# Antia, a natural anti-oxidant product, attenuates cognitive dysfunction in streptozotocin-induced mouse model of sporadic Alzheimer's disease targeting the amyloidogenic, inflammatory, autophagy and oxidative stress pathways

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Key words: Alzheimer's, autophagy, oxidative stress, Antia, amyloid beta

# Abstract

**Background:** Many neurodegenerative diseases such as Alzheimer's disease are associated with oxidative stress. Therefore, antioxidant therapy has been suggested for the prevention and treatment of neurodegenerative diseases. In this study, we investigate the ability of the anti-oxidant Antia to exert a protective effect against sporadic Alzheimer's disease induced in mice. Antia is a natural product that is extracted from edible yamabushitake mushroom, the gotsukora plant and diosgenin after treatment with MRN-100 (an iron-based fluid).

**Methods:** Single intracerebroventricular (ICV) injection of streptozotocin (STZ) (3mg/kg) was used for induction of sporadic Alzheimer's disease in mice. Antia was injected intraperitoneally (IP) in 3 doses (25, 50 and 100 mg/kg/day) for 21 days. Neurobehavioural tests were carried out within 24h after the last day of injection. Afterwards, mice were sacrificed by cervical dislocation and decapitation. The hippocampi were rapidly excised, weighted and then homogenized to be used for estimation of biochemical parameters.

**Results:** Treatment with Antia significantly improved mice performance on the Morris water maze. In addition, biochemical analysis showed that Antia exerts a protective effect for several compounds, including GSH, MDA, NF-kB, IL-6, TNF-a, and Amyloid-beta. Further studies with Western blot showed the protective effect of Antia for the JAK2/STAT3 pathway.

**Conclusion:** Antia exerts a significant protection against cognitive dysfunction induced by ICV-STZ injection. This effect is achieved through targeting the amyloidogenic, inflammatory and oxidative stress pathways. The JAK2/STAT3 pathway played a protective role for neuroinflammatory and neurodegenerative diseases such as SAD.



**Acknowledgement:** Antia was provided by ACM Co., Ltd, Japan.

*Keywords:* Alzheimer's disease, Antia, JAK2/STAT3, …

## 1) Background

Age-related neurological disorders such as Alzheimer's disease (AD) are on the rise. AD is a neurodegenerative disorder characterized by a progressive decline of memory and cognition, and it is the most common cause of dementia, accounting for 60-80% of all cases (Patterson:2018). The most common type of AD in the elderly, sporadic Alzheimer's disease (SAD), is associated with progressive neurodegeneration of the central nervous system (Blennow et al. 2006). Several pathways have been examined as possible targets for SAD, including the oxidative stress, amyloidogenic, inflammatory, and autophagy pathways.

The appearance of oxidative stress markers is one of the earliest changes in AD brains, preceding the accumulation of visible amyloid deposits and neurofibrillary tangles (Nunomura et al., 2000). Oxidative stress is implicated in many disorders like chronic inflammation, AD, and Parkinson's disease (Polidori, 2004). Neurons in the brain are at extremely high risk of excessive generation of reactive oxygen species (ROS) and oxidative damage since they show high oxygen consumption and energy production (Bélanger et al 2011).

In AD brains, normally solid amyloid-β (Aβ) and tau proteins assemble into amyloid-like filaments called plaques and tangles. It is currently unresolved how Aβ accumulates in the central nervous system and initiates cell disease, but a suggested mechanism by which Aβ may damage and cause neuronal death includes ROS generation during Aβ self-aggregation. When this occurs on the membrane of neurons in vitro, it ultimately leads to depolarization of the synaptic membrane, excessive calcium influx, and mitochondrial impairment (Mattson 2004, Flagmeier et al 2017).

Neurodegenerative diseases such as AD are also accompanied by neuroinflammation. The transcription factor NF–κB has been found to play a crucial role in the inflammatory response of neurons. Under normal physiological conditions, NF-κB forms a cytoplasmic complex with its inhibitor IκBα as an inactive form, but when stimulated, NF-κB can induce the transcription of inflammatory target genes such as cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). In addition, neuroinflammation has been linked with autophagy in neurodegenerative diseases. Pathological disruption of autophagy can cause an initiation or exacerbation of neuroinflammation and, conversely, neuroinflammation can induce an autophagic deficit that exacerbates neurodegeneration (Zheng et al., 2013). In human AD, as well as in mouse models of AD, autophagy has been found to be decreased and to contribute to

the pathological accumulation of tau aggregates (Zare-Shahabadi et al., 2015). Autophagy is known to be regulated by mTOR, the mammalian target of rapamycin, and mTOR inhibition has been shown to prevent neuroinflammation in a mouse model of cerebral palsy (Srivastava et al., 2016). Moreover, it has been demonstrated that GSK-3β inhibition suppressed neuroinflammation in the cortices of rats subjected to ischemic brain injury by activating autophagy (Zhou et al., 2011).

Pharmacological management of AD has been limited to date. Long-term usage of non-steroidal anti-inflammatory drugs (NSAIDs) were thought in 2007 to be associated with a reduced likelihood of developing AD (Szekely et al 2007). Evidence also suggested the notion that NSAIDs could reduce inflammation related to amyloid plaques, but trials were suspended due to high adverse events (Hsu and Marshall, 2017). There are no medications or supplements that have been shown to decrease risk of AD (Hsu D, Marshall GA, 2017), and unfortunately, current FDA-approved AD treatments only offer symptomatic relief and are unable to delay or cure the disease (Patterson:2018).

Recently, antioxidants have received increased attention in preventing the onset of AD by reducing oxidative stress insult (Markesbery 2018, Gugliandolo:2017). Furthermore, the use and search for drugs and dietary supplements from plants have accelerated in recent years, due in part to the health benefits that have been found in phytochemicals whose uses have been documented in traditional medicine (Choi et al., 2005). For example, components of the traditional Chinese medicinal mushroom called yamabushitake promote nerve growth factor synthesis in cultured astrocytes (Kawagishi et al., 1990; Kawagishi et al., 1994) as well as improving mild cognitive impairment in humans (Mori et al., 2009). The gotsukora plant has traditionally been used for dementia and memory improvement (Nadkarni 1954; Kirtikar, 1987), and its extracts have been shown to improve memory retention in rodents (Gupta et al., 2003), alter amyloid beta pathology in the hippocampus of a mouse model of AD, and modulate the oxidative stress response implicated in neurodegenerative changes that occur with AD (Dhanasekaran et al., 2009). Diosgenin, a plant-derived steroidal sapogenin, has been shown to exert anti-cancer effects (McGeer and McGeer, 2013), improve aging-related cognitive deficits (Zhu et al., 2007), and relieve diabetic neuropathy (Bush 2008). Recently, it was proved that diosgenin improved memory function and reduced axonal degeneration in AD mouse models (Nadkarni, 1954, Tohda et al., 2013).

In this study, we examine the cogno-protective effects of an anti-oxidant product called Antia whose components include yamabushitake, gotsukora, and diosgenin. These components are treated together with the hydroferrate fluid MRN-100 to generate Antia. Previous research on MRN-100 has shown it to protect against age-associated oxidative stress (Badr El-Din et al., 2010) and against oxidative damage in endothelial cells as well as murine and human leukemia cells (Lin and Girotti, 1997). Recent studies on Antia have shown its ability to reverse oxidative-stress-induced mitochondrial dysfunction in human peripheral blood lymphocytes (Ghoneum 2019). In light of the above-mentioned neuro-protective effects of Antia's plant components, we hypothesized that Antia would have beneficial effects on the pathways relevant to AD, namely the oxidative stress, amyloidogenic, inflammatory, and autophagy pathways. We studied the effect of Antia on mice induced with SAD via intracerebroventricular (ICV) injection of streptozotocin; this is a well-established animal model of SAD based on brain resistance to insulin (Salkovic-Petrisic et al., 2013) and imitates the age-related pathology of SAD in humans like memory impairment, oxidative stress, neuroinflammation and neurodegeneration (Kamat et al., 2016). Here we present behavioral, biochemical, and western blot experiments in support of our hypothesis.

## 2. Materials and methods

### 2.1. Animals

Adult male albino mice weighing 25-30 g were provided by the animal facility of Faculty of Pharmacy Cairo University, Egypt, and then were allowed to acclimate for one week before conducting the study. Animals were housed in controlled environmental conditions of constant temperature (25 ± 2 °C), relative humidity of 60 ± 10%, and light/dark cycle (12/12-h). Standard chow diet and water were allowed ad libitum. All efforts were utilized to minimize animal suffering and to reduce the number of animals used. This study was approved by the Ethics Committee for Animal Experimentation (Faculty of Pharmacy, Cairo University) and complies with the recommendations of *the National Institutes of Health Guide for Care and Use of Laboratory Animals* (2011).

