# Chapter 4: Effect of rosmarinic acid against combined stress-induced cardiac abnormalities in Wistar rats validated using in-silico and experimental studies

## 4.1 Introduction

In abundant preclinical and clinical studies, researchers showed that stress in early states of life may disturb the neuroendocrine system such as HPA-axis that contributes to depression and cardiac abnormalities in the adolescence and adulthood period [461-463]. Additionally, these stages of life are sensitive period of psychological, physiological, and behavioral changes which play a vital role in neurodevelopment and psychiatric ailments in both rodents and humans [464].

In our study, we used combined stress model including maternal separation (MS) model and chronic unpredictable stress (CUS) model to induce depressive-like behavior in rodents [465-469]. MS combined CUS work as a significant stress model for the induction of depression that may cause heart abnormalities [470, 471]. The combined stress model represents a situation where an individual unfortunately experiences both stress in the childhood and later in the life. A recent report suggested that childhood maltreatment (child neglect and abuse) and unpredictable stress may badly impact on health in a gender dependent [472].

National comorbidity study indicated that females have higher risk of depression whereas, males have higher risk of cardiac diseases [472]. However, childhood maltreatment increases the further chances of depression in males and cardiac disease in females [472, 473]. Therefore, childhood maltreatment may be considered as an independent risk factor for depression and cardiac diseases in the later life. In the previous study, we showed that CUS increased cardiac abnormalities in Wistar rats (Chapter 2). Similarly, other researchers reported that stress models may cause myocardial fibrosis, hypertrophy, increased density of vascular structure, and cardiovascular diseases [343, 345, 474].

In depression and cardiac diseases, humoral changes and overactivity of automatic nervous system are major link which converge into endothelial dysfunction that initiate cardiac anomalies [475, 476]. In addition, depression and cardiac abnormalities share several pathophysiological features [223, 360]. Briefly, overactivation of HPA-axis is the major cause of imbalance in sympathetic system, cytokines, and platelet aggregation that may cause cardiac dysfunctions [472, 477]. Moreover, imbalance in neurotrophic, serotonergic, and antioxidative system are major cause of inflammation, vasoconstriction, and hypertension [225, 226, 478, 479]. In antioxidative system imbalance, stress reduces the functions of antioxidative systems (superoxide dismutase, glutathione, catalase) whereas, increased free radicals are the roots for abnormal matrix metalloproteinase-2 (MMP-2) and degradation of cytoskeletal proteins causing cardiac dysfunction [227, 467].

Our previous studies (chapter 2 and chapter 3) stated that treatment with rosmarinic acid manage depression-associated cardiac anomalies in two separate stressor models. However, the effects of dual stress model (MS combined CUS) on cardiac functions has not been studied yet. Therefore, in this study, we hypothesized that with the early life stress and adulthood stress may worsen the cardiac anomalies and prophylactic administration of rosmarinic acid may reduce the severity of cardiac anomalies. To test this hypothesis, we designed experiments incorporating dual stress models (MS and CUS) to induce depression associated cardiac abnormalities. As per previous study, experiments were performed to estimate different parameters including forced swim test (immobility period), sucrose preference test (anhedonia behavior), body weight (low/high), adrenal gland hyperplasia, plasma BDNF, serotonin level in prefrontal cortex, plasma corticosterone *via* High-Performance Liquid Chromatography-Ultraviolet (HPLC-UV), ECG parameters (P-wave, QRS-complex, T-

wave, RR-interval, PR-interval, ST-segment, and QT-interval), cardiac biomarkers including creatine kinase-MB (CKMB), lactate dehydrogenase (LDH), cardiac troponin-I (cTn-I), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), MMP2 level in heart tissue, platelet aggregation using adenosine di-phosphate (ADP) and thrombin agent, oxidative stress parameters (SOD activity, lipid peroxidation, and nitrite level), gene expressions of IL-10, NF-kB, and TNF-α, myocardial tissue necrosis, and separation of myocardial fibres.

