



Tannin as a Multifunctional Natural Agent in Hyperglycemic Conditions: Neuroprotective and Immunoregulatory Actions Comparable to Metformin

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10.18805/ajdfr.DRF-639

ABSTRACT

Background: Tannin exhibits potent antioxidant and anti-inflammatory properties. This study aimed to investigate the protective effects of Tannin on neuroendocrine and its correlation with hippocampal and immunological alterations in Streptozotocin-induced diabetic mice.

Methods: Sixty Adult male albino mice were divided into six groups, including control, high-dose Tannin-sole (300 mg/kg) and Streptozotocin-induced diabetic groups (150 mg/kg, *intraperitoneally*) treated with metformin (250 mg/kg), low-and high-dose Tannin (200, 300 mg/kg, respectively, gavage) for 28 days. Fasting blood glucose (FBG) was monitored weekly (4weeks) and serum cortisol, noradrenaline and acetylcholine levels were quantified using ELISA. Brain tissues were examined histologically to assess neuronal integrity (HandE and Cresyl Violet staining) and apoptosis (TUNEL). Spleen tissues were analyzed by HandE and Trichrome Masson staining, while, CD4⁺ /CD8⁺ T-cell distribution was determined by immunofluorescence.

Result: Tannin treatment significantly reduced FBG in hyperglycemic mice in a time-dependent manner ($p < 0.05$), comparable to metformin. The stress-associated biomarkers (cortisol, noradrenaline and acetylcholine) were markedly ($p < 0.05$) reduced in Tannin-treated groups, with significant ($p < 0.05$) reduction in noradrenaline compared to the metformin-received group. Also, significantly ($p < 0.05$) improved pyramidal cells' integrity and Nissl body storage and reduced apoptosis. High-dose Tannin further restored immune homeostasis in spleen tissue by attenuating CD4⁺ and CD8⁺ T-cell cellularity.

Key words: Hippocampus, Hyperglycemia, Metformin, Mice model, Spleen, Tannin.

INTRODUCTION

Diabetes is a metabolic disease with a complications extended far beyond glucose metabolism (Mahmoudian *et al.*, 2013). It impairs brain physiology, contributing to neurodegenerative processes (Trojsi *et al.*, 2018). Chronic hyperglycemia activates immune cells in the brain leading to destruction of some healthy neurons, leading to impairments in memory and cognitive functions (Hardigan *et al.*, 2016), confirmed by that impaired hippocampal neurogenesis and cognitive deficits are associated with decreased acetylcholine levels (Yang *et al.*, 2025), observed in diabetic conditions reflecting diminished cholinergic transmission (Fernández-Cabezudo *et al.*, 2019), together with elevation in glucocorticoids (Cortisol) (Joseph and Golden, 2017; Moon *et al.*, 2014; Sun and Wang, 2023) and circulating noradrenaline during diabetes (Ahriculesei *et al.*, 2025), correlates with oxidative stress, impairs glucose utilization in neurons and promotes hippocampal cell apoptosis (Stranahan *et al.*, 2008). Moreover, this abnormal immune response extended to different vital organs, including spleen, particularly the white pulp, which is rich in lymphocytes and essential for mounting effective immune responses (Tsalamandris *et al.*, 2019).

Considering these positive impacts of tannins, the present study conducted to investigate the protective role of tannins extracted from *Rhus coriaria* L. (sumac), on the key neuroendocrine and immunological parameters in

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How to cite this article: Abed, A.K., Delirezh, N. and Mahdi, L.H. (2026). Tannin as a Multifunctional Natural Agent in Hyperglycemic Conditions: Neuroprotective and Immunoregulatory Actions Comparable to Metformin. *Asian Journal of Dairy and Food Research*. 45(4): 601-609. doi: 10.18805/ajdfr.DRF-639.

Submitted: 27-12-2025 **Accepted:** 12-02-2026 **Online:** 20-02-2026

hyperglycemic mice alongside histopathological examination of the hippocampus to determine neuronal integrity and pyramidal cell preservation were assessed. In addition, the role of tannins in spleen histopathology and CD4⁺ and CD8⁺ T cell populations tested to evaluate the impact of treatment on immune regulation.

MATERIALS AND METHODS

Animals grouping and treatments

In this experimental study, 60 adult male albino mice (weight~35 g) were randomly assigned to six groups (10 each), divided to three main groups including.

C group= Control group (received no treatment).

HDT-sole group= High Dose Tannin (300 mg/kg of Tannin was administered orally for 28 days) and Diabetes-induced groups, the animals in this group were induced hyperglycemia by Streptozotocin (STZ) injection, (SigmaAldrich, LOT: WXB2887V) interperitoneally (150 mg/kg), then the blood sugar was checked after 72 h and the blood glucose above 250 mg/dl were considered as Diabetes. The Diabetes-induced groups were then divided into 4 groups.

