Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i4.6065

FRACTIONS OF *ZIZIPHUS JUJUBA* HONEY ATTENUATES STREPTOZOTOCIN INDUCED HYPERGLYCEMIA MEDIATED COGNITIVE DYSFUNCTION IN ADULT MICE

Zaid Ullah[1*], Asad Ullah[2], Akhtar Muhammad[3], Shahid Ali Shah[4]

[1*, 2, 3]Department of Chemistry, Islamia College Peshawar, Khyber Pakhtunkhwa, Pakistan.
[4]Department of Biology, The University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan.

***Correspondent Author:** Zaid Ullah
*Email: zaid@icp.edu.pk

Abstract

Purpose: There are several evidences showing that hyperglycemic conditions have adverse effects on the brain causing synapse and cognitive deficits along with Alzheimer disease (AD) neuropathology. This study was investigated to examine the neurotherapeutic potential of three different fractions extracted with chloroform (Ch), ethyl acetate (EA) and n-butyl alcohol (n-BuOH)of *Ziziphus jujuba* Honey against Streptozotocin (STZ) induced hyperglycemia mediated cognitive dysfunction in adult mice.

Methods: Male albino mice were randomly placed into five groups i.e. normal mice, STZ treated (90 mg/kg) mice, STZ+Ch, STZ+EA and STZ+n-BuOH mice (30 mg/kg) for a period of three weeks. Different behavioral tests and biochemical analysis such as Morris water maze test, Y-maze test, GTT, total cholesterol, HDL, LDL, VLDL and TGL were performed to observe the effect of these fractions on memory dysfunction induced by STZ.

Results: A single injection of STZ at a dose of (90 mg/kg) administered intraperitonealy induces hyperglycemia followed by the activation of Toll-like receptor 4 (TLR4 receptors) and ultimately phosphorylated NF-kB (p-NF-kB) activation in the brain of adult albino mice. Moreover, STZ administration significantly caused both pre- and post-synapse loss and cognitive decline in adult albino mice. On the other hand, the administration of three different fractions (Chloroform, Ethyl acetate and n-butyl alcohol) of *Ziziphus Jujuba* Honey (30 mg/kg daily for three weeks) significantly decreased STZ induced high blood glucose, negatively modulated TLR4 receptors and inhibiting p-NF-kB activation in the brain of adult albino mice.

Conclusion: Taken together, all the results suggest that these three different fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* Honey are natural, safe and potent agents against hyperglycemic induced cognitive impairment and associated neurodegenerative diseases.

Keywords: *Ziziphus Jujuba* Honey, STZ, Cognitive dysfunction, Chloroform, ethyl acetate, n-butyl alcohol, TLR4.

1. Introduction

Diabetes mellitus (DM) is one of the major and complex metabolisms associated disorder spread throughout the globe and affecting almost 450 million populations. The characteristics of this disorder are long term hyperglycemia, insulin resistance or less or no production of insulin due to damaged *β*-cells of pancreas (Chen et al., 2017). Basically, there are two main types of diabetes mellitus (DM) i.e., type-1 and type-2. Both are very different from each other in clinical presentation and disease progression (Chen et al., 2017). Type 1 diabetes is mostly present in non-adult child and adults. This disease is an autoimmune featured with damaged *β*-cells of pancreas by action of both lymphocytes and macrophages leading to hyperglycemia, usually results lower down the insulin (Barcala et al., 2013). Various animal models have been reported to mimic diabetes in human both types to examine and understand the pathophysiology of disease. In this regard Streptozotocin (STZ) has been reported to produce conditions like type 2 in the experimental animals such as rats or mice. Accumulating evidences reported that diabetes certainly damages central nervous system (CNS) (Liu et al., 2013). So, both Alzheimer disease (AD) and DM shares some key features such as oxidative stress and neuroinflammation, accompanied by changed cell signaling pathways, and these are the well-known mechanisms brings damages to the brains (Alfaris et al., 2021).