Further, we performed *in-silico* studies (network pharmacology and docking) to identify and validate primary drug targets which are shared by both depression and cardiac abnormalities. Network pharmacology establishes a network of "compound-targets/disease-targets" which is an integrative *in-silico* approach that identifies the symbiotic therapeutic mechanisms. This evolution shifted to "network target-multiple components-therapeutics" mode from "one target-one drug" mode paradigm. In the current epoch of large data, by simply comprehending the combinatorial characteristic of rosmarinic acid and their mechanisms of action [480]. Network pharmacology not only explores the molecular complexity of rosmarinic acid and other natural products but also offers systematic association of natural products with the complicated diseases [481]. Thus, network pharmacology has become a popular research tool to exploit the complexity of all natural products in the management and treatment of complex diseases [482].

## 4.2 Materials and methods

### 4.2.1 *In silico* study

#### 4.2.1.1 Network Pharmacology

The target genes and rosmarinic acid targets specific to depression-associated cardiac genes were retrieved from GeneCards database [483]. The GeneCards was used to obtained information related to target genes associated with depression, MI, and rosmarinic acid (accessed on 6 November 2022). GeneCards is an integrated database on human genes and annotation associated with target genes. Furthermore, for identification of common targets in depression, MI, and rosmarinic acid we used Venn diagram analysis as described previously [484]. The target genes of rosmarinic acid were compared with the disease (depression and MI) genes individually in VENNY 2.1 and common genes were selected for further analysis. These common genes were further analysed on ShinyGO analysis. In gene enrichment analysis, we used ShinyGO to analyse the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway resultant protein associated protein-protein interaction (accessed on 6 November 2022). Biological, molecular, and cellular processes were used to describe and analyse genomic information *via* ShinyGO. Gene function annotation information were retrieved by KEGG pathway which also involve in the metabolic pathways of targets associated depression and MI.

#### 4.2.1.2 Docking

The Auto Dock Tool 1.5.7, PyMOL, and Discovery studio 2021 were used for performing the computational investigation as described previously [484]. Matrix metalloproteinase (MMP-2; PDB ID: 7xgj), signal transducer and activator of transcription 3 (STAT3 for IL10; PDB ID: 6tlc), tropomyosin receptor kinase B (TrkB

for BDNF; PDB ID: 4asz), platelet 2y12 (P2Y12 for ADP; PDB ID: 4ntj), and thromboxane A2 (TXA2 for thrombin; PDB ID: 6iiv) were obtained from Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (PDB). To interpret the affinity of the ligands to the receptor, the lowest value of free binding energy and its Ki values were used.

### 4.2.2 Animals and Housing

Before experimentation, animals (Wistar rats) were acclimatized for seven days in animal house. Animals were obtained from Central Animal House (IMS, BHU), Varanasi, Uttar Pradesh, India. Experiments were performed according to the CPCSEA guidelines, Forest and Climate Changes, Government of India IIT(BHU)/IAEC/2023/069 During the whole experiment, experimental rats had received food pellets and water *ad libitum*.

### 4.2.3 Experimental design

After acclimatization period, rats were divided into different experimental groups (n = 6, nine group) and experiments were performed up to 75 days (*Figure 23*). During the experiment, MS combined CUS stress was applied for 75 days and treatments were administered orally. On the final day (day 75), animals were anesthetized using $CO_2$, and blood was collected through retro-orbital puncture and platelet aggregation study was performed as described previously [485]. After blood collection, plasma and serum were separated as previously described and stored at −20 °C for estimation of corticosterone, CKMB, LDH, cTn-I, AST, and ALT levels. The heart was excised, blotted with filter paper, and weighed. PFC part of brain was separated for the estimation of serotonin level as previously described [250]. The adrenal glands were

isolated to estimate adrenal hyperplasia. Oxidative stress was also determined in both heart and brain tissue homogenate as described previously (Chapter 2 and Chapter 3).

