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# EFFECT OF LONG -TERM CONSUMPTION OF SUCROSE, ASPARTAME AND STEVIA SWEETENERS ON HIPPOCAMPUS ANTIOXIDANT DEFENSE STATUS

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## ABSTRACT
Stevia leaf extract has the potential to be used as a non-caloric natural sweetener instead of the synthetic sweetener aspartame due to its antioxidant potency in controlling free radicals with resultant health benefits on brain hippocampus. In addition, stevia has no serious toxic effects making it a perfect sugar non caloric substitute. For this purpose, male albino rats were divided into four groups, control group receiving drinking water, sucrose group receiving 10% sucrose solution, aspartame group receiving a dose of 75 mg aspartame/kg body weight /day and stevia group receiving a dose of 40 mg/kg body weight /day. The results of this study revealed that long term supplementation of sucrose and aspartame to rats produced a significant decrease in total antioxidant, glutathione, Na+-K+ ATPase and total protein of hippocampus tissue. This was accompanied by significant decrease in serum creatine kinase–brain type after 90 days of either sucrose or aspartame supplementation. On the other hand, hippocampus catalase, malondialdehyde, nitric oxide elucidated a significant increase in their mean values in either sucrose or aspartame groups compared to the control group. Moreover, long term sucrose and aspartame consumption activated the immune-expressions of Caspase 3 and Bax protein in hippocampus tissue. Stevia leaf extract supplementation to experimental animals verified near to normal control results in all the investigated parameters. Additionally, stevia has no serious genotoxic effects making it a perfect sugar non-calorie substitute. So, it could be used as antioxidant agent for controlling free radicals in many neurodegenerative diseases.

**KEYWORDS:** Hippocampus, Sucrose, Aspartame, Stevia.

## 1. INTRODUCTION
Sucrose is a natural caloric sweetener extensively used in human nourishment owing to its pleasant taste, nutritive value. Sucrose intake is higher among children, adolescents and adults. High levels of sucrose consumption is associated with depression and psychological distress.[1] Rodent models who over-consumed different sugars were less motivated which was viewed as a central symptom in neuropsychiatric disorders.[2] One of the brain regions of particular interest is the hippocampus because it is a critical region for decision-making and various forms of long term memory and spatial navigation.[3] Daily sucrose consumption may pose a risk for the development of mental health disorders, impairing neurocognitive functions thus affecting decision-making and memory.[4] Deficits in cognitive flexibility and hippocampal-dependent memory have been found in healthy middle-aged and young adults with higher intake of refined sugar than those with less intake, independent of age and body mass index.[5] In addition, excessive intake of sucrose enriched diet may be a cause and consequence to body weight gain and metabolic syndrome. This is done by interfering with hippocampal dependent higher order processes including episodic memory, sensitivity to internal hunger and satiety signals thus forgetting what we have eaten.[6] Owing to the high sucrose demand and its related adverse effects, non-nutritive sweeteners either synthetic or natural were used to substitute sugars in diverse food products.[7] People trying to lose weight or patients with diabetes, including children use aspartame frequently without the risk of consuming extra calories served by normal sugar-based products.[8] Aspartame is an artificial low-calorie sweetener which has been extensively studied because of its presence in many sugar free products. Reports on effects of aspartame on the brain is of great interest as the worldwide consumption of aspartame-containing products is on rise especially in

children and young adults.[9] Aspartame metabolites cause changes in brain's hippocampus antioxidant defense system leading to deleterious damage to brain tissue and the development of various neurological diseases.[10] The demand of natural antioxidant compounds for protecting the damage of oxidative stress has received increasing attention. Stevia (*Stevia rebaudiana* Bertoni) is a perennial shrub of the *Asteraceae* family which has been historically used as a sweetener in South America.[11] Stevia leaf extract has antioxidant, anti-tumor, anti-inflammatory properties which makes it an ideal and economical plant source sugar substitute with potential benefits on human health.[12] In addition, stevia leaf extract exerts a memory-preservative effect in cognitive deficits of rats possibly through its effect on brain antioxidant system.[13]

The aim of the present study was to investigate the effects of sucrose as a natural high caloric sweetener on brain's hippocampus tissue as well as to find out whether substituting sucrose with the low caloric sweeteners aspartame or stevia leaf extract will have beneficial effects on the antioxidant defense system in hippocampus tissue of rats.