D-sole group= Diabetes sole group with no treatment.

D+M group= Diabetes + Metformin (following the hyperglycemia induction, treated with metformin at a dose of 250 mg/kg, orally for 28 days).

D+LDT group= Diabetes+Low Dose Tannin group (following the hyperglycemia induction, treated with Tannin at a dose of 200 mg/kg, orally for 28 days).

D+HDT group= Diabetes+High Dose Tannin group (following the hyperglycemia induction, treated with Tannin at a dose of 300 mg/kg, orally for 28 days).

The study conducted in RASTA Private Medical Research Institute during the period 02.05.2024 to 10.06.2025. The study was approved by Iranian Research Institute for Information Science and Technology (Ethical Approval Letter No. 1799361 on 31.02.2024). All mice were procured from the Animal Center of Urmia University (ACUU) and maintained under standardized conditions. At the end of experiment, the blood samples were collected via cardiac puncture under anesthesia from mice in different groups, serum samples were separated and stored at (-80°C) for further biochemical analyses.

Fasting blood glucose measurement

During the experiment period the FBG levels were monitored weekly for four weeks using a glucometer (Accu-Chek, Roche Diagnostics, Germany).

Analysis of measured biochemical factors

As per manufacturer's instructions, commercial ELISA kit from Invitrogen (USA) were used to measure corticosterone (Cat: EELR016), noradrenaline (Cat No: EEL010) and acetylcholine (Cat No: EEL014).

Hematoxyline and Eosin (H and E) staining of brain and spleen tissues

At the end of the experiments, tissue sections from spleen and brain were dissected and fixed in 10% formalin. The fixed samples were processed to generate formalin-fixed, paraffin-embedded (FFPE) tissue blocks, which were cut into 5 µm slices using a rotary microtome (HistoRange LKB, Sweden). The sections were then prepared for histological staining. Deparaffinization, gradual rehydration and staining cell nuclei with hematoxyline.

Cresyl violet staining of hippocampus

To investigate neurodegenerative changes, the 5 µm thick sections were prepared to visualize neuronal cell bodies

and examine the neurons with Nissl bodies. Sections were placed in xylene and stepwise descending alcohols to deparaffinize and rehydrate, respectively. The slides were then immersed in 0.1% crystal violet with a pH of 3.5 to 3.8 at room temperature. Excess stain was removed with a brief rinse in distilled water, followed by differentiation in 95% ethanol to reduce background staining. Slides were then dehydrated in absolute alcohol, cleared in xylene and mounted. Light microscopic evaluation (Nexcope, Model: NE620) was done to assess neuronal Nissl body deposit in hippocampal pyramidal cells and it was reported as the percentage of stained pyramidal cells representing the presence of Nissl body.

Masson's trichrome staining of spleen tissue

The 5 µm-thick sections were first deparaffinized and rehydrated through a graded series of alcohols, followed by washing in distilled water. Next, the sections were re-fixed in Bouin solution for 1 h at 56°C to enhance staining quality. The sections were then stained in Wiegert's iron hematoxylin working solution. Following this, the sections were stained with Biebrich scarlet-acid Fuchsin solution for 2 min.

Immunofluorescence staining of tissue

For antigen retrieval, slides were heated in 10 mM sodium citrate buffer (pH 7.2) for 15 min to unmask target epitopes. To minimize nonspecific binding, sections were treated with a blocking solution (5% normal serum and 0.3% Triton X-100) for 30 min at room temperature. Primary antibodies targeting CD4 (Santa Cruz Biotechnology, Cat No: sc-19641) and CD8 (Santa Cruz Biotechnology, Cat No: sc-1177), which are conjugated to a fluorophore, were diluted as recommended, applied and left overnight at 4°C. After PBS washes, the prepared slides were stored in darkness and analyzed under a fluorescence microscope (Canada Smart Tech, Canada).

TUNEL staining of brain tissue

The TUNEL (Terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay conducted to assess the hippocampus apoptosis with the InSitu cell death detection kit (Roche, Cat. No. 11684817910, Germany).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (V10, USA). The data of each group were expressed as mean±SD and p-value<0.05 was considered statistically significant using one-way ANOVA followed by Tukey's post hoc test was used.