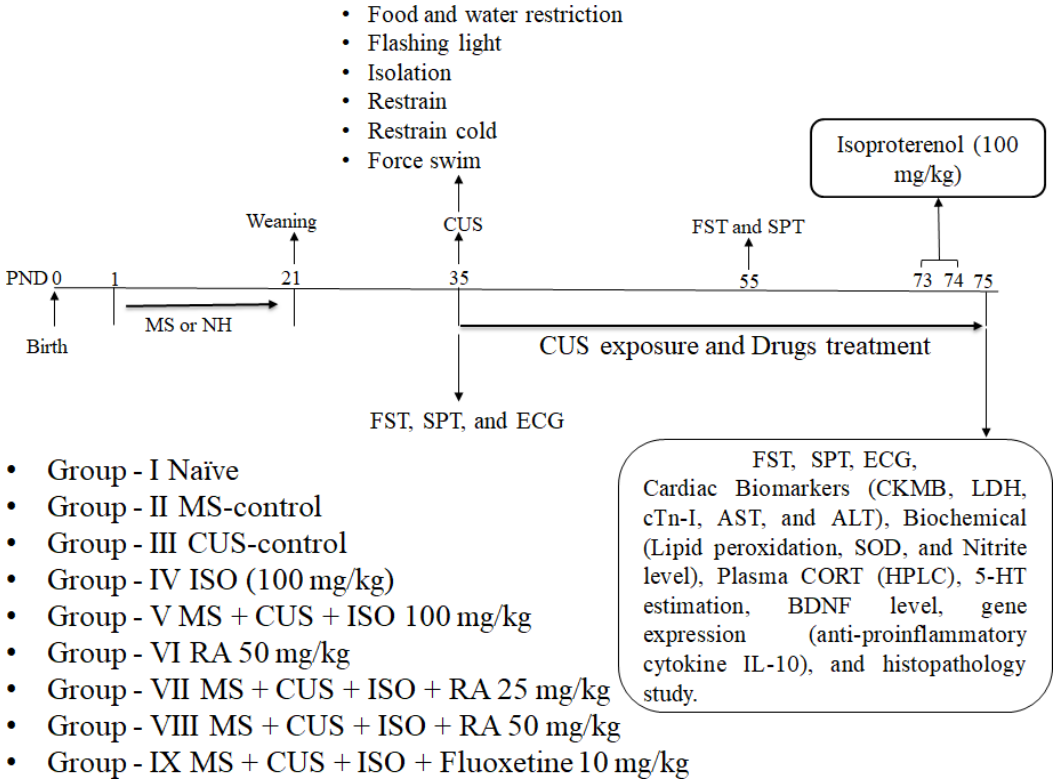



Figure 23: Schematic representation of experimental design.

RA; Rosmarinic Acid, PND; Postnatal Days, MS; Maternal Separation; NH; Non-Handled, CUS; Chronic Unpredictable Stress, ISO; Isoproterenol, FST; Forced Swim Test, SPT; Sucrose Preference Test, ECG; Electrocardiography, CK-MB; Creatin Kinase-MB, LDH; cTn-I; Cardiac Troponin-I, AST; Aspartate Aminotransferase, ALT; Alanine Aminotransferase, SOD; Superoxide Dismutase, Lactate Dehydrogenase, GSH; Glutathione, CORT; Corticosterone, HPLC; High Performance Liquid Chromatography, 5-HT; 5-hydroxytryptamine, BDNF; Brain Derived Neurotrophic Factor, IL-10; Interleukin-10

### 4.2.3.1 Maternal separation and CUS exposure

The protocol for MS and CUS exposure, we followed the procedure as described previously (Chapter 1 and Chapter 2). Briefly, after birth, the MS group pups were separated from their mother from post-natal day 1 (PND1) to post-natal day 21

(PND21) for 3 h (9:30 AM to 12:30 PM) each day. Except control group, all other groups received CUS exposure from day 35 to day 75. All animals were exposed to stressors for 40 days as per the CUS paradigm described in Chapter 2 of this thesis.

#### 4.2.3.2 Drug preparation and treatment

Rosmarinic acid (Sigma Aldrich, India), fluoxetine (Umang Pharmacy, IMS BHU, Varanasi), and isoproterenol (100 mg/kg) (Sigma, St. Louis, MO, USA) were procured. Drug preparation, doses, and treatments were performed as per described in Chapter 3.

#### 4.2.3.3 Forced swim test and sucrose preference test

The forced swim test and sucrose preference test were conducted on day 35, 55, and day 75 to measure the immobility period and anhedonia symptom, respectively as described in Chapter 2.