### 2.2. Chemicals

STZ was purchased from Sigma–Aldrich Co. (St Louis, MO, USA). STZ was dissolved in saline solution (0.9 % NaCl) and injected ICV at a volume of 10 µL by the free hand method. Antia was dissolved in saline solution in three doses; 25mg/kg equivalent to the adult dose (4 tablets/day), 50 mg/kg and 100 mg/kg then, administered intraperitoneally (i.p.) at the volume of 0.1 ml/20g mouse. Fresh drug solutions were prepared on each day of experimentation. Control group received saline injections of the same volume and through the same routes of administration. All other chemicals were of the highest analytical grade.

**2.3. Antia**

Antia is a natural compound derived from a variety of mushrooms and plants, including the edible Yamabushitake mushroom, the Gotsukora plant, and Diosgenin (an extract from the tubers of Dioscorea wild yam). The ingredients are treated with an iron-based fluid called MRN-100. MRN-100 is made from phytosin and is an iron-based compound derived from bivalent and trivalent ferrates (hydroferrate fluid). The chemical composition of Antia is still under active investigation. Antia was provided by ACM Co., Ltd, Japan. For this study, Antia was prepared in distilled water (DW) with the concentration of $Fe^{2+}$ and $Fe^{3+}$ ions at about $2 \times 10^{-12}$ mol/L (36). For the present study, we used concentrations of 0.0, 0.1275, 0.3825, and 1.1475 mg/ml.

**2.4. Induction of SAD**

SAD was induced by ICV injection of STZ (3 mg/kg) into the lateral ventricle of mice according to the freehand procedure (Pelleymounter et al., 2002), and updated by Warnock et al., 2010, to avoid the probability of cerebral vein penetration. Mice were anesthetized with thiopental (5 mg/kg, i.p.), then the head was stabilized using downward pressure above the ears and the needle was inserted directly through the skin and skull into the lateral ventricle which was targeted by visualizing an equilateral triangle between the eyes and the center of the skull to locate the bregma, allowing the needle to be inserted about 1mm lateral to this point. Mice behaved normally 1 min following the injection.

**2.5. Experimental design**

Mice were randomly divided into five groups, each containing 12 animals. Group I (Control): mice received ICV and intraperitoneal (i.p.) saline injection once and for 21 consecutive days, respectively and served as normal control group. Group II (STZ): mice received STZ (3 mg/kg, ICV) once and served as a model for SAD (Mehla et al., 2012). Group III (STZ+Antia 1): mice received STZ (3 mg/kg, ICV) followed by Antia (25 mg/kg, i.p) after five hours then every day for 21 consecutive days. Group IV (STZ+Antia 2): mice received STZ (3 mg/kg, ICV) followed by Antia (50 mg/kg, i.p) after five hours then every day for 21 consecutive days. Group V (STZ+Antia 3): mice received STZ (3 mg/kg, ICV) followed by Antia 3(100 mg/kg, i.p) after five hours then every day for 21 consecutive days. Twenty-four hours after the end of the treatments, neurobehavioral tests were carried out including: object recognition, and Morris water maze tests (MWM), arranged in sequence from the least stressful test to the most stressful test. To minimize possible circadian variability; all testing was conducted during the animals' light cycle under top illumination.

### 2.6. Behavioral assessments

#### 2.6.1. Object recognition test

The object recognition test is used to assess long-term memory and estimate cognition (Ennaceur, 2010). In this study, the performed test took place in three consecutive days. On the first day (the habituation phase), each mouse was individually placed in a wooden box of dimensions 30x30x30 cm for 30 min in order to adapt to the surrounding environment. The second day was designated for the familiarization or training, where two wooden cubes identical in shape, color and size were placed in opposite corners within the box, 2 cm from the walls. Each mouse was placed in the middle of the box and was left to explore these two objects for 10 min. On the third day, testing took place, one of the two identical cubes was replaced by a novel object different in shape, size and color was added. Each mouse was exposed again to two different objects for 5 min. Objects added were cleaned with 70% ethanol between experiments with animals to ensure that the behavior was not guided by odor cues. All objects and locations were adjusted to decrease potential biases due to inclinations for particular locations or objects. A mouse could not displace the objects and the subjects were always placed into the box confronting the same wall. The animals' behavior was video-recorded and the following parameters were calculated:

1) Discrimination index: Difference in time exploring the novel and familiar objects divided by the total time spent exploring both objects (This result varies between +1 and -1 where a positive score indicates more time spent with the novel object, a negative score shows more time spent with the familiar object, and a zero score indicates a null preference).
2) Recognition index: Time spent by the animal exploring the novel object as percentage of the total exploration time for both objects.

### 2.6.2. Morris water maze test

The MWM test is used to investigate spatial learning and memory in laboratory mice (Morris, 1981). The maze consisted of stainless-steel circular tanks (210 cm in diameter, 51 cm high) divided into four quadrants and filled with water (25 ± 2ºC) to a depth of 35 cm. A submerged platform (10 cm width, 28 cm in height), painted in black, was placed inside the target quadrant, 2 cm below the water surface. The platform was kept at a consistent position during the time of training and the test. A purple-colored non-toxic dye was added to make the water opaque so that the platform was made invisible. Memory-acquisition trials (120 s/trail) were performed two times a day for four consecutive days, with an interval of at least 15 min between the trials. During each acquisition trial, animals were left free to locate the hidden platform in the target quadrant. Once the mouse located the platform, it was left there for additional 20 s to rest, while if an animal failed to reach the platform within 120 s, it was gently guided to the platform and kept there for 20 s. The mean escape latency was calculated as the time taken by each rat to find the hidden platform and was used as an index of acquisition or learning. On the fifth day, the mice were subjected to a probe-trial session where the platform was taken away from the pool and each rat was allowed to probe the pool for 60 s. The time spent by each rat in the target quadrant in which the hidden platform was previously placed, was recorded as an indicator of retrieval or memory.

## 2.7. Brain processing

After behavioral testing, mice were euthanized by cervical dislocation and brains were rapidly dissected, washed with ice-cold saline. The hippocampi (n=6) were excised from each brain on an ice-cold glass plate. The hippocampus was homogenized in ice-cold saline to prepare 10% homogenates that were divided into several aliquots and stored at -80 ºC. The other hippocampus was stored at -80 ºC to be used for Western blot analysis.

### 2.8. Biochemical measurements

#### 2.8.1. Determination of oxidative stress and inflammatory biomarkers

Hippocampal lipid peroxidation was estimated by measuring the level of malondialdehyde (MDA). MDA was determined by measuring the thiobarbituric acid reactive substances, according to the method described by Uchiyama and Mihara, 1978. Moreover, the brain glutathione (GSH) content was spectrophotometrically determined using Ellman's reagent, according to the method described by Beutler et al.,1963. The results are expressed as Mmol/mg protein.

***Enzyme-Linked Immunosorbent assay***

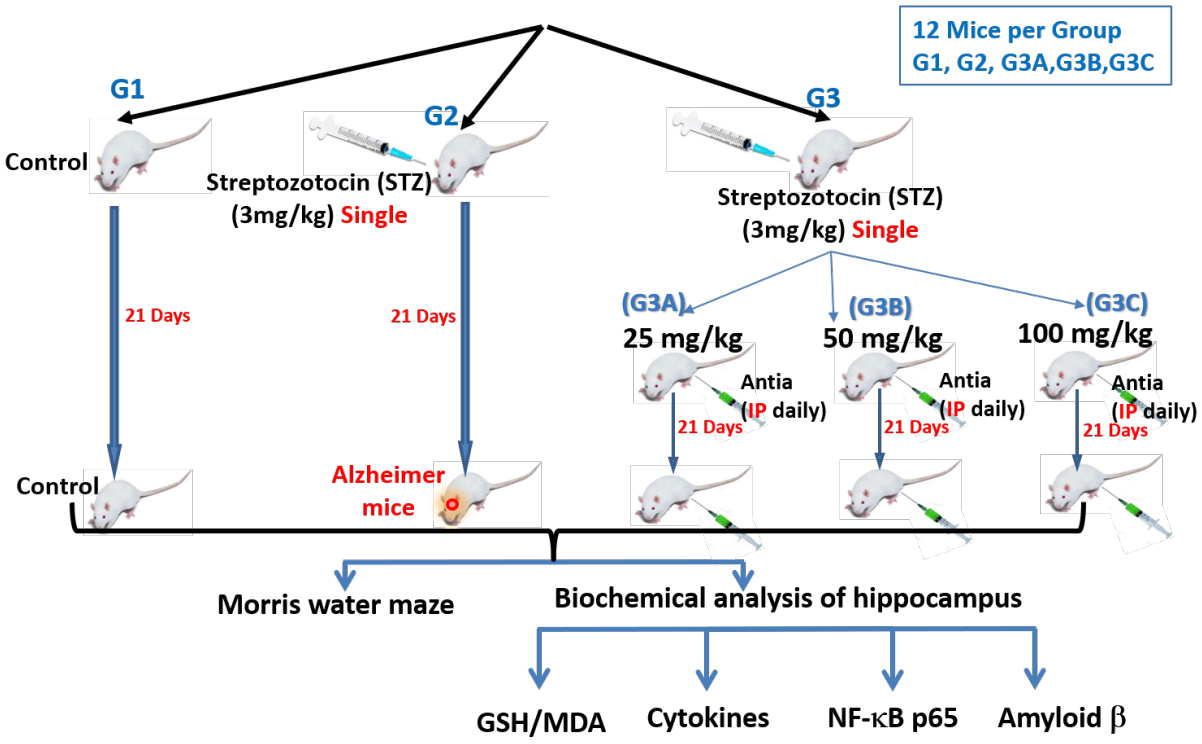
Hippocampal TNF-α and IL-6 levels were estimated using rat ELISA kits purchased from RayBiotech Inc. (Norcross, Georgia, USA), R&D Systems Inc. (Minneapolis, USA), respectively. The procedures were performed according to the manufacturer's instructions. The results are presented as pg/mg protein for TNF-α and IL-6.