Honey is the main historical product of apiculture to the mankind. Its importance and drug like features are already documented in major religions and civilization throughout the world benefits of honey have been indicated in every culture and religion of the world (Wootton et al., 1978; Grossman 1985). Its importance and therapeutic characteristics are due to inclusion of different biological and pharmacological active ingredients. It has been in several reports previously that honey is an ample source of more than 200 substances such as mainly sugars in solid form, contains enzymes, vitamins (Riboflavin, Niacin, Pantothenic acid, Pyridoxine, Folate, flavonoids, polyphenols, amino acids, Proteins, organic acids, carotenoids and minerals (White et al., 1957a; White et al., 1957b; Wilson et al., 2011; Eteraf et al., 2013) are the major vitamins present in the honey. *Ziziphus jujuba*, belongs to Rhamnaceae family is a medicinal plant known for traditional herbal medicine and its various parts such as fruits, seeds, and leaves having anti-inflammatory and neuroprotective abilities historically used for treating several diseases including typhoid fever, furuncle, sleep disorders, diarrhea, and pain (Misra et al., 1972; Smith et al., 1987; Zaia et al., 2005; Tsikas 2007; Kandeda et al., 2017).

To date, there is no study on the diabetes induced neuroprotective effects of different fractions (Chloroform, Ethyl acetate and n-butyl alcohol) of *Ziziphus Jujuba* Honey caused by STZ in mice, therefore, in the current study we have used STZ induced hyperglycemia mediated cognitive dysfunction in adult mice.

2. Materials and Methods

2.1. Chemicals

STZ (Streptozotocin), PBS (Phosphate Buffer Saline) tablets [Cat. No; P4417-50TAB], SDS [Sodium Dodecyl Sulphate], Acrylamide, Bisacrylamide, Ammonium per Sulphate (APS), Tris base, Sodium Chloride, Potassium Chloride, were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Daejung Chemicals & Metals Co. Ltd (Gyeonggi-do, Shiheung, South Korea) respectively.

2.2. Fractionation of Honey

Ziziphus jujuba honey (1kg) was suspended in water and extracted with chloroform, ethyl acetate and N-butyl alcohol using separating funnel to obtain the three fractions.

2.3. Mice and Their Grouping

During this study adult male albino mice (8 weeks old) were randomly placed into five groups as given below. These mice were purchased from VRI (Veterinary Research Institute), Peshawar,

Pakistan and brought to Neuro Molecular Medicines Research Center (NMMRC, Peshawar). Then the mice were allowed to acclimatize to the environment. Mice were separately placed in their respective cages (Biobase China). Their grouping was,

1. Normal mice
2. STZ treated mice (90mg/kg)
3. STZ (90mg/kg) + Chloroform extract treated mice (30mg/kg)
4. STZ (90mg/kg) + Ethyl acetate extract treated (30mg/kg)
5. STZ (90mg/kg) + N-butyl alcohol extract treated (30mg/kg)

The male mice (30–32 g average body weight) were placed in the breeding room with a 12/12-h light/dark cycle at 25 ±°C temperature, provided with water and food ad libitum.

2.4. Behavioral Tests

To examine the positive effects of three different fractions (Chloroform, Ethyl acetate and n-butyl alcohol) of *Ziziphus Jujuba* Honey on memory dysfunction induced by STZ, behavioral tests were performed. STZ was intraperitonealy (i.p.) injected to the adult albino mice with or without honey fractions. All the animals were randomized into four groups, and the person conducting the behavioral testing was totally blinded to the treatment groups during the course of the experiment.

2.4.1. Morris Water Maze Test

The Morris water maze (MWM) test was conducted to investigate the hippocampal-dependent long-time spatial learning abilities. The mice were first trained twice a day for 3 days. Then the escape latency of the mice to find the submerged platform was noted for 60 seconds. In case of failure of the mice to find the platform, the mice were manually directed to the platform and stay there for 10 sec. This practice was continued until day 5, and every day has its own data (seconds) for the three different experimental groups. After giving them rest for 2 days, the mice were allowed for probe test to find the platform which was hidden and their time spent in the target quadrant was noted.

2.4.2. Y-Maze test

The Y-maze test was conducted as reported (Kraeuter A. K. et al., 2019). Y-maze is an apparatus having 3 arms of 50 x 10 x 20 (cm^3L x W x H) lying at angle of 120^0. Every time mice were allowed for 10 minutes to adjust to the new environment. Then the mice were allowed to explore the maze for 8 minutes by keeping it in the center of the maze. The mice total arm entries and successive number of triplets were noted by software, and the percentage of alternations was calculated by the formula as [successive triplet sets/total number of arm entries-2] x 100. The percentage of alternations was positively correlated with spatial working memory function.