RESULTS AND DISCUSSION

Fasting blood sugar profile across four weeks

FBS levels were significantly ($p<0.0001$) higher in D-sole group compared to controls across all experimental weeks. At week 1, mice in the D-sole group exhibited FBS levels exceeding 350 mg/dl, which remained high until

week 4, whereas control mice remained at levels below 120 mg/dl. High-dose Tannin in non-diabetic mice did not change the FBS levels compared to the control group. FBS values following treatment with HDT, LDT and metformin in diabetic groups did not differ significantly during the first week. However, FBS was significantly reduced in treated-hyperglycemic mice relative to untreated diabetic mice from week 1 onwards. Glucose levels were remarkably reduced in week 2 after treatment with HDT ($p<0.001$), LDT ($p<0.01$) and Metformin ($p<0.01$). It was reduced in time-dependent manner at all treated groups reaching 180 mg/dl by week 4 and was significant ($p<0.0001$) compared to the D-sole group. No significant difference was observed among the treated groups at all time periods. The data indicate persistent hyperglycemia in untreated diabetic mice and demonstrate a significant, time-dependent hypoglycemic effect of tannin at both low and high doses, representing the anti-hyperglycemia effect of tannin (Fig 1).

Tannins moderated stress-associated markers

Compared to control group, cortisol ($p<0.0001$) and noradrenaline ($p<0.001$) levels were significantly elevated. When low or high dose of tannin used, tannin has efficiently mitigated cortisol and noradrenaline levels versus diabetes ($p<0.0001$) group without tannin. For noradrenaline, this reduction was significantly ($p<0.001$)

higher in the D+HDT group even when compared to D+M group. Compared to control group or D+M group, both HDT ($p<0.0001$) and LDT ($p<0.001$) significantly restored Ach levels to nearly normal values, reflecting that tannin induced neuroprotective modulating neurotransmitter levels in diabetic patients (Fig 2).

Neurohistoprotective effects of tannin treatment

H and E staining analyses revealed distinct histopathological alterations in hippocampus sections of D-sole mice compared to the control group. In the CA1 and CA2 regions, the D-sole group demonstrated marked neuronal damage, characterized by pyknotic nuclei (PN), vacuolation (Vac) and disorganized pyramidal cell layers (DO). Additionally, perivascular edema (Ed) and thinning of the pyramidal layer (ThL) were evident.

The dentate gyrus (DG) of the D-sole group displayed increased pyknotic nuclei. Also, decreased cell population was detected in the dentate gyrus area in this group. The HDT non-diabetic mice demonstrated histological features similar ($p>0.05$) to those in the control group (Fig 3A). Quantitative scoring confirmed significant increases in pyknotic nuclei in both CA1 and CA2 regions of diabetic mice compared to controls ($p<0.0001$).

Similarly, vacuolation and disorganized pyramidal cell layer scores were remarkably higher in both CA1 and CA2 of diabetes ($p<0.0001$). Perivascular edema was also

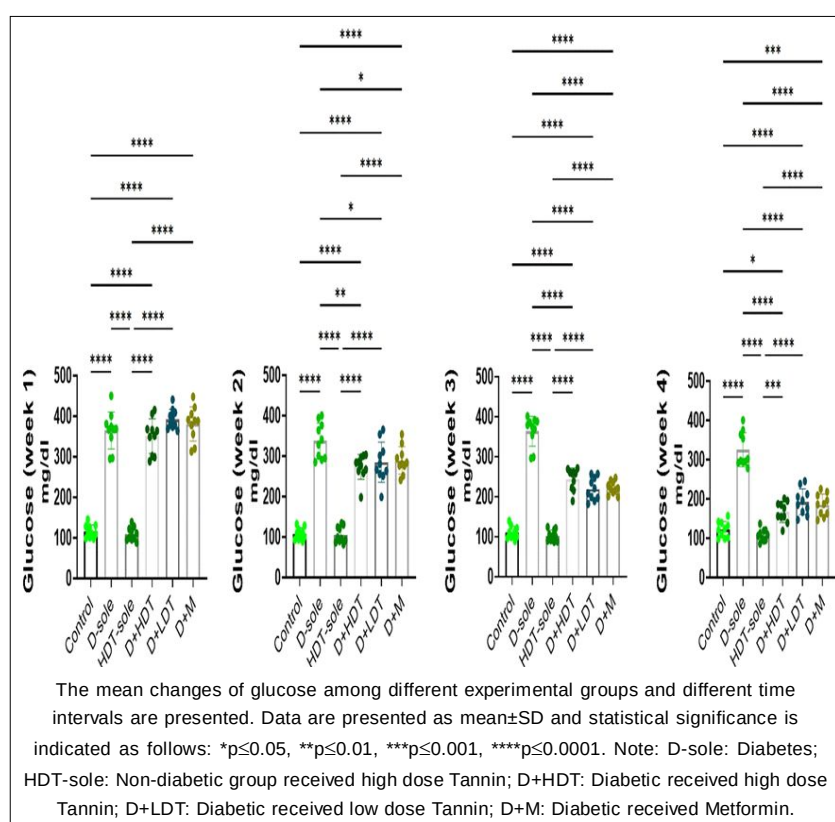


Fig 1: Effects of tannin and metformin treatments on glucose levels under diabetic conditions during the 1st, 2nd, 3rd and 4th weeks.

