# INVESTIGATION OF THE EFFECT OF STINGING NETTLE EXTRACT ON NEUROLOGICAL ALTERATIONS DURING COMORBIDITY OF DEPRESSION AND TYPE 2 DIABETES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

**THE DEGREE OF DOCTOR OF PHILOSOPHY**

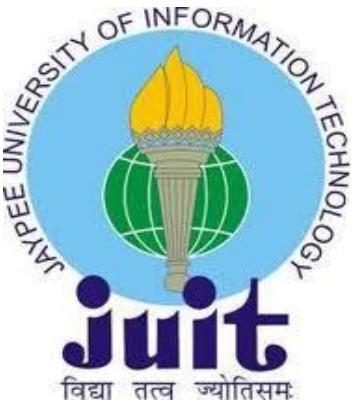
**IN**

**PHARMACEUTICAL SCIENCES**

**BY**


## SITA SHARAN PATEL

**ENROLLMENT NO. 116753**


**UNDER THE SUPERVISION OF**

**DR. MALAIRAMAN UDAYABANU**


**DEPARTMENT OF PHARMACY**
**JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY**
**WAKNAGHAT, SOLAN**
**HIMACHAL PRADESH-173234, INDIA**


**MARCH 2016**

# CERTIFICATE

This is to certify that the thesis entitled, **"Investigation of the effect of stinging nettle extract on neurological alterations during comorbidity of depression and type 2 diabetes"** which is being submitted by Sita Sharan Patel for the award of degree of Doctor of Philosophy in Pharmaceutical Sciences by the Jaypee University of Information Technology at Waknaghat, is a record of the candidate's own work, carried out by him under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY • WAKNAGHAT • JUIT

**Dr. M. Udayabanu**
Assistant Professor
Department of Pharmacy
Jaypee University of Information Technology
Waknaghat, Solan, Himachal Pradesh,
India- 173234

# DECLARATION

I certify that

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor.

b. The work has not been submitted to any other organization for any degree or diploma.

c. Whenever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

S. Patel.

**Sita Sharan Patel**

# *ACKNOWLEDGEMENTS*

*I deem it my privilege and honor to place and record my gratitude and indebtedness to the following without whose guidance, support and concern I would not have been able to complete my Ph. D thesis.*

*I feel privileged to express my deep sense of reverence and gratitude to my revered mentor,* ***Dr. M. Udayabanu****, for his support, immaculate guidance, constructive criticism, constant encouragement and providing requisite facilities to carry on my research which otherwise would have remained incomplete. His nurturing and caring concern has been a stimulus which I will always cherish. I also appreciate him untiring efforts during the entire tenure of my research work and patience during writing of this thesis. I have no words to express my gratitude for everything he has contributed in my Ph. D and without his blessings it was surely impossible for me to finish my work. I thank him from bottom of my heart.*

*I emphatically express my loyal and venerable thanks to* ***Brig. (Retd.) Balbir Singh*** *(Director, JUIT) for providing opportunity to pursue a Doctorate Degree and advanced lab infrastructure to accomplish this scientific venture of my life. It gives me immense pleasure to express my gratitude to* ***Balbir Sir*** *for his ever smiling disposition coming to my rescue in solving my problems and suggestions which helped me in maintaining my confidence.*

*I am very much grateful to Vice Chancellor, JUIT and* ***Prof. T. S. Lamba*** *(Dean, JUIT) as well as JUIT administration for their valuable guidance, constant support and cooperation.*

*I gratefully acknowledge the help rendered by* ***Prof. R. S. Chauhan*** *(HOD, Dept. of BT, BI & Pharmacy) for his encouragement, timely help and cooperation throughout my research work.*

*I also feel indebted to the advisor,* ***Professor Anju Katyal*** *(Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi) for her valuable advice, unconditional support and providing me the research facilities available in her department during the conduct of research work.*

*I am grateful to Council of Scientific and Industrial Research* ***(C.S.I.R.)*** *for financial assistance as senior research fellowship. Indian Council of Medical Research* ***(I.C.M.R.)*** *for the approval of project as senior research fellowship. Defence Research and Development Organisation* ***(D.R.D.O.)*** *for funding during my research work.*

*This document would have remained an infant had it not received its necessary 'diet' in the form of comments, suggestions etc. by* ***Dr. Harish Changotra, Dr. Jata Shankar and Dr. Hemant Sood****. I am short of words in expressing my thanks to them, for their innovative ideas that shaped this document.*

*I wish to convey my sincere thanks to all the faculty members of Department of Biotechnology, Bioinformatics and Pharmacy, for their help and guidance at the various stages of this study.*

*I m also thankful to all the members of technical and non-technical staff of the department, especially* ***Mrs. Sonika Gupta, Mrs. Somlata Sharma, Mrs. Mamta, Mr. Ravikant, Mr. Baleshwar*** *and* ***Mr. Kamlesh*** *for their assistants and valuable contributions.*

*I am fortunate to have friends who have always stood beside me. I extend my heartfelt thanks to* ***Arun, Vineet, Deepika, Sahil, Kaveri, Archit and Anjali*** *for their sustained support and ever needed cooperation.*

*It is my pleasure to express my gratitude to all research scholars of the Biotechnology, Bioinformatics and Pharmacy Department for keeping me blessed with best wishes.*

*The successful culmination of this thesis could not have been possible without the blessing of* ***my loving parents****. Without their help I would have been greatly incapacitated. All may not be mentioned, but no one is forgotten.*

***Sita Sharan Patel***

# *TABLE OF CONTENTS*

**5.5. Depression mediated insulin resistance and hypercorticosteronemia and the effect of UD**

**5.6. Effect of UD extract on depression mediated alteration in hippocampal insulin signaling pathway**

**5.7. The effect of UD extract on depression mediated impairment in Smo-Gli pathway and synaptic plasticity**

**5.8. Depression mediated alteration in hippocampal and striatal cholinergic system and the effect of UD and FLX**

**5.9. Effect of UD on depression mediated impairment in ATG and neuronal survival**

**5.10. Effect of UD extract on CUMS-induced oxidative and nitrative stress**

**5.11. Accumulation of stinging nettle and St. John's wort constituents on whole brain after CUMS paradigm**

**5.12. Effect of UD extract on STZ induced hyperglycemia, hypoinsulinemia and insulin resistance**

**5.13. Effect of UD extract on diabetes mediated alteration in body weight and water intake**

**5.14. Diabetes mediated depressive like behaviour and motor function deficit and the effect of UD extract**

**5.15. Effect of UD extract on diabetes mediated cognitive deficit**

**5.16. Effect of UD extract on hippocampal insulin signaling pathway**

**5.17. Effect of UD extract on diabetes mediated impairment in hippocampal GLUT4 membrane translocation**

**5.18. Effect of UD extract on hippocampal cholinergic system**

**5.19. Effect of UD extract on oxidative stress, inflammation and neuronal survival**

**5.20. Immunofluorescence study of TNFα on hippocampal section of chronically stressed mice**

**5.21. Histopathology study on hippocampal section of chronically stressed mice**

**5.22. Immunofluorescence study of TNFα on hippocampal section of STZ induced diabetic mice**

**5.23. Histopathology study on hippocampal section of STZ induced diabetic mice**

# List of Tables

# LIST OF FIGURES

sec maximum duration, 30 s intervals); I2- immobilization (4 h); T2- tail pinch (3 min); O1- overnight illumination with wet cage; C2- cold swim (6°C, 3 min); I3- immobilization (5 h); OT- overnight illumination with tilted cage.

**Figure 14:** Experimental design for the effect of UD against STZ induced neurological alterations. UD = *Urtica dioica* extract, STZ = Streptozotocin.

**Figure 15:** Transverse section of Stinging nettle leaf stained with safranin solution (40X).

**Figure 16:** Transverse section of St. John's wort (*Hypericum perforatum*) leaf stained with safranin solution and viewed at 10X **(A)** and 40X **(B)**.

**Figure 17:** Negative ion LC-MS spectrum of hydro-alcoholic extract of UD leaves showing peak at *m/z* 153.02 (gentisic acid), *m/z* 177.02 (esculetin), *m/z* 191.04 (scopoletin), *m/z* 301.04 (quercetin) and *m/z* 609.51 (rutin).

**Figure 18:** HPLC chromatogram of standard scopoletin **(A)** and crude hydro-alcoholic extract of UD leaves **(B)**.

**Figure 19:** Negative ion LC-MS chromatogram of hyperforin (*m/z* 535.38) **(A)** and hypericin (*m/z* 503.08) **(B)** in hydro-alcoholic extract of St. John's wort.

**Figure 20:** Typical HPLC chromatograms of the standard hypericin (CAS 548-04-9) **(A)**, hyperforin (CAS 11079-53-1) **(B)**, 50% methanolic extract of St John's wort **(C)**, 75% methanolic extract of St John's wort **(D)** and 25% methanolic extract of St John's wort **(E)** with detection wavelength set at 200-800 nm using PDA detector.

**Figure 21:** Effect of UD extract on CUMS-induced behavioural alterations in FST **(A)** and TST **(B)**. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; FST = forced swim test; TST = tail suspension test.

**Figure 22:** Effect of UD extract on CUMS-induced behavioural alteration in SPT: SPT base line **(A)**, SPT at week one **(B)**, SPT week at two **(C)** and SPT at week three **(D)**. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001.

CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; SPT = sucrose preference test.

**Figure 23:** Overall effects of CUMS and drug treatment on SPT. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + UD. **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX =

**Figure 24:** Effect of UD extract on CUMS-induced behavioural alterations in Morris water maze task **(A)** and probe trial (number of crossings) **(B)**. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 25:** Effect of UD extract on CUMS-induced behavioural alteration in PA task: PA task base line **(A)**, PA task at week one **(B)**, PA task at week two **(C)** and PA task at week three **(D)**. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD; $^{\Psi}$CTRL vs. CTRL + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; PA task = passive avoidance step through task.

**Figure 26:** Overall effects of CUMS and UD treatment on PA task. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + UD; $^{\Psi}$CTRL vs. CTRL + UD. *p < 0.05, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; UD = *Urtica dioica* extract; PA task = passive avoidance step through task.

**Figure 27:** Effect of UD extract on CUMS-induced alterations in locomotor activity in actophotometer using bar graph **(A)** and locomotor performance using line graph **(B)**. Data were mean ± SEM values (n=6). Significant differences: $^{*/\#}$CTRL vs. CUMS. **p < 0.01. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 28:** Effect of UD extract on CUMS-induced alterations in the level of fasting blood glucose **(A)**, oral glucose tolerance test **(B)**, plasma corticosterone **(C)** and serum insulin **(D)**.

Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 29:** Effect of UD extract on CUMS-induced alterations in the mRNA expression of hippocampal PPARγ **(A)**, IR **(B)**, ILGF 1r **(C)**, GLP1 **(D)**, IRS1 **(E)** and IRS2 **(F)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + ROSI and CUMS + UD, aCUMS + FLX vs. CUMS + UD, cCUMS + HYP vs. CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 30:** Effect of UD extract on CUMS-induced alterations in the mRNA expression of hippocampal PI3K **(A)**, PKB **(B)**, GLUT4 **(C)**, INSG1 **(D)** and MAPK1 **(E)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD; ΨCTRL vs. CTRL + HYP and CTRL + UD, aCUMS + FLX vs. CUMS + UD; cCUMS + HYP vs. CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 31:** Effect of UD extract on CUMS-induced alteration in the content of hippocampal GLUT4 membrane protein**.** Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + ROSI. ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; ROSI = rosiglitazone; UD = *Urtica dioica* extract; GLUT4 = glucose transporter type 4.

**Figure 32:** Effect of UD on CUMS-induced alterations in hippocampal Shh mRNA **(A)**, Ptch1 mRNA **(B)**, Smo mRNA **(C)** and Gli1 mRNA **(D)** expression. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + HYP and CUMS + UD; ΨCTRL vs. CTRL + HYP and CTRL + UD, αCTRL + FLX vs. CTRL + UD; βCTRL + ROSI vs. CTRL + UD; aCUMS + FLX vs. CUMS + UD; bCUMS + ROSI vs. CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic

unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 33:** Effect of UD on CUMS-induced alterations in hippocampal Hhip mRNA **(A)**, cyclin D1 mRNA **(B)**, BDNF mRNA **(C)** and TrkB mRNA **(D)** expression. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD; $^{+}$CTRL vs. CUMS + HYP. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 34:** Effect of UD on Gli1 **(A)** and Ptch1 **(B)** mRNA expression in hippocampal slices pre-treated with Smo antagonist cyclopamine. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. Cyc 5μM; *CTRL vs. Pur 1μM, HYP 50μg, HYP 100μg, UD 250μg; $^{\alpha}$HYP 50μg and UD 125μg vs. HYP 50μg + Cyc 5μM and UD 125μg + Cyc 5μM; $^{\beta}$HYP 100μg and UD 250μg vs. HYP 100μg + Cyc 5μM and UD 250μg + Cyc 5μM; $^{+}$Pur 1μM vs. Cyc 5μM+ Pur 1μM, HYP 50μg + Cyc 5μM, HYP 100μg + Cyc 5μM, UD 125μg + Cyc 5μM and UD 250μg + Cyc 5μM. ***p < 0.001. CTRL = control; Cyc = cyclopamine; Pur = purmorphamine; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 35:** The effect of UD on CUMS-induced alterations mAChRs expression: mAChR1 mRNA in hippocampus **(A)**, mAChR4 mRNA in hippocampus **(B)**, mAChR1 mRNA in striatum **(C)** and mAChR4 mRNA in striatum **(D)**. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX and CUMS + UD; *p < 0.05, **p < 0.01. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 36:** The effect of UD on CUMS-induced alterations in the expression of mAChR1 protein in hippocampus **(A)**, mAChR4 protein in hippocampus **(B)**, mAChR4 protein in striatum **(C)**, AChE protein in hippocampus **(D)** and ChAT protein in hippocampus **(E)**. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + UD and CUMS vs. CUMS + FLX. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; UD = *Urtica dioica* extract; FLX = fluoxetine.

**Figure 37:** Effect of UD on CUMS-induced alterations in the mRNA expression of hippocampal BCL2 **(A)**, AIP2 **(B)**, ATG5 **(C)** and ATG7 **(D)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 38:** Effect of UD on CUMS-induced alterations in the mRNA expression of hippocampal iNOS **(A)**, IL6 **(B)** and TNFα **(C)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 39:** Effect of UD on CUMS-induced alterations in TBARS level **(A)**, nitric oxide level **(B)**, catalase level **(C)** and total thiol level **(D)** in plasma. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; TBARS = thiobarbituric acid-reactive substances; NO = nitric oxide.

**Figure 40:** HPLC chromatogram of standard scopoletin **(A)** as well as whole brain homogenate of chronically stressed mice treated with *Urtica dioica* extract **(B)**.

**Figure 41:** HPLC chromatogram of standard hyperforin **(A),** hypericin **(B)** as well as whole brain homogenate of chronically stressed mice treated with St. John's wort extract **(C)**.

Orally administered Stinging nettle extract accumulated as its constituent scopoletin (0.002%) in the brain tissue of mice after the last dose followed by 12 hr fasting (Figure 40).

**Figure 42:** Effect of UD extract on STZ induced alteration in fasting blood glucose level **(A)**, serum insulin level **(B)** and oral glucose tolerance test **(C)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p

**Figure 43:** Effect of UD extract on STZ induced alteration in body weight **(A)** and water intake **(B)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ;

*STZ vs. STZ + UD50 and STZ + ROSI. **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 44:** Effect of UD extract on STZ induced depressive like behaviour in forced swim test **(A),** depressive like behaviour in tail suspension test **(B)** and locomotor deficit in actophotometer test **(C)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 45:** Effect of UD extract on STZ induced behavioural alteration in Morris water maze task **(A),** probe trial **(B)** and passive avoidance step through task **(C)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 46:** Effect of UD extract on diabetes-mediated alterations in the mRNA expression of hippocampal PPARγ **(A)**, IR **(B)**, ILGF 1r **(C)**, GLP1 **(D)**, IRS1 **(E)** and IRS2 **(F)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI; ΨCTRL vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 47:** Effect of UD extract on diabetes-mediated alterations in the mRNA expression of hippocampal PI3K **(A)**, PKB **(B)**, GLUT4 **(C)**, INSG1 **(D)** and MAPK1 **(E)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 48:** Effect of UD extract on STZ-induced alterations in the content of hippocampal GLUT4 protein in cytosol **(A)** and plasma membrane by immunblot **(B)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD and STZ + ROSI. *p < 0.05. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 49:** *In vitro* stimulation of hippocampal slices by UD and insulin increases the association of GLUT4 membrane protein. Data were mean ± SEM values (n=4). Significant

differences: #Veh vs. Ins; *Ins vs. Ins + LY294002; +UD vs. UD + LY294002.Veh = vehicle; Ins = insulin; UD125 & UD250 = *Urtica dioica* extract 125 µg & 250 µg.

**Figure 50:** The effect of UD on diabetes mediated alterations in mAChRs expression: mAChR1 mRNA in hippocampus **(A)**, mAChR4 mRNA in hippocampus **(B)**, mAChR1 mRNA in striatum **(C)** and mAChR4 mRNA in striatum **(D)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 51:** The effect of UD on diabetes mediated alterations in protein expression of mAChR1 in hippocampus **(A)**, mAChR4 in striatum **(B)**, AChE in hippocampus **(C)** and ChAT in hippocampus **(D)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 52:** The effect of UD on diabetes mediated alterations in the mRNA expression of BDNF **(A)**, TrkB **(B)** and cyclin D1 **(C)** in hippocampus. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 53:** The effect of UD on diabetes mediated alterations in the mRNA expression of BCL2 **(A)**, AIP2 **(B)**, ATG5 **(C)**, ATG7 **(D)**, iNOS **(E)**, IL6 **(F)** and TNFα **(G)** in hippocampus. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 54:** Effect of UD on diabetes mediated alterations in TBARS level **(A)**, nitric oxide level **(B)**, catalase level **(C)** and total thiol level **(D)** in plasma. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 55:** Effect of UD on CUMS mediated alterations in TNF-α expression using immunofluorescence study. CTRL = control; CUMS = chronic unpredictable mild stress;

FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 56:** Effect of UD on CUMS mediated neuronal damage using histopathology study. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

**Figure 57:** Effect of UD on diabetes induced alterations in TNF-α expression using immunofluorescence study. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 58:** Effect of UD on diabetes mediated neuronal damage using histopathology study. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

**Figure 59:** An overview on mechanism of action of UD extract: Shh signaling in normal control mice (A), stressed mice (B), stressed mice treated with UD or HYP extract (C) and normal control mice treated with UD or HYP extract (D).

**Abstract**

Clinically, depression and diabetes are co-morbid. Diabetes makes the symptoms of depression worse. Depression reduces overall physical and mental health, not only by increasing the risk for diabetes but making diabetic symptoms worse. Both depression and diabetes are the risk factor for cognitive impairment. Cholinergic system, autophagy (ATG), insulin signaling pathway and sonic hedgehog (Shh) pathway are involved in many regulatory processes, including learning and memory. Stinging nettle (*Urtica dioica*, UD) extract has been claimed for its beneficial effects against depression, diabetes and cognition. The present study was performed to evaluate whether chronic unpredictable mild stress (CUMS) or diabetes mediated cognitive deficit is associated with dysfunction in cholinergic system, ATG, insulin signaling and Shh pathway. In addition, standardized UD extract was used to evaluate its effect on CUMS or diabetes mediated neuronal dysfunction. Rosiglitazone, fluoxetine and St. John's wort were used as standard drugs for comparison. CUMS (3 weeks) and multiple dose of streptozotocin (STZ) (50 mg/kg, i.p. for 5 consecutive days) resulted in depressive like behaviour, cognitive impairments and hypolocomotion in mice. CUMS induced insulin resistance and hypercorticosteronemia in mice. CUMS and diabetes impaired insulin signaling pathway, ATG and muscarinic cholinergic system in the hippocampus. In addition, CUMS impaired Smoothened (Smo)-Glioma associated oncogene-1 (Gli1) pathway in the hippocampus. CUMS and diabetes downregulated muscarinic cholinergic receptor-4 (mAChR4) expression in striatum but not in hippocampus. Both CUMS and diabetes were associated with oxidative stress, inflammation and apoptosis. Chronic UD treatment (50 mg/kg, p.o.) significantly reverted CUMS and diabetes mediated cognitive impairment, depressive like behaviour and insulin resistance. UD reduced hypercorticosteronemia in stressed mice. Chronic UD administration modulated insulin signaling pathway, ATG and muscarinic cholinergic system in the hippocampus of chronically stressed and diabetic mice. Chronic UD administration effectively modulated hippocampal Smo-Gli1 pathway in stressed mice. UD treatment significantly reduced hyperglycemia, body weight loss and polydypsia in diabetic mice. UD administration significantly ameliorated hippocampal glucose transporter-4 (GLUT4) membrane translocation in diabetic but not in stressed mice. UD administration did not modulate mAChR4 expression in striatum and hypolocomotion. Chronic UD administration attenuated oxidative stress, inflammation and apoptosis in stressed and diabetic mice. These results suggest that chronic administration of UD extract might prove to be effective for depression and diabetes related neurological disorders.

# CHAPTER 1

## INTRODUCTION

## 1. Introduction

Depression is a mood disorder in which feelings of sadness, anger or frustration interfere with the daily life for weeks or longer. The symptoms of depression includes agitation, restlessness and anger, irritability, becoming isolated, fatigue and lack of energy, hopeless and helpless feeling, worthless, self-hate, loss of interest or pleasure in activities that were once enjoyed, sleep-wake abnormalities, thoughts of death or suicide etc [1].

Depression is associated with the hyperactivity of the hypothalamic-pituitary-adrenal axis (HPA axis) [2-3]. The hormone cortisol is the end product of activation of the HPA axis. It has been reported that uncontrollable stressors likely to be associated with elevations in cortisol level [3]. Hyperactivation of HPA axis is known to induce neurodegenerative process, reduce neurogenesis and associated with cognitive dysfunction [4-5]. Prolonged elevation of cortisol inhibits insulin secretion from pancreatic β-cells, decrease glucose uptake and utilization, stimulates glucagon secretion, hepatic glucose production, decreased body weight and induces type 2 diabetes like state [6-7].

The chronic unpredictable mild stress model (CUMS) is a widely used model for induction of depressive like behaviour in rodents, which consists of the repeated exposure to an array of unpredictable and mild stressors over a sustained period of time [8-9]. Unpredictable mild foot shock stress for 21 days induced significant hyperglycaemia, glucose intolerance, hypercorticosteronemia, gastric ulcerations, male sexual dysfunction, immunosuppression, cognitive deficits and mental depression in rats [10]. Exposure to 21 days of CUMS significantly reduced brain-pancreas relative protein and induced hyperglycemia and hypoinsulinemia [11].

Clinically, depression and diabetes are co-morbid. Diabetes make the symptoms of depression worse [12]. At the same time, depression reduces overall physical and mental health, not only increasing the risk for diabetes but making diabetes symptoms worse. Studies have shown that people with depression and diabetes have more severe diabetes symptoms than people who do not have diabetes [13]. In animal models, depressive phenotype is associated with hyperglycaemia, insulin resistance, hypercorticosteronemia, cognitive deficits, immunosuppression and hypoinsulinemia [10-11].

Hyperglycemia is associated with oxidative stress in hippocampus, resulting in neurological disorders such as memory impairment, depressive like behaviour and anxiety [14-15]. Hypercorticosteronemia induces over production of reactive oxygen species and elevation of cytosolic calcium, and subsequent increase in the calcium dependent death in neuronal cells [16]. Evidence suggest that elevated corticosterone level as observed in stress, induces dysregulation of insulin receptor (IR), thereby decreased metabolic activities and plasticity of hippocampal neurons resulting in cognitive dysfunction [17-19].

Chronic activation of the HPA axis lead to condition called glucocorticoid resistance where immune cells are no longer to hear cortisol signal, thus leading to increases in both cortisol level and inflammation [3]. Reactive nitrogen species such as nitric oxide (NO) has been implicated in stress mediated inflammation and cognitive deficit [20]. In addition, NO impairs autophagy (ATG) in rat primary cortical neurons which is known to modulate neuronal health [21].

The hippocampus region of brain that regulates learning and memory processes is vulnerable to oxidative stress and hypercortisolemia [22-24], during the depressive episodes [25]. Hippocampal muscarinic receptors are known to modulate cognition. Thus, hippocampal cholinergic dysfunctions might underlie depression associated cognitive impairments [25]. Administration of selective muscarinic acetylcholine receptor 1 (mAChR1) antagonists induces spatial memory impairment [26]. mAChR4 function as presynaptic autoreceptor in hippocampal neurons to inhibit acetylcholine (ACh) release [27], which is known to be involved in learning and memory processes [28]. Upregulation of mAChR4 in striatal neurons inhibits locomotor activity in mice [29]. Chronic stress activates acetylcholinesterase (AChE), resulting in reduced amount of ACh in synaptic cleft [30]. Depression dysregulate brain derived neurotrophic factor (BDNF) and mitogen activated protein kinase (MAPK) levels, which is known for its modulation in synaptic plasticity [31-32].

Depression results in increased neurodegeneration and decreased hippocampal neurogenesis [33]. It has been demonstrated that glucocorticoid treatment induces arrest of the neural cell cycle [34] and apoptosis in neuronal progenitors and mature neurons [35]. Suppression of neurogenesis affects mood [5], fear conditioning, synaptic plasticity [36] and memory [37]. Sonic hedgehog (Shh), a mitogenic protein, has been implicated in neurological disorders. It has been demonstrated that, dysregulation of Shh co-receptors like patched (Ptch) and

smoothened (Smo) within the dentate gyrus subfield of the hippocampus is associated with depressive phenotype [38].

Diabetes can be caused by low insulin, resistance to insulin, or both [39-40]. Prolonged hyperglycemia often associated with damage to nerves in the body that occurs due to high blood sugar level [41]. In the central nervous system, diabetes exacerbates depression, phobias, anorexia and cognitive deficit [42-43]. Clinically, patients with diabetes are at increased risk of developing depression and cognitive impairment as compared to the general population [44-45]. Diabetes induces oxidative stress and inflammation in the hippocampal neurons resulting in neurodegeneration [14, 46]. Animal models also revealed the persistence of depression and neurocognitive impairment during chronic diabetes. Streptozotocin (STZ) induced diabetes showed depressive like behaviour [47-48] and cognitive deficit in rodents [49].

STZ is an antibiotic [50], induces hyperglycaemia by its direct cytotoxic action on the pancreatic β-cells [51-52]. In STZ treated mice, changes in spinal terminals of calcitonin gene-related peptide in sensory neurons were observed 4 weeks after diabetes and progressively worsened with time [53]. STZ induced chronic diabetes induced depressive like behaviour in animals. In addition, STZ induced diabetic animals showed a significant hypolocomotion [54] and cognitive dysfunction [55].

Many signaling pathway has been involved in the pathogenesis of diabetic neuropathy. Dysfunctioning of neuronal peroxisome proliferator-activated receptor-γ (PPARγ) was observed in cognitive dysfunction [56]. A recent study reported that, downregulation of PPARγ levels in the hippocampus of diabetic mice [57] is associated with depressive like behaviour in forced swim test [58]. Further, downregulation of IR expression in hypothalamus induces depressive like behaviour in rats [59]. Diabetic neuropathies in brain are exacerbated by severe deficiency of BDNF with depressive behaviour [60].

Diabetes induces oxidative stress and inflammation in hippocampus [14]. Chronic diabetes is known to elevate the level of NO. NO induces inflammation of hippocampal neurons [30]. NO derived from inducible form of nitric oxide synthase (iNOS) contribute to the depressive like behaviors in mice due to neurodegenerative effects in the cerebral cortex [20].

Choline acetyltransferase (ChAT) and AChE are responsible for the synthesis and metabolism of ACh. Evidence suggests that ChAT, a specific marker for functional state of cholinergic neurons [61], activity is reduced during STZ administration resulting in cognitive deficit [62-63]. STZ increases AChE activity in hippocampus resulting in learning and memory deficit [64]. Previous study suggest that STZ-induced diabetes significantly downregulated the expression of mAChR1 in hippocampus termed cholinergic dysfunction [64]. Chronic diabetes resulted in motor activity deficit [65]. Upregulation of mAChR4 in the striatum inhibits dopaminergic-D1 receptor-induced locomotor stimulation in mice [29]. Autophagic dysfunction has also been observed in diabetes mice [66].

Saint John's wort also known as *Hypericum perforatum* is a flowering plant, belonging to the family Hypericaceae [67-68]. The herb of St. John's wort has been used from centuries to treat various ailments including depression [69-70]. Clinical trials in patients revealed that Hypericum extract is superior for depressive disorder and their effectiveness is similar to that of standard antidepressants with fewer side effects [71]. Hypericum extract improves spatial memory [72], proliferation of progenitor cells and dendritic spine, restored the synaptic plasticity [73] and Alzheimer's pathology [74]. Hypericum extract is a suitable alternative for the management of depressive disorder associated with cognitive impairment [75]. St. John's Wort and its active constituent hyperforin protect rat as well as human islets against cytokine mediated β-cell injury in type 1 diabetes [76]. Hypericum administration significantly reduced hyerglycemia in STZ treated diabetic rats. Clinically, Saint John's wort induces photosensitivity and has interaction with many drugs [67].

In randomized controlled trial, fluoxetine (FLX) exerted neurocognitive improvement in patients with moderate depression [77]. FLX attenuated impaired cognition in depressed rodents as well [78]. FLX upregulated BDNF levels in hippocampus and modulated adult neurogenesis and depressive-like behaviour [79]. FLX treatment reduced the level of glycated hemoglobin during co-mobidity depression and diabetes in rodents. FLX ameliorated spatial learning and showed hypoglycaemic and antidepressant effects during CUMS associated diabetes [80]. FLX is also prescribed for variety of pathological conditions including mood and eating disorders [81], obsessive compulsive disorders [82], depression in the elderly [83] and dysthymia [84]. In view of pathogenesis in type 2 diabetes mellitus and brain disease, antidiabetic medications known to positively modulate the metabolism of neuronal cell,

which could be of clinical importance for the treatment of neurological disorders [85]. Rosiglitazone (ROSI) is a thiazolidinedione class of oral antidiabetic drug for therapy of type 2 diabetes mellitus [86-87]. ROSI increases glucose transport in adipose tissue and muscle by enhancing synthesis and translocation of glucose transporters [88-89]. ROSI showed central anti-diabetic action against D-glucose fed and STZ-induced diabetes in rodents [90]. ROSI is known to protect cognitive impairment in individuals with type 2 diabetes [91]. ROSI improves cognition and memory performance in patients with mild Alzheimer disease and animal models of Alzheimer disease [92]. It has been reported that, insulin sensitizing drug ROSI exhibited significant declines in depression severity associated with insulin resistance [93]. Mice administered with ROSI showed increased hippocampal neurogenesis during depression [94].

Currently available antidepressants are known to improve neurocognitive functions depending on clinical, social and emotional factors [95]. Studies have shown that, therapy with antidepressants is correlated with an increased risk of suicidal behaviour in those aged under 25 [96]. Sexual side effects are also common with the use of FLX, such as loss of sexual drive, failure to reach orgasm and erectile dysfunction [97]. Antidepressant medication use increases the risk of hyperglycemia and diabetes mellitus. The incidence of diagnosed diabetes is higher among antidepressant users than nonusers [98-99]. Many currently available antidiabetic drugs are known to induce hypoglycaemia and associated cognitive impairment [100]. It has been reported that patients receiving chronic pioglitazone therapy showed increased incidence of bladder cancer compared to general population [101-102]. Chronic rosiglitazone therapy is associated with an increased incidence of myocardial infarction and heart failure in type 2 diabetic patients [103].

*Urtica dioica* (UD) is an herbaceous plant and commonly known as stinging nettle, belonging to the family Urticaceae [104-105]. Administration of UD leaves repaired pancreatic tissue damage in STZ induced diabetic rats [106]. In earlier studies, UD administration significantly reduced hyperglycemia, insulin resistance and hyperlipidemia in fructose induced diabetic rats [107]. In clinical trial, UD is reported to have glycemic control in type 2 diabetic patients by lowering the levels of fasting and postprandial blood glucose [108]. Earlier studies reported that administration of diabetic patients with UD significantly increased antioxidant capacity and reduced inflammatory stress and glycated hemoglobin [109-110].

# CHAPTER 2

## REVIEW OF LITERATURES

## 2. Review of Literature

### 2.1. Depression

A World Health Organization survey from primary healthcare system suggests a wide variation in prevalence of depressive disorder across 14 countries (from 1.6% to 26.3%) [111]. Depression is a mental health problem. It is a mood disorder in which feelings of sadness, anger or frustration interfere with the daily life for weeks or longer. Depression may be brought by alcohol or drug abuse, certain medical conditions (including underactive thyroid, cancer or long-term pain), steroidal drugs, abnormal sleeping, stressful life events, such as death of someone close to the family, divorce, loneliness (common in the elderly), relationship breakup etc. The symptoms of depression includes agitation, restlessness and anger, irritability, becoming isolated, fatigue and lack of energy, hopeless and helpless feeling, worthless, self-hate, loss of interest or pleasure in activities that were once enjoyed, change in appetite, often with weight loss or weight gain, thoughts of death or suicide, trouble concentrating, trouble sleeping or sleeping too much etc [1]. Cognitive impairments and diabetes are often observed during depression, in addition to mood disturbances and other motor, autonomic, endocrine and sleep-wake abnormalities [11, 112].

### 2.2. Co-morbidity of depression and diabetes with cognitive dysfunction

Many physiological alterations occur in the body during major depression. Many tissues, hormones, neurotransmitters and cytokines work together in order to rescue a stress response to maintain normal homeostasis [113-114]. The most important anatomical structures of brain involved are the hypothalamus, pituitary and the adrenal gland, constitute the HPA axis. Amygdala and prefrontal cortex also modulate the HPA axis. Depression is associated with the hyperactivity of the HPA axis [2-3]. During depressive episodes or stressful situations the hippocampus, amygdala and the prefrontal cortex are decreased in size, similarly to the effects shown during stressful conditions. The hormone cortisol is the end product of activation of the HPA axis. A meta-analysis investigated cortisol responses to stress, found that uncontrollable stressors likely to be associated with elevations in cortisol [3]. Stressors evoked drastic elevations in cortisol are important for energy mobilization to deal with impending threats; however, elevated levels of corticosteroids may also lead to deleterious health outcomes via associated increases in inflammation, oxidative stress, brain atrophy etc. Numerous studies have been conducted on structural effects of the glucocorticoids in

hippocampus and beyond. It has been reported that, glucocorticoids beyond a certain threshold level rendered corticosteroid receptor-bearing hippocampal neurons more vulnerable to excitotoxic neurotransmitter such as glutamate. A direct neurodegenerative effects of corticosteroids in hippocampal neurons, leading to reduced cell numbers. Hyperactivation of HPA axis is known to induce neuritic neurodegenerative process, reduce neurogenesis and associated with cognitive dysfunction [4-5].

The hormone insulin is secreted by the pancreatic β-cells, plays a major role in the stage that controls differentiation, especially by stem cells, into almost all of the cells that compose the body or the organ systems including hippocampus. Prolonged elevation of glucocorticoids inhibits insulin secretion from pancreatic β-cells, decrease glucose uptake and utilization, stimulates glucagon secretion, hepatic glucose production, decreased body weight and induces type 2 diabetes like state [6-7].

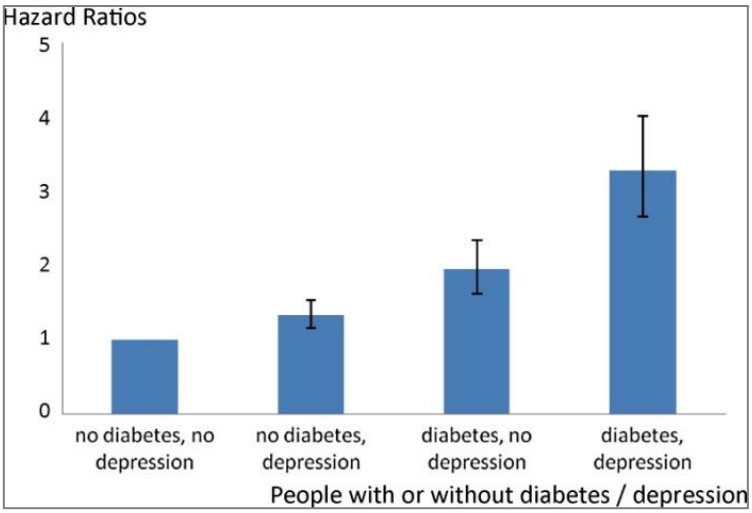



**Figure 1:** Effect of depression on all cause mortality in patients with diabetes, reprinted from [45], Copyright © 2015, with permission from Elsevier.

Literature showed that depression and diabetes are co-morbid, but it is not yet known whether depression increases the risk of diabetes or diabetes increases the risk of depression. Recent studies suggest that both cases are possible. In addition to possibly increasing the risk for depression, diabetes might make the symptoms of depression worse. The stress of managing diabetes daily and the effects of diabetes on the brain exacerbates the symptoms of depression [12]. At the same time, some symptoms of depression may reduce overall physical and mental health, not only increasing the risk for diabetes but making diabetes symptoms worse.

Studies have shown that people with depression and diabetes have more severe diabetes symptoms than people who do not have diabetes [6-7, 13].

It has been hypothesised that, increased risk of type 2 diabetes in depressive patients is believed to be the result from increased counter regulatory hormone mechanism, alterations in glucose transportation and increased inflammation [115]. These physiological alterations thought to contribute to insulin resistance and pancreatic β-cell dysfunction, resulting in the development of type 2 diabetes. Clinically, the symptoms depression is known to increases the risk of pre-diabetes and type 2 diabetes [116-119]. In a cohort study, 11,615 nondiabetic patients with depression symptoms (including fatigue, sleep abnormalities, feelings of hopelessness, increased irritability and loss of libido) were selected and followed for 6 years. Results of this cohort study revealed that depressive symptoms induced the incidence of type 2 diabetes and this relation was explained by metabolic, demographic, and lifestyle factors [119]. In another study, 2127 Swedish middle aged men and 3100 women with psychological distress were included and followed-up for 8-10 years, 245 men and 177 women showed pre-diabetes, and 103 men and 57 women detected with type 2 diabetes [116]. Earlier data reported that depression with co-morbid diabetes have higher hazard ratio (Figure 1). In animal models, depressive phenotype also induced significant hyperglycaemia, glucose intolerance, hypercorticosteronemia, cognitive deficits, immunosuppression and hypoinsulinemia [10-11]. Hyperglycemia induces oxidative stress in hippocampus, resulting in neurological disorder such as memory impairment, depression and anxiety [14-15]. Besides, hypercorticosteronemia associated with over production of reactive oxygen species and elevation of cytosolic calcium, resulting in subsequent increase in the calcium dependent death in neuronal cells [16]. Evidence suggests that elevated corticosterone level as observed in stress, induces dysregulation of IR stimulated trafficking of glucose transporter-4 (GLUT4), thereby decreased metabolic activities and plasticity of hippocampal neurons resulting in cognitive dysfunction [17-19]. In animal models, CUMS exhibits insulin resistance, hypoinsulinemia and hyperglycemia [11, 120]. Insulin acts as a growth factor in the brain and is a neuroprotective, activates the dendritic sprouting, regeneration and proliferation of stem cells. The impairment of insulin signaling in hippocampus might facilitate the development of Alzheimer's disease [121]. The complex pathophysiology of cognitive dysfunction during co-morbidity of depression and diabetes has been illustrated in Figure 2.

Although, cortisol is typically considered as anti-inflammatory, however chronic activation of the HPA axis lead to condition called glucocorticoid resistance where immune cells are no longer to hear cortisol signal, thus leading to increases in both cortisol and inflammation [3]. Reactive nitrogen species such as NO has been implicated in stress mediated inflammation and cognitive deficit [20]. In addition, NO impairs ATG by inhibiting the activity of S-nitrosylation substrates in rat primary cortical neurons [21]. In ATG deficient mice and flies, neurodegeneration is accompanied by the accumulation of ubiquitylated protein aggregates, similar to those observed in human cognitive decline [122]. Chronic fatigue syndrome, the symptom of depression is associated with decreased hippocampal BDNF mRNA expression and exacerbated hippocampal apoptosis and brain atrophy [123]. Study revealed that cognitive decline is associated with biological markers such as brain atrophy, circulating levels of BDNF and insulin-like growth factor 1 [124]. Earlier data showed that an intervening stress free period can reverse hippocampal atrophy and the associated hippocampus-dependent behavioural deficits [125].

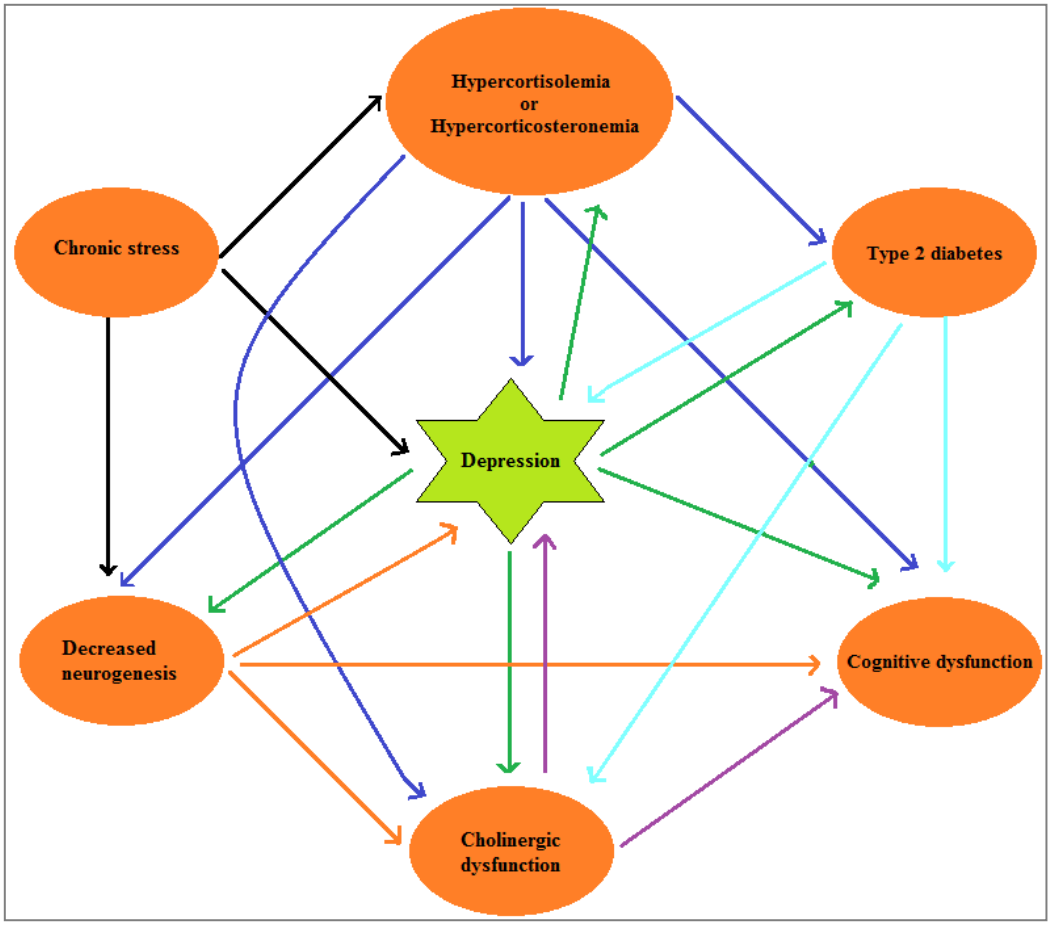


**Figure 2:** The complex pathophysiology of cognitive dysfunction during co-morbidity of depression and diabetes. Hypercortisolemia, depression, diabetes and neuronal dysfunction have been implicated as major causes of cognitive impairment.

Large number of report underlines the connection between a high glucocorticoid level in the blood, a depressed like state and cognitive dysfunctions. Psychiatric and cognitive symptoms resembling major depression have been observed in patients receiving chronic glucocorticoid therapy as well as in patients suffering from Cushing's disease [126-128]. Modulation of neuronal plasticity by corticosteroids has been established. One example is the induction of long term potentiation (LTP) in the hippocampus, which shows a specific pattern: LTP is observed when corticosteroids are kept within normal physiological levels [129], but altered when corticosteroid levels are elevated (e.g. during stress) or presumably both glucocorticoid receptors and mineralocorticoid receptors are occupied [4]. LTP is a persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse in the hippocampus, an important process for learning and memory formation. The hippocampus region of brain that regulates learning and memory processes is vulnerable to oxidative stress and hypercortisolemia [22-24], during the course of depression [25]. Hippocampal muscarinic receptors are known to modulate cognition. Thus, hippocampal cholinergic dysfunctions might underlie depression associated cognitive impairments [25]. In the central nervous system, mAChR1 is mainly coupled to phosphoinositide pathway through $G_q$ protein, whereas mAChR4 preferentially coupled to the inhibition of stimulated adenylyl cyclase through $G_{i/0}$ [130]. The mAChR1 has been considered one of the neurotransmitter receptors regulating hippocampal synaptic plasticity, which is known play a critical role in learning and memory. Cholinomimetic drug carbachol enhanced LTP of excitatory synaptic transmission in mouse hippocampal slices and this enhancing effect was abolished in mAChR1 knock-out mice [131]. Administration of selective mAChR1 antagonists induces spatial memory impairment in Morris water maze task [26]. Besides, mAChR4 function as presynaptic autoreceptor in hippocampal neurons to inhibit ACh release [27], which is known to be involved in learning and memory processes [28]. It has been reported that, upregulation of mAChR4 in striatal neurons inhibits locomotor activity in mice [29]. Apart from alteration in mAChRs, chronic stress activates AChE, resulting in reduced amount of ACh in synaptic cleft [30]. Direct acting mAChR1 agonists and indirect acting muscarinic cholinergic agonists such as AChE inhibitors, have shown cognition-enhancing properties [132]. Further, selective

blockade of mAChR4 induces presynaptic autoreceptor desensitization in hippocampal neurons and have a valuable therapeutic target in cognitive functions [27]. Besides muscarinic cholinergic system, it has been reported that depression modulates BDNF and MAPK levels, which is known for its modulation in synaptic plasticity and transmission [31-32]. Further, abnormalities in the hippocampal cholinergic system could represent depression phenotype [133].

Since the number of dentate gyrus hippocampal cells is also dependent on postnatal neurogenesis, observations that stress or hypercorticolism negatively regulate hippocampal neurogenesis [134]. Depression results in increased neurodegeneration and decreased hippocampal neurogenesis [33]. It has also been demonstrated that glucocorticoid treatment induces arrest of the neural cell cycle [34] and apoptosis in neuronal progenitors and mature neurons [35]. On the basis of numerous observations, it has been shown that high glucocorticoid levels initiate dendritic atrophy and synaptic loss in hippocampal neurons [125, 135-136]. Suppression of neurogenesis affects mood [5], fear conditioning, synaptic plasticity [36] and memory [37]. Shh, a mitogenic protein considered widely in cancer biology, has been implicated in neurological disorders and its vital role in neuronal regeneration has attracted several researchers globally in the field of neuroscience. An overview of Shh signaling has been given in Figure 3.

In the absence of Shh ligand, downstream hedgehog signaling is maintained in a repressed state by the activity of hedgehog receptor Ptch. Ptch is 12-transmembrane domain protein (display transporter like structure), whose intracellular loop is localized at the base of primary cilium [137-138]. Although the mechanism by which Ptch represses the downstream signal transduction has not been fully elucidated, report has shown that, free Ptch (unbound by Shh) acts sub-stoichiometrically to suppress 7-transmembrane domain protein-Smo activity in primary cilium and thus is critical in specifying the level of pathway activity [139]. Like Ptch, hedgehog-interacting protein (Hhip) is one of the inhibitory ligands that bind to Shh with high affinity and participate in an inhibitory mechanism of hedgehog signal by sequestering, modifying or degrading the Shh ligand at the cell surface [140-141]. Ptch induces rapid receptor-mediated endocytosis of Shh targeted by lysosomes for degradation [142], whereas Hhip appears to only physically sequester Shh at the cell surface [143]. Gli signals are also negatively regulated by binding to cytoplasmic protein suppressor of fused

(Sufu) [144-146]. Sufu protects full-length glioma-associated oncogenes (Gli) proteins from speckle-type POZ protein-cullin3 (SPOP-Cul3) mediated ubiquitination and degradation by the proteasome. In this way, Sufu functions as an adaptor to protect a pool of Gli2 and Gli3 that can be converted into activators and repressors. This aspect of hedgehog pathway is evolutionarily conserved and independent of the cilium [147-149]. Furthermore, without Shh ligand, the full length Gli2 and Gli3 translocate at low levels into and out of cilia, where protein kinase A (PKA) phosphorylate them at four to six sites in cilia [147, 150-151]. The phosphorylated residues provide a binding site for centrosomal β-transducin repeat-containing protein, which in turn recruits Skp1/Cullin1/F-box-ubiquitin ligase complex to target full-length, ~190-kDa Gli3 and ~185-kDa Gli2 for cleavage via the ubiquitin-proteasome pathway to generate the ~83-kDa Gli3 (Gli3R) and up to some extent ~78-kDa Gli2 (Gli2R) N-terminal repressor form, respectively, while the C terminals are assumed to be completely degraded [147, 152-153]. Gli3R then translocate to the nucleus, bind with hedgehog gene promoters and repress target gene expression prior to being degraded by speckle-type POZ protein-cullin3 (SPOP-Cul3) ligase complex [147].

The repression exerted by Ptch on Smo is relieved, when Shh binds Ptch, simultaneous localization of Smo to cilia occur [137, 144, 154]. Activation of Smo and its translocation to the primary cilium involve association of Smo with G-protein coupled receptor kinase 2 (GRK2) and β-arrestins and anterior-grade trafficking motor kinesin-II protein (Kif3A) [155]. Reports suggest that, phosphorylation of Smo by the GRK2 and recruitment of β-arrestin results in endocytosis of Smo in clathrin-coated pits [156].

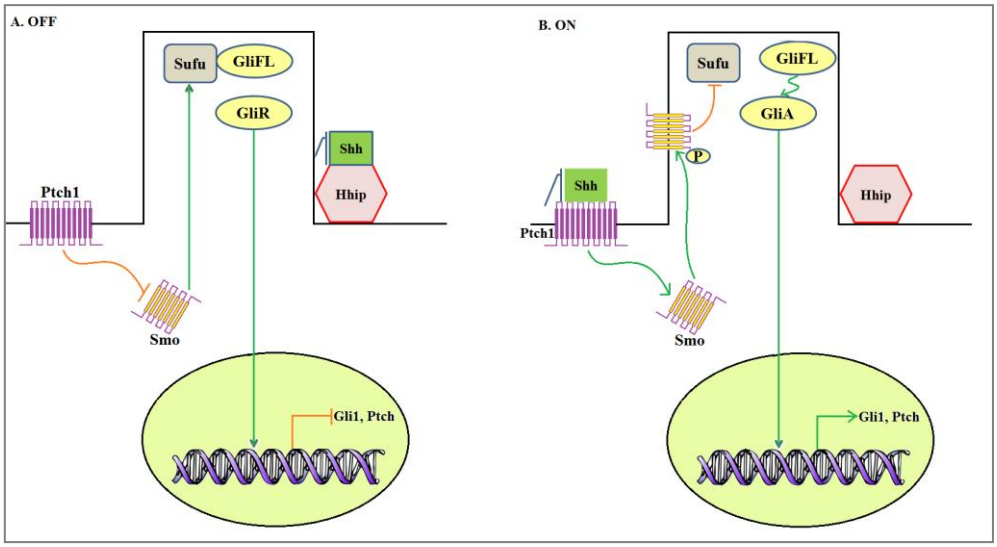


**Figure 3:** An overview of Shh signaling. In the absence of Shh ligand the downstream signaling is off **(A)** and in the presence of Shh the downstream signaling is activated **(B)**.

A recent study demonstrated that, serine/threonine kinases (CK1α) also plays a key role in phosphorylation of mammalian Smo, in which Shh signaling recruits CK1α to initiate Smo phosphorylation, and phosphorylation further increases the binding of CK1α and GRK2 to Smo, forming a positive feedback loop that further increase the level of Smo phosphorylation [157]. Further, β-arrestin promotes translocation of active (phosphorylated) Smo into the cilia by mediating its interaction with the Kif3A in mammalian cells [155, 158]. It has been recently proposed that Evc/Evc2, the products of two human disease genes responsible for the Ellis-van Creveld syndrome interact with phosphorylated Smo to form Smo/Evc/Evc2 complex, further transduces the hedgehog signal to activate Gli by antagonizing Sufu in the primary cilium [159-160]. However, the mechanism by which Shh dissociates Gli-Sufu complex is not well understood, but there is some evidence suggests that the Sufu-full length Gli2 and Sufu-full length Gli3 complexes accumulate in the primary cilium following exposure to Shh, and they independently synthesis new proteins. This leads to immediate trafficking of full length Gli2 and Gli3 into the primary cilium followed by their phosphorylation and dissociation from Sufu. These modifications allow full length Gli2 and Gli3 to be converted into Gli activator (GliA) forms [161-163]. At the same time, PKA phosphorylation is inhibited, preventing βTrCP/Cul binding and processing [147]. In contrast, pharmacological activation of PKA mediates Gli phosphorylation resulting into decline in GliA levels [164]. As result of these complex events, the full length activator form of Gli, also reffered as GliA, migrate towards the nucleus, where it binds with target gene promoter, leading to the activation of transcription factors of the Gli family (Gli1-3) and to the transcription of target genes including Ptch and Gli1 themselves [144, 165-166]. Following this, GliA is ubiquitinated by the SPOP/Cul3 interaction and degraded by proteasome [147]. Gli1 constitutes a convenient readout for the pathway activation, amplifies the hedgehog response and is itself a hedgehog-target gene [167]. Gli2 mainly functions as a transcription activator; however, it was shown to have repressor functions in some specific contexts such as skeletal muscle and CNS development [167-168]. Gli3 retains a bipotential activity, functions as a transcriptional repressor in dorsal interneuron, but also exerts activator functions during embryonic development [144, 167-169].

Shh interacts with its receptor Ptch1, which is observed to be present in the adult basal forebrain [170] and hippocampus [171]. The role of Shh on the proliferation of the cholinergic neurons [170] and induction of ATG [172] imply its critical role in neuronal/memory dysfunction associated with neurological disorders. Autophagy is a highly conserved pathway for degradation, by which intracellular macromolecules are delivered to lysosomes, where they are degraded into biologically active monomers such as amino acids that are subsequently recycled to maintain cellular homeostasis [173].

One of the etiological hypothesis attempting to explain the pathogenesis of depression is the monoamine theory, which hypothesised that depression is the result of decreased availability of monoamine neurotransmitters such as serotonin (5HT) and norepinephrine (NE) in the central nervous system [174]. Monoamine oxidases are the family of enzymes that metabolize the monoamines. This hypothesis derived from the observation that drugs acting on the synaptic concentrations of monoamines can attenuate the symptoms of depression, suggests that depletion of monoamine neurotransmitters is the important cause of depressive symptoms. Currently the etiology of depression seems to involve the monoaminergic receptors and the downstream signaling events that this receptors trigger, including the gene expression. At molecular level the abnormality would be in the signal transduction cascade or in appropriate gene expression [175].

A decline in hippocampal neurogenesis is observed following 5HT and/or NE depletion, while increased hippocampal neurogenesis is seen following elevation of monoamine levels [38, 176-178]. It has been reported that combined depletion of both 5HT and NE with para-chlorophenylalanine resulted in a significant decrease in Smo and Ptch mRNA levels within the dentate gyrus subfield of the hippocampus. However, selective depletion of 5HT, using the serotonergic neurotoxin 5,7-dihyrdroxytryptamine or NE using the noradrenergic neurotoxin DSP-4, did not alter expression of Shh and its co-receptors Smo and Ptch [38].

### 2.3. Drug therapy

Antidepressants are the class of drugs used primarily in the management of depressive and anxiety disorders. This class of drugs is also used for the control of sexual dysfunction, eating disorders, enuresis, aggression and some personality disorders [179]. The most important classes of current antidepressant therapy are the selective serotonin reuptake inhibitors

(SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), NE-dopamine reuptake inhibitors (NDRI) and monoamine oxidase inhibitors (MAOIs). Other drugs used for the treatment of depression include buprenorphine [180], low dose of antipsychotics [181] and St. John's wort [71]. On the basis of literature the list of drugs comes under antidepressants are reported on Table 1 [179, 182] .

**Table 1:** Classification of major antidepressant drugs.

| S. No. | Class | Drugs |
|---|---|---|
| 1. | TCAs | Amitriptyline, Butriptyline, Clomipramine, Desipramine, Dosulepin, Doxepin, Imipramine, Iprindole, Lofepramine, Nortriptyline, Protriptyline, Trimipramine |
| 2. | SNRIs | Venlafaxine, Milnacipran, Duloxetine, Levomilnacipran, Desvenlafaxine, Sibutramine |
| 3. | SSRIs | Citalopram, Escitalopram, Fluoxetine, Fluvoxamine, Paroxetine, Sertraline |
| 4. | MAOIs | Isocarboxazid, Nialamide, Phenelzine, Tranylcypromine, Moclobemide, Pirlindole, Toloxatone, Rasagiline, Selegiline |
| 5. | NDRI | Amineptine, Bupropion, Desoxypipradrol, Dexmethylphenidate, Difemetorex, Ethylphenidate, Fencamfamine, Prolintane, Tametraline |

Antidepressants are also known to improve neurocognitive functions depending on clinical, social and emotional factors [95]. Antidepressant medications involve in serotonin modulation has the potential to cause serotonin toxicity which induce mania, restlessness, agitation, emotional liability, insomnia and confusion [183-184]. MAOIs have pronounced (sometimes fatal) interactions with a wide variety of medications and over the counter drugs. If taken with foods that contain high amount tyramine (e.g. cheese, cured meats or yeast extracts), they may induce a potentially lethal hypertensive crisis. At lower doses the person bothered only by headache due to an increase in blood pressure [185]. The use of SSRIs during pregnancy is associated with an increased risk of spontaneous abortion of about 1.7-fold and associated with pre-term birth and low birth weight [96, 186]. Studies have shown that, therapy with antidepressants is correlated with an increased risk of suicidal behaviour

and thinking (suicidality) in those aged under 25 [96]. Sexual side effects are also common with the use of SSRIs, such as loss of sexual drive, failure to reach orgasm and erectile dysfunction. These effects are usually reversible, these sexual side effects can, in rare cases, last for months or years after the drug has been completely withdrawn [97]. Antidepressant medication use increases the risk of hyperglycemia and diabetes mellitus. The incidence of diagnosed diabetes is higher among antidepressant users than nonusers [98-99].

### 2.4. Diabetes

Diabetes mellitus, commonly referred to as diabetes, is a chronic disease in which the body cannot regulate the amount of sugar in the blood. Insulin is a peptide hormone produced by the pancreatic β-cells to control blood sugar. Diabetes can be caused by low insulin, resistance to insulin, or both. A sugar also called glucose enters the blood stream and act as source of fuel for the body. An organ called the pancreas contains β-cells makes insulin. An important role of insulin is to transport glucose from the blood stream into muscle, fat and liver cells, where it can be stored or used as fuel [39-40]. The disease burden of diabetes is very high and rising in every country, indicated by global rise in the prevalence of obesity and unhealthy lifestyles. Literature showed that global prevalence of 382 million people with diabetes in 2013, expected to rise 592 million by 2035 [187].

There are two types of diabetes namely type 1 and type 2. Type 1 diabetes is a chronic disease in which there is a high level of glucose in the blood.

Type 1 diabetes can occur at any age and often diagnosed in children, adolescents or young adults. With type 1 diabetes, pancreatic β-cells did not produce insulin. In absence of insulin, glucose builds up in the blood stream instead of moving into cytoplasm. This build up of glucose in the blood is called hyperglycemia. The body is unable to utilize the glucose as fuel and leads to the symptoms of type 1 diabetes. The exact cause of type 1 diabetes is not known; however, most likely it is considered as an autoimmune disorder. In this condition, immune system mistakenly attacks and destroys healthy tissue. Any infection can also trigger the body to mistakenly attack the cells in the pancreas that makes the hormone insulin. The tendency to develop autoimmune diseases, including type 1 diabetes, can run down through families [39, 188-189].

The symptoms may be the first signs of type 1 diabetes include being very thirsty, feeling hungry, feeling tired all the time, having blurry eyesight, feeling numbness or tingling in feet, losing weight without trying, urinating more often as well as urinating at night or bedwetting in children who were dry overnight before the symptom. The serious warning symptoms of type 1 diabetes occurs when blood sugar is very high, which include deep and rapid breathing, dry skin and mouth, flushed face, fruity breath odour, nausea or vomiting and stomach pain [39].

Type 2 diabetes is much more common and most often occurs in adulthood, but because of high obesity rates, teens and young adults are also diagnosed with this disease. Type 2 diabetes is a chronic metabolic disease in which there is a high level of sugar in the blood. Insulin is a peptide hormone produced by the pancreatic β-cells. The location of pancreas is below and behind the stomach. Insulin is required to transport blood glucose into cells. Inside the cells, glucose is utilized and stored as fuel for later use. When type 2 diabetes occur fat, liver and muscle cells do not respond correctly to the hormone insulin. This is called insulin resistance. As the result of insulin resistance, blood glucose does not influx in these cells to be stored for energy, and thus a high level of glucose builds up in the blood, which is termed as hyperglycemia. Family history and genes may also play a role in the pathogenesis of type 2 diabetes. Low physical activity, poor diet and excess body weight around the waist increases the chance of getting this disease. People with type 2 diabetes often have no symptoms at first or they may not have symptoms for many years. Early symptoms of diabetes has been considered as bladder, kidney, skin or other infections that are more frequent or heal slowly, fatigue, hunger, increased thirst, increased urination, blurred vision, erectile dysfunction, pain or numbness in the feet or hands [39, 190-193].

### 2.5. Diabetes and central nervous system

Prolonged hyperglycemia often associated with the number of complications such as diabetic neuropathy, retinopathy, nephropathy, cardiomyopathy etc. Diabetic neuropathy is damage to nerves in the body that occurs due to high blood sugar level [41]. In the central nervous system, diabetes exacerbates depression, phobias, anorexia [42-43] and reduces complex reasoning skills [44]. Clinically, patients with diabetes are at increased risk of developing depression and cognitive impairment as compared to the general population [44-45]. Further, diabetes accelerates the progression from mild cognitive impairment to severe dementia

[194]. Diabetes induces oxidative stress and inflammation in the hippocampal neurons resulting in neurodegeneration [14, 46].

In clinical settings, there was a close relationship between diabetes and depression [45]. Genesis of cognitive deficits in diabetic patients is very complex. A meta-analysis was conducted on 42 published studies inclusive of 21,351 adults and found that the prevalence of co-morbid depression was higher in diabetic women (28%) than in diabetic men (18%), in uncontrolled studies (30%) than in controlled studies (21%), in clinical samples (32%) than in community (20%) samples and when assessed by self-report questionnaires (31%) than in diagnostic interviews (11%) [45, 195]. The, worldwide estimations of depression prevalence among individuals with diabetes mellitus appear to vary with the type of diabetes and developed and developing nations. Depression is highly prevalent among people with diabetes and the prevalence rate varied greatly by demographic condition and diabetes types [196]. In U.S., data from the 2006 Behavioral Risk Factor Surveillance System by telephone survey of U.S. adults aged 18 and older, found that age adjusted depression rate was 8.3%, ranging from a low of 2.0% to a high of 28.8% among the 50 states [196]. In follow-up study it was found that about 45% of all diabetes patients had undiagnosed depression [197]. Glycated hemoglobin (HbA1c) is a form of hemoglobin that is measured primarily to observe the average plasma glucose level over prolonged periods of time. It is formed by non-enzymatic glycation pathway by exposure of hemoglobin's to plasma glucose. Clinical study reported co-morbidity of depression and diabetes in terms of HbA1c levels (Figure 4) [45].