2.5. Glucose Tolerance Test (GTT)

To conduct GTT the mice were kept on fastening for 6-8 hrs, and their blood glucose level was checked indicating as at zero time. Then 200 mg/kg dextrose was injected to all the five groups and then their blood glucose level was analyzed with the help of glucometer after a regular interval of 15, 30, 60, 120 and 180 minutes respectively.

2.6. Biochemical analysis of Serum

Once the drug treatment was finished the animals were sacrificed and blood collected was used for biochemical analysis i.e., total cholesterol, HDL, LDL, VLDL and TGL.

2.7. Western Blotting Analysis

To conduct western blot analysis the mice were sacrificed as reported previously (Mahmood, T., & Yang, P. C. 2012), after completion of treatment. The whole mice brains were quickly collected, and then the hippocampus part was separated with great care and dipped in RNA later solution with PBS in 1:1 on ice. Then the hippocampus part was homogenized in tissue extraction protein reagent T-

PER (Thermo Scientific) solution. Bio-Rad protein assay tests were conducted to note the absorbance at 595 nm, in order to analyze the protein concentration. Every sample proteins were normalized to 30 µg/ group and 12-15% SDS PAGE electrophoresis gel was performed. Then the proteins were transferred to PVDF membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA) through semi dry transblot (Bio Rad). Different primary antibodies such as mouse derived (anti-actin, anti-PSD95, anti-SYP, anti-TLR4 and anti-p-NF-kB) monoclonal antibodies from (Santa Cruz, CA, USA) followed by HRP conjugated anti-mouse (Santa Cruz, CA, USA) secondary antibody were employed. Then the results were developed on the X-rays.

2.8. Statistical Analysis

All the X-rays of results were scanned and compiled and their statistical analyses were done through specified computer-based software. It includes image J, Prism 5 graph Pad, Adobe Photoshop etc. The density of proteins is expressed in arbitrary units (A.U.s) as the mean ± S.E.M. $^{\#}$significantly different from normal saline treated and STZ significantly different from STZ treated mice and honeyfractions, respectively; *, **, ***, #, ##, ### $P < 0.01$.

3. Results

3.1. Fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* Honey reduced STZ-induced hyperglycemia and hypercholesterolemia in adult Albino Mice

A single dose of STZ caused hyperglycemia in mice; the blood of all experimental animals was examined randomly. These findings demonstrate that STZ considerably raised the random blood glucose level of these animals (Fig. 1A). Additionally, STZ also suppressed their capability to tolerate the exogenously administered glucose (Fig. 1B) that they were supplied during the glucose tolerance test (GGT). These findings indicate that STZ may lead to diabetes in mice followed by hypercholesterolemia, which is characterized by elevated levels of triglycerides, LDL, VLDL and relatively low levels of HDL in mice's blood. However, different fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of Ziziphus Jujube Honey significantly decreased random as well as GTT elevated blood sugar levels and STZ induced hyperglycemia and hypercholesterolemia in adult albino mice (Fig. 1A-G).