significantly increased in the D-sole group ($p<0.05$), these markers were attenuated in LDT, HDT and D+M groups. The quantitative scoring showed that the neuronal damages in the CA1 region, including PN (D+M vs D+sole: $p<0.01$; D+LDT and D+HDT vs D+sole: $p<0.001$), DO and Vac, were significantly ($P<0.0001$) decreased in LDT, HDT and D+M groups in comparison with those in the D-sole group. Similarly, the histopathologic alterations such as PN and DO were attenuated in the CA2 region (D+M and D+HDT vs D+sole: $p<0.0001$; D+LDT vs D+sole: $p<0.01$). However, reductions in CA2 vacuolation and perivascular edema observed in the treated groups were not statistically significant. Regarding the dentate gyrus pyknotic nuclei, both LDT and HDT (LDT: $p<0.01$; HDT: $p<0.05$) and D+M ($p<0.001$) decreased the pyknosis relative to the D-sole group, reflecting that diabetes induces severe histopathological damage in hippocampal sub-regions and tannin supplementation could ameliorate neuronal injury and structural disorganization (Fig 3B).

Tannin-mediated neuropreservation-nissl staining

Compared to control group, Nissl body-positive hippocampus neurons significantly ($p<0.0001$) reduced in diabetic group reaching down to 20%, reflecting severe neurodegeneration induced by diabetes mellitus. This neurodegeneration was successfully blocked by tannin ($p<0.0001$ vs diabetes), particularly THD group which preserved normal Nissl deposition comparable ($P>0.05$) to controls. Similarly, M+D and LDT group have also maintained

neuronal Nissl body preservation (Metformin: $p<0.01$ and LDT: $p<0.05$ vs diabetes). These results reflect that tannin supplementation protected against diabetes induced neurodegeneration in dose dependent manner (Fig 4).

Tannin attenuation of hippocampus neuronal apoptosis

TUNEL staining of D-sole revealed a marked ($p<0.0001$) increase in TUNEL-positive cells, indicating a significant elevation in neuronal apoptosis compared to the control group, reflecting that diabetes induced extensive apoptotic labeling in the hippocampus of diabetic mice, consistent with diabetes-induced neuronal degeneration. The HDT alone showed a distribution of TUNEL-positive cells comparable to that of the control group, suggesting no cytotoxic or pro-apoptotic effect of tannin itself. Furthermore, the findings of D+M group ($p<0.0001$), LDT ($p<0.0001$), or HDT ($p<0.0001$) markedly reduced the number of TUNEL-positive cells compared to D-sole group. Among the treated groups, HDT exerted the strongest anti-apoptotic effect compared to LDT ($p<0.001$) and D+M ($p<0.01$), with apoptotic cell numbers approaching control values. These findings indicate that tannin supplementation, particularly at higher doses, effectively protects hippocampal neurons against diabetes-induced apoptosis, demonstrating potent neuroprotective potential comparable to or exceeding that of metformin (Fig 5).

Tannin spleen protection after diabetes

Stained by H&E, the spleen sections from control and HDT-non-diabetic revealed well-preserved splenic