Both diabetes and depression are the risk factor for neurocognitive impairment. How depression exacerbates diabetes has been covered in our earlier section. Study conducted in India revealed that 48% of diabetic patients with co-morbid depression showed cognitive impairment [198]. The basic pathophysiology of diabetes and depression with cognitive dysfunction has been shown in Figure 5. Animal models also revealed the persistence of depression and neurocognitive impairment during chronic diabetes. In experimental studies, STZ induced diabetic animals showed depressive like behaviour in Porsolt's forced swim test [47-48] and cognitive deficit in Morris water maze task [49].

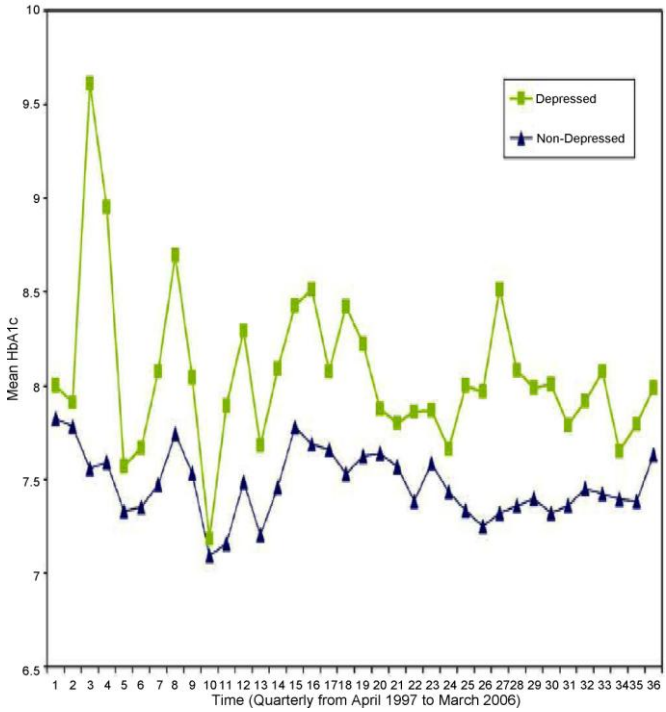



**Figure 4:** Comparison of unadjusted mean HbA1c over time among depressed and non-depressed adults with diabetes, reprinted from [45], Copyright © 2015, with permission from Elsevier.

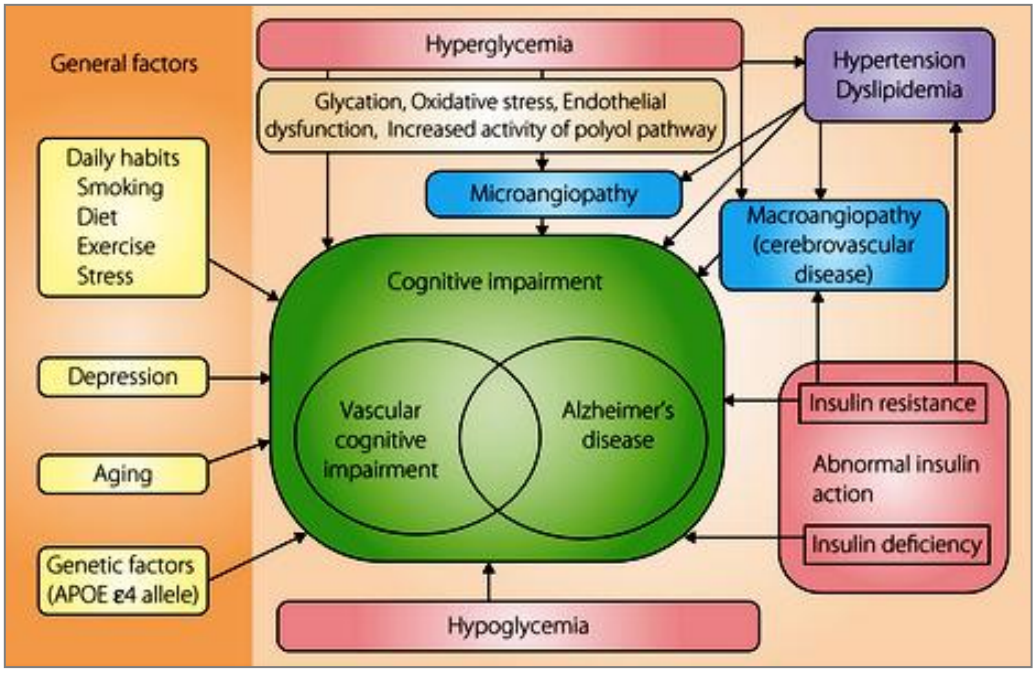



**Figure 5:** The possible mechanistic contribution of cognitive impairment seen in diabetes mellitus, stress and depression. Hyperglycemia, hypoglycaemia, depression, dyslipidemia and

abnormal insulin action have been implicated as major causes of cognitive impairment in diabetic patients, but many other factors, such as those shown in the Figure, are also involved. APOE, apolipoprotein E.Reprinted from [100], Copyright © 2015, with permission from John Wiley and Sons.

Many signaling pathway has been involved in the pathogenesis of diabetic neuropathy. ROSI, a PPARγ agonist is known to improve neuronal insulin receptor functioning in rat hippocampus during insulin resistance (Figure 6) [199]. Further, dysfunctioning of neuronal PPARγ receptor was observed in cognitive dysfunction [56].

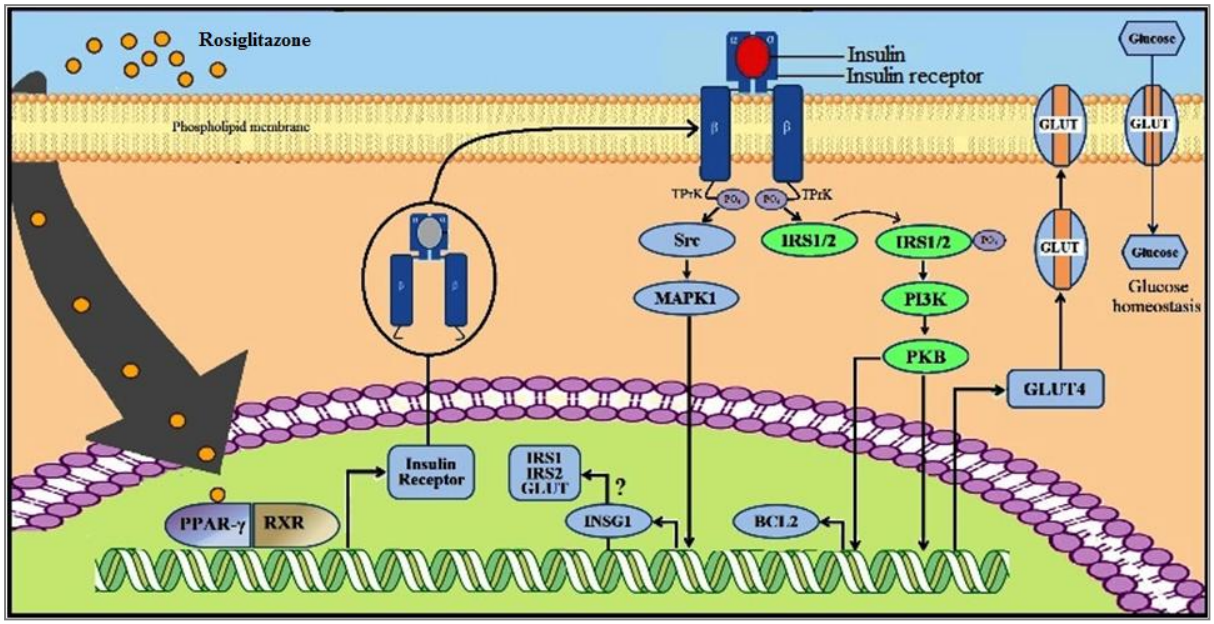



**Figure 6:** An overview on neuronal intracellular signaling of insulin receptor. IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; PI3K, phosphatidylinositol 3 kinase; GLUT4, glucose transporter type 4; INSG1, insulin induced gene 1; PPARγ, peroxisome proliferator activated receptor-γ; MAPK1, mitogen activated protein kinase 1; PKB, protein kinase B; BCL2, B-cell lymphoma 2.

During neuronal activity, insulin binds to the α-subunit of the IR and activates the tyrosine kinase residue of the β-subunit (TrkB) with subsequent activation of intracellular signaling cascades. Activation of the IR→Shc (Src homology collagen peptide)→MAPK pathway induces gene expression, that are required for cellular glucose homeostasis and synapse growth [121, 200]. IR has a direct effect on modulation of neurotransmission which influence

cognitive processes via insulin receptor substrate 1/2 (IRS1/2)→PI3K (phosphatidylinositol 3-kinase)→cyclic phosphodiesterase 3β (cPD3B) pathway [121]. Activation of IR→IRS1/2→PI3K→PDK (phosphoinositide dependent kinase)→protein kinase B (Akt/PKB) pathway suppresses the induction of apoptosis [201-202] and induces translocation of GLUT4 in the hippocampal neurons [203]. Insulin-like growth factor 1 receptor (ILGF 1r) is also known to stimulate IRS1/2→PI3K→Akt/PKB and Shc→MAPK pathways resulting in memory consolidation [204]. IR mediated translocation of GLUT4 in hippocampal neurons rapidly increases glucose uptake and utilization during neuronal activity, which is associated with hippocampal dependent learning and memory formation [17]. Conversely, cellular and metabolic alterations in the hippocampus caused by alteration in insulin signaling cascade is associated with cognitive deficit [205]. Experimental evidence suggests that, a decrease in the insulin receptor signaling cascade during diabetes [121]. Study revealed that both protein and mRNA levels of BDNF were severely reduced after STZ treatment. A recent study reported that downregulation of PPARγ levels in the hippocampus of diabetic mice [57] is associated with depressive like behaviour in forced swim test [58]. Further, downregulation of IR expression in hypothalamus induces depressive like behaviour in rats [59]. Diabetic neuropathies in brain are associated with severe deficiency of BDNF with depressive behaviour [60].

There is a correlation between BDNF, diabetes and Shh signaling. Diabetes induces oxidative stress and inflammation in hippocampus [14]. Oxidative stress is a process by which exposure of reactive oxygen intermediates, such as hydrogen peroxide ($H_2O_2$), superoxide anion ($O^{2-}$), NO and hydroxyl ($OH^-$) radical's damage the proteins and nucleic acids. The levels reactive oxygen species are neutralized by antioxidant defense system in the body [206]. NO induces inflammation of hippocampal neurons [30]. NO derived from iNOS contribute to the depressive like behaviors in mice due to neurodegenerative effects in the cerebral cortex [20]. Diabetes reduces coetaneous Shh signaling and associated with delayed wound healing potential in children. Dysregulation of Shh signaling was associated with impaired NO function and wound healing. Activation of Shh pathway is known to upregulates two important factors, namely BDNF and vascular endothelial growth factor. Shh signaling attenuates the effect of oxidative stress on cortical neurons and has potential role in neurodegenerative disorders [206].

Shh signaling is known to modulate cellular and neurochemical homeostasis in the adult nigrostriatal circuit. It has been reported that, the interruption of Shh signaling emerging from dopaminergic neurons of the mesostriatal circuit causes progressive adult onset degeneration in cholinergic, dopaminergic and fast spiking GABAergic neurons. Further, the imbalance between cholinergic and dopaminergic transmission leads to motor deficits indicative of Parkinson's disease [207].

ACh is necessary for proper functioning of cholinergic neurons in hippocampus and regulates the process of learning and memory [30, 208]. ChAT and AChE are responsible for the synthesis and metabolism of ACh. Evidence suggests that ChAT, a specific marker for functional state of cholinergic neurons [61], activity is reduced during STZ administration resulting in cognitive deficit [62-63]. It was reported that, STZ increases AChE activity in hippocampus resulting in cognitive deficit [64]. Previous study suggest that STZ-induced diabetes significantly downregulated the expression of mAChR1 in hippocampus termed cholinergic dysfunction [64]. Chronic diabetes resulted in motor control activity deficit [65]. Upregulation of mAChR4 in the striatum inhibits dopaminergic-D1 receptor-induced locomotor stimulation in mice [29]. Autophagic dysfunction has also been observed in diabetes mice [66].

### 2.6. Drug therapy

Antidiabetic drugs are used to treat diabetes by lowering the level of blood glucose. Apart from insulin, exenatide, pramlintide and liraglutide, all other drugs are administered orally and thus called oral antihyperglycemic agents. Type 1 diabetes is caused by the lack of insulin hence insulin injection is required to control type 1 diabetes. Type 2 diabetes is caused by insulin resistance [209]. The major categories of drugs used to control type 2 diabetes have been reported in Table 2 [209-210].

| S. No. | Class | Drugs |
|---|---|---|
| 1. | Sulfonylureas | Tolbutamide, Acetohexamide, Tolazamide, Chlorpropamide, Glipizide, Glyburide, Glimepiride, Gliclazide, Gliquidone |
| 2. | Biguanides | Metformin, Phenformin, Buformin |
| 3. | Thiazolidinediones | Rosiglitazone, Pioglitazone, Troglitazone |

| | | |
|---|---|---|
| 4. | Meglitinides | Repaglinide, Nateglinide |
| 5. | Alpha-glucosidase inhibitors | Miglitol, Acarbose, Voglibose |
| 6. | Others | Exenatide, Liraglutide, Taspoglutide, Lixisenatide, Vildagliptin, Sitagliptin, Saxagliptin, Linagliptin |

Sulfonylureas are act by stimulating or depolarizing pancreatic β-cells to facilitate insulin release. Biguanides acts on the liver cells to reduce gluconeogenesis and attenuate insulin resistance via increasing 5'AMP-activated protein kinase signalling. Thiazolidinediones is known to reduce insulin resistance by PPARγ gene transcription. Alpha glucosidase inhibitors delay the digestion of starch from the intestine and restrict intestinal absorption. These agents are effective only in the earliest stages of glucose intolerance, but might be helpful in combination with other oral antihyperglycemics. Meglitinides analogues depolarize the pancreas to produce insulin and are often called short acting secretagogues. Meglitidines act on the potassium channels as sulfonylureas but at different binding site. Dipeptidyl peptidase-4 (DPP4) inhibitors increase the concentration of incretin and glucagon like peptide-1 (GLP 1) in blood and thus induce insulin release [209, 211-212].

In view of pathogenesis in type 2 diabetes mellitus and brain disease, antidiabetic medications also known to positively modulate the metabolism of neuronal cell, which could be of clinical importance for the treatment of neurological disorders [85]. Type 2 diabetes mellitus affects neuronal cells by affecting neuronal metabolism, neuronal viability and behaviour. GLP 1 receptor (GLP 1r) agonists, biguanides and thiazolidinediones can attenuate hyperglycemia periphery and counteract the CNS complications of type 2 diabetes (Figure 7).

These drugs also have ameliorative effects on the central nervous system diseases [213-215]. The mechanisms of actions of these drugs in the brain pathology are being to be investigated. They might have direct effect on brain cells or act indirectly by modulating whole body metabolism [85].

Study demonstrated that ROSI possesses antidepressant like activity in behavioral models [216]. Glyburide potentiates the effect of antidepressants in the forced swimming test [217].

Chronic administration of metformin and milnacipran reduces the co-morbidity of depression and diabetes in patients [218]. GLP 1 is known to exert neuroprotective effects against cognitive deficits in individuals with depressive disorders [219]. The randomized controlled trials summarized current and ongoing research on the management of depression in patients with diabetes mellitus revealed that, there is no single treatment that consistently leads to better therapeutic outcomes in patients with co-morbid depression and diabetes [220-221].

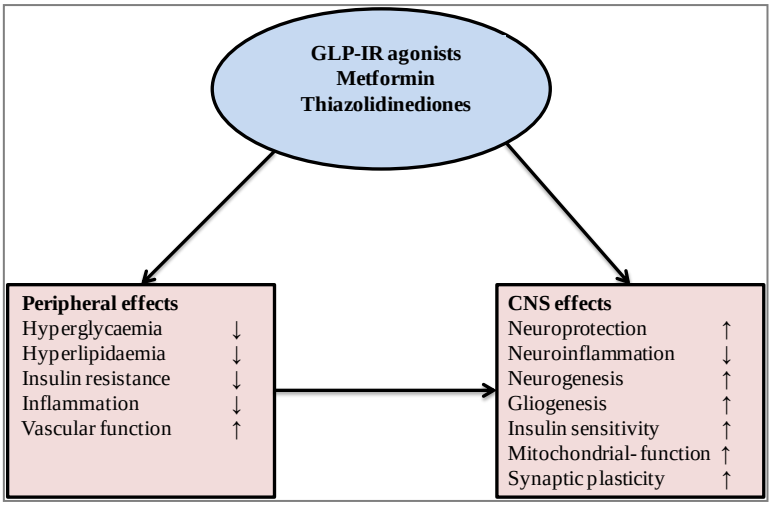



**Figure 7:** Summary of the effects of antidiabetics on the peripheral and central nervous system. Reprinted from [85], Copyright © 2015, with permission from Elsevier.

Metformin is known to induce neuroprotection against cytotoxic stress and improved insulin sensitivity in neuronal cell lines [222-223]. Metformin also induces neurogenesis in the rodent brain and improved spatial memory formation [224]. Results of clinical study showed that patients with type 2 diabetes mellitus with Alzheimer's disease receiving metformin had lower rate of cognitive dysfunction than patients not receiving metformin [213]. In a randomized double blind trial ROSI, a thiazolidinedione, or glyburide, a sulfonylurea, was combined with metformin, showed improvement in glycemic control as well as working memory in type 2 diabetic patients after 24 weeks [225]. In randomized controlled trial study, subjects were administered with either repaglinide or glibenclamide as oral hypoglycemic agents for 1 year, cognitive function test showed greater decline in cognitive performance in the glibenclamide group [226]. However, a population based case control trial showed that patients with type 2 diabetes mellitus receiving metformin had a slightly higher risk of developing Alzheimer's disease than those who did not receive the metformin [227].

Thiazolidinediones act via PPARϒ receptor. Its regulation depends on transcriptional coactivators peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Downregulation of PGC-1α leads to mitochondrial dysfunctions and associated with increased oxidative stress that are commonly observed in type 2 diabetic patients and neurological disorders [228-230]. GLP 1r activation increases the cellular insulin sensitivity by amplifying insulin signaling [231]. Further, GLP 1r agonists are known to counteract neuroinflammation, enhance neurosynaptic transmission and memory performance [232-233]. The potential cellular targets of major antidiabetic drugs in the central nervous system have been reported in Figure 8.

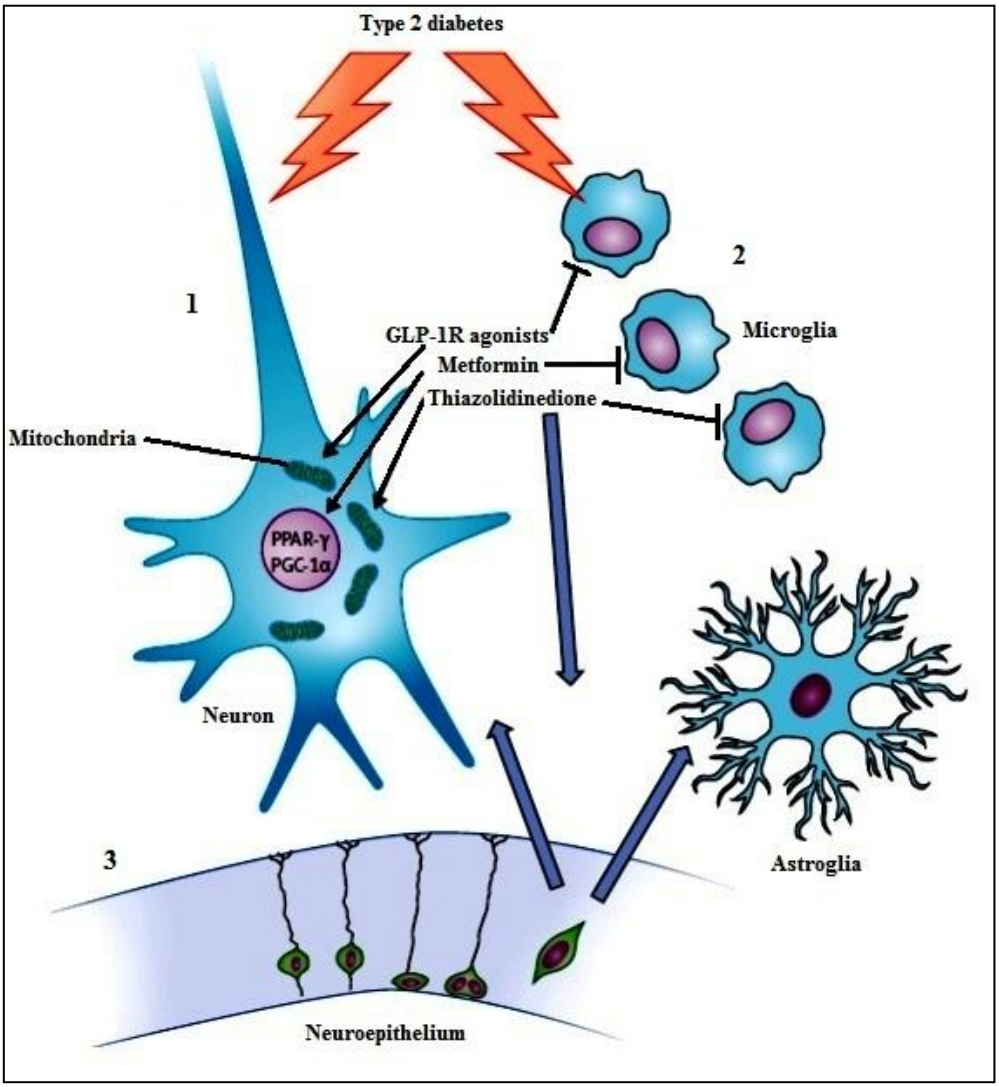



**Figure 8:** The potential cellular targets of major antidiabetic drugs in the central nervous system. Drugs used to treat type 2 diabetes mellitus have various targets in the central

nervous system, including neuronal cells (1), glial cells including both microglia and astroglia (2) and neuronal precursor cells (3). These drugs exert neuroprotective effects by acting at the level of gene transcription, mitochondria and cell signalling cascades. These drugs block the effect of chronic diabetes on microglia. Evidence suggests that these drugs can stimulate neurogenesis in the epithelium and contribute to tissue regeneration and repair. Reprinted from [85], Copyright © 2015, with permission from Elsevier.

Thiazolidinediones, like ROSI and pioglitazone are the potent insulin sensitizers in type 2 diabetes mellitus that effectively reduces hyperglycemia as well as fatty acids [234]. Clinical trials report showed that ROSI improves memory performance in Alzheimer's patients [235]. Another study revealed that ROSI did not improve cognitive performance in patients with Alzheimer's disease [236]. Cognitive impairment has been observed in patients receiving insulin therapy [237-238]. There have been reports that agents ameliorating incretin effects will have protective effects on neurons and could be effective in preventing dementia [239-240].

Many antidiabetic drugs are known to induce hypoglycaemia. Besides, hypoglycaemia is associated cognitive impairment [100]. Sulfonylurea causes an average of 2-4 kg weight gain and hypoglycaemia. Adverse effects of repaglinide includes, upper respiratory infection, dizziness, athralgia, back pain, diarrhea and hypoglycaemia [241-242]. Sitagliptin is associated with constipation, influenza, nasopharyngitis, upper respiratory infection, headache and cough. Acarbose elicited flatulence, abdominal pain, diarrhea, dyspepsia and nausea [241]. It has been reported that patients receiving chronic pioglitazone therapy showed increased incidence of bladder cancer compared to general population [101-102]. Chronic ROSI therapy is associated with an increased incidence of myocardial infarction and heart failure in type 2 diabetic patients [103].

### 2.7. Animal models of depression

The chronic unpredictable mild stress model (CUMS; also referred to as chronic variable or intermittent stress), is a widely used rodent model of depression, which consists of the repeated exposure to an array of unpredictable and mild stressors over a sustained period of time (ranging from 10 days to 8 weeks). The CUMS model was originally developed by Paul Willner in the late 1980s based on both clinical and preclinical research regarding the

etiology of depression [8-9]. In humans, chronic exposure to uncontrollable and unpredictable life stressors is often said to be a major participant in the development of depressive disorders [243-244]. Based on this knowledge, earlier studies demonstrated that exposure of rodents to severe stressors resulted in a reduction in physical activity and of their consumption of rewarding, palatable substances namely sucrose [9]. This reduction in sucrose consumption was believed to be similar to the impairments in reward processing, which is commonly anhedonia, a core symptom of major depression. Therefore, the chronic mild stress model was developed, which consisted of repeated exposure of rodents to a series of unpredictable "microstressors", making the more reliable model for depression [245]. The endpoint of this model focused exclusively on sucrose intake and preference as well as decreased physical activity, which was also used as an endpoint, which was believed to relate with the deficit in hedonic impact in these rodents. The validity of this model was evaluated by the fact that this reduction in hedonic impact was reversible by chronic treatment with antidepressant agents, which mimicked the time course required for clinical effectiveness [246-247]. One major argument regarding the reductions in sucrose intake was simply a reflection of the reduced body weight and food consumption that are typically concurrent with chronic mild stress exposure [248]. However, subsequent studies controlled the body weight changes and demonstrated deficits in sucrose preference (as opposed to intake) argued against this proposition [249-250].

During the last two decades, there has been an explosion of behavioural research, which has extended the behavioural endpoints of this model with other models of depression beyond hedonic processing and reward salience. For example, exposure of animals to CUMS enhances immobility in the forced swim and learned helplessness test, decreases the frequency of male sexual and aggressive behaviors, reduces self-care and increases rapid eye movement sleep latency [251]. Thus, despite a few anomalous findings from some laboratories, and regardless of some continuing controversy about the reliability of this model from laboratory to laboratory, the CUMS model has been widely accepted as a valid model of depression in rodents.

Another variable that comes into light is the duration of CUMS. The original CUMS paradigm [247] was developed as an 8-week paradigm that would allow 3 weeks of initial stress exposure prior to the onset of antidepressant treatment and then continue for the next 5

weeks. The rationale behind this outcome from a clinical standpoint is that, the depressive behaviour must be established before the onset of antidepressant therapy. However, recent studies demonstrates that many of the effects of CUMS, especially the robust and reliable effects, are present as early as 10 days following the onset of CUMS exposure and are nearly all present following 3 weeks of CUMS [245]. For example, unpredictable mild foot shock stress for 21 days induced significant hyperglycaemia, glucose intolerance, hypercorticosteronemia, gastric ulcerations, male sexual dysfunction, immunosuppression, cognitive deficits and mental depression in rats [10]. Exposure to 21 days of CUMS significantly reduced brain-pancreas relative protein, accompanied by an increase in levels of blood sugar with hypoinsulinemia [11].

An important variable that comes into light from literature is the precise post-CUMS time interval when measurements should ideally be considered. Most studies conducted the CUMS model performed their tissue extraction on the day following the last day of CUMS, a point at which residual effects of CUMS appeared, but acute effects of stress exposure would be absent given the lag time since the last stress exposure. However, when studies employed a longer rest period following CUMS exposure (such as 1 week following CUMS termination) most of these effects were not present [252-253]. These findings are encouraging in the sense that, they suggest the recovery of functions following the cessation of stress exposures, they also highlighted the importance of consistency in experimental methodology and the importance of standardizing time points of analysis. In addition, termination of animals for longer time intervals, such as 1 or 2 weeks post-CUMS or longer, would provide important information on how long-lasting the effects of CUMS and/or antidepressant therapy are present on specific outcome measures. Whether effects are transient or long-lasting is important in extrapolating the adverse effects of chronic stressors and therapeutic effect of antidepressants from the animal model to the human situation [245].

Apart from CUMS, tail suspension and forced swim test models, other models have also been described. Behavioural changes after neonatal clomipramine treatment produces changes in adult rats that resemble endogenous depression in man. Not only clomipramine, but also other psychotropic drugs induced changes in the behaviour of adult rats after treatment in neonatal age. However, the specificity of this procedure to evaluate potential antidepressant compounds remains to be established [254]. A selective inhibition of mouse-killing behaviour

in rats by antidepressants has also been investigated as a test model. The test can be use to evaluate antidepressants such as tricyclics and monoamine oxidase inhibitors [255]. In this test neuroleptics and benzodiazepines are active in doses which impair motor performance [256]. Major drawback of this test was might be the large numbers of mice are required to be sacrifice by rats during trails. Antidepressants like drugs block the re-uptake of biogenic amines into nervous tissue. In this way, the toxic effects of norepinephrine are potentiated. Hence the test for antidepressant activity based on potentiation of norepinephrine toxicity was investigated [256]. The critical assessment of this method was that, several antidepressants block not only the uptake of noradrenaline, but also of dopamine and of serotonin, which might interfere with the potency and nature of drugs. It has been reported that apomorphine induces emesis in man and in other species, like dogs. Treatment of rodents with apomorphine causes compulsive gnawing behaviour instead of vomiting. The compulsive gnawing behaviour in mice by apomorphine is due to stimulation of dopaminergic system. Centrally acting anticholinergics shift the balance between acetylcholine and dopamine resulting in potentiation of the apomorphine effect. Therefore, many drugs with psychotropic activity are known to possess apomorphine-synergistic effect. This enhancement was also found after the administration of tricyclic antidepressants. Critical assessment of this test was that, not only antidepressants, but also centrally acting anticholinergics and antihistaminics are active during the test [256-257]. However, the test has the advantage of its simplicity without any pretraining of the rodents.

## 2.8. Animal models of diabetes

Animal models have been extensively used in diabetes research. In the 1880s, von Mering was working on the absorption of fat from the intestine when Minkowski removed the pancreas of a dog. The animal developed polydipsia and polyuria, and was found to have diabetes mellitus. Thereafter, many experiments on rabbits and dogs followed, although history has given the special place to Marjorie, one of the dogs used by Banting and Best in their experiments on the isolation and purification of the peptide insulin in the 1920s. Marjorie was the most famous experimental animal in history, only to be superseded by the Dolly sheep in recent years [258]. Recently, most experiments are carried out in rodents [259-260], although some studies are still conducted on larger animals [258, 261].

Several toxins including STZ and alloxan induce hyperglycaemia in rats and mice. Since the initial findings in 1943 of alloxan induced β-cell toxicity in rabbits, this compound has long been used for inducing diabetes. Alloxan is a uric acid derivative and is highly unstable in water at neutral pH, but stable at pH 3. Alloxan acts by selectively destroying the pancreatic beta cells leading to hypoinsulinemia, hyperglycaemia and ketosis [262]. Alloxan causes diabetes in many rodent and non-rodent animals and is most preferably used in case of rabbit because of the ineffectiveness of STZ in rabbits for induction of diabetes and development of well characterized diabetic complications [262-264]. However, guineapig and musk shrew have been reported to be resistant to the action of alloxan due to unknown mechanisms [262]. Because of low stability of alloxan, relatively very shorter half-life (less than 1 min) and acidic nature of the solution, intravenous route of administration is preferred. The alloxan treated animal's exhibit severe hyperglycaemia, glucosuria, hyperlipidemia, polyphagia, polydypsia and other symptoms of diabetes and also develop various complications such as neuropathy, cardiomyopathy, as well as marked retinopathy etc. Alloxan use is disadvantageous as the percentage incidence of diabetes is quite variable among the experimental groups. In addition, the incidence of ketosis and resulting mortality is very high. Because of these drawbacks, alloxan is now almost replaced by STZ for induction of diabetes in laboratory animals [261].

Streptozotocin is an antibiotic derived from *Streptomyces achromogenes* and is structurally to the glucosamine derivative of nitrosourea. Rakieten et al. first demonstrated the diabetogenic property of STZ in dogs and rats [50]. Like alloxan, it induces hyperglycaemia by its direct cytotoxic action on the pancreatic β-cells [51-52]. Its nitrosourea moiety is responsible for destruction of pancreatic β-cells, while deoxyglucose moiety facilitates transport across the cellular membrane. Like alloxan, the participation of free radicals generation and resulting in alteration of endogenous scavengers of these radicals has been reported in STZ diabetogenecity. In addition, STZ causes alkylation or breakage of DNA strands and subsequent increase in the activity of poly-ADP-ribose synthetase, an enzyme depleting nicotinamide adenine dinucleotide in β-cells, resulting in energy deprivation and death of β-cells. These hypotheses have been confirmed by different studies in which the administration of various chemicals such as free radical scavengers and poly ADP-ribosyl synthase inhibitors, concomitantly or before STZ injection prevent or lessen the severity of the induction of diabetes [51, 265-266]. STZ is a preferred chemical agent to induce

experimental diabetes because it has some advantages over alloxan such as, relatively longer half-life (15 min), sustained hyperglycaemia for longer duration and the development of well characterized diabetic complications with limited incidence of ketosis as well as mortality [51]. Alloxan and STZ diabetic animals are widely used for screening the compounds including natural products for their insulinomimetic, insulinotropic, hypoglycaemic, antihyperglycaemic activities [261]. It has been reported that STZ induces both type 1 diabetes and type 2 diabetes [267-268]. Experimental evidence suggests that, high doses of STZ induce rapid and complete insulin deficiency resembling type 1 diabetes. However, multiple lower doses of STZ, which cause partial destruction of β-cells, can be used to produce type 2 diabetes [269]. In STZ treated mice, changes in spinal terminals of calcitonin gene-related peptide in sensory neurons were observed 4 weeks after diabetes and progressively worsened with time (6-7 weeks) [53]. With increasing duration of diabetes from 7 to 9 weeks, there is a loss in cutaneous C-fiber innervations [270] and decrease in motor nerve conduction velocity, sensory nerve conduction velocity as well as hypoalgesia [271-272]. Besides, STZ induced chronic diabetes showed depressive-like behaviour when submitted to the forced swim test, which is a predictive animal model of depression. It has been reported that, depressive like behaviour in diabetic animals was due to alteration of monoamine levels in brain as well as oxidative stress and inflammation. In addition, STZ induced diabetic animals showed a significant hypolocomotion with respect to control animals [54]. In another study, STZ induced diabetic animals showed cognitive dysfunction in a spatial version of the Morris water maze test. It has been suggested that STZ exacerbates cognitive ability in animals by down-regulating the expressions of BDNF and cAMP responsive element binding protein and by inducing hippocampus neuronal apoptosis [55].

Apart from chemically induced hyperglycemia, other animal models of type 2 diabetes has also been known and divided into following major categories. (1) Spontaneous or genetically derived diabetic animals, which includes both obese and non-obese diabetic models. Obese type type-2 diabetic models include *ob/ob* mouse, *db/db* mouse, KK mouse, KK/$A^y$ mouse, NZO mouse, NONcNZO10 mouse, TSOD mouse, M16 mouse, Zucker fatty rat, ZDF rat, SHR/N-cp rat, JCR/LA-cp rat, OLETF rat, Obese rhesus monkey. Non-obese type 2 diabetic models include, Cohen diabetic rat, GK rats, Torri rat Non obese C57BL/6, ALS/Lt mouse. (2) Surgical diabetes by partial pancreatectomized animals. (3) Transgenic/knock-out diabetic animals such as transgenic or knockout mice involving genes of insulin and insulin receptor

and its components of downstream insulin signaling e.g. IRS-1, IRS-2, GLUT4 and others like PPARϒ tissue specific knockout mouse, glucokinase or GLUT2 gene knockout mice, human islet amyloid polypeptide overexpressed rat (HIP rat) [261].

## 2.9. **Stinging nettle (*Urtica dioica*)**

### 2.9.1. Description

*Urtica dioica* (UD) is indigenous to Asia and Africa, but also found in all temperate regions of the world including Australia, Europe and America. It is mainly found in the North Western Himalayas from Kashmir to Kumaon at the altitudes of 2100-3200 m. Vernacular names of the plant are Bichu Butti (Hindi and Punjabi), Vrishchhiyaa shaaka (Sanskrit), Anjuraa (Unani) and Shisuun in (Kumaon). UD is an herbaceous plant and commonly known as stinging nettle (Figure 9) belonging to the family Urticaceae. Leaves are opposite, oblong or ovate and toothed. The colour of upper surface is dark green and underside paler or light green. Flowers are small, incomplete and green [273].

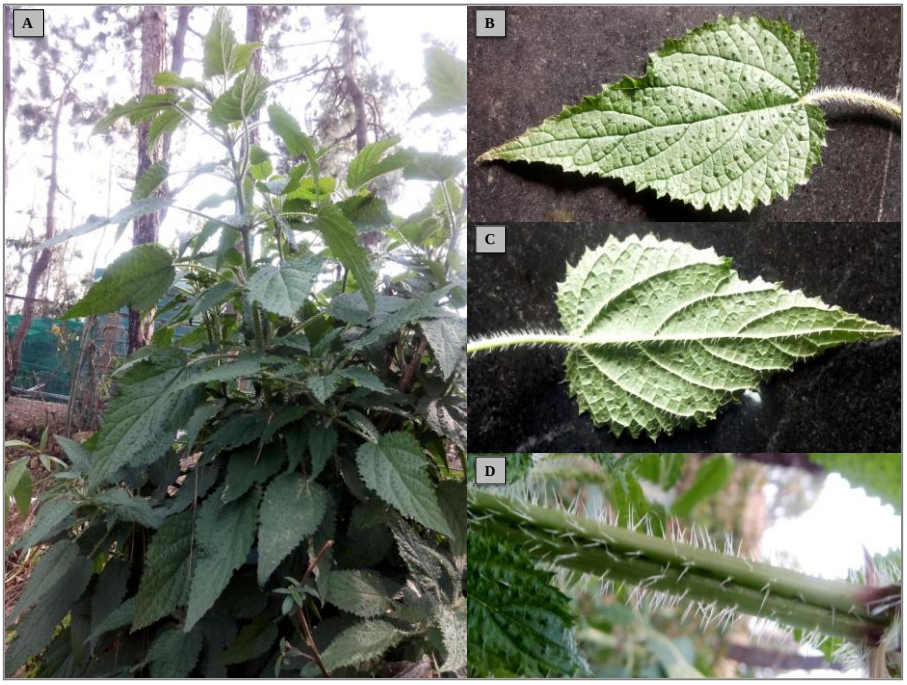



**Figure 9:** Parts of stinging nettle plant. *Urtica dioica* whole plant **(A)**, leaf upper surface **(B)**, leaf lower surface **(C)** and stem with trichomes **(D)**.

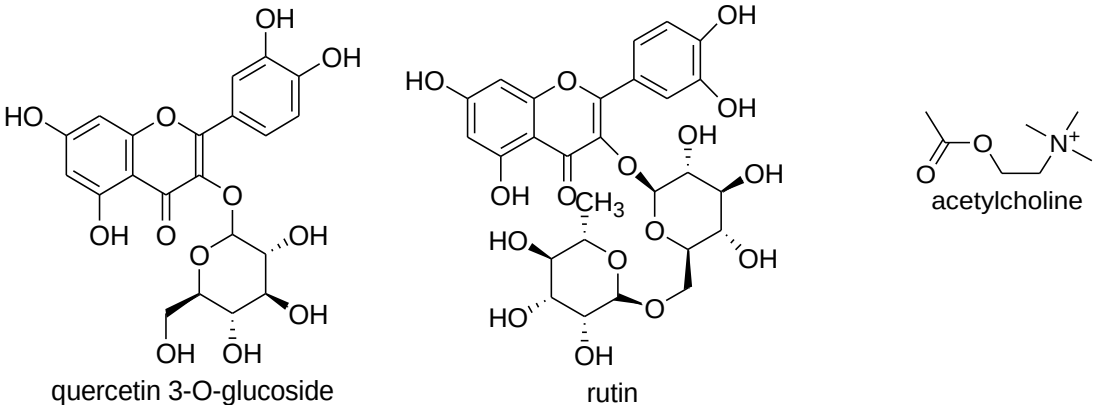
Both leaves and stems are covered with glandular trichomes that contain ACh, formic acid, 5HT and histamine. The plants cause intense skin irritation if touched [104]. Transverse

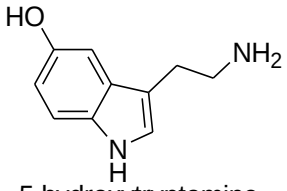
section of nettle leaf has a layer of upper and lower epidermis, underside of the leaf contain more stomata. Stinging nettle leaf contains glandular and non glandular trichomes. Mesophyll occupied by 5 to 6 row of spongy parenchyma embedded with rosette and cluster type of calcium oxalate crystals [105].

### 2.9.2. Phytochemical Studies

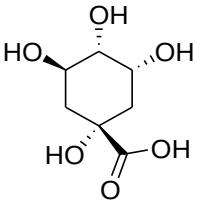
A large number of chemical compounds belonging to different chemical classes including fatty acids, phenylpropanes, coumarins, phenolics, flavonoids, lignans, ceramides, terpenes, lectins, triterpenes and sterols have been isolated from the herb of UD [273-276]. Flowers of UD contains p-hydroxybenzoic acid, gentisic acid, protocatechuic acid, vanillic acid, quinic acid, caffeic acid, ferulic acid, 5-O-caffeoylquinic acid, esculetin, scopoletin, chrysoeriol, kaempferol, isorhamnetin, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, quercitrin, rutin and amentoflavon. UD leaves are known to contain cholineacetyltransferase, 5HT, ACh, p-hydroxybenzoic acid, gentisic acid, protocatechuic acid, quinic acid, caffeic acid, ferulic acid, 5-O-caffeoylquinic acid, esculetin, scopoletin, chrysoeriol, rutin and amentoflavon. UD root contains p-hydroxybenzoic acid, quinic acid, p-coumaric acid, caffeic acid, ferulic acid, 5-O-caffeoylquinic acid, esculetin, scopoletin, secoisolariciresinol, chrysoeriol and rutin. UD stem contains p-hydroxybenzoic acid, quinic acid, p-coumaric acid, caffeic acid, ferulic acid, 5-O-caffeoylquinic acid, esculetin, scopoletin, chrysoeriol, quercetin 3-O-glucoside and rutin (Figure 10) [277-280].

**Figure 10:** Structure of the some chemical constituents present on stinging nettle.

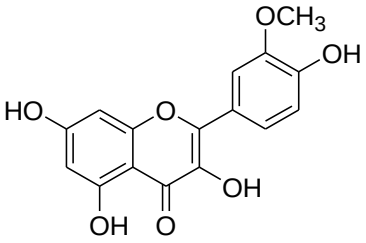




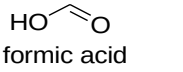
5 hydroxytryptamine



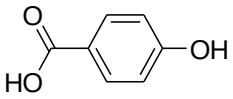
quinic acid



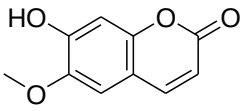
isorhamnetin



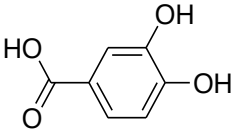
formic acid



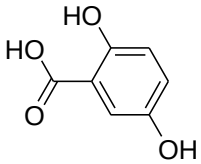
p-hydroxybenzoic acid



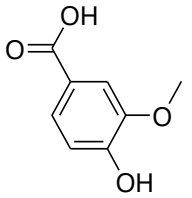
scopoletin



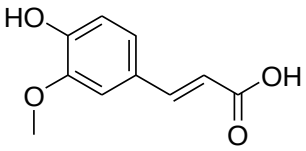
protocatechuic acid



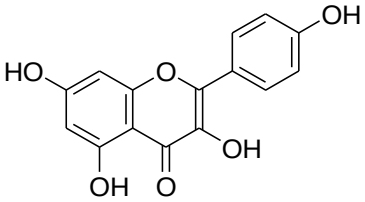
gentisic acid



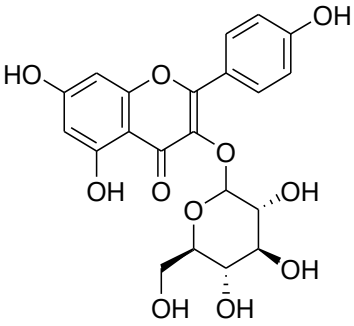
vanillic acid



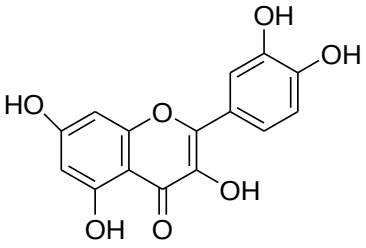
ferulic acid



kaempferol



kaempferol 3-O-glucoside



quercetin

### *2.9.3. Pharmacology*

Stinging nettle extract has been known to decrease the level of blood glucose. Administration of alcoholic and aqueous extract of UD leaves repaired pancreatic tissue damage induced by STZ in rats [106]. A polyherbal mixture containing UD, *Cinnamomum zeylanicum*, *Nigella sativa colocynthis*, *Citrullus Juglans regia*, *Olea europaea*, *Vaccinium arctostaphylos*, *Trigonella foenum*, *Allium sativum*, *Punica granatum*, *Salvia officinalis* and *Teucrium polium* reduced fasting blood glucose level, water intake, urine output and hyperlipidemia in diabetic rats [281]. Kadan et al., demonstared that the antihyperglycemic effect of hydroalcoholic

extracts of UD was mediated through GLUT4 membrane translocation in L6-GLUT4myc cells [282]. UD extract significantly reduced serum glucose, insulin, low-density lipoprotein (LDL) and leptin, and LDL/HDL (high-density lipoprotein) ratio and insulin resistance in fructose induced diabetic rats [107]. The aqueous extract of UD leaves significantly attenuated blood glucose level during oral glucose tolerance test in rodents [283].

In a randomized double blind placebo controlled clinical trial, UD is reported to have glycemic control in type 2 diabetic patients by lowering the levels of fasting and postprandial blood glucose [108]. Earlier studies reported that administration of diabetic patients with UD significantly increased antioxidant capacity and reduced inflammatory stress and glycated hemoglobin [109-110].

It has been demonstrated that UD extract compensate astrocytes loss and granule cell density in the dentate gyrus, which can attenuate cognitive impairment in diabetic rat [284]. Nettle supplementation has been known to reverse the brain injury in rats caused by N-methyl-D-aspartate (NMDA) receptor. Nettle supplementation reduced the level of free radicals and DNA binding activity of nuclear factor kappa-B. Nettle supplementation has also been known to possess antiapoptotic effect, thereby promoting cell survival in the brain. These data demonstrated that nettle supplementation improves antioxidant capacities, downregulates inflammatory transcription factors and ameliorate learning performance in passive avoidance step through task [285-286]. Hydroalcoholic extract of stinging nettle significantly lowered the activity of cytochrome $P_{450}$, lactate dehydrogenase, nicotinamide adenine dinucleotide, cytochrome $P_{450}$ reductase, total sulfhydryl groups, nonprotein sulfhydryl groups and protein bound sulfhydryl groups. UD extract effectively improved glutathione S-transferase, DT-diaphorase, superoxide dismutase and catalase activity in forestomach and superoxide dismutase and catalase activity the lungs [287].

UD leaves extract showed significant hepatoprotective activity against carbon tetracloride induced liver toxicity in rats by decreasing lipid peroxidation and increasing antioxidant defense system [288]. Treatment of UD seeds extract restored the aflatoxin induced imbalance between malondialdehyde and antioxidant system in rat liver [289]. Methanolic extract of UD showed defensive role against cisplatin induced toxicity in tumor bearing mice by decreasing alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, lactate dehydrogenase, creatinine, protein oxidation and lipid peroxidation, as well as

increasing catalase, reduced glutathione, glutathione peroxidase, superoxide dismutase and glutathione S-transferase status resulted in nephroprotective, hepatoprotective and antioxidant activity [290]. UD seeds extract showed protective effect against ischemia/reperfusion induced morphological changes in rat liver [291].

Tourniquets are used to provide bloodless field for many surgical procedure by minimizing the blood loss. They might induce ischemia/reperfusion injury locally or systematically. In one experiment tourniquet application in rats was used to induce oxidative stress in muscle tissues and the effect of UD extract was evaluated. Results of this study demonstrated that UD extract has a potential to restore ischemic muscle tissues via antioxidant mechanism [292]. UD extract is also known to possess hypotensive, natriuretic and diuretic effects in rats which were comparable to furosemide [293]. In a Langendorff perfused rat heart experiment, aqueous extract of stinging nettle exerts vasoconstriction effect on aorta via stimulation of alpha1 adrenergic receptors. Further, stinging nettle strongly induced bradycardia which might be responsible for hypotensive action [294].

Methanolic extract of stinging nettle dose dependently inhibited the carrageenan induced paw edema as well as acetic acid-induced abdominal twitches in mice; the highest effective dose of stinging nettle was 400 mg/kg [295]. In another experiment, aqueous extract of stinging nettle dose dependently inhibited (50, 100 and 200 mg/kg) the acetic acid induced writhing in mice [296]. Activation of nuclear factor kappa B is increased in several inflammatory diseases and responsible for the upregulation of many pro-inflammatory genes. Results of the earlier study demonstrated that UD leaves extract have potential to inhibit cytokines production as well as inflammation and arthritis by blocking nuclear factor kappa B pathway [293].

Aqueous extract of UD showed immunomodulatory and antiinflammatory effects on the murine splenocytes and murine peritoneal macrophages. It was demonstrated that UD extract activated the proliferation of T-lymphocytes and decreased nitric oxide production in lipopolysaccharide induced macrophages without affecting the cell viability [297].

A randomized double blind clinical trial was performed to evaluate the effect of stinging nettle plant in the symptoms of benign prostatic hyperplasia. Nettle supplementation significantly relived the clinical symptoms in patients with benign prostate hyperplasia

compared to placebo without any side effect [298]. Further, in a prospective, randomized double blind, placebo controlled, crossover study UD administration significantly reduced the symptoms of benign prostatic hyperplasia [299-300]. UD extract in combination with Sabal extract showed clinically relevant benefit in patients with lower urinary tract symptoms [301]. A protein fraction from the aerial parts of stinging nettle elicited antimutagenic and antioxidant activity in cell lines of human hepatoma HepG2 cells [302]. Extract of stinging nettle attenuated testosterone induced prostatic hyperplasia in rodents [278]. The 20% methanolic extract of UD significantly inhibited benign prostatic hyperplasia induced by implanting urogenital sinus into the ventral prostate gland of mouse [303].

The crude UD extract exhibited significant antimicrobial activity against Gram-positive bacteria [304]. UD extract exhibited promising antibacterial activity against fast growing, non pathogenic *Mycobacterium semegmatis* bacteria in disk diffusion method [305]. In earlier study, UD extract also showed promising effect against *C. michiganensis* and *Xanthomonas vesicatoria* bacteria [306]. Aqueous extract of UD showed antifungal effect against *A. alternate* and *R. solani* [307]. N-acetylglucosamine, the specific lectin from stinging nettle is the potent and selective inhibitor of cytomegalovirus and human immunodeficiency virus replication *in vitro* [308].

The $LD_{50}$ value of UD leaf extract was observed as 3.625 g/kg in mice. Higher dose (>750 mg/kg) was associated with hypothermia and reduced muscular tone. In toxicity studies, 50 mg/kg of hydroalcoholic extract of UD was administered orally to rabbits for 10 days. Results of this study revealed that UD extract caused occasional diarrhoea and reduced 40% bodyweight [273]. However, UD extract did not show significant toxicity after long term use in clinical trials [309-310].

## 2.10. St. John's wort (*Hypericum perforatum*)

### 2.10.1. Description

Saint John's wort also known as *Hypericum perforatum* is a flowering plant of the genus Hypericum belonging to the family Hypericaceae (Figure 11). Common name of *Hypericum perforatum* derived from its traditional flowering as well as harvesting on 24 June St. John's day. St. John's wort is also known as Johns wort, Goat weed, Rosin rose, Klamath weed and Tipton weed. *Hypericum perforatum* is a branching shrubby herb. The branches and stems of

*Hypericum perforatum* are densely covered by oblong and smooth margined leaves. Leaves are green in colour with black spots which is visible against light. Leaves exerts distinct and balsamic odour with astringent and bitter taste. The aerial part of mature plant produces several dozen of five petaled yellow colour flowers. The edges of petals are covered through black dots. Crushed flowers of St. John's wort produce a blood red colour pigment. The species is native of Europe but has also spread to temperate regions of Asia, Africa, Australia and North and South America. Due to photosensitivity reaction, *Hypericum perforatum* is listed as a noxious weed in the United States [67-68].

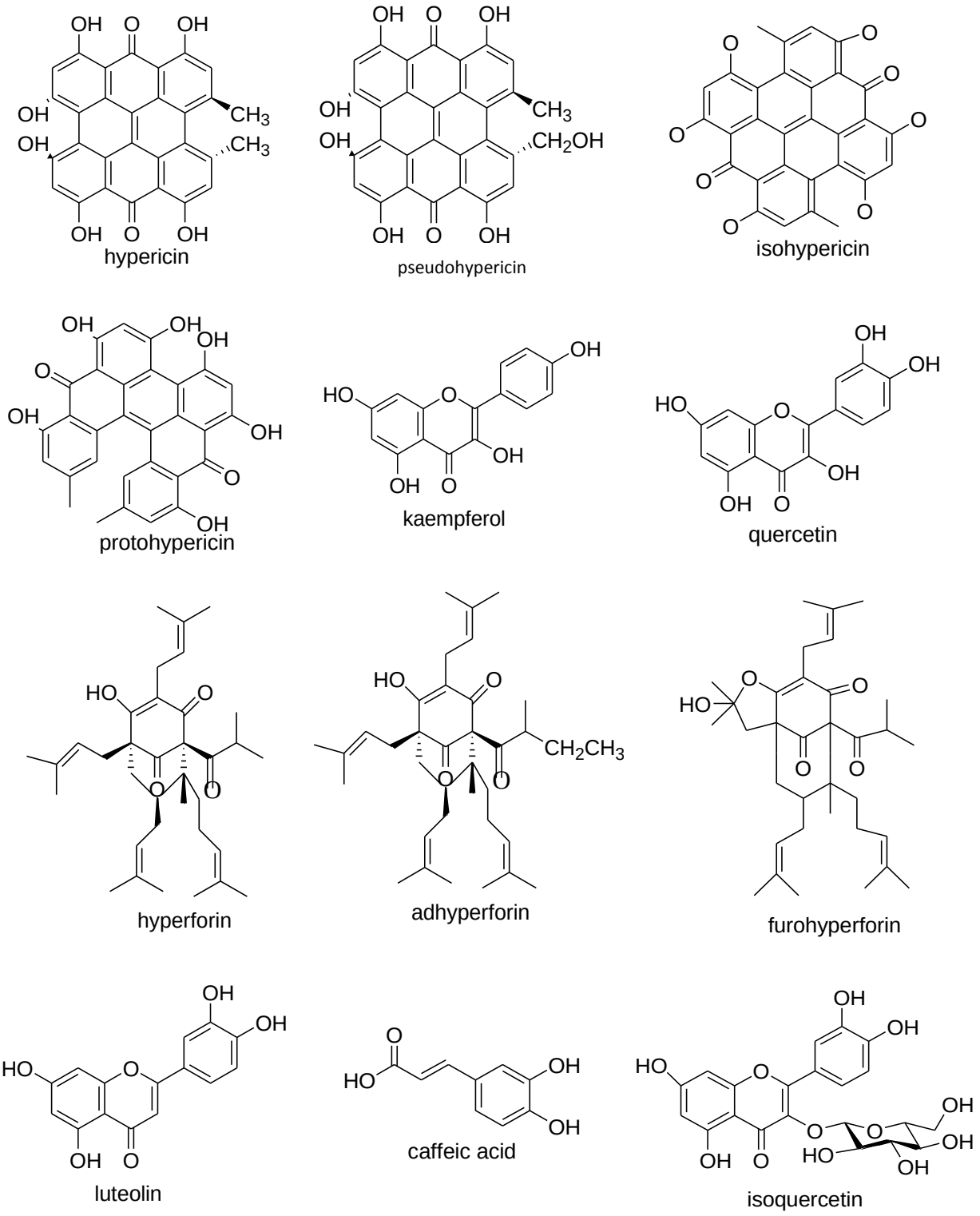
**Figure 11:** Parts of Saint John's wort plant. St. John's wort whole plant **(A)** and flower **(B)**.

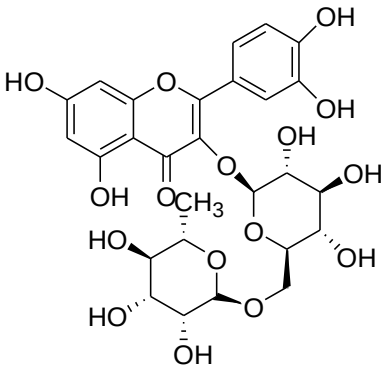

### 2.10.2. Phytochemical Studies

The most common chemical constituent naphthodianthrones include hypericin, isohypericin pseudohypericin and protohypericin [311]. Flavonoids content of *Hypericum perforatum* ranging from 7.0% in stems to 12.0% in flowers and leaves. Kaempferol, quercetin, luteolin, hyperside, isoquercitrin, rutin, amentoflavone, hyperin, myricetin, rutin and miquelianin are the important flavonoids of this plant [312-315]. Extracts of *Hypericum perforatum* contains lipophilic compounds including hyperforin, adhyperforin and furohyperforin. Essential oils of *Hypericum perforatum* consist of mono and sesquiterpenes i.e. 2-methyl-octane, pinene, terpineol, myrecene, caryophyllene, geranil and limonene. Additional components of *Hypericum perforatum* contain chlorogenic acid, caffeic acid, p-coumaric acid, nicotinic acid, myristic acid, palmitic acid, stearic acid, carotenoids, pectin, choline, long-chain alcohols and

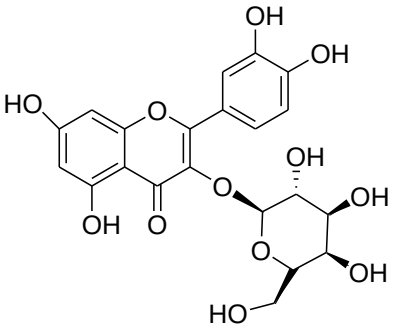
hydrocarbons. Several amino acids like cysteine, leucine, glutamine, lysine and γ-aminobutyric acid have also been isolated from *Hypericum perforatum* extract [67, 316-317].

**Figure 12:** Structure of the some chemical constituents present on St. John's wort.

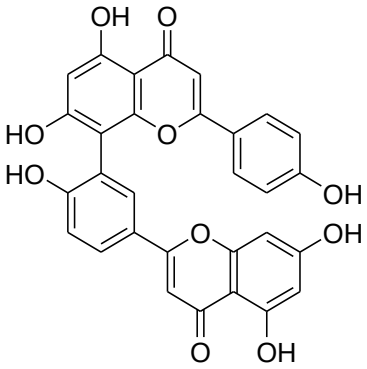




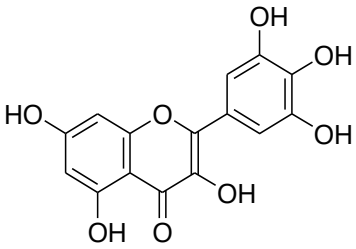

rutin



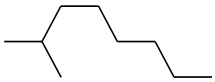

hyperoside



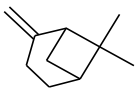

amentoflavone



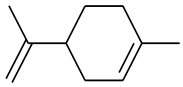

myricetin



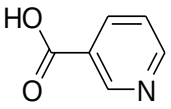
2-methyl-octane



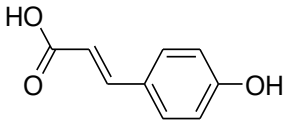
pinene



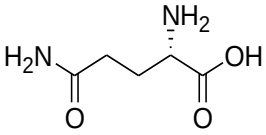
limonene



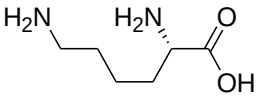

nicotinic acid



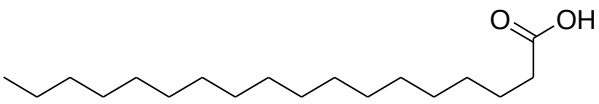

p-coumaric acid



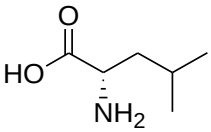

glutamine



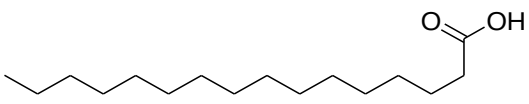

lysine



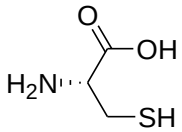

stearic acid



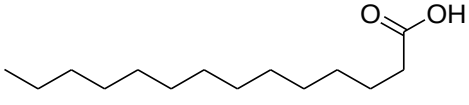

leucine



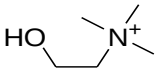

palmitic acid




cysteine




myristic acid




choline

### 2.10.3. Pharmacology

The herb of St. John's wort has been used from centuries to treat various ailments. In Europe, St. John's wort is commonly prescribed for the treatment of depression and in United States, it is available as over the counter herbal supplement [69]. Different controlled clinical trials showed its effectiveness in the treatment of mild-to-moderate depression [70]. Randomised and double blind controlled trials in 5489 patients demonstrated that Hypericum extract is superior to placebo in patients with depressive disorder and their effectiveness is similar to that of standard antidepressants with fewer side effects [71]. Evidence suggests that the antidepressant activity of Hypericum extract is mediated through inhibition of the reuptake of synaptosomal neurotransmitters such as 5HT, dopamine, NE [318] and modulation of neuronal excitability via GABAergic mechanisms [319].

Earlier study suggested that hypericin is the main active constituent of Hypericum extract, stimulates capillary blood flow, resulting in antidepressant effect [67]. Hypericin is also known to strongly inhibits the enzyme monoamine oxidases (MAO) [320-321]. MAO enzyme is involved in the metabolism and degradation of amine neurotransmitters in the synapse. Hypericin also modulate the levels of dopamine via sigma receptors. Hypericin modulate neuronal action potential by antagonizing benzodiazepine, gamma amino butyric acid A, gamma amino butyric acid B (GABA-B), adenosine and inositol triphosphate receptors [67]. Later studies suggest that, hypericin can not by itself completely responsible for the antidepressant effect. Follow-up study demonstrated that hyperforin is responsible for antidepressant action [322]. Hyperforin is a powerful reuptake inhibitor of 5HT, dopamine, NE, GABA and L-glutamate in synaptic cleft with $IC_{50}$ values between 0.05-0.1 μg/ml [323-326]. Hyperforin alleviates the symptoms of depression by blocking the the reuptake of 5HT from the synaptic cleft [327].

Hypericum extract improves hippocampus dependent spatial memory [72], proliferation of progenitor cells and dendritic spine in hippocampal neurons, and restored the synaptic plasticity [328]. Recent study reported that St. John's Wort reduces Alzheimer's pathology by facilitating blood brain barrier ABCC1 transporter protein expression and microglia activation [74]. St. John's wort attenuated object recognition memory impairment caused by chronic restraint stress in rodents [72]. It has been reported that Hypericum extract is a better

alternative for the management of depression associated with cognitive impairment than other antidepressants known to possess anticholinergic side effects such as sedation and delirium. Hypericum extract also increased the retrieval of memory in passive avoidance task [75]. Hypericum extract significantly attenuated scopolamine and sodium nitrite-induced impaired cognition in active avoidance task [329].

St. John's Wort and its active constituent hyperforin protect rat as well as human islets against cytokine mediated β-cell injury in type 1 diabetes [76]. Hypericum administration significantly reduced hyerglycemia in STZ treated diabetic rats. It also reduced depression and anxiety in type 2 diabetes and might be the potential candidate for management of comorbidities caused by depression, anxiety and diabetes [48]. It has been demonstrated that Hypericum extract restored psychiatric illness such as depressive moods, cognitive deficits, locomotion and sleeping disturbances associated with STZ induced diabetes [330]. Extract of *Hypericum perforatum* is known to modulate antioxidant status and cholinergic system, thereby ameliorate acquisition and retrieval processes in passive avoidance task [331]. In addition, Hypericum extract significantly induced insulin release in rats with nicotinamide-STZ induced type 2 diabetes [332]. In contrast, Hypericum extract significantly inhibits the differentiation of adipocyte and induces insulin resistance in mature 3T3-L1 adipocytes [333].