#### 4.2.3.4 Body weight

Body weight (weight loss or weight gain) is an vital parameter for the measurement of depressive-like behavior in rodents and humans [244, 486]. The body weights of all rats were noted throughout the experiment.

### 4.2.4 Electrocardiography

Electrocardiography was performed at day 35, 55, and day 75 during experimentation as described previously (Chapter 3). Briefly, ECG was recorded using 3 electrodes (red, black, and green) per rat (LabScribe software, iWorx-B3G) [246, 247].

### 4.2.4 Determination of heart weight index

All rats were sacrificed, the heart was excised, blotted with filter paper, and weighed. The heart weight index was interpreted as HWI = heart weight/body weight as per previous study [487].

### 4.2.5 Biochemical Analysis

#### 4.2.5.1 Estimation of plasma corticosterone using HPLC-UV

Corticosterone is an important core stress biomarker used in the assessment of depression in rodents [256-258]. Here, the concentration of corticosterone was quantified in plasma using HPLC-UV detector (Agilent), with minor changes, using dexamethasone (DXM) instead of 19-nortestosterone as internal standard according to previous studies [488-490]. Briefly, a known quantity of DXM in 0.5 mL plasma extracted with dichloromethane (5 mL). The dichloromethane extract was dried by evaporation and mixed with 0.1 mL of mobile phase. The chromatographic separation was conducted using a mobile phase consisted of $CH_3OH$: $H_2O$ (90:10), at flow rate 1.4 mL/minute, and corticosterone levels were detected at 250 nm wavelength. From a standard curve, the unknown plasma corticosterone levels were estimated and expressed as µg/mL.

#### 4.2.5.2 Enzyme linked immunosorbent assay and diagnostic kits

In rodents, a decreased concentration of BDNF and serotonin indicated as biomarkers of depressive-like behavior [250, 259]. In our study, the serotonin in PFC (ng/g) (L.D.N. Gm. & Co, Germany), BDNF in plasma (pg/mL) (My BioSource Inc.), cTn-I in serum (pg/mL) (Abcam, UK), and MMP-2 in heart tissue (ng/g) (IBL, USA) were assessed using commercially available ELISA kits as per manufacturer's instructions. Further, cardiac abnormalities of rats were evaluated by measuring

biomarkers such as CKMB, LDH, AST, and ALT levels in plasma and heart weight index (HWI). Diagnostic kits for CKMB, LDH, AST, and ALT were purchased from Accuax Biomedical, Gujarat, India. All the kits were used according to manufacturer's instructions and results were expressed as IU/L.

#### 4.2.5.3 Estimation of imbalance in oxidative stress and antioxidative systems

In psychiatric disorders and cardiac anomalies, imbalance in antioxidative systems is a major pathological issue that leads to oxidative stress and cell death [260]. Lipid peroxidation and SOD assay was performed as described in our previous studies (Chapter 2). In this chapter, the nitrite level was also measured in the brain and heart tissues. The brain and heart tissue homogenates were prepared and nitrite level were measured using Griess reaction method as described previously [491]. Briefly, 100 µL sample of brain and heart homogenates were mixed with 100 µL of Griess reagent (sulfanilic acid and N-(1-naphthyl) ethylenediamine) to form azo dye (Roche Diagnostic). After 10 min of incubation (at 25°C), the absorbances were recorded at 540 nm using iMark microplate reader (Bio-Rad Laboratories, USA). The unknown nitrite levels were calculated from a standard curve and expressed as mmol/mg protein. Lowry method was used to quantified the protein content as described previously [265].

#### 4.2.5.4 Platelet and platelet rich plasma (PRP) isolation

The platelet and PRP were isolated from blood according to a previous study [485]. Briefly, blood was collected in sodium citrate tube (citrate: blood; 1:9, 3.8%) *via* cardiac puncture method. Then, blood was centrifuged (1000 RPM for 10 min, 37 °C) to obtain PRP. The remaining blood was further centrifuged (1000 RPM for 10 min, 37 °C) to obtain poor plasma protein (PPP). Then, the collected PRP centrifuged (350 g for 10 min) again to separate platelet from plasma.