## 2. MATERIALS AND METHODS

### 2.1. Experimental animals

Thirty-two male albino rats of the strain *Rattus norvegicus* weighing (100-110) grams were bred and kept under conventional conditions at the experimental animal research unit of Medical Research Center and Bilharzia, Faculty of Medicine, Ain Shams University. Rats were maintained under a 12:12 light/dark cycle with a temperature of 23–25 C◦. Fresh tap water was available all time. Animals were acclimatized to laboratory conditions for a week before the commencement of the study. All animal procedures were made in accordance with the National Institutes of Health (NIH) protocol accepted by Ain Shams University.

### 2.2. Experimental treatments and dosage

Sucrose (El-Nasr pharmaceutical chemicals Company, Egypt) was supplemented to rats as 10% sucrose solution dissolved in distilled water (w/v) according to Kendig et al.[14] This sucrose solution provided a caloric density of (approx. 0.4 kcal/g) similar to the commercially available sugar drinks.

Aspartame (1-methyl N-L-a- aspartyl -L-phenylalanine**)** was purchased as tablet formulation from (Amrya for pharmaceutical industries Alexandria, Egypt). Each tablet contained 20 mg of aspartame (one tablet equal teaspoonful of sugar and 0.4 calorie). Tablets were dissolved in distilled water and given orally to rats at a dose of 75 mg/kg body weight /day days.[15]

Stevia leaf extract extracted from the leaves of *Stevia rebaudiana* Bertoni was purchased as a powder of stevia leaf extract from Alpha Nexa Nutritionals Ltd, Kent, U.S.A. Stevia leaf extract was dissolved in distilled water and given orally as the calculated human therapeutic dose according to Paget and Barnes[16] at a dose of 40 mg/kg body weight /day. All experimental regimens were supplemented to rats by oral gavage for 90 consecutive days.

### 2.3. Animal grouping and sampling

Rats were divided into four experimental groups; control group receiving distilled water. The second group represented the sucrose group, the third group served as the aspartame group and the fourth group represented the stevia group. Eight animals from each group were dissected after 90 days of experimental study. At the end of the study period, rats were anesthetized under ether inhalation and blood was collected by cardiac puncture. Blood samples were collected and left to clot in empty, dry clean tubes. Serum was collected then divided into small aliquots to avoid the repeating of thawing and freezing then stored at -20°C until analysis. Serum was then used for the determination of the serum creatine kinase-brain type (CK-BB). The brain was immediately removed and washed with ice-cold phosphate buffered saline, dropped onto an ice-cold glass plate leaving the olfactory bulbs behind. After removing the brain, hippocampus was separated from the brain on ice-cold glass plate. Hippocampus homogenates were prepared as (10%w/v) using ice-cold phosphate buffer saline (pH 7.4) in a Teflon-glass tissue homogenizer. Samples were centrifuged separately in a refrigerated centrifuge at 3000 rpm for 15 min. The supernatant was used for analyzing different investigated parameters.

### 2.4. Determination of oxidative stress markers, Na+ /K+ ATPase, total protein in hippocampus tissue

Brain's hippocampus tissue homogenates were used for the determination of total antioxidant activity according to the method described by Koracevic et al.[17]. Malondialdhyde (MDA) was measured using thiobarbituric acid (TBA) assay modified according to the method of Draper and Hadley.[18] Catalase (CAT) activity in brain's hippocampus tissue was determined by the method of Bock et al.[19] Brain's hippocampus tissue glutathione (GSH) content was assayed according to Prins and Loose [20]. Nitric oxide (NO) level was measured by the determination of total nitrite concentration in the sample (R&D systems, Minneapolis, USA) adopted by Green et al. [21]. The activity of Na+/K+ ATPase was measured according to Üner et al.[22] The total protein concentration in hippocampus was evaluated using reagent kits obtained from Diamond Diagnostic, Cairo, Egypt.[23]

### 2.5. Determination of kinetic creatine kinase brain type (CK-BB) activity

Creatine Kinase, brain type was performed by Kinetic UV method according to the method of Zilva and Pannall.[24] The kit was purchased from Vitro Scientific, Hannover, Germany.

