated that SA significantly decreased Cas-3 immune-positive neurons and SA is effective against apoptosis rate either by increasing the total antioxidant activity in the hippocampus or by inhibiting free radicals. The neuroprotective activity of SA in biological systems is leading to the reduced rate of oxidative damage, glial activation, apoptosis, ROS/RNS and neurodegeneration in CA1/3 pyramidal neurons. SA treatment against DTM effectively induced antioxidant enzymes in the hippocampus at 25 mg/kg/day and give rise to neural tissues less susceptible to oxidative damage.

There was a statistically significant decrease in the body weight in DTM induced rats which may have resulted due to the time of administration, excessive salivation, loss of appetite or diarrhea. This derogate is in consistence with the results of Kavlock et al., they stated a 20% decrement in the total body weight in rats after DTM exposure (Kavlock et al., 1996). Several authors also noticed a similar decline in total body weight in Wistar rats respectively after oral administration of DTM (Elbetieha et al., 2001; Madsen et al., 1996). Similarly, in the present study, total body weight was significantly decreased by DTM in Wistar rats at week 1st to 8th of exposure ($p < 0.05$). The maximum total body weight gain was found in the SA group and these findings demonstrate that SA had a positive impact on DTM by increasing food consumption.

After the SA treatment at the dose of 25 mg/kg/day, there is an improvement and neutralization of neurotoxicity caused by 1.28 mg/kg/day of DTM. This polyphenol has many useful and sanative

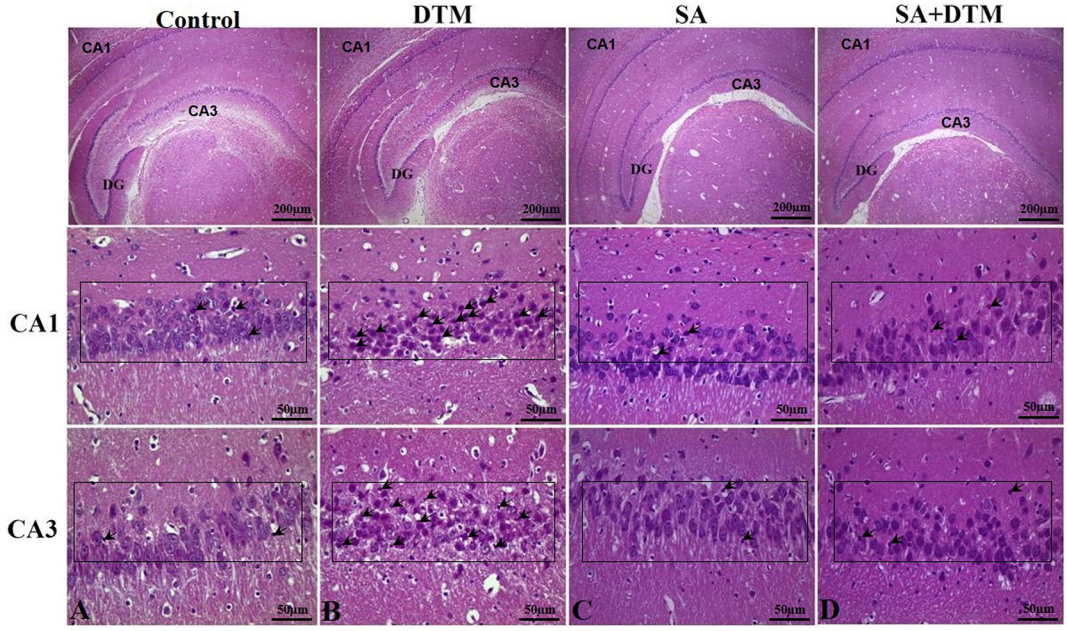

Fig. 7. Histopathological changes in the CA1/3 of the different groups (haematoxylin and eosin). Haematoxylin and eosin staining indicated that the DTM exhibited increased neuronal damage for both the CA1/3. A. Hippocampus of control (C) group showing normal histological view with less apoptotic neurons (arrows). B. CA1/3 of DTM treated group showing apoptosis, degeneration and necrosis of neurons (arrows) with vacuolization, glial degeneration, disorganization, nuclear pyknosis, degeneration of eosinophilic cytoplasm and neural Nissl shrinkage. C. SA group showing normal pyramidal neurons and less apoptotic cells (arrows). D. SA + DTM group showing few numbers of degenerated and apoptotic neurons (arrows).

characters, forestall oxidative damage, encourages the synthesis of dopamine and enhances the antioxidant system in the hippocampus for its ability to neutralize free radicals and oxidative stress. The histopathological sections of the hippocampus ratify these findings. In conclusion, our study has indicated that the sub-chronic DTM exposure can damage the CA1/3 pyramidal neurons by disrupting glutamine levels or balance mechanism between antioxidant and oxidant status. This will cause many neurodegenerative diseases but the oral supplementation of SA neutralized the neurodegeneration, improved the learning and memory and inactivated the cas-3 stimulated by DTM. It was concluded that SA had positive effects on learning and strengthened the learning and memory process via different pathways. The major findings are that DTM immunoreactivity is increased in the nucleus of hippocampal pyramidal neurons in the DTM induced rat hippocampus, and the levels