#### 2.8.2. Western blot analysis

After protein solutions were extracted from the brain tissues, equal amounts of protein (20–30 µg of total protein) were separated by SDS-PAGE (10% acrylamide gel) and transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system. Immunodetection of Western blots was conducted by incubating the membranes at room temperature for 1 h with blocking solution comprised of 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 3% bovine serum albumin. Membranes were incubated overnight at 4 °C with one of the following primary antibodies: P- JAK2 (Tyr 1007/1008), P-STAT3 (Tyr 705), IκB-α, GSK-3β mTOR,, COX2and β-actin obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). After washing, peroxidase-labelled secondary antibodies were added, and the membranes were incubated at room temperature for 1 h. The band intensity was analyzed using ChemiDoc™ imaging system with Image LabTM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results are presented as arbitrary units after normalization to levels of the β-actin protein.

***Determination of protein content***

Protein content was measured according to the method of Bradford. All the results were expressed as tissue concentration per mg protein.




### 2.9. Statistical analysis

The data are presented as means ± S.E. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. The GraphPad Prism software (version 6; GraphPad Software, Inc., San Diego, CA, USA) was used to perform the statistical analysis and create the graphical presentations. The level of significance was set to $p < 0.05$ for all statistical tests.

## 3. Results

The effects of Antia on the behavioral and biochemical functions of ICV-STZ treated mice are described in the following sections, which include 1) neurobehavioral tests, and 2) biochemical analysis of the hippocampal content.

### 3.1 Neurobehavioral analysis

The effects of STZ and Antia (25, 50 and 100 mg/kg) on neurobehavioral tests were carried out within 24h after the last day of Antia injection.

### 3.1.1 Antia enhances recognition memory

Morris water maze was used to examine the possible protective effect of Antia treatment on ICV-STZ injected mice. The effect of Antia on mean escape latency (MEL) in Morris water maze is illustrated in Figure 2A. Mice in different groups took different times to escape on day 2. Alzheimer's mice took 1.63 times as long to escape on day 2 as compared to control mice. On the other hand, Alzheimer's mice with Antia took only 1.08 times as long as control mice on day 2. These results were further confirmed in the subsequent days 3 and 4.

### 3.1.2 Time spent in target quadrant

Studies on the effect of Antia on time spent in target quadrant in Morris water maze showed that Alzheimer's mice spent only 25.4% of the time in the quadrant as compared to control mice, while Alzheimer's mice with 25, 50, and 100 mg/kg of Antia spent 72.5%, 75.8%, and 85.4% of the time, respectively, as compared to control mice (Figure 2B).

### 3.1.3 Discrimination and preference indices in Novel Object Recognition test

The effect of STZ and Antia on the discrimination and preference indices in Novel Object Recognition test (NOR) was examined. The discrimination index was declined in STZ-induced sporadic AD mice when compared to control group, but significantly increased after Antia (25, 50 and 100mg/kg) administration as compared to STZ group in a dose dependent manner. In addition, the time spent exploring the novel object was lower in ICV-STZ injected mice by 63% compared to control group, which reflects lower preference index. On the other hand, Antia (25, 50 and 100mg/kg) administration normalized the preference index, indicating that Antia-treated mice preferred the novel object over the familiar object in a dose dependent manner (Figure 2C).

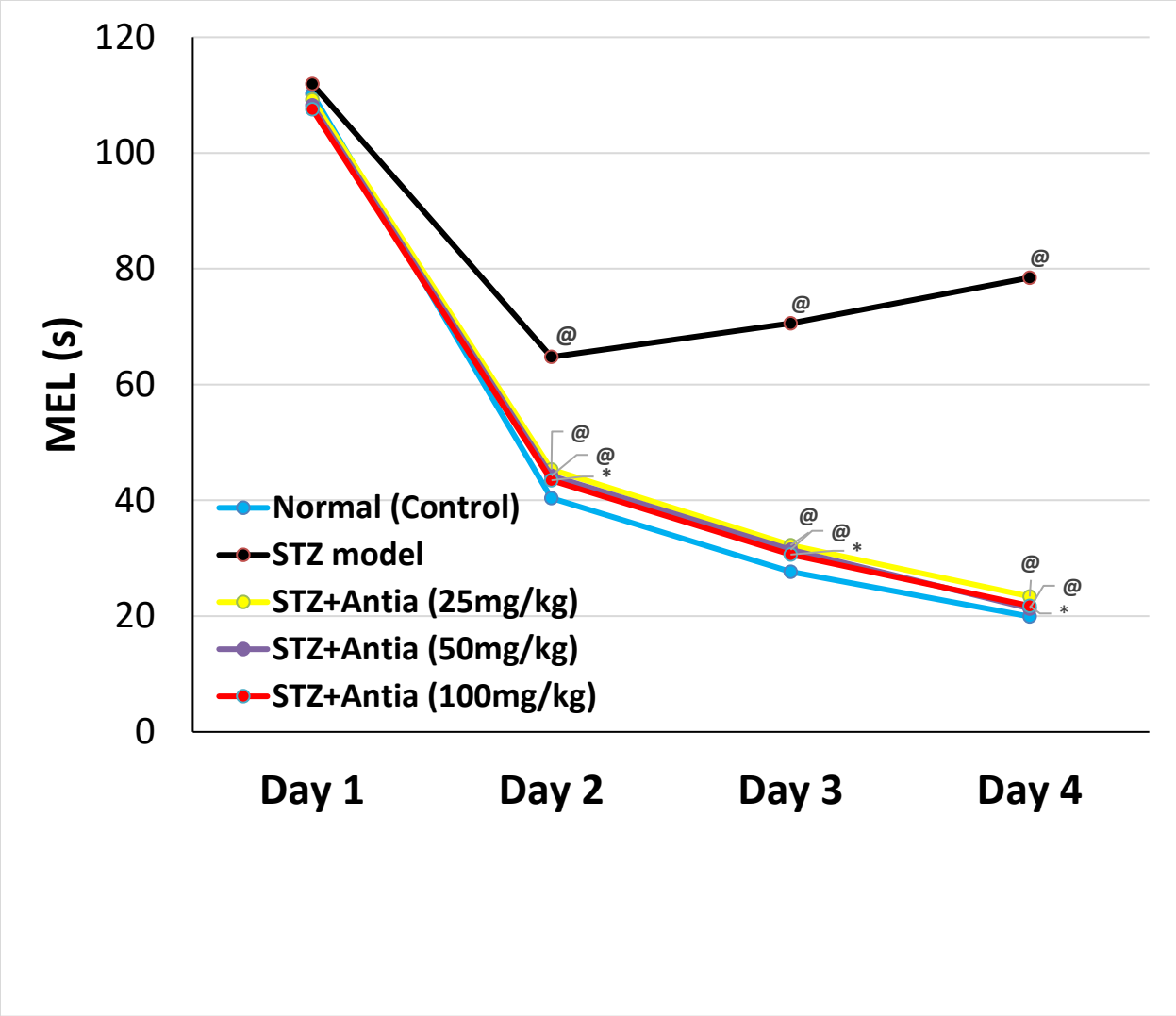



Figure 2A: Effect of Antia on mean escape latency (MEL) in Morris water maze in ICV-STZ injected mice.
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$