**2.6. Immunohistochemical staining and quantitative evaluations of Caspase-3 and Bax protein**

Hippocampus tissue sections were deparaffinized, rehydrated and washed twice times in buffer according to the staining protocol of Petrosyan et al.[25] Hippocampus tissue sections were incubated in hydrogen peroxide for 10 minutes and washed. Primary antibodies and antibody enhancers of Caspase 3 and Bax protein were then applied and incubated according to manufacturer's recommended protocol. Peroxidase-compatible chromogen was then applied, incubated and washed. Counter stain was applied and covered using an aqueous mounting media. The immune stained sections were examined using light microscope to assess the prevalence of positive cases and location of immunostaining within hippocampus tissue. Tumor cells were fascin positive if there was cytoplasmic or nuclear staining (brown color). Immuno-reactivity was quantitatively evaluated by estimating the percentage of positive immune stained cells using computed image analysis (Leica Qwin software 500, Germany).

**2. 7. Statistical Analysis**

Data were expressed as means± standard error of means (SE). All the recorded data were analyzed using the Statistical Processor System Support (SPSS) version 10 computer program. The significance of differences between means of control animals and all treated rats were analyzed using one-way analysis of variance (ANOVA) test. * is considered statistically significant at ($P < 0.05$) for all experimental groups in comparison with the control group.

## 3. RESULTS AND DISCUSSION

Long term supplementation of sucrose and aspartame to rats produced a ($P < 0.05$) decreases in total antioxidant, GSH, Na+-K+ ATPase and total protein activities in hippocampus tissue. This was accompanied by significant decrease in serum CK-BB after 90 days of either sucrose or aspartame supplementation. On the other hand, hippocampus CAT, MDA, NO elucidated significant ($P < 0.05$) increases in their mean values in either sucrose or aspartame groups compared to the control group. The most significant ($P < 0.05$) changes in the pre-mentioned parameters were clearly elucidated in aspartame group (Table1).