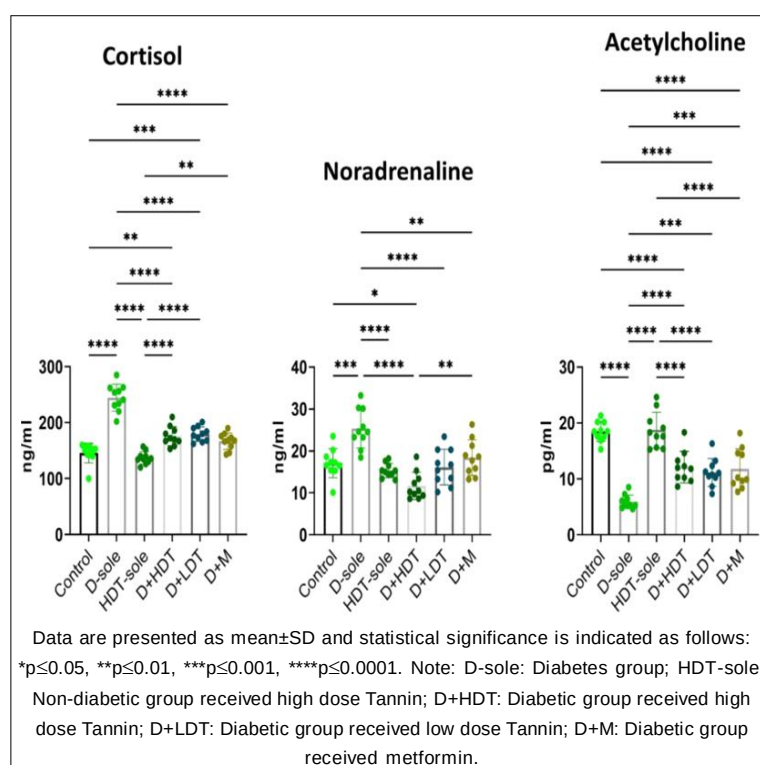


Fig 2: The mean changes of cortisol, noradrenaline and acetylcholine levels among different experimental groups are presented.

architecture characterized by distinct white pulp (WP) and red pulp (RP) regions. The spleen sections from D-sole mice and LDT-diabetic groups demonstrated enlargement and hypercellularity in the WP, indicating an intense immune response. The D+M and HDT have demonstrated relatively preserved tissue architectures with reduced evidence of hypercellularity compared with untreated diabetic mice.

Stained by Masson's trichrome, the spleen sections demonstrated normal collagen distribution within the splenic parenchyma in all groups, indicating that the observed architectural changes were not associated with fibrotic remodeling (Fig 6A).

Tannin maintained normal T cells distribution

The results of immunofluorescent staining of spleen sections revealed that CD4⁺ and CD8⁺ T in the control and HDT in non-diabetic mice were similarly distributed pattern. However, the immunoreactivity of CD4⁺ and CD8⁺ T were highly increased on diabetes group reflecting increased infiltration into the WP and RP regions. The CD4⁺ and CD8⁺

cells remained high in the LDT and D+M groups, respectively. However, CD4⁺ T cells slightly decreased in the D+M group, whereas in TLD group CD8⁺ T-cells were mildly reduced compared to the D-sole group.

The elevated T-cell density was particularly prominent in the D-sole, LDT and D+M groups, suggesting an inflammatory or immune-activated state. In contrast, the HDT group demonstrated a more balanced distribution and lower density of CD4⁺ and CD8⁺ T cells mimicking control pattern, reflecting the efficacy of tannin attenuation of splenic immune activation (Fig 6A). The software analyses of the green fluorescent intensity representing the CD4 and CD8 presence and distribution, have confirmed these observations. The histogram showed elevated CD4 and CD8 values in the D-sole group in comparison with the control group. (Fig 6B, C).

The results revealed that tannin regulate glycemic control comparable to that of metformin, this euglycemic effects may be attributed not only to its enzymatic inhibition and insulin-sensitizing effects but also to its potent