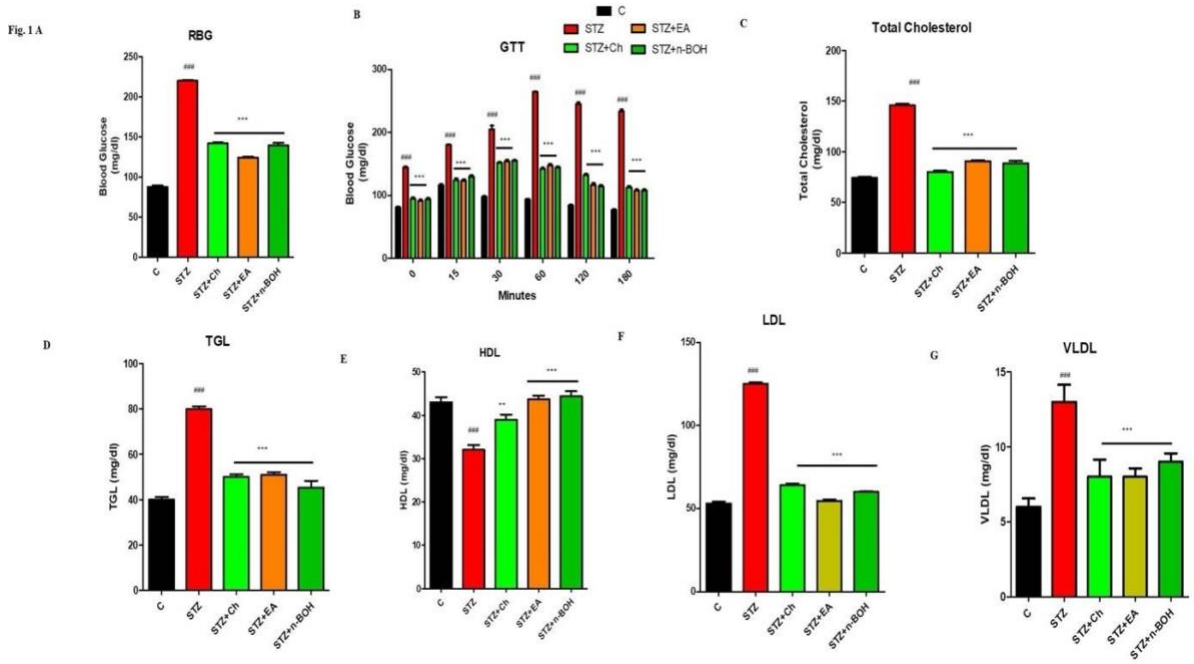

Fig. 1: Honey fractions reduced hyperglycemia in adult mice.

Shown are the results of **(A)** random blood glucose **(B)** GTT **(C)** total cholesterol **(D)** TGL **(E)** LDL **(F)** VLDL and (**G**) HDL in the serum of experimental animals. These experiments were replicated three times. Significance of control vs STZ is expressed as #, while * denotes STZ vs STZ plus Honey Fractions $^{**, \#\#}p\leq0.01$ & $^{***, \#\#\#}p\leq0.001$.

3.2. Fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* Honey improved STZ induced synaptotoxicity in adult albino mice

Elegant investigations have shown that STZ intoxication is quite likely to occur at both pre- and post-neuronal synapse. Both pre-synapse proteins (synaptophysin-SYP) as well as post-synapse density protein (PSD95) were determined using the immunoblot technique. The western blot analysis data shown in figure 2A indicate that STZ injection suppressed pre- as well as post-synapse protein in adult male mice brain. We employed different fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* honey to determine its protective effects on the neural synapse and the outcomes demonstrated that HSM greatly increased the expression of protein SYP as well as PSD95in the homogenized samples of mice brain figure 2A-C.