These data suggest that sucrose consumption has adverse effects on hippocampus tissue of brain. In accordance with these results, Reichelt et al.[4] confirmed that when rats were exposed to sucrose for 2 hours a day for a long duration during adolescence, they exhibited various cognitive deficits. In brain tissue, the disaccharide sucrose can easily cross the blood brain barrier because it is uncharged molecule, not subjected to protein binding, metabolically stable after parenteral administration, falling into the molecular weight range of most small molecule drugs.[26] That is why concurrent exposure to high sucrose diets has been shown to reduce hippocampal dendritic spine density with reduction in hippocampal neurogenesis.[5] The present study also revealed altered antioxidant defense system with increased oxidative stress in hippocampus tissue of rats supplementing sucrose for 90 days (Table 1). 10% sucrose solution supplementation to rats is sufficient to impair hippocampal neuro-inflammatory markers TNF-α and IL-1β. Up regulation of these neuro-inflammatory markers emphasizes the increased oxidative stress in brain tissue [27].These results confirm that oxidative stress and inflammation may both contribute to the hippocampal-dependent memory impairments seen in sucrose supplemented rats. Also, the increased levels of oxidative stress and decreased antioxidant defense system suggests that the brain is more susceptible to oxidative stress than other organs because it contains high levels of redox-active iron and copper enhancing its vulnerability to oxidative stress. Brain also has little potential to replenish damaged cells since it is composed mostly of terminally differentiated neurons and glia.[28]Thus to counteract the high demand of sucrose which is related to the incidence of obesity and memory impairment, non-nutritive sweeteners either artificial or natural were used to substitute sugars in diverse food products.[7] Aspartame is one of the most widely used artificial non caloric sweeteners. It is metabolized in the gastrointestinal tract to aspartic acid, methanol and phenylalanine. Methanol is later oxidized to formaldehyde and then to formic acid.[15] Additionally, the results of the present study clarified that oral aspartame ingestion for 90 days designated a significant increase in CAT, MDA and NO with a significant decrease in total antioxidant and GSH in hippocampus tissue and serum CK-BB. These alterations in hippocampus oxidant antioxidant system may be due to the accumulation of methanol which is oxidized to formaldehyde and formates inhibiting cytochrome oxidase complex at the end of the respiratory chain in brain mitochondria.[15] In addition, the high ATP demand of the brain makes it consume $O_2$ rapidly interfering with mitochondrial function which leads to the elevated levels of $O_2^{\bullet-}$ formation.[29] The significant elevated levels of MDA and NO in hippocampus tissue of aspartame supplemented rats presented here in this study may be also attributed to the elevated levels of inflammatory agents and cytokines which in turn increases free radicals production and decreases endogenous antioxidants. High levels of MDA and NO have been linked with neurotoxicity [30]. Glutathione depletion in the hippocampus tissue has been significantly shown in this study which disrupts short-term spatial memory [31]. GSH is an important nonenzymatic antioxidant that plays an important role in the detoxification of toxic chemicals of endogenous and exogenous origin. When the brain suffers from oxidative stress it has lower levels catalase and GSH.[10] The inhibition of glutathione related enzymes in hippocampus tissue may be due to methanol intoxication produced by aspartame administration. Also, the metabolism of methanol and formaldehyde depends on GSH. A decrease in glutathione levels reduces their

metabolism, thereby increasing their toxicity.[32] In addition, phenylalanine produces oxidation of sulfhydryl groups from cysteine residues to form disulfide which potentially alters the redox state of proteins leading to their inactivation which also explains the significant decrease in hippocampus proteins [10]. Similarly, Formaldehyde radicals react with the amino acids of soluble proteins, leading to the formation of hydroxymethyl derivatives or protein peroxides via reacting with free radicals.[33] Furthermore, aspartic acid is an excitatory neurotransmitter in brain resulting from long term aspartame administration. The increase in aspartic acid and phenylalanine levels in brain could an explanation for the increased oxidative stress observed after aspartame ingestion.[34] The results of the current study were confirmed by Abbott et al.[35] who verified that long term aspartame ingestion for 90 days resulted in cumulative toxic high amounts of phenylalanine. Phenylalanine competes for a binding site on the neutral amino acid transporter because this is the only way it can cross the blood–brain barrier. Aspartic acid and phenylalanine deteriorate the neurons of the brain when they cross the blood brain barrier.[36] Furthermore, when phenylalanine crosses the blood–brain barrier it causes severe changes in the production of important neurotransmitters which in turn influences brain monoamine synthesis and turn over.[36] The results of the present study again elucidated a significant decrease in hippocampus Na+ K+-ATPase enzyme after aspartame ingestion for 90 days. Na+ K+-ATPase enzyme is critical for maintaining ionic gradients in neurons and could be involved with memory. The observed enzyme inhibition after 90 days of aspartame ingestion may be due to an increase in reactive oxygen species production.[37] Stevia leaf extract supplementation to experimental animals verified near to normal control results in all the investigated parameters ( Table 1).

**Table 1: Mean ±SE of total antioxidant, GSH, CAT, MDA, NO, Na+ /K+ ATPase, total protein in rat brain's hippocampus tissue as well as and serum creatine kinase brain type in experimental groups.**