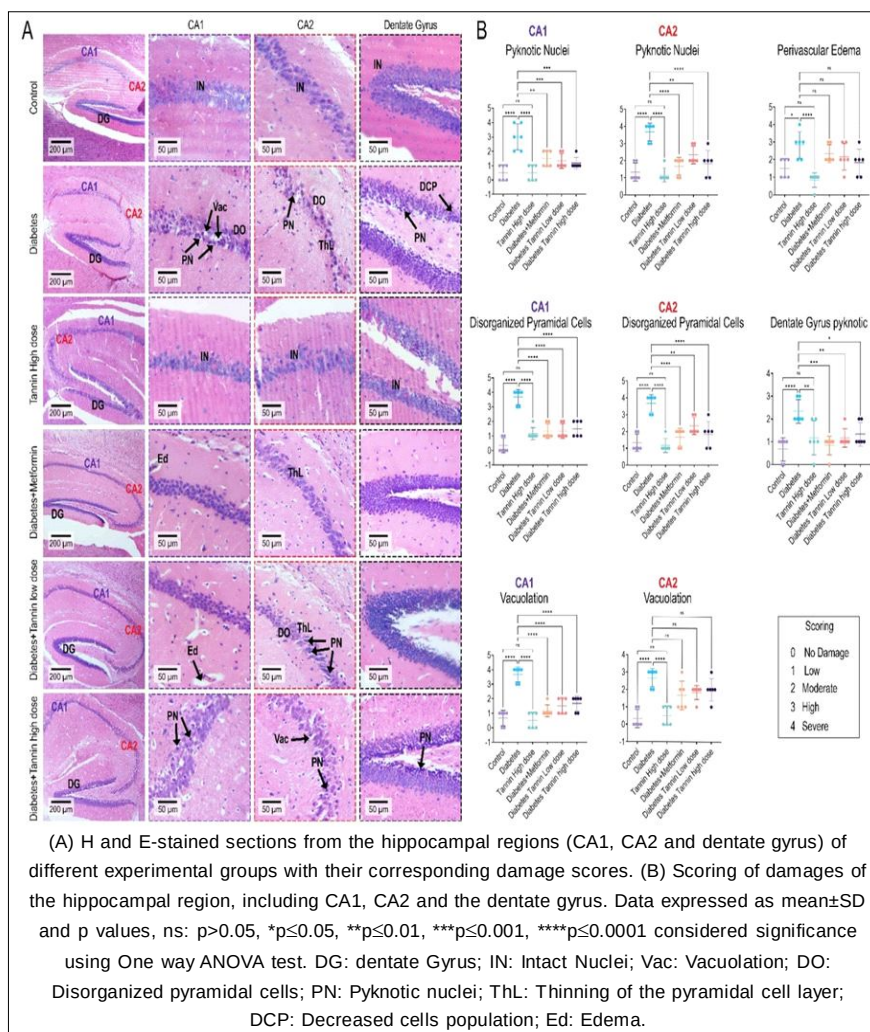


Fig 3: Tannin and metformin protection against diabetes-induced histopathological alterations of the hippocampus region.

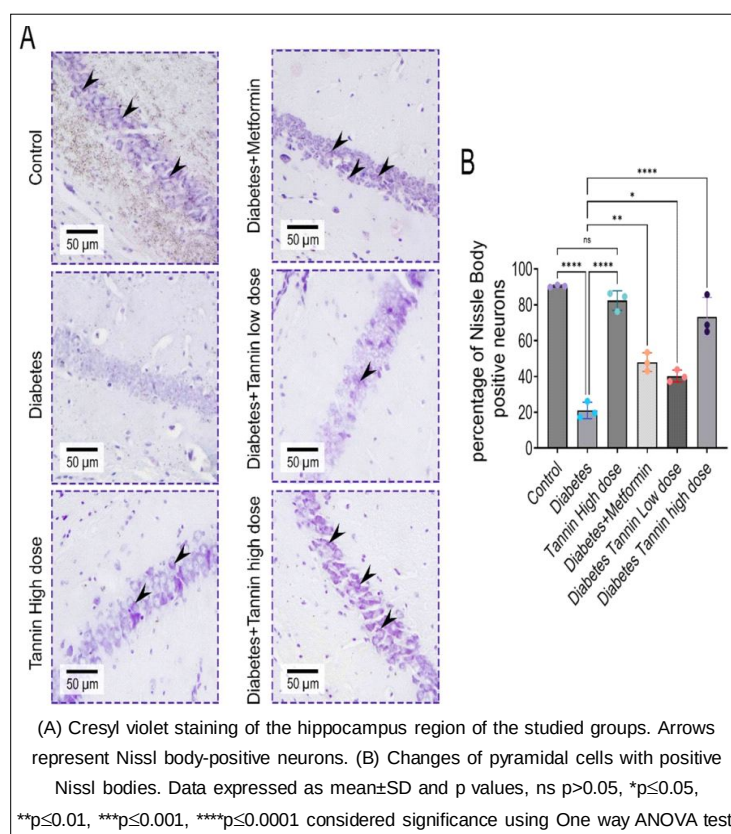


Fig 4: Tannin and metformin protection against diabetes-induced positive nissl body of the hippocampal pyramidal cells.