#### 4.2.5.5 *In-vitro* platelet aggregation

*In-vitro* platelet aggregation was performed after incubating PRP (200 µL) with rosmarinic acid (30 µM), Dabigatran (10 µM), clopidogrel (3 µM), or di-methyl sulphoxide (DMSO, 200 µL, 0.5 % v/v) as per previous study [485, 492-494]. PRP absorbance was measured at 570 nm using microplate reader before the initiation of aggregation step. Then, for initiation of platelet aggregation, we added adenosine diphosphate (ADP) (10 µM) or thrombin (0.05 U/mL) and incubated for 5 min at 37 °C with continuous shaking before recording absorbance at 570 nm. The inhibitory drug concentrations against ADP- and thrombin-induced aggregation were calculated (using non-linear regression analysis in GraphPad Prism) as described previously [485]. As a blank, absorbance of PPP was recorded and aggregation was calculated by the following formula:

$$Platelet\ aggregation\ rate\ PAR\ (\%) =$$

$$PRP\ abs\ (\mathrm{BS}) - \mathrm{PRP\ abs\ (AS)}/(PRP\ abs\ (\mathrm{BS})\ - PPP\ abs) * 100$$

#### 4.2.5.6 RNA isolation and gene expression

The expression of anti-inflammatory cytokine IL-10 was examined *via* reverse transcriptase-polymerase chain reaction (RT-PCR) method. According to manufacturer's instructions, total RNA was isolated from the heart tissue homogenate using Trizol (RNA isolation reagent) purchased from Takara, Clontech. RNA concentration and purity were computed by Nanodrop 1000 technologies and A260/A280 ratio, respectively. For reverse transcription, 2 µg total RNA was used for cDNA synthesis using PCR buffer, Oligo dT (100 ng), reverse transcriptase (20 U/µL), and dNTP mixture (20mM). AB17500 fast system was used for gene expression and PCR reaction as described previously [495]. Briefly, real time mix (10 µL) was

prepared using SYBER green master mix (5 µL), cDNA (1 µL), nuclease free water (6 µL), RNase inhibitor (1 µL), and reverse/forward primers (0.5 µL) for each cytokine separately. PCR condition were set at 50 ºC for initial incubation (2 min), followed by denaturation (at 95 ºC for 10 min), and 40 cycles at 95 ºC, 60 ºC, and 72 ºC for 15 sec, 1 min, and 40 sec, respectively. The fold change was calculated as previously described [496]. The following primers were used: IL-10 (Forward; AAGGGGGCGAGTGTAACAAG, Reverse; CCAGGTGCAGAATAGCTCCC), NF-kB (Forward; ATCAAAGAGCTGGTGGAGGC, Reverse; GAAGGCTGCCTGGATCACTT), TNFα (Forward; GCTTGTGGGGTCCGTGAAT, Reverse; GTAACTGGAGGAGAATGGGGC), and GAPDH (Forward; GGAAATGAGAGAGGCCCAGCTAC, Reverse; CTAGCTCAAGGGCGCAGAGG). The gel electrophoresis was performed according to the previous study [497]. Briefly, 1% w/v agarose gel was used and staining was done by ethidium bromide in 0.5 µg/mL and images were captured by Gel Doc™ EZ (Bio-Rad Laboratories, Hercules, CA, USA). The Image Lab version 5.2.1 software (Bio-Rad Gel Doc™ EZ) was used to notify the band intensity.

#### 4.2.5.7 Histopathology

Histopathology was done on three representative heart samples from each treatment group. Each tissue was immediately washed with cold saline and fixed in buffered formalin (10%) for one week at 25-27 ºC and then embedded in paraffin. Then, these samples were sectioned into 4 µm thick sections using cryotome (Cryostat MEV, SLEE medical GmbH, Germany). Finally, the sections were stained using haematoxylin and eosin dye as described previously [498, 499].

### 4.2.6 Statistical analysis

Statistical analysis was done using GraphPad Prism version 7.03 (GraphPad Software Inc., USA). Results were represented as the mean ± SD (standard deviation). $IC_{50}$ values of the RA against platelet aggregating agents (ADP and thrombin) were calculated by non-linear regression analysis. Data sets with one independent variable were analysed through one-way analysis of variance under Tukey's multiple comparison test and P value was set at 0.05.