| Parameters | Control | Sucrose | Aspartame | Stevia |
|---|---|---|---|---|
| **Total antioxidant** (mmol/liter) | 1.59±0.0363 | 0.85* ±0.0317 | 0.30* ±0.0258 | 1.63±0.0546 |
| **GSH** (mg GSH/g tissue) | 1.12±0.024 | 0.70*±0.026 | 0.32*±0.031 | 1.08±0.033 |
| **CAT** (µmol/sec/g tissue) | 36.29±0.890 | 37.44*±0.180 | 52.40* ±0.211 | 35.87±0.343 |
| **MDA** (µmol/g tissue) | 0.48±0.0141 | 0.51*±0.0074 | 0.63*±0.0148 | 0.46±0.0070 |
| **NO** (µmol/g) | 64.70±0.149 | 65.48*±0.172 | 66.96*±0.088 | 64.28±0.329 |
| **Na+-K+ ATPase** (µmol/g protein/minute) | 249.55±11.4 | 200.05*±6.66 | 125.80*±7.85 | 231.80±7.80 |
| **Total protein** (g/L) | 8.12±0.169 | 5.06*±0.122 | 2.42*±0.201 | 7.93±0.098 |
| **CK-BB** (U/l) | 7.01±0.207 | 5.63*±0.098 | 4.90*±0.105 | 6.89±0.173 |

** is considered statistically significant at P < 0.05 for all experimental groups in comparison with control*.

Activated Caspase 3 and Bax protein expressions were studied in table 2 and figures 1 and 2 respectively. The sucrose treated animals manifested a marked significant increase in activated Caspase 3 (Fig 1-b) and Bax protein (Fig 2-b) expressions in their hippocampus tissues compared with their corresponding controls respectively (Fig 1-a and Fig 2-a). Similarly, aspartame treated animals elucidated a significant 5.6-fold increase in the Bax protein expression (Table 2) and a 6.9-fold increase in activated Caspase 3 in their hippocampus tissues (Table 2). Figure 1-c showed a significant number of neuron cells with immune reaction for activated Caspase 3 and Bax protein (Fig 2-c) as brown coloured neuronal cells that were considered positive cells in the hippocampus tissue of animals supplementing aspartame for 90 days. Moreover, nuclear immunostaining was also observed in addition to the cytoplasmic ones in both immunohistochemical stains. On the contrary, animals supplementing stevia leaf extract did not produce a marked significant increase either in Caspase 3 (Fig 1-d) or Bax protein expressions (Fig 2-d) when compared to their corresponding controls (Table2). It is well known that neural death occurs according to an apoptotic program. The up regulation of Bax with the activation of Caspase 3 leads to the apoptotic damage of neurons in different brain regions, which resulted from the increased production of free radicals and increased oxidative damage to brain proteins resulting in neuronal toxicity.[15] Additionally, the depletion of intracellular brain GSH reported in this study is an additional factor for the observed apoptotic changes. More to the point, the accumulated free radicals in brain tissue can also attack DNA strands to induce breaks which are the base modifications of mutations.[38] *Stevia rebaudiana* is a natural sweetening herb that is 250- to 300-fold sweeter than saccharose. Stevia leaf extract is a source of low calorie and high potency natural sweeteners. Steviosides, rebaudioside A and polyphenols are the major components found in stevia leaf extract possessing significant reactive oxygen species scavenging activity. These active compounds act through inhibiting oxidant enzymes and promoting anti-oxidant defense system.[39] Furthermore steviosides rich in stevia plant reduced the breakdown and degradation of ascorbic acid thus form

protection against radical attack to the different body organs.[40] The results of the present study elucidated near to normal values of oxidative parameters and $Na^+$ $K^+$-ATPase enzyme activity in hippocampus tissue of rats supplementing stevia supplemented rats. Barroso et al.[41] showed that pretreatment of stevia leaves stimulated SOD and CAT to reverse oxidative damage. Similarly, stevia leaf extract may have improving effect on memory performance by inhibiting acetylcholinesterase activity.[13] In addition, steviol glycosides have strong anti-inflammatory, antioxidant capacities by suppressing some proinflammatory cytokines and preventing the activation of the NF kappa β. Furthermore, GSH concentration is proved to be increased in brain tissue of animals administering stevia.[39] The results of the present study confirmed that Caspase-3 and Bax proteins showed nearly normal expressing values after administrating stevia leaf extract for 90 days. Reactive oxygen species can damage proteins and lipids as well as the mitochondrial DNA. Mutations of mitochondrial DNA can lead to changes in the respiratory chain, which causes a reduction in its efficiency and thus produces more reactive oxygen species, creating a vicious circle leading to apoptosis of cells. In addition, mutations within the mitochondrial genome accumulate in common neurodegenerative diseases such as Parkinson's and Alzheimer's disease in which NO plays a critical pathophysiological role. Steviosides show radical scavenging activity against reactive oxygen species resulting from neurodegenerative diseases.[40]