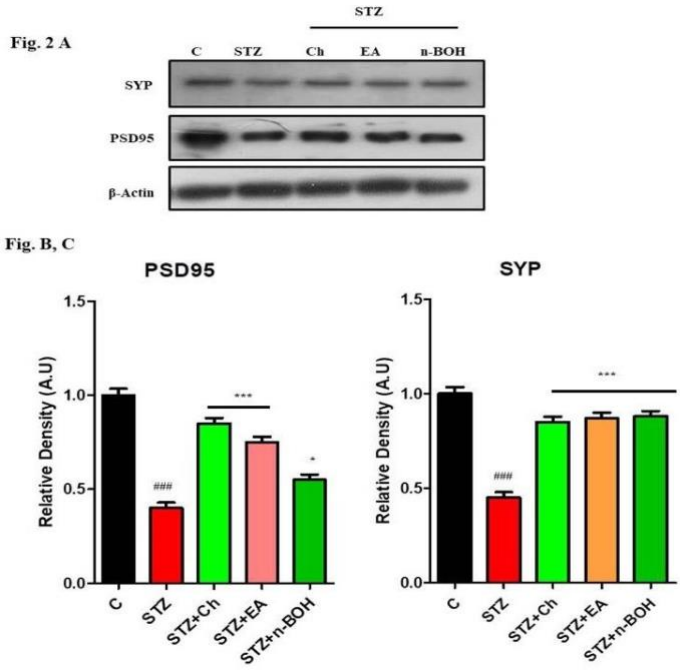

Fig. 2: Honey fractions improved both pre and post synapse against STZ in mice

Shown are the immunoblot of **(A)** SYP and PSD95 along with their respective relative densities histograms **(B, C).** The results were expressed in arbitrary unit (A.U) and were determined using Image J software and histogram indicates mean in A.U ± SEM. Significance of control vs STZ is expressed as #, while * denotes STZ vs STZ plus Honey Fractions $^{**, \#\#}p\leq0.01$ & $^{***, \#\#\#}p\leq0.001$.

3.3. Fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* Honey improved STZ induced memory and behavior impairment in adult albino mice

Studies suggests that STZ as the cause of behavior and memory impairment in animal model. All the mice injected with STZ, STZ plus honey fractions as well as control group were subjected to two most common behavior tests, the Y-maze test and Morris water maze test (MWM). During the MWM test, the mice received two days training twice a day. Following a one-day rest, mean escape latencies of mice were measured over the course of five days. According to daily observations, control mice were found to have much lower mean escape latencies from day 1 to day 5 and that these animals' mean escape latencies were consistently dropping as shown in figure 3A.

The STZ induced mice exhibited higher mean escape latencies from day 1 to day 5. The mean escape latencies were on the higher side, despite a slight decrease from day 1 to day 5 in those latencies. It's interesting to note that animals treated with honey fractions have significantly decreased mean escape latencies between day 1 and day 5. Even though honey fractions treated mice improves behavior and lowers mean escape latencies from day 1 to day 5, their escaping times were remained longer than those of the control animals as seen in figure 3A.

The mice received one day rest and were then subjected to probe test wherein the platform was kept hidden. The mice were permitted to locate the hidden platform and the time duration spent in the target area was recorded. The animals of control group remained in the target quadrants for a longer period of time than the STZ-treated animals, which stayed there for a much shorter period of time asreflected by. In contrast, honey fractions-treated animals spent a considerably longer duration in the target quadrant but as a whole they remained for less period of time than the control animals as shownin the figure 3B.

Finally, the Y-maze test was performed on these animals and the % age at which spontaneous alternation occurred was documented which depends on the spatial memory. The control mice in the Y-maze test were again found to have a high % age of spontaneous alteration whereas mice treated with STZ spent considerably short period of time as indicated in the figure X. On the other hand, honey fractions and STZ treated mice in the Y-maze test demonstrated a significant % age of spontaneous alteration as shown in the given figure 3C.