## 4.3 Results

### 4.3.1 Network Pharmacology Analysis

#### *4.3.1.1 Target prediction results*

In this study, categories of targets are related to depression, myocardial infarction, and rosmarinic acid were retrieved from different public databases. According to GeneCard, a total of 13320 targets for depression, 4820 for MI, and 58 for rosmarinic acid were found, from which the 49 common targets were identified by performing a Venn diagram analysis (*Figure 24*). These common targets were included for further studies.

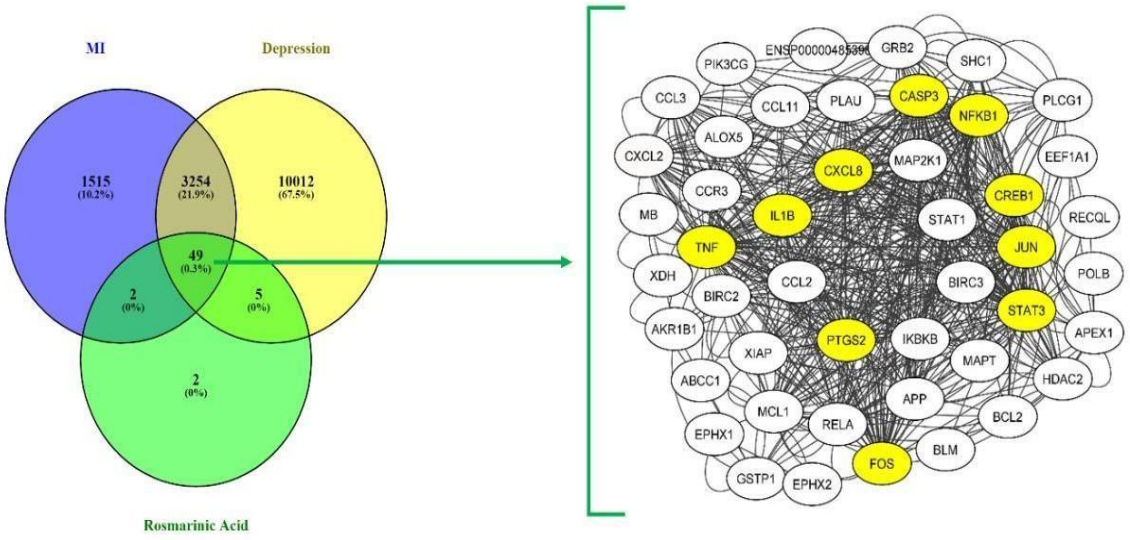



Figure 24: Venn diagram of targeted genes with GeneCard database for MI, depression, and rosmarinic acid showed protein-protein interaction of 49 target genes.

Top 10 nodes represented by yellow nodes based on the degree.

#### *4.3.1.2* Protein–protein interaction

The STRING protein–protein interactions of 49 target genes were found. The interacted STRING protein–protein network has 49 nodes (proteins) connected by 296 edges (interaction) with a local clustering coefficient of 0.679 and an average node degree of 12.3. Less than 1 p-value (PPI enrichment) was found, thus suggesting that the resultant network has more significantly interaction than excepted. With the help of network analyser plugin of CytoScape, we performed the topological analysis such as degree, betweenness centrality of target genes, and closeness centrality. The top 10 target genes based on topological analysis were TNF, STAT3, JUN, IL1B, CASP3, PTGS2, CXCL8, FOS, CREB1, NFKB1 (indicated with yellow nodes in *Figure 24*). With a considerably high value of topological analysis, these target genes can be considered as nodes with hub nodes, high interaction, and nodes with potential control, respectively and obtain the highest degree top 10 genes.

#### *4.3.1.3* Gene Enrichment Analysis

The Shiny GO operating interface for GO enrichment was set at $p$-value $\leq 0.05$ and analyzed target genes in three areas: GO biological, GO molecular, and GO cellular. The obtained top 10 GO biological, GO molecular, and GO cellular processes encompassing target genes are shown in *Figure 25*. In the Shiny GO webserver for the KEGG pathway, similar operating settings were used. The top 10 enriched pathways involving target genes are represented in a dot plot, as shown in *Figure 25*. Out of 49 target genes, 23 target genes were involved in KEGG pathways of MI and depression, as shown in *Figure 26*. These 23 genes were majorly related to TNF signalling pathway.