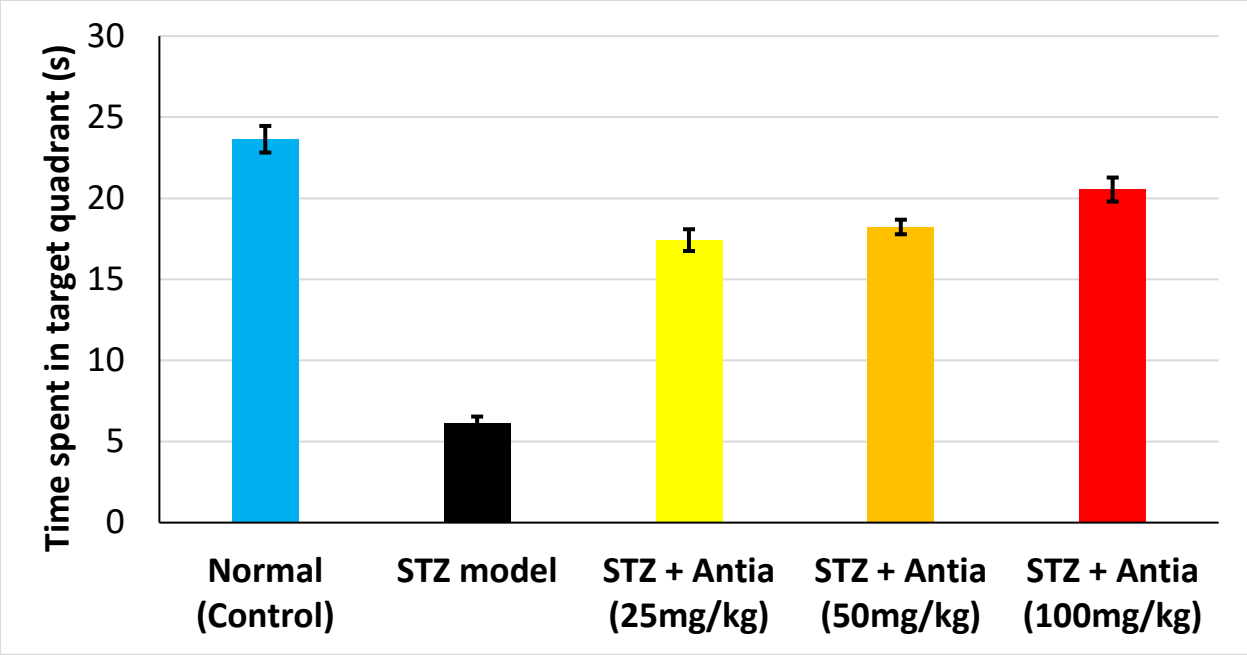



Figure 2B: Effect of Antia on time spent in target quadrant in Morris water maze in ICV-STZ injected mice

* Significantly different from normal group at $p<0.05$

@ Significantly different from ICV-STZ group at $p<0.05$

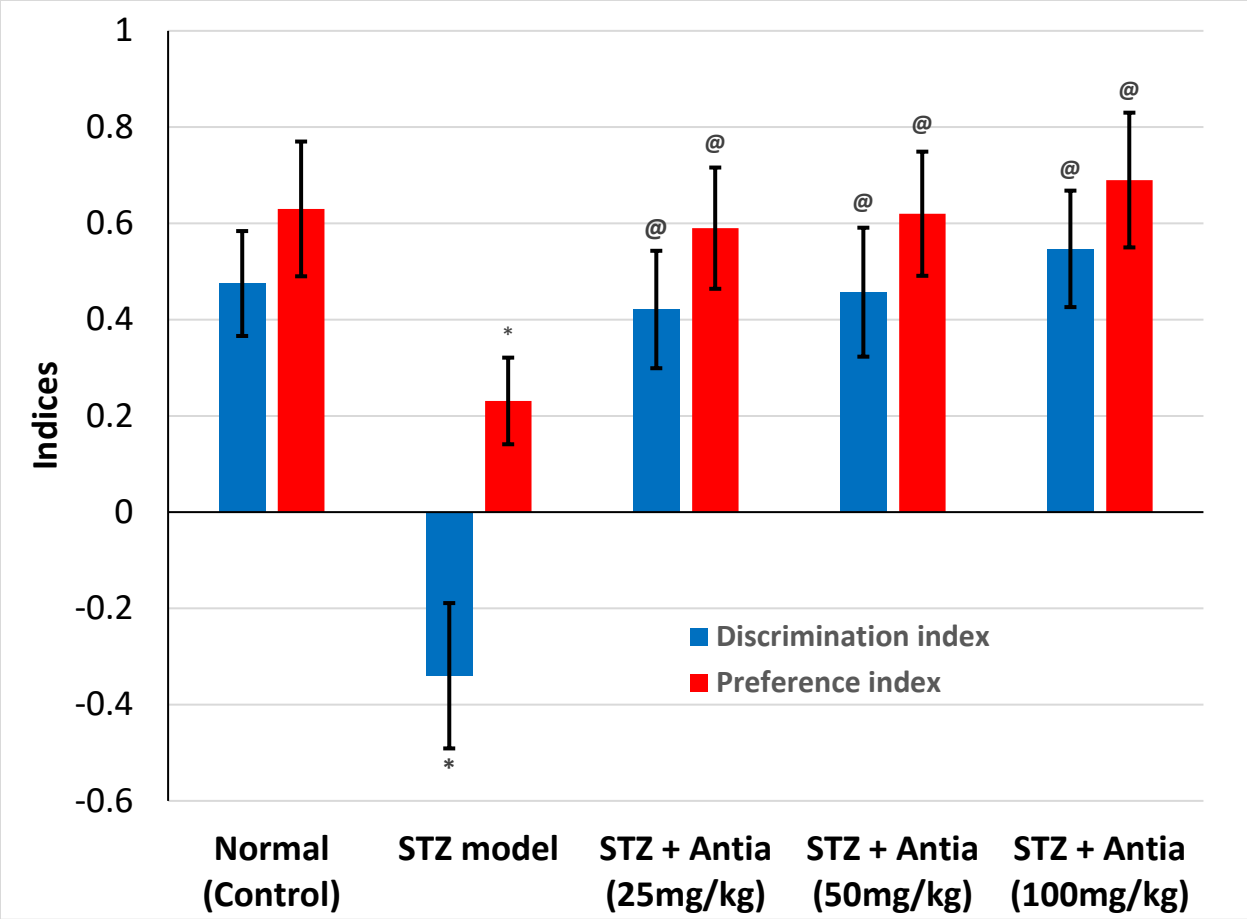


Figure 2C: Effect of Antia on cognitive function of ICV-STZ injected mice in the novel object recognition test
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$

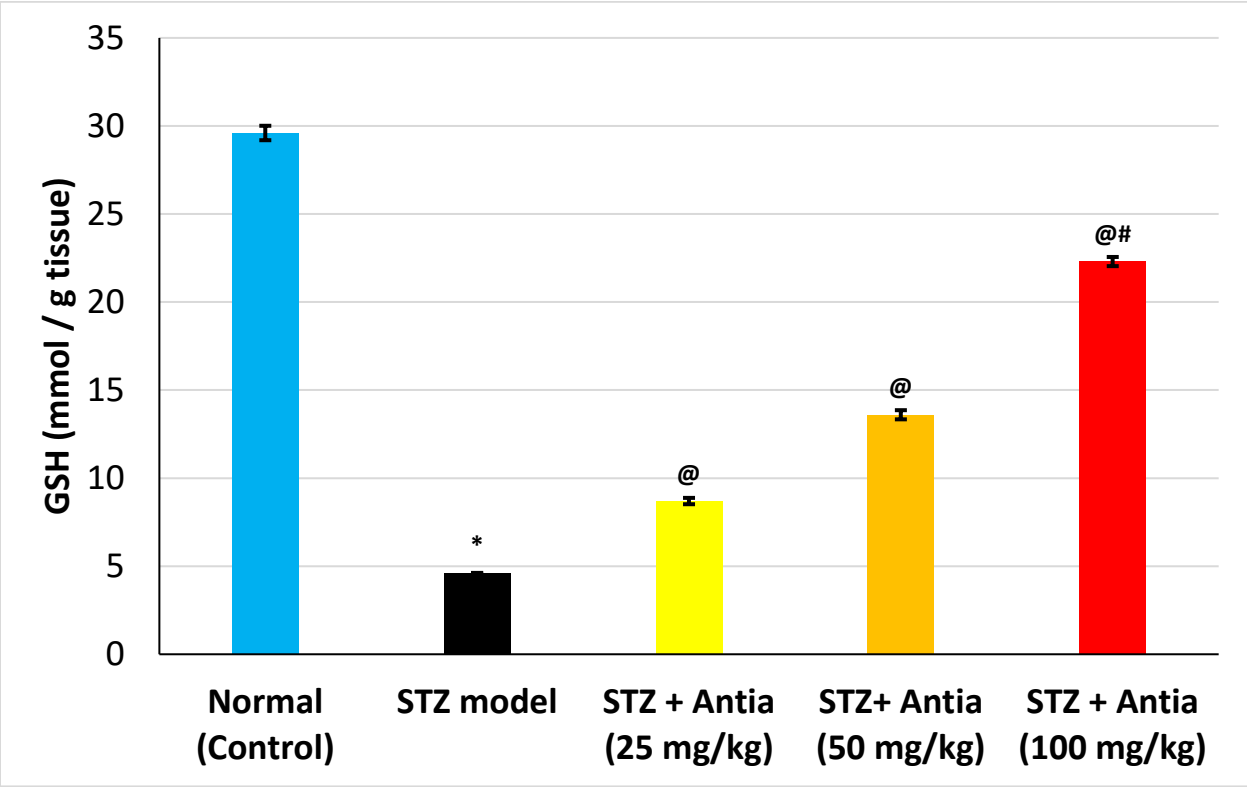
**3.2 Biochemical analysis in the hippocampal content.**

We performed several biochemical analysis in the hippocampal content in ICV-STZ treated mice order to examine the ability of Antia to attenuate the amyloidogenic, inflammatory, autophagy and oxidative stress pathways.