Hydroalcoholic extract of *Hypericum perforatum* showed analgesic effect against acetic acid induced abdominal constriction in mice via opioid receptor activation [334]. It has been reported that Hypericum extract attenuates physical withdrawal signs in opium dependence [335] comparable to clonidine [336].

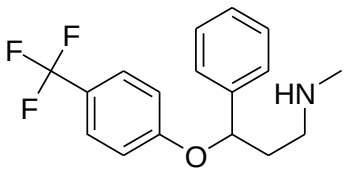
In a randomized, double blind and vehicle controlled study, Hypericum cream significantly attenuated UV induced erythema and this effect was mediated by the constituent hyperforin, a powerful free radical scavenger [337]. The mechanisms for antiinflammatory response of Hypericum was found to be due to downregulation of interleukin-6 (IL6), cyclooxygenase-2 and inducible nitric oxide synthase expressions [67]. Hypericum extract is also known to possess cardioprotective [338], wound healing [339], anticancer [67] and antihyperlipidemic [340] activities. An herbal mixture containing *Urtica dioica* and *Hypericum perforatum*

effectively reduced the redness, distortion, swelling and ankylosis of the joint in muramyl dipeptide/collagen induced arthritis in rats [341].

The most common adverse effects associated with the normal dose of St. John's Wort are gastrointestinal symptoms, dizziness, confusion, allergic reactions, lethargy, restlessness and dryness of the mouth. St. John's wort also induced neuropathy and mania. Hypericin, an active constituent of St. John's Wort has elicited phototoxic dermititis in high doses. Normal doses of St. John's Wort taken for treatment of depression did not show any significant phototoxic effects [67]. It has been widely accepted that, St. John's wort extract induces P-glycoprotein along with a series of drug metabolizing enzymes cytochrome $P_{450s}$ (CYPs) including $CYP_{3A4}$ and $CYP_{2C9}$, and participates in drug-drug interaction [342-344].

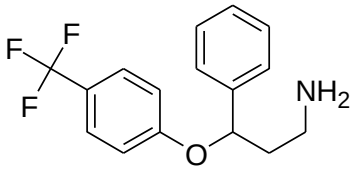
### 2.11. Fluoxetine (FLX)

The systematic (IUPAC) name of FLX is (RS) N-methyl-3-phenyl-3-[4-(trifluoro-methyl) phenoxy] propan-1-amine.



Fluoxetine

The U.S. Food and Drug Administration have approved the FLX for treatment of depressive disorder in December 1987. FLX is a bicyclic derivative of phenyl propylamine. FLX is the most widely used SSRI and prescribed for a variety of neurological disorders including mood and eating disorders, depression in elderly and obsessive compulsive disorders. FLX seems to facilitate the serotonergic transmission in central nervous system via downregulation of pre-synaptic autoreceptors. The dosage of 20 mg/day has been found to be effective for neurological disorders. The oral bioavailability of FLX is 72% and $C_{max}$ (15-55 µg/L) reached in 6 to 8 hours. FLX is highly (94%) bound to plasma proteins, mostly $\alpha_1$-glycoprotein and albumin. Volume of distribution of FLX is 12-43 L/kg. Its elimination half-life is 1-4 days. Its clearance is 36-50 L/h (urine and faeces). FLX is metabolised to norfluoxetine via *O*-demethylation. Norfluoxetine has potency and selectivity similar to that of parent compound [345-347].



Norfluoxetine

In a meta-analysis, FLX was administered to 2635 adult and 960 geriatric patients with depressive disorder. In all age of depressive patients, FLX showed significant improvement relative to placebo. Improvement rate was largest for adult patients receiving FLX (35% greater than placebo) and the remission rate was 30.1% [83]. In randomized controlled trials, FLX exerted neurocognitive improvement in patients with moderate depression [77]. FLX attenuated impaired cognitive behaviour in depressed rodents as well [78]. FLX increased neurotrophins (vascular endothelial growth factor and brain derived neurotrophic factor) levels in hippocampus and modulated adult neurogenesis and depressive-like behaviour in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxin induced brain lesions [79].

Study revealed that, clinical dose of FLX for the treatment of depression symptoms induced hypoglycaemia in type 1 diabetic patient and prompted a progressive reduction of the insulin dose. The insulin requirement was reduced during FLX therapy while the level of HbA1c remained stable. However, FLX withdrawal increased insulin requirements progressively to the patients usual dose [348]. FLX treatment reduced the level of HbA1c during co-mobidity depression and diabetes in rodents. FLX ameliorated spatial learning in Morris water maze task, and showed hypoglycaemic and antidepressant effects in a model of CUMS in rats with diabetes mellitus [80]. In contrast, FLX treatment reduced glucose mediated insulin secretion. This decreased pancreatic β-cell function concomitant with increased oxidative stress, which further contributes to decreased mitochondrial electron-transport chain enzyme activity in pancreas. This study revealed that FLX administration during depressive episodes increases the onset type 2 diabetes by causing oxidative stress in β-cells [349]. Another study suggested that, FLX inhibits insulin secretion, induces unfolded protein response and apoptotic process, triggers β-cell death and promotes insulin resistance in murine islets or Min6 β-cell line [350]. An analysis of published case reports further revealed the association between antidepressants therapy and glucose dysregulation. In these reports, FLX was associated with hypoglycaemia [351].

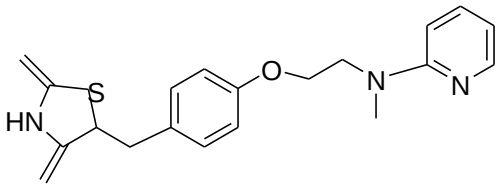
FLX is also prescribed for variety of pathological conditions including mood and eating disorders [81], obsessive compulsive disorders [82], depression in the elderly [83] and dysthymia [84].

FLX may precipitate side effects including nervousness, dryness of mouth, nausea, sore throat, drowsiness, weakness, tremor, akathisia, anorexia, weight loss, changes in sexual drive, excessive sweating, hives, rashes, fever, joint pain, swelling of the throat, face, tongue, eyes, lips, hands, ankles, feet and lower legs, difficulty in swallowing or breathing, confusion, fast or irregular heartbeat, severe muscle stiffness, hallucinations and seizures [352-355]. The discontinuation of FLX was associated with dizziness, insomnia, fatigue, light headedness, agitation, nausea and sensory disturbances. These symptoms last for around three weeks and disappeared by restarting FLX dose or any other antidepressant having similar pharmacological profile [356].

FLX inhibits many drug metabolizing isozymes of cytochrome $P_{450}$ group. FLX is the potent inhibitor of $CYP_{2D6}$ and mild to moderate inhibitor of $CYP_{2B6}$, $CYP_{1A2}$, $CYP_{2C9}$, $CYP_{2C19}$, and $CYP_{3A4}$ [357]. Its use is avoided in patients receiving other medications such as monoamine oxidase inhibitors, methamphetamine, tricyclic antidepressants, buspirone, triptans, serotonin-norepinephrine-reuptake inhibitors and other SSRIs due exacerbation of FLX side effects [358-359].

## 2.12. Rosiglitazone (ROSI)

The systematic (IUPAC) name of ROSI is (RS)-5-[4-(2-[methyl(pyridin-2-yl) amino] ethoxy) benzyl] thiazolidine-2,4-dione.




Rosiglitazone

ROSI is thiazolidinedione class of drug, a potential oral antidiabetic drug for therapy of type 2 diabetes mellitus. The dosage of 4 mg/day has been found to be effective for type 2 diabetes. The oral bioavailability of ROSI is 99% and $C_{max}$ (427.68 ng/ml) reached in 1 hours. ROSI is highly (99.8%) bound to plasma proteins mostly $\alpha_1$-glycoprotein. Volume of

distribution of ROSI is 17.6 L/kg. $CYP_{2C8}$ and $CYP_{2C9}$ are responsible for metabolism of ROSI in liver by *N*-demethylation and hydroxylation. Its elimination half-life is 4.45 hrs. Its clearance is renal (64%) as well as fecal (23%) [86-87].

ROSI is the selective agonist for nuclear PPARϒ receptor. It also increases the gene expression of PPARϒ receptor. ROSI bind to PPARϒ, which activates insulin receptor gene expression that regulate carbohydrate, protein and lipid metabolism. ROSI require insulin for their action. ROSI increases glucose transport influx in adipose tissue and muscle by enhancing synthesis and translocation of glucose transporters [88-89].

In a trial, individuals (5,269) with impaired fasting glucose and/or impaired glucose tolerance were administered with ROSI. After three years, 982 participants had oral glucose tolerance tests at baseline. ROSI significantly improved β-cells functions and attenuated insulin resistance in type 2 diabetic patients [360]. In animal model, ROSI showed central anti-diabetic action against D-glucose fed and STZ-induced diabetes [90]. In another investigation, ROSI improved hyperglycemia and insulin resistance in high fat diet and STZ-nicotinamide induced type 2 diabetes in mice [361]. Earlier report demonstrated that, ROSI administration significantly decreased fasting blood glucose and pancreatic levels of tumor necrosis factor alpha (TNF-α), interferon gamma and NO in cyclosporin A and multiple lower doses of STZ induced diabetic mice. In the similar experiment, the level of insulin in serum was increased after ROSI treatment. ROSI treatment induced regeneration of pancreatic islets and attenuated the effect of CD4 and CD8 T-cells in pancreas. ROSI also showed anti-inflammatory effect in autoimmune disease mediated diabetes [362]. Study revealed that ROSI administration attenuated lipopolysaccharide induced IL-1β and interferon gamma secretion from peritoneal cells and enhanced islet engraftment [363].

Studies suggested that insulin resistance induces cognitive deficit in individuals with type 2 diabetes mellitus. ROSI is known to protect cognitive impairment in individuals with type 2 diabetes [91]. Study revealed that ROSI improves cognition and memory performance in patients with mild Alzheimer disease and animal models of Alzheimer disease [92]. ROSI improved hippocampus dependent cognitive impairment in some Alzheimer disease patients and attenuated cognitive deficits in a mouse model amyloidosis [364]. Clinical report suggests that, ROSI does not ameliorate cognition when used as adjunctive therapy with AChE inhibitors in patients with mild to moderate Alzheimer's disease [365]. Further,

administration of ROSI induced cognitive impairment in some patients with type 2 diabetes [366].

Earlier cross sectional studies suggested that insulin resistance is associated with affective disorders. It has been documented that, insulin sensitizing drug ROSI exhibited significant declines in depression severity associated with insulin resistance [93]. ROSI is known to have antidepressant activity in both human and rodents. ROSI upregulated fibroblast growth factor 2 expression in neurotrophic factor-$\alpha_1$ dependent manner in the hippocampus of stressed mice. Mice administered with ROSI showed increased hippocampal neurogenesis in depressed mice [94]. ROSI improved glucose tolerance and normalized hyperglycemia in *db/db* mice. ROSI significantly increased mobility time in forced swim test in *db/db* mice, suggested antidepressant like effect. In the open field task, ROSI did not modulate locomotor activity of *db/db* mice [367]. ROSI significantly reduced hypercorticosteronemia in animal model of depression, thereby improved mobility in forced swim and tail suspension test [216]. ROSI exhibited antiinfammatory activity in the hippocampus by significantly inhibiting the expression of TNFα, CD40 and microglial activation [368].

ROSI may precipitate side effects including runny nose and other cold symptoms, headache, back pain, sore throat, pain in the jaw, arm, neck, back, or stomach, lightheadedness, chest pain, anorexia, nausea, vomiting, dark urine, changes in vision, yellowing of the skin or eyes, vision loss, dizziness, pale skin, hoarseness, swelling of the eyes, lips, face, tongue or throat, hives, difficulty in swallowing or breathing, itching, blisters, fever, bone fractures, hypoglycaemia, hepatotoxicity, myocardial infarction and death [89, 346, 369-372].

# CHAPTER 3

## OBJECTIVE OF THE STUDY

## 3. Objectives of the study

- To study the effect of chronic unpredictable mild stress induced depression as a risk factor for diabetes and associated behavioural dysfunction.
- To investigate the effect of *Urtica dioica* (stinging nettle) on stress induced insulin resistance and behavioural dysfunction.
- To study the involvement of cholinergic system, insulin signaling cascade, autophagy and sonic hedgehog signaling pathway in the hippocampus region of brain during depressive like behaviour and the effect of *Urtica dioica*.
- To evaluate the effect of *Urtica dioica* on streptozotocin induced chronic diabetes and its associated depressive like behaviour and cognitive dysfunction.
- To evaluate the effect of *Urtica dioica* extract on diabetes associated molecular alterations in the hippocampus including insulin signaling pathway, cholinergic system, autophagy and inflammation.

# CHAPTER 4

## MATERIALS AND METHODS

## 4. Materials and methods

### 4.1. Collection, identification and standardization of plant material

UD leaves was collected from the North Western Himalayan region and authenticated from Department of Forest Product, Dr. Y.S. Parmar University of Horticulture & Forestry, INDIA (voucher specimen number 12399). *Hypericum perforatum* was also collected from the North Western Himalayan region and authenticated from Department of Forest Product, Dr. Y.S. Parmar University of Horticulture & Forestry, India (voucher specimen number 13505). Leaves of UD and aerial parts of *Hypericum perforatum* were air-dried under shade, pulverized and passed through 40 mesh sieves followed by extraction.

#### 4.1.1. Leaf microscopy

For microscopical studies free hand sections were taken using a sharp razor. The sections were cleared by warming with a few drops of chloral hydrate and stained with safranin solution. The sections were then mounted in glycerin with cover slip for microscopical observations [373].

#### 4.1.2. Powder Analysis

For powder analysis the parts of the plant collected and washed thoroughly with water to remove any unwanted matter. This was further dried in the shade. After complete drying, it was powdered and passed through sieve no. 60. This was further subjected with different reagents like, phluroglucinol and conc. HCl (1:1), iodine solution for the presence of the constituents like lignin, starch and calcium oxalate crystals [373].

### 4.2. Determination of Ash values

Ash values are helpful in determining the quality and purity of the crude drug especially in powdered form. The object of ashing vegetable drug is to remove all traces of organic matter that may otherwise interfere in an analytical determination. On incineration, a crude drug normally leaves an ash consisting of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. The total ash of a crude drug reflects the care taken in its preparation. Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Water soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water.

### 4.2.1. Total ash

About 3 gm of the powdered drug was accurately weighed and taken in a silica crucible, which was previously ignited and weighed. The powdered drug was spread in a fine even layer at the bottom of the tarred crucible. The crucible was kept inside the muffle furnace and the temperature increased to make it dull red hot until free from carbon. The crucible was cooled, kept in a dessicator and weighed. The total ash value of the entire plant was noted [374].

### 4.2.2. Acid insoluble ash

The ash obtained (as described above) was boiled with 25 ml of dilute HCl for 5 minutes. The insoluble ash was collected in ash less filter paper and washed with hot water. The insoluble matter was transferred into tarred silica crucible, ignited and weighed. The percentage of acid insoluble ash was calculated [374].

### 4.2.3. Water insoluble ash

The total ash obtained was boiled with 25 ml of water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 minutes at temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated [374].

## 4.3. Determination of extractive values

Extractive values of a crude drug determine the amount of active constituents extracted with solvents from a given amount of medicinal plant material. It is employed for materials for which no suitable chemical or biological assay exists.

### 4.3.1. Alcohol soluble extractive

Five gm of the coarse powder (60-80 mesh) of the crude drug (shade dried) was macerated with 100 ml of 90% alcohol, in a closed flask for a duration of 24 hrs, shaking the flask

frequently during the process and finally allowed to stand for 18 hrs. The solution was filtered rapidly, taking precaution against loss of alcohol. The filtered solution of 25ml was evaporated to dryness in a tarred flat bottomed petri-dish. The percentage of alcohol soluble extract was determined [374].

### 4.3.2. Water soluble extractive value

The procedure adopted for water soluble extractive values was similar to that adopted for the determination of alcohol soluble extractive. Instead of alcohol, water was used as solvent. The values were noted [374].

## 4.4. Determination of moisture content

Drying plays a very important role in the quality as well as purity of the material. Moisture will lead to the activation of enzymes and gives suitable condition, to the proliferation of living micro-organisms. Method: Two grams of the air dried crude drug was accurately weighed in a tarred watch glass. The drug was kept in hot air oven at 105°c and dried for a period until constant weight obtained. The difference in weight gives the moisture content of the drug [374].

## 4.5. Extraction

The extraction of UD leaves and the aerial parts of *Hypericum perforatum* L. (4×50 g) was performed at room temperature, with constant shaking during 48 h, using methanol and water (1:1) as solvent. The extract thus obtained was filtered, centrifuged, evaporated under reduced pressure (Heidolph-Vap, Germany) and lyophilized. UD gave a yield of 17.9% (w/w) of crude hydro-alcoholic extract that was used for the study. *Hypericum perforatum* gave a yield of 8.6% (w/w) of crude hydro-alcoholic extract that was used for the study.

## 4.6. Phytochemical evaluation

The concentrated extracts were subjected to chemical test as per the methods mentioned below for the identification of the various constituents [374].

### 4.6.1. Detection of alkaloids

Solvent free extract, 50mg is stirred with few ml of dilute hydrochloric acid & filtered. The filtrate is tested carefully with various alkaloidal reagents as follows:

Mayer's test: To a few ml of filtrate, a drop or two of Mayer's reagent was added by the side of the test tube. A white or creamy ppt indicates test as positive.

Dragendorff's test: To a few ml of filtrate, 1 or 2ml of Dragendorff's reagent was added by the side of the test tube. A prominent yellow ppt indicates test as positive.

### 4.6.2. Detection of carbohydrates and glycosides

The extract (100mg) was dissolved in 10ml of water & filtered. The filtrate was subjected to the following test:

Molish's test: To 2 ml of filtrate, 2 drops of alcoholic solution of alpha-naphthol was added, the mixture was shaken well & 1ml of conc. $H_2SO_4$ was added slowly along the side of the test tube & allowed to stand. A violet ring indicates the presence of carbohydrate.

Fehling's test: 1ml of filtrate is boiled on water bath with 1ml each of Fehling solution A & Fehling solution B; a red ppt indicates the presence of sugar.

Barfoed's test: To 1 ml of filtrate, 1 ml of Barfoed's reagent was added & heated on a water bath for 2 min. Red ppt. indicates presence of sugar.

Legal's test: 50mg of extract was dissolved in pyridine, sodium nitroprusside solution was added & made alkaline using 10% NaOH & presence of glycoside is indicated by pink color.

Keller-Killiani test: To an extract of drug in glacial acetic acid, few drops of ferric chloride & conc. sulfuric acid were added. A reddish brown color was formed at the junction of two layers & the upper layer turns bluish green.

Ferric Chloride Test: The extract (50mg) was dissolved in 5ml of distilled water. To this few drops of natural 5% ferric chloride solution was added. A dark green color indicates glycoside.

### 4.6.3. Detection of saponins

The extract (50 mg) was diluted with distilled water & made upto 20ml. The suspension was shaken for 15 min. A layer of 2 cm of foam indicates the presence of saponins.

### 4.6.4. Detection of phytosterols

Liebermann- Burchard's test: Extract (50 mg) + 2 ml acetic anhydride. To this 1-2 drops of conc. Sulfuric acid is added, along the sides of the test tube. An array of color changes shows the presence of phytosterols.

### 4.6.5. Detection of phenolic compounds and flavonoids

Gelatin Test: The extract (50mg) was dissolved in 5ml of distilled water & 2ml of 10% sodium chloride solution was added. White ppt. indicates the presence of phenolic compounds.

Lead Acetate Test: The extract (50mg) was dissolved in distilled water & to this; 3ml of 10% lead acetate solution was added. A bulky white ppt. indicates the presence of phenolic compounds.

Alkaline reagent test: An aqueous solution of the extract was treated with 10% $NH_4OH$ solution. Yellow fluorescence indicates the presence of flavonoids.

### 4.6.6. Detection of proteins & amino Acids

The extract (100mg) was dissolved in 10ml of distilled water & filter through Whatman filter paper no-1 & the filtrate was subjected to tests for proteins & amino acids.

Biuret test: An aliquot of filtrate was treated with one drop of 2% copper sulphate solution. To this 1ml of ethanol (95%) was added, followed by excess of potassium hydroxide palate. Pink color in the ethanolic layer indicates the presence of proteins.

Ninhydrin test: Two drops of ninhydrin solution (10mg of ninhydrin in 200 ml of acetone) was added to 2ml of aqueous filtrate. A characteristic purple color indicates presence of amino acids.

### 4.6.7. Detection of fixed oils & fats

Spot test: Press a small quantity of extract separately between filter papers. Oil stains on the paper indicate the presence of fixed oil.

Saponification test: Added a few drops of 0.5N alcoholic KOH to a small quantity of extract along with a drop of phenolphthalein. Heated the mixture on water bath for 1-2 hr. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils & fats.

#### 4.6.8. Detection of gums & mucilage

Extract (100 mg) was dissolved in 10 ml of distilled water & to this 25 ml of absolute alcohol was added with constant stirring. White or cloudy ppt. indicates the presence of gums & mucilage.

### 4.7. Specific chemical test for stinging nettle extract

Moistened dried extract was taken into test tubes and covered with filter paper shocked in dilute NaOH and kept in water bath. After some time, filter paper was exposed to UV light.

In our preliminary experiment, 5HT in UD extract was determined with Folin-Ciocalteu reagent in presence of sodium carbonate.

### 4.8. High-performance liquid chromatography and liquid chromatography-mass spectrometry

#### 4.8.1. Identification of scopoletin in UD leaves extract by HPLC

Identification and quantification of scopoletin was performed by the method described earlier with some modifications. Briefly, UD extract was ground into powder. Scopoletin in crude hydroalcoholic extract of UD was determined by RP-HPLC, using ODS2 column (Waters Spherisorb ODS2 Column, 80Å, 5 µm, 4.6 mm X 250 mm), 0.5% glacial acetic acid in methanol-water (26:55) as mobile phase and UV detector at 310 nm [375]. Finally, the chromatogram of crude UD extract was compared with standard scopoletin (sc-206059, Santa Cruz Biotech).

#### 4.8.2. Identification of active constituents in UD leaves extract by LC-MS analysis

Extract was diluted with mobile phase solvents A (0.05% aqueous formic acid) and B (methanol), premixed in ratio of 1:1, to obtain a final concentration of 2 mg/ml. Five micro

litre was injected into the system (Q-ToF Micro Waters), and compounds were separated on C18 rapid resolution column held at 50 °C. Mobile phase was delivered at flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min). Eluted components were detected by MS, using the ion source parameters as follows: nebulization gas ($N_2$) pressure 40 psi, drying gas ($N_2$) flow 9 L/min and temperature 350 °C, capillary voltage 4 kV, negative polarity [277].

### 4.8.3. Identification of hyperforin and hypericin in St. John's wort extract by HPLC

Analysis was carried out using ODS2 column (Waters Spherisorb ODS2 Column, 80Å, 5 µm, 4.6 mm X 250 mm). The mobile phase consisted of water (A, containing 20% methanol and 0.5% TFA) and acetonitrile (B, containing 10% methanol and 0.5% TFA). Our analysis followed a linear gradient program. Initial conditions were 90% A, 0-20 min; changed to 30% A, 20-25 min; to 10% A, 25-30 min; to 0% A kept to 60 min; 60-65 min, went back to 90% A. The flow-rate was kept at 1 mL/min, and the injection volume was 10 µl. The peak in the HPLC chromatogram of St. John's Wort extract was tentatively identified by comparing the retention time and UV spectra of the peaks in the samples with those of reference standards using photo diode array detector at 200-800 nm [376].

### 4.8.4. Identification of hyperforin and hypericin in St. John's wort extract by LC-MS

The separation was achieved using RP-HPLC $C_{18}$ column. A neutral mobile phase was used in order to run negative ionization mode. The mobile phases were: (A) a mixture of 20mM ammonium acetate and acetonitrile in a ratio of 9:1; (B) acetonitrile. The flow rate was 1 mL/min with a gradient elution beginning with 50% B, changing linearly to 100% B from 2 to 22 min, and then holding 15 min before returning to the initial condition. The injection volume was 15 µl and the total run time was 50 min. A diode array detector was used to monitor UV-vis signals in a spectrum ranging from 200 to 800 nm. HPLC elute was subjected to electrospray ionization source in MS. Negative ionization modes was operated during the acquisition to confirm the molecular weight determination. The tune conditions were set as follows: source voltage 5 kV, capillary voltage 3V, sheath gas flow 80, auxiliary gas flow 20 and capillary temperature 300 °C. The mass range from 250 to 1000 atomic mass unit was acquired with collision energy of 40 on the selected ions in the MS/MS studies [377].

## 4.9. Animals

Male Swiss albino mice (aged 8-10 weeks) weighing 25-30 g was housed under a 12 h light/dark cycle (the lights were on from 6 a.m. to 6 p.m.) at 26 ± 2 °C. The animals had access to food and water *ad libitum*. All animal experiments were carried out in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines and Institutional Animal Ethical Clearance. All efforts were made to minimize animal suffering, to reduce the number of animals used.

## 4.10. CUMS procedure and drug administration

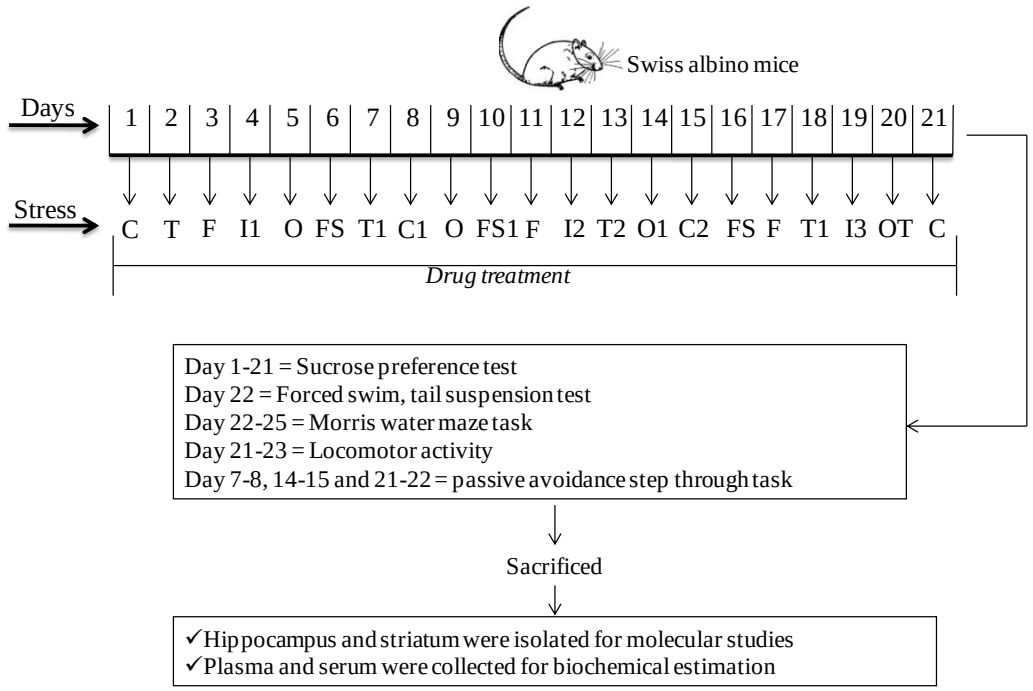



**Figure 13:** CUS procedure and experimental design- C- cold swim (8°C, 3 min); T- tail pinch (1 min); F- food and water deprivation (24 h); I1- immobilization (3 h); O- overnight illumination; FS- foot shock (20 trials, 0.5 mA, 5.0 sec maximum duration, 1 min intervals); T1- tail pinch (2 min); C1- cold swim (10°C, 5 min); FS1- foot shock (20 trials, 0.5 mA, 5.0 sec maximum duration, 30 s intervals); I2- immobilization (4 h); T2- tail pinch (3 min); O1- overnight illumination with wet cage; C2- cold swim (6°C, 3 min); I3- immobilization (5 h); OT- overnight illumination with tilted cage.

Animals were divided into groups and treated as follows: group I received 0.3% caboxymethyl-cellulose (0.3% CMC) and served as control; group II was exposed to CUMS

and received vehicle (0.3% CMC); group III was subjected to CUMS and received FLX (10 mg/kg); group IV received FLX (10 mg/kg); group V was subjected to CUMS and received ROSI (5 mg/kg); group VI received ROSI (5 mg/kg); group VII was subjected to CUMS and received Hypericum extract (350 mg/kg); group VIII received Hypericum extract (350 mg/kg); group IX was subjected to CUMS and received UD extract (50 mg/kg); group X received UD extract (50 mg/kg). All drugs used in the study were administered by gastrointestinal gavage (p.o.). Drugs were administered between 5:00 p.m. and 7:00 p.m. once a day for 21 consecutive days. Dose of St. John's wort, UD, FLX and ROSI used in the present experiment were selected from previous results [72, 378-380]. In the present study HYP, FLX and ROSI were used as standard drug.

The animals were subjected to CUMS paradigm as described previously [381-382] with some modifications. Animals undergo stress paradigm once a day over a period of 21 days (Figure 13).

Stressors were administered at any time of the day. After 3 weeks of CUMS and drug treatment, animals were submitted to different behavioural studies to access depressive like behaviour, cognition and locomotion. Immediately after behavioural studies, the animals were euthanized; blood was collected via retro-orbital puncture in tubes containing 10% sodium citrate and centrifuged at 1000 g for 20 min at 4 °C. Serum was also collected for measuring the level of insulin. Plasma samples were collected for biochemical estimation. Immediately after cervical dislocation, hippocampus and striatum were dissected and frozen at -80 °C for molecular studies.

### 4.11. STZ induced diabetes and drug treatment

Animals were divided into two groups, viz., G1- normal control and G2- animals were administered with STZ (50 mg/kg, i.p.) for five consecutive days. The animals which showed the blood glucose level ≥200 mg/dl were considered for the study. Animals did not show hyperglycemia was removed from the study. The diabetic animals were then randomly divided into three groups, viz., G2 – STZ, G3 – STZ + hydro-alcoholic extract of UD (50 mg/kg) and G4 – STZ + ROSI (5 mg/kg). Hydro-alcoholic extract of UD or ROSI or vehicle (0.3% CMC in water for injection) was administered through oral gavage once daily from the

6th day after STZ injection till 60th day. After drug treatment, animals were subjected to behavioural studies.

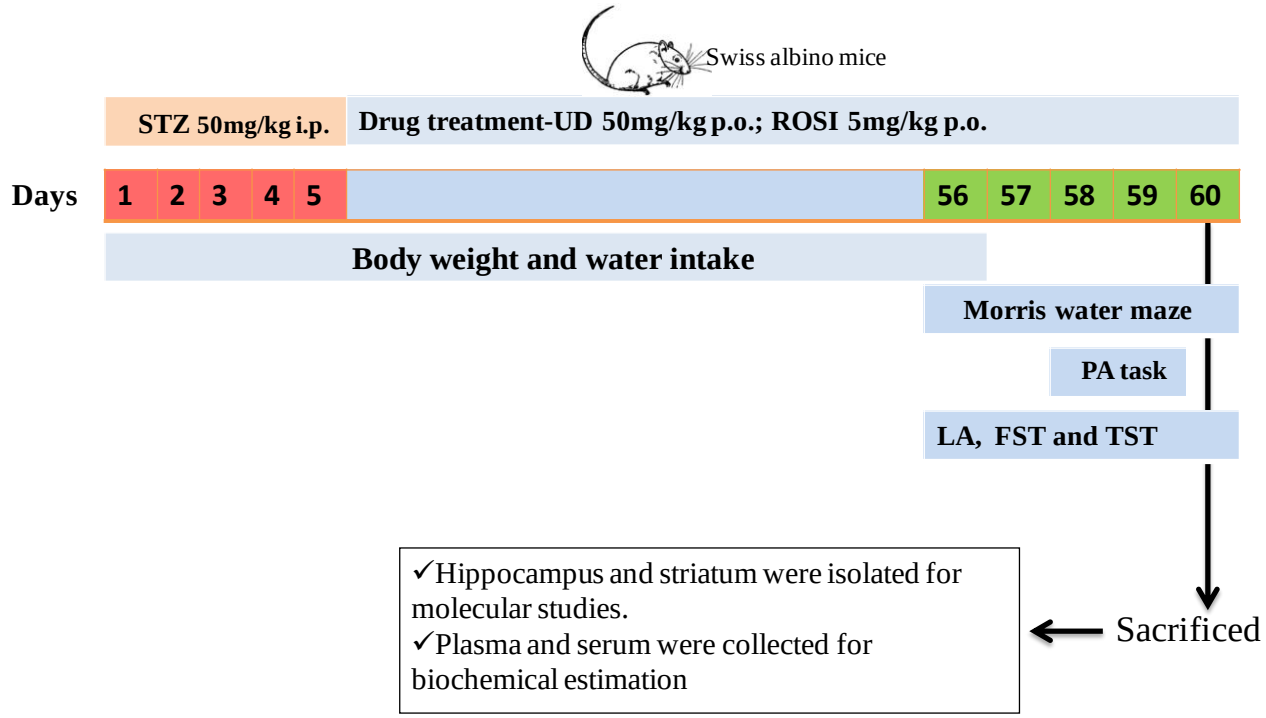



**Figure 14:** Experimental design for the effect of UD against STZ induced neurological alterations. PA = passive avoidance step through task, LA = locomotor activity, FST = forced swim test, TST= tail suspension test, UD = *Urtica dioica* extract, ROSI = rosiglitazone.

Immediately after behavioural studies, the animals were euthanized; blood and serum were collected and aliquots were stored at -80°C for biochemical estimations. Finally, hippocampus and striatum dissected and used for molecular studies (Figure 14). Apart from this water intake and body weights were constantly measured throughout the study.

## 4.12. Behavioural studies

### 4.12.1. Forced swim test (FST)

The animals were individually forced to swim in a cylinder with radius 24 cm and height 25 cm filled with water (26±2 °C) up to a height of 18 cm. An animal was considered immobile whenever it remained floating passively in the water in a slightly hunched but upright position and its nose just above the water surface. The total immobility period of each animal during the 6 min test was recorded [383].

### 4.12.2. Tail suspension test (TST)

The animals were individually suspended on the edge of a shelf, 60 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. Animals were considered immobile when they hang passively and completely motionless. The duration of immobility was recorded for the periods of 6 min during the test [384].

### 4.12.3. Sucrose preference test (SPT)

SPT was conducted as described previously [385]. Briefly, mice were habituated for 48 hours to 1% sucrose, and following a 4 hr deprivation, the preference for sucrose (1%) or water (identical bottles) was determined for 1 h. Sucrose habituation was performed during base line but not during CUMS. Sucrose preference was determined regularly and calculated using formula.

Sucrose preference (%) = [Sucrose intake/(Sucrose intake + Water intake)]x100 .

### 4.12.4. Morris water maze task (MWM)

Spatial memory was assessed using MWM, which consisted of a white circular pool of 1 m diameter, filled with water at room temperature and has a submerged transparent escape platform kept 1 cm below the water surface. The pool was made opaque with addition of nontoxic water-soluble white paint, which makes the submerged platform invisible to the mice. The pool was divided into four hypothetical quadrants. Each mouse was individually allowed to swim freely (habituation trial) in the maze for 5 min (without platform) on day 21. During training trial (day 22-25) the platform was positioned in the centre of a quadrant and each mouse was released facing toward the wall of the pool in the randomly selected quadrant. The mice were allowed to search the platform spontaneously within 60 s. Mice that failed to find the submerged platform within 60 s were placed onto the platform by the experimenter and allow to remain on the platform for 5 s (learning trial). Each mouse received four learning session per day with 10 min interval between each trial. In each trial, the time taken by the mouse to find the hidden platform was recorded as escape latency. Probe trial test was conducted on day 25 to evaluate the index of memory in which the number of crossings across the platform area [386].

### 4.12.5. Passive avoidance step-through (PA) task

PA task was used to evaluate the associative memory, which consisted of a 25 cm long box partitioned into light (10 cm × 14 cm × 16 cm) and dark (10 cm × 10 cm × 16 cm) compartments. The light compartment was illuminated with 60 W bulb, kept 60 cm above the apparatus. Initially, all mice were given one habituation trial to explore both compartments for 120 s. On day 1 of acquisition trial the animals were allowed to explore the light chamber for 5 s. The guillotine door was opened and time taken by mouse to enter the dark chamber was recorded as step through latency (STL). Each animal received an inescapable electric shock for 2 s on a grid floor in the dark chamber. On day 2 of acquisition, memory retention was tested for each mouse in the same manner, but the shock was not delivered [386].

### 4.12.6. Locomotor activity

The locomotor activity in mice was assessed by using digital actophotometer (Inco Ambala, India). Animals were individually placed at the centre of the square arena (35cm×35cm) of apparatus. After an initial familiarization period (3 minutes), the digital locomotor score was recorded for the next 10 minutes [387].

### 4.12.7. Real-time quantitative reverse transcription PCR

qPCR amplifications were performed in an CFX96$^{TM}$ Real-Time PCR Detection System (Bio-Rad) using the iQ$^{TM}$ SYBR green supermix (Bio-Rad). Reactions were carried out in total volumes of 12.5 µl and included 2.5 pM of each primer (Table 3) and 1 µl of diluted cDNA template containing 100 ng cDNA. The thermal cycler conditions for cDNA amplification were as follows: Step 1, 95°C for 3:00 min; Step 2, 95°C for 10 s, 52-58°C for 30 s and 72°C for 2:20 s (35 cycles). GAPDH was used as an internal control and thermal cycler conditions for GAPDH was as follows: Step 1, 95°C for 3:00 min; Step 2, 95°C for 10 s, 57.6°C for 30 s and 72°C for 2:20 s (35 cycles).

## 4.13. Immunoblot

Briefly, hippocampus and striatum were homogenized in 400µl ice-cold RIPA buffer containing protease inhibitor cocktail (MP Biomedicals, LLC, Cat # 158837), extracted on ice for 1 h and then centrifuged at 4°C 1,000xg for 10 min. The supernatant was collected, centrifuged at 16,000xg for 15 min to isolate cytosolic fraction for GLUT4, ChAT and AChE protein expression. The pellet was resuspended in 100µl cell lysis RIPA buffer containing 1%

Triton X-100, extracted on ice for 1 h, centrifuged at 4°C 16,000xg for 15 min and the supernatant was collected as a crude membrane fraction [388] for mAChRs and GLUT4 expression.

Thirty microgram of total protein from tissue homogenate was denatured with Laemmli loading buffer by heating at 95°C for 5 min. The Bradford method was used to measure concentration of protein using BSA as a standard. The samples were resolved on 8-10% sodium dodecyl sulphate-polyacrylamide gel and electroblotted to nitrocellulose membrane using semi-dry transblot. Blots were blocked with 3% BSA in PBS overnight at 4°C, and then incubated with the respective primary antibodies: mAChR1 (1:3500) (~51. kDa), mAChR4 (1:3500) (~72. kDa), ChAT (1:3500) (~72. kDa), AChE (1:3500) (~55. kDa), GLUT4 (~55. kDa) in PBS for 2.5 h at room temperature. GAPDH (1:3500) (~35. kDa) was used as an internal control. After washing, the membranes were incubated with respective HRP conjugated sheep anti-rabbit / goat anti-mouse IgG (1:5000, Santa Cruz Biotechnology, Inc.) secondary antibodies. Donkey anti-goat IgG-HRP (1:5000), sc-2033 was used as secondary antibody for GLUT4 goat polyclonal IgG (1:3500), sc-1606. The membranes were developed with 0.06% 3,3′-diaminobenzidine tetrahydrochloride, 0.025% $CoCl_2$ in PBS and 0.01% $H_2O_2$. The blot images were captured and band density analysis was performed using densitometer (GS-800 Calibrated densitometer, BioRad).

### 4.14. Biochemical estimation

#### 4.14.1. Estimation of blood glucose level

Blood glucose levels were measured in blood samples collected from the tail vein using Accu-check (Roach Diagnostics GmbH, Germany) blood glucose monitoring system. Oral glucose tolerance test (OGTT) was performed on 16 h fasted mice using 2 g glucose/kg body weight. In all groups, blood was collected from the animals by tail snipping at 0, 0.5, 1.0, 1.5 and 2.0 h after glucose load. The results of blood glucose values were expressed in milligrams per deciliter of blood.

#### 4.14.2. Estimation of serum insulin level

The levels of serum insulin were determined by chemiluminescent immunoassay using a commercially available kit (AccuLite CLIA Microwells, Monobind Inc., USA).

### 4.14.3. Estimation of corticosterone

An HPLC-UV system was used for quantification of plasma corticosterone, using dexamethasone as an internal standard. Briefly, 500µl of plasma containing known quantity of dexamethasone was extracted with 5 ml of dichloromethane (DCM). The DCM extract was evaporated to dryness and dissolved in 100µl of mobile phase. Twenty microliter of extract was injected into HPLC system for quantification. Mobile phase consisted of methanol:water (70:30) at a flow rate of 1.2 ml/min and corticosterone was detected at 250 nm [389].

### 4.14.4. Thiobarbituric acid reactive substances (TBARS)

The level of plasma TBARS was determined as per method described previously with some modifications. The mixture consists of 100µl of plasma, 0.1 ml of 8% sodium dodecyl sulphate, 1.0 ml of 20% acetic acid (pH 3.5) and 1.0 ml of 0.67% thiobarbituric acid. The mixture was boiled at 95 ºC for 60 min and then cooled. 1.0 ml of double-distilled water and 5.0 ml of n-butanol:pyridine (15:1, v/v) mixture were added and centrifuged at 5000 rpm for 10 min. The absorbance of organic layer was measured at 540 nm using spectrophotometer [390].

### 4.14.5. Plasma nitric oxide (NO)

The plasma NO was determined as nitrite plus nitrate. The NO is an unstable molecule, which spontaneously oxidizes to nitrite and nitrate ($NOx^-$). The nitrates were reduced to nitrite by using 2% ammonium molybdate and 4% ferrous ammonium sulphate and quantified by using Greiss reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride) at 540 nm spectrophotometrically [391].

### 4.14.6. Catalase level

Catalase level was measured by the previously described method. A total of 0.1 ml of plasma was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7). The reaction was started by the addition of 1 ml freshly prepared 30 mM $H_2O_2$. The rate of decomposition of $H_2O_2$ was measured spectrophotometrically at 240 nm [15].

### 4.14.7. Total thiol level

The level of total thiol in plasma was determined as per method described earlier with some modifications. The reaction mixture consists of 100 µl of plasma, 900 µl of 2 mM $Na_2EDTA$ in 0.2 M $Na_2HPO_4$ and 20 µl of 10 mM DTNB in 0.2 M $Na_2HPO_4$, incubated at room temperature for 5 min and absorbance was measured at 412 nm [392].

### 4.15. *In vitro* assays on hippocampal slices

Control mice were decapitated, the hippocampus were isolated and sliced into 500 µm sections using a tissue slicer. All incubations were performed at room temperature. Slices were distributed into the 6-well tissue culture plates containing artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.7 mM KCl, 1.25 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 0.5 mM $CaCl_2$, 7 mM $MgSO_4$, 4 µM ketamine, pH 7.4). The plates were then incubated (Fisher Scientific, Model 371) with the supply of 95% $O_2$ and 5% $CO_2$. After pre-incubation period of 1 hour, tissues were separated into different experimental groups.

To study the effect of UD on Smo-Gli pathway, the tissues were divided into 13 groups: 1) control group, which was incubated with aCSF for 30 minutes; 2) cyclopamine 2.5 µM group, which was incubated with aCSF for 15 minutes and then with cyclopamine (final concentration 2.5 µM) for additional 15 minutes; 3) cyclopamine 5 µM group, which was incubated with aCSF for 15 minutes and then with cyclopamine (final concentration 5 µM) for additional 15 minutes; 4) purmorphamine 1 µM group, which was incubated with aCSF for 15 minutes and then with purmorphamine (final concentration 1 µM) for additional 15 minutes; 5) cyclopamine 5 µM + purmorphamine 1 µM group, which was incubated with cyclopamine (final concentration 5 µM) for 15 minutes and then with purmorphamine (final concentration 1 µM) for an additional 15 minutes; 6) Hypericum 50 µg group, which was incubated with aCSF for 15 minutes and then with Hypericum extract (final concentration 50 µg) for additional 15 minutes; 7) Hypericum 100 µg group, which was incubated with aCSF for 15 minutes and then with Hypericum extract (final concentration 100 µg) for additional 15 minutes; 8) Hypericum 50 µg + cyclopamine 5 µM group, which was incubated with cyclopamine (final concentration 5 µM) for 15 minutes and then with Hypericum extract (final concentration 50 µg) for an additional 15 minutes; 9) Hypericum 100 µg + cyclopamine 5 µM group, which was incubated with cyclopamine (final concentration 5 µM) for 15 minutes and then with Hypericum extract (final concentration 100 µg) for an additional 15 minutes; 10) UD 125 µg group, which was incubated with aCSF for 15 minutes and then with

UD extract (final concentration 125 µg) for additional 15 minutes; 11) UD 250 µg group, which was incubated with aCSF for 15 minutes and then with UD extract (final concentration 250 µg) for additional 15 minutes; 12) UD 125 µg + cyclopamine 5 µM group, which was incubated with cyclopamine (final concentration 5 µM) for 15 minutes and then with UD extract (final concentration 125 µg) for an additional 15 minutes; 13) UD 250 µg + cyclopamine 5 µM group, which was incubated with cyclopamine (final concentration 5 µM) for 15 minutes and then with UD extract (final concentration 250 µg) for an additional 15 minutes. After the incubation periods, slices were homogenized with trizol reagent for molecular studies.

To study the effect of UD on GLUT4 membrane translocation, the tissues were divided into 7 experimental groups: 1) Vehicle group, which was incubated with aCSF for 30 minutes; 2) Insulin group, which was incubated with aCSF for 15 minutes and then with insulin (final concentration 1 µM) for an additional 15 minutes; 3) LY294002 + Insulin group, which was incubated with LY294002 (final concentration 50 µM) for 15 minutes and then with insulin (final concentration 1 µM) for an additional 15 minutes; 4) LY294002 + UD125 group, which was incubated with LY294002 (final concentration 50 µM) for 15 minutes and then with UD extract (final concentration 125 µg) for an additional 15 minutes; 5) LY294002 + UD250 group, which was incubated with LY294002 (final concentration 50 µM) for 15 minutes and then with UD extract (final concentration 250 µg) for an additional 15 minutes; 6) UD125 group, which was incubated with aCSF for 15 minutes and then with UD extract (final concentration 125 µg) for additional 15 minutes; 7) UD250 group, which was incubated with aCSF for 15 minutes and then with UD extract (final concentration 250 µg) for additional 15 minutes. Isolation of membrane containing fractions from these incubations was performed as described above.

### 4.16. Identification of hyperforin, hypericin and scopoletin on brain homogenate of stressed mice

Whole brain tissue was homogenized in 2 ml of PBS. An HPLC-UV system was used for identification of hyperforin, hypericin and scopoletin in brain homogenate. Briefly, 500 µl of the tissue homogenate was extracted with 5 ml of DCM. The DCM extract was evaporated to dryness and dissolved in 100 µl of mobile phase. Thereafter, twenty microliter was injected

into HPLC system for quantification. The identification of hyperforin, hypericin and scopoletin in brain homogenate was performed as described above.

### 4.17. Immunofluorescence & Histopathology

The mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and intracardially perfused with 0.1 Molar PBS (pH 7.4) followed by 4% paraformaldehyde contained in PBS. There after the whole brain was isolated and stored in 30% sucrose and 10% glycerol solution at -80°C. Hippocampal sections of 4 µm were made using cryotome (CM 1850 Leica, Heidelberg, Germany). For immunofluorescence study, the hippocampal sections were permeabilized with 0.1% IPEGAL CA 630 in PBS for 10 min and then blocked with 3% BSA in PBS for 1 h at room temperature followed by overnight incubation with primary antibody- rabbit polyclonal IgG anti-mouse TNF-α (1:100) (eBioscience, USA). Thereafter, the sections were washed thrice with PBS and incubated with fluorescein isothiocyanate (FITC) labeled respective secondary antibody (Santa-cruz biotech) for 1:30 hour in a dark place and counterstained with DAPI. The sections were mounted using glycerol/PBS (9:1), observed at 40X objectives on Olympus microscope and images were captured. For histopathology study, the hippocampal sections were subjected to hematoxylin and eosin staining and images were captured at 40X using Olympus microscope.

### 4.18. *Statistical analysis*

All the data were expressed as mean ± SEM. The statistical significance was assessed by one-way analysis of variance followed by Tukey's post-hoc test with a confidence level of $p<0.05$.

**Table 3:** Sequence of oligonucleotides used for qRT-PCR.

| S No. | Gene | Forward primer 5'→3' | Reverse primer 5'→3' |
|---|---|---|---|
| 1. | PPARγ | AGG GCG ATC TTG ACA GGA AA | CGA AAC TGG CAC CCT TGA AA |
| 2. | IR | TTT GTC ATG GAT GGA GGC TA | CCT CAT CTT GGG GTT GAA CT |
| 3. | ILGF 1r | GTG GGG GCT GCT CGT GTT TCT C | GAT CAC CGT GCA GTT TTC CA |
| 4. | GLP1 | TCA GAG ACG GTG CAG AAA TGG | ATC AAA GGT CCG GTT GCA GAA |
| 5. | IRS1 | CGA TGG CTT CTC AGA CGT G | CAG CCC GCT TGT TGA TGT TG |
| 6. | IRS2 | CTG CGT CCT CTC CCA AAG TG | GGG GTC ATG GGC ATG TAG C |
| 7. | PI3K | CGA GAG TGT CGT CAC AGT GTC | TGT TCG CTT CCA CAA ACA CAG |
| 8. | PKB | TGC CCA CAC GCT TAC TGA GA | CAA AGC AGA GGC GGT CGT |
| 9. | GLUT4 | GAT GGG CTT TCT CCG TCC | GTG TGG CAA GAG TTC AGT GG |
| 10. | INSG1 | CAC GAC CAC GTC TGG AAC TAT | TGA GAA GAG CAC TAG GCT CCG |
| 11. | MAPK1 | CCC TTA GAC ACT GTG ACG GT | CAC AGT CCC AAA GCC ACA AA |
| 12. | Shh | CAA GTA CGG CAT GCT GGC TC | AAG GTG AGG AAG TCG CTG TA |
| 13. | Ptch1 | AGG CGC TAA TGT TCT GAC CA | CCT CCT GCC AAT GCA TAT AC |

| S No. | Gene | Forward primer 5'→3' | Reverse primer 5'→3' |
|---|---|---|---|
| 14. | Smo | GAC TCC GTG AGT GGC ATC TG | GTG GCA GCT GAA GGT GAT GA |
| 15. | Gli1 | AAG CCT GAG CCT GAG TCT GT | GGT CAC TGG CAT TGC TAA AG |
| 16. | Cyclin D1 | GCG TAC CCT GAC ACC AAT CTC | ACT TGA AGT AAG ATA CGG AGG GC |
| 17. | BDNF | ATG TCT ATG AGG GTT CGG CG | GCG AGT TCC AGT GCC TTT TG |
| 18. | TrkB | ACT GTG AGA GGC AAC CCC AA | ATC ACC AGC AGG CAG AAT CC |
| 19. | mAChR1 | AGT CCC AAC ATC ACC GTC TTG | CAG GTT GCC TGT CAC TGT AGC |
| 20. | mAChR4 | ATG GCG AAC TTC ACA CCT GTC | CTG TCG CAA TGA ACA CCA TCT |
| 21. | BCL2 | GGC TGA GCA CTA CCT TCA GTA | TGG CGG TAT CTA TGG ATT CCA C |
| 22. | AIP2 | AGC TTG GTG TCT GTT CTC TGT | TGG AGG GAA GAT AGG TCC CAC |
| 23. | ATG5 | CTC GCT AGA TGG AAC CAC | AGT GGT CCT GTG TGT CTC |
| 24. | ATG7 | TGG CTG CTA CTT CTG CAA TGA TGT | CAG GAC AGA GAC CAT CAG CTC CAC |
| 25. | iNOS | GTT CTC AGC CCA ACA ATA CAA GA | GTG GAC GGG TCG ATG TCA C |
| 26. | IL6 | GGT GCC CTG CCA GTA TTC TC | GGC TCC CAA CAC AGG ATG A |
| 27. | TNFα | CCC TCA CAC TCA GAT CAT CTT CT | GCT ACG ACG TGG GCT ACA G |

| S No. | Gene | Forward primer 5'→3' | Reverse primer 5'→3' |
|---|---|---|---|
| 28. | Hhip | GGG TCA CAT CTT GGG ATT TG | GAG GCA CTT GTT CGG TCT GA |
| 29. | GAPDH | TTC ACC ACC ATG GAG AAG GC | GGC ATG GAC TGT GGT CAT GA |

# CHAPTER 5

## RESULTS

## 5. Results

### 5.1. Standardization of plant materials

Microscopy of UD leaf showed straight and sharp stinging hair **(A)**, upper epidermis **(B)**, lower epidermis **(C)**, spongy parenchyma **(D)** and vascular bundle **(E)** (Figure 15).

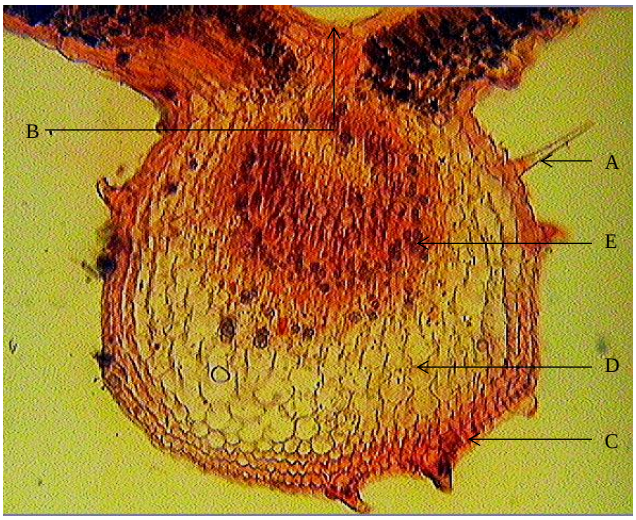



**Figure 15:** Transverse section of Stinging nettle leaf stained with safranin solution (40X).

Microscopy of St. John's wort leaf showed very thin mid rib, oil gland, upper epidermis, lower epidermis and vascular bundle (Figure 16).

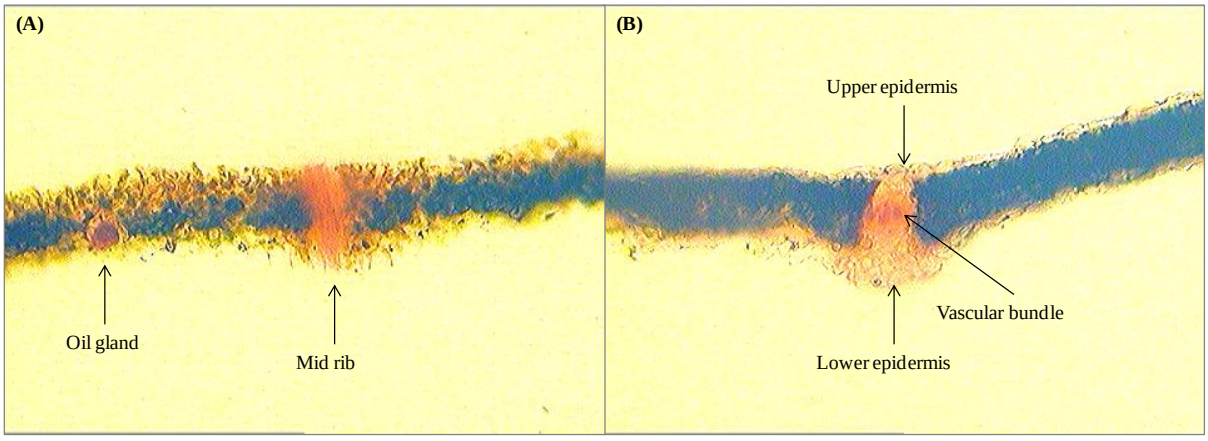



**Figure 16:** Transverse section of St. John's wort (*Hypericum perforatum*) leaf stained with safranin solution and viewed at 10X **(A)** and 40X **(B)**.

The powder microscopy of UD leaves revealed the presence of sharp pointed unicellular trichomes, prismatic and raphide crystals of calcium oxalate, paracytic or rubiaceous stomata,

septed fibers, elongated cork cells, epidermal cells, collenchymal cells with starch and starch granules. Powder microscopy of St. John's wort leaves revealed the presence of large oil glands, pitted and thick walled fibers, fragments of vascular bundles, collenchymal cells and starch granules. We did not observe the presence of trichomes and calcium oxalate crystals in the powder of St. John's wort leaves.

Physical constant like ash values, extractive values and moisture content are reported in Table 4 to Table 6.

**Table 4:** Ash values (% w/w)

| S No. | Physical constant | Stinging nettle | *H. perforatum* |
|---|---|---|---|
| 1. | Total ash | 21.2 | 6.2 |
| 2. | Acid insoluble ash | 3.04 | 2.4 |
| 3. | Water insoluble ash | 3.3 | 3.2 |

**Table 5:** Extractive values (% w/w)

| S No. | Extracts | Extractive value of Stinging nettle | Extractive value of *H. perforatum* |
|---|---|---|---|
| 1. | Water soluble extractives | 23.3 | 14.2 |
| 2. | Alcohol soluble extractives | 25.3 | 14.02 |

**Table 6:** Moisture content

| S No. | Plant materials | Initial weight (gm) | Final weight (gm) | Moisture content (%) |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 1. | S. nettle | 0.151 | 0.140 | 7.28 |
| 2. | *H. perforatum* | 0.161 | 0.155 | 3.72 |

Phytochemical evaluation on UD extract revealed the presence of alkaloids, carbohydrates, glycosides, phenolics, flavonoids, proteins and amino acids (Table 7).

**Table 7:** Phytochemical evaluation of plant extracts

| **S No.** | **Phytochemical test** | **S. nettle extract** | **SJW extract** | **S No.** | **Phytochemical test** | **S. nettle extract** | **SJW extract** |
|---|---|---|---|---|---|---|---|
| **1.** | Alkaloids | | | **5.** | Phenolics & flavonoids | | |
| | a) Mayer's test | - | - | | a) Ferric chloride test | + | + |
| | b) Dragendorff's test | ++ | + | | b) Gelatin test | - | - |
| | | | | | c) Lead acetate test | + | - |
| | | | | | d) Alkaline reagent | + | - |
| **2.** | Carbohydrates & Glycosides | | | **6.** | Proteins & Amino acids | | |
| | a) Molish test | + | + | | a) Biuret test | + | + |
| | b) Fehling test A | - | - | | b) Ninhydrin test | + | + |
| | c) Fehling test B | + | + | | | | |
| | d) Barfoed test | - | - | | | | |
| | e) Legal test | - | - | | | | |
| | f) Kellar-Killiani test | - | + | | | | |
| | g) Ferric chloride test | + | + | | | | |
| **3.** | Saponins | | | **7.** | Gum & Mucilage | | |
| | a) Foam test | - | - | | a) Alcohol 95% test | - | - |
| **4.** | Phytosteroids | | | **8.** | Fixed oils & fats | | |
| | a) Liebermann-Burchards test | - | - | | a) Spot test | - | - |
| | | | | | b) Saponification test | - | - |

### 5.1.1. Specific chemical tests for stinging nettle extract:

Moistened dried extract was taken into test tubes and covered with filter paper shocked in dilute NaOH and kept in water bath. After some time, filter paper was exposed to UV light. It showed yellowish-green fluorescence and indicated the presence of scopoletin in UD extract.

In our preliminary experiment, 5HT in UD extract showed an intense blue colored chromogen with Folin-Ciocalteu reagent in presence of sodium carbonate.

### 5.1.2. LC-MS and HPLC analysis

Mass calculated for $C_{10}H_8O_4$ scopoletin, exact mass: 192.04, found 191.04 (M-1); $C_7H_6O_4$ gentisic acid, exact mass: 154.02, found 153.02 (M-1); $C_9H_6O_4$ esculetin, exact mass: 178.02, found 177.02 (M-1); $C_{15}H_{10}O_7$ quercetin, exact mass: 302.04, found 301.04 (M-1) and $C_{27}H_{30}O_{16}$ rutin, exact mass: 610.51, found 609.51 (M-1) in LC-MS analysis (Figure 17).

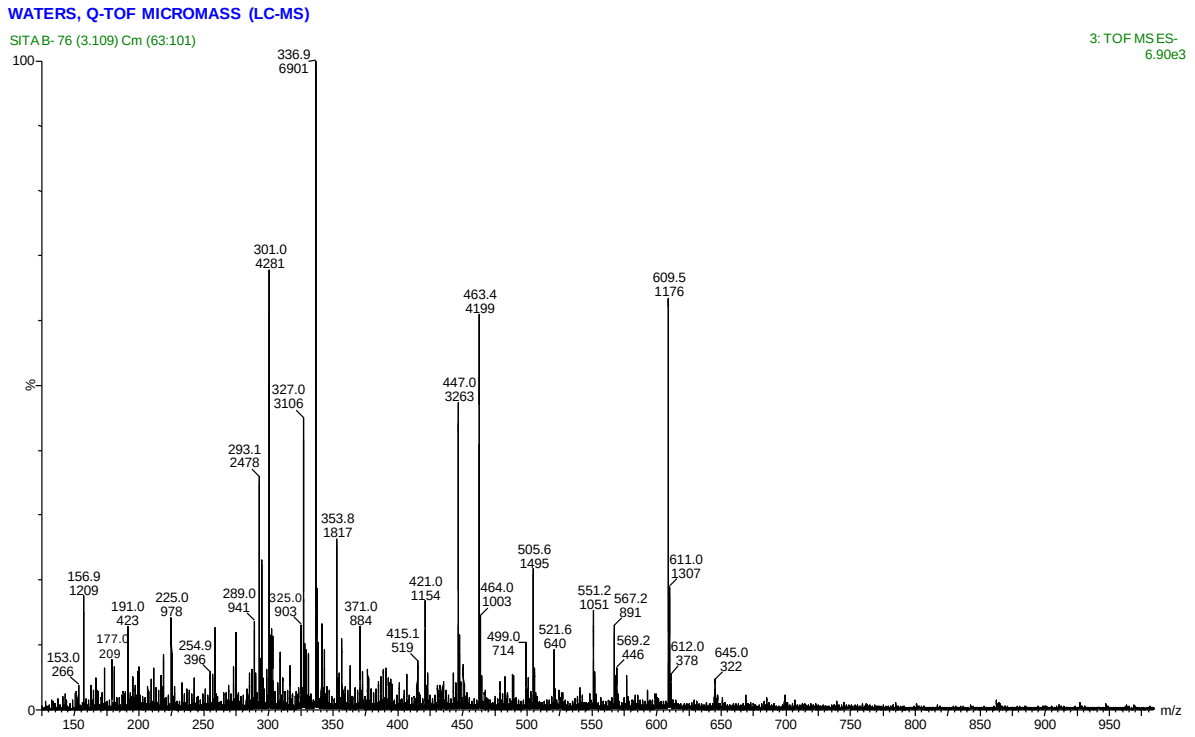



**Figure 17:** Negative ion LC-MS spectrum of hydro-alcoholic extract of UD leaves showing peak at *m/z* 153.02 (gentisic acid), *m/z* 177.02 (esculetin), *m/z* 191.04 (scopoletin), *m/z* 301.04 (quercetin) and *m/z* 609.51 (rutin).

In HPLC analysis, the crude hydro-alcoholic extract of UD leaves showed peak ($t_R$=14.254) corresponding to standard scopoletin ($t_R$=14.296). Herein, we observed that the crude UD extract contains 6.51% of scopoletin (Figure 18 A and B).