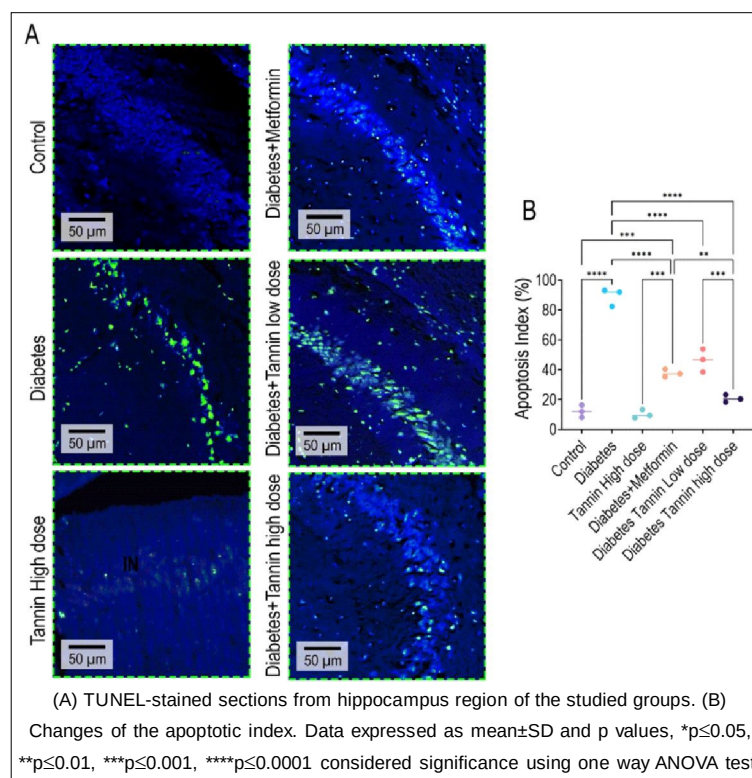


Fig 5: Tannin and metformin protection against diabetes-induced apoptosis of the hippocampal pyramidal cells.

antioxidant and anti-inflammatory properties, which alleviate oxidative stress-induced insulin resistance (Lahrizi *et al.*, 2024; Sahakyan *et al.*, 2022). Tannin-rich enhanced peripheral glucose uptake and improve insulin sensitivity in diabetic rodents, leading to reduced systemic glucose levels (Barik *et al.*, 2025; Sahakyan *et al.*, 2022). In our STZ-induced diabetic model, metformin effectively lowered fasting blood glucose levels, this antidiabetic effect is largely mediated through AMPK activation, leading to inhibition of hepatic gluconeogenesis and enhancement of peripheral glucose uptake and insulin sensitivity (Goel *et al.*, 2022).

Hyperglycemic increased cortisol and noradrenaline levels, exacerbating insulin resistance and contribute to metabolic dysfunction (Mosili *et al.*, 2024), triggering oxidative stress and neuroinflammation (Khan *et al.*, 2024), promoting gluconeogenesis, oxidative stress and inflammation (Knezevic *et al.*, 2023), creating a cycle that worsens metabolic dysregulation and neuronal vulnerability. Tannin used attenuated diabetes-induced elevation of cortisol and noradrenaline, with the amelioration

effect on elevated noradrenaline. Importantly, the HDT exhibited a greater reduction in noradrenaline levels than the metformin-treated group, highlighting tannins as promising multifunctional agents with superior efficacy to metformin in restoring neuroendocrine balance and mitigating diabetes-related stress responses.

The present study demonstrated that diabetes reduced acetylcholine levels, this finding aligns with earlier studies showing that chronic hyperglycemia impairs acetylcholine synthesis and accelerate acetylcholinesterase activity, leading to deficits in cholinergic signaling and cognitive dysfunction in diabetic models (Benloughmari *et al.*, 2025; Otsuka *et al.*, 2024). Previous studies have shown that increased cortisol negatively affects the hippocampus region of the brain (Jie *et al.*, 2025; Platero *et al.*, 2021) and the observed neurochemical alterations, the study also evaluated histopathological changes in the hippocampus region of the brain, including neuronal morphology and Nissl body integrity, to assess structural correlates of diabetes-induced damage and tannin-mediated neuroprotection.