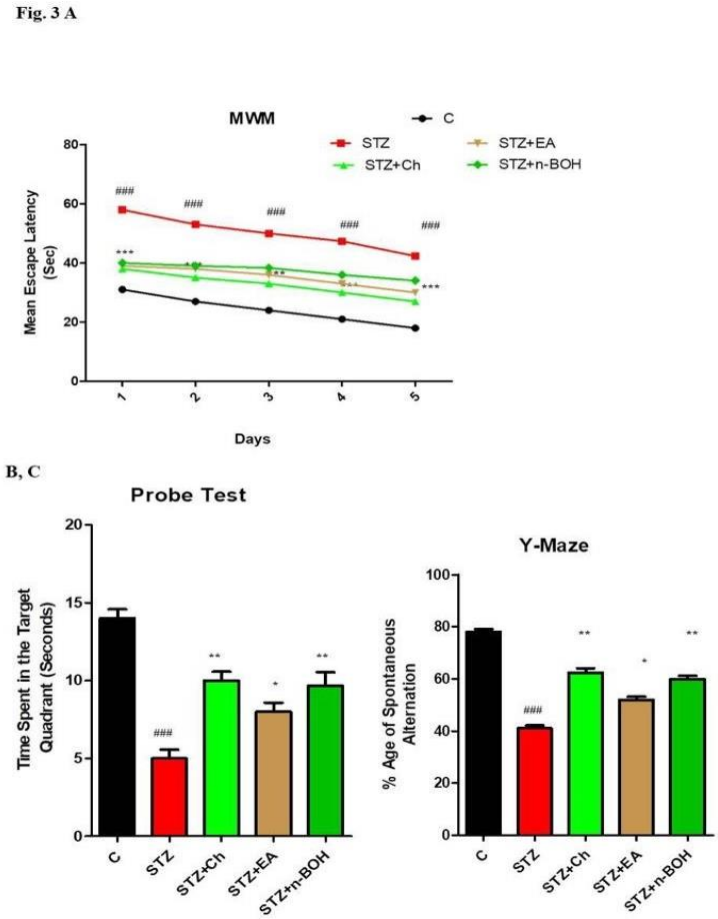

Fig. 3: Honey fractions restored memory dysfunction against STZ in mice

Shown are results of behavioral tests are given as **(A)** mean escape latency in MWM **(B)** probe test and **(C)** %age spontaneous alteration in Y-Maze test. Significance of control vs STZ is expressed as #, while * denotes STZ vs STZ plus Honey Fractions $^{**, ##}p\leq0.01$ & $^{***, ###}p\leq0.001$.

3.4. Fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* Honey negatively modulated TLR4 to inhibit NF-kB against STZ in adult albino Mice

All experimental animals' brain homogenates were analyzed using western blot method. Previously it has been reported that hyperglycemia leads to elevate TLR4 and p-NF-kB protein expression. Similarly, in the current study our immunoblot results also reveal that STZ significantly induced TLR4 receptor activation in adult albino mice brain. These events ultimately enhanced the expressionof NF-kB as shown in the image 4. On the other hand, different fractions (Chloroform, Ethyl acetateand N-butyl alcohol) of Ziziphus Jujube Honey significantly inhibited TLR4 receptor activation and p-NF-kB protein in adult albino mice. The proposed mechanism of Different fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of Ziziphus Jujube Honey against STZ is shown in the figure 4A-C.

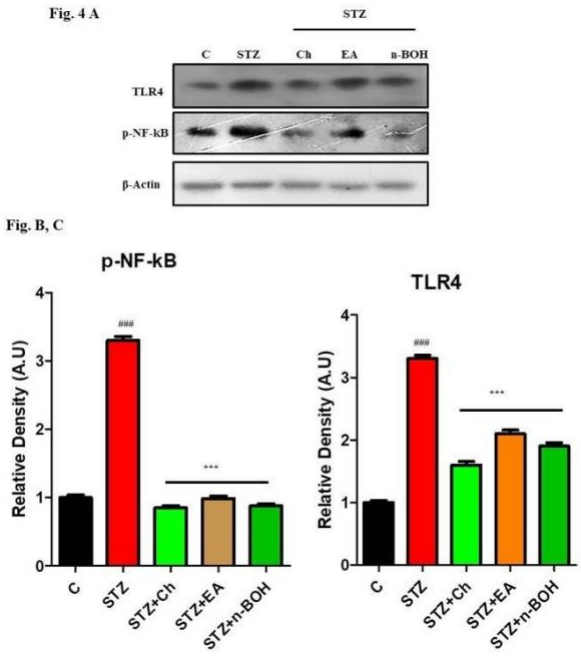

Fig. 4: Honey fractions inhibited TLR4 receptors proteins to abrogate STZ induced neuroinflammation in mice.

The immune-blot analysis of **(A)** TLR4 and neuro-inflammatory marker NF-kB along with histograms of respective relative densities is shown in **(B-D)**. β-Actin was used as the loading control with both. The results were expressed in arbitrary unit (A.U) and were determined using Image J software and histogram indicate mean in A.U ± SEM. Significance of control vs STZ is expressed as #, while * denotes STZ vs STZ + Honey fractions. $^{**,\,\#\#}p\leq0.01$ & $^{***,\,\#\#\#}p\leq0.001$.

4. Discussion

Current research reveals for the first time that different fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* Honey exhibits neuroprotective potential in an animal model of STZ-mediated hyperglycemia. During the study, it is found that different fractions (Chloroform, Ethyl acetate and N-butyl alcohol) of *Ziziphus Jujuba* honey can ameliorate STZ induced hyperglycemia mediated neuroinflammation. Moreover, it can also restore memory dysfunction as well as both pre- and post-synapse in adult male albino mice. Most interestingly it is to note that honey fractions in the current work negatively modulated TLR4 receptors accompanied by the inhibition of p-NF-kB proteins to limit the neuroinflammatory burden against STZ in adult albino mice.