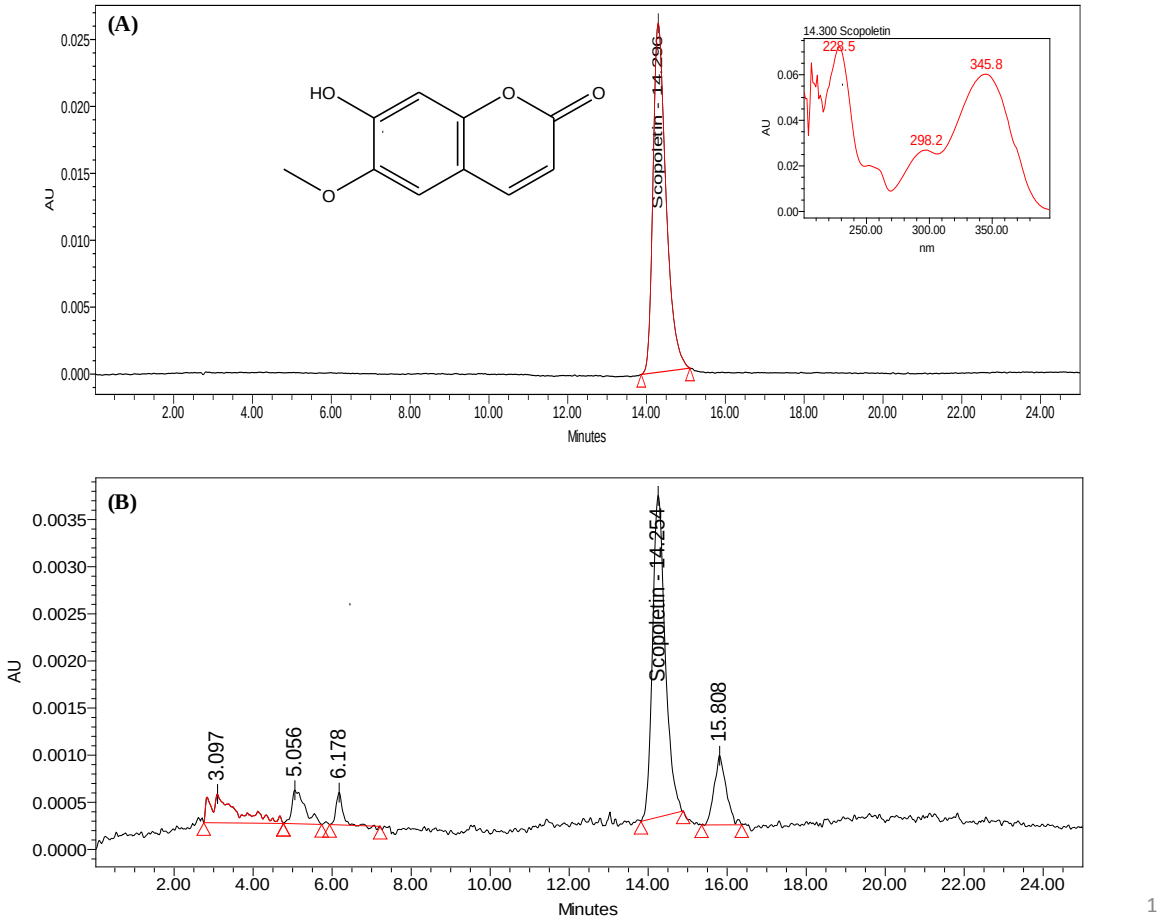



**Figure 18:** HPLC chromatogram of standard scopoletin **(A)** and crude hydro-alcoholic extract of UD leaves **(B)**.

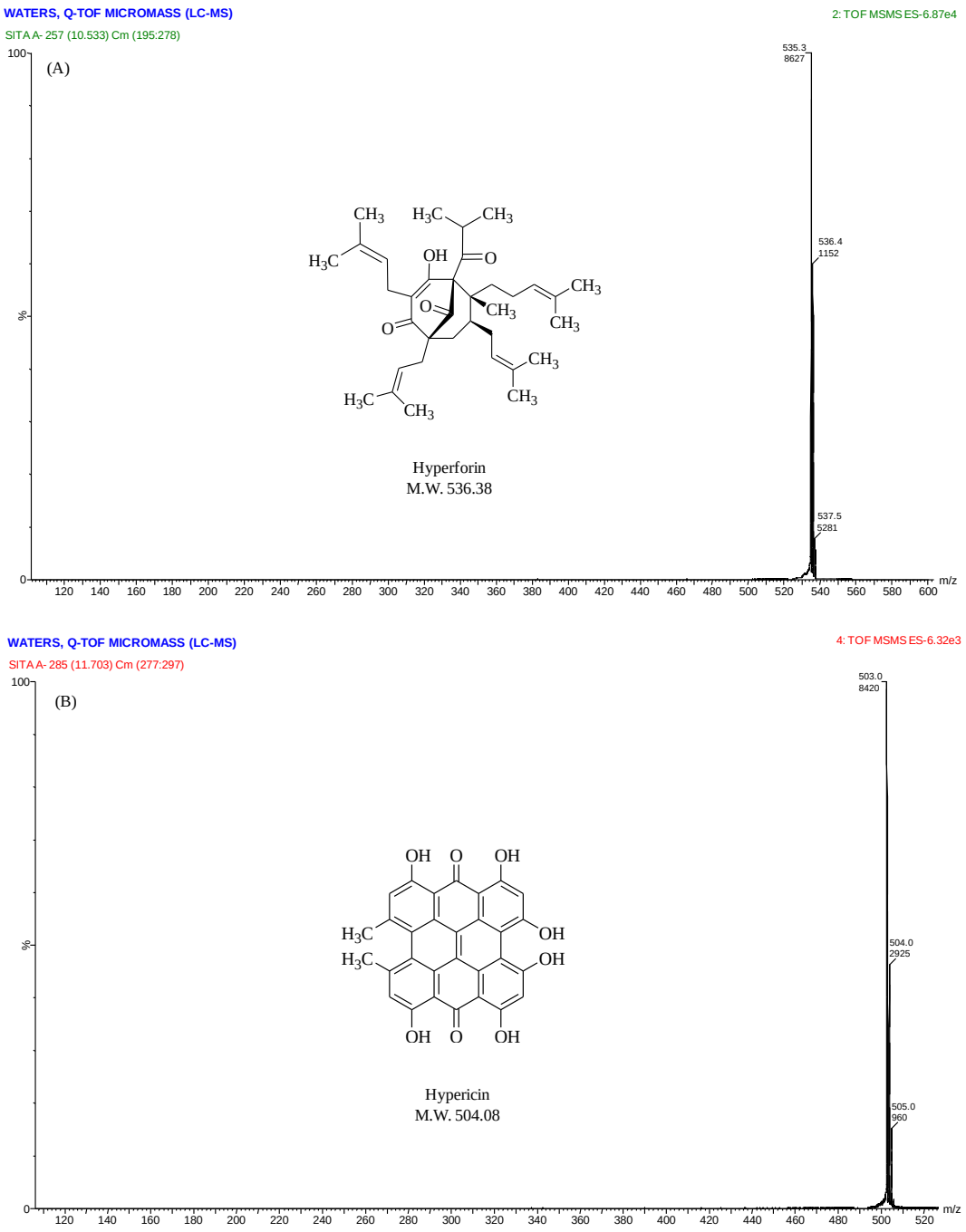
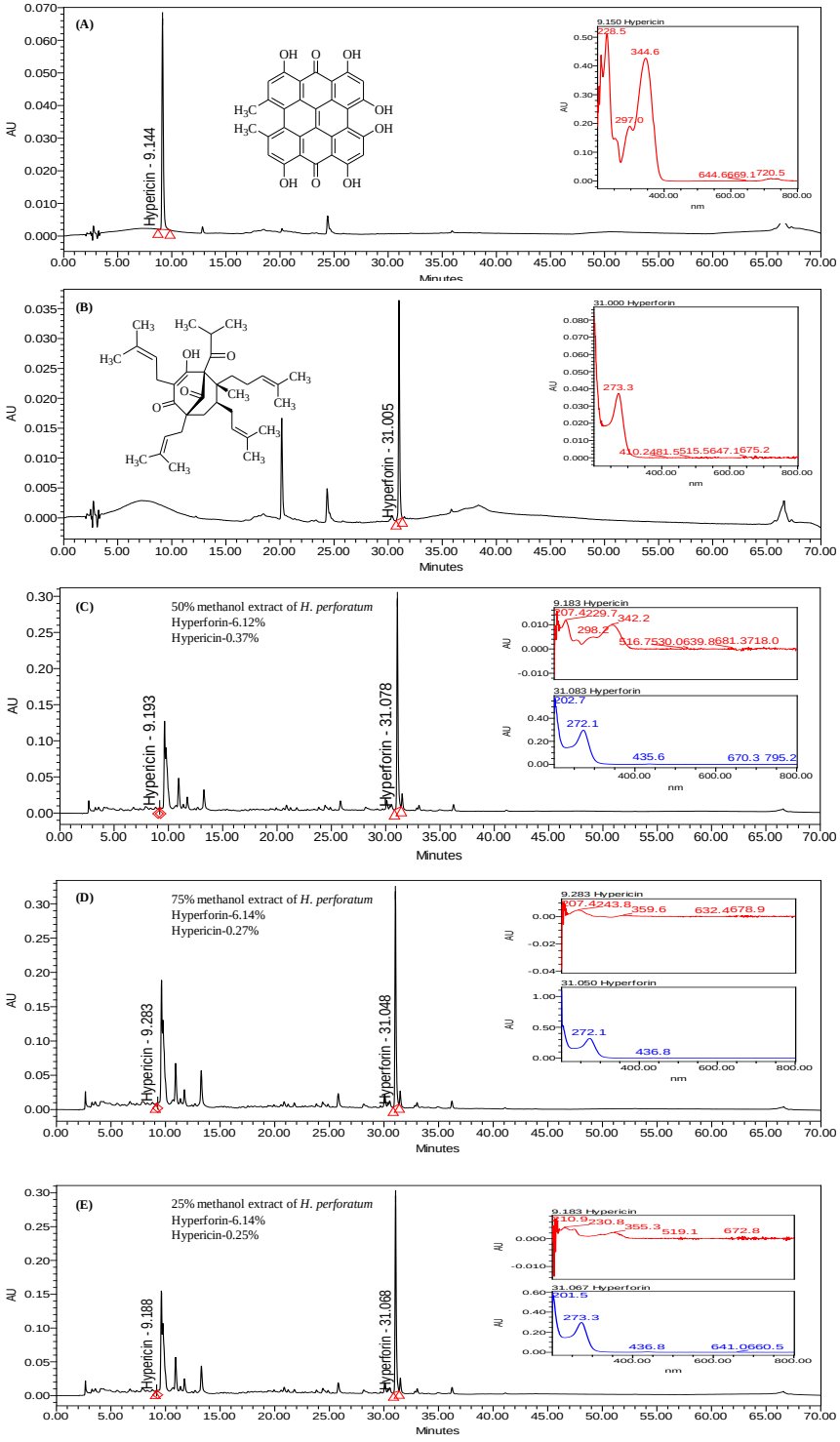



**Figure 19:** Negative ion LC-MS chromatogram of hyperforin (*m/z* 535.38) **(A)** and hypericin (*m/z* 503.08) **(B)** in hydro-alcoholic extract of St. John's wort.

Mass calculated for $C_{35}H_{52}O_4$ hyperforin, exact mass: 536.38, found 535.38 (M-1) and $C_{30}H_{16}O_8$ hypericin, exact mass: 504.08, found 503.08 (M-1) in LC-MS analysis (Figure 19).

**Figure 20:** Typical HPLC chromatograms of the standard hypericin (CAS 548-04-9) **(A)**, hyperforin (CAS 11079-53-1) **(B)**, 50% methanolic extract of St John's wort **(C)**, 75% methanolic extract of St John's wort **(D)** and 25% methanolic extract of St John's wort **(E)** with detection wavelength set at 200-800 nm using PDA detector.



HPLC chromatograms of standard hypericin and hyperforin were reported in Figure 20A and 20B, respectively. Quantitative HPLC analysis revealed the presence of hyperforin (6.12%) and hypericin (0.37%) in Hypericum extract (methanol: water, 1:1) (Figure 20C). 75% methanolic extract of St. John's wort showed 6.14% of hyperforin and 0.27% of hypericin (Figure 20D). 25% methanolic extract of St. John's wort showed 6.14% of hyperforin and 0.25% of hypericin (Figure 20E).

## 5.2. Assessment of depressive like behaviour in stressed mice and the effect of UD extract

Chronic stress significantly increased the duration of immobility in FST ($p<0.001$ vs. CTRL). Chronic UD treatment significantly improved ($p<0.01$ vs. CUMS) the mobility period in FST comparable to FLX ($p<0.01$ vs. CUMS). Duration of immobility was also significantly reduced by chronic treatment with ROSI ($p<0.05$ vs. CUMS) and HYP ($p<0.05$ vs. CUMS) in FST. There were no significant alterations in the duration of immobility between CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL) (Figure 21A).

CUMS significantly increased the duration of immobility in TST ($p<0.01$ vs. CTRL). Chronic UD treatment significantly improved ($p<0.001$ vs. CUMS) the mobility period in stressed mice subjected to TST. Duration of immobility was also significantly reduced by chronic treatment with FLX ($p<0.01$ vs. CUMS), ROSI ($p<0.01$ vs. CUMS) and HYP ($p<0.05$ vs. CUMS) in TST. There were no significant alterations in the mobility periods between CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL) (Figure 21B).

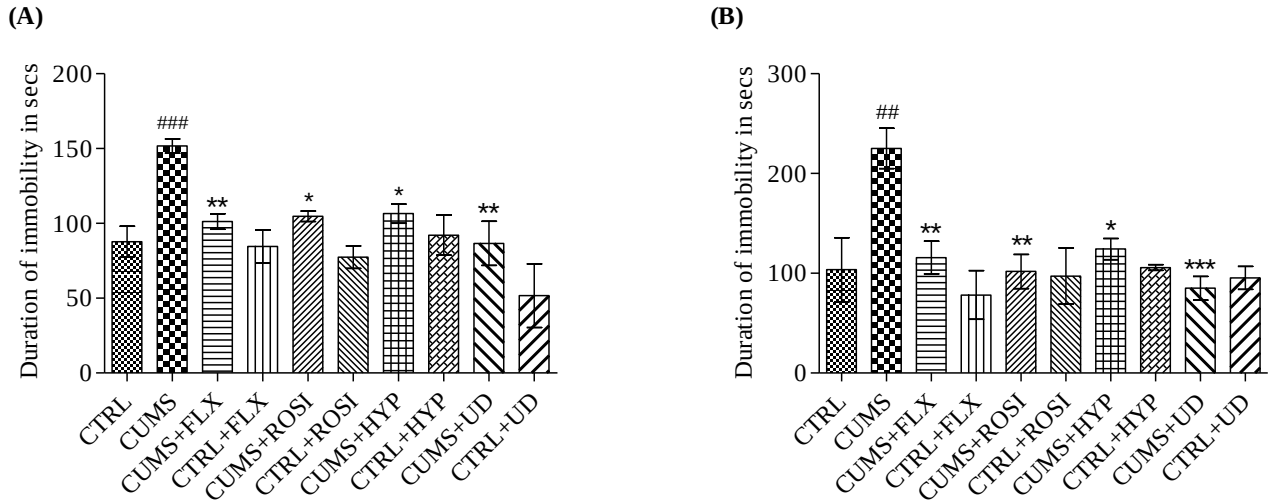


**Figure 21:** Effect of UD extract on CUMS-induced behavioural alterations in FST **(A)** and TST **(B)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; FST = forced swim test; TST = tail suspension test.

Before CUMS and drug treatment, the basal level of sucrose preference was not significantly altered among the groups (p>0.05) (Figure 22A). Further, one week of chronic stress or drug treatment did not modulate sucrose preference (p>0.05). In addition, there were no significant alterations in the sucrose preference between CTRL treated with FLX, ROSI, HYP and UD (p>0.05 vs. CTRL) (Figure 22B) on one week of stress exposure.

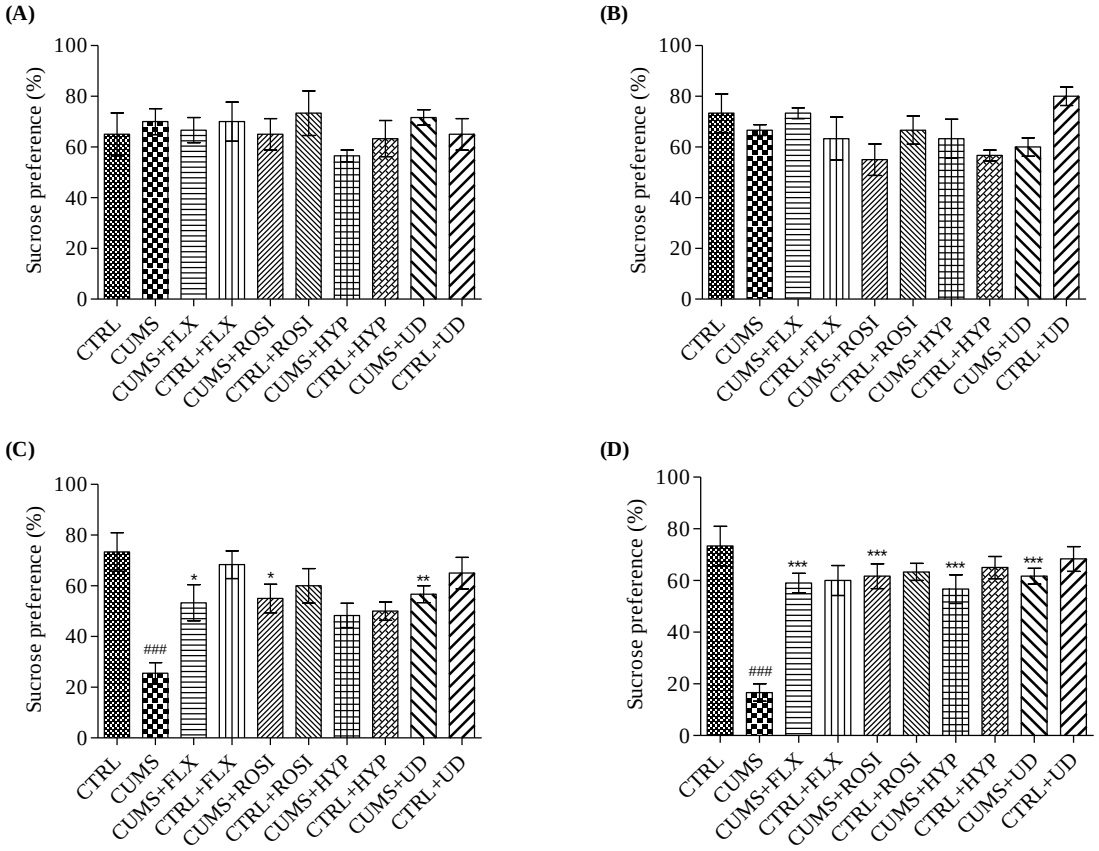



**Figure 22:** Effect of UD extract on CUMS-induced behavioural alteration in SPT: SPT base line **(A)**, SPT at week one **(B)**, SPT week at two **(C)** and SPT at week three **(D)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001.

CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; SPT = sucrose preference test.

Stress exposure significantly reduced the sucrose intake in mice on week two ($p<0.001$ vs. CTRL). Chronic UD administration significantly ameliorated sucrose preference in stressed mice on week two ($p<0.01$ vs. CUMS). Sucrose preference was also significantly ameliorated by chronic treatment with FLX ($p<0.05$ vs. CUMS) and ROSI ($p<0.05$ vs. CUMS). Hypericum extract did not modulate sucrose preference on week two ($p>0.05$ vs. CUMS). There were no significant alterations in the sucrose preference between CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL) (Figure 22C) on week two of CUMS exposure.

CUMS exposure significantly reduced the sucrose intake in mice on week three ($p<0.001$ vs. CTRL). Chronic UD administration significantly ameliorated sucrose preference in stressed mice on week three ($p<0.001$ vs. CUMS) comparable to FLX, ROSI and HYP treatment. Sucrose preference was also significantly ameliorated by chronic treatment with FLX ($p<0.001$ vs. CUMS), ROSI ($p<0.001$ vs. CUMS) and HYP ($p<0.001$ vs. CUMS). In addition, there were no significant alterations in the sucrose preference between CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL) at the end of CUMS exposure (Figure 22D).

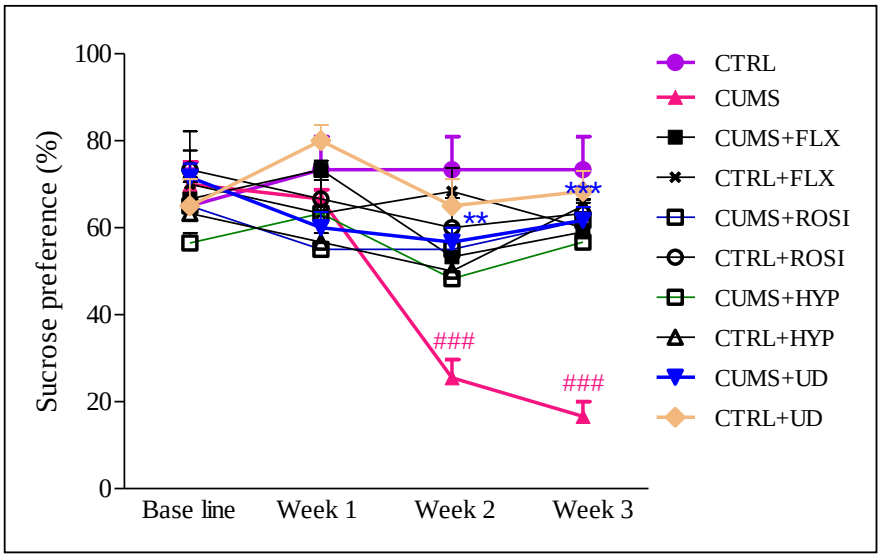



**Figure 23:** Overall effects of CUMS and drug treatment on SPT. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + UD. **p <

0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; SPT = sucrose preference test.

When combined SPT data from base line to week three, we observed that CUMS gradually declined the sucrose preference after week one onwards and significantly reduced after week two ($p<0.001$ vs. CTRL) and at the end of CUMS exposure ($p<0.001$ vs. CTRL). Conversely, chronic UD treatment significantly induced sucrose preference after week two ($p<0.01$ vs. CUMS) and at the end of CUMS paradigm ($p<0.001$ vs. CUMS). In addition, there were no significant alteration between CTRL and CTRL treated with UD ($p>0.05$) (Figure 23).

### 5.3. Effect of UD extract on depression mediated cognitive deficit

On day 1 of training (day 22), all the animals showed similar learning behaviour and there was no significant alteration in the learning pattern between the groups ($p>0.05$) in Morris water maze task. The control animals showed improvement in learning between trials from day 23-25 as evident from decrease in the escape latency, while chronically stressed mice did not show any significant improvement in learning between the trials. During training trial, CUMS significantly increased the escape latency on day 3 ($p<0.001$ vs. CTRL) and day 4 ($p<0.001$ vs. CTRL). Chronic UD treatment significantly decreased the escape latency on day 3 ($p<0.001$ vs. CUMS) and day 4 ($p<0.001$ vs. CUMS) in stressed animals comparable to chronic FLX, ROSI and HYP treatment ($p<0.001$ vs. CUMS). In addition, there were no significant alteration between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$) (Figure 24A).

In the probe trial, number of crossings across the platform area was significantly decreased in stressed mice ($p<0.01$ vs. CTRL). Chronic UD treatment significantly increased the number of crossings across the platform area in stressed mice ($p<0.001$ vs. CUMS), and this effect was comparable to chronic ROSI treated stressed mice ($p<0.001$ vs. CUMS). Chronic FLX and HYP treatment also significantly increased the number of crossings across the platform area ($p<0.05$ vs. CUMS and $p<0.01$ vs. CUMS, respectively) (Figure 24B). In addition, there were no significant alteration between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL).

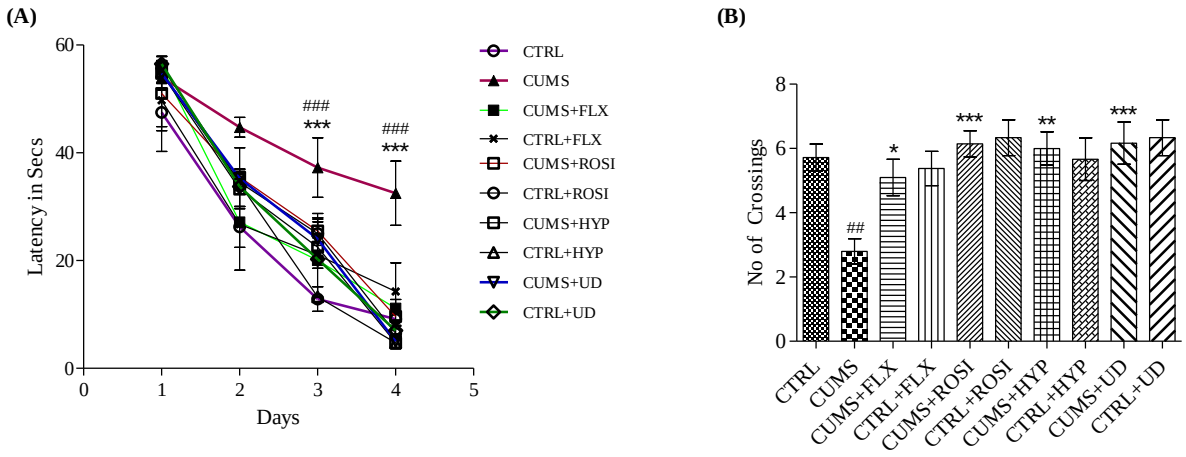



**Figure 24:** Effect of UD extract on CUMS-induced behavioural alterations in Morris water maze task **(A)** and probe trial (number of crossings) **(B)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

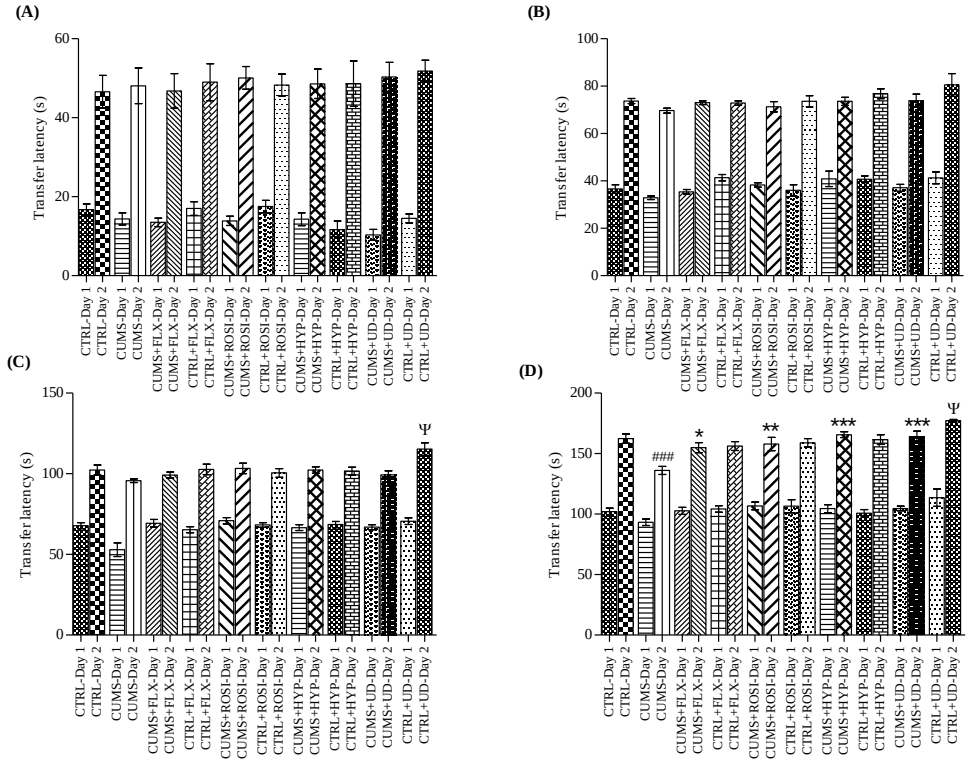



**Figure 25:** Effect of UD extract on CUMS-induced behavioural alteration in PA task: PA task base line **(A)**, PA task at week one **(B)**, PA task at week two **(C)** and PA task at week

three **(D)**. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD; $^{\Psi}$CTRL vs. CTRL + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; PA task = passive avoidance step through task.

In PA task, before CUMS and drug treatment, the base line values of step through latency (STL) or transfer latency during memory retention trial was not significantly altered among the groups (p>0.05) (Figure 25A). One week of chronic stress or drug treatment did not modulate STL during memory retention trial (p>0.05). In addition, there were no significant alterations in the STL during memory retention trial between CTRL and CTRL treated with FLX, ROSI, HYP and UD (p>0.05 vs. CTRL) after one week of stress exposure (Figure 25B).

Neither CUMS nor drugs treated stressed mice modulated STL during memory retention trial after week 2 (p>0.05). There were no significant alterations in the STL during memory retention trial between CTRL and CTRL treated with FLX, ROSI and HYP (p>0.05 vs. CTRL), while chronic UD treatment significantly increased the STL in control animals (p<0.05 vs. CTRL) after week two (Figure 25C). Chronically stressed mice showed no significant alteration (p>0.05) in STL on day 23 (memory retention trial) when compared with their respective day 22 (acquisition trial) STL at the end of CUMS exposure. During memory retention trial, the stressed animals showed significantly decreased (p<0.001 vs. CTRL) STL. During memory retention trial, chronic UD administration significantly increased the STL (p<0.001 vs. CUMS) in stressed mice, and the effect was comparable to chronic HYP treatment (p<0.001 vs. CUMS). Chronic FLX and ROSI treatment also significantly increased the STL during memory retention trial after three weeks of CUMS exposure (p<0.05 vs. CUMS and p<0.01 vs. CUMS, respectively). There were no significant alterations in the STL during memory retention trial between CTRL and CTRL treated with FLX, ROSI and HYP (p>0.05 vs. CTRL), while chronic UD administration significantly increased the STL in control animals (p<0.05 vs. CTRL) after week three (Figure 25D).

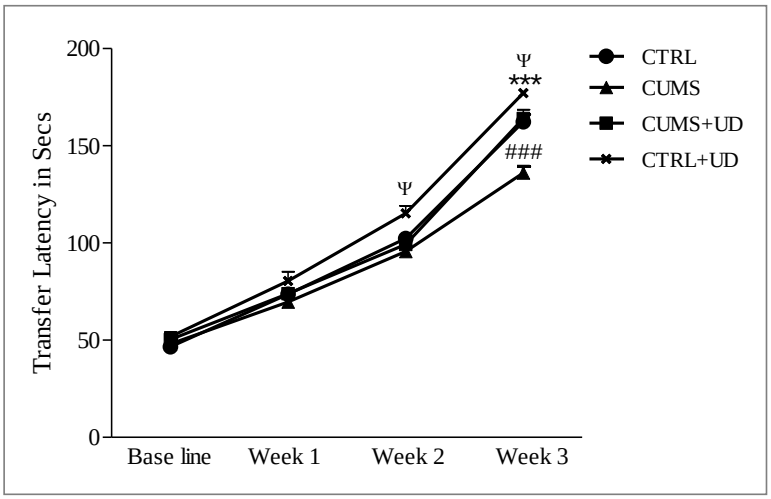



**Figure 26:** Overall effects of CUMS and UD treatment on PA task. Data were mean ± SEM values (n=6). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + UD; $^{\Psi}$CTRL vs. CTRL + UD. *p < 0.05, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; UD = *Urtica dioica* extract; PA task = passive avoidance step through task.

When combined PA task data of memory retention trial from base line to week three, we observed that CUMS exposure did not modulate the STL after week one and week two ($p>0.05$ vs. CTRL), while significantly declined the STL after week three ($p<0.001$ vs. CTRL). Conversely, chronic UD treatment significantly increased transfer latency after week three of CUMS paradigm ($p<0.001$ vs. CUMS). Chronic UD treatment did not modulate STL on stressed mice between week two and week three ($p>0.05$ vs. CUMS). In addition, chronic UD treatment gradually increased the STL from week one onwards, while significantly improved ($p<0.05$ vs. CTRL) after week two and week three in control animals (Figure 26).

### 5.4. Effect of UD extract on depression mediated locomotor deficit

In actophotometer test, before CUMS and drug treatment, the base line values of locomotor activity score was not significantly altered among the groups ($p>0.05$). One week of stress paradigm or drug treatment did not modulate locomotor activity scores ($p>0.05$) in mice. Further, stress paradigm or drug treatment did not modulate locomotor activity scores in mice after week two ($p>0.05$). However, CUMS exposure significantly reduced the locomotor activity score after week three ($p<0.01$ vs. CTRL). Chronic UD, FLX, ROSI and HYP treatment did not reverse locomotor activity in stressed mice ($p>0.05$ vs. CUMS) after week three. There were no significant alterations in the locomotor activity score between CTRL

and CTRL treated with FLX, ROSI, HYP and UD (p>0.05 vs. CTRL) after week three (Figure 27A).

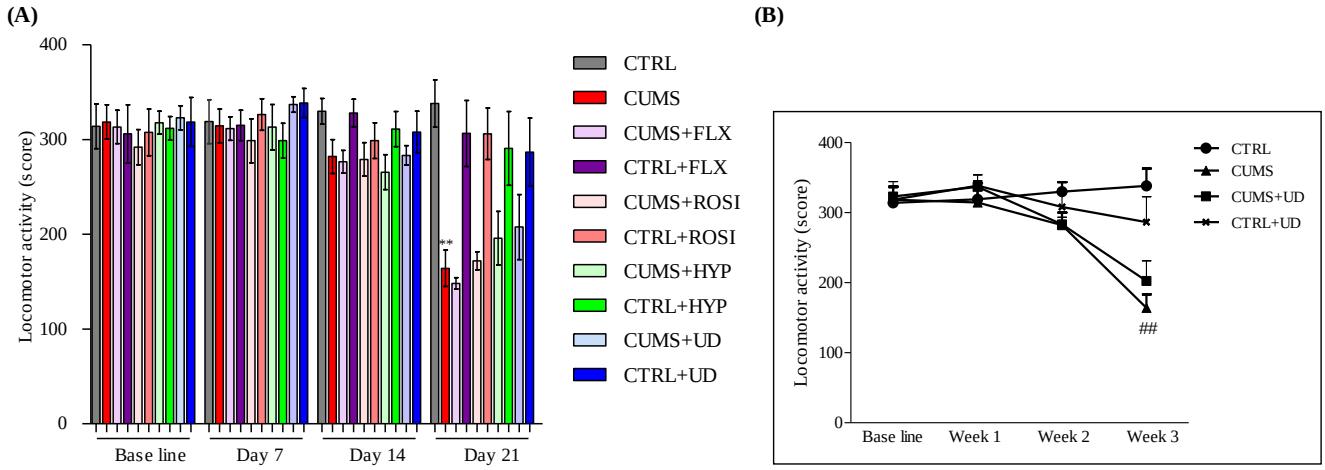



**Figure 27:** Effect of UD extract on CUMS-induced alterations in locomotor activity in actophotometer using bar graph **(A)** and locomotor performance using line graph **(B)**. Data were mean ± SEM values (n=6). Significant differences: $*^{/\#}$CTRL vs. CUMS. **p < 0.01. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

When drawn the overall performance of locomotor activity using line graph, observed that CUMS exposure started dysregulation of locomotor activity from week one onwards, while significantly reduced after week three of CUMS exposure (p<0.01 vs. CTRL). Chronic UD treatment did not significantly reverse locomotor activity score in chronically stressed mice (p>0.05 vs. CUMS) at any point. There were no significant alterations in the locomotor activity between CTRL and CTRL treated UD (p>0.05 vs. CTRL) during the study (Figure 27B).

## 5.5. Depression mediated insulin resistance and hypercorticosteronemia and the effect of UD

Three weeks of CUMS exposure did not significantly increase the level of fasting blood glucose in mice (p>0.05 vs. CTRL). Further, chronic UD, FLX, ROSI and HYP administration did not modulate the level of fasting blood glucose in control and stressed mice (p>0.05) (Figure 28A).

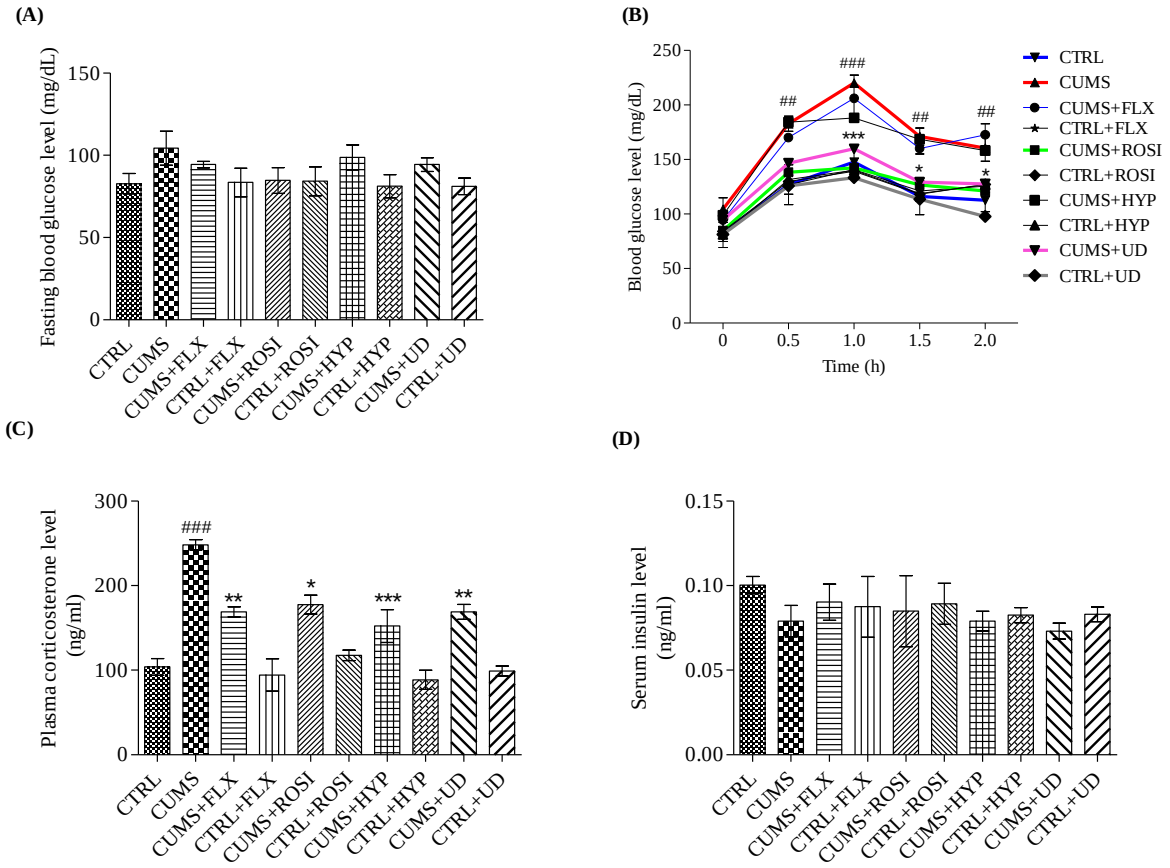



**Figure 28:** Effect of UD extract on CUMS-induced alterations in the level of fasting blood glucose **(A)**, oral glucose tolerance test **(B)**, plasma corticosterone **(C)** and serum insulin **(D)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

While, in oral glucose test, CUMS significantly increased the level of blood glucose after 0.5 h ($p<0.01$ vs. CTRL), 1.0 h ($p<0.001$ vs. CTRL), 1.5 h ($p<0.01$ vs. CTRL) and 2 hr ($p<0.01$ vs. CTRL) resulting in insulin resistance. Chronic UD administration significantly reduced the glucose load after 1.0 h ($p<0.001$ vs. CUMS), 1.5 h ($p<0.05$ vs. CUMS) and 2 h ($p<0.05$ vs. CUMS) comparable to ROSI. However, chronic FLX and HYP administration did not modulate the level of blood glucose in stressed mice ($p>0.05$ vs. CUMS). There were no

significant alterations in the level of blood glucose at any interval between CTRL and CTRL treated UD, FLX, ROSI and HYP ($p>0.05$ vs. CTRL) (Figure 28B).

CUMS significantly increased the level of plasma corticosterone in mice ($p<0.001$ vs. CTRL). Chronic UD administration significantly attenuated CUMS induced hypercorticosteronemia ($p<0.01$ vs. CUMS) in stressed mice comparable to chronic FLX administration ($p<0.01$ vs. CUMS). Chronic ROSI and HYP administration also significantly reduced the level of plasma corticosterone in stressed mice ($p<0.05$ vs. CUMS and $p<0.001$ vs. CUMS, respectively). There were no significant alterations in the level of plasma corticosterone between CTRL and CTRL treated UD, FLX, ROSI and HYP ($p>0.05$ vs. CTRL) (Figure 28C).

Three weeks of CUMS exposure did not significantly decreased the level of serum insulin under non-fasting condition ($p>0.05$ vs. CTRL). Further, chronic UD, FLX, ROSI and HYP administration did not modulate the level of serum insulin in control and stressed mice ($p>0.05$) (Figure 28D).

### 5.6. Effect of UD extract on depression mediated alteration in hippocampal insulin signaling pathway

CUMS induced depressed mice showed significant downregulation in hippocampal PPARγ mRNA expression ($p<0.01$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal PPARγ mRNA expression in stressed mice ($p<0.01$ vs. CUMS) comparable to ROSI ($p<0.01$ vs. CUMS), while chronic FLX and HYP treatment did not reverse the expression level of PPARγ mRNA in stressed mice ($p>0.05$ vs. CUMS). Chronic UD administration significantly upregulated the mRNA expression of PPARγ in stressed mice as compared to FLX ($p<0.01$ vs. CUMS+FLX) and HYP administration ($p<0.05$ vs. CUMS+HYP) (Figure 29A). In addition, chronic UD, FLX, ROSI and HYP administration did not modulate the expression of PPARγ mRNA in control mice ($p>0.05$ vs. CTRL).

Hippocampal IR mRNA expression was significantly downregulated ($p<0.05$ vs. CTRL) in chronically stressed animals. Chronic UD treatment significantly upregulated the mRNA expression of IR in stressed mice ($p<0.05$ vs. CUMS) comparable to ROSI ($p<0.05$ vs. CUMS). However, chronic FLX and HYP administration did not modulate the mRNA expression of IR in stressed mice ($p>0.05$ vs. CUMS). Chronic UD administration

significantly upregulated the mRNA expression of IR in stressed mice as compared to FLX ($p<0.01$ vs. CUMS+FLX) and HYP administration ($p<0.001$ vs. CUMS+HYP) (Figure 29B). There were no significant alterations in the mRNA expression of IR between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL).

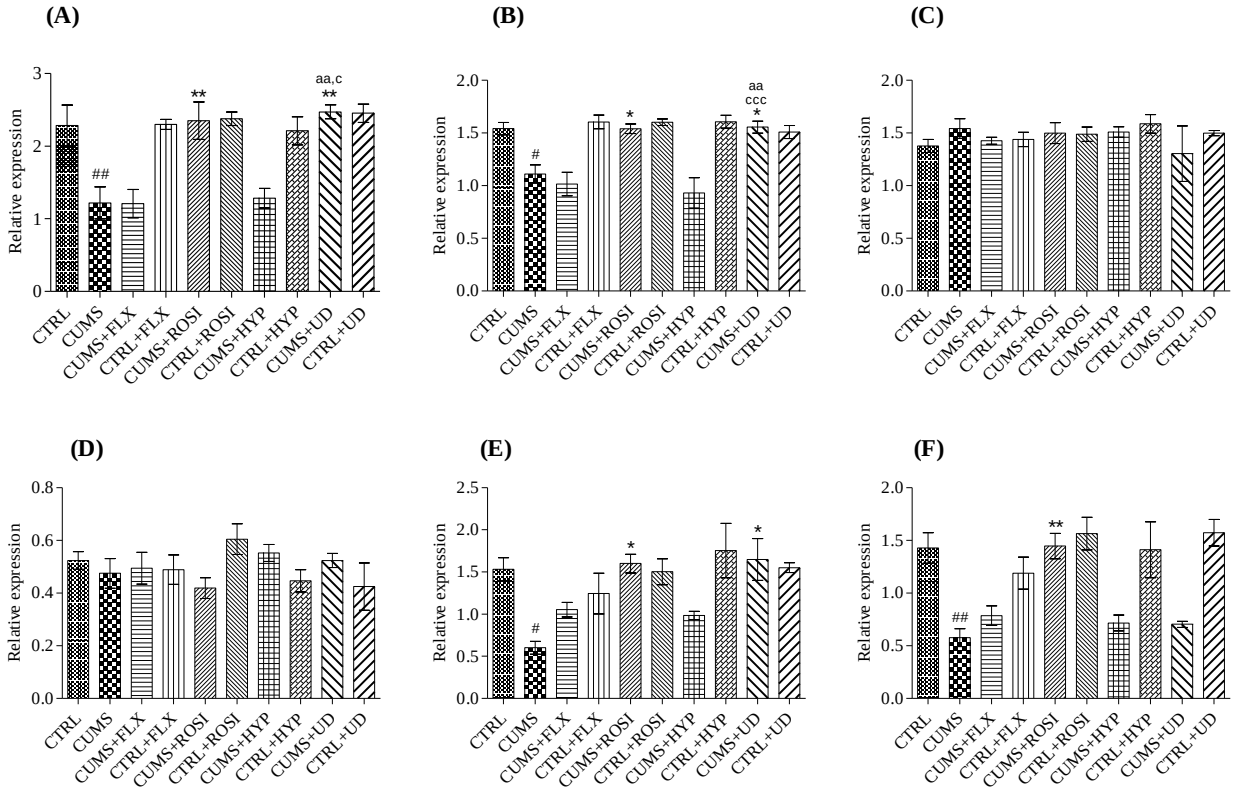



**Figure 29:** Effect of UD extract on CUMS-induced alterations in the mRNA expression of hippocampal PPARγ **(A)**, IR **(B)**, ILGF 1r **(C)**, GLP1 **(D)**, IRS1 **(E)** and IRS2 **(F)**. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + ROSI and CUMS + UD, $^{a}$CUMS + FLX vs. CUMS + UD, $^{c}$CUMS + HYP vs. CUMS + UD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

Three weeks of CUMS exposure did not regulate the expression level of hippocampal ILGF 1r mRNA ($p>0.05$ vs. CTRL) in stressed mice. Further, chronic UD, FLX, ROSI and HYP administration did not alter the expression of ILGF 1r mRNA in control and stressed mice ($p>0.05$) (Figure 29C).

CUMS induced depressed mice did not show significant alteration in mRNA expression of hippocampal GLP1 ($p>0.05$ vs. CTRL) in stressed mice. In addition, chronic UD, FLX, ROSI and HYP administration did not alter the expression of GLP1 mRNA in control and stressed mice ($p>0.05$) (Figure 29D).

CUMS induced depressed mice showed significant downregulation in hippocampal IRS1 mRNA expression ($p<0.05$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal IRS1 mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to ROSI ($p<0.05$ vs. CUMS), while chronic FLX and HYP treatment did not reverse the expression level of IRS1 mRNA in stressed mice ($p>0.05$ vs. CUMS) (Figure 29E). In addition, chronic UD, FLX, ROSI and HYP administration did not regulate the expression of IRS1 mRNA in control mice ($p>0.05$ vs. CTRL).

Hippocampal IRS2 mRNA expression was significantly downregulated ($p<0.01$ vs. CTRL) in chronically stressed animals. Chronic ROSI treatment significantly upregulated the mRNA expression of IRS2 in stressed mice ($p<0.01$ vs. CUMS), while chronic UD, FLX and HYP administration did not regulate the mRNA expression of IRS2 in stressed mice ($p>0.05$ vs. CUMS) (Figure 29F). There were no significant alterations in the mRNA expression of IRS2 between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL).

CUMS induced depressed mice showed significant downregulation in hippocampal PI3K mRNA expression ($p<0.01$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal PI3K mRNA expression in stressed mice ($p<0.01$ vs. CUMS) comparable to ROSI ($p<0.05$ vs. CUMS), while chronic FLX and HYP treatment did not reverse the expression level of PI3K mRNA in stressed mice ($p>0.05$ vs. CUMS). UD administration significantly upregulated the mRNA expression of PI3K in stressed mice as compared to FLX ($p<0.001$ vs. CUMS+FLX) and HYP administration ($p<0.001$ vs. CUMS+HYP) (Figure 30A). In addition, chronic UD, FLX, ROSI and HYP administration did not regulate the expression of PI3K mRNA in control mice ($p>0.05$ vs. CTRL).

Hippocampal PKB mRNA expression was significantly downregulated ($p<0.05$ vs. CTRL) in chronically stressed animals. Chronic UD treatment significantly upregulated the mRNA expression of PKB in stressed mice ($p<0.05$ vs. CUMS) comparable to ROSI ($p<0.05$ vs. CUMS), while chronic FLX and HYP administration did not regulate the mRNA expression

of PKB in stressed mice (p>0.05 vs. CUMS). UD administration significantly upregulated the mRNA expression of PKB in stressed mice as compared to HYP administration (p<0.05 vs. CUMS+HYP) (Figure 30B). There were no significant alterations in the mRNA expression of PKB between CTRL and CTRL treated with FLX, ROSI, HYP and UD (p>0.05 vs. CTRL).

CUMS induced depressed mice showed significant downregulation in hippocampal GLUT4 mRNA expression (p<0.01 vs. CTRL). Chronic UD administration significantly upregulated the hippocampal GLUT4 mRNA expression in stressed mice (p<0.01 vs. CUMS) comparable to ROSI (p<0.001 vs. CUMS), while chronic FLX and HYP treatment did not reverse the expression level of GLUT4 mRNA in stressed mice (p>0.05 vs. CUMS). UD administration significantly upregulated the mRNA expression of GLUT4 in stressed mice as compared to FLX (p<0.01 vs. CUMS+FLX) and HYP administration (p<0.01 vs. CUMS+HYP) (Figure 30C). In addition, chronic UD, FLX, ROSI and HYP administration did not regulate the expression of GLUT4 mRNA in control mice (p>0.05 vs. CTRL).

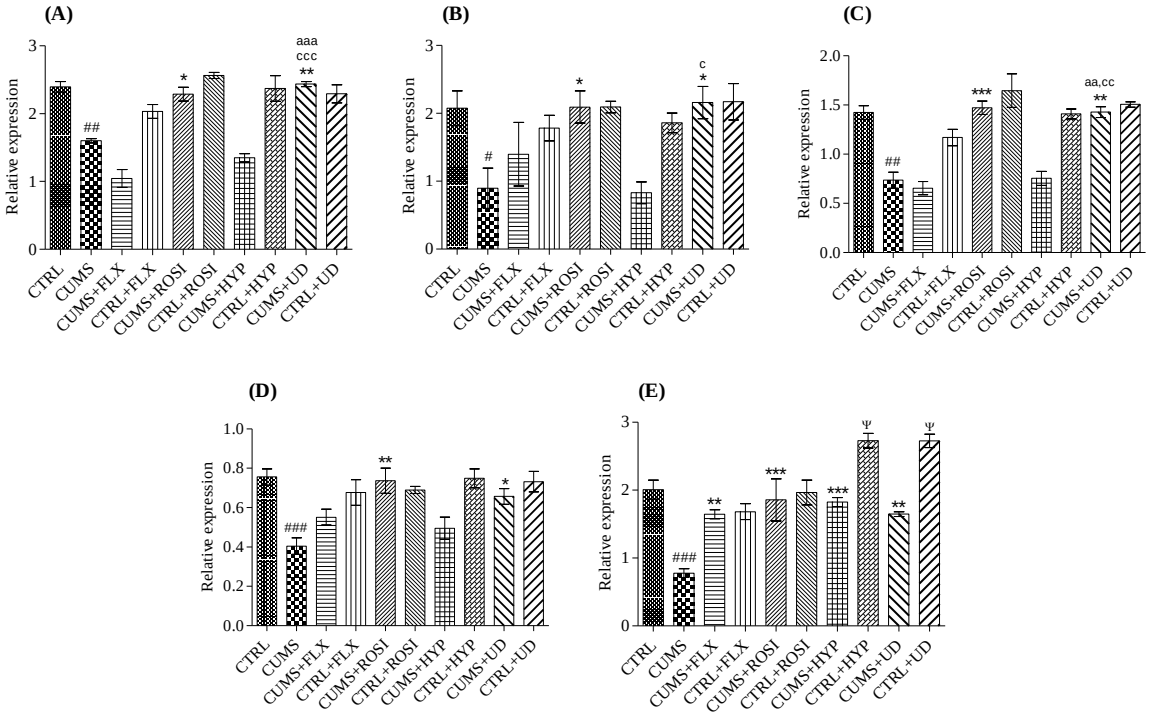



**Figure 30:** Effect of UD extract on CUMS-induced alterations in the mRNA expression of hippocampal PI3K **(A)**, PKB **(B)**, GLUT4 **(C)**, INSG1 **(D)** and MAPK1 **(E)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX,

CUMS + ROSI, CUMS + HYP and CUMS + UD; $^{\Psi}$CTRL vs. CTRL + HYP and CTRL + UD, $^{a}$CUMS + FLX vs. CUMS + UD; $^{c}$CUMS + HYP vs. CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

Hippocampal INSG1 mRNA expression was significantly downregulated (p<0.001 vs. CTRL) in chronically stressed animals. Chronic UD treatment significantly upregulated the mRNA expression of INSG1 in stressed mice (p<0.05 vs. CUMS) comparable to ROSI (p<0.01 vs. CUMS), while chronic FLX and HYP administration did not regulate the mRNA expression of INSG1 in stressed mice (p>0.05 vs. CUMS) (Figure 30D). There were no significant alterations in the mRNA expression of INSG1 between CTRL and CTRL treated with FLX, ROSI, HYP and UD (p>0.05 vs. CTRL).

CUMS induced depressed mice showed significant downregulation in hippocampal MAPK1 mRNA expression (p<0.001 vs. CTRL). Chronic UD administration significantly upregulated the hippocampal MAPK1 mRNA expression in stressed mice (p<0.01 vs. CUMS) comparable to FLX (p<0.01 vs. CUMS). Chronic ROSI and HYP treatment also significantly upregulated the expression level of MAPK1 mRNA in stressed mice (p<0.001 vs. CUMS). In addition, chronic FLX and ROSI administration did not regulate the expression of MAPK1 mRNA in control mice (p>0.05 vs. CTRL), while UD and HYP treatment significantly upregulated the mRNA expression of MAPK1 in control mice (p<0.05 vs. CTRL) (Figure 30E).

Hippocampal GLUT4 membrane protein was significantly decreased (p<0.001 vs. CTRL) in chronically stressed animals. Chronic UD treatment did not significantly increased the content of GLUT4 membrane protein in stressed mice (p>0.05 vs. CUMS). There was no significant alteration in the content of GLUT4 membrane protein between CTRL and CTRL treated with UD (p>0.05 vs. CTRL). Chronic ROSI administration significantly increased the content of hippocampal GLUT4 membrane protein in stressed mice (p<0.001 vs. CUMS). There was no significant alteration in the GLUT4 membrane protein between CTRL and CTRL treated with ROSI (p>0.05 vs. CTRL) (Figure 31).

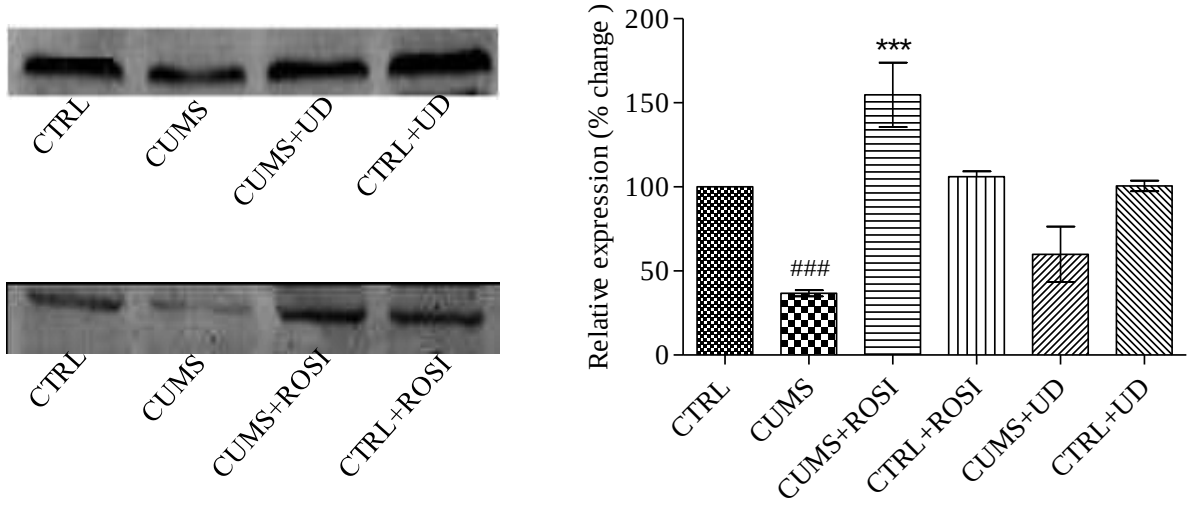



**Figure 31:** Effect of UD extract on CUMS-induced alteration in the content of hippocampal GLUT4 membrane protein**.** Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + ROSI. ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; ROSI = rosiglitazone; UD = *Urtica dioica* extract; GLUT4 = glucose transporter type 4.

## 5.7. The effect of UD extract on depression mediated impairment in Smo-Gli pathway and synaptic plasticity

Three weeks of CUMS exposure did not regulate the expression level of hippocampal Shh mRNA ($p>0.05$ vs. CTRL) in stressed mice. Further, chronic UD, FLX, ROSI and HYP administration did not alter the expression of Shh mRNA in control and stressed mice ($p>0.05$) (Figure 32A).

CUMS induced depressed mice showed no significant alteration in hippocampal Ptch1 mRNA expression ($p>0.05$ vs. CTRL). Chronic UD, FLX, ROSI and HYP administration did not modulate the hippocampal Ptch1 mRNA expression in stressed mice ($p>0.05$ vs. CUMS). Chronic FLX and ROSI administration did not modulate the expression of Ptch1 mRNA in control mice ($p>0.05$ vs. CTRL), while UD treatment significantly upregulated the mRNA expression of Ptch1 in control mice ($p<0.01$ vs. CTRL, CTRL + FLX and CTRL + ROSI). Chronic HYP administration significantly increased the mRNA expression of Ptch1 in control mice ($p<0.05$ vs. CTRL) (Figure 32B).

CUMS induced depressed mice showed significant downregulation in hippocampal Smo mRNA expression ($p<0.05$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal Smo mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to HYP ($p<0.05$ vs. CUMS). Chronic FLX and ROSI treatment did not modulate the expression level of Smo mRNA in stressed mice ($p>0.05$ vs. CUMS). Chronic FLX and ROSI administration did not modulate the expression of Smo mRNA in control mice ($p>0.05$ vs. CTRL), while UD and HYP treatment significantly upregulated the mRNA expression of Smo in control mice ($p<0.05$ vs. CTRL and $p<0.01$ vs. CTRL, respectively). UD treatment significantly increased the mRNA expression of Smo in control mice ($p<0.05$ vs. CTRL + FLX and CTRL + ROSI) (Figure 32C).

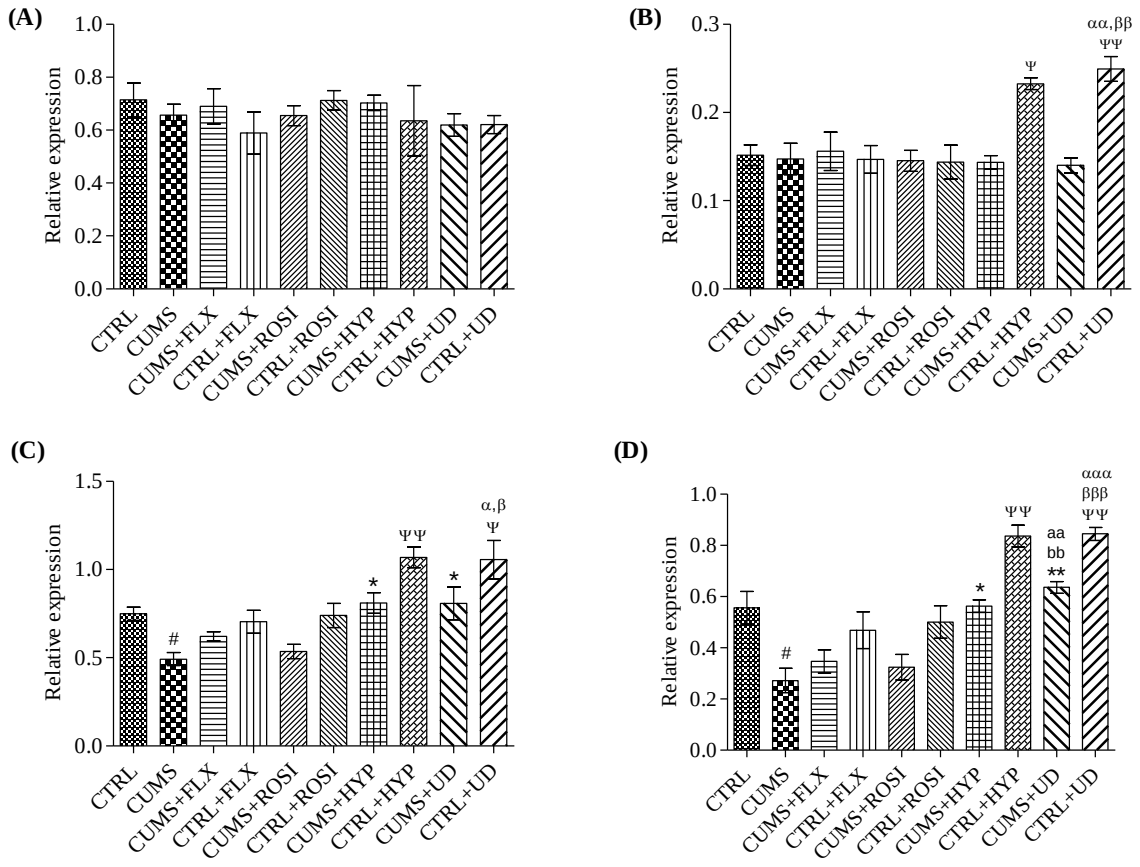



**Figure 32:** Effect of UD on CUMS-induced alterations in hippocampal Shh mRNA **(A)**, Ptch1 mRNA **(B)**, Smo mRNA **(C)** and Gli1 mRNA **(D)** expression. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. CUMS; *CUMS vs. CUMS + HYP and CUMS + UD; $^{\Psi}$CTRL vs. CTRL + HYP and CTRL + UD, $^{\alpha}$CTRL + FLX vs. CTRL + UD; $^{\beta}$CTRL + ROSI vs. CTRL + UD; $^{a}$CUMS + FLX vs. CUMS + UD; $^{b}$CUMS + ROSI vs.

CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

CUMS induced depressed mice showed significant downregulation in hippocampal Gli1 mRNA expression ($p<0.05$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal Gli1 mRNA expression in stressed mice ($p<0.01$ vs. CUMS) comparable to HYP ($p<0.05$ vs. CUMS). Chronic UD administration significantly increased the level of Gli1 in stressed mice as compared to FLX and ROSI treated stressed mice ($p<0.01$ vs. CUMS + FLX and CUMS + ROSI). Chronic FLX and ROSI treatment did not modulate the expression level of Gli1 mRNA in stressed mice ($p>0.05$ vs. CUMS). Chronic FLX and ROSI administration did not modulate the expression of Gli1 mRNA in control mice ($p>0.05$ vs. CTRL), while UD treatment significantly upregulated the mRNA expression of Gli1 in control mice ($p<0.01$ vs. CTRL) ($p<0.001$ vs. CTRL + FLX and CTRL + ROSI). Chronic HYP treatment significantly increased the mRNA expression of Gli1 in control mice ($p<0.01$ vs. CTRL) (Figure 32D).