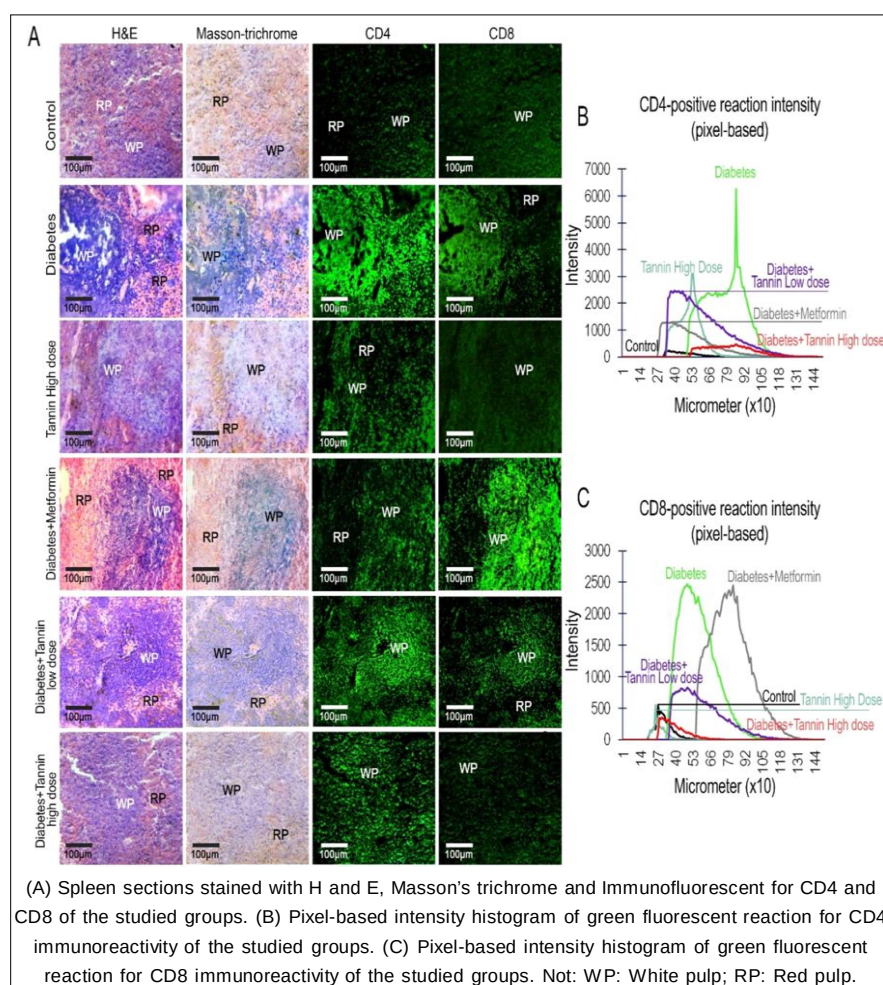


Fig 6: Tannin and metformin protection against diabetes-induced histological and immunological alterations of spleen.

Histological findings demonstrated that hyperglycemia induced marked neuronal degeneration (pyknotic nuclei, vacuolation and disorganized pyramidal layers) in the hippocampal CA1, CA2 and dentate gyrus regions, confirmed by increased neuronal apoptosis via increased TUNEL-positive cells in the hippocampus region, reflecting extensive DNA fragmentation and neuronal apoptosis as a hyperglycemia-induced neurodegeneration (Gupta *et al.*, 2023). The LDT and HDT have blocked degenerative alterations and protected hippocampal structure alongside restored protein synthesis indicated by Nissl bodies recovery from diabetic damage in comparable activity with metformin (Bhati *et al.*, 2023) and reduced TUNEL-positive pyramidal cells, reflecting vital neuronal protection (Gorman, 2008), the protection was tannin-dose dependent, perhaps the mechanism is linked to enhanced antioxidant defenses through Nrf2 pathway activation and inhibition of NF- κ B-mediated inflammation (Sahakyan *et al.*, 2022).

In order to find out the contribution of systemic immune alterations associated with diabetes, the spleen was assessed, reflecting systemic inflammatory role in diabetes (Alahmari and Al-Doaiss, 2025). The LDT and HDT restored normal splenic structure with minimal hyperplasia in dose dependent manner. This improvement congruent with earlier study of polyphenols anti-inflammatory effects in chronic conditions such as diabetes (Yahfoufi *et al.*, 2018). Metformin-treated mice also showed protected spleen structure, reflecting the regulatory effect of metformin on glucose homeostasis (Raj and Rani, 2024).

The HDT or LDT-treatment restored immunoregulatory status by restoring nearly normal CD4⁺ and CD8⁺, normalizing of immune response, while CD4⁺ and CD8⁺ T-cell increased in diabetic mice, this increment could be secondary to the diabetes induced increased cortisol (Knezevic *et al.*, 2023). This immunomodulatory effect could be explained based on previous findings that tannin-rich extracts inhibit NF- κ B signaling and downregulate proinflammatory cytokines such as IFN- γ and IL-17, thereby reducing T-cell and derived cytokines (Piazza *et al.*, 2022). The balanced CD4⁺/CD8⁺ ratio observed after high-dose tannin treatment supports its role in re-establishing immune homeostasis and limiting excessive immune activation seen in diabetic pathology. These findings complement the histological observations and highlight Tannin's dual role as both an anti-inflammatory and immunoregulatory compound.

CONCLUSION

Tannin offered protective role against streptozotocin-induced hyperglycemia through metabolic, neurological and immunological pathways. Tannin improved the hyperglycemic-related alterations, improved glycemic control, perhaps reducing systemic oxidative stress, which in turn ameliorated neuronal and splenic damage, evidenced by restored neuronal morphology and Nissl body storage of the hippocampus region, reduced apoptosis index, as well as restored CD4⁺ and CD8⁺ T-cell homeostasis.

normalization of cortisol and neurotransmitters may have further stabilized the neuroimmune axis, creating a feedback loop that reinforces both metabolic and immune regulation.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this research paper.

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