**3.2.1 Antia increases Glutathione (GSH) and decreases Malondialdehyde (MDA).**

Studies on the protective effect of Antia treatments on levels of glutathione (GSH) and malondialdehyde (MDA) hippocampal content were carried out. Results in Figure 3A show that Alzheimer's mice had a GSH level that was 15.5% of the GSH level of control mice. On the other hand, Alzheimer's mice with Antia showed an elevation in the GSH content in a dose dependent manner that was maximized at 78.7% of the control GSH level for 100 mg/kg Antia treatment. Results of the levels of MDA hippocampal content showed significantly higher levels

of MDA in ICV-STZ injected mice as compared with control mice by a factor of 4.3 fold. On the other hand, Alzheimer's mice with Antia showed an elevation in the MDA content of only 3.5 fold, 2.5 fold, and 1.8 fold for mice receiving Antia at doses of 25, 50 and 100 mg/kg respectively (Figure 3B).



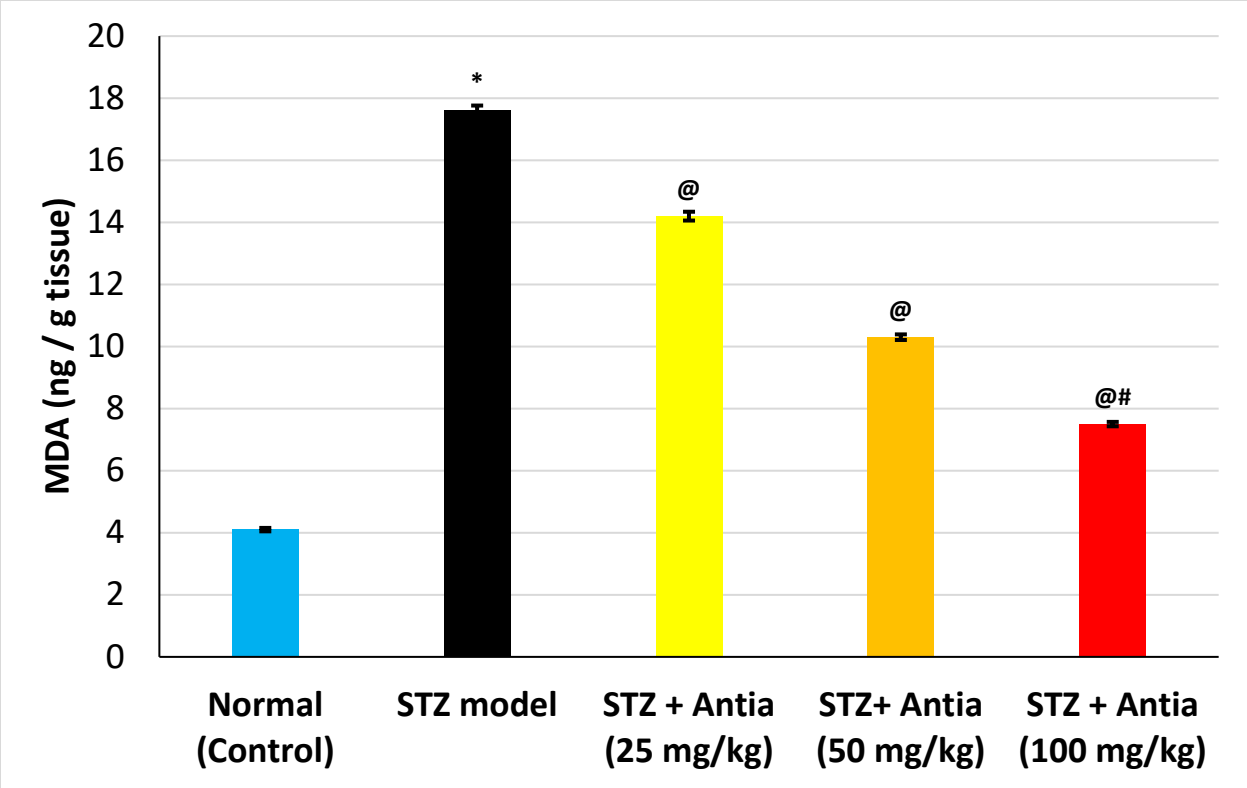



Figure 3A&B. Effect of Antia on GSH and MDA hippocampal content in ICV-STZ injected mice.
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

### 3.2.2 Antia reduces levels of Tumor Necrotic Factor Alpha (TNF-α), Interleukin 6 (IL-6), and NF-kB p65

Effect of ICV-STZ injection on hippocampal content of anti-inflammatory cytokines was examined in the presence and absence of Antia treatment. Two cytokines were examined: Tumor Necrotic Factor Alpha (TNF-α) and Interleukin 6 (IL-6). Results in Figure 4 show that STZ model mice exhibited significant increase in the expression of TNF-α and IL-6 cytokines as compared with control mice, but treatment with Antia suppressed this induction in a dose dependent fashion that reached the level of control at 100 mg/kg.

Similar trend was seen in the hippocampal content of NF-kB p65. Results in Figure 4 also showed increased levels of NF-kB p65 in the Alzheimer's mice, and its gradual decrease in Alzheimer's mice with Antia.

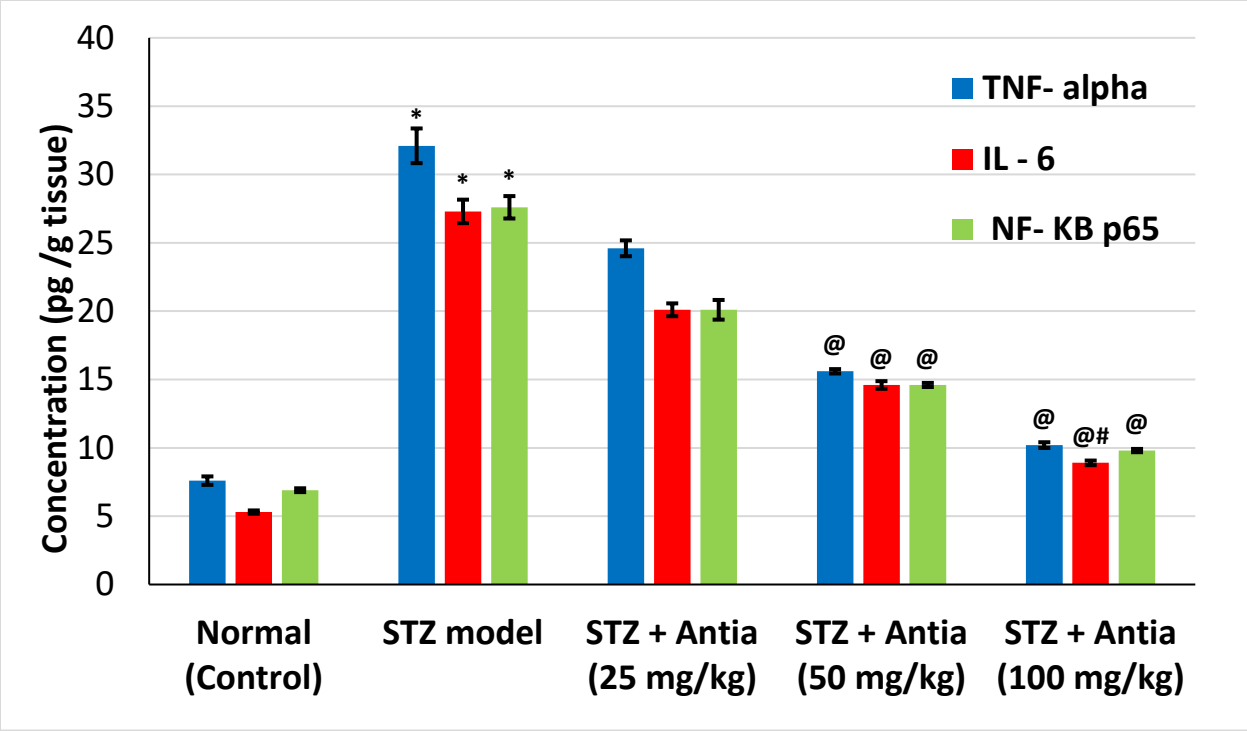



Figure 4 Effect of Antia on TNF- alpha, IL – 6 and NF- KB p65 hippocampal content in ICV-STZ injected mice.
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

### 3.2.3 Antia decreased Amyloid β expression.

Amyloid β make up the plaques of Alzheimer's disease where these normally solid proteins assemble into amyloid-like filaments, therefore we examined the effect of Antia on Amyloid $\beta_{1-42}$ hippocampal content in ICV-STZ injected mice. Results depicted in Figure 5 show that STZ model mice exhibited about 4 fold increase the expression of Amyloid β as compared with control mice. It is of interest to note that the levels of Amyloid β were significantly decreased in Alzheimer's mice with Antia. The effect was a dose dependent and reached its lowest levels at 100 mg/kg.