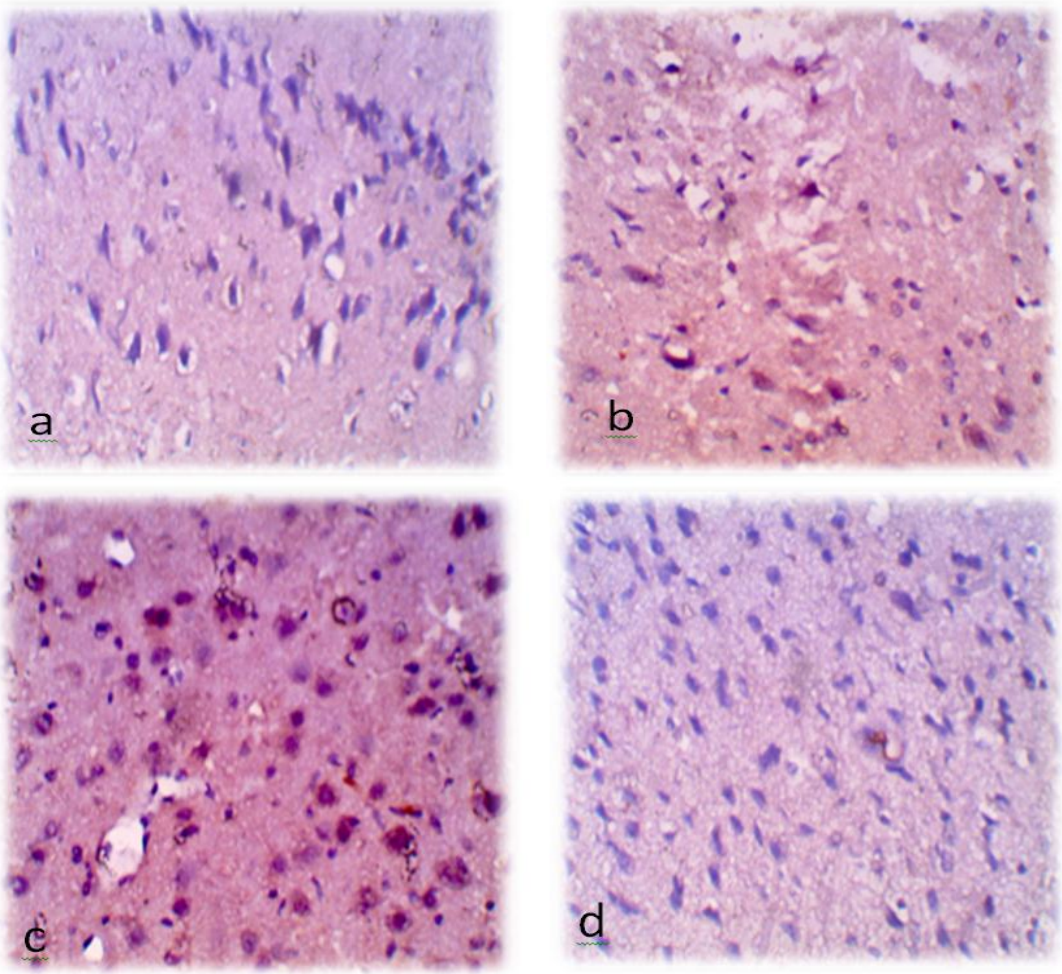


**Figure 1: Caspase immunoreactivity in hippocampus tissue of control (a), sucrose (b), aspartame (c) and stevia (d) supplemented rats.**