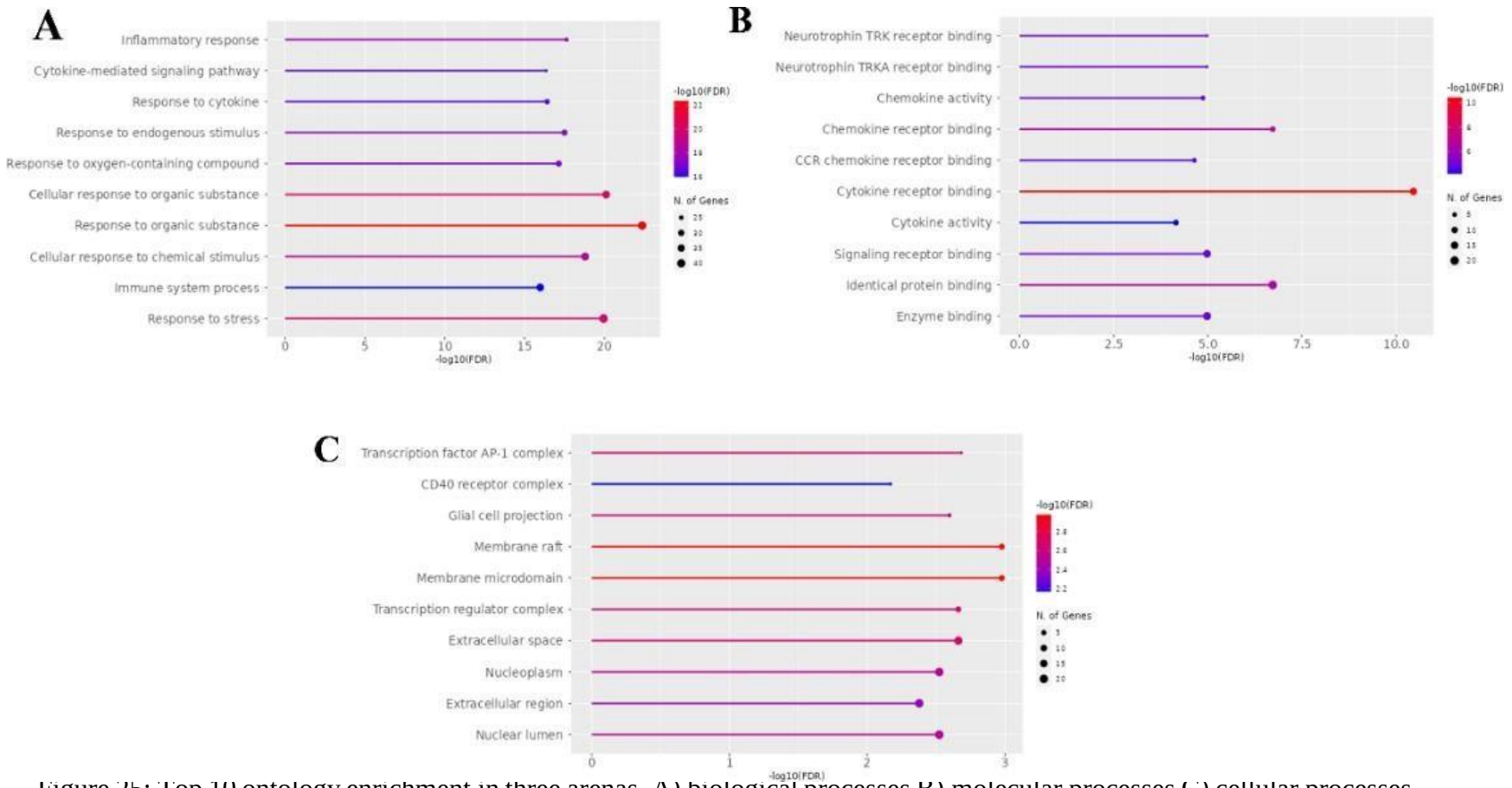



Figure 25: Top 10 ontology enrichment in three arenas, A) biological processes B) molecular processes C) cellular processes