Three weeks of CUMS exposure did not regulate the expression level of hippocampal Hhip mRNA ($p>0.05$ vs. CTRL) in stressed mice. Further, chronic UD, FLX and ROSI administration did not alter the expression of Hhip mRNA in control and stressed mice ($p>0.05$). Chronic HYP administration significantly downregulated the mRNA expression of Hhip in stressed mice ($p<0.01$ vs. CTRL and $p<0.05$ vs. CUMS) (Figure 33A).

CUMS induced depressed mice showed significant downregulation in hippocampal cyclin D1 mRNA expression ($p<0.05$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal cyclin D1 mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to HYP ($p<0.01$ vs. CUMS). Chronic FLX and ROSI treatment did not regulate the expression level of cyclin D1 mRNA in stressed mice ($p>0.05$ vs. CUMS). Chronic UD, FLX, ROSI and HYP administration did not regulate the expression of cyclin D1 mRNA in control mice ($p>0.05$ vs. CTRL) (Figure 33B).

Hippocampal BDNF mRNA expression was significantly downregulated ($p<0.01$ vs. CTRL) in chronically stressed animals. Chronic UD and ROSI treatment did not modulate the mRNA expression of BDNF in stressed mice ($p>0.05$ vs. CUMS), while chronic FLX and HYP

administration significantly upregulated the mRNA expression of BDNF in stressed mice ($p<0.01$ vs. CUMS and $p<0.001$ vs. CUMS, respectively) (Figure 33C). There were no significant alterations in the mRNA expression of BDNF between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL).

CUMS induced depressed mice showed significant downregulation in hippocampal TrkB mRNA expression ($p<0.01$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal TrkB mRNA expression in stressed mice ($p<0.05$ vs. CUMS). Also, chronic FLX, ROSI and HYP treatment significantly upregulated the expression level of TrkB mRNA in stressed mice ($p<0.01$ vs. CUMS). Further, chronic FLX, ROSI, HYP and UD administration did not regulate the expression of TrkB mRNA in control mice ($p>0.05$ vs. CTRL) (Figure 33D).

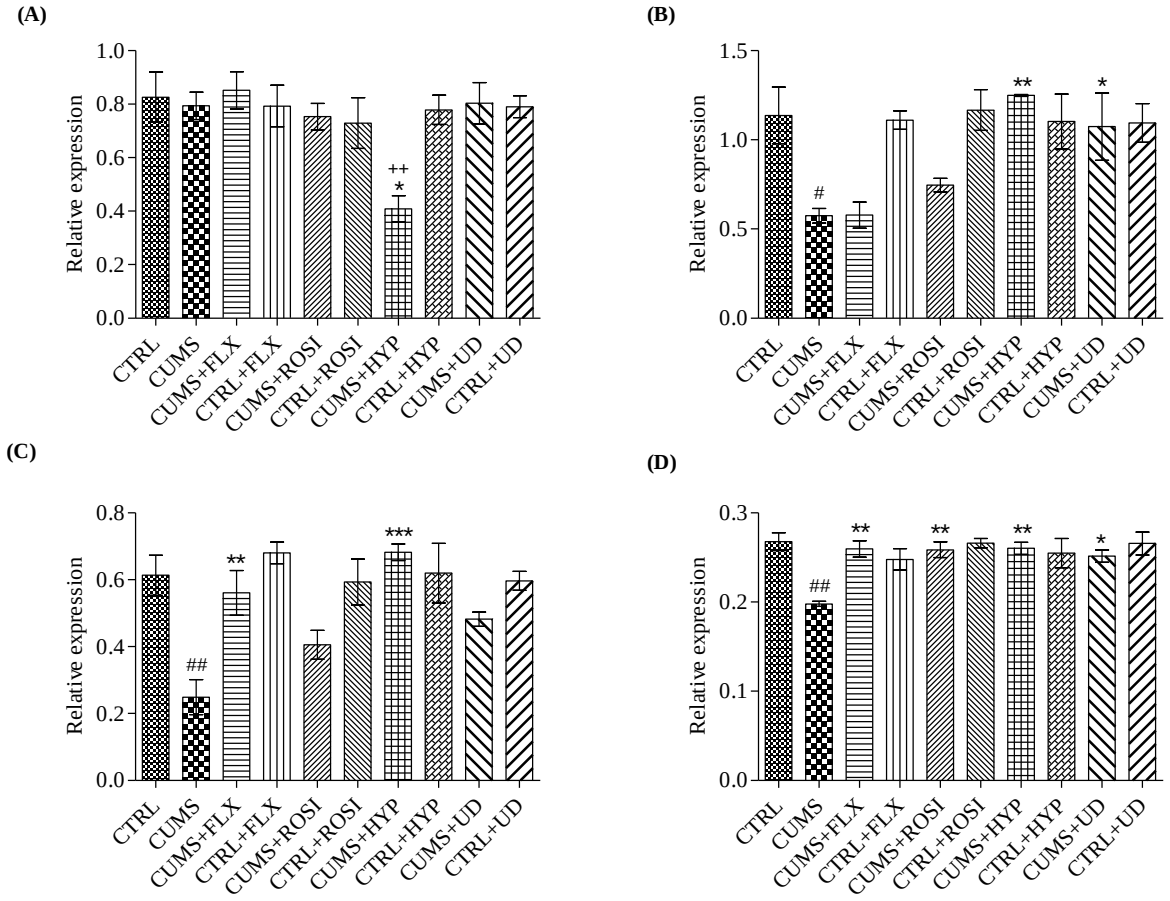



**Figure 33:** Effect of UD on CUMS-induced alterations in hippocampal Hhip mRNA **(A)**, cyclin D1 mRNA **(B)**, BDNF mRNA **(C)** and TrkB mRNA **(D)** expression. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX,

CUMS + ROSI, CUMS + HYP and CUMS + UD; $^{+}$CTRL vs. CUMS + HYP. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

Cyclopamine (Cyc) 2.5µM did not alter the mRNA expression of Gli1 (p>0.05 vs. CTRL), while Cyc 5µM significantly downregulated (p<0.001 vs. CTRL) the mRNA expression of Gli1 in hippocampal slices. Acute purmorphamine (Pur) 1µM significantly upregulated the mRNA expression of Gli1 in hippocampal slices (p<0.001 vs. CTRL), while co-treatment with Cyc 5µM the level of Gli1 mRNA was significantly downregulated (p<0.001 vs. Pur 1µM). Both dosage (50 and 100µg) of HYP significantly upregulated the mRNA expression of Gli1 in hippocampal slices (p<0.001 vs. CTRL), while this effect was significantly blocked in presence of Cyc 5µM (p<0.001 vs. Pur 1µM) and (p<0.001 vs. HYP 50 and 100µg). UD 125µg treatment did not modulate the Gli1 mRNA expression in hippocampal slices (p>0.05 vs. CTRL). UD 250µg treatment significantly upregulated the mRNA expression of Gli1 in hippocampal slices (p<0.001 vs. CTRL), while pre-administration with Cyc 5µM blocked the effect of UD 125µg and UD 250µg on Gli1 mRNA expression (p<0.001 vs. Pur 1µM) and (p<0.001 vs. UD125 and 250µg) (Figure 34A).

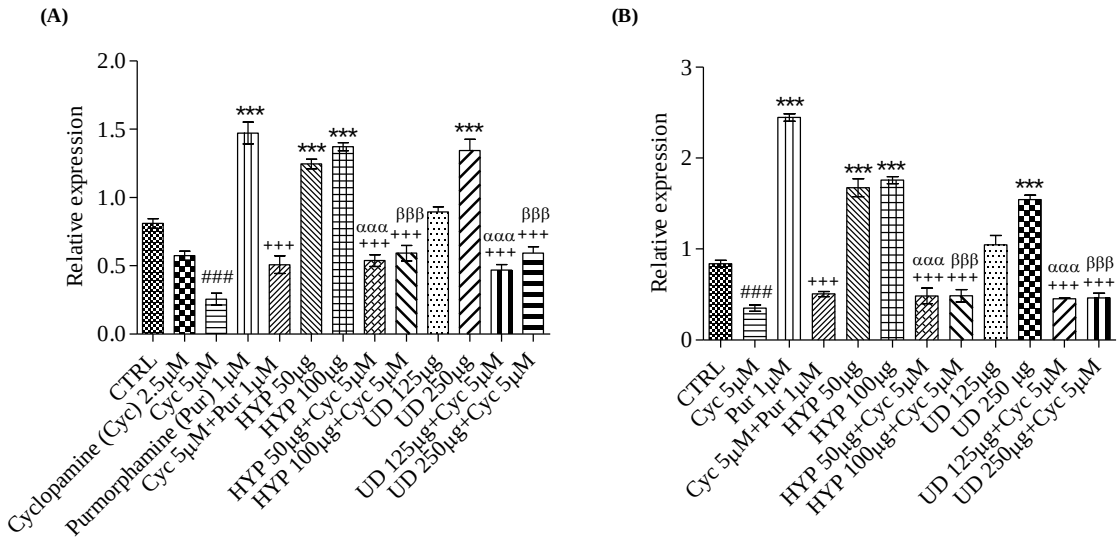



**Figure 34:** Effect of UD on Gli1 **(A)** and Ptch1 **(B)** mRNA expression in hippocampal slices pre-treated with Smo antagonist cyclopamine. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. Cyc 5µM; *CTRL vs. Pur 1µM, HYP 50µg, HYP 100µg,

UD 250µg; $^{\alpha}$HYP 50µg and UD 125µg vs. HYP 50µg + Cyc 5µM and UD 125µg + Cyc 5µM; $^{\beta}$HYP 100µg and UD 250µg vs. HYP 100µg + Cyc 5µM and UD 250µg + Cyc 5µM; $^{+}$Pur 1µM vs. Cyc 5µM+ Pur 1µM, HYP 50µg + Cyc 5µM, HYP 100µg + Cyc 5µM, UD 125µg + Cyc 5µM and UD 250µg + Cyc 5µM. ***p < 0.001. CTRL = control; Cyc = cyclopamine; Pur = purmorphamine; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

5µM of Cyc treatment significantly downregulated (p<0.001 vs. CTRL) the mRNA expression of Ptch1 in hippocampal slices. Acute Pur 1µM significantly upregulated the mRNA expression of Ptch1 in hippocampal slices (p<0.001 vs. CTRL), while pre-treatment with Cyc 5µM the level of Ptch1 mRNA was significantly downregulated (p<0.001 vs. Pur 1µM). Both dosage (50 and 100µg) of HYP significantly upregulated the mRNA expression of Ptch1 in hippocampal slices (p<0.001 vs. CTRL), while this effect was significantly blocked in presence of Cyc 5µM (p<0.001 vs. Pur 1µM) and (p<0.001 vs. HYP 50 and 100µg). UD 125µg treatment did not modulate the Ptch1 mRNA expression in hippocampal slices (p>0.05 vs. CTRL). UD 250µg treatment significantly upregulated the mRNA expression of Ptch1 in hippocampal slices (p<0.001 vs. CTRL), while pre-administration with Cyc 5µM blocked the effect of UD 125µg and UD 250µg on Ptch1 mRNA expression (p<0.001 vs. Pur 1µM) and (p<0.001 vs. UD125 and 250µg) (Figure 34B).

## 5.8. Depression mediated alteration in hippocampal and striatal cholinergic system and the effect of UD and FLX

CUMS induced depressed mice showed significant downregulation in hippocampal mAChR1 mRNA expression (p<0.01 vs. CTRL). Chronic UD administration significantly upregulated the hippocampal mAChR1 mRNA expression in stressed mice (p<0.05 vs. CUMS) comparable to FLX (p<0.05 vs. CUMS). Chronic ROSI and HYP treatment did not modulate the expression level of mAChR1 mRNA in stressed mice (p>0.05 vs. CUMS). In addition, chronic FLX, ROSI, UD and HYP administration did not regulate the expression of mAChR1 mRNA in control mice (p>0.05 vs. CTRL (Figure 35A).

Three weeks of CUMS exposure did not regulate the expression level of hippocampal mAChR4 mRNA (p>0.05 vs. CTRL) in stressed mice. Further, chronic UD, FLX, ROSI and

HYP administration did not alter the expression of hippocampal mAChR4 mRNA in control and stressed mice ($p>0.05$) (Figure 35B).

CUMS induced depressed mice did not show significant alteration in mRNA expression of striatal mAChR1 ($p>0.05$ vs. CTRL) in stressed mice. In addition, chronic UD, FLX, ROSI and HYP administration did not alter the expression of striatal mAChR1 mRNA in control and stressed mice ($p>0.05$) (Figure 35C).

CUMS induced depressed mice showed significant upregulation in striatal mAChR4 mRNA expression ($p<0.05$ vs. CTRL). Besides, chronic UD, FLX, ROSI and HYP administration did not significantly alter the expression of striatal mAChR4 mRNA in control and stressed mice ($p>0.05$) (Figure 35D).

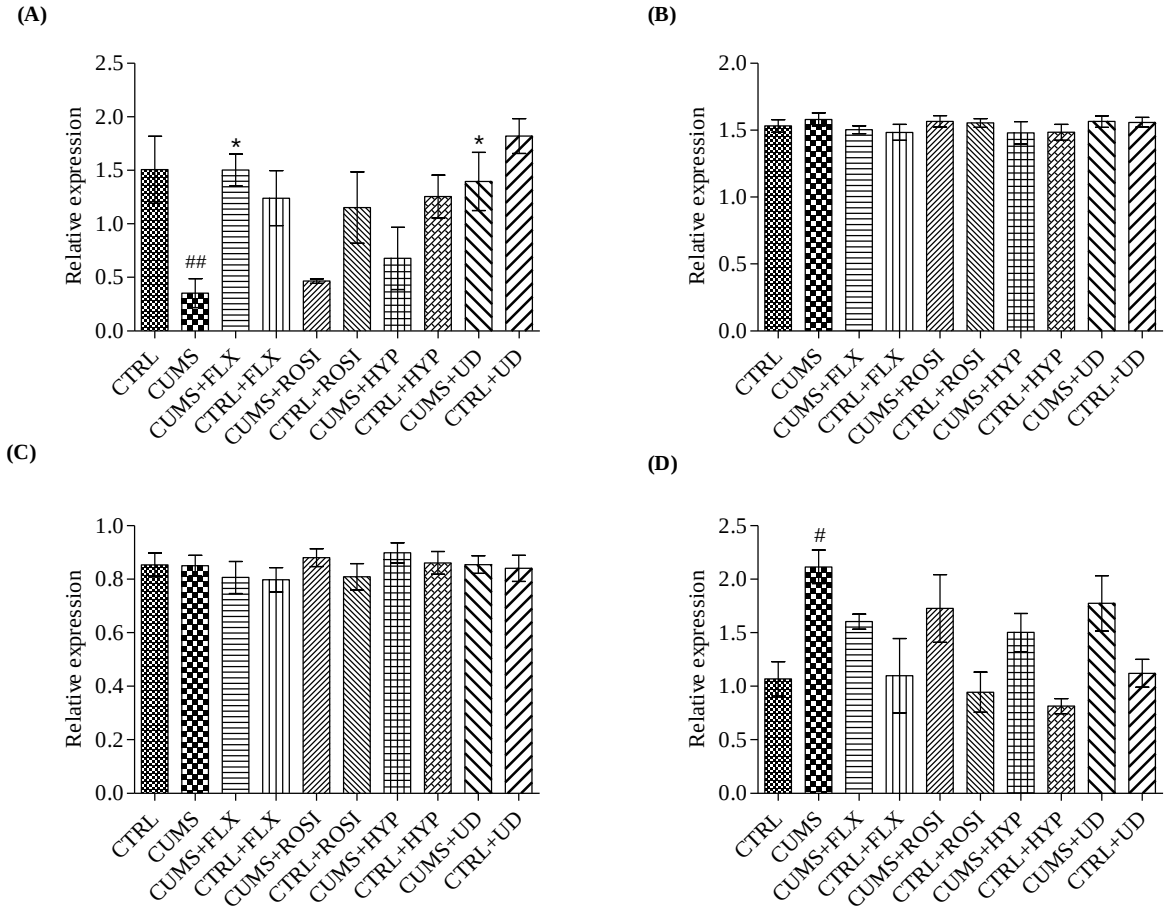



**Figure 35:** The effect of UD on CUMS-induced alterations mAChRs expression: mAChR1 mRNA in hippocampus **(A)**, mAChR4 mRNA in hippocampus **(B)**, mAChR1 mRNA in striatum **(C)** and mAChR4 mRNA in striatum **(D)**. Data were mean ± SEM values (n=4).

Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX and CUMS + UD; *p < 0.05, **p < 0.01. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

Herein, we observed that CUMS differentially alters the protein expression of mAChRs in hippocampus and striatum. Hippocampal mAChR1 protein expression was significantly downregulated ($p<0.01$ vs. CTRL) in chronically stressed animals. UD treatment significantly upregulated the protein expression of mAChR1 in stressed mice ($p<0.05$ vs. CUMS). There was no significant alteration in protein expression between control and control treated with UD ($p>0.05$ vs. CTRL). Chronic FLX treatment significantly upregulated the protein expression of mAChR1 in stressed mice ($p<0.05$ vs. CUMS). There was no significant alteration in protein expression between control and control treated with FLX ($p>0.05$ vs. CTRL) (Figure 36A).

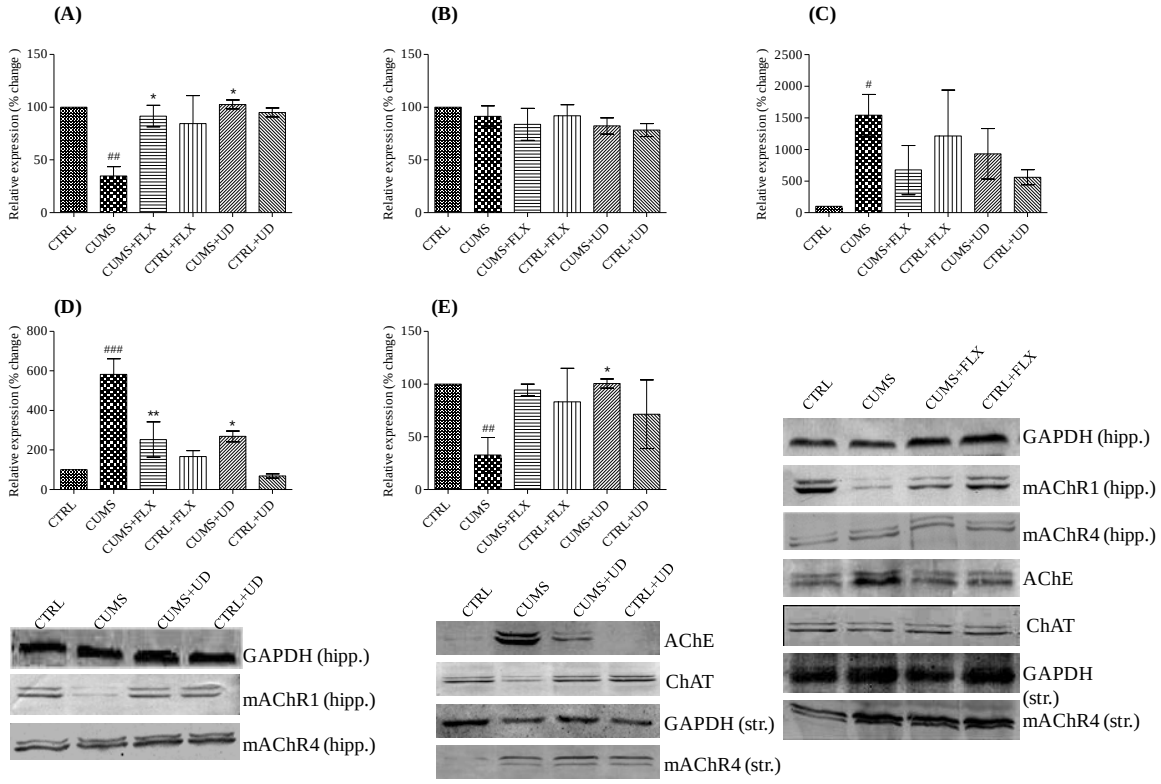



**Figure 36:** The effect of UD on CUMS-induced alterations in the expression of mAChR1 protein in hippocampus **(A)**, mAChR4 protein in hippocampus **(B)**, mAChR4 protein in striatum **(C)**, AChE protein in hippocampus **(D)** and ChAT protein in hippocampus **(E)**. Data

were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + UD and CUMS vs. CUMS + FLX. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; UD = *Urtica dioica* extract; FLX = fluoxetine.

CUMS showed insignificant alteration in protein expression of mAChR4 in hippocampus as compared to control animals (p>0.05 vs. CTRL). Chronic UD and FLX treatment did not modulate the level of hippocampal mAChR4 protein in stressed and non-stressed animals (Figure 36B).

CUMS induced depressed mice showed significant upregulation in striatal mAChR4 protein expression (p<0.05 vs. CTRL). Chronic UD administration did not significantly alter the expression of striatal mAChR4 protein in stressed mice and non stressed mice (p>0.05 vs. CUMS). Chronic FLX administration did not modulate the expression of striatal mAChR4 protein in stressed and control mice (p>0.05 vs. CUMS) (Figure 36C).

Chronically stressed mice showed significant upregulation in hippocampal AChE protein expression (p<0.001 vs. CTRL). Chronic UD administration significantly downregulated the hippocampal AChE expression in stressed mice (p<0.05 vs. CUMS). Chronic UD administration did not modulate the hippocampal AChE expression in control mice (p>0.05 vs. CTRL). Chronic FLX administration significantly downregulated the hippocampal AChE expression in stressed mice (p<0.01 vs. CUMS). Chronic FLX administration did not modulate the hippocampal AChE expression in control mice (p>0.05 vs. CTRL) (Figure 36D).

Hippocampal ChAT protein expression was significantly downregulated (p<0.01 vs. CTRL) in chronically stressed mice. UD treatment significantly upregulated the protein expression of ChAT in stressed mice (p<0.05 vs. CUMS). There was no significant alteration in ChAT protein expression between control and control treated with UD (p>0.05 vs. CTRL). FLX treatment insignificantly upregulated the protein expression of ChAT in stressed mice (p>0.05 vs. CUMS). There was no significant alteration in ChAT protein expression between control and control treated with FLX (p>0.05 vs. CTRL) (Figure 36E).

## 5.9. Effect of UD on depression mediated impairment in ATG and neuronal survival

CUMS induced depressed mice showed significant downregulation in hippocampal BCL2 mRNA expression ($p<0.01$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal BCL2 mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to ROSI and HYP ($p<0.05$ vs. CUMS). Chronic FLX treatment also significantly upregulated the expression level of BCL2 mRNA in stressed mice ($p<0.001$ vs. CUMS) (Figure 37A). In addition, chronic UD, FLX, ROSI and HYP administration did not regulate the expression of BCL2 mRNA in control mice ($p>0.05$ vs. CTRL).

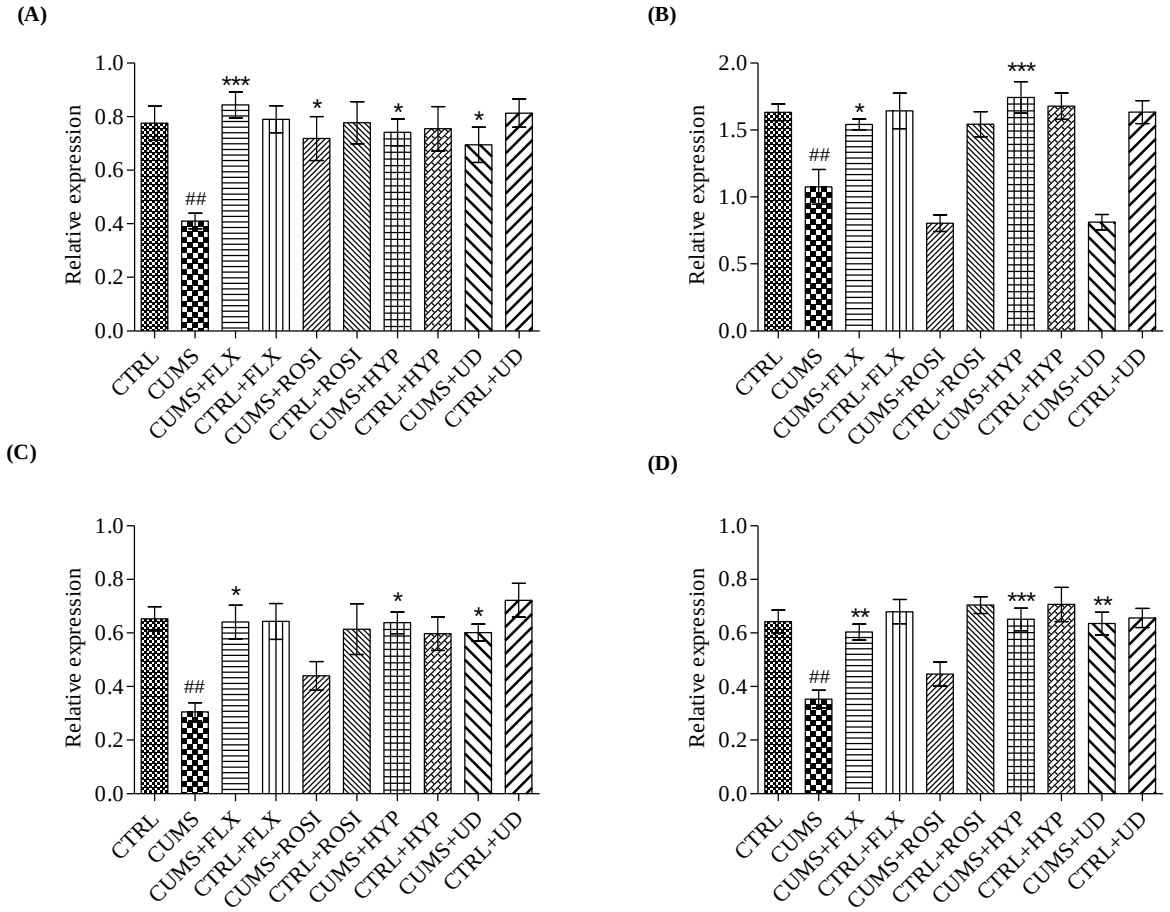



**Figure 37:** Effect of UD on CUMS-induced alterations in the mRNA expression of hippocampal BCL2 **(A)**, AIP2 **(B)**, ATG5 **(C)** and ATG7 **(D)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CTRL =

control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

Hippocampal AIP2 mRNA expression was significantly downregulated ($p<0.01$ vs. CTRL) in chronically stressed animals. Chronic UD and ROSI treatment did not modulate the mRNA expression of AIP2 in stressed mice ($p>0.05$ vs. CUMS), while chronic FLX and HYP administration significantly upregulated the mRNA expression of AIP2 in stressed mice ($p<0.05$ vs. CUMS and $p<0.001$ vs. CUMS, respectively) (Figure 37B). There were no significant alterations in the mRNA expression of AIP2 between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL).

CUMS induced depressed mice showed significant downregulation in hippocampal ATG5 mRNA expression ($p<0.01$ vs. CTRL). Chronic UD administration significantly upregulated the hippocampal ATG5 mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to FLX and HYP ($p<0.05$ vs. CUMS), while chronic ROSI administration did not modulate the expression level of ATG5 in stressed mice ($p>0.05$ vs. CUMS) (Figure 37C). In addition, chronic UD, FLX, ROSI and HYP administration did not regulate the expression of ATG5 mRNA in control mice ($p>0.05$ vs. CTRL).

Hippocampal ATG7 mRNA expression was significantly downregulated ($p<0.01$ vs. CTRL) in chronically stressed mice. Chronic ROSI treatment did not modulate the mRNA expression of ATG7 in stressed mice ($p>0.05$ vs. CUMS), while chronic FLX, UD and HYP administration significantly upregulated the mRNA expression of ATG7 in stressed mice ($p<0.01$ vs. CUMS, $p<0.01$ vs. CUMS and $p<0.001$ vs. CUMS, respectively) (Figure 37D). There were no significant alterations in the mRNA expression of ATG7 between CTRL and CTRL treated with FLX, ROSI, HYP and UD ($p>0.05$ vs. CTRL).

### 5.10. Effect of UD extract on CUMS-induced oxidative and nitrative stress

CUMS induced depressed mice showed significant upregulation in hippocampal iNOS mRNA expression ($p<0.001$ vs. CTRL). Chronic UD administration significantly downregulated the hippocampal iNOS mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to FLX ($p<0.05$ vs. CUMS) and ROSI ($p<0.05$ vs. CUMS). Also, chronic HYP treatment significantly downregulated the expression level of iNOS mRNA in stressed mice ($p<0.01$ vs. CUMS). Further, chronic FLX, ROSI, HYP and UD administration

did not regulate the expression of iNOS mRNA in control mice (p>0.05 vs. CTRL) (Figure 38A).

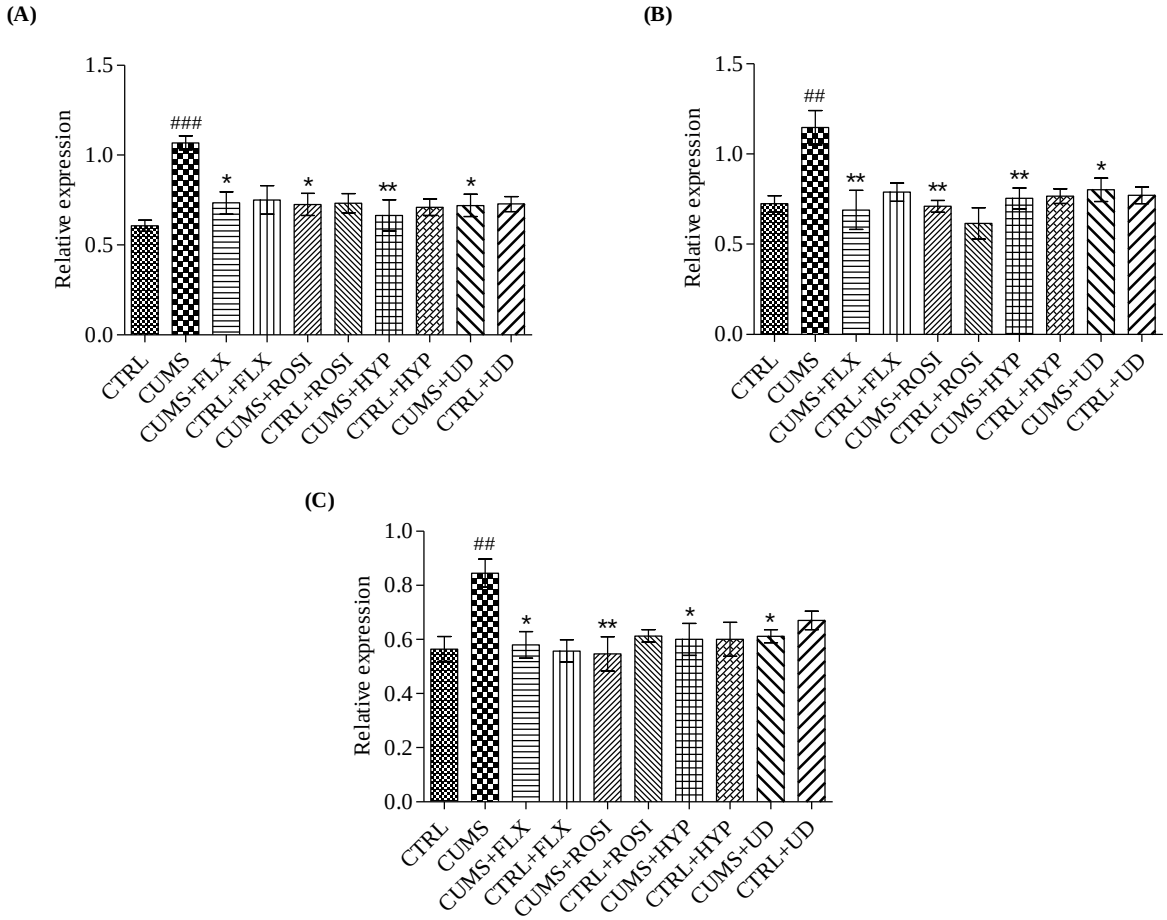



**Figure 38:** Effect of UD on CUMS-induced alterations in the mRNA expression of hippocampal iNOS **(A)**, IL6 **(B)** and TNFα **(C)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

Hippocampal IL6 mRNA expression was significantly upregulated (p<0.01 vs. CTRL) in chronically stressed mice. Chronic UD administration significantly downregulated the hippocampal IL6 mRNA expression in stressed mice (p<0.05 vs. CUMS). Also, chronic HYP, FLX and ROSI treatment significantly downregulated the expression level of IL6 mRNA in stressed mice (p<0.01 vs. CUMS). Further, chronic FLX, ROSI, HYP and UD

administration did not regulate the expression of IL6 mRNA in control mice ($p>0.05$ vs. CTRL) (Figure 38B).

CUMS induced depressed mice showed significant upregulation in hippocampal TNFα mRNA expression ($p<0.01$ vs. CTRL). Chronic UD administration significantly downregulated the hippocampal TNFα mRNA expression in stressed mice ($p<0.05$ vs. CUMS) comparable to FLX ($p<0.05$ vs. CUMS) and HYP ($p<0.05$ vs. CUMS). Also, chronic ROSI treatment significantly downregulated the expression level of TNFα mRNA in stressed mice ($p<0.01$ vs. CUMS). Further, chronic FLX, ROSI, HYP and UD administration did not modulate the expression of TNFα mRNA in control mice ($p>0.05$ vs. CTRL) (Figure 38C).

CUMS significantly increased the level of plasma thiobarbituric acid-reactive substances (TBARS) in mice ($p<0.01$ vs. CTRL). Chronic UD administration insignificantly attenuated CUMS induced elevated level of TBARS ($p>0.05$ vs. CUMS) in stressed mice. Chronic FLX ($p<0.05$ vs. CUMS) and ROSI ($p<0.05$ vs. CUMS) administration reduced the level of TBARS in stressed mice. Chronic HYP administration also significantly reduced the level of plasma TBARS in stressed mice ($p<0.001$ vs. CUMS). There were no significant alterations in the level of plasma TBARS between CTRL and CTRL treated UD, FLX, ROSI and HYP ($p>0.05$ vs. CTRL) (Figure 39A).

Level of plasma nitric oxide (NO) was significantly increased in chronically stressed mice ($p<0.001$ vs. CTRL). Chronic UD administration significantly attenuated CUMS induced elevated level of NO ($p<0.05$ vs. CUMS) in stressed mice comparable to chronic ROSI ($p<0.05$ vs. CUMS) administration. Chronic HYP and FLX administration also significantly reduced the level of plasma NO in stressed mice ($p<0.001$ vs. CUMS and $p<0.01$ vs. CUMS, respectively). There were no significant alterations in the level of plasma NO between CTRL and CTRL treated UD, FLX, ROSI and HYP ($p>0.05$ vs. CTRL) (Figure 39B).

CUMS significantly decreased the level of plasma catalase in mice ($p<0.01$ vs. CTRL). Chronic UD administration significantly increased the level of plasma catalase ($p<0.05$ vs. CUMS) in stressed mice comparable to chronic FLX ($p<0.05$ vs. CUMS) administration. Chronic ROSI and HYP administration also significantly increased the level of plasma catalase in stressed mice ($p<0.01$ vs. CUMS and $p<0.001$ vs. CUMS). There were no

significant alterations in the level of plasma catalase between CTRL and CTRL treated UD, FLX, ROSI and HYP (p>0.05 vs. CTRL) (Figure 39C).

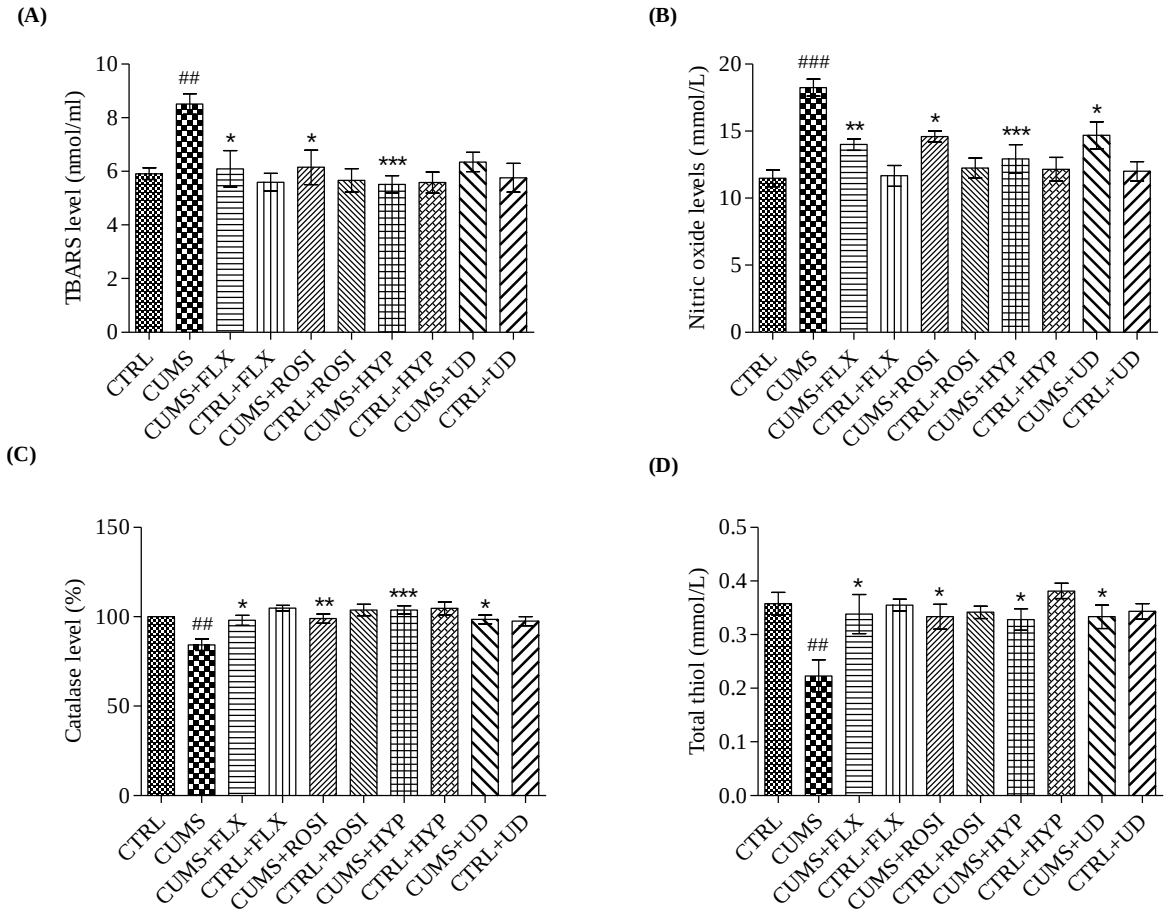



**Figure 39:** Effect of UD on CUMS-induced alterations in TBARS level **(A)**, nitric oxide level **(B)**, catalase level **(C)** and total thiol level **(D)** in plasma. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. CUMS; *CUMS vs. CUMS + FLX, CUMS + ROSI, CUMS + HYP and CUMS + UD. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract; TBARS = thiobarbituric acid-reactive substances; NO = nitric oxide.

Level of plasma total thiol was significantly decreased in chronically stressed mice (p<0.01 vs. CTRL). Chronic UD administration significantly increased the level of plasma total thiol (p<0.05 vs. CUMS) in stressed mice comparable to chronic ROSI (p<0.05 vs. CUMS), FLX

(p<0.05 vs. CUMS) and HYP (p<0.05 vs. CUMS) administration. There were no significant alterations in the level of plasma total thiol between CTRL and CTRL treated with UD, FLX, ROSI and HYP (p>0.05 vs. CTRL) (Figure 39D).

## 5.11. Accumulation of stinging nettle and St. John's wort constituents on whole brain after CUMS paradigm

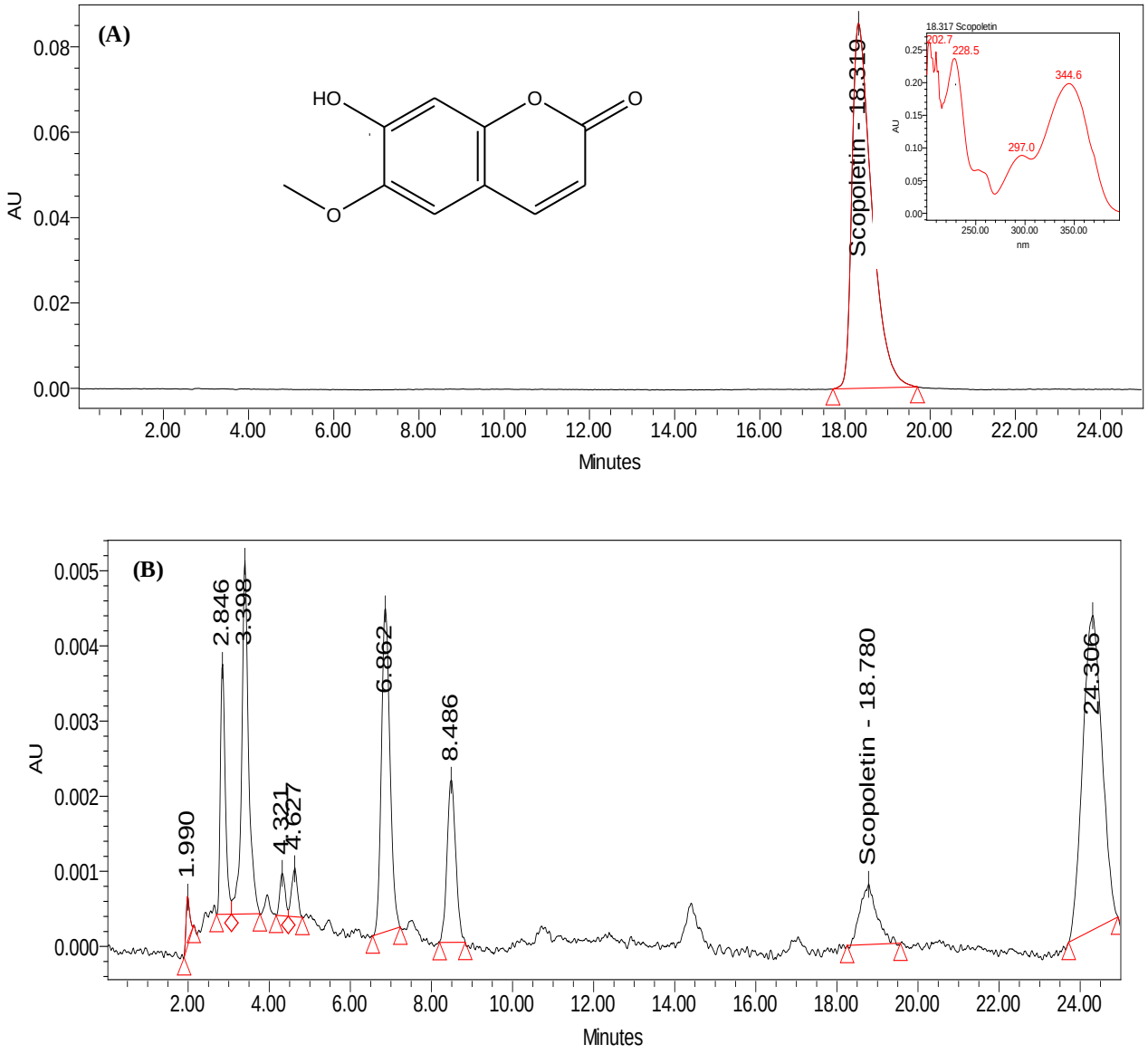



**Figure 40:** HPLC chromatogram of standard scopoletin **(A)** as well as whole brain homogenate of chronically stressed mice treated with *Urtica dioica* extract **(B)**.

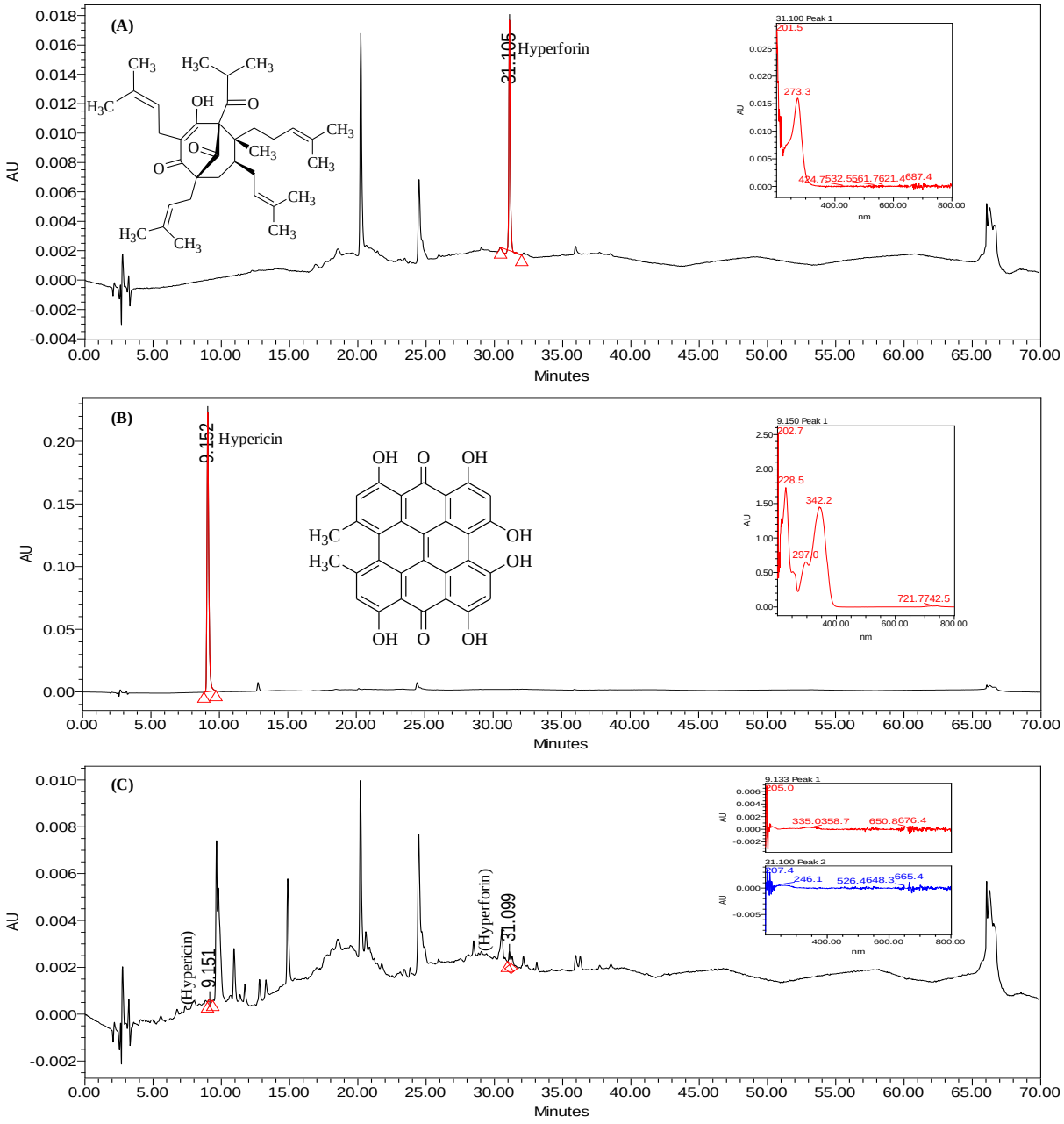



**Figure 41:** HPLC chromatogram of standard hyperforin **(A),** hypericin **(B)** as well as whole brain homogenate of chronically stressed mice treated with St. John's wort extract **(C)**.

Orally administered Stinging nettle extract accumulated as its constituent scopoletin (0.002%) in the brain tissue of mice after the last dose followed by 12 hr fasting (Figure 40).

Orally administered Hypericum extract accumulated as hyperforin-0.18% & hypericin-0.06% in the brain tissue of mice after the last dose followed by 12 hr fasting (Figure 41).

## 5.12. Effect of UD extract on STZ induced hyperglycemia, hypoinsulinemia and insulin resistance

STZ significantly elevated the level of fasting blood glucose (≥210 mg/dl) ($p<0.001$ vs. CTRL) and decreased circulating insulin level ($p<0.001$ vs. CTRL) in experimental animals. Treatment with UD to diabetic mice significantly ($p<0.05$ vs. STZ) reduced the blood glucose level comparable to ROSI ($p<0.05$ vs. STZ) (Figure 42A). Further, there was a significant increase in circulating insulin level in UD ($p<0.01$ vs. STZ) and ROSI ($p<0.05$ vs. STZ) treated diabetic mice as compared to diabetic animals (Figure 42B).

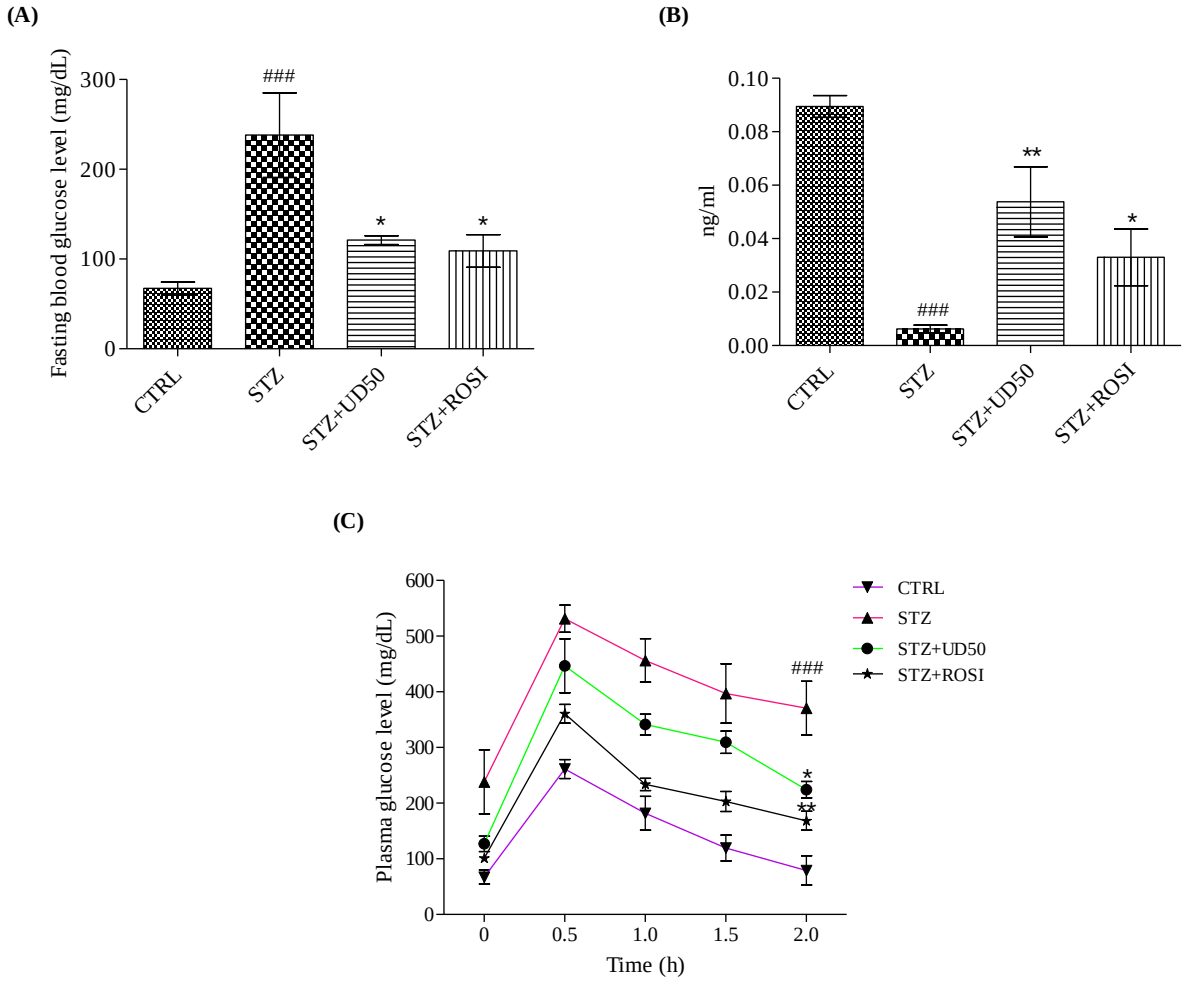



**Figure 42:** Effect of UD extract on STZ induced alteration in fasting blood glucose level **(A)**, serum insulin level **(B)** and oral glucose tolerance test **(C)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p

< 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

In oral glucose tolerance test, the blood glucose level at 0 time was different among the groups like fasting blood glucose. Glucose challenge dramatically raised the blood glucose level of STZ group compared with control group at 0.5-2.0 h intervals indicating insulin resistance. Chronic UD and ROSI treatment significantly reduced the level of blood glucose ($p<0.05$ vs. STZ and $p<0.01$ vs. STZ, respectively) at 2 h as compared to STZ group (Figure 42C).

## 5.13. Effect of UD extract on diabetes mediated alteration in body weight and water intake

After STZ injection, animals exhibited decreased body weight ($p<0.01$ vs. CTRL) as compared with control mice. Chronic treatment with UD and ROSI to diabetic mice after 5th–60th day significantly ($p<0.01$ vs. STZ) ameliorated body weight loss as compared with STZ treated diabetic mice (Figure 43A).

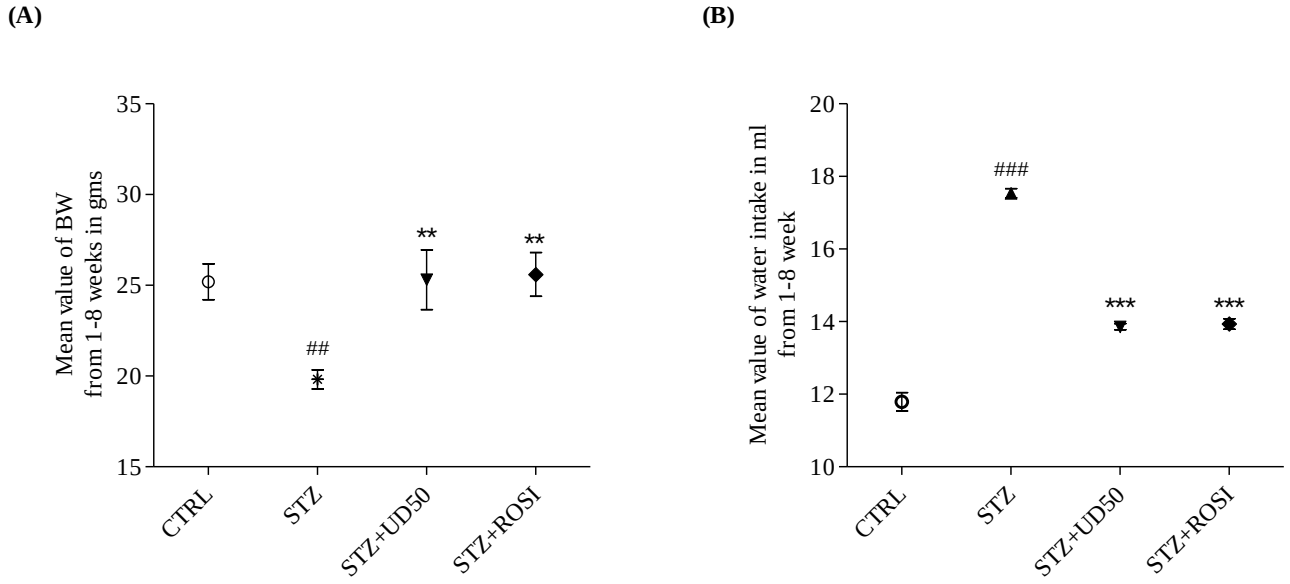



**Figure 43:** Effect of UD extract on STZ induced alteration in body weight **(A)** and water intake **(B)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

In our study, long standing diabetes significantly ($p<0.001$ vs. CTRL) increased the water intake as compared to normal control mice, which is a classic sign of diabetes. Chronic administration of UD to diabetic mice significantly ($p<0.001$ vs. STZ) reduced the water intake when compared with STZ-diabetic mice. ROSI also reversed the water intake in STZ induced diabetic mice significantly ($p<0.001$ vs. STZ) (Figure 43B).

## 5.14. Diabetes mediated depressive like behaviour and motor function deficit and the effect of UD extract

STZ induced diabetic mice showed significant increase in the duration of immobility in forced swim test ($p<0.001$ vs. CTRL). Chronic UD administration significantly reduced the duration of immobility in diabetic mice ($p<0.001$ vs. STZ). Also, chronic ROSI treatment significantly reduced the duration of immobility in diabetic mice ($p<0.001$ vs. STZ) (Figure 44A).

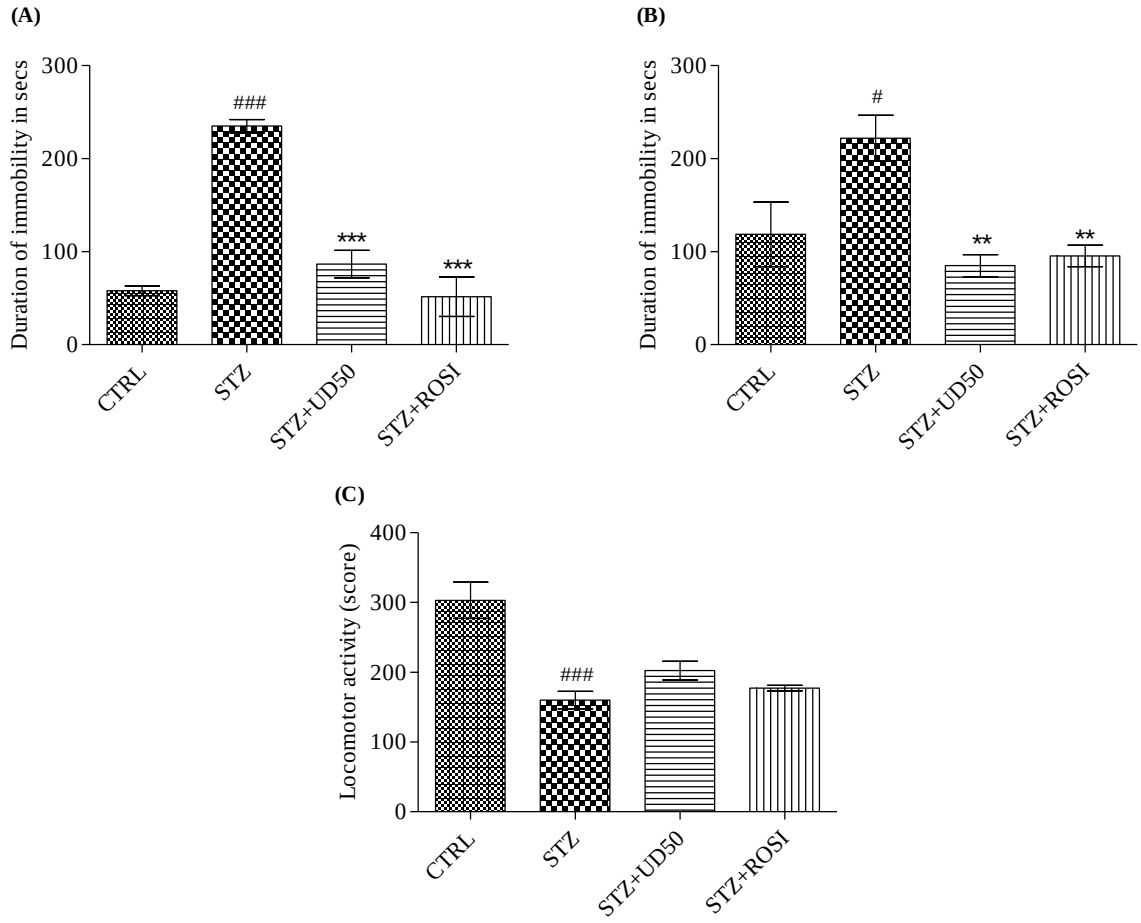



**Figure 44:** Effect of UD extract on STZ induced depressive like behaviour in forced swim

test **(A),** depressive like behaviour in tail suspension test **(B)** and locomotor deficit in actophotometer test **(C)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

Chronically diabetic mice showed significant increase in the duration of immobility in tail suspension test ($p<0.05$ vs. CTRL). Chronic UD administration significantly reduced the duration of immobility in diabetic mice ($p<0.01$ vs. STZ). Also, chronic ROSI treatment significantly reduced the duration of immobility in diabetic mice ($p<0.01$ vs. STZ) (Figure 44B).

STZ induced diabetes significantly reduced the locomotor activity in mice during actophotometer test ($p<0.001$ vs. CTRL). Chronic UD treatment did not modulate the locomotor activity in diabetic mice ($p>0.05$ vs. STZ). Chronic ROSI administration also did not revere diabetes induced hypolocomotion ($p>0.05$ vs. STZ) (Figure 44C).

### 5.15. Effect of UD extract on diabetes mediated cognitive deficit

In Morris water maze task, diabetic mice showed significantly increased escape latency on day 57 ($p<0.05$ vs. CTRL), 58 ($p<0.01$ vs. CTRL) and 59 ($p<0.001$ vs. CTRL) as compared to the control animals (Figure 45A). The control animals showed significant improvement in learning between trials from day 57 to day 59 as evident from the decrease in the escape latency. The diabetic animals did not show any significant alteration in learning between the trials and the number of crossings across the platform area was also significantly decreased ($p<0.001$ vs. CTRL) as compared to normal animals during the probe trial (on day 60). UD and ROSI treatment significantly decreased the escape latency on day 58 ($p<0.05$ vs. STZ) and 59 ($p<0.001$ vs. STZ), whereas improved the number of crossings ($p<0.05$ vs. STZ) across the platform area (probe trial) as compared to STZ induced diabetic mice (Figure 45B).

In passive avoidance step through task, diabetic mice showed no alteration in STL on day 2 (memory retention trial) when compared with their respective day 1 (acquisition trail) STL ($p>0.05$). During memory retention trial on day 2 the diabetic animals showed decreased STL as compared to normal animals ($p<0.01$ vs. CTRL Day 2). Further, chronic treatment with

UD and ROSI significantly increased ($p<0.05$ vs. STZ Day 2) the STL on day 2 as compared with STZ induced diabetic mice (Figure 45C).