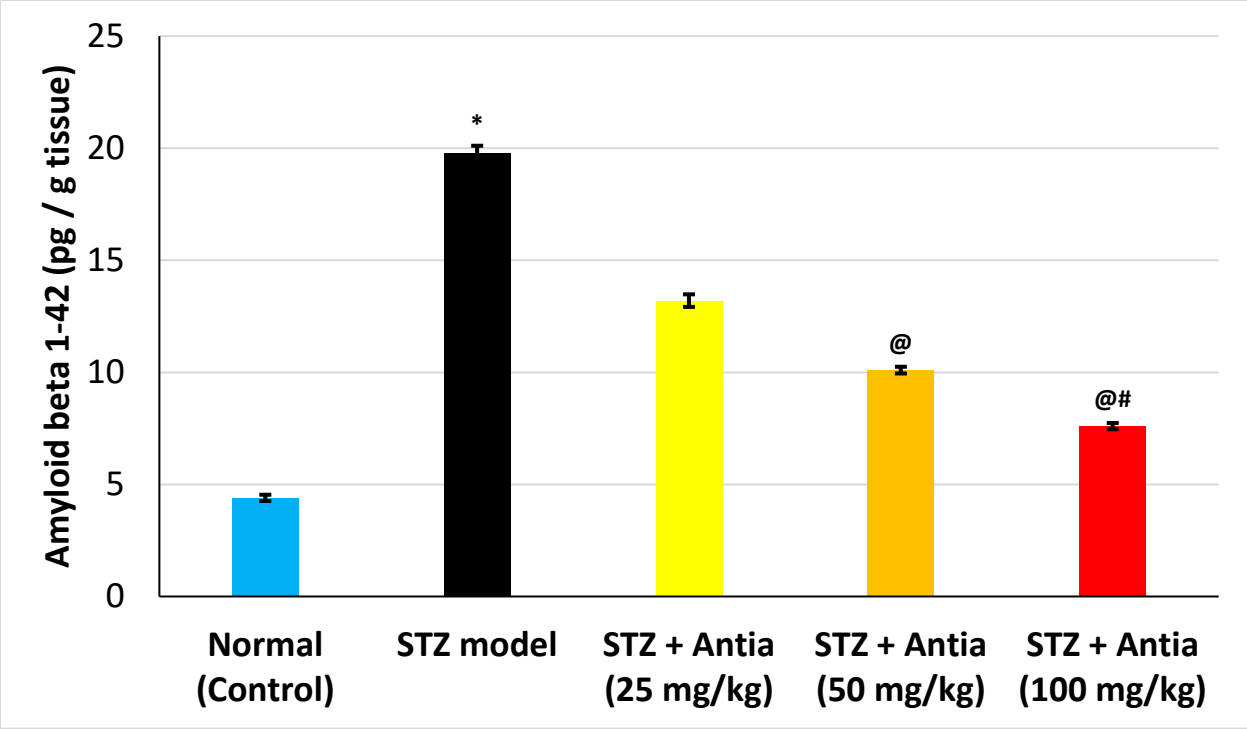


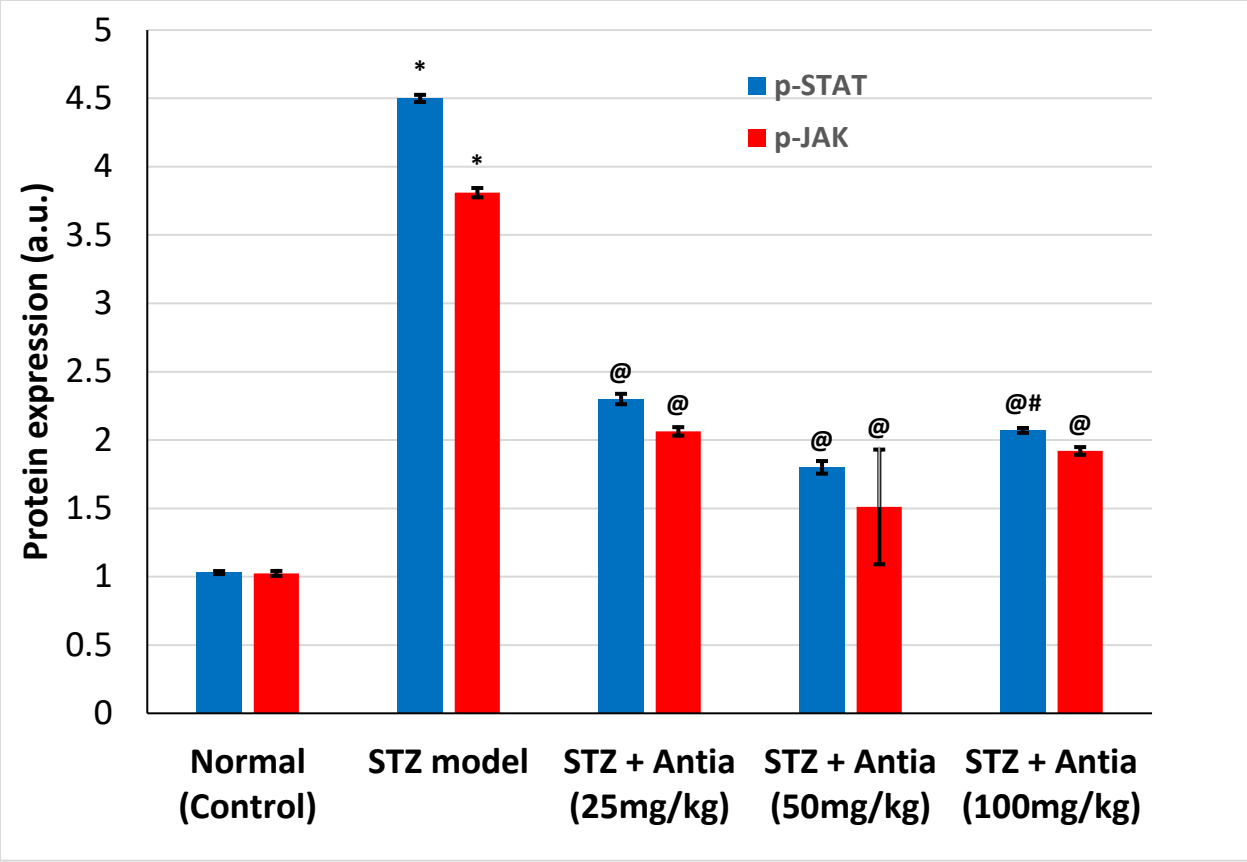

Figure 5: Effect of Antia on Amyloid $\beta_{1-42}$ hippocampal content in ICV-STZ injected mice.
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

### 3.3 Effect of Antia on the phosphorylated STAT protein expression

The levels of phosphorylation of STAT and JAK protein expression is well established method used in Alzheimer's Research**.** We examined whether treatment with Antia suppresses the phosphorylation of STAT expression in STZ- mice. As expected the levels of phosphorylation of STAT protein expression was significantly reduced as compared with control mice. However treatment of STZ- mice with Antia resulted in significant inhibition in the phosphorylation level of STAT3 (Figure 6A).

### 3.4 Effect of Antia on the phosphorylation of JAK2 protein expression

Similar trend of results were observed with JAK2 protein expression. Treatment with Antia caused a significant inhibition in the phosphorylation level of JAK2 due to of STZ injection (Figure 6A). These results indicate the protective effect of Antia for the JAK2/STAT3 pathway.



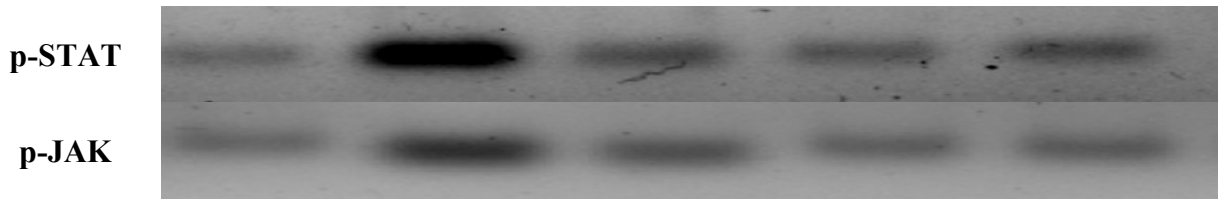




Figure 6A: Effect of Antia on the phosphorylated STAT and JAK protein expression in the hippocampi of ICV-STZ injected mice
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

### 3.5 Effect of Antia on the phosphorylated GSK3β and IKBα protein expression

Earlier studies showed that Glycogen synthase kinase-3 (GSK3) phosphorylates tau protein, the principal component of neurofibrillary tangles, inhibition of GSK-3a offers a new approach to reduce the formation of both amyloid plaques and neurofibrillary tangles, two pathological hallmarks of Alzheimer's disease (Phiel et al 2003). Results in Figure 6B show that Alzheimer's mice had a high expression of GSK3β level that was 7 folds of the GSK3β level of

control mice. On the other hand, treatment with Antia caused dramatic inhibition in the expression of GSK3β that was about 3 fold of the control.