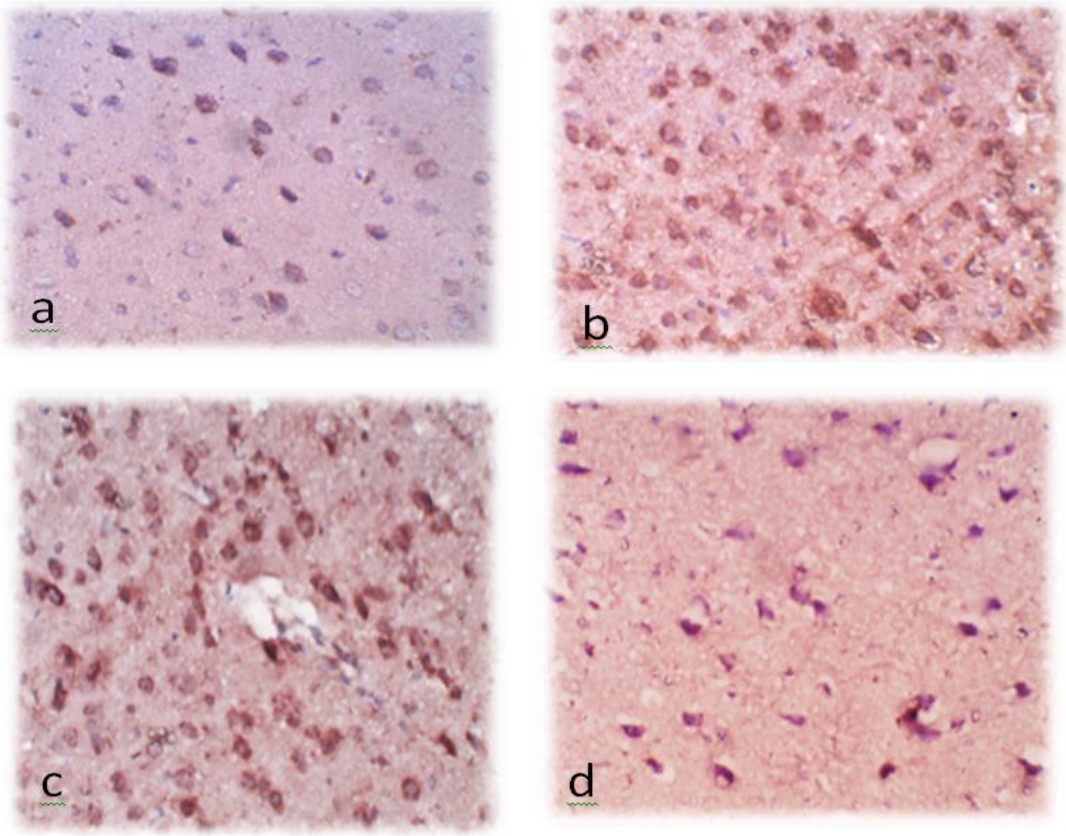


**Figure 2: Bax protein immunoreactivity in hippocampus tissue of control (a), sucrose (b), aspartame (c) and stevia (d) supplemented rats.**

**Table 2: Immunohistochemical analysis of Caspase 3 and Bax protein expressions in experimental groups.**

| | Control | Sucrose | Aspartame | Stevia |
|---|---|---|---|---|
| **Caspase 3** | 5.09±0.795 | 19.29*a±2.07 | 35.15*a±2.58 | 6.91±1.58 |
| **Bax protein** | 7.01±0.691 | 24.13*a±1.99 | 39.40*a±2.09 | 8.34±1.56 |

** is considered statistically significant at P < 0.05 for all experimental groups in comparison with control*

## CONCLUSION

The results of the present study emphasize on substituting high caloric sweetener sucrose with the low caloric sweetener stevia leaf extract instead of aspartame. It also highlights on the antioxidant neuroprotective action of long-term supplementation of stevia leaf extract sweetener on brain hippocampus tissue.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest concerning this article.

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