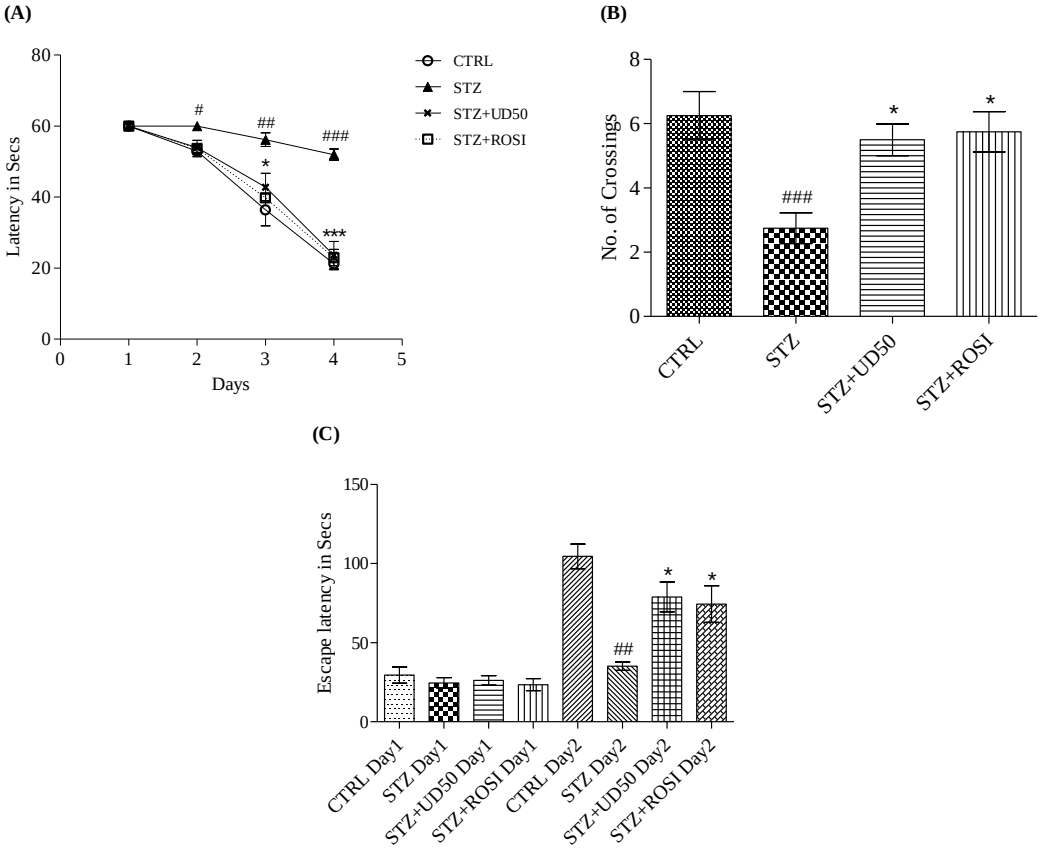



**Figure 45:** Effect of UD extract on STZ induced behavioural alteration in Morris water maze task **(A),** probe trial **(B)** and passive avoidance step through task **(C)**. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

### 5.16. Effect of UD extract on hippocampal insulin signaling pathway

PPARγ mRNA expression was not significantly altered in the STZ induced diabetic mice as compared to control mice ($p>0.05$ vs. CTRL). Chronic ROSI and UD administration significantly increased ($p<0.05$ vs. CTRL) the mRNA expression of hippocampal PPARγ in STZ induced diabetic mice (Figure 46A).

STZ induced diabetic mice showed significant decrease ($p<0.05$ vs. CTRL) in the IR mRNA expression as compared to control mice. Chronic UD and ROSI treatment significantly increased ($p<0.01$ vs. STZ) the mRNA expression of IR in diabetic mice (Figure 46B).

Hippocampal ILGF 1r mRNA expression was not significantly altered in chronically diabetic mice ($p>0.05$ vs. CTRL). Chronic UD and ROSI treatment did not regulate the mRNA expression of hippocampal ILGF 1r mRNA expression in diabetic mice ($p>0.05$ vs. STZ) (Figure 46C).

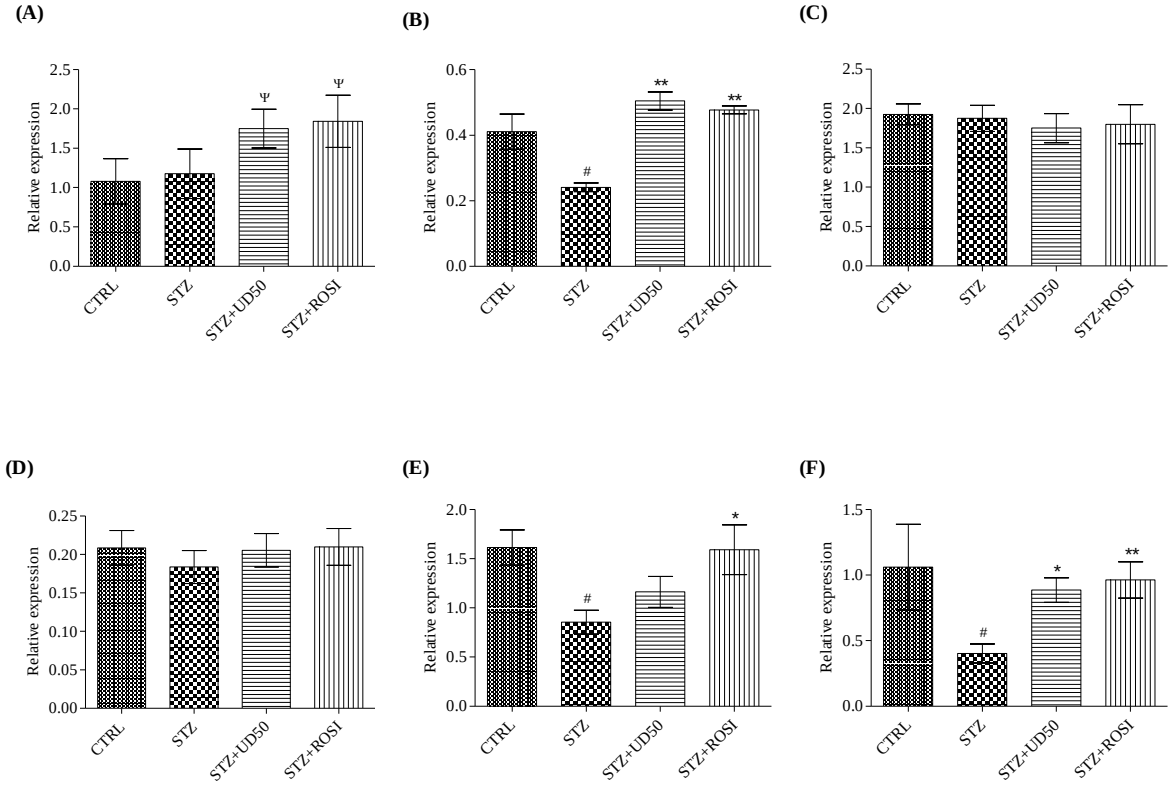



**Figure 46:** Effect of UD extract on diabetes-mediated alterations in the mRNA expression of hippocampal PPARγ **(A)**, IR **(B)**, ILGF 1r **(C)**, GLP1 **(D)**, IRS1 **(E)** and IRS2 **(F)**. Data were mean ± SEM values (n=4). Significant differences: $^{\#}$CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI; $^{\Psi}$CTRL vs. STZ + UD50 and STZ + ROSI. *$p < 0.05$, **$p < 0.01$. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

STZ induced diabetic mice showed no significant alteration in mRNA expression of hippocampal GLP1 ($p>0.05$ vs. CTRL). Chronic UD and ROSI treatment did not modulate the mRNA expression of hippocampal GLP1 in diabetic mice ($p>0.05$ vs. STZ) (Figure 46D).

In the present study, STZ induced diabetic mice showed significant decrease ($p<0.05$ vs. CTRL) in the hippocampal IRS1 mRNA expression as compared to control animals. Chronic UD treatment did not regulate ($p>0.05$ vs. STZ) the mRNA expression of hippocampal IRS1 in diabetic mice. Chronic ROSI treatment significantly ($p<0.05$ vs. STZ) increased the mRNA expression of hippocampal IRS1 in diabetic mice (Figure 46E).

IRS2 mRNA expression was significantly downregulated ($p<0.05$ vs. CTRL) in the hippocampus of diabetic mice as compared to control mice. Further, chronic UD and ROSI administration significantly increased the mRNA expression of hippocampal IRS2 in diabetic mice ($p<0.05$ vs. STZ and $p<0.01$ vs. STZ, respectively) (Figure 46F).

PI3K mRNA expression was significantly downregulated ($p<0.05$ vs. CTRL) in the hippocampus of diabetic mice as compared to control mice. Further, chronic UD or ROSI treatment significantly increased ($p<0.01$ vs. STZ) the mRNA expression of hippocampal PI3K in diabetic mice (Figure 47A).

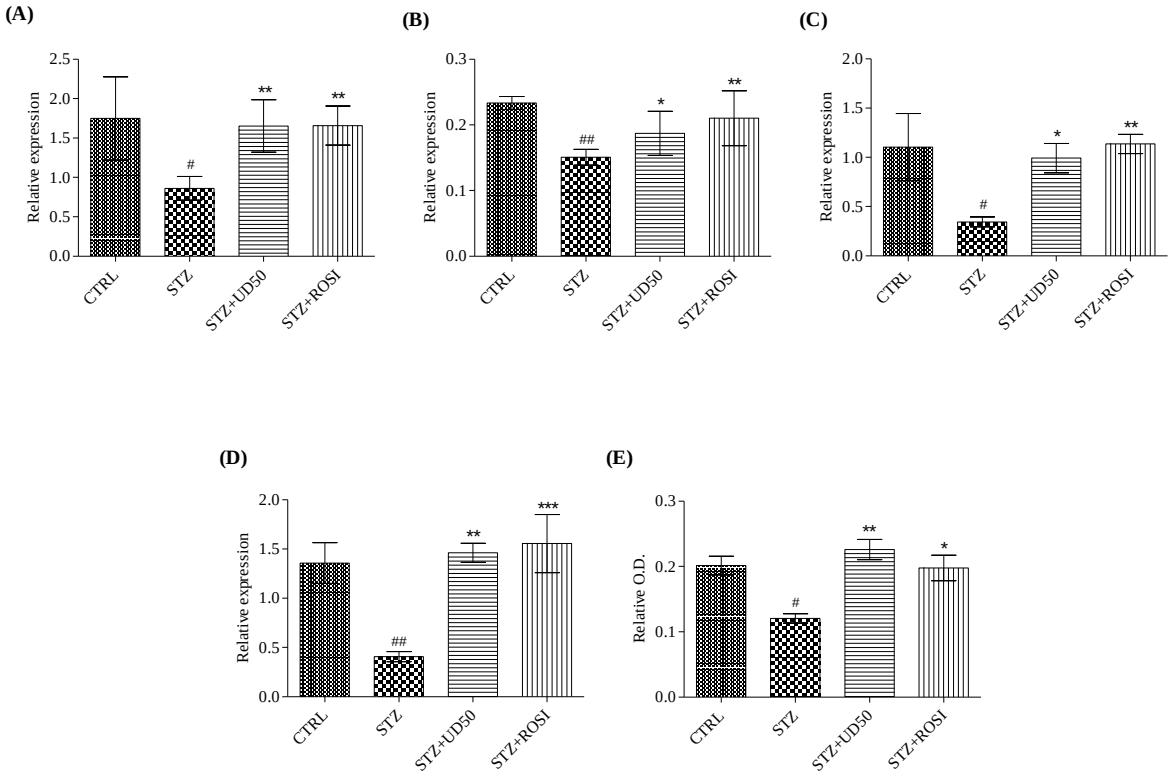



**Figure 47:** Effect of UD extract on diabetes-mediated alterations in the mRNA expression of hippocampal PI3K **(A)**, PKB **(B)**, GLUT4 **(C)**, INSG1 **(D)** and MAPK1 **(E)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and

STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

PKB mRNA expression was significantly downregulated (p<0.01 vs. CTRL) in STZ treated mice as compared to control mice. Chronic UD and ROSI administration significantly increased (p<0.05 vs. STZ and p<0.01 vs. STZ, respectively) the mRNA expression of PKB in hippocampus as compared to STZ induced diabetic mice (Figure 47B).

GLUT4 mRNA expression was significantly downregulated (p<0.05 vs. CTRL) in STZ treated animals as compared to control animals. Chronic UD and ROSI treatment significantly increased the mRNA expression of GLUT4 (p<0.05 vs. STZ and p<0.01 vs. STZ, respectively) in the hippocampus as compared to STZ treated animals (Figure 47C).

STZ induced diabetic mice showed significant downregulation (p<0.01 vs. CTRL) in hippocampal INSG1 mRNA expression as compared to control animals. Chronic UD and ROSI administration significantly increased (p<0.01 vs. STZ and p<0.001 vs. STZ, respectively) the mRNA expression of hippocampal INSG1 in diabetic animals (Figure 47D).

STZ induced diabetic mice showed significant downregulation (p<0.05 vs. CTRL) in the MAPK1 mRNA expression as compared to control mice. Chronic UD and ROSI treatment significantly increased (p<0.01 vs. STZ and p<0.05 vs. STZ, respectively) the mRNA expression of hippocampal MAPK1 in diabetic mice (Figure 47E).

## 5.17. Effect of UD extract on diabetes mediated impairment in hippocampal GLUT4 membrane translocation

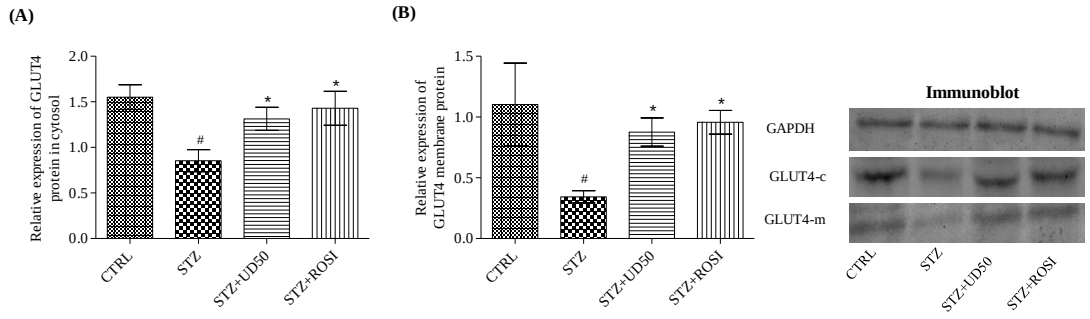



**Figure 48:** Effect of UD extract on STZ-induced alterations in the content of hippocampal GLUT4 protein in cytosol **(A)** and plasma membrane by immunblot **(B)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD and STZ +

ROSI. *p < 0.05. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

STZ induced diabetic mice showed significant downregulation (p<0.05 vs. CTRL) in the level of cytosolic GLUT4 protein as compared to control animals. Besides, chronic UD and ROSI treatment significantly increased the expression of cytosolic GLUT4 protein in diabetic animals (p<0.05 vs. STZ) (Figure 48A). The relative abundance of plasma membrane GLUT4 protein was significantly reduced (p<0.05 vs. CTRL) in STZ induced diabetic mice when compared to control mice. Chronic UD and ROSI treatment significantly increased (p<0.05 vs. STZ) the abundance of plasma membrane GLUT4 protein in diabetic mice (Figure 48B).

Hippocampal slices were prepared and treated with vehicle (artificial cerebrospinal fluid), insulin or UD plus LY294002 (PI3K inhibitor). Insulin, UD 125µg and UD 250µg treatment increased hippocampal GLUT4 plasma membrane association, an effect that was blocked by the acute treatment with PI3kinase inhibitor LY294002 (Figure 49).

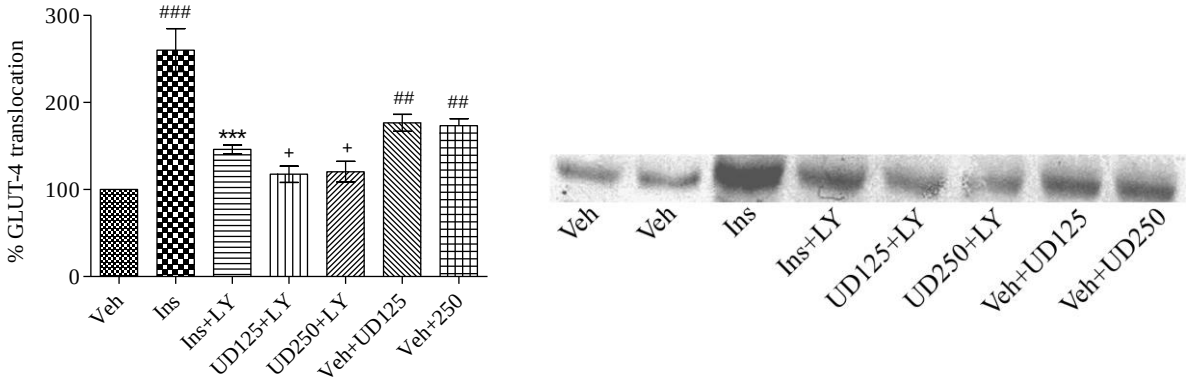



**Figure 49:** *In vitro* stimulation of hippocampal slices by UD and insulin increases the association of GLUT4 membrane protein. Data were mean ± SEM values (n=4). Significant differences: #Veh vs. Ins; *Ins vs. Ins + LY294002; +UD vs. UD + LY294002.Veh = vehicle; Ins = insulin; UD125 & UD250 = *Urtica dioica* extract 125 µg & 250 µg.

### 5.18. Effect of UD extract on hippocampal cholinergic system

STZ induced diabetic mice showed significant downregulation (p<0.05 vs. CTRL) in the mAChR1 mRNA expression in hippocampus. Chronic UD and ROSI treatment significantly

increased (p<0.01 vs. STZ) the mRNA expression of hippocampal mAChR1 in diabetic mice (Figure 50A).

Chronic diabetes did not regulate the mRNA expression of mAChR4 in hippocampus (p>0.05 vs. CTRL). Further, chronic UD and ROSI treatment did not modulate hippocampal mAChR4 mRNA expression in diabetic mice (p>0.05 vs. STZ) (Figure 50B).

Chronic diabetes did not regulate the mRNA expression of mAChR1 in striatum (p>0.05 vs. CTRL). Further, chronic UD and ROSI treatment did not modulate striatal mAChR1 mRNA expression in diabetic mice (p>0.05 vs. STZ) (Figure 50C).

Striatal mAChR4 mRNA expression was significantly upregulated (p<0.05 vs. CTRL) in STZ treated animals as compared to control animals. Chronic UD and ROSI treatment did not reverse striatal mAChR4 mRNA expression in diabetic mice (Figure 50D).

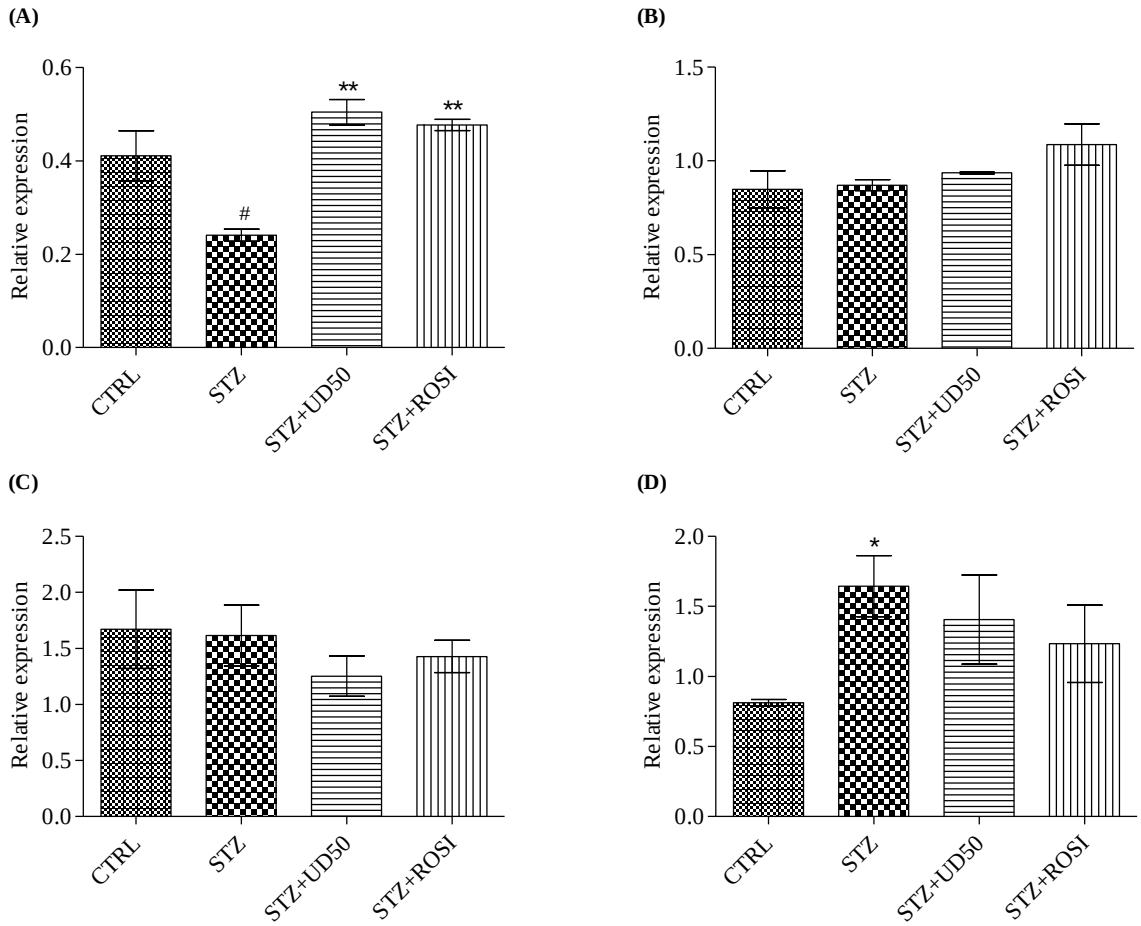



**Figure 50:** The effect of UD on diabetes mediated alterations in mAChRs expression: mAChR1 mRNA in hippocampus **(A)**, mAChR4 mRNA in hippocampus **(B)**, mAChR1

mRNA in striatum **(C)** and mAChR4 mRNA in striatum **(D)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

STZ induced diabetic mice significantly downregulated the protein (p<0.01 vs. CTRL) expression of mAChR1 in hippocampus as compared to control mice. Chronic UD and ROSI administration significantly increased the protein (p<0.05 vs. STZ) expression of hippocampal mAChR1 in diabetic mice (Figure 51A).

Striatal mAChR4 protein expression was significantly upregulated (p<0.05 vs. CTRL) in STZ treated diabetic animals as compared to control animals. Chronic UD and ROSI treatment did not modulate striatal mAChR4 protein expression in diabetic mice (Figure 51B).

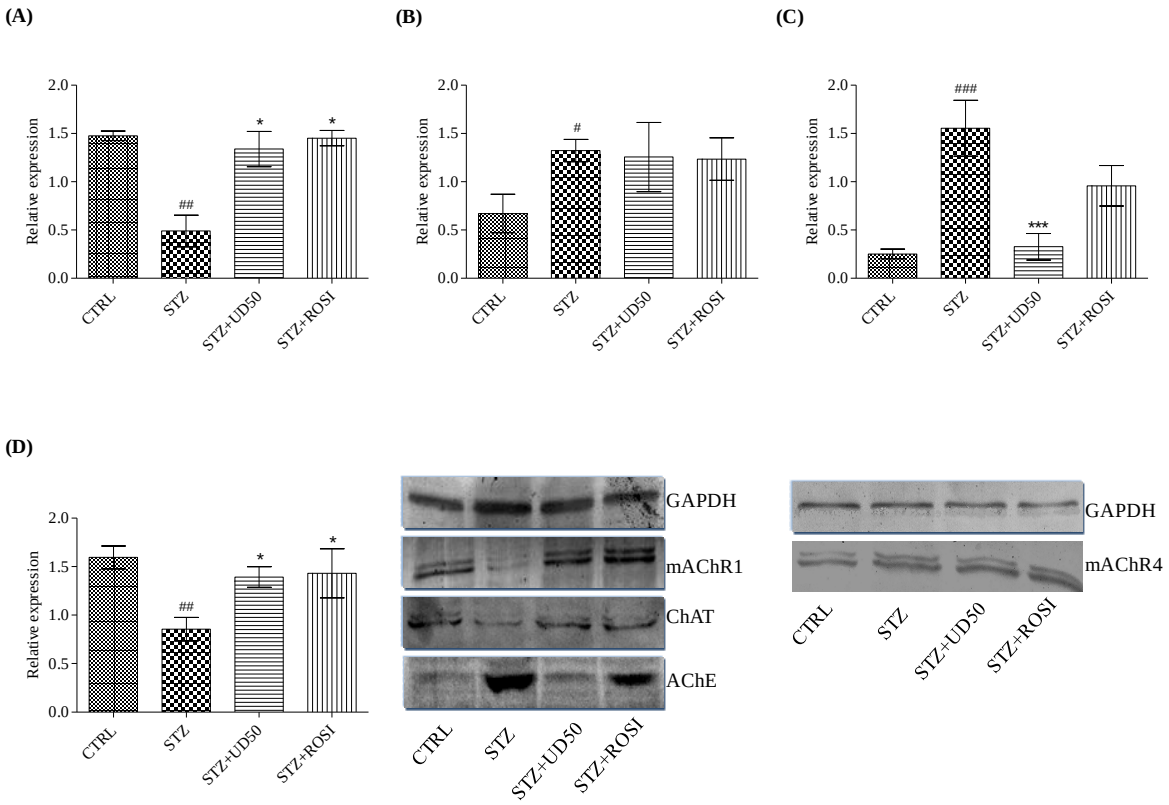



**Figure 51:** The effect of UD on diabetes mediated alterations in protein expression of mAChR1 in hippocampus **(A)**, mAChR4 in striatum **(B)**, AChE in hippocampus **(C)** and ChAT in hippocampus **(D)**. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p <

0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

STZ induced diabetic mice significantly upregulated the protein ($p<0.001$ vs. CTRL) expression of AChE in hippocampus as compared to control mice. Chronic UD administration significantly decreased ($p<0.001$ vs. STZ), while chronic ROSI administration did not modulate ($p>0.05$ vs. STZ) the protein expression of hippocampal AChE in diabetic mice (Figure 51C).

ChAT protein expression was significantly downregulated in the hippocampus of chronically diabetic mice ($p<0.01$ vs. CTRL). Chronic UD and ROSI administration significantly increased the protein expression of ChAT in diabetic mice ($p>0.05$ vs. STZ) (Figure 51D).

### 5.19. Effect of UD extract on oxidative stress, inflammation and neuronal survival

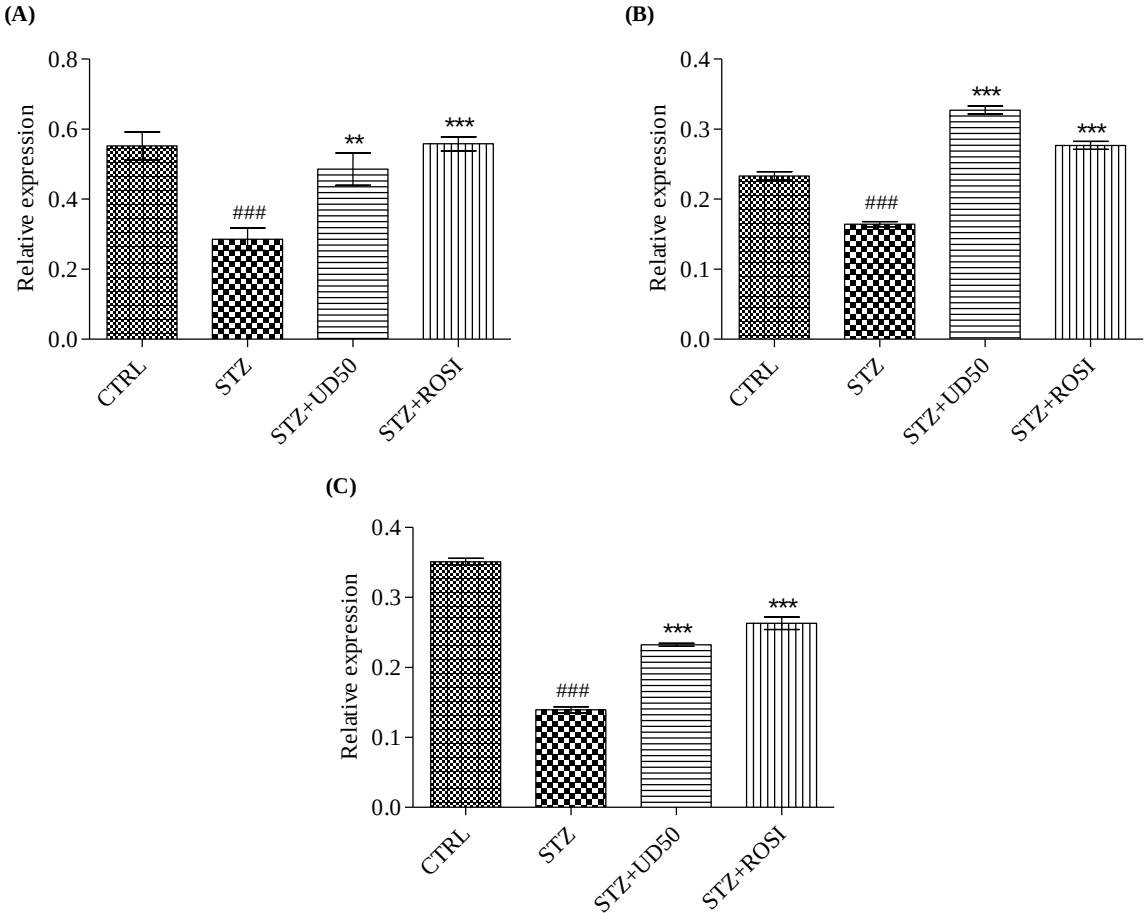



**Figure 52:** The effect of UD on diabetes mediated alterations in the mRNA expression of BDNF **(A)**, TrkB **(B)** and cyclin D1 **(C)** in hippocampus. Data were mean ± SEM values

(n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

BDNF mRNA expression was significantly downregulated in the hippocampus of chronically diabetic mice (p<0.001 vs. CTRL). Chronic UD and ROSI administration significantly increased the mRNA expression of BDNF in diabetic mice (p<0.01 vs. STZ and p<0.001 vs. STZ) (Figure 52A).

STZ induced diabetic mice significantly downregulated the mRNA (p<0.001 vs. CTRL) expression of TrkB in hippocampus as compared to control mice. Chronic UD and ROSI administration significantly increased the mRNA (p<0.001 vs. STZ) expression of hippocampal TrkB in diabetic mice (Figure 52B).

Cyclin D1 mRNA expression was significantly downregulated in the hippocampus of chronically diabetic mice (p<0.001 vs. CTRL). Chronic UD and ROSI administration significantly increased the mRNA expression of cyclin D1 in diabetic mice (p<0.001 vs. STZ) (Figure 52C).

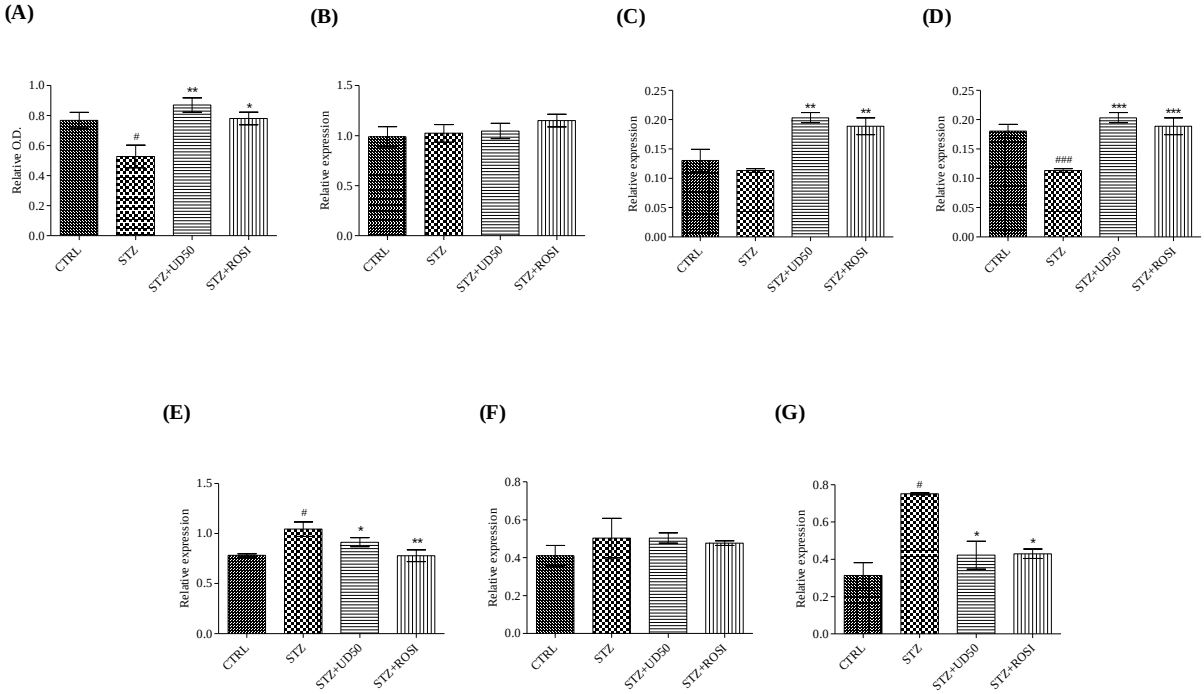



**Figure 53:** The effect of UD on diabetes mediated alterations in the mRNA expression of BCL2 **(A)**, AIP2 **(B)**, ATG5 **(C)**, ATG7 **(D)**, iNOS **(E)**, IL6 **(F)** and TNFα **(G)** in

hippocampus. Data were mean ± SEM values (n=4). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01, ***p < 0.001. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

BCL2 mRNA expression was significantly downregulated in the hippocampus of chronically diabetic mice ($p<0.05$ vs. CTRL). Chronic UD and ROSI administration significantly increased the mRNA expression of BCL2 in diabetic mice ($p<0.01$ vs. STZ and $p<0.05$ vs. STZ) (Figure 53A).

Chronic diabetes did not alter the mRNA expression of AIP2 in hippocampus ($p>0.05$ vs. CTRL). Chronic UD and ROSI treatment had no effect on the level of AIP2 in hippocampus of diabetic mice ($p>0.05$ vs. STZ) (Figure 53B).

STZ induced diabetic mice insignificantly modulated the mRNA ($p>0.05$ vs. CTRL) expression of ATG5 in hippocampus as compared to control mice. Chronic UD and ROSI administration significantly increased the mRNA ($p<0.01$ vs. STZ) expression of hippocampal ATG5 in diabetic mice (Figure 53C).

ATG7 mRNA expression was significantly downregulated in the hippocampus of chronically diabetic mice ($p<0.001$ vs. CTRL). Chronic UD and ROSI administration significantly increased the mRNA expression of ATG7 in diabetic mice ($p<0.001$ vs. STZ) (Figure 53D).

STZ induced diabetic mice significantly upregulated the mRNA ($p<0.05$ vs. CTRL) expression of iNOS in hippocampus as compared to control mice. Chronic UD and ROSI administration significantly decreased the mRNA expression of hippocampal iNOS in diabetic mice ($p<0.05$ vs. STZ and $p<0.01$ vs. STZ) (Figure 53E).

Chronic diabetes did not alter the mRNA expression of IL6 in hippocampus ($p>0.05$ vs. CTRL). Chronic UD and ROSI treatment had no effect on the level of IL6 in hippocampus of diabetic mice ($p>0.05$ vs. STZ) (Figure 53F).

STZ induced diabetic mice significantly upregulated the mRNA ($p<0.05$ vs. CTRL) expression of TNFα in hippocampus as compared to control mice. Chronic UD and ROSI administration significantly decreased the mRNA expression of hippocampal TNFα in diabetic mice ($p<0.05$ vs. STZ) (Figure 53G).

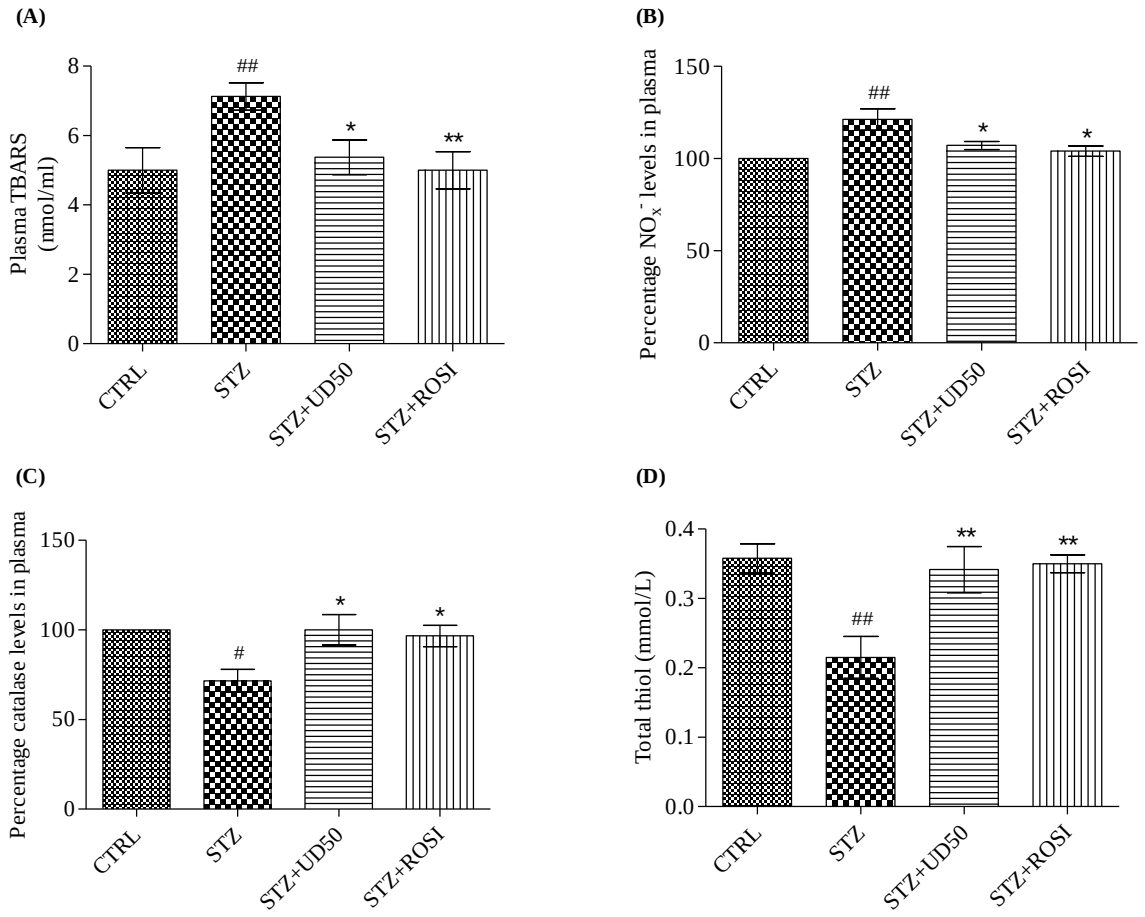



**Figure 54:** Effect of UD on diabetes mediated alterations in TBARS level **(A)**, nitric oxide level **(B)**, catalase level **(C)** and total thiol level **(D)** in plasma. Data were mean ± SEM values (n=6). Significant differences: #CTRL vs. STZ; *STZ vs. STZ + UD50 and STZ + ROSI. *p < 0.05, **p < 0.01. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

The level of plasma TBARS was significantly increased (p<0.01 vs. CTRL) in diabetic animals as compared to control animals. Chronic treatment with UD and ROSI significantly decreased (p<0.05 vs. STZ and p<0.01 vs. STZ, respectively) the elevated level of TBARS in diabetic animals (Figure 54A).

STZ-induced long standing diabetes significantly elevated (p<0.01 vs. CTRL) the level of plasma NO as compared to control animals. Chronic UD and ROSI administration significantly attenuated (p<0.05 vs. STZ) the elevated level of plasma NO in diabetic mice (Figure 54B).

STZ-induced diabetes significantly decreased the level of catalase ($p<0.05$ vs. CTRL) in plasma as compared to control animals. UD and ROSI treatment significantly increased ($p<0.05$ vs. STZ) the level of plasma catalase in diabetic mice (Figure 54C).

STZ-induced diabetic mice showed significant decrease in the level of total thiol ($p<0.01$ vs. CTRL) in plasma as compared control animals. Chronic UD and ROSI treatment significantly improved ($p<0.01$ vs. STZ) the level of plasma total thiol in diabetic mice (Figure 54D).

## 5.20. Immunofluorescence study of TNFα on hippocampal section of chronically stressed mice

Control mice showed normal nucleus on DAPI staining which were altered in CUMS group. Chronic stress increased the fluorescence of TNF-α whereas FLX, ROSI, HYP and UD treatment reversed it (Figure 55).

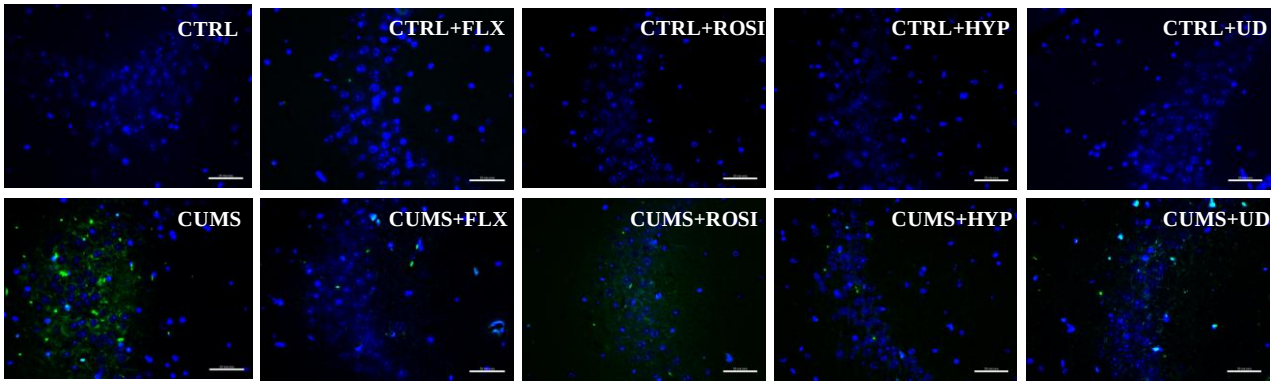



**Figure 55:** Effect of UD on CUMS mediated alterations in TNF-α expression using immunofluorescence study. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

## 5.21. Histopathology study on hippocampal section of chronically stressed mice

Control mice showed normal cellular morphology which were altered in CUMS group. Chronic stress increased the neuronal damage as indicated by arrow whereas FLX, ROSI, HYP and UD treatment reversed it (Figure 56).

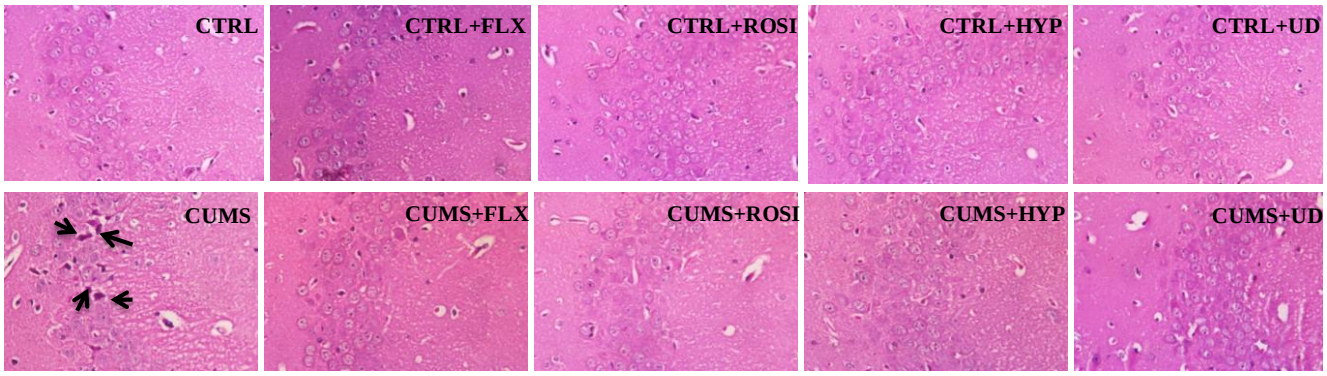



**Figure 56:** Effect of UD on CUMS mediated neuronal damage using histopathology study. CTRL = control; CUMS = chronic unpredictable mild stress; FLX = fluoxetine; ROSI = rosiglitazone; HYP = *Hypericum perforatum* extract; UD = *Urtica dioica* extract.

## 5.22. Immunofluorescence study of TNFα on hippocampal section of STZ induced diabetic mice

Control mice showed normal nucleus on DAPI staining which were altered in STZ group. Chronic diabetes increased the expression of TNF-α whereas UD and ROSI treatment reversed it (Figure 57).

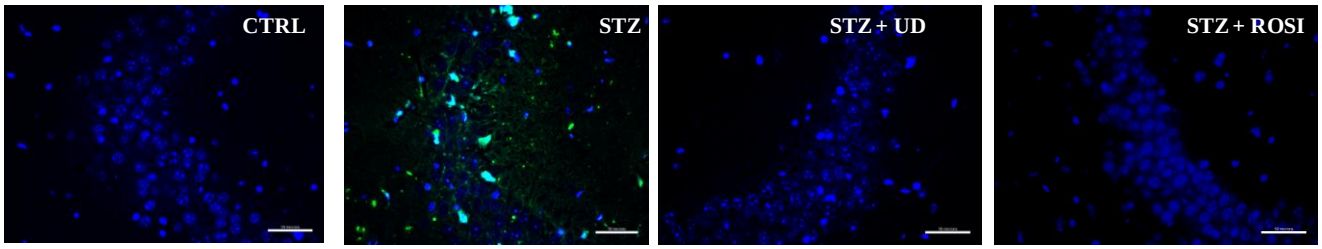



**Figure 57:** Effect of UD on diabetes induced alterations in TNF-α expression using immunofluorescence study. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

## 5.23. Histopathology study on hippocampal section of STZ induced diabetic mice

Control mice showed normal cellular morphology which were altered in diabetic group. Chronic diabetes increased the neuronal damage as indicated by arrow whereas ROSI and UD treatment reversed it (Figure 58).

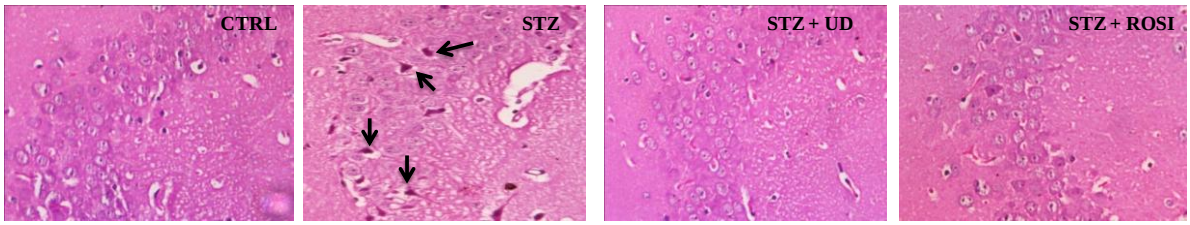



**Figure 58:** Effect of UD on diabetes mediated neuronal damage using histopathology study. CTRL = control; STZ = streptozotocin; UD50 = *Urtica dioica* extract 50 mg/kg; ROSI = rosiglitazone.

# CHAPTER 6

## DISCUSSION

## 6. Discussion

Microscopy of UD leaf showed straight and sharp trichome, upper epidermis, lower epidermis, spongy parenchyma and vascular bundle. Microscopy of St. John's wort leaf showed very thin mid rib, oil gland, upper epidermis, lower epidermis as well as vascular bundle. Dried UD leaves showed total ash (21.2% w/w), acid insoluble ash (3.04% w/w), water insoluble ash (3.3% w/w), water soluble extractives (23.3% w/w) and alcohol soluble extractives (25.3% w/w). UD leaves extract contains 7.28% of moisture. Dried St. John's wort leaves showed total ash (6.2% w/w), acid insoluble ash (2.4% w/w), water insoluble ash (3.2% w/w), water soluble extractives (14.2% w/w) and alcohol soluble extractives (14.02% w/w). St. John's wort leaves extract contains 3.72% of moisture. Phytochemical evaluation revealed the presence of alkaloids, carbohydrates, glycosides, phenolics, flavonoids, proteins and amino acids in UD and St. John's wort extracts. In our preliminary experiments, UD leaves extract showed the presence of 5HT and scopoletin. LC-MS analysis on hydroalcoholic extract of St. John's wort revealed the presence of hyperforin and hypericin. High performance liquid chromatography also revealed the presence of scopoletin (6.51µg/mg) in crude UD leaves extract. In HPLC chromatogram, 75% hydroalcoholic extract of St. John's wort contains 6.14% of hyperforin and 0.27% of hypericin. Hyperforin and hypericin content in 25% hydroalcoholic extract of St. John's wort was calculated as 6.14% and 0.25% respectively. 50% hydroalcoholic extract of St. John's wort was standardized to 6.12% hyperforin and 0.37% hypericin in HPLC analysis and used at the dose of 350 mg/kg in all experiments.

Chronic stress often precipitates or present co-morbidly with depression and affects the normal physiological state of the body, interfering with emotional, cognitive and physical aspects of the health. Anhedonia, a disability to experience pleasures, is a major symptom of depression [393]. It has been reported that hedonic deficits can also be induced by chronic unpredictable stress in rodents [394-395]. Thus, Katz and his co-workers subjected animals to 21 day stress paradigm, including electric shocks, swimming in cold water, immobilization and other strong stimuli, resulted in decrease of sucrose consumption and finally interpreted as a sign of hedonic deficit [382]. Using behavioural animal models, after termination of stressors, it was observed that anhedonia is associated with increased floating during forced swimming and decreased novel object exploration [394]. In the present study, sucrose

consumption was significantly declined from week 2 onwards in stressed mice. Exposure of mice to CUMS resulted in increased duration of immobility in FST and TST, indicating depressive like behaviour. While, depressive like behaviour in stressed mice was attenuated by administration with FLX, ROSI, HYP and UD. Current study confirms and extends previous findings, in which UD extract has antidepressant like property [391, 396] but UD *per se* did not show any alteration in the duration of immobility and sucrose consumption. Chronic FLX, ROSI and HYP treatment did not mimic or induce depressive like behaviour in control animals. In LC-MS analysis, we observed the presence of scopoletin, gentisic acid, esculetin, quercetin and rutin in hydroalcoholic extract of UD leaves. In earlier study, scopoletin administration reduced the depressive like behaviour in forced swim test via modulation of monoaminergic, adrenergic and dopaminergic system [397]. The flavonoids quercetin and rutin showed antidepressive and neuroprotective action in rats similar to that of clinically used drug moclobemide [398]. HYP is known to possess potent antidepressant property [72, 399]. HYP contain dozens of biologically active substances, among which hyperforin (a lipophilic phloroglucinol) and hypericin (a naphthodianthrone) have been extensively studied. Hyperforin is a potent reuptake inhibitor of neurotransmitters such as serotonin, noradrenaline, dopamine, γ-aminobutyric acid and L-glutamate from the synaptic cleft. Hypericin was found to strongly inhibit monoamine oxidase A and B [67]. FLX reduced hedonic deficit and increased mobility in FST resulted in antidepressant action [400]. ROSI is known to induce neurogenesis and attenuate depressive like behaviour in stressed animals [94].

The influence of stress on cognitive functions has been well documented. Clinically, stress or depression has been reported to induce neurocognitive dysfunctions [401]. Chronic exposure to unpredictable stressors has been reported to induce disruption of spatial and associative memory [402-403]. In line with the literature, we observed that CUMS increased the escape latency in MWM task. This increase in escape latency is a reliable index of spatial memory dysfunction in stressed mice [402]. In addition, we observed that CUMS-induced depression led to associative memory dysfunction in mice evident from decreased step through latency in PA task on week three. Chronic administration of UD, FLX, HYP and ROSI to chronically stressed mice significantly reduced the escape latency in MWM task and ameliorated number of crossings in probe trials. Further, UD, FLX, HYP and ROSI treatment significantly reversed associative memory dysfunction associated with depressive like behaviour on week

three. Interestingly, chronic UD treatment significantly ameliorated associative memory in control animals as well from week two onwards. But, FLX, ROSI and HYP per se did not modulate spatial and associative memory in control animals. UD is known to reduce learning and memory deficits in hypercorticosteronemic mice [391]. Quercetin protects against intracerebroventricular colchicines induced cognitive dysfunctions in rats [404]. Rutin ameliorate head trauma-induced impaired spatial navigation in Morris water maze test [405]. The coumarin, scopoletin increases the gene expression of PPARγ [406]. Recent studies suggested that PPARγ agonist ROSI improves learning and memory in both human and animal models [199, 407]. Esculetin did not reduce amnesia in passive avoidance task [408]. St John's Wort was found to have a novel type of antidepressant with memory enhancing properties [72, 409] and hyperforin is involved in its cognitive effects [410]. Further, reduced Alzheimer's disease pathology by St John's Wort is independent of hyperforin content is also on record [74]. FLX attenuated impaired cognition induced by chronic mild stress in MWM task [411].

The influence of chronic stress on motor function has been established. Chronic unpredictable stress is known to induce motor function deficit [394, 412]. In the present study, CUMS modulated the motor function in mice. Chronically depressed mice showed defective muscle coordination in rota rod test from week two onwards. Locomotor activity in depressed mice was reduced on week three. Chronic UD, FLX, HYP and ROSI administration to depressed or normal mice showed no alteration in locomotor activity.

The influence of chronic stress or depression on incidence of diabetes has been well established. Clinically, depression has been reported to induce type 2 diabetes mellitus [119]. In animal models, CUMS exhibited insulin resistance, hypoinsulinemia and hyperglycemia [11, 120]. In the present study, 21 day of CUMS significantly induced insulin resistance in mice. However the level of fasting blood glucose and serum insulin was not significantly altered. Chronic UD and ROSI administration significantly reduced insulin resistance in stressed mice, while FLX and HYP did not modulate insulin resistance. Besides, chronic FLX, ROSI, HYP and UD administration did not alter the level of serum insulin and blood glucose in control mice. Current follow-up study confirms and extends previous findings, in which UD extract has shown antidiabetic property [106, 378].

Evidences have indicated a frequent co-existence of depressive like behaviour and diabetes mellitus. Both diseases are associated with similar alterations in the structure and function of the central nervous system cells and with similar disturbances in cognitive processes [413]. The major neuro-endocrine responses to chronic stress involve the release of glucocorticoids from the adrenal cortex. It has been reported that, chronic supraphysiological level glucocorticoids induces depressogenic-like effect and hyperglycemia in animal models [128, 391]. In addition, excess of glucocorticoids impaired the insulin action and glucose homeostasis in hippocampal neurons, resulting in disturbed synaptic plasticity [413]. In the present study we observed that, CUMS significantly increased the level of plasma corticosterone and induced memory deficit in mice. Evidences suggest that hypercorticosteronemia deleteriously affects the survival and function of hippocampal neurons, and associated with diminished memory and mood [414]. In the present study, chronic UD, FLX, ROSI and HYP treatment significantly reversed stress-induced hypercorticosteronemia in mice. It has been demonstrated that UD extract attenuated hypercorticosteronemia and hyperglycemia in dexamethasone induced behavioural deficits [391]. ROSI is known to improve uptake of glucose in 3T3-L1 adipocyte with insulin resistance induced by hypercorticosteronemia [415]. Chronic UD, ROSI, FLX and HYP administration did not modulate the level of plasma corticosterone in normal mice.

The molecular mechanism underlying the memory improvement of UD extract remains unclear. However, it is well accepted that exposure to chronic stress induces alteration in crucial neuronal pathways. Earlier studies have shown the presence of not only the insulin but also IR and GLUT4 in the certain regions of brain such as cerebral cortex, olfactory bulb, cerebellum, hippocampus and hypothalamus. Indeed, alterations in insulin signaling mechanism of depressive and diabetic mice have been seen in the hippocampus region that is involved in cognitive process [121, 391, 413].

Accumulated evidences suggest that, insulin signaling pathway is important not only for metabolic process or neurotransmitter homeostasis in the hippocampus but also for proper synaptic plasticity, energy metabolism, neuroprotection and cognitive function [416-417]. We observed that, CUMS-induced depressed mice have insulin resistance which might be due to hypercorticosteronemia or downregulation of IR. Both hypercorticosteronemia or downregulation of IR led to dysregulation of insulin signaling components like IRS1, IRS2,

PI3K, PKB, MAPK1, INSG1 and GLUT4, resulting in defective neuroprotection, neuronal development and memory formation [121, 418]. A recent study demonstrated that, both chronic hypercorticosteronemia and diabetes are associated with downregulation of PPARγ expression in hippocampus and hypothalamus, which also contribute to the cognitive impairment [419]. Consistent with these findings, herein we observed that, CUMS-induced depression with comorbid insulin resistance significantly downregulated the mRNA expressions of PPARγ, IR, IRS1, IRS2, PI3K, GLUT4, PKB, MAPK1 and INSG1 in the hippocampus region of mice brain under non fasting condition. However, we did not observe any significant downregulation in the levels of ILGF 1r and GLP1 mRNA in hippocampus. A recently published study demonstrated that dysregulation of insulin signaling components in hippocampus could well play a role in development of neurological disorders, as it leaves neurons more exposed to toxic influences [121]. In the present study, chronic UD treatment significantly upregulated the mRNA expression hippocampal PPARγ and restored stress-induced alterations in mRNA involved in insulin signaling pathway, and the effect of UD was comparable to chronic ROSI administration. However, UD had no effect on the level of hippocampal IRS2 mRNA after CUMS procedure. Chronic FLX and HYP treatment did not modulate hippocampal insulin signaling cascade in depressed and non-depressed mice, while ameliorated MAPK1 mRNA expression in stressed mice. Chronic UD and ROSI administration did not modulate insulin signaling cascade in control animals. However, UD and ROSI were associated with upregulation of MAPK1 mRNA expression in control animals. The coumarin, scopoletin in UD [277] increases the gene expression of PPARγ and reduces insulin resistance [406]. Recent studies suggested that PPARγ agonist ROSI improves learning and memory in both human and animal models [199, 407].

In the central nervous system, the level of mRNA and cytosolic GLUT4 protein was reduced during depression with diabetes [391, 420]. Herein we observed that, CUMS-induced depressed mice with insulin resistance showed reduced translocation of GLUT4 protein to the hippocampal plasma membrane. Chronic UD administration did not induce GLUT4 membrane translocation, while ROSI administration ameliorated GLUT4 content in depressed mice. Both chronic UD and ROSI treatment did not alter GLUT4 translocation in control mice. Amelioration of GLUT4 membrane translocation in hippocampal neurons induces neurocognitive improvement [18].

Chronic stress is known to affect hippocampal neurogenesis precipitating cognitive impairment [33]. Experimental evidences suggest that depression exhibit decreased adult hippocampal neurogenesis and treatment with antidepressant drugs restores hippocampal neurogenesis [421]. Sonic hedgehog, a regulator of adult hippocampal neurogenesis is essential for the maintenance of the adult neurogenic niches [422]. The expression of Shh co-receptors Ptch and Smo are seen in dentate gyrus subfield including the subgranular zone of hippocampus. Paracrine or autocrine activation of canonical Shh signaling is initiated by binding of Shh to their receptor Ptch, mediating the expression of Gli transcription factor via activation of Smo [422-423]. These facts suggest that Shh signaling might be a major determinant of depression mediated impairment in neurogenesis and cognition. In the present study CUMS, FLX, ROSI, HYP and UD treatment did not modulate the mRNA expression of Shh in hippocampus. Also, the level of Shh co-receptor Ptch1 was not modulated by chronic FLX and ROSI administration. The level of Ptch1 mRNA expression was ameliorated by chronic HYP and UD administration in control animals. Chronic UD and ROSI administration did not modulate mRNA expression of Ptch1 in depressed animals. It was observed that CUMS reduced the mRNA expression of Smo and Gli1 in the hippocampus. Gli1 is a target gene of Shh signaling pathway. Chronic UD and HYP administration led to significant improvement in Smo and Gli1 mRNA expression in both depressed and non-depressed mice. Chronic FLX and ROSI administration did not modulate Smo and Gli1 mRNA expression in control and depressed mice. Only HYP administered depressed mice showed downregulation of hippocampal Hhip mRNA, a negative regulator of Shh signaling. A recent in vitro study suggests that depletion of both NE & 5HT, a clinical condition of depression resulted in the modulation of Shh signaling but not in the case of selective depletion [38]. Hyperforin is known to inhibit the reuptake of both 5HT and NA in synaptic cleft [67]. We report, CUMS, HYP or UD treatment did not modulate canonical Shh signaling. CUMS dysregulate non-canonical Shh signaling in hippocampus as observed through downregulation of Smo and Gli mRNA. It has been reported that hedgehog signaling is activated by non-canonical Src kinase pathway [424]. Further, activation of Src-MAPK pathway induces neuronal survival, neuronal repair and regeneration in hippocampus [121]. We observed that chronic UD and HYP administration significantly increased the mRNA expression of MAPK1 in control and stressed mice. We observed that, pretreatment of hippocampus slices with cyclopamine significantly downregulated the mRNA expression of

Gli1 and Ptch1. Neither UD nor HYP could reverse cyclopamine induced downregulation of Gli1 and Ptch1 mRNA in hippocampal slices when compared with normal and purmorphamine groups, suggests that UD and HYP treatment modulates Smo-Gli pathway (Figure 59). HYP treatment showed better efficacy for hedgehog signaling then chronic UD treatment in stressed mice.

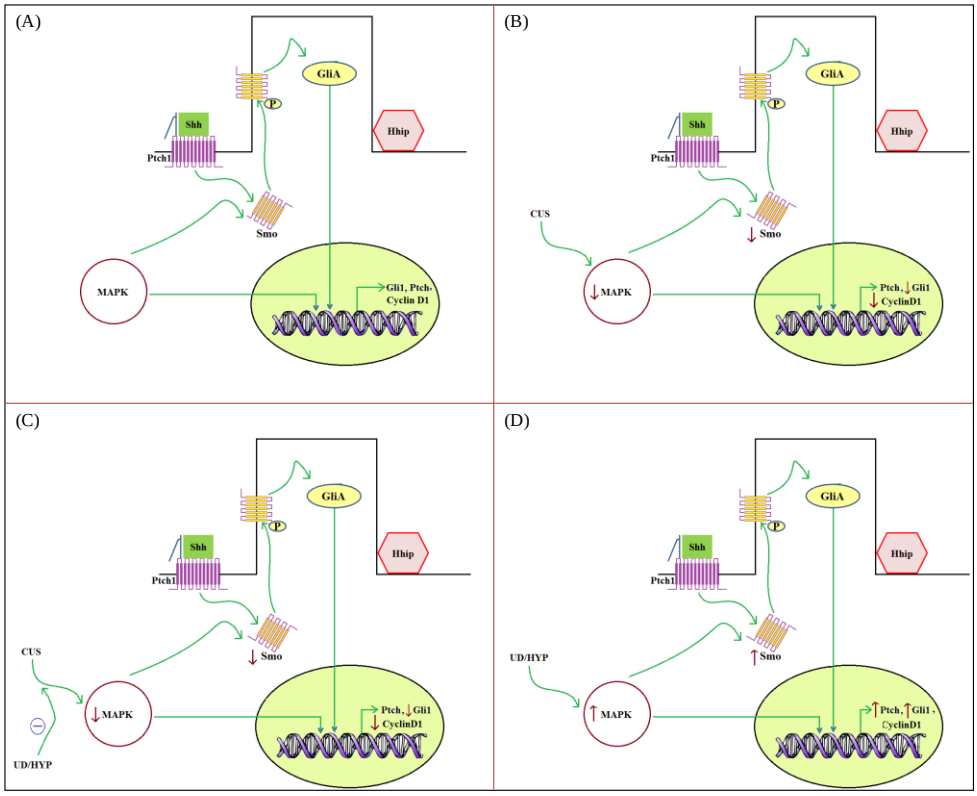



**Figure 59:** An overview on mechanism of action of UD extract: Shh signaling in normal control mice (A), stressed mice (B), stressed mice treated with UD or HYP extract (C) and normal control mice treated with UD or HYP extract (D).