Results in Figure 6B also show that Alzheimer's mice had a high expression of IKBα level that was 6.5 folds of the IKBα level of control mice. On the other hand, treatment with Antia caused dramatic inhibition in the expression of IKBα that was about 2.8 fold of the control.

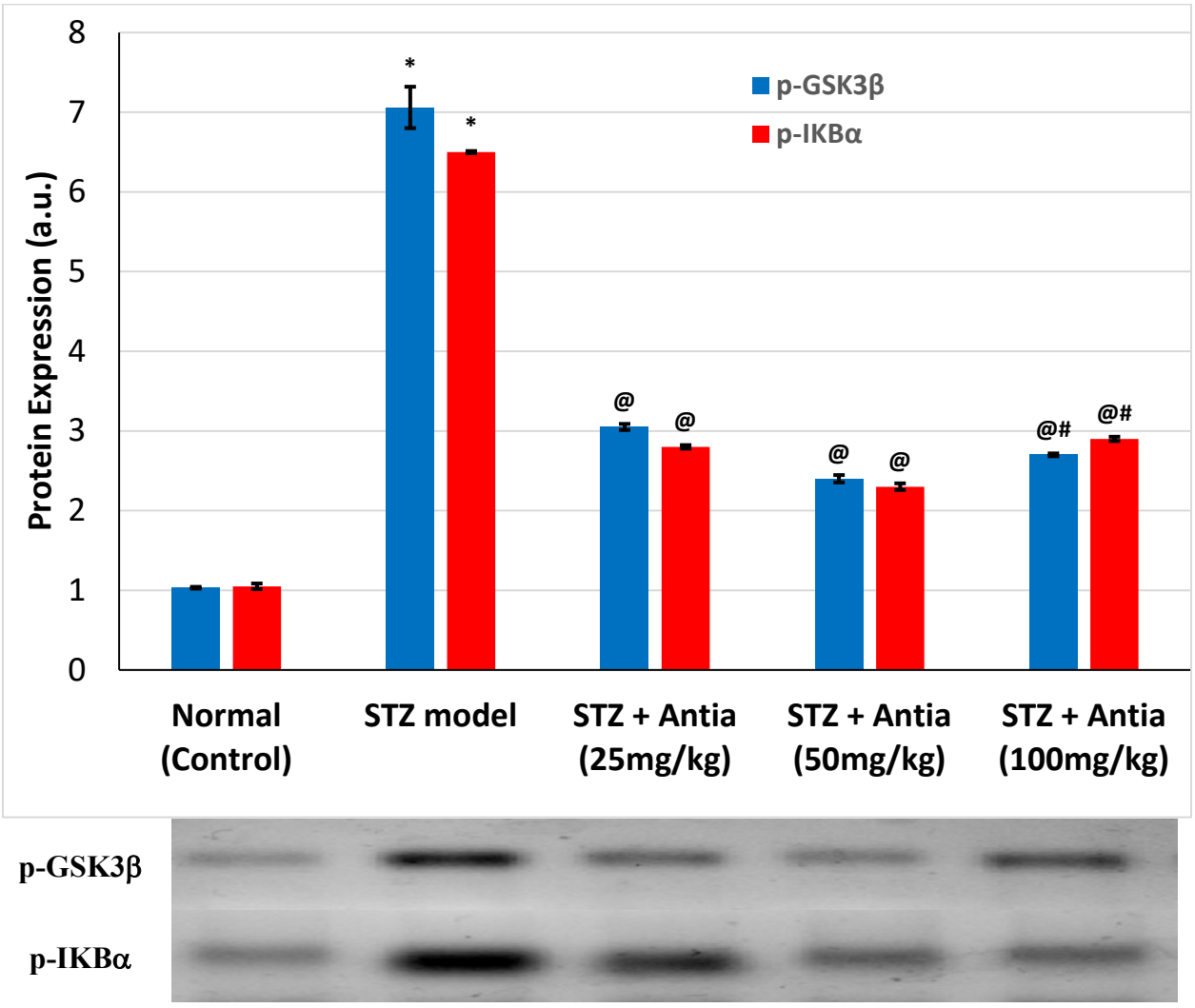



Figure 6B: Effect of Antia on the phosphorylated GSK3β and IKBα protein expression in the hippocampi of ICV-STZ injected mice
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

### 3.6 Effect of Antia on the mTOR and p-AKT protein expression.

Several studies showed that the mammalian target of rapamycin (mTOR) may play a role in Amyloid β and tau induced neurodegeneration (Oddo 2012). Earlier studies showed higher levels of mTOR phosphorylated at Ser2481 in the medial temporal cortex of AD cases compared to control cases (Li et al 2005, Griffin et al 2005).Results in Figure 6C showed that STZ injected mice exhibited significant increased levels of the mTOR and p-AKT protein expression that were 5x and 6x greater than the level of control mice, respectively, but treatment with Antia reversed that increase and brought it close to that of the control values.

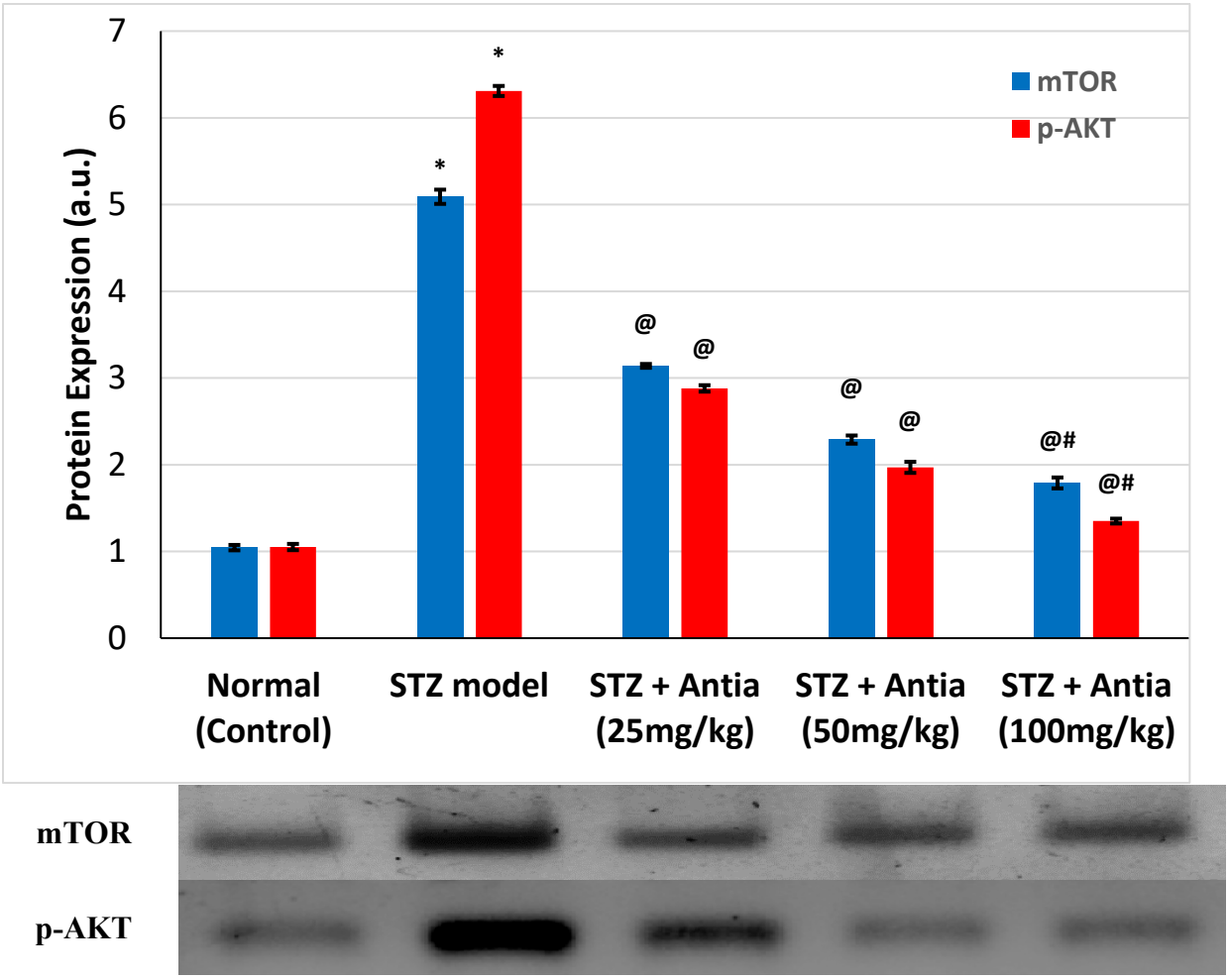



Figure 6C: Effect of Antia on the mTOR and p-AKT protein expression in the hippocampi of ICV-STZ injected mice
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

### 3.7 Antia inhibits the upregulation of COX-2 protein expression.

COX-2 is a key enzyme in the inflammatory processes. Results in Figure 6D show that Alzheimer's mice exhibited significant induction in COX-2 expression, that was 600% of the COX-2 level of control mice, however treatment with Antia significantly reduced the expression of COX-2 to 150%-300%.