Neurological diseases are now become one of the big issues health these days to human beings. The neurological disorders burden enhanced suddenly not only in the countries belong to third-world but also throughout the world (Carroll et al., 2019). These diseases include Alzheimer's disease (AD),

Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), and stroke damages main sites of the brain. The main reason of the brain damage is the burden of oxidative stress induced by the aggregation of free radicals and decline of antioxidants (Salim 2017). The main reason of neuronal death protection is the increasing the levels of antioxidants can be effectual against these neurodegenerative diseases (Hamd et al., 2009). In this regard honey contains various antioxidants such as polyphenols and flavonoids. Almost all types of honey contain these bioactive phytochemicals in different ranges. Both these phytochemical including polyphenols and flavonoids rescue neurons against oxidative stress and reduce much neuronal neurotoxicity by altering several signaling pathways (Ramezani et al., 2016).

Most importantly we have observed in the current study that the three different fractions of *Ziziphus Jujuba* honey significantly improved the neuronal synapse and memory and cognitive decline in the brains of mice as administration of STZ impaired it by increasing hyperglycemia. Our both MWM and Y-maze results shows that three different fractions of *Ziziphus Jujuba* honey restored the cognitive deficits by overcoming the hyperglycemia, hypercholesterinmia and hyperlipidemia. In most of the honey present flavonoids such as Myricetin and Kaempferol, and the studies have shown that both are neuroprotective and improving the memory. Myricetin restored the dysfunctions of cognition and enhanced the memory against STZ-induced animal AD models [18]. Administration of myricetin attenuates the damage induced by oxidative stress in the hippocampus part of experimental mice. This is because it increased the activities of antioxidative enzymes (Wang et al., 2017). Additionally, myricetin shown anti-inflammatory potential by inhibiting NF-κB followed by the inhibition of NLRP3 inflammasome complex. Similarly, Kaempferol present in the honey ameliorates cognitive decline by reducing oxidative stress and neuroinflammatory markers in model of dementia. (Chen et al., 2019; Chanput et al., 2016).

Fig. 5

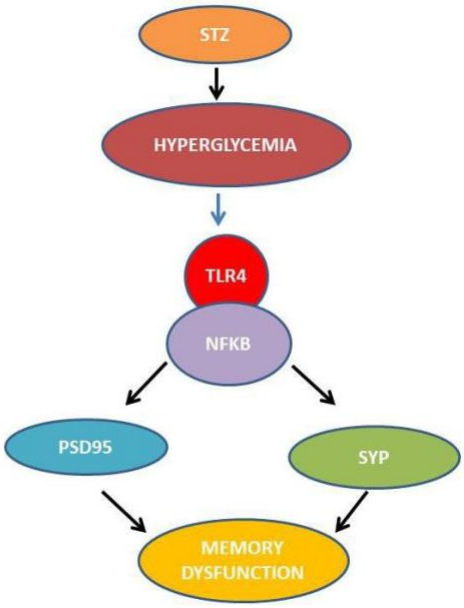

Fig. 5: The proposed mechanism of Honey fractions against STZ induced hyperglycemia mediated memory impairment in adult mice brain.

The signaling pathway depicts the proposed mechanism of honey fractions against STZ induced hyperglycemia mediated cognitive decline in adult mice brain. It shows that how honey fractions in a TLR4/NF-kB dependent mechanism rescued adult mice brain against STZ mice model.

5. Conclusion

In short, this study reveals that three different fractions of *Ziziphus Jujuba* honey significantly attenuated STZ induced hyperglycemia induced neuroinflammation. Moreover, we have also shown

that three different fractions of *Ziziphus Jujuba* honey reduced the STZ induced damaging effect on the neuronal synapse and memory deficits in animal model. A very in-depth study is warranted to know the mechanism of neuroprotective ability of three different fractions of *Ziziphus Jujuba* honey in neurodegenerative diseases.

Statements & Declarations

Competing Interest

All authors declare they have no financial interest.

Consent to Publish

All authors agreed to publish this article.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

Not applicable

Availability of data materials

All data generated or analyzed during this study are included in this published article.

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