BDNF is reported to strongly influence synaptic morphogenesis, neuronal survival, long-term potentiation of brain synapses as well as adult hippocampal neurogenesis [425-426]. In the present study, CUMS significantly decreased the mRNA expressions of BDNF, its receptor TrkB in hippocampus. Chronic UD, ROSI, FLX and HYP treatment normalized the mRNA expression of TrkB in stressed mice, while UD and ROSI treatment did not modulate the mRNA expression of BDNF in hippocampus. Upon binding to BDNF, the receptor TrkB activates MAPK/PI3K/cAMP-response element binding (CREB) protein pathway, resulting in cell survival, growth and synaptic plasticity [427-428]. It was reported that activation of

CREB protein and MAPK which further mediate the expression of cyclin D1[429-430]. Cyclin D1 is co-localized with newly generated neurons in murine hippocampus led to neurogenesis [431]. Herein we observed that, cyclin D1 mRNA expression was downregulated, while chronic HYP and UD treatment significantly upregulated cyclin D1 mRNA expression in stressed mice. Further, it could be hypothesized that UD and HYP might have mediated alteration in cyclin D1 expression partly by TrkB/MAPK/PI3K/cAMP-CREB protein pathway and non-canonical hedgehog pathway.

ACh is required for proper functioning of cholinergic neurons in hippocampus which regulates the process of learning and memory [208, 432]. ChAT and AChE are responsible for the biosynthesis and metabolism of ACh. Evidence suggests that ChAT, a specific marker for functional state of cholinergic neurons in the central nervous systems [61], activity is reduced during cognitive impairment [62]. A decrease in the ChAT expression was noted in the dorsal hippocampus of stressed mice [433]. Earlier reports suggest that CUMS increases AChE expression as well as activity in the hippocampus resulting into cognitive impairments [30, 434-435]. In the present study, CUMS resulted in upregulation of AChE whereas downregulation of ChAT protein expression in hippocampus. Chronic FLX treatment did not modulate ChAT expression in the hippocampus, indicating that FLX had no effect on ACh biosynthesis in control and depressed mice. While, UD administration significantly elevated ChAT expression in stressed mice. UD extract did not modulate ChAT expression in control animals. Besides, chronic UD and FLX administration downregulated the level of hippocampal AChE in depressed mice. Chronic UD and FLX administration had no effect on AChE expression in control animals. It could be speculated that, the modulation of CUMS mediated cognitive impairments by UD and FLX could be the result of potentiation of ACh action at the neuronal junction of hippocampus. Our results are consistent with previous report which suggests that FLX reduce the degradation rate of ACh by decreasing the activity of AChE [436]. Further, both scopoletin and rutin have been known to reduce AChE activity in the hippocampus [405, 437]. Cholinergic system is known to be closely related to memory process, in particular, muscarinic type of acetylcholine receptors play key roles in regulating the activity of vital functions of the central nervous system including hippocampus [438]. They are present in high concentrations in the prefrontal cortex and the hippocampus [439] and have an integral role in spatial learning & memory [386]. ACh in synaptic cleft bind to mAChRs of either pre or post synaptic neurons. In the central nervous system, mAChR1 is

predominantly expressed in post-synaptic neurons whereas mAChR4 functions as pre-synaptic autoreceptor [130]. We observed that CUMS differentially altered the mRNA and protein levels of mAChR subtypes in hippocampus, in which mAChR1 subtype was downregulated whereas mAChR4 expressions was not modulated in stressed mice. Earlier reports suggest that mAChR1 metabotropic muscarinic receptor represent a possible target for amelioration of affective disorder-associated memory dysfunctions [25]. mAChR1 is known to be involved in cognitive function and neurogenesis [440]. Endogenous ACh rescues N-methyl-D-aspartate induced long lasting hippocampal cell damage via activation of mAChR1. Post-treatments with tacrine reduced the N-methyl-D-aspartate induced long lasting hippocampal damage. The effect of tracine was blocked by mAChR1 receptor antagonist pirenzepine, but not by a muscarinic mAChR3 receptor antagonist, darifenacin or nicotinic receptor antagonist, mecamylamine, suggested that mAChR1 rescue the hippocampal exitotocicity induced by N-methyl-D-aspartate [441]. mAChR1 mediate action through stimulation of phospholipase C, which ultimately mobilize $Ca^{2+}$ from intracellular stores and activate protein kinase C (PKC). PKC is essential for the induction and maintenance of long-term potentiation, a putative cellular mechanism of memory formation [130]. Beside, even type of muscarinic receptor (mAChR4, autoreceptor) mediates inhibition of adenylate cyclase resulting in decreased cyclic AMP (cAMP) levels. cAMP is known to regulate memory formation through activation of cAMP-sensitive protein kinase A [130]. Autoreceptors, act by reducing the level of acetylcholine at synaptic cleft which further leads to reduce neurogenesis [442]. In the present study, chronic UD and FLX treatment significantly increased the expression of hippocampal mAChR1 in stressed mice, but UD and FLX *per se* did not modulate mAChR1 level. Chronic HYP and ROSI did not modulate mAChR1 mRNA expression in hippocampus. Chronic UD, FLX, ROSI and HYP treatment had no effect on hippocampal mAChR4 expression in control and stressed mice. Besides, mAChR4 mRNA and protein expression was significantly upregulated, while mAChR1 mRNA was unaffected in the striatum of chronically stressed mice. Chronic FLX, ROSI, HYP and UD treatment did not modulate mRNA expression of striatal mAChR1 and mAChR4 in depressed or non-depressed mice. UD and FLX did not reduce upregulation of striatal mAChR4 protein in depressed mice. Chronic FLX administration did not modulate striatal mAChR4, while UD significantly upregulated mAChR4 protein in control mice.

Striatum is densely innervated by cholinergic interneurons, co-expressed with dopaminergic D1 receptor that is crucial for motor behaviour [443]. The dopaminergic D1 receptor activates, whereas mAChR4 inhibits, adenylyl cyclase and thereby modulates cAMP production through the trimeric $G_{\alpha s}$ and $G_{\alpha i/o}$ proteins, respectively [444]. Activation of mAChR4 in the striatum inhibits D1 receptor-induced locomotor stimulation in mice [29]. Our observations are consistent with previous study suggesting that major depression results in motor control system deficit [445]. Hence, it could be speculated that, decreased locomotor activity in depressed mice might be the result of enhanced striatal mAChR4 expression.

It has been well accepted that, chronic exposure to unpredictable stressors induces oxidative stress, inflammation and apoptosis, which further exacerbates the dysfunctioning of cholinergic neurons in hippocampus, resulting in cognitive impairments [30, 446]. In this regard, the effect of UD on depression mediated impairment in ATG and neuronal survival was evaluated and compared with FLX, ROSI and HYP. Evidence suggests that, neuronal damage or apoptosis is associated with memory performance in rodents with neuropsychiatric disorder [447]. Social stress increases the number of apoptotic cells in the hippocampus and temporal cortex [448], whereas antidepressants upregulate BCl2 expression and improve neural plasticity [447]. The ability of AIP2 to inhibit caspase-2 indicates that it occupies a unique position in programmed cell death initiated by endoplasmic reticulum stress [449]. In the present study CUMS significantly downregulated the level of hippocampal BCl2 and AIP2 mRNA. Besides, chronic UD, FLX, ROSI and HYP treatment significantly upregulated hippocampal BCL2 in stressed mice, but UD, FLX, ROSI and HYP *per se* did not regulate the expression of BCL2 mRNA. In addition, chronic FLX and HYP administration ameliorated AIP2 mRNA in stressed mice, while UD and ROSI did not modulate it.

In contrast to apoptosis, ATG has a protective role against the pathogenesis of a number of neurodegenerative diseases. In the present study, CUMS downregulated the level of ATG5 and ATG7 mRNA in hippocampus. ATG5 & ATG7 are involved in autophagosome formation, where ATG5 is covalently conjugated to ATG12 in an ubiquitin-like conjugation reaction and play a role in promoting efficient ATG8 lipidation. ATG7 is an essential component of the core autophagy machinery that functions as an activating enzyme for both ATG12 and ATG8 during autophagosome formation. It is a highly conserved pathway for degradation, by which intracellular macromolecules are delivered to lysosomes, where they

are degraded into biologically active monomers such as amino acids that are subsequently recycled to maintain cellular homeostasis. Report suggests that dysfunctions in autophagy develop neuronal damage accompanied by ubiquitinated protein aggregates, indicating that basal levels of autophagy are essential for neuronal health [173]. Herein we report, dysregulation of ATG in hippocampus during depression might induce cognitive dysfunction. In the present study chronic UD, FLX, HYP but not ROSI ameliorated the level of ATG5 & ATG7 in chronically stressed mice. In addition chronic UD, FLX, ROSI and HYP administration did not modulate ATG expression in control mice.

CUMS plays an important role in induction of various clinical disorders by modulating monoaminergic-serotoninergic system and antioxidant defense mechanism. Dysregulation of monoaminergic-serotoninergic system in central nervous system interact with the oxidative load during chronic unpredictable stress [450]. In the present study, CUMS resulted in elevation in the level of oxidative and nitrative stress markers in plasma, as indicated by raise in the levels of TBARS and NO, and depletion of antioxidants such as catalase and total thiol. Chronic treatment with UD, ROSI, FLX and HYP reversed CUMS-induced alteration in the level of catalase along with attenuation of oxidative and nitrative stress. However, chronic UD treatment did not significantly reverse the level of plasma TBARS in stresses mice.

Oxidative insult is known to induce cytokine proteins including ILs and TNFs [451]. Increased expression of iNOS in hippocampus make significant contributions to CUMS induced depressive like behaviour [452]. Cytokines are known to mediate inflammatory processes observed during neurodegenerative diseases such as vascular dementia and Alzheimer's disease [451]. Herein, CUMS induced depressed mice showed significantly elevated level of inflammatory mediator's viz. iNOS, TNFα and IL6 in the hippocampus. Besides, chronic UD, FLX, ROSI and HYP administration reversed upregulation of inflammatory mediator's production in depressed mice. In earlier study, ROSI decreased chronic repeated immobilization stress induced accumulation of oxidative and inflammatory mediators in rat brain [453]. ROSI mediated anti-inflammatory action in hippocampus via attenuation of NO and IL1β production in rats [454]. FLX treatment reduces oxidative stress in the hippocampus of rat neonates [455]. FLX treatment decreased the immunoreactivity of COX1 and iNOS in the hippocampus of Bacillus Calmette-Guerin induced depression in mice [456]. Study demonstrated the anti-oxidant and anti-inflammatory effects of HYP

extract [457]. Chronic UD, FLX, ROSI and HYP treatment did not modulate oxidative stress and inflammation in control animals.

Orally administered HYP extract accumulated as hyperforin and hypericin in the brain tissue of depressed mice after last dose followed by 8hr fasting. Further, hyperforin and hypericin are well known antidepressant [67]. Similarly, orally administered UD extract accumulated as scopoletin in the brain tissue of depressed mice after last dose followed by 8hr fasting. Scopoletin is known to attenuate depressive like behaviour in mice via modulation of monoaminergic system [397].

We also evaluated the effect of UD and ROSI extract against diabetes mediated neurological disorders in mice. We used multiple lower doses of STZ to induce diabetes. STZ-induced diabetes is a well described model of diabetes mellitus in experimental animals. β-cell toxicity and diabetogenic properties of STZ is mediated through various mechanisms including uptake of STZ in β-cells by glucose transporter type 2 and increased oxidative stress due to generation of free radicals [458]. Experimental evidences suggest that, high doses of STZ induce rapid and complete insulin deficiency resembling type 1 diabetes. However, multiple lower doses of STZ, which cause partial destruction of β-cells, can be used to produce type 2 diabetes [269]. Diabetes mellitus is one of the most common metabolic disorder, clinically characterized by insulin deficiency, hyperglycemia and associated with a number of complications such as diabetic neuropathy, nephropathy, retinopathy, cardiomyopathy, etc [459]. It is also well defined that long standing diabetes leads to depression [42], dementia [460], and sexual dysfunction [461]. Polydypsia and body weight loss are clinical symptoms associated with diabetes in both experimental animals and patients [462]. Accordingly, in the present study, multiple lower doses of STZ treatment significantly elevated the fasting and postprandial blood glucose in experimental animals. We observed that STZ induced diabetes resulted in increased water intake and body weight loss determined from 1st week to 8th week as compared to normal animals. Initially, there was no significant loss in body weight for the first two weeks but started declining from the third week. Further, it was observed that the circulating insulin level was reduced in STZ induced diabetic animals. The current therapy for diabetic neuropathy as approved by FDA is restricted to painful neuropathy, where the memory dysfunction is spared, but their side effect restricts its long term use. Treatment with UD and ROSI significantly reversed the STZ

induced alteration in bodyweight, water intake, blood glucose and serum insulin level. Current study confirms and extends previous findings, in which UD extract has anti-diabetic property [106, 378]. It has been reported that, scopoletin an active constituent of UD has potential to inhibit hyperglycemia in rodents [463]. Quercetin, rutin and esculetin are also known to possess antidiabetic effect in rodents [464-467]. Rutin ameliorates glucose homeostasis during diabetes by altering glycolytic and gluconeogenic enzymes [468]. Quercetin, a flavonoids induces regeneration of the pancreatic islets and increases insulin release in diabetic animals, thus exerting beneficial effects against diabetes [469].

After eight week post STZ treatment, diabetic mice showed depressive like behaviour in TST and FST. Our results are consistent with previous study demonstrated that, STZ induced diabetes associated with depressive like behaviour [470-472]. Earlier reports have shown that STZ induced diabetes in rodent's exhibit motor system control deficit [471, 473]. In the present study chronic UD and ROSI administration significantly reduced the depressive like behaviour but did not reverse motor deficit in diabetic mice. The chemical constituents of UD like scopoletin, rutin and quercetin is reported to have antidepressant like effect [397-398]. In the FST, ROSI treatment decreased immobility period and increased climbing, while unaffected the motor activity in the open field test during depression [216].

The current study demonstrated that long standing diabetes caused impairment in acquisition and retrieval processes (spatial memory), as observed by performance in the Morris water maze test, which is in line with previous reports [474]. It was also observed that STZ induced long standing diabetes leads to associative memory dysfunction in mice evident from decreased step through latency in PA task. In the present study, chronic treatment with either UD or ROSI significantly improved the spatial and associative memory dysfunction associated with long standing diabetes. In line with our current results it was reported that ROSI improves learning and memory in both human and animal models [199].

The mechanism underlying memory dysfunction is not well understood, but evidences suggest that, dysfunction in the insulin signaling system alters hippocampal synaptic neurotransmission and cognition [121].

It is well accepted that, exposure to STZ induces alteration in crucial neuronal pathways. Alterations in insulin signaling pathway have been observed in the hippocampus of diabetic

mice [121]. Hence, the effect of UD extract on hippocampal insulin signaling pathway was investigated in diabetic mice. STZ induced diabetic mice have elevated level of blood glucose which might be due to hypoinsulinemia or insulin resistance. In both cases, the downstream insulin signaling components like IR, IRS1/2, PI3K, PKB, MAPK1, INSG1 and GLUT4 are impaired, alongside deficits in neuronal development and memory formation [121, 418]. A recent study reported that PPARγ levels are downregulated in the hippocampus of diabetic mice [57]. It was reported that the level of ILGF1 was downregulated in the hippocampus of chronically diabetic mice [475]. GLP1 protects hippocampal neurons against diabetes associated advanced glycation end product insult [476]. Herein we observed that STZ induced diabetes significantly downregulated the mRNA expression of IR, IRS1/2, PI3K, PKB, MAPK1, INSG1 and GLUT4 in the hippocampus. We did not observe any significant alteration in the mRNA expression of hippocampal PPARγ, GLP1 and ILGF 1r in diabetic mice. It was reported that impairment of insulin signaling in the hippocampus could well play a role in the development of neurodegenerative disorders, as it leaves neurons more exposed to toxic influences (glucotoxicity) [121]. We observed that chronic treatment with UD or ROSI upregulated PPARγ gene expression and restored diabetes induced alteration in insulin signaling components viz., IR, IRS, PI3K, PKB, MAPK1, INSG1 and GLUT4 in hippocampus, while UD did not modulate the expression of IRS1 in diabetic mice. UD and ROSI did not regulate GLP1 and ILGF 1r in diabetic mice. Earlier study demonstrated that UD extract upregulated the expression level of GLUT4 gene as well as reduced hypercorticosteronemia and depressive like behaviour in dexamethasone induced diabetic mice [391]. Evidence suggests that, scopoletin, an active constituent of UD increases the gene expression of PPARγ, a target receptor for ROSI [406]. Quercetin upregulates expressions of PPARγ [477] and attenuates diabetes associated cognitive deficit [478]. Rutin is known to increase the expression of PPARγ in skeletal muscles of *db/db* mice [479]. Esculetin treatment attenuates the downregulation of PPARγ in diabetic kidney disease [480]. PPARγ agonist mediates insulin sensitizing actions in various cell types and attenuates memory deficits in STZ-induced diabetic mice [57, 481].

Cognitive benefit is achievable with pharmacological interventions targeting glycemic control [225]. ROSI treatment increases IR expression via PPARγ gene transcription resulting in improved insulin sensitivity [482]. During neuronal activity, IR→Src→MAPK pathway activates gene transcription. These genes code for proteins that are required for glucose

homeostasis, cell growth, synapse growth and for cell repair and maintenance [121]. For example, induction of INSG1 via MAPK pathway plays a role in cellular glucose homeostasis through unknown mechanism [200]. Further, activation of IR suppresses apoptosis through stimulation of IRS1/2 binding to PI3K, and activation of PI3K and Akt/PKB [121, 202]. We observed that, diabetes significantly downregulated the level of anti-apoptotic BCl2 gene in the hippocampus, whereas ROSI or UD treatment significantly reversed BCl2 downregulation. It has been demonstrated that, ROSI protects the neuronal cells against advanced glycation end products-induced injury via its anti-apoptotic property, that seems to be mediated by PPARγ activation, and suggested a beneficial role of ROSI in the treatment of Alzheimer's disease [483]. Herein, UD extract upregulated hippocampal BCL2 expression in diabetic mice, which might be through upregulation of PPARγ. Further, esculetin is known to activate the MAPK and PI3K/Akt pathways [484].

On the other hand, IRS1 and 2 activations are associated with cellular glucose homeostasis. This is achieved by activating Akt/PKB dependent transcription and translocation of GLUT4 [121, 485]. The level of cytosolic GLUT4 protein [420] and PI3K-dependent translocation of hippocampal GLUT4 to the plasma membrane was reduced during diabetes [18, 486], which might contribute to cognitive impairment [18]. In the present study, STZ induced diabetic mice showed reduced level of GLUT4 protein in the cytosolic fraction of hippocampus. Moreover, GLUT4 translocation to the hippocampal plasma membrane was significantly reduced in diabetic mice. Besides, chronic UD and ROSI treatment significantly restored diabetes mediated alteration in GLUT4 content in cytosol and their translocation to the hippocampal plasma membrane. Previous study demonstrated that, ROSI induces insulin-stimulated glucose uptake as a result of increased GLUT4 translocation to the plasma membrane [482]. Exposure of L6-GLUT4myc muscle cell with UD extract led to significant increase in GLUT4 content in plasma membranes [282]. In agreement with this, the active constituents of UD extract like scopoletin, rutin, quercetin and esculetin modulates the level of PPARγ gene [477, 479-480], which is known to activate IR→IRS1/2→PI3K→Akt/PKB pathway and induces GLUT4 translocation in hippocampal neurons [18, 121, 485]. Interestingly, quercetin inhibited insulin mediated GLUT4 translocation under normal conditions, but when insulin resistance, quercetin facilitated PI3K signaling and improved insulin sensitivity in 3T3-L1 cells [487]. UD and insulin treatment increased plasma

membrane association of GLUT4 in hippocampal slices, an effect that was blocked by the PI3K inhibitor LY294002.

Evidence suggests that ChAT, a specific marker for functional state of cholinergic neurons [61], activity is reduced during STZ administration resulting in cognitive deficit [62-63]. It was reported that, STZ increases AChE activity in hippocampus resulting in cognitive deficit [64]. In the present study, chronic diabetes resulted in downregulation of ChAT whereas upregulation of AChE expression in the hippocampus. UD treatment for 8 weeks reversed the diabetes mediated modulation in ChAT and AChE levels. Scopoletin inhibits AChE activity, potentiates acetylcholine release from synaptosomes, amplifies hippocampal long-term potentiation and attenuates anticholinergic- and age-impaired cognitive deficit [437]. Rutin has also been known to reduce AChE activity in the hippocampus [405]. Besides, ROSI effectively modulated ChAT expression but did not show significant effect against AChE level.

The diverse function of mAChR1 and its occurrence in the hippocampus, suggests that mAChR1 play a key role in learning and memory [28] and is known to transiently enhance long-term potentiation resulting in neurocognitive improvement [488]. Administration of selective mAChR1 antagonists induces spatial memory deficit [26]. In the present study, chronic diabetes significantly reduced the expression of mAChR1 in hippocampus, which is in line with a previous study suggesting that STZ-induced diabetes significantly downregulated the expression of mAChR1 in hippocampus [64]. Besides, chronic UD and ROSI treatment significantly upregulated hippocampal mAChR1 expression in diabetic mice. mAChR4 act by reducing the level of ACh at synaptic cleft led to reduce hippocampal neurogenesis [442]. In the present study, chronic UD, STZ and ROSI administration did not modulate the mRNA level of mAChR4 in hippocampus. Our results suggest that neurocognitive improvement by UD treatment might be due to the modulation of muscarinic cholinergic system in hippocampus. Quercetin modulates cholinergic neurotransmission in the hippocampus [489-490] and attenuates memory impairment and anxiogenic-like behaviour in rodents [489]. Chronic diabetes significantly increased mAChR4 expression in striatum along with hypolocomotion in mice. Our observations are consistent with previous studies suggesting that diabetes results in motor control system deficit [65]. Both chronic UD and ROSI treatment could not reverse diabetes-mediated alteration in striatal mAChR4

expression. Further, UD and ROSI treatment had no effect on diabetes mediated hypolocomotion.

It has been reported that, BDNF mRNA expression was downregulated in the hippocampus of diabetic rats and associated with cognitive impairment in novel object recognition task [491]. In addition, chronic type 2 diabetes affected brain plasticity evidenced by dysregulation of BDNF-TrkB pathway [492]. BDNF is well known to modulate neuroprotective effect in hippocampus [491]. Cyclin D1 is known to induce neural stem cells proliferation and differentiation in the rat brain [493]. Herein, we observed that STZ induced diabetes significantly downregulated the mRNA expression of BDNF, TrkB and cyclin D1 in the hippocampus. Chronic UD and ROSI administration significantly attenuated diabetes mediated alteration in BDNF, TrkB and cyclin D1 expression in the hippocampus.

Growing body of evidence demonstrate that, oxidative stress, inflammation and apoptosis plays a central role in the onset and subsequent neurological complications of diabetes [14, 494]. Oxidative stress, inflammation and apoptosis have also been known to exacerbate cognitive impairment [30, 495]. Earlier study showed that inflammation increases cytokine production and decreased the levels of autophagy markers in the cortex and hippocampus [496]. In the present study, chronic diabetes induced inflammation as evidenced by upregulation of TNFα and iNOS expression. Further, the level of ATG7 was significantly downregulated in chronically diabetic mice. Chronic diabetes did not modulate the level of AIP2, ATG5 and IL6 in the hippocampus. STZ significantly elevated the level of TBARS and NO in plasma. The level of antioxidant catalase and total thiols were significantly decreased in diabetic mice. Chronic treatment with UD and ROSI significantly reversed diabetes-induced alteration in the level of antioxidants (catalase and total thiol) along with attenuation of oxidative stress, inflammation and apoptosis. UD and ROSI did not regulate the level of AIP2 and IL6 in the hippocampus of diabetic mice. UD extract has shown antioxidant and antiinflammatory effects in rodents [286]. Both chronic stress and diabetes increased the inflammation in CA3 region of hippocampus as indicated by increase fluorescence of TNFα. CUMS and STZ also induced neuronal damage in CA3 region of hippocampus. Chronic UD administration reversed neuroinflammation as well as neuronal damage in chronically stressed and diabetic mice. It has been reported that, the active constituents of UD leaves extract exerts antioxidant and neuroprotective effects during

diabetes. Quercetin reduces oxidative stress in hippocampus during chronic diabetes [497]. Quercetin is also known to reduce apoptosis in the hippocampus of adult rats [498]. Esculetin exerts protective effect during diabetes by attenuating hyperglycemia-induced oxidative stress and antioxidant competence [467]. Esculetin also inhibits N-methyl-D-aspartate induced neurotoxicity in primary cortical cultures [499]. Gentisic acid inhibits glucose autoxidation [500], which is known to induce protein oxidative damage [501]. Rutin plays a positive role in glucose metabolism and antioxidant status during diabetes [468]. Rutin also protects hippocampal neurons from inflammation and apoptosis during cognitive deficits [405].

# CHAPTER 7

## CONCLUSION

## 7. Conclusion

In conclusion, UD modulated muscarinic cholinergic system, autophagy, Smo-Gli pathway, insulin signaling pathway and glucose homeostasis in hippocampus resulting in neurocognitive improvement during co-morbidity of depression and diabetes. In addition, UD presents a notable anti-inflammatory, antioxidant and anti-apoptotic effects, which appears to be related with the protection of hippocampal neurons during chronic stress and diabetes. Phytochemical analysis revealed the presence of flavonoids and phenolics like scopoletin, gentisic acid, esculetin, quercetin and rutin in the UD extract, which might possibly involved in the anti-inflammatory, antioxidant and anti-apoptotic effects. UD extract might prove to be effective for the chronic stress and diabetes related neurological disorders. Future studies are warranted to understand the role of bioactive constituents like scopoletin, gentisic acid, esculetin, quercetin and rutin in stress and/or diabetes mediated neurological disorders.

# CHAPTER 8

## SUMMARY

## 8. Summary

Chronic unpredictable mild stress (21 days) was associated with hypercorticosteronemia and insulin resistance but not hyperglycemia or hypoinsulinemia. Chronic unpredictable mild stress induced depressive like behaviour, spatial and associative memory deficit, and hypolocomotion. Stinging nettle reversed stress mediated hypercorticosteronemia, insulin resistance, depressive like behaviour, spatial and associative memory deficit. Stinging nettle did not modulate hypolocomotion in stressed mice. Effects of HYP and FLX were similar to that of UD treatment but they did not modulate insulin resistance. Chronic stress exacerbated muscarinic cholinergic system, insulin signaling pathway, autophagy and Smo-Gli pathway in the hippocampus. Chronic stress induced oxidative stress and inflammation which were reversed by chronic stinging nettle treatment comparable to FLX, ROSI and HYP. Stinging nettle extract modulated stress induced alteration in muscarinic cholinergic system, insulin signaling pathway, autophagy and Smo-Gli pathway in the hippocampus. Stinging nettle did not modulate stress induced alteration in glucose transporter type-4 membrane translocation in hippocampus whereas ROSI significantly modulated the GLUT4 translocation. FLX and HYP treatment did not modulate insulin resistance and insulin signaling pathway. FLX and ROSI did not modulate Smo-Gli pathway. ROSI and HYP treatment did not modulate muscarinic cholinergic system. Streptozotocin induced chronic diabetes showed hyperglycemia, hypoinsulinemia, insulin resistance, polydypsia and body weight loss which were reversed by chronic stinging nettle treatment. Streptozotocin induced comorbidity of diabetes and depression. Further comorbidity was associated with cognitive dysfunction, locomotion deficit. Stinging nettle reversed diabetes induced depression and cognitive dysfunction. Stinging nettle did not modulate hypolocomotion in diabetic mice. Chronic diabetes induced oxidative stress and inflammation which were reversed by chronic stinging nettle treatment. Stinging nettle extract modulated diabetes mediated alteration in muscarinic cholinergic system, insulin signaling pathway and autophagy in the hippocampus. Stinging nettle extract did not modulate muscarinic receptors expression in striatum. The effect of UD was similar to that of ROSI treatment however ROSI did not modulate acetylcholinesterase expression in hippocampus.

# CHAPTER 9

## REFERENCES

## References


1. Association, A.P., *Diagnostic and statistical manual of mental disorders, (DSM-5®)*. 2013: American Psychiatric Pub.
2. Sapolsky, R.M., L.C. Krey, and B.S. McEwen, *The Neuroendocrinology of Stress and Aging: The Glucocorticoid Cascade Hypothesis*.* Endocrine reviews, 1986. **7**(3): p. 284-301.
3. Pariante, C.M. and A.H. Miller, *Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment.* Biological psychiatry, 2001. **49**(5): p. 391-404.
4. Sousa, N., J.J. Cerqueira, and O.F. Almeida, *Corticosteroid receptors and neuroplasticity.* Brain research reviews, 2008. **57**(2): p. 561-570.
5. Rossetti, C., O. Halfon, and B. Boutrel, *Controversies about a common etiology for eating and mood disorders.* Frontiers in psychology, 2014. **5**.
6. Ghaisas, M., et al., *Effect of< i> Tectona grandis</i> Linn. on dexamethasone-induced insulin resistance in mice.* Journal of ethnopharmacology, 2009. **122**(2): p. 304-307.
7. Jatwa, R., et al., *Amelioration of Corticosteroid-Induced Type 2 Diabetes Mellitus by Rosiglitazone Is Possibly Mediated through Stimulation of Thyroid Function and Inhibition of Tissue Lipid Peroxidation in Mice.* Basic & clinical pharmacology & toxicology, 2007. **101**(3): p. 177-180.
8. Kessler, R.C., R.H. Price, and C.B. Wortman, *Social factors in psychopathology: Stress, social support, and coping processes.* Annual review of psychology, 1985. **36**(1): p. 531-572.
9. Katz, R.J., *Animal model of depression: pharmacological sensitivity of a hedonic deficit.* Pharmacology Biochemistry and Behavior, 1982. **16**(6): p. 965-968.
10. Bhattacharya, S. and A. Muruganandam, *Adaptogenic activity of< i> Withania somnifera</i>: an experimental study using a rat model of chronic stress.* Pharmacology Biochemistry and Behavior, 2003. **75**(3): p. 547-555.
11. Lin, Y.-H., et al., *Effect of chronic unpredictable mild stress on brain–pancreas relative protein in rat brain and pancreas.* Behavioural brain research, 2005. **165**(1): p. 63-71.

12. Golden, S.H., et al., *Examining a bidirectional association between depressive symptoms and diabetes.* Jama, 2008. **299**(23): p. 2751-2759.
13. Egede, L.E., D. Zheng, and K. Simpson, *Comorbid depression is associated with increased health care use and expenditures in individuals with diabetes.* Diabetes Care, 2002. **25**(3): p. 464-470.
14. Alipour, M., I. Salehi, and F.G. Soufi, *Effect of exercise on diabetes-induced oxidative stress in the rat hippocampus.* Iranian Red Crescent medical journal, 2012. **14**(4): p. 222.
15. Kuhad, A. and K. Chopra, *Curcumin attenuates diabetic encephalopathy in rats: behavioral and biochemical evidences.* European journal of pharmacology, 2007. **576**(1): p. 34-42.
16. Suwanjang, W., et al., *Melatonin attenuates dexamethasone toxicity-induced oxidative stress, calpain and caspase activation in human neuroblastoma SH-SY5Y cells.* The Journal of steroid biochemistry and molecular biology, 2013. **138**: p. 116-122.
17. Grillo, C., et al., *Insulin-stimulated translocation of GLUT4 to the plasma membrane in rat hippocampus is PI3-kinase dependent.* Brain research, 2009. **1296**: p. 35-45.
18. Piroli, G.G., et al., *Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus.* Neuroendocrinology, 2007. **85**(2): p. 71-80.
19. Huang, X., et al., *Down-regulation of insulin receptor substrates (IRS)-1 and IRS-2 and Src homologous and collagen-like protein Shc gene expression by insulin in skeletal muscle is not associated with insulin resistance or type 2 diabetes.* The Journal of Clinical Endocrinology & Metabolism, 2002. **87**(1): p. 255-259.
20. Peng, Y.-L., et al., *Inducible nitric oxide synthase is involved in the modulation of depressive behaviors induced by unpredictable chronic mild stress.* J. Neuroinflammation, 2012. **9**(75).
21. Sarkar, S., et al., *Complex inhibitory effects of nitric oxide on autophagy.* Molecular cell, 2011. **43**(1): p. 19-32.
22. Udayabanu, M., et al., *Nitric oxide associated with iNOS expression inhibits acetylcholinesterase activity and induces memory impairment during acute hypobaric hypoxia.* Brain research, 2008. **1230**: p. 138-149.

23. Aykaç, A., et al., *The change in muscarinic receptor subtypes in different brain regions of rats treated with fluoxetine or propranolol in a model of post-traumatic stress disorder.* Behavioural brain research, 2012. **232**(1): p. 124-129.
24. Liu, D., et al., *Resveratrol prevents impaired cognition induced by chronic unpredictable mild stress in rats.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2014. **49**: p. 21-29.
25. Dagyte, G., J.A. Den Boer, and A. Trentani, *The cholinergic system and depression.* Behav Brain Res, 2011. **221**(2): p. 574-82.
26. Hunter, A. and F. Roberts, *The effect of pirenzepine on spatial learning in the Morris water maze.* Pharmacology Biochemistry and Behavior, 1988. **30**(2): p. 519-523.
27. Liu, J., et al., *GRK5 deficiency leads to reduced hippocampal acetylcholine level via impaired presynaptic M2/M4 autoreceptor desensitization.* Journal of Biological Chemistry, 2009. **284**(29): p. 19564-19571.
28. Drever, B.D., G. Riedel, and B. Platt, *The cholinergic system and hippocampal plasticity.* Behavioural brain research, 2011. **221**(2): p. 505-514.
29. Gomeza, J., et al., *Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M4 muscarinic acetylcholine receptor knockout mice.* Proceedings of the National Academy of Sciences, 1999. **96**(18): p. 10483-10488.
30. Rinwa, P. and A. Kumar, *Modulation of nitrergic signalling pathway by American ginseng attenuates chronic unpredictable stress-induced cognitive impairment, neuroinflammation, and biochemical alterations.* Naunyn-Schmiedeberg's archives of pharmacology, 2014. **387**(2): p. 129-141.
31. Gottschalk, W.A., et al., *Signaling mechanisms mediating BDNF modulation of synaptic plasticity in the hippocampus.* Learning & Memory, 1999. **6**(3): p. 243-256.
32. Zhang, L., et al., *Curcumin produces antidepressant effects via activating MAPK/ERK-dependent brain-derived neurotrophic factor expression in the amygdala of mice.* Behavioural brain research, 2012. **235**(1): p. 67-72.
33. Becker, S. and J.M. Wojtowicz, *A model of hippocampal neurogenesis in memory and mood disorders.* Trends in cognitive sciences, 2007. **11**(2): p. 70-76.
34. Crochemore, C., et al., *Enhancement of p53 activity and inhibition of neural cell proliferation by glucocorticoid receptor activation.* The FASEB journal, 2002. **16**(8): p. 761-770.

35. Crochemore, C., et al., *Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation.* Molecular psychiatry, 2005. **10**(8): p. 790-798.
36. Saxe, M.D., et al., *Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus.* Proceedings of the National Academy of Sciences, 2006. **103**(46): p. 17501-17506.
37. Winocur, G., et al., *Inhibition of neurogenesis interferes with hippocampus-dependent memory function.* Hippocampus, 2006. **16**(3): p. 296-304.
38. Rajendran, R., et al., *Monoaminergic regulation of Sonic hedgehog signaling cascade expression in the adult rat hippocampus.* Neuroscience letters, 2009. **453**(3): p. 190-194.
39. Association, A.D., *Standards of medical care in diabetes—2014.* Diabetes Care, 2014. **37**(Supplement 1): p. S14-S80.
40. Alberti, K.G.M.M. and P.f. Zimmet, *Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation.* Diabetic medicine, 1998. **15**(7): p. 539-553.
41. Danaei, G., et al., *Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Blood Glucose) National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants.* Lancet, 2011. **378**(9785): p. 31-40.
42. Lustman, P.J., L.S. Griffith, and R.E. Clouse, *Depression in adults with diabetes: results of 5-yr follow-up study.* Diabetes Care, 1988. **11**(8): p. 605-612.
43. Nouwen, A., et al., *Prevalence of Depression in Individuals With Impaired Glucose Metabolism or Undiagnosed Diabetes A systematic review and meta-analysis of the European Depression in Diabetes (EDID) Research Consortium.* Diabetes Care, 2011. **34**(3): p. 752-762.
44. Lupien, S.B., E.J. Bluhm, and D.N. Ishii, *Systemic insulin-like growth factor-I administration prevents cognitive impairment in diabetic rats, and brain IGF regulates learning/memory in normal adult rats.* Journal of neuroscience research, 2003. **74**(4): p. 512-523.

45. Egede, L.E. and C. Ellis, *Diabetes and depression: global perspectives.* Diabetes research and clinical practice, 2010. **87**(3): p. 302-312.
46. Saravanan, G. and P. Ponmurugan, *Antidiabetic effect of S-allylcysteine: Effect on Thyroid hormone and circulatory antioxidant system in experimental diabetic rats.* Journal of Diabetes and its Complications, 2012. **26**(4): p. 280-285.
47. Celikyurt, I.K., et al., *Exenatide treatment exerts anxiolytic-and antidepressant-like effects and reverses neuropathy in a mouse model of type-2 diabetes.* Medical science monitor basic research, 2014. **20**: p. 112.
48. Husain, G.M., et al., *Beneficial effect of Hypericum perforatum on depression and anxiety in a type 2 diabetic rat model.* Acta poloniae pharmaceutica, 2011. **68**(6): p. 913-918.
49. Jiang, L.Y., et al., *PPARγ agonist pioglitazone reverses memory impairment and biochemical changes in a mouse model of type 2 diabetes mellitus.* CNS neuroscience & therapeutics, 2012. **18**(8): p. 659-666.
50. Rakieten, N., M. Rakieten, and M. Nadkarni, *Studies on the diabetogenic action of streptozotocin (NSC-37917).* Cancer chemotherapy reports. Part 1, 1963. **29**: p. 91-98.
51. Oztürk, Y., V.M. Altan, and N. Yildizoğlu-Ari, *Effects of experimental diabetes and insulin on smooth muscle functions.* Pharmacological Reviews, 1996. **48**(1): p. 69-112.
52. Srivastava, L., P. Bora, and S. Bhatt, *Diabetogenic action of streptozotocin.* Trends in Pharmacological Sciences, 1982. **3**: p. 376-378.
53. Akkina, S., C. Patterson, and D. Wright, *GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice.* Experimental neurology, 2001. **167**(1): p. 173-182.
54. El-Marasy, S.A., et al., *Anti-depressant effect of hesperidin in diabetic rats.* Canadian journal of physiology and pharmacology, 2014. **92**(11): p. 945-952.
55. Ma, L.-y., et al., *Ghrelin-attenuated cognitive dysfunction in streptozotocin-induced diabetic rats.* Alzheimer Disease & Associated Disorders, 2011. **25**(4): p. 352-363.
56. Greene-Schloesser, D., et al., *The peroxisomal proliferator-activated receptor (PPAR) α agonist, fenofibrate, prevents fractionated whole-brain irradiation-induced cognitive impairment.* Radiation research, 2014. **181**(1): p. 33-44.

57. Liu, L.-p., et al., *Pioglitazone ameliorates memory deficits in streptozotocin-induced diabetic mice by reducing brain β-amyloid through PPARγ activation.* Acta Pharmacologica Sinica, 2013. **34**(4): p. 455-463.
58. Sadaghiani, M.S., et al., *Antidepressant-like effect of pioglitazone in the forced swimming test in mice: the role of PPAR-gamma receptor and nitric oxide pathway.* Behavioural brain research, 2011. **224**(2): p. 336-343.
59. Grillo, C.A., et al., *Downregulation of hypothalamic insulin receptor expression elicits depressive-like behaviors in rats.* Behavioural brain research, 2011. **222**(1): p. 230-235.
60. Nitta, A., et al., *Diabetic neuropathies in brain are induced by deficiency of BDNF.* Neurotoxicology and teratology, 2002. **24**(5): p. 695-701.
61. Oda, Y., *Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system.* Pathology international, 1999. **49**(11): p. 921-937.
62. Prickaerts, J., et al., *Spatial discrimination learning and choline acetyltransferase activity in streptozotocin-treated rats: effects of chronic treatment with acetyl-L-carnitine.* Brain research, 1995. **674**(1): p. 142-146.
63. Ahmed, M.E., et al., *Amelioration of cognitive impairment and neurodegeneration by catechin hydrate in rat model of streptozotocin-induced experimental dementia of Alzheimer's type.* Neurochemistry international, 2013. **62**(4): p. 492-501.
64. Sherin, A., et al., *Cholinergic and GABAergic receptor functional deficit in the hippocampus of insulin-induced hypoglycemic and streptozotocin-induced diabetic rats.* Neuroscience, 2012. **202**: p. 69-76.
65. Kou, Z.-Z., et al., *Alterations in the neural circuits from peripheral afferents to the spinal cord: possible implications for diabetic polyneuropathy in streptozotocin-induced type 1 diabetic rats.* Frontiers in neural circuits, 2014. **8**.
66. Wang, B., et al., *Resveratrol-enhanced autophagic flux ameliorates myocardial oxidative stress injury in diabetic mice.* Journal of cellular and molecular medicine, 2014. **18**(8): p. 1599-1611.
67. Benzie, I.F., et al., *Medical Attributes of St. John's Wort (Hypericum perforatum).* 2011.
68. Vattikuti, U. and V. Ciddi, *An overview on Hypericum perforatum Linn.* Natural Product Radiance, 2005. **4**(5): p. 368-381.

69. Shelton, R.C., *St John's wort (Hypericum perforatum) in major depression.* The Journal of clinical psychiatry, 2008. **70**: p. 23-27.
70. Kasper, S., et al., *Efficacy and tolerability of Hypericum extract for the treatment of mild to moderate depression.* European neuropsychopharmacology, 2010. **20**(11): p. 747-765.
71. Linde, K., M.M. Berner, and L. Kriston, *St John's wort for major depression.* The Cochrane Library, 2008.
72. Trofimiuk, E., A. Walesiuk, and J.J. Braszko, *St John's wort (Hypericum perforatum) diminishes cognitive impairment caused by the chronic restraint stress in rats.* Pharmacological research, 2005. **51**(3): p. 239-246.
73. Crupi, R., et al., *Hypericum perforatum treatment: effect on behaviour and neurogenesis in a chronic stress model in mice.* BMC complementary and alternative medicine, 2011. **11**(1): p. 7.
74. Hofrichter, J., et al., *Reduced Alzheimer's disease pathology by St. John's wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice.* Current Alzheimer research, 2013. **10**(10): p. 1057.
75. Khalifa, A.E., *Hypericum perforatum as a nootropic drug: enhancement of retrieval memory of a passive avoidance conditioning paradigm in mice.* Journal of ethnopharmacology, 2001. **76**(1): p. 49-57.
76. Novelli, M., et al., *St. John's wort extract and hyperforin protect rat and human pancreatic islets against cytokine toxicity.* Acta diabetologica, 2014. **51**(1): p. 113-121.
77. Bastos, A.G., L.S.P. Guimarães, and C.M. Trentini, *Neurocognitive changes in depressed patients in psychodynamic psychotherapy, therapy with fluoxetine and combination therapy.* Journal of affective disorders, 2013. **151**(3): p. 1066-1075.
78. Sun, X.-P., et al., *Antidepressant-like effects and memory enhancement of a herbal formula in mice exposed to chronic mild stress.* Neuroscience bulletin, 2013. **29**(6): p. 737-744.
79. Lesemann, A., et al., *MPTP-induced hippocampal effects on serotonin, dopamine, neurotrophins, adult neurogenesis and depression-like behavior are partially influenced by fluoxetine in adult mice.* Brain research, 2012. **1457**: p. 51-69.

80. Wang, Y.H., et al., *Hypoglycemic and anti-depressant effects of Zuogui Jiangtang Jieyu formulation in a model of unpredictable chronic mild stress in rats with diabetes mellitus.* Experimental and therapeutic medicine, 2014. **8**(1): p. 281-285.
81. Milano, W., et al., *The pharmacological options in the treatment of eating disorders.* ISRN pharmacology, 2013. **2013**.
82. Manna, S. and S.N. Umathe, *Paracetamol potentiates the antidepressant-like and anticompulsive-like effects of fluoxetine.* Behavioural pharmacology, 2014.
83. Gibbons, R.D., et al., *Benefits from antidepressants: synthesis of 6-week patient-level outcomes from double-blind placebo-controlled randomized trials of fluoxetine and venlafaxine.* Archives of general psychiatry, 2012. **69**(6): p. 572-579.
84. Bersani, G., et al., *l-Acetylcarnitine in dysthymic disorder in elderly patients: A double-blind, multicenter, controlled randomized study vs. fluoxetine.* European neuropsychopharmacology, 2013. **23**(10): p. 1219-1225.
85. Patrone, C., O. Eriksson, and D. Lindholm, *Diabetes drugs and neurological disorders: new views and therapeutic possibilities.* The Lancet Diabetes & Endocrinology, 2014. **2**(3): p. 256-262.
86. Baldwin, S., S. Clarke, and R. Chenery, *Characterization of the cytochrome P450 enzymes involved in the in vitro metabolism of rosiglitazone.* British journal of clinical pharmacology, 1999. **48**: p. 424-432.
87. Yu, J., et al., *Bioequivalence and comparison of pharmacokinetic properties of 4-mg tablet formulations of rosiglitazone hydrochloride and rosiglitazone maleate: a single-dose, randomized, open-label, two-period crossover study in healthy adult male Chinese volunteers.* Clinical therapeutics, 2008. **30**(12): p. 2272-2279.
88. Hardman, J. and L. Limberd, *Insulin, oral hypoglycemic agents and the pharmacology of the endocrine pancreas.* Goodman and Gilman's: The Pharmacological basis of Therapeutics, 2001: p. 1383-1399.
89. Tripathi, K., *Essentials of medical pharmacology*. 2013: JP Medical Ltd.
90. Sim, Y.-B., et al., *Central anti-diabetic action of biguanide and thizolidinediones in D-glucose fed and streptozotocin-treated mouse models.* Neuroscience letters, 2012. **528**(1): p. 73-77.

91. Abbatecola, A.M., et al., *Rosiglitazone and cognitive stability in older individuals with type 2 diabetes and mild cognitive impairment.* Diabetes Care, 2010. **33**(8): p. 1706-1711.
92. Araki, A., *[Dementia and insulin resistance in patients with diabetes mellitus]*. Nihon rinsho. Japanese journal of clinical medicine, 2010. **68**(3): p. 569-574.
93. Rasgon, N.L., et al., *Rosiglitazone add-on in treatment of depressed patients with insulin resistance: a pilot study.* The Scientific World Journal, 2010. **10**: p. 321-328.
94. Cheng, Y., et al., *Neurotrophic factor-α1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone.* Molecular psychiatry, 2014.
95. Biringer, E., A. Rongve, and A. Lund, *A review of modern antidepressants' effects on neurocognitive function.* Current Psychiatry Reviews, 2009. **5**(3): p. 164-174.
96. Nikfar, S., et al., *Increasing the risk of spontaneous abortion and major malformations in newborns following use of serotonin reuptake inhibitors during pregnancy: A systematic review and updated meta-analysis.* Daru, 2012. **20**(1): p. 75.
97. Csoka, A., A. Bahrick, and O.P. Mehtonen, *Persistent sexual dysfunction after discontinuation of selective serotonin reuptake inhibitors.* The journal of sexual medicine, 2008. **5**(1): p. 227-233.
98. Kivimäki, M., et al., *Antidepressant Medication Use and Risk of Hyperglycemia and Diabetes Mellitus—A Noncausal Association?* Biological psychiatry, 2011. **70**(10): p. 978-984.
99. Kivimäki, M. and G. Batty, *Antidepressant drug use and future diabetes risk.* Diabetologia, 2012. **55**(1): p. 10-12.
100. Kawamura, T., T. Umemura, and N. Hotta, *Cognitive impairment in diabetic patients: Can diabetic control prevent cognitive decline?* Journal of diabetes investigation, 2012. **3**(5): p. 413-423.
101. Lewis, J.D., et al., *Risk of bladder cancer among diabetic patients treated with pioglitazone interim report of a longitudinal cohort study.* Diabetes Care, 2011. **34**(4): p. 916-922.
102. Ferwana, M., et al., *Pioglitazone and risk of bladder cancer: a meta-analysis of controlled studies.* Diabetic medicine, 2013. **30**(9): p. 1026-1032.

103. Chen, X., L. Yang, and S. Zhai, *Risk of cardiovascular disease and all-cause mortality among diabetic patients prescribed rosiglitazone or pioglitazone: a meta-analysis of retrospective cohort studies.* Chinese medical journal, 2012. **125**(23): p. 4301-4306.
104. Asgarpanah, J. and R. Mohajerani, *Phytochemistry and pharmacologic properties of Urtica dioica L.* J Med Plant Res, 2012. **6**: p. 5714-9.
105. Khare, V., et al., *Pharmacognostic Evaluation and Antioxidant Activity of Urtica dioica L.* 2012.
106. Qujeq, D., et al., *Effect of Urtica dioica leaf alcoholic and aqueous extracts on the number and the diameter of the islets in diabetic rats.* International journal of molecular and cellular medicine, 2013. **2**(1): p. 21.
107. Ahangarpour, A., M. Mohammadian, and M. Dianat, *Antidiabetic effect of hydroalcholic urtica dioica leaf extract in Male Rats with Fructose-Induced Insulin Resistance.* Iranian journal of medical sciences, 2012. **37**(3): p. 181.
108. Kianbakht, S., F. Khalighi-Sigaroodi, and F.H. Dabaghian, *Improved glycemic control in patients with advanced type 2 diabetes mellitus taking Urtica dioica leaf extract: a randomized double-blind placebo-controlled clinical trial.* Clinical laboratory, 2012. **59**(9-10): p. 1071-1076.
109. Namazi, N., et al., *The effect of hydro alcoholic Nettle (Urtica dioica) extracts on insulin sensitivity and some inflammatory indicators in patients with type 2 diabetes: a randomized double-blind control trial.* Pakistan journal of biological sciences: PJBS, 2011. **14**(15): p. 775-779.
110. Namazi, N., A. Tarighat, and A. Bahrami, *The effect of hydro alcoholic nettle (Urtica dioica) extract on oxidative stress in patients with type 2 diabetes: a randomized double-blind clinical trial.* Pakistan journal of biological sciences: PJBS, 2012. **15**(2): p. 98-102.
111. Bentley, S.M., G.L. Pagalilauan, and S.A. Simpson, *Major Depression.* Medical Clinics of North America, 2014. **98**(5): p. 981-1005.
112. Dagytė, G., J.A. Den Boer, and A. Trentani, *The cholinergic system and depression.* Behavioural brain research, 2011. **221**(2): p. 574-582.
113. Nestler, E.J., et al., *Neurobiology of depression.* Neuron, 2002. **34**(1): p. 13-25.

114. Krishnan, V. and E.J. Nestler, *The molecular neurobiology of depression.* Nature, 2008. **455**(7215): p. 894-902.
115. Musselman, D.L., et al., *Relationship of depression to diabetes types 1 and 2: epidemiology, biology, and treatment.* Biological psychiatry, 2003. **54**(3): p. 317-329.
116. Eriksson, A.K., et al., *Psychological distress and risk of pre-diabetes and Type 2 diabetes in a prospective study of Swedish middle-aged men and women.* Diabetic medicine, 2008. **25**(7): p. 834-842.
117. Kawakami, N., et al., *Depressive symptoms and occurrence of type 2 diabetes among Japanese men.* Diabetes Care, 1999. **22**(7): p. 1071-1076.
118. Palinkas, L., P. Lee, and E. Barrett-Connor, *A prospective study of type 2 diabetes and depressive symptoms in the elderly: the Rancho Bernardo Study.* Diabetic medicine, 2004. **21**(11): p. 1185-1191.
119. Golden, S.H., et al., *Depressive symptoms and the risk of type 2 diabetes the atherosclerosis risk in communities study.* Diabetes Care, 2004. **27**(2): p. 429-435.
120. Pan, Y., et al., *Impaired hypothalamic insulin signaling in CUMS rats: Restored by icariin and fluoxetine through inhibiting CRF system.* Psychoneuroendocrinology, 2013. **38**(1): p. 122-134.
121. Hlscher, C., *Diabetes as a risk factor for Alzheimer's disease: insulin signalling impairment in the brain as an alternative model of Alzheimer's disease.* Biochemical Society Transactions, 2011. **39**(4): p. 891.
122. Bishop, N.A., T. Lu, and B.A. Yankner, *Neural mechanisms of ageing and cognitive decline.* Nature, 2010. **464**(7288): p. 529-535.
123. Chen, R., et al., *Brain atrophy in a murine model of chronic fatigue syndrome and beneficial effect of Hochu-ekki-to (TJ-41).* Neurochemical research, 2008. **33**(9): p. 1759-1767.
124. Paulsen, J.S., *Cognitive impairment in Huntington disease: diagnosis and treatment.* Current neurology and neuroscience reports, 2011. **11**(5): p. 474-483.
125. Sousa, N., et al., *Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement.* Neuroscience, 2000. **97**(2): p. 253-266.
126. Brown, E.S. and T. Suppes, *Mood symptoms during corticosteroid therapy: a review.* Harvard review of psychiatry, 1998. **5**(5): p. 239-246.

127. Kelly, W.F., et al., *Cushing's syndrome and depression--a prospective study of 26 patients.* The British Journal of Psychiatry, 1983. **142**(1): p. 16-19.
128. Wróbel, A., et al., *The depressogenic-like effect of acute and chronic treatment with dexamethasone and its influence on the activity of antidepressant drugs in the forced swim test in adult mice.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2014. **54**: p. 243-248.
129. Diamond, D.M., et al., *Inverted-U relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation.* Hippocampus, 1992. **2**(4): p. 421-430.
130. Hamilton, S.E. and N.M. Nathanson, *The M1 receptor is required for muscarinic activation of mitogen-activated protein (MAP) kinase in murine cerebral cortical neurons.* Journal of Biological Chemistry, 2001. **276**(19): p. 15850-15853.
131. Shinoe, T., et al., *Modulation of synaptic plasticity by physiological activation of M1 muscarinic acetylcholine receptors in the mouse hippocampus.* The Journal of neuroscience, 2005. **25**(48): p. 11194-11200.
132. Bubser, M., et al., *Muscarinic receptor pharmacology and circuitry for the modulation of cognition,* in *Muscarinic Receptors*. 2012, Springer. p. 121-166.
133. Mineur, Y.S., et al., *Cholinergic signaling in the hippocampus regulates social stress resilience and anxiety-and depression-like behavior.* Proceedings of the National Academy of Sciences, 2013. **110**(9): p. 3573-3578.
134. Gould, E., et al., *Adrenal steroids regulate postnatal development of the rat dentate gyrus: II. Effects of glucocorticoids and mineralocorticoids on cell birth.* Journal of Comparative Neurology, 1991. **313**(3): p. 486-493.
135. Watanabe, Y., E. Gould, and B.S. McEwen, *Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons.* Brain research, 1992. **588**(2): p. 341-345.
136. Magariños, A.M., J.M.G. Verdugo, and B.S. McEwen, *Chronic stress alters synaptic terminal structure in hippocampus.* Proceedings of the National Academy of Sciences, 1997. **94**(25): p. 14002-14008.
137. Rohatgi, R., L. Milenkovic, and M.P. Scott, *Patched1 regulates hedgehog signaling at the primary cilium.* Science, 2007. **317**(5836): p. 372-376.

138. Aberger, F., et al., *Canonical and noncanonical Hedgehog/GLI signaling in hematological malignancies.* Vitamins and hormones, 2011. **88**: p. 25-54.
139. Taipale, J., et al., *Patched acts catalytically to suppress the activity of Smoothened.* Nature, 2002. **418**(6900): p. 892-896.
140. Jeong, J. and A.P. McMahon, *Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1*. Development, 2005. **132**(1): p. 143-154.
141. Yan, G.N., et al., *Glioma stem cells enhance endothelial cell migration and proliferation via the Hedgehog pathway.* Oncol Lett, 2013. **6**(5): p. 1524-1530.
142. Incardona, J.P., et al., *Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1*. Proceedings of the National Academy of Sciences, 2000. **97**(22): p. 12044-12049.
143. Traiffort, E., E. Angot, and M. Ruat, *Sonic Hedgehog signaling in the mammalian brain.* J Neurochem, 2010. **113**(3): p. 576-90.
144. Ruiz i Altaba, A., C. Mas, and B. Stecca, *The Gli code: an information nexus regulating cell fate, stemness and cancer.* Trends Cell Biol, 2007. **17**(9): p. 438-47.
145. Cheng, S.Y. and S. Yue, *Role and regulation of human tumor suppressor SUFU in Hedgehog signaling.* Advances in cancer research, 2008. **101**: p. 29-43.
146. Tostar, U., et al., *Novel Mechanism of Action on Hedgehog Signaling by a Suppressor of Fused Carboxy Terminal Variant.* PLoS One, 2012. **7**(5): p. e37761.
147. Wen, X., et al., *Kinetics of hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation.* Molecular and cellular biology, 2010. **30**(8): p. 1910-1922.
148. Chen, M.-H., et al., *Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved.* Genes & development, 2009. **23**(16): p. 1910-1928.
149. Jiang, J. and C.-c. Hui, *Hedgehog signaling in development and cancer.* Developmental cell, 2008. **15**(6): p. 801-812.
150. Pan, Y., et al., *Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation.* Molecular and cellular biology, 2006. **26**(9): p. 3365-3377.

151. Tempé, D., et al., *Multisite protein kinase A and glycogen synthase kinase 3β phosphorylation leads to Gli3 ubiquitination by SCFβTrCP.* Molecular and cellular biology, 2006. **26**(11): p. 4316-4326.
152. Christopher, K.J., et al., *Forward genetics uncovers Transmembrane protein 107 as a novel factor required for ciliogenesis and Sonic hedgehog signaling.* Developmental biology, 2012. **368**(2): p. 382-392.
153. Robbins, D.J., D.L. Fei, and N.A. Riobo, *The hedgehog signal transduction network.* Science signaling, 2012. **5**(246): p. re6.
154. Milenkovic, L., M.P. Scott, and R. Rohatgi, *Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium.* The Journal of cell biology, 2009. **187**(3): p. 365-374.
155. Kovacs, J.J., et al., *β-Arrestin–Mediated Localization of Smoothened to the Primary Cilium.* Science, 2008. **320**(5884): p. 1777-1781.
156. Chen, W., et al., *Activity-dependent internalization of smoothened mediated by ß-arrestin 2 and GRK2.* Science, 2004. **306**(5705): p. 2257-2260.
157. Chen, Y., et al., *Sonic Hedgehog dependent phosphorylation by CK1α and GRK2 is required for ciliary accumulation and activation of smoothened.* PLoS biology, 2011. **9**(6): p. e1001083.
158. Evron, T., et al., *Growth Arrest Specific 8 (Gas8) and G protein-coupled receptor kinase 2 (GRK2) cooperate in the control of Smoothened signaling.* Journal of Biological Chemistry, 2011. **286**(31): p. 27676-27686.
159. Caparrós-Martín, J.A., et al., *The ciliary Evc/Evc2 complex interacts with Smo and controls Hedgehog pathway activity in chondrocytes by regulating Sufu/Gli3 dissociation and Gli3 trafficking in primary cilia.* Human Molecular Genetics, 2013. **22**(1): p. 124-139.
160. Yang, C., et al., *Smoothened transduces Hedgehog signal by forming a complex with Evc/Evc2.* Cell research, 2012.
161. Robbins, D.J., D.L. Fei, and N.A. Riobo, *The hedgehog signal transduction network.* Sci Signal, 2012. **5**(246): p. re6.
162. Humke, E.W., et al., *The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins.* Genes Dev, 2010. **24**(7): p. 670-82.

163. Tukachinsky, H., L.V. Lopez, and A. Salic, *A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu–Gli protein complexes.* The Journal of cell biology, 2010. **191**(2): p. 415-428.
164. Wolff, F., et al., *Imiquimod directly inhibits Hedgehog signalling by stimulating adenosine receptor/protein kinase A-mediated GLI phosphorylation.* Oncogene, 2013.
165. Blotta, S., et al., *Canonical and noncanonical Hedgehog pathway in the pathogenesis of multiple myeloma.* Blood, 2012. **120**(25): p. 5002-5013.
166. Ruat, M., et al., *Hedgehog trafficking, cilia and brain functions.* Differentiation, 2012. **83**(2): p. S97-104.
167. Riobo, N.A. and D.R. Manning, *Pathways of signal transduction employed by vertebrate Hedgehogs.* Biochem J, 2007. **403**(3): p. 369-79.
168. Voronova, A., et al., *Ascl1/Mash1 is a novel target of Gli2 during Gli2-induced neurogenesis in P19 EC cells.* PLoS One, 2011. **6**(4): p. e19174.
169. McDermott, A., et al., *Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation.* Development, 2005. **132**(2): p. 345-357.
170. Reilly, J.O., et al., *Cooperative effects of Sonic Hedgehog and NGF on basal forebrain cholinergic neurons.* Molecular and Cellular Neuroscience, 2002. **19**(1): p. 88-96.
171. Petralia, R.S., et al., *Subcellular localization of patched and smoothened, the receptors for sonic hedgehog signaling, in the hippocampal neuron.* Journal of Comparative Neurology, 2011. **519**(18): p. 3684-3699.
172. Petralia, R.S., et al., *Sonic hedgehog promotes autophagy in hippocampal neurons.* Biology open, 2013. **2**(5): p. 499-504.
173. Cherra 3rd, S.J. and C.T. Chu, *Autophagy in neuroprotection and neurodegeneration: a question of balance.* 2008.
174. Haase, J. and E. Brown, *Integrating the monoamine, neurotrophin and cytokine hypotheses of depression—A central role for the serotonin transporter?* Pharmacology & therapeutics, 2014.
175. Pasquini, M., I. Berardelli, and M. Biondi, *Ethiopathogenesis of depressive disorders.* Clin Pract Epidemiol Ment Health, 2014. **10**: p. 166-71.

176. Brezun, J. and A. Daszuta, *Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats.* Neuroscience, 1999. **89**(4): p. 999-1002.

177. Jha, S., et al., *Selective serotonin depletion does not regulate hippocampal neurogenesis in the adult rat brain: Differential effects of< i> p</i>-chlorophenylalanine and 5, 7-dihydroxytryptamine.* Brain research, 2006. **1075**(1): p. 48-59.

178. Kulkarni, V.A., S. Jha, and V.A. Vaidya, *Depletion of norepinephrine decreases the proliferation, but does not influence the survival and differentiation, of granule cell progenitors in the adult rat hippocampus.* European Journal of Neuroscience, 2002. **16**(10): p. 2008-2012.

179. Avasthi, A., S. Grover, and M. Aggarwal, *Research on antidepressants in India.* Indian journal of psychiatry, 2010. **52**(Suppl1): p. S341.

180. Bodkin, J.A., et al., *Buprenorphine treatment of refractory depression.* Journal of clinical psychopharmacology, 1995. **15**(1): p. 49-57.

181. Wheeler, V.J., A.M. Mortimer, and P.J. Tyson, *Conventional antipsychotic prescription in unipolar depression, I: an audit and recommendations for practice.* The Journal of clinical psychiatry, 2003. **64**(5): p. 568-574.

182. Evans, E.A. and M.A. Sullivan, *Abuse and misuse of antidepressants.* Substance abuse and rehabilitation, 2014. **5**: p. 107.

183. Birmes, P., et al., *Serotonin syndrome: a brief review.* Canadian Medical Association Journal, 2003. **168**(11): p. 1439-1442.

184. Boyer, E.W. and M. Shannon, *The serotonin syndrome.* New England Journal of Medicine, 2005. **352**(11): p. 1112-1120.

185. Rao, T.S. and V.K. Yeragani, *Hypertensive crisis and cheese.* Indian journal of psychiatry, 2009. **51**(1): p. 65.

186. Rahimi, R., S. Nikfar, and M. Abdollahi, *Pregnancy outcomes following exposure to serotonin reuptake inhibitors: a meta-analysis of clinical trials.* Reproductive Toxicology, 2006. **22**(4): p. 571-575.