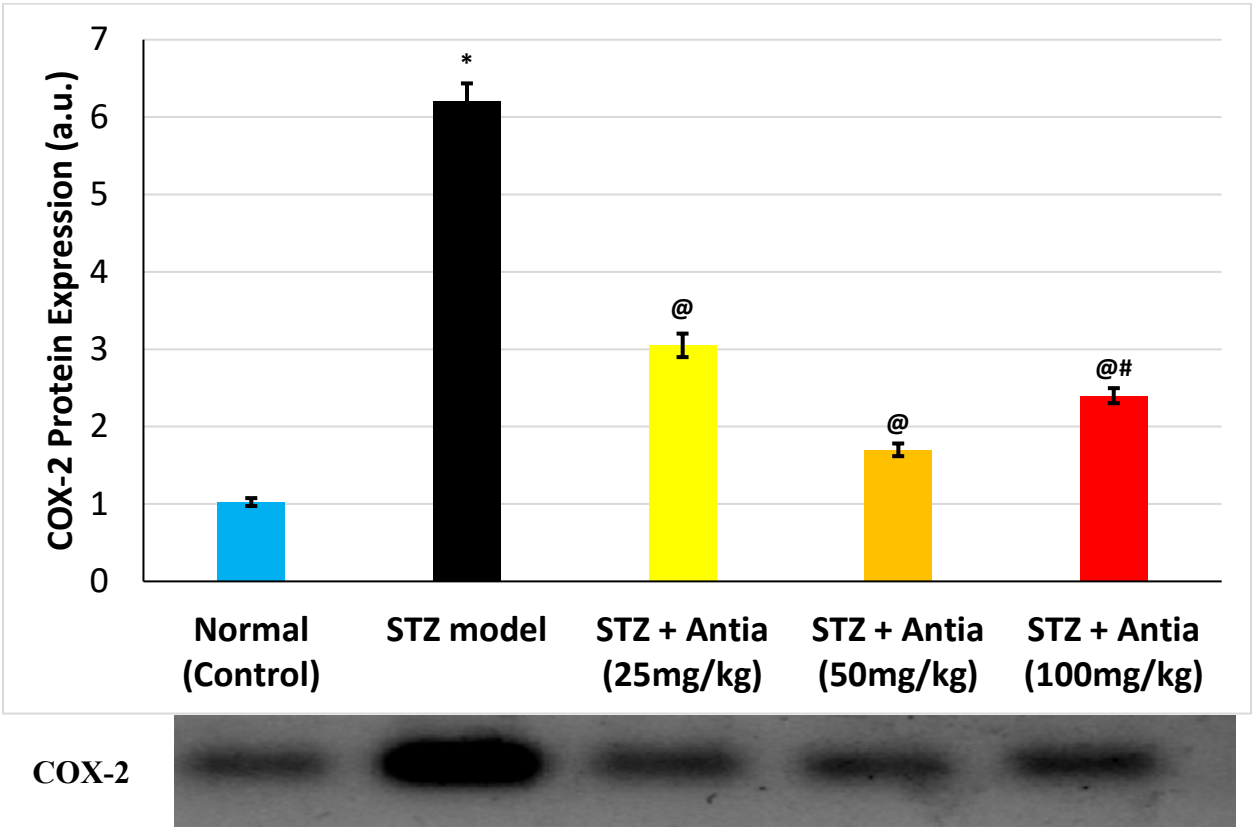



Figure 6D: Effect of Antia on the COX-2 protein expression in the hippocampi of ICV-STZ injected mice
* Significantly different from normal group at $p<0.05$
@ Significantly different from ICV-STZ group at $p<0.05$
# Significantly different from Antia (25 mg/kg) at $p<0.05$
$ Significantly different from Antia (50 mg/kg) at $p<0.05$

## 4) Discussion

Results of the present study demonstrated the ability of the anti-oxidant Antia to exert a protective effect against SAD induced in mice. Antia attenuates cognitive dysfunction in mouse model via targeting several linked pathways such as the amyloidogenic, inflammatory, autophagy and oxidative stress pathway.

In the present study, induction of SAD in mice by STZ induced a significant cognitive decline in the Morris water maze and NOR tests. ICV injection of STZ is an experimental model that mimics the progressive pathology of SAD similar to human brain (Kamat et al., 2016). STZ-treated mice showed significant learning and memory deficits as shown by the noticed inability of mice to discriminate between familiar and novel objects as demonstrated in Morris water maze and NOR tasks. This is in harmony with previous studies (Halawany et al., 2017, Abdel Rasheed et al., 2018). However, the profound elevation in escape latency during the acquisition trial and the time spent in the target quadrant during probe trail in Morris water maze test as well as the increase in discrimination and preference indices in NOR proved that Antia prevented the STZ-induced impairments of spatial and short term memory. This improvement in the object recognition memory deficit could be attributed to the previously proven effects of several of Antia's ingredients. For example, it has been shown that diosgenin has an anti-amyloidogenic effect (Lecanu et al., 2010; Tohda et al., 2013) and that Hericium erinaceus has a strong neuroprotective effect against neuronal loss and dementia in AD (Nagai et al., 2006; Kawagishi and Zhuang, 2008). Furthermore, oral administration of dried Yamabushitake mushroom powder has been demonstrated to be effective in improving mild cognitive impairment in humans (Mori et al., 2009).

STZ administration exhibited significant increase in the expression of hippocampal content of NF-κB and anti-inflammatory cytokines; tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). NF-κB plays a crucial role in the inflammatory responses in neurons where it induces the transcription of inflammatory target genes including, cyclooxygenase-2 (COX-2), interleukin-1β, IL-6 and TNF-α (Xiao et al., 2005). TNF-α is involved in systemic inflammation, in particular, it is involved in AD-related brain neuroinflammation, as well as amyloidogenesis via β-secretase regulation. Moreover, profound neuropathological changes such as Parkinson's and Alzheimer's disease are associated with increased IL-6 expression in the brain (Alam, 2016). NF-κB has also been shown to regulate BACE-1 expression level, the rate

limiting enzyme responsible for the production of Amyloid β  Our results showed that Antia reduces levels of TNF-α, IL-6, and NF-kB in Alzheimer's mice in a dose dependent  manner. Additionally, Alzheimer's mice with Antia demonstrated dramatic inhibition in the expression of phosphorylated STAT3 and JAK2, which are linked to TNF-α production (Huang et al 2008, Nishiki et al 2004).

Neuroinflammation has been linked to a deficit of autophagy, which may contribute to neurodegeneration (Zheng et al., 2013). The mammalian target of rapamycin (mTOR) is known to regulate autophagy, along with protein kinase B (Akt) (Jung et al., 2010). Several studies emphasize the close relationship between mTOR signaling and the presence of Aβ plaques and cognitive impairment in AD (Paccalin et al 2006, Cai et al 2012, Pozueta et al 2013, Lafay-Chebassier et al 2005). Furthermore, in human and rat studies of AD, autophagy activation has been linked to GSK-3β inhibitors and its deficit has been found to contribute to the pathological accumulation of Tau aggregates ((Zhou et al., 2011, Zare-Shahabadi et al., 2015). Our results show that treatment with Antia reversed the high expression of mTOR, Akt, IKBα and GSK-3β level due to STZ injection and brought it to that of control.

The constituents of Antia have been shown to possess various neuro-regenerative and protective properties. Yamabushitake mushrooms were shown to synthesize nerve growth factor (Mori:2008,Ma:2010,Spelman:2017); gotsukora extracts reduced the beta-amyloid levels in the Alzheimer's-stricken brains of laboratory animals (Dhanasekaran:2009); diosgenin enhances the cognitive performance of mice (Tohda:2013).  Further, we recently found *in vitro* that Antia has the ability to reverse oxidative-stress-induced mitochondrial dysfunction and apoptosis in human peripheral blood lymphocytes (Ghoneum:2019).  Previous studies have shown the beneficial anti-oxidant properties of MRN-100, the hydroferrate fluid that is used to treat Antia's constituents, to increase brain levels of GSH, superoxide dismutase, catalase and glutathione peroxidase, and inhibit of the levels of oxidative stress biomarkers including malondialdehyde (MDA), nitric oxide, and total free radicals (Badr El-Din:2010). Previous research showed that the generation of reactive oxygen species (ROS) via Amyloid β during self-aggregation may damage and cause neuronal death (Ahmad  et al 2017). Glutathione (GSH) is an anti-oxidant that has the ability to prevent damage caused by ROS and may protect against oxidative and neurotoxic degeneration of oligometric amyloid beta (Monks:1999,Lasierra-Cirujeda:2013).

Results of this study showed that Antia increases GSH and decreases MDA levels in Alzheimer's mice.

## 5) Conclusions

It could be concluded from the present study that Antia exerts a significant protection against cognitive dysfunction induced by intracerebroventricular injection of streptozotocin. This effect is achieved through targeting the amyloidogenic, inflammatory and oxidative stress pathways. The JAK2/STAT3 pathway played a protective role for neuroinflammatory and neurodegenerative diseases such as SAD.

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