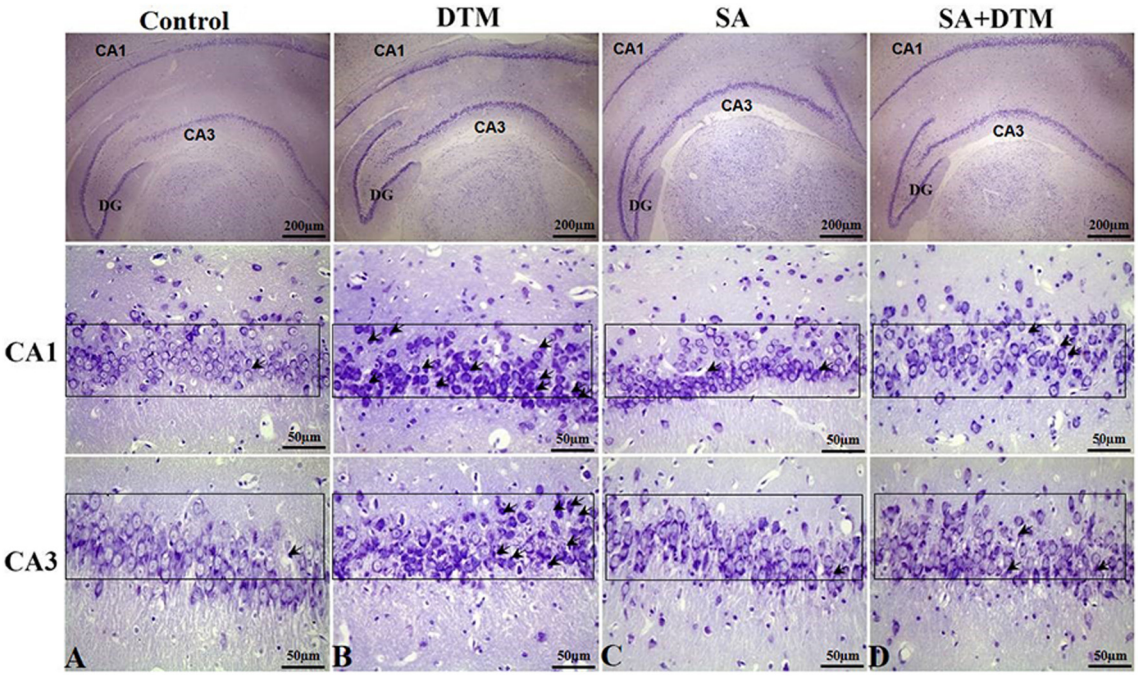

Fig. 8. Histopathological changes in the CA1/3 of the different groups (cresyl violet). A. Control (C) group showing normal pyramidal neurons and individual necrosis (arrow). B. DTM group indicating apoptosis, neurodegeneration, necrosis of neurons (arrows) with gliosis with a condensed chromatins, Nissl shrinkage and intensive basophils. C. SA group showing of individual pyramidal neuron necrosis (arrows). D. SA + DTM group showing few numbers of degenerated and necrosed neurons (arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of glutamine are altered by following the progression of excitotoxicity. It would help identify the precise molecular mechanism involved in DTM induced cell death in pyramidal neurons. Our results may provide a novel concept for developing new therapeutic strategies against sub-chronic DTM-induced toxicity. To identify, however, the precise molecular mechanism involved in the SA-mediated neuronal tract in neurotoxicity, investigations will be required.

5. Conclusion

The data stated that oral SA administration caused considerable neuroprotective effects against sub-chronic DTM intoxication and alleviated its severity both on biochemical and histopathological parameters. Antioxidant SA treatment may inhibit the ROS/RNS-induced oxidative damage, apoptosis of CA1/3 pyramidal neurons, neurodegeneration and degenerated cellular disorganization. No studies have investigated the neuroprotective role of SA treatment on short and long-term memory or its neuroprotective effect against sub-chronic DTM neurotoxicity. The parameters in this study provide a useful basis for further development of SA as an alternative therapy.

Authors' contributions

Fatoş Belgin Yildirim, Narin Derin, Mutay Aslan, and Eren Ogut participated in the study design, Eren Ogut, Rahime Sekerci, Guven Akcay performed the drug administrations, learning and memory test, surgical operations. Mutay Aslan evaluated the biochemical parameters and helped to draft the manuscript. Narin Derin evaluated the learning and memory parameters and drafted the manuscript. Eren Ogut and Leyla Sati performed the histopathological and immunohistochemical analysis. All authors read and approved the final manuscript.

Transparency document

The Transparency document associated with this article can be found in online version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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