187. Forouhi, N.G. and N.J. Wareham, *Epidemiology of diabetes.* Medicine (Abingdon), 2014. **42**(12): p. 698-702.

188. Precechtelova, J., et al., *Type I Diabetes Mellitus: Genetic Factors and Presumptive Enteroviral Etiology or Protection.* Journal of pathogens, 2014. **2014**.
189. Antonelli, A., et al., *Hepatitis C virus infection and type 1 and type 2 diabetes mellitus.* World journal of diabetes, 2014. **5**(5): p. 586.
190. Stumvoll, M., et al., *Association of the TG Polymorphism in adiponectin (Exon 2) with obesity and insulin sensitivity interaction with family history of type 2 diabetes.* Diabetes, 2002. **51**(1): p. 37-41.
191. Scheen, A., *Pathophysiology of type 2 diabetes.* Acta Clinica Belgica, 2003. **58**(6): p. 335-341.
192. Scheen, A.J., *Treatment of type 2 diabetes.* Acta Clinica Belgica, 2003. **58**(5): p. 318-324.
193. Petersen, K.F. and G.I. Shulman, *Etiology of insulin resistance.* The American journal of medicine, 2006. **119**(5): p. S10-S16.
194. Yates, K.F., et al., *Impact of Metabolic Syndrome on Cognition and Brain A Selected Review of the Literature.* Arteriosclerosis, thrombosis, and vascular biology, 2012. **32**(9): p. 2060-2067.
195. Anderson, R.J., et al., *The prevalence of comorbid depression in adults with diabetes a meta-analysis.* Diabetes Care, 2001. **24**(6): p. 1069-1078.
196. Li, C., et al., *Prevalence of Depression Among US Adults With Diabetes Findings from the 2006 Behavioral Risk Factor Surveillance System.* Diabetes Care, 2008. **31**(1): p. 105-107.
197. Li, C., et al., *Prevalence and correlates of undiagnosed depression among US adults with diabetes: the Behavioral Risk Factor Surveillance System, 2006.* Diabetes research and clinical practice, 2009. **83**(2): p. 268-279.
198. Solanki, R., V. Dubey, and D. Munshi, *Neurocognitive impairment and comorbid depression in patients of diabetes mellitus.* International journal of diabetes in developing countries, 2009. **29**(3): p. 133.
199. Pipatpiboon, N., et al., *PPARγ agonist improves neuronal insulin receptor function in hippocampus and brain mitochondria function in rats with insulin resistance induced by long term high-fat diets.* Endocrinology, 2011. **153**(1): p. 329-338.
200. Krapivner, S., et al., *Human evidence for the involvement of insulin-induced gene 1 in the regulation of plasma glucose concentration.* Diabetologia, 2007. **50**(1): p. 94-102.

201. Fernando, R.N., et al., *Distribution and cellular localization of insulin-regulated aminopeptidase in the rat central nervous system.* Journal of Comparative Neurology, 2005. **487**(4): p. 372-390.
202. Schubert, M., et al., *Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation.* The Journal of neuroscience, 2003. **23**(18): p. 7084-7092.
203. Grilloa, G.G.P.C.A., et al., *Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus.* Neuroendocrinology, 2007. **85**: p. 71-80.
204. Alberini, C.M. and D.Y. Chen, *Memory enhancement: consolidation, reconsolidation and insulin-like growth factor 2.* Trends in neurosciences, 2012. **35**(5): p. 274-283.
205. Shingo, A.S., et al., *Cognitive decline in STZ-3V rats is largely due to dysfunctional insulin signalling through the dentate gyrus.* Behavioural brain research, 2012. **229**(2): p. 378-383.
206. Al-Ayadhi, L.Y., *Relationship between Sonic hedgehog protein, brain-derived neurotrophic factor and oxidative stress in autism spectrum disorders.* Neurochemical research, 2012. **37**(2): p. 394-400.
207. Gonzalez-Reyes, L.E., et al., *Sonic hedgehog maintains cellular and neurochemical homeostasis in the adult nigrostriatal circuit.* Neuron, 2012. **75**(2): p. 306-319.
208. Yuede, C.M., H. Dong, and J.G. Csernansky, *Anti-dementia drugs and hippocampal-dependent memory in rodents.* Behavioural pharmacology, 2007. **18**(5-6): p. 347.
209. Inzucchi, S.E., *Oral antihyperglycemic therapy for type 2 diabetes: scientific review.* Jama, 2002. **287**(3): p. 360-372.
210. Krentz, A.J. and C.J. Bailey, *Oral antidiabetic agents.* Drugs, 2005. **65**(3): p. 385-411.
211. Nathan, D.M., et al., *Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes.* Diabetes Care, 2009. **32**(1): p. 193-203.
212. Richter, B., et al., *Dipeptidyl peptidase-4 (DPP-4) inhibitors for type 2 diabetes mellitus.* The Cochrane Library, 2008.

213. Domínguez, R.O., et al., *Type 2 diabetes and/or its treatment leads to less cognitive impairment in Alzheimer's disease patients.* Diabetes research and clinical practice, 2012. **98**(1): p. 68-74.
214. Lee, E.Y., et al., *Rosiglitazone, a PPAR-γ agonist, protects against striatal dopaminergic neurodegeneration induced by 6-OHDA lesions in the substantia nigra of rats.* Toxicology letters, 2012. **213**(3): p. 332-344.
215. Schintu, N., et al., *PPAR-gamma-mediated neuroprotection in a chronic mouse model of Parkinson's disease.* European Journal of Neuroscience, 2009. **29**(5): p. 954-963.
216. Ahmed, A.A.E., N.M. Al-Rasheed, and N.M. Al-Rasheed, *Antidepressant-like effects of rosiglitazone, a PPARγ agonist, in the rat forced swim and mouse tail suspension tests.* Behavioural pharmacology, 2009. **20**(7): p. 635-642.
217. Guo, W., et al., *Additive effects of glyburide and antidepressants in the forced swimming test: evidence for the involvement of potassium channel blockade.* Pharmacology Biochemistry and Behavior, 1996. **54**(4): p. 725-730.
218. Hofmann, P., *Treatment of patients with comorbid depression and diabetes with metformin and milnacipran.* Neuropsychiatric disease and treatment, 2010. **6**(Suppl I): p. 9.
219. McIntyre, R.S., et al., *The neuroprotective effects of GLP-1: possible treatments for cognitive deficits in individuals with mood disorders.* Behavioural brain research, 2013. **237**: p. 164-171.
220. Petrak, F. and S. Herpertz, *Treatment of depression in diabetes: an update.* Current opinion in psychiatry, 2009. **22**(2): p. 211-217.
221. Heeramun-Aubeeluck, A., Z. Lu, and Y. Luo, *Comorbidity of Depression and Diabetes: In a Nutshell.* Psychology, 2012. **3**(09): p. 787.
222. El-Mir, M.-Y., et al., *Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons.* Journal of Molecular Neuroscience, 2008. **34**(1): p. 77-87.
223. Gupta, A., B. Bisht, and C.S. Dey, *Peripheral insulin-sensitizer drug metformin ameliorates neuronal insulin resistance and Alzheimer's-like changes.* Neuropharmacology, 2011. **60**(6): p. 910-920.

224. Wang, J., et al., *Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation.* Cell stem cell, 2012. **11**(1): p. 23-35.
225. Ryan, C.M., et al., *Improving metabolic control leads to better working memory in adults with type 2 diabetes.* Diabetes Care, 2006. **29**(2): p. 345-351.
226. Abbatecola, A., et al., *Postprandial plasma glucose excursions and cognitive functioning in aged type 2 diabetics.* Neurology, 2006. **67**(2): p. 235-240.
227. Imfeld, P., et al., *Metformin, Other Antidiabetic Drugs, and Risk of Alzheimer's Disease: A Population-Based Case–Control Study.* Journal of the American Geriatrics Society, 2012. **60**(5): p. 916-921.
228. Lin, J., C. Handschin, and B.M. Spiegelman, *Metabolic control through the PGC-1 family of transcription coactivators.* Cell metabolism, 2005. **1**(6): p. 361-370.
229. Aviles-Olmos, I., et al., *Parkinson's disease, insulin resistance and novel agents of neuroprotection.* Brain, 2013. **136**(2): p. 374-384.
230. Cui, L., et al., *Transcriptional repression of PGC-1α by mutant huntingtin leads to mitochondrial dysfunction and neurodegeneration.* Cell, 2006. **127**(1): p. 59-69.
231. Gao, H., et al., *GLP-1 amplifies insulin signaling by up-regulation of IRβ, IRS-1 and Glut4 in 3T3-L1 adipocytes.* Endocrine, 2007. **32**(1): p. 90-95.
232. During, M.J., et al., *Glucagon-like peptide-1 receptor is involved in learning and neuroprotection.* Nature medicine, 2003. **9**(9): p. 1173-1179.
233. Salcedo, I., et al., *Neuroprotective and neurotrophic actions of glucagon-like peptide-1: an emerging opportunity to treat neurodegenerative and cerebrovascular disorders.* British journal of pharmacology, 2012. **166**(5): p. 1586-1599.
234. Mangialasche, F., et al., *Alzheimer's disease: clinical trials and drug development.* The Lancet Neurology, 2010. **9**(7): p. 702-716.
235. Watson, G.S., et al., *Preserved cognition in patients with early Alzheimer disease and amnestic mild cognitive impairment during treatment with rosiglitazone: a preliminary study.* The American journal of geriatric psychiatry, 2005. **13**(11): p. 950-958.
236. Miller, B.W., K.C. Willett, and A.R. Desilets, *Rosiglitazone and pioglitazone for the treatment of Alzheimer's disease.* Annals of Pharmacotherapy, 2011. **45**(11): p. 1416-1424.

237. Ott, A., et al., *Diabetes mellitus and the risk of dementia The Rotterdam Study.* Neurology, 1999. **53**(9): p. 1937-1937.
238. Feil, D.G., et al., *Risk of hypoglycemia in older veterans with dementia and cognitive impairment: implications for practice and policy.* Journal of the American Geriatrics Society, 2011. **59**(12): p. 2263-2272.
239. Himeno, T., et al., *Beneficial effects of exendin-4 on experimental polyneuropathy in diabetic mice.* Diabetes, 2011. **60**(9): p. 2397-2406.
240. Bomfim, T.R., et al., *An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease–associated Aβ oligomers.* The Journal of clinical investigation, 2012. **122**(4): p. 1339.
241. Stein, S.A., E.M. Lamos, and S.N. Davis, *A review of the efficacy and safety of oral antidiabetic drugs.* Expert opinion on drug safety, 2013. **12**(2): p. 153-175.
242. Lee, I.-T., W.H.-H. Sheu, and S. Liu, *Hypoglycemia Probably Due to Accidental Intake of Repaglinide.* Chang Gung medical journal, 2002. **25**(11): p. 783-786.
243. Kessler, R.C., *The effects of stressful life events on depression.* 1997.
244. Kendler, K., L. Karkowski, and C. Prescott, *The assessment of dependence in the study of stressful life events: validation using a twin design.* Psychological medicine, 1999. **29**(06): p. 1455-1460.
245. Hill, M.N., et al., *Neurobiology of chronic mild stress: parallels to major depression.* Neuroscience & Biobehavioral Reviews, 2012. **36**(9): p. 2085-2117.
246. Willner, P., *Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS.* Neuropsychobiology, 2005. **52**(2): p. 90-110.
247. Willner, P., et al., *Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant.* Psychopharmacology, 1987. **93**(3): p. 358-364.
248. Matthews, K., N. Forbes, and I.C. Reid, *Sucrose consumption as an hedonic measure following chronic unpredictable mild stress.* Physiology & behavior, 1995. **57**(2): p. 241-248.
249. Grønli, J., et al., *Chronic mild stress affects sucrose intake and sleep in rats.* Behavioural brain research, 2004. **150**(1): p. 139-147.

250. Willner, P., et al., *Decreased hedonic responsiveness following chronic mild stress is not secondary to loss of body weight.* Physiology & behavior, 1996. **60**(1): p. 129-134.
251. Willner, P., *Animal models of depression: validity and applications.* Advances in biochemical psychopharmacology, 1995. **49**: p. 19.
252. Vancassel, S., et al., *n-3 polyunsaturated fatty acid supplementation reverses stress-induced modifications on brain monoamine levels in mice.* Journal of lipid research, 2008. **49**(2): p. 340-348.
253. Häidkind, R., et al., *Effects of partial locus coeruleus denervation and chronic mild stress on behaviour and monoamine neurochemistry in the rat.* European neuropsychopharmacology, 2003. **13**(1): p. 19-28.
254. Vogel, G., et al., *Animal depression model by neonatal clomipramine: reduction of shock induced aggression.* Pharmacology Biochemistry and Behavior, 1988. **31**(1): p. 103-106.
255. Horovitz, Z., et al., *Effects of drugs on the mouse-killing (muricide) test and its relationship to amygdaloid function.* International journal of neuropharmacology, 1966. **5**(6): p. 405-IN7.
256. Vogel, H.G., *Drug Discovery and Evaluation: Pharmacological Assays; Including a CD-ROM*. 2002: Springer.
257. Randall, P.K., *Quantification of dopaminergic supersensitization using apomorphine-induced behavior in the mouse.* Life sciences, 1985. **37**(15): p. 1419-1423.
258. Rees, D. and J. Alcolado, *Animal models of diabetes mellitus.* Diabetic medicine, 2005. **22**(4): p. 359-370.
259. Dey, P., et al., *Assessment of anti-diabetic activity of an ethnopharmacological plant< i> Nerium oleander</i> through alloxan induced diabetes in mice.* Journal of ethnopharmacology, 2014.
260. Yao, X.-g., et al., *BBT improved glucose homeostasis by ameliorating β-cell dysfunction in type 2 diabetic mice.* Journal of Endocrinology, 2015: p. JOE-14-0721.
261. Srinivasan, K. and P. Ramarao, *Animal models in type 2 diabetes research: an overview*. The Indian journal of medical research, 2012. **136**(1): p. 0.
262. RERUP, C.C., *Drugs producing diabetes through damage of the insulin secreting cells.* Pharmacological Reviews, 1970. **22**(4): p. 485-518.

263. Kasiviswanath, R., A. Ramesh, and K.E. Kumar, *Hypoglycemic and antihyperglycemic effect of Gmelina asiatica LINN. in normal and in alloxan induced diabetic rats.* Biological and Pharmaceutical Bulletin, 2005. **28**(4): p. 729-732.
264. Sheng, X.-Q., K.-X. Huang, and H.-B. Xu, *Influence of alloxan-induced diabetes and selenite treatment on blood glucose and glutathione levels in mice.* Journal of trace elements in medicine and biology, 2005. **18**(3): p. 261-267.
265. Ho, E., G. Chen, and T.M. Bray, *Alpha-Phenyl-< i> tert</i>-butylnitrone (PBN) inhibits NFκB activation offering protection against chemically induced diabetes.* Free Radical Biology and Medicine, 2000. **28**(4): p. 604-614.
266. Juárez-Reyes, K., et al., *Hypoglycemic, antihyperglycemic, and antioxidant effects of the edible plant< i> Anoda cristata</i>*. Journal of ethnopharmacology, 2015. **161**: p. 36-45.
267. Nain, P., et al., *Antidiabetic and antioxidant potential of< i> Emblica officinalis</i> Gaertn. leaves extract in streptozotocin-induced type-2 diabetes mellitus (T2DM) rats.* Journal of ethnopharmacology, 2012. **142**(1): p. 65-71.
268. Ventura-Sobrevilla, J., et al. *Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice*. in *Proceedings of the Western Pharmacology Society*. 2011.
269. Ning, Y., et al., *Ranolazine increases β-cell survival and improves glucose homeostasis in low-dose streptozotocin-induced diabetes in mice.* Journal of Pharmacology and Experimental Therapeutics, 2011. **337**(1): p. 50-58.
270. Christianson, J.A., et al., *Beneficial actions of neurotrophin treatment on diabetes-induced hypoalgesia in mice.* The Journal of Pain, 2003. **4**(9): p. 493-504.
271. Demiot, C., et al., *Aldose reductase pathway inhibition improved vascular and C-fiber functions, allowing for pressure-induced vasodilation restoration during severe diabetic neuropathy.* Diabetes, 2006. **55**(5): p. 1478-1483.
272. Sigaudo-Roussel, D., B. Fromy, and J.L. Saumet, *Diabetic neuropathy in animal models.* Drug Discovery Today: Disease Models, 2007. **4**(1): p. 39-44.
273. Joshi, B.C., M. Mukhija, and A.N. Kalia, *Pharmacognostical review of Urtica dioica L.* International Journal of Green Pharmacy, 2014. **8**(4): p. 201.

274. Krystofova, O., et al., *Effects of various doses of selenite on stinging nettle (Urtica dioica L.).* International journal of environmental research and public health, 2010. **7**(10): p. 3804-3815.
275. Wetherilt, H., *Evaluation of Urtica species as potential sources of important nutrients.* Developments in food science, 1992.
276. Gül, S., et al., *Chemical composition and in vitro cytotoxic, genotoxic effects of essential oil from Urtica dioica L.* Bulletin of environmental contamination and toxicology, 2012. **88**(5): p. 666-671.
277. Orčić, D., et al., *Quantitative determination of plant phenolics in Urtica dioica extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection.* Food chemistry, 2014. **143**: p. 48-53.
278. Nahata, A. and V. Dixit, *Ameliorative effects of stinging nettle (Urtica dioica) on testosterone-induced prostatic hyperplasia in rats.* Andrologia, 2012. **44**(s1): p. 396-409.
279. Otles, S. and B. Yalcin, *Phenolic compounds analysis of root, stalk, and leaves of nettle.* The Scientific World Journal, 2012. **2012**.
280. Wessler, I., et al., *The biological role of non-neuronal acetylcholine in plants and humans.* Japanese journal of pharmacology, 2001. **85**(1): p. 2-10.
281. Ghorbani, A., et al., *Antihyperlipidemic effect of a polyherbal mixture in streptozotocin-induced diabetic rats.* Journal of lipids, 2013. **2013**.
282. Kadan, S., et al., *In vitro evaluations of cytotoxicity of eight antidiabetic medicinal plants and their effect on GLUT4 translocation.* Evidence-Based Complementary and Alternative Medicine, 2013. **2013**.
283. Dar, S.A., et al., *Pharmacological and toxicological evaluation of Urtica dioica.* Pharmaceutical biology, 2013. **51**(2): p. 170-180.
284. Fazeli, S., et al., *The granule cell density of the dentate gyrus following administration of Urtica dioica extract to young diabetic rats.* Folia morphologica, 2008. **67**(3): p. 196-204.
285. Toldy, A., et al., *The beneficial effects of nettle supplementation and exercise on brain lesion and memory in rat.* The Journal of nutritional biochemistry, 2009. **20**(12): p. 974-981.

286. Toldy, A., et al., *The effect of exercise and nettle supplementation on oxidative stress markers in the rat brain.* Brain research bulletin, 2005. **65**(6): p. 487-493.
287. Özen, T. and H. Korkmaz, *Modulatory effect of Urtica dioica L.(Urticaceae) leaf extract on biotransformation enzyme systems, antioxidant enzymes, lactate dehydrogenase and lipid peroxidation in mice.* Phytomedicine, 2003. **10**(5): p. 405-415.
288. Kanter, M., O. Coskun, and M. Budancamanak, *Hepatoprotective effects of Nigella sativa L and Urtica dioica L on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride-treated rats.* World Journal of Gastroenterology, 2005. **11**(42): p. 6684.
289. Yener, Z., et al., *Effects of Urtica dioica L. seed on lipid peroxidation, antioxidants and liver pathology in aflatoxin-induced tissue injury in rats.* Food and Chemical Toxicology, 2009. **47**(2): p. 418-424.
290. Özkol, H., et al., *Ameliorative influence of Urtica dioica L against cisplatin-induced toxicity in mice bearing Ehrlich ascites carcinoma.* Drug and chemical toxicology, 2012. **35**(3): p. 251-257.
291. Kandis, H., et al., *Effects of Urtica dioica on hepatic ischemia-reperfusion injury in rats.* Clinics, 2010. **65**(12): p. 1357-1361.
292. Cetinus, E., et al., *The role of urtica dioica (urticaceae) in the prevention of oxidative stress caused by tourniquet application in rats.* The Tohoku journal of experimental medicine, 2005. **205**(3): p. 215-221.
293. Tahri, A., et al., *Acute diuretic, natriuretic and hypotensive effects of a continuous perfusion of aqueous extract of Urtica dioica in the rat.* Journal of ethnopharmacology, 2000. **73**(1): p. 95-100.
294. Legssyer, A., et al., *Cardiovascular effects of Urtica dioica L. in isolated rat heart and aorta.* Phytotherapy Research, 2002. **16**(6): p. 503-507.
295. Hajhashemi, V. and V. Klooshani, *Antinociceptive and anti-inflammatory effects of Urtica dioica leaf extract in animal models.* Avicenna Journal of Phytomedicine, 2013. **3**(2): p. 193.
296. Gülçin, I., et al., *Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (Urtica dioica L.).* Journal of ethnopharmacology, 2004. **90**(2): p. 205-215.

297. Harput, U.S., I. Saracoglu, and Y. Ogihara, *Stimulation of lymphocyte proliferation and inhibition of nitric oxide production by aqueous Urtica dioica extract.* Phytotherapy Research, 2005. **19**(4): p. 346-348.
298. Ghorbanibirgani, A., A. Khalili, and L. Zamani, *The efficacy of stinging nettle (Urtica dioica) in patients with benign prostatic hyperplasia: a randomized double-blind study in 100 patients.* Iran Red Crescent Med J, 2013. **15**(1): p. 9-10.
299. Safarinejad, M.R., *Urtica dioica for treatment of benign prostatic hyperplasia: a prospective, randomized, double-blind, placebo-controlled, crossover study.* Journal of herbal pharmacotherapy, 2005. **5**(4): p. 1-11.
300. Melo, E., et al., *Evaluating the efficiency of a combination of Pygeum africanum and stinging nettle (Urtica dioica) extracts in treating benign prostatic hyperplasia (BPH): double-blind, randomized, placebo controlled trial.* Int Braz J Urol, 2002. **28**(5): p. 418-425.
301. Lopatkin, N., et al., *Efficacy and safety of a combination of Sabal and Urtica extract in lower urinary tract symptoms—long-term follow-up of a placebo-controlled, double-blind, multicenter trial.* International urology and nephrology, 2007. **39**(4): p. 1137-1146.
302. Di Sotto, A., et al., *Antimutagenic and antioxidant activity of a protein fraction from aerial parts of Urtica dioica.* Pharmaceutical biology, 2014(0): p. 1-4.
303. Lichius, J.J. and C. Muth, *The inhibiting effects of Urtica dioica root extracts on experimentally induced prostatic hyperplasia in the mouse.* Planta medica, 1997. **63**(4): p. 307-310.
304. Modarresi-Chahardehi, A., et al., *Screening antimicrobial activity of various extracts of Urtica dioica.* Revista de Biología Tropical, 2012. **60**(4): p. 1567-1576.
305. Singh, R., et al., *Anti-mycobacterial screening of five Indian medicinal plants and partial purification of active extracts of Cassia sophera and Urtica dioica.* Asian Pacific journal of tropical medicine, 2013. **6**(5): p. 366-371.
306. Körpe, D.A., et al., *High-antibacterial activity of Urtica spp. seed extracts on food and plant pathogenic bacteria.* International journal of food sciences and nutrition, 2013. **64**(3): p. 355-362.
307. Hadizadeh, I., B. Peivastegan, and M. Kolahi, *Antifungal activity of nettle (Urtica dioica L.), colocynth (Citrullus colocynthis L. Schrad), oleander (Nerium oleander L.)*

*and konar (Ziziphus spina-christi L.) extracts on plants pathogenic fungi.* Pakistan journal of biological sciences: PJBS, 2009. **12**(1): p. 58-63.

308. Balzarini, J., et al., *The mannose-specific plant lectins from Cymbidium hybrid and Epipactis helleborine and the (N-acetylglucosamine) n-specific plant lectin from Urtica dioica are potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication in vitro.* Antiviral research, 1992. **18**(2): p. 191-207.
309. Lopatkin, N., et al., *Long-term efficacy and safety of a combination of sabal and urtica extract for lower urinary tract symptoms—a placebo-controlled, double-blind, multicenter trial.* World journal of urology, 2005. **23**(2): p. 139-146.
310. Bondarenko, B., et al., *Long-term efficacy and safety of PRO 160/120 (a combination of sabal and urtica extract) in patients with lower urinary tract symptoms (LUTS).* Phytomedicine, 2003. **10**: p. 53-55.
311. Piperopoulos, G., et al., *Determination of naphthodianthrones in plant extracts from Hypericum perforatum L. by liquid chromatography–electrospray mass spectrometry.* Journal of Chromatography B: Biomedical Sciences and Applications, 1997. **695**(2): p. 309-316.
312. Zou, Y., Y. Lu, and D. Wei, *Antioxidant activity of a flavonoid-rich extract of Hypericum perforatum L. in vitro.* Journal of Agricultural and Food Chemistry, 2004. **52**(16): p. 5032-5039.
313. Butterweck, V., et al., *Flavonoids from Hypericum perforatum show antidepressant activity in the forced swimming test.* Planta medica, 2000. **66**(1): p. 3-6.
314. Zou, Y., Y. Lu, and D. Wei, *Hypocholesterolemic effects of a flavonoid-rich extract of Hypericum perforatum L. in rats fed a cholesterol-rich diet.* Journal of Agricultural and Food Chemistry, 2005. **53**(7): p. 2462-2466.
315. Tatsis, E.C., et al., *Identification of the major constituents of Hypericum perforatum by LC/SPE/NMR and/or LC/MS.* Phytochemistry, 2007. **68**(3): p. 383-393.
316. Schwob, I., et al., *Changes in essential oil composition in Saint John's wort (Hypericum perforatum L.) aerial parts during its phenological cycle.* Biochemical systematics and ecology, 2004. **32**(8): p. 735-745.
317. Greeson, J.M., B. Sanford, and D.A. Monti, *St. John's wort (Hypericum perforatum): a review of the current pharmacological, toxicological, and clinical literature.* Psychopharmacology, 2001. **153**(4): p. 402-414.

318. Müller, W., et al., *Effects of hypericum extract (LI 160) in biochemical models of antidepressant activity.* Pharmacopsychiatry, 1997. **30**: p. 102-107.
319. Langosch, J., et al., *St John's wort (Hypericum perforatum) modulates evoked potentials in guinea pig hippocampal slices via AMPA and GABA receptors.* European neuropsychopharmacology, 2002. **12**(3): p. 209-216.
320. Bouron, A. and E. Lorrain, *[Cellular and molecular effects of the antidepressant hyperforin on brain cells: Review of the literature]*. L'Encephale, 2014. **40**(2): p. 108-113.
321. Barnes, J., L.A. Anderson, and J.D. Phillipson, *St John's wort (Hypericum perforatum L.): a review of its chemistry, pharmacology and clinical properties.* Journal of pharmacy and pharmacology, 2001. **53**(5): p. 583-600.
322. Cervo, L., et al., *Role of hyperforin in the antidepressant-like activity of Hypericum perforatum extracts.* Psychopharmacology, 2002. **164**(4): p. 423-428.
323. Zanoli, P., *Role of hyperforin in the pharmacological activities of St. John's Wort.* CNS drug reviews, 2004. **10**(3): p. 203-218.
324. Müller, W., A. Singer, and M. Wonnemann, *Hyperforin--antidepressant activity by a novel mechanism of action.* Pharmacopsychiatry, 2001. **34**: p. S98-102.
325. Vormfelde, S.V., W. Poser, and B. Gaster, *Hyperforin in extracts of St John's wort (Hypericum perforatum) for depression.* Archives of internal medicine, 2000. **160**(16): p. 2548.
326. Chatterjee, S., et al., *Antidepressant activity of Hypericum perforatum and hyperforin: the neglected possibility.* Pharmacopsychiatry, 1998. **31**: p. 7-15.
327. Jones, B.J. and T.P. Blackburn, *The medical benefit of 5-HT research.* Pharmacology Biochemistry and Behavior, 2002. **71**(4): p. 555-568.
328. Crupi, R., et al., *Hypericum perforatum treatment: effect on behaviour and neurogenesis in a chronic stress model in mice.* BMC Complement Altern Med, 2011. **11**: p. 7.
329. Kumar, V., et al., *Effect of Indian Hypericum perforatum Linn on animal models of cognitive dysfunction.* Journal of ethnopharmacology, 2000. **72**(1): p. 119-128.
330. Can, O.D., Y. Oztürk, and U.D. Ozkay, *Effects of insulin and St. John's Wort treatments on anxiety, locomotory activity, depression, and active learning*

*parameters of streptozotocin-diabetic rats.* Planta medica, 2011. **77**(18): p. 1970-1976.

331. Hasanein, P. and S. Shahidi, *Effects of Hypericum perforatum extract on diabetes-induced learning and memory impairment in rats.* Phytotherapy Research, 2011. **25**(4): p. 544-549.
332. Husain, G.M., P.N. Singh, and V. Kumar, *Beneficial effects of a standardized Hypericum perforatum extract in rats with experimentally induced hyperglycemia.* Drug discoveries & therapeutics, 2009. **3**(5): p. 215-220.
333. Amini, Z., et al., *St. John's Wort inhibits adipocyte differentiation and induces insulin resistance in adipocytes.* Biochemical and biophysical research communications, 2009. **388**(1): p. 146-149.
334. Subhan, F., et al., *Antagonism of antinociceptive effect of hydro-ethanolic extract of Hypericum perforatum Linn. by a non selective opioid receptor antagonist, naloxone.* Pakistan journal of biological sciences: PJBS, 2007. **10**(5): p. 792-796.
335. Khan, M., et al., *Nature cures nature: Hypericum perforatum attenuates physical withdrawal signs in opium dependent rats.* Pharmaceutical biology, 2013. **52**(5): p. 586-590.
336. Feily, A. and N. Abbasi, *The inhibitory effect of Hypericum perforatum extract on morphine withdrawal syndrome in rat and comparison with clonidine.* Phytotherapy Research, 2009. **23**(11): p. 1549-1552.
337. Meinke, M.C., et al., *In vivo photoprotective and anti-inflammatory effect of hyperforin is associated with high antioxidant activity in vitro and ex vivo.* European Journal of Pharmaceutics and Biopharmaceutics, 2012. **81**(2): p. 346-350.
338. Dubey, G., et al., *Individuals at Risk of Coronary Heart Disease (CHD), its Prevention and Management by an Indigenous Compound.* Ancient science of life, 2000. **20**(1-2): p. 48.
339. Prisăcaru, A.I., et al., *Evaluation of the wound-healing effect of a novel Hypericum perforatum ointment in skin injury.* Rom J Morphol Embryol, 2013. **54**(4): p. 1053-9.
340. You, M.-K., et al., *Effect of St. John's Wort (Hypericum perforatum) on obesity, lipid metabolism and uterine epithelial proliferation in ovariectomized rats.* Nutrition research and practice, 2014. **8**(3): p. 292-296.

341. Khalifeh, M.S., et al., *Clinical and histopathological evaluation of MDP/collagen induced arthritis rat model (MCIA) after treatment with Urtica dioica, Plantago major and Hypericum perforatum L herbal mixture.* Experimental animals, 2008. **57**(2): p. 101-110.
342. Izzo, A., *Drug interactions with St. John's Wort (Hypericum perforatum): a review of the clinical evidence.* International journal of clinical pharmacology and therapeutics, 2004. **42**(3): p. 139-148.
343. Di, Y.M., et al., *Clinical Drugs that Interact with St. John's Wort and mplication in Drug Development.* Current pharmaceutical design, 2008. **14**(17): p. 1723-1742.
344. Zhou, S.-F. and X. Lai, *An update on clinical drug interactions with the herbal antidepressant St. John's wort.* Current drug metabolism, 2008. **9**(5): p. 394-409.
345. Altamura, A.C., A.R. Moro, and M. Percudani, *Clinical pharmacokinetics of fluoxetine.* Clinical pharmacokinetics, 1994. **26**(3): p. 201-214.
346. Katzung, B.G., S.B. Masters, and A.J. Trevor, *Basic & clinical pharmacology*. 2004.
347. Goodman, L.S., *Goodman and Gilman's the pharmacological basis of therapeutics*. Vol. 1549. 1996: McGraw-Hill New York.
348. Biagetti, B. and R. Corcoy, *Hypoglycemia associated with fluoxetine treatment in a patient with type 1 diabetes.* World Journal of Clinical Cases: WJCC, 2013. **1**(5): p. 169.
349. De Long, N.E., et al., *Fluoxetine-induced pancreatic beta cell dysfunction: New insight into the benefits of folic acid in the treatment of depression.* Journal of affective disorders, 2014. **166**: p. 6-13.
350. Isaac, R., et al., *Selective serotonin reuptake inhibitors (SSRIs) inhibit insulin secretion and action in pancreatic β cells.* Journal of Biological Chemistry, 2013. **288**(8): p. 5682-5693.
351. Khoza, S. and J.C. Barner, *Glucose dysregulation associated with antidepressant agents: an analysis of 17 published case reports.* International journal of clinical pharmacy, 2011. **33**(3): p. 484-492.
352. Wernicke, J., *The side effect profile and safety of fluoxetine.* The Journal of clinical psychiatry, 1985. **46**(3 Pt 2): p. 59-67.

353. Meltzer, H., et al., *Extrapyramidal side effects and increased serum prolactin following fluoxetine, a new antidepressant.* Journal of neural transmission, 1979. **45**(2): p. 165-175.
354. Brambilla, P., et al., *Side-effect profile of fluoxetine in comparison with other SSRIs, tricyclic and newer antidepressants: a meta-analysis of clinical trial data.* Pharmacopsychiatry, 2005(38): p. 69-77.
355. Lane, R.M., *SSRI-induced extrapyramidal side-effects and akathisia: implications for treatment.* Journal of psychopharmacology, 1998. **12**(2): p. 192-214.
356. Zajecka, J., K.A. Tracy, and S. Mitchell, *Discontinuation symptoms after treatment with serotonin reuptake inhibitors: a literature review.* Journal of Clinical Psychiatry, 1997. **58**(7): p. 291-297.
357. Ciraulo, D.A. and R.I. Shader, *Pharmacotherapy of depression*. 2004: Springer.
358. Ciraulo, D.A. and R.I. Shader, *Fluoxetine drug-drug interactions: I. Antidepressants and antipsychotics.* Journal of clinical psychopharmacology, 1990. **10**(1): p. 48-50.
359. Sternbach, H., *The serotonin syndrome.* Am J Psychiatry, 1991. **148**(6): p. 705-713.
360. Hanley, A.J., et al., *Effect of Rosiglitazone and Ramipril on β-Cell Function in People With Impaired Glucose Tolerance or Impaired Fasting Glucose The DREAM trial.* Diabetes Care, 2010. **33**(3): p. 608-613.
361. Tahara, A., A. Matsuyama-Yokono, and M. Shibasaki, *Effects of antidiabetic drugs in high-fat diet and streptozotocin–nicotinamide-induced type 2 diabetic mice.* European journal of pharmacology, 2011. **655**(1): p. 108-116.
362. Awara, W.M., et al., *Insulinotropic and anti-inflammatory effects of rosiglitazone in experimental autoimmune diabetes.* The Review of Diabetic Studies, 2005. **2**(3): p. 146.
363. Hsu, B.-S., et al. *Enhancing islet engraftment with rosiglitazone*. in *Transplantation proceedings*. 2005: Elsevier.
364. Jahrling, J.B., et al., *PPARγ Recruitment to Active ERK during Memory Consolidation Is Required for Alzheimer's Disease-Related Cognitive Enhancement.* The Journal of neuroscience, 2014. **34**(11): p. 4054-4063.
365. Harrington, C., et al., *Rosiglitazone does not improve cognition or global function when used as adjunctive therapy to AChE inhibitors in mild-to-moderate Alzheimer's disease: two phase 3 studies.* Current Alzheimer research, 2011. **8**(5): p. 592-606.

366. Seaquist, E.R., et al., *Effect of thiazolidinediones and insulin on cognitive outcomes in ACCORD-MIND.* Journal of Diabetes and its Complications, 2013. **27**(5): p. 485-491.
367. Sharma, A.N., K.M. Elased, and J.B. Lucot, *Rosiglitazone treatment reversed depression-but not psychosis-like behavior of db/db diabetic mice.* Journal of psychopharmacology, 2012. **26**(5): p. 724-732.
368. Sun, H., et al., *Peroxisome proliferator-activated receptor gamma agonist, rosiglitazone, suppresses CD40 expression and attenuates inflammatory responses after lithium pilocarpine-induced status epilepticus in rats.* International Journal of Developmental Neuroscience, 2008. **26**(5): p. 505-515.
369. Nissen, S.E. and K. Wolski, *Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes.* New England Journal of Medicine, 2007. **356**(24): p. 2457-2471.
370. Singh, S., Y.K. Loke, and C.D. Furberg, *Long-term risk of cardiovascular events with rosiglitazone: a meta-analysis.* Jama, 2007. **298**(10): p. 1189-1195.
371. Loke, Y.K., S. Singh, and C.D. Furberg, *Long-term use of thiazolidinediones and fractures in type 2 diabetes: a meta-analysis.* Canadian Medical Association Journal, 2009. **180**(1): p. 32-39.
372. Mannucci, E., et al., *Cardiac safety profile of rosiglitazone: a comprehensive meta-analysis of randomized clinical trials.* International journal of cardiology, 2010. **143**(2): p. 135-140.
373. Evans, W.C., *Trease and Evans' pharmacognosy*. 2009: Elsevier Health Sciences.
374. Kokate, C., A. Purohit, and S. Gokhale, *Pharmacognosy.* Nirali prakashan, 2006. **42**.
375. Li, M. and M. Lu, *[Determination of total scopoletin in Caulis Erycibes by RP-HPLC]*. Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica, 1999. **24**(1): p. 41-3, 64.
376. Li, W. and J.F. Fitzloff, *High performance liquid chromatographic analysis of St. John's wort with photodiode array detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 2001. **765**(1): p. 99-105.
377. Liu, F., et al., *Liquid chromatography–mass spectrometry studies of St. John's wort methanol extraction: active constituents and their transformation.* Journal of pharmaceutical and biomedical analysis, 2005. **37**(2): p. 303-312.

378. Ahangarpour, A., M. Mohammadian, and M. Dianat, *Antidiabetic effect of hydroalcholic urticadioica leaf extract in male rats with fructose-induced insulin resistance.* Iran J Med Sci, 2012. **37**(3): p. 181-6.
379. Dulawa, S.C., et al., *Effects of chronic fluoxetine in animal models of anxiety and depression.* Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology, 2004. **29**(7): p. 1321-1330.
380. Lee, K.S., et al., *Modulation of airway remodeling and airway inflammation by peroxisome proliferator-activated receptor γ in a murine model of toluene diisocyanate-induced asthma.* The journal of Immunology, 2006. **177**(8): p. 5248-5257.
381. Bhutani, M.K., M. Bishnoi, and S.K. Kulkarni, *Anti-depressant like effect of curcumin and its combination with piperine in unpredictable chronic stress-induced behavioral, biochemical and neurochemical changes.* Pharmacology Biochemistry and Behavior, 2009. **92**(1): p. 39-43.
382. Katz, R.J., *Animal models and human depressive disorders.* Neuroscience & Biobehavioral Reviews, 1981. **5**(2): p. 231-246.
383. Kulkarni, S. and A.K. Mehta, *Purine nucleoside—mediated immobility in mice: reversal by antidepressants.* Psychopharmacology, 1985. **85**(4): p. 460-463.
384. Steru, L., et al., *The tail suspension test: a new method for screening antidepressants in mice.* Psychopharmacology, 1985. **85**(3): p. 367-370.
385. Banasr, M. and R.S. Duman, *Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors.* Biological psychiatry, 2008. **64**(10): p. 863-870.
386. Udayabanu, M., D. Kumaran, and A. Katyal, *Free chelatable zinc modulates the cholinergic function during hypobaric hypoxia-induced neuronal damage: an in vivo study.* Neuroscience, 2012. **202**: p. 434-445.
387. Mahesh, R., et al., *Antidepressant-like activity of (4-phenylpiperazin-1-yl)(quinoxalin-2-yl) methanone (4a), a novel 5-HT3 receptor antagonist: An investigation in behaviour-based rodent models of depression.* Indian journal of pharmacology, 2012. **44**(5): p. 560.
388. Potapenko, E.S., et al., *Altered astrocyte glutamate transporter regulation of hypothalamic neurosecretory neurons in heart failure rats.* American Journal of

Physiology-Regulatory, Integrative and Comparative Physiology, 2012. **303**(3): p. R291-R300.

389. Sheikh, N., et al., *Effect of Bacopa monniera on stress induced changes in plasma corticosterone and brain monoamines in rats.* Journal of ethnopharmacology, 2007. **111**(3): p. 671-676.
390. Ohkawa, H., N. Ohishi, and K. Yagi, *Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction.* Analytical biochemistry, 1979. **95**(2): p. 351-358.
391. Patel, S.S. and M. Udayabanu, *Urtica dioica extract attenuates depressive like behavior and associative memory dysfunction in dexamethasone induced diabetic mice.* Metabolic brain disease, 2014. **29**(1): p. 121-130.
392. Muttigi, M.S., et al., *Glutathione S-Transferase activity and total thiol status in chronic alcohol abusers before and 30 days after alcohol abstinence.* Online Journal of Health and Allied Sciences, 2009. **8**(1).
393. Hamilton, M., *Development of a rating scale for primary depressive illness.* British journal of social and clinical psychology, 1967. **6**(4): p. 278-296.
394. Strekalova, T., et al., *Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration.* Neuropsychopharmacology, 2004. **29**(11): p. 2007-2017.
395. Harkin, A., D.D. Houlihan, and J.P. Kelly, *Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine.* Journal of psychopharmacology, 2002. **16**(2): p. 115-123.
396. Chahardehi, A.M., et al., *Antidepressant-like Effects of Elatostema umbellatum and Urtica dioica in Mice Using Forced Swim Test and Tail Suspension Test.* Annual Research & Review in Biology, 2014. **4**(14): p. 2396-2405.
397. Capra, J.C., et al., *Antidepressant-like effect of scopoletin, a coumarin isolated from Polygala sabulosa (Polygalaceae) in mice: evidence for the involvement of monoaminergic systems.* European journal of pharmacology, 2010. **643**(2): p. 232-238.
398. Dimpfel, W., *Rat electropharmacograms of the flavonoids rutin and quercetin in comparison to those of moclobemide and clinically used reference drugs suggest antidepressive and/or neuroprotective action.* Phytomedicine, 2009. **16**(4): p. 287-294.

399. Bukhari, I. and A. Dar, *Behavioral profile of Hypericum perforatum (St. John's Wort) extract. A comparison with standard antidepressants in animal models of depression.* European review for medical and pharmacological sciences, 2013. **17**(8): p. 1082-1089.

400. Borges Filho, C., et al., *Chronic unpredictable mild stress decreases BDNF and NGF levels and Na+, K+-ATPase activity in the hippocampus and prefrontal cortex of mice: Antidepressant effect of chrysin.* Neuroscience, 2015.

401. Shimizu, Y., et al., *Neurocognitive impairments and quality of life in unemployed patients with remitted major depressive disorder.* Psychiatry research, 2013. **210**(3): p. 913-918.

402. Bisaz, R., M. Schachner, and C. Sandi, *Causal evidence for the involvement of the neural cell adhesion molecule, NCAM, in chronic stress-induced cognitive impairments.* Hippocampus, 2011. **21**(1): p. 56-71.

403. Gumuslu, E., et al., *The effects of tianeptine, olanzapine and fluoxetine on the cognitive behaviors of unpredictable chronic mild stress-exposed mice.* Drug research, 2013. **63**(10): p. 532-539.

404. Kumar, A., et al., *Protective effect of quercetin against ICV colchicine-induced cognitive dysfunctions and oxidative damage in rats.* Phytotherapy Research, 2008. **22**(12): p. 1563-1569.

405. Kumar, A., P. Rinwa, and H. Dhar, *Possible nitric oxide modulation in the protective effects of rutin against experimental head trauma–induced cognitive deficits: behavioral, biochemical, and molecular correlates.* journal of surgical research, 2014. **188**(1): p. 268-279.

406. Zhang, W., et al., *Amelioration of insulin resistance by scopoletin in high-glucose-induced, insulin-resistant HepG2 cells.* Hormone and metabolic research= Hormon- und Stoffwechselforschung= Hormones et metabolisme, 2010. **42**(13): p. 930-935.

407. O'Reilly, J.-A. and M. Lynch, *Rosiglitazone Improves Spatial Memory and Decreases Insoluble Aβ1–42 in APP/PS1 Mice.* Journal of Neuroimmune Pharmacology, 2012. **7**(1): p. 140-144.

408. Hölscher, C., *Inhibitors of cyclooxygenases produce amnesia for a passive avoidance task in the chick.* European Journal of Neuroscience, 1995. **7**(6): p. 1360-1365.

409. Johnson, D., et al., *Effects of hypericum extract LI 160 compared with maprotiline on resting EEG and evoked potentials in 24 volunteers.* Journal of geriatric psychiatry and neurology, 1994. **7**(1): p. S44-S46.
410. Klusa, V., et al., *Hypericum extract and hyperforin: memory-enhancing properties in rodents.* Pharmacopsychiatry, 2001. **34**: p. S61-9.
411. Song, L., et al., *Impairment of the spatial learning and memory induced by learned helplessness and chronic mild stress.* Pharmacology Biochemistry and Behavior, 2006. **83**(2): p. 186-193.
412. Metz, G.A., N.M. Jadavji, and L.K. Smith, *Modulation of motor function by stress: a novel concept of the effects of stress and corticosterone on behavior.* European Journal of Neuroscience, 2005. **22**(5): p. 1190-1200.
413. Detka, J., et al., *Neuroendocrine link between stress, depression and diabetes.* Pharmacological Reports, 2013. **65**(6): p. 1591-1600.
414. Haynes, L., et al., *Dexamethasone induces limited apoptosis and extensive sublethal damage to specific subregions of the striatum and hippocampus: implications for mood disorders.* Neuroscience, 2001. **104**(1): p. 57-69.
415. Yang, G., et al., *[Mechanism of rosiglitasone to improve glucose-uptake of 3T3-L1 adipocyte with insulin resistance induced by dexamethasone and insulin]*. Sichuan da xue xue bao. Yi xue ban= Journal of Sichuan University. Medical science edition, 2007. **38**(5): p. 816-818.
416. Datusalia, A.K. and S.S. Sharma, *Amelioration of diabetes-induced cognitive deficits by GSK-3β inhibition is attributed to modulation of neurotransmitters and neuroinflammation.* Molecular neurobiology, 2014. **50**(2): p. 390-405.
417. Mielke, J.G. and Y. Wang, *Insulin, synaptic function, and opportunities for neuroprotection.* Progress in molecular biology and translational science, 2010. **98**: p. 133-186.
418. Agrawal, R., et al., *Insulin receptor signaling in rat hippocampus: a study in STZ (ICV) induced memory deficit model.* European neuropsychopharmacology, 2011. **21**(3): p. 261-273.
419. Qi, W.-w., et al., *Hyperglycemia Induces the Variations of 11* Experimental diabetes research, 2012. **2012**.

420. Vannucci, S.J., et al., *GLUT4 glucose transporter expression in rodent brain: effect of diabetes.* Brain research, 1998. **797**(1): p. 1-11.
421. Malberg, J.E., et al., *Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus.* The Journal of neuroscience, 2000. **20**(24): p. 9104-9110.
422. Traiffort, E., E. Angot, and M. Ruat, *Sonic Hedgehog signaling in the mammalian brain.* Journal of neurochemistry, 2010. **113**(3): p. 576-590.
423. Ruat, M., et al., *Hedgehog trafficking, cilia and brain functions.* Differentiation, 2012. **83**(2): p. S97-S104.
424. Ruat, M., et al., *Targeting of Smoothened for therapeutic gain.* Trends in Pharmacological Sciences, 2014. **35**(5): p. 237-246.
425. Binder, D.K. and H.E. Scharfman, *Brain-derived neurotrophic factor.* Growth Factors, 2004. **22**(3): p. 123-31.
426. Schmidt, H.D. and R.S. Duman, *The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior.* Behavioural pharmacology, 2007. **18**(5-6): p. 391-418.
427. Autry, A.E. and L.M. Monteggia, *Brain-derived neurotrophic factor and neuropsychiatric disorders.* Pharmacological Reviews, 2012. **64**(2): p. 238-258.
428. Chen, M., et al., *Norepinephrine induces BDNF and activates the PI-3K and MAPK cascades in embryonic hippocampal neurons.* Cellular signalling, 2007. **19**(1): p. 114-128.
429. Balmanno, K. and S.J. Cook, *Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells.* Oncogene, 1999. **18**(20): p. 3085-3097.
430. Zusso, M., et al., *Fluoxetine-induced proliferation and differentiation of neural progenitor cells isolated from rat postnatal cerebellum.* Biochemical pharmacology, 2008. **76**(3): p. 391-403.
431. Harry, G.J., et al., *Trimethyltin-induced neurogenesis in the murine hippocampus.* Neurotoxicity research, 2003. **5**(8): p. 623-627.
432. Gold, P.E., *Acetylcholine modulation of neural systems involved in learning and memory.* Neurobiology of learning and memory, 2003. **80**(3): p. 194-210.
433. Zhao, T., et al., *Effects of chronic social defeat stress on behavior and choline acetyltransferase, 78-kDa glucose-regulated protein, and CCAAT/enhancer-binding*

*protein (C/EBP) homologous protein in adult mice.* Psychopharmacology, 2013. **228**(2): p. 217-230.

434. Dang, H., et al., *Preventive action of Kai Xin San aqueous extract on depressive-like symptoms and cognition deficit induced by chronic mild stress.* Experimental Biology and Medicine, 2009. **234**(7): p. 785-793.
435. Maratha, S.R. and N. Mahadevan, *Memory enhancing activity of naringin in unstressed and stressed mice: possible cholinergic and nitriergic modulation.* Neurochemical research, 2012. **37**(10): p. 2206-2212.
436. Müller, T.C., et al., *Antidepressants inhibit human acetylcholinesterase and butyrylcholinesterase activity.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2002. **1587**(1): p. 92-98.
437. Hornick, A., et al., *The coumarin scopoletin potentiates acetylcholine release from synaptosomes, amplifies hippocampal long-term potentiation and ameliorates anticholinergic-and age-impaired memory.* Neuroscience, 2011. **197**: p. 280-292.
438. Norimoto, H., et al., *Muscarinic receptor activation disrupts hippocampal sharp wave-ripples.* Brain research, 2012. **1461**: p. 1-9.
439. Nathanson, N.M., *Synthesis, trafficking, and localization of muscarinic acetylcholine receptors.* Pharmacology & therapeutics, 2008. **119**(1): p. 33-43.
440. Van Kampen, J.M. and C.B. Eckman, *Agonist-induced restoration of hippocampal neurogenesis and cognitive improvement in a model of cholinergic denervation.* Neuropharmacology, 2010. **58**(6): p. 921-929.
441. Inada, C., et al., *Endogenous acetylcholine rescues NMDA-induced long-lasting hippocampal cell damage via stimulation of muscarinic M 1 receptors: Elucidation using organic hippocampal slice cultures.* European journal of pharmacology, 2013. **699**(1): p. 150-159.
442. Veena, J., B.S. Rao, and B. Srikumar, *Regulation of adult neurogenesis in the hippocampus by stress, acetylcholine and dopamine.* Journal of natural science, biology, and medicine, 2011. **2**(1): p. 26.
443. Volpicelli-Daley, L.A., et al., *Altered striatal function and muscarinic cholinergic receptors in acetylcholinesterase knockout mice.* Molecular pharmacology, 2003. **64**(6): p. 1309-1316.

444. Stein, I.S. and J.W. Hell, *CaMKII hunkers down on the muscarinic M4 receptor to help curb cocaine-induced hyperlocomotion.* The EMBO journal, 2010. **29**(12): p. 1943-1945.

445. Overstreet, D.H., *The Flinders sensitive line rats: a genetic animal model of depression.* Neuroscience & Biobehavioral Reviews, 1993. **17**(1): p. 51-68.

446. Zhang, X., et al., *Effects of Ning Shen Ling Granule (宁神灵冲剂) and dehydroepiandrosterone on cognitive function in mice undergoing chronic mild stress.* Chinese journal of integrative medicine, 2007. **13**: p. 46-49.

447. Manji, H.K., W.C. Drevets, and D.S. Charney, *The cellular neurobiology of depression.* Nature medicine, 2001. **7**(5): p. 541-547.

448. Lucassen, P.J., et al., *Chronic psychosocial stress differentially affects apoptosis in hippocampal subregions and cortex of the adult tree shrew.* European Journal of Neuroscience, 2001. **14**(1): p. 161-166.

449. Cheung, H.H., et al., *Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs.* Experimental cell research, 2006. **312**(12): p. 2347-2357.

450. Ahmad, A., et al., *Alterations in monoamine levels and oxidative systems in frontal cortex, striatum, and hippocampus of the rat brain during chronic unpredictable stress.* Stress: The International Journal on the Biology of Stress, 2010. **13**(4): p. 356-365.

451. Wilson, C.J., C.E. Finch, and H.J. Cohen, *Cytokines and cognition—the case for a head-to-toe inflammatory paradigm.* Journal of the American Geriatrics Society, 2002. **50**(12): p. 2041-2056.

452. Lian, T. and S. An, *[Antidepressant effect of microinjection of neuropeptide Y into the hippocampus is mediated by decreased expression of nitric oxide synthase]*. Sheng li xue bao:[Acta physiologica Sinica], 2010. **62**(3): p. 237-246.

453. García-Bueno, B., et al., *Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats.* Biological psychiatry, 2005. **57**(8): p. 885-894.

454. Loane, D.J., et al., *Interleukin-4 mediates the neuroprotective effects of rosiglitazone in the aged brain.* Neurobiology of aging, 2009. **30**(6): p. 920-931.

455. da Silva, A.I., et al., *Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life.* Canadian journal of physiology and pharmacology, 2014. **92**(4): p. 330-337.
456. Saleh, L.A., et al., *Ibuprofen suppresses depressive like behavior induced by BCG inoculation in mice: role of nitric oxide and prostaglandin.* Pharmacology Biochemistry and Behavior, 2014. **125**: p. 29-39.
457. Uchida, S., et al., *Antinociceptive effects of St. John's wort, Harpagophytum procumbens extract and Grape seed proanthocyanidins extract in mice.* Biological and Pharmaceutical Bulletin, 2008. **31**(2): p. 240-245.
458. Friederich, M., P. Hansell, and F. Palm, *Diabetes, oxidative stress, nitric oxide and mitochondria function.* Current diabetes reviews, 2009. **5**(2): p. 120-144.
459. Mathis, D., L. Vence, and C. Benoist, *beta-Cell death during progression to diabetes.* Nature, 2001. **414**(6865): p. 792-8.
460. Ryan, C.M., *Neurobehavioral complications of type I diabetes: examination of possible risk factors.* Diabetes Care, 1988. **11**(1): p. 86-93.
461. F Adeniyi, A., J. O Adeleye, and C. Y Adeniyi, *Diabetes, sexual dysfunction and therapeutic exercise: a 20 year review.* Current diabetes reviews, 2010. **6**(4): p. 201-206.
462. Singh, J., et al., *Suppl 2: Medicinal Chemistry of the Anti-Diabetic Effects of Momordica Charantia: Active Constituents and Modes of Actions.* The open medicinal chemistry journal, 2011. **5**: p. 70.
463. Panda, S. and A. Kar, *Evaluation of the antithyroid, antioxidative and antihyperglycemic activity of scopoletin from Aegle marmelos leaves in hyperthyroid rats.* Phytotherapy Research, 2006. **20**(12): p. 1103-1105.
464. Eid, H., et al., *The molecular basis of the antidiabetic action of quercetin in cultured skeletal muscle cells and hepatocytes.* Pharmacognosy Magazine, 2015. **11**(41): p. 74.
465. Niture, N.T., A.A. Ansari, and S.R. Naik, *Anti-hyperglycemic activity of rutin in streptozotocin-induced diabetic rats: an effect mediated through cytokines, antioxidants and lipid biomarkers.* Indian journal of experimental biology, 2014. **52**(7): p. 720-727.

466. Kang, K.S., et al., *Protective Effect of Esculin on Streptozotocin-Induced Diabetic Renal Damage in Mice.* Journal of Agricultural and Food Chemistry, 2014. **62**(9): p. 2069-2076.

467. Prabakaran, D. and N. Ashokkumar, *Protective effect of esculetin on hyperglycemia-mediated oxidative damage in the hepatic and renal tissues of experimental diabetic rats.* Biochimie, 2013. **95**(2): p. 366-373.

468. Prince, P. and N. Kamalakkannan, *Rutin improves glucose homeostasis in streptozotocin diabetic tissues by altering glycolytic and gluconeogenic enzymes.* Journal of biochemical and molecular toxicology, 2006. **20**(2): p. 96-102.

469. Vessal, M., M. Hemmati, and M. Vasei, *Antidiabetic effects of quercetin in streptozocin-induced diabetic rats.* Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 2003. **135**(3): p. 357-364.

470. Miyata, S., S. Hirano, and J. Kamei, *Abnormal benzodiazepine receptor function in the depressive-like behavior of diabetic mice.* Pharmacology Biochemistry and Behavior, 2005. **82**(4): p. 615-620.

471. Hirano, S., S. Miyata, and J. Kamei, *Antidepressant-like effect of leptin in streptozotocin-induced diabetic mice.* Pharmacology Biochemistry and Behavior, 2007. **86**(1): p. 27-31.

472. Chen, C., et al., *Contribution of neural cell death to depressive phenotypes of streptozotocin-induced diabetic mice.* Disease models & mechanisms, 2014. **7**(6): p. 723-730.

473. Grzeda, E., R. Wiśniewska, and K. Wiśniewski, *Effect of an NMDA receptor agonist on T-maze and passive avoidance test in 12-week streptozotocin-induced diabetic rats.* Pharmacological reports: PR, 2006. **59**(6): p. 656-663.

474. Wang, T., et al., *Danshensu ameliorates the cognitive decline in streptozotocin-induced diabetic mice by attenuating advanced glycation end product-mediated neuroinflammation.* Journal of neuroimmunology, 2012. **245**(1): p. 79-86.

475. Chen, Z., et al., *Effect of sericin on diabetic hippocampal growth hormone/insulin-like growth factor 1 axis.* Neural regeneration research, 2013. **8**(19): p. 1756.

476. Chen, S., et al., *Glucagon-like peptide-1 protects hippocampal neurons against advanced glycation end product-induced tau hyperphosphorylation.* Neuroscience, 2014. **256**: p. 137-146.

477. Lee, S.-M., et al., *Quercetin up-regulates expressions of peroxisome proliferator-activated receptor γ, liver X receptor α, and ATP binding cassette transporter A1 genes and increases cholesterol efflux in human macrophage cell line.* Nutrition Research, 2013. **33**(2): p. 136-143.
478. Bhutada, P., et al., *Ameliorative effect of quercetin on memory dysfunction in streptozotocin-induced diabetic rats.* Neurobiology of learning and memory, 2010. **94**(3): p. 293-302.
479. Cai, Y., et al., *Effects of rutin on the expression of PPARγ in skeletal muscles of db/db mice.* Planta medica, 2012. **78**(9): p. 861-865.
480. Surse, V.M., J. Gupta, and K. Tikoo, *Esculetin induced changes in Mmp13 and Bmp6 gene expression and histone H3 modifications attenuate development of glomerulosclerosis in diabetic rats.* Journal of molecular endocrinology, 2011. **46**(3): p. 245-254.
481. Kavak, S., et al., *The effects of rosiglitazone on oxidative stress and lipid profile in left ventricular muscles of diabetic rats.* Cell biochemistry and function, 2008. **26**(4): p. 478-485.
482. Hernandez, R., T. Teruel, and M. Lorenzo, *Rosiglitazone produces insulin sensitisation by increasing expression of the insulin receptor and its tyrosine kinase activity in brown adipocytes.* Diabetologia, 2003. **46**(12): p. 1618-1628.
483. Wang, L., et al., *Rosiglitazone protects neuroblastoma cells against advanced glycation end products-induced injury.* Acta Pharmacologica Sinica, 2011. **32**(8): p. 991-998.
484. Park, J.H., et al., *Esculetin promotes type I procollagen expression in human dermal fibroblasts through MAPK and PI3K/Akt pathways.* Molecular and cellular biochemistry, 2012. **368**(1-2): p. 61-67.
485. Gandhi, G.R., et al., *Insulin sensitization via partial agonism of PPARγ and glucose uptake through translocation and activation of GLUT4 in PI3K/p-Akt signaling pathway by embelin in type 2 diabetic rats.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2013. **1830**(1): p. 2243-2255.
486. Kennedy, J.W., et al., *Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes.* Diabetes, 1999. **48**(5): p. 1192-1197.

487. Xu, M., et al., *Quercetin differently regulates insulin-mediated glucose transporter 4 translocation under basal and inflammatory conditions in adipocytes.* Molecular nutrition & food research, 2014. **58**(5): p. 931-941.
488. Anisuzzaman, A.S.M., et al., *Novel contribution of cell surface and intracellular M1-muscarinic acetylcholine receptors to synaptic plasticity in hippocampus.* Journal of neurochemistry, 2013. **126**(3): p. 360-371.
489. Abdalla, F.H., et al., *Quercetin protects the impairment of memory and anxiogenic-like behavior in rats exposed to cadmium: Possible involvement of the acetylcholinesterase and Na+, K+-ATPase activities.* Physiology & behavior, 2014. **135**: p. 152-167.
490. Beckmann, D.V., et al., *Neuroprotective role of quercetin in locomotor activities and cholinergic neurotransmission in rats experimentally demyelinated with ethidium bromide.* Life sciences, 2014. **103**(2): p. 79-87.
491. Caletti, G., et al., *Antidepressant dose of taurine increases mRNA expression of GABA A receptor α 2 subunit and BDNF in the hippocampus of diabetic rats.* Behavioural brain research, 2015.
492. Agrawal, R., et al., *Deterioration of plasticity and metabolic homeostasis in the brain of the UCD-T2DM rat model of naturally occurring type-2 diabetes.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2014. **1842**(9): p. 1313-1323.
493. Tiwari, S.K., et al., *Inhibitory Effects of Bisphenol-A on Neural Stem Cells Proliferation and Differentiation in the Rat Brain Are Dependent on Wnt/β-Catenin Pathway.* Molecular neurobiology, 2014: p. 1-23.
494. Yonguc, G.N., et al., *Grape seed extract has superior beneficial effects than vitamin E on oxidative stress and apoptosis in the hippocampus of streptozotocin induced diabetic rats.* Gene, 2015. **555**(2): p. 119-126.
495. Sun, L.-J., et al., *Fish oil modulates glycogen synthase kinase-3 signaling pathway in diabetes-induced hippocampal neurons apoptosis.* Brain research, 2014. **1574**: p. 37-49.
496. François, A., et al., *Impairment of autophagy in the central nervous system during lipopolysaccharide-induced inflammatory stress in mice.* Mol. Brain, 2014. **7**(56): p. 10.1186.

497. Santi, A., et al., *Effects of quercetin on oxidative stress biomarkers in methimazole-induced hypothyroid rats.* Experimental and clinical endocrinology & diabetes: official journal, German Society of Endocrinology [and] German Diabetes Association, 2014. **122**(9): p. 533-539.

498. Selvakumar, K., et al., *Protective role of quercetin on PCBs-induced oxidative stress and apoptosis in hippocampus of adult rats.* Neurochemical research, 2012. **37**(4): p. 708-721.

499. Lee, C.-R., et al., *Esculetin inhibits N-methyl-D-aspartate neurotoxicity via glutathione preservation in primary cortical cultures.* Laboratory animal research, 2011. **27**(3): p. 259-263.

500. Exner, M., et al., *The salicylate metabolite gentisic acid, but not the parent drug, inhibits glucose autoxidation-mediated atherogenic modification of low density lipoprotein.* FEBS letters, 2000. **470**(1): p. 47-50.

501. Wolff, S.P. and R. Dean, *Glucose autoxidation and protein modification. The potential role of'autoxidative glycosylation'in diabetes.* Biochem. j, 1987. **245**: p. 243-250.

*******