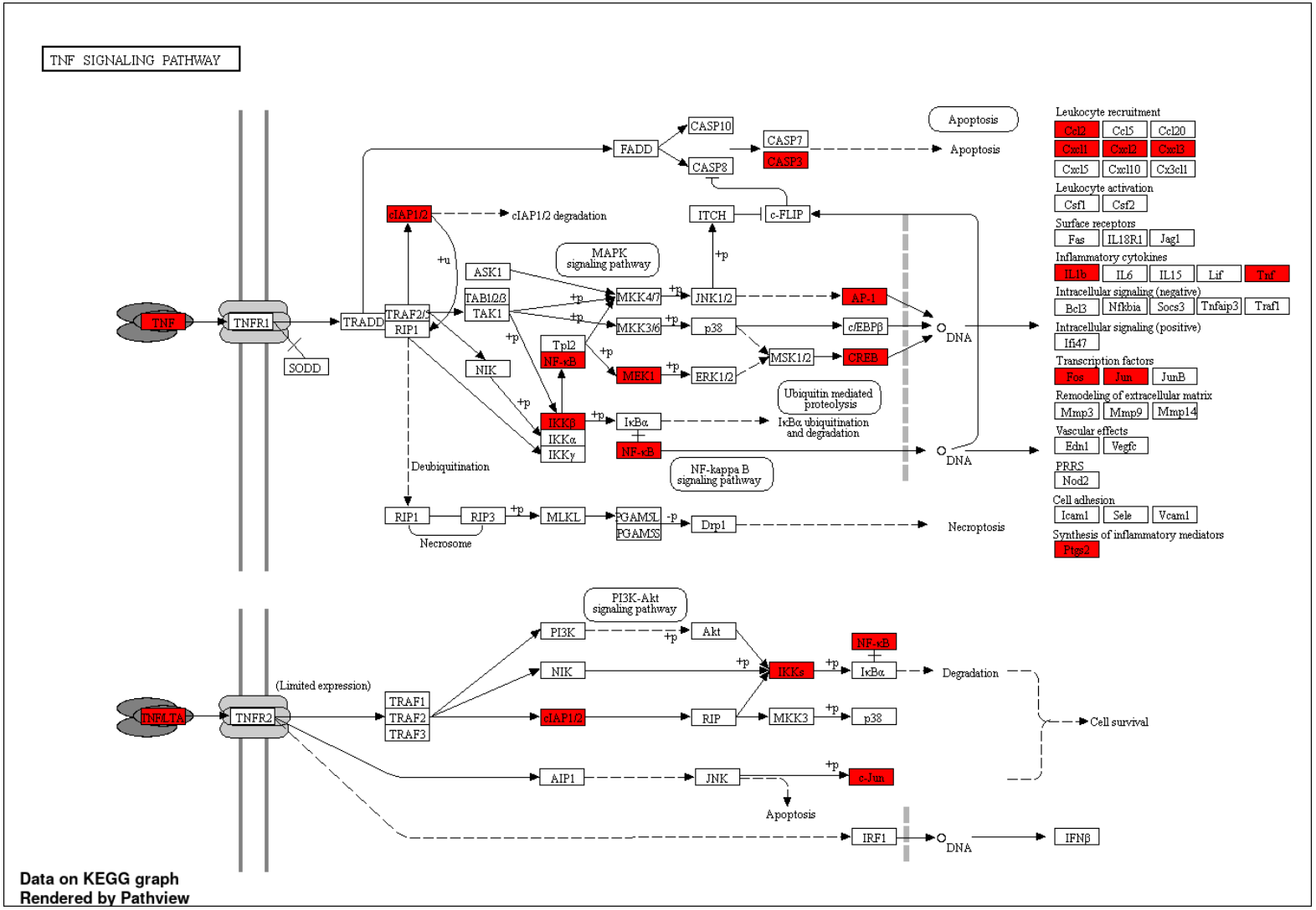



Figure 26: KEGG pathway of TNF signaling pathway in MI, depression, and rosmarinic acid.

### 4.3.2 Docking

#### 4.3.2.1 Rosmarinic acid exhibited binding affinity with MMP2, STAT3, TrkB, P2Y12, and TXA2 receptors

In this study we measured the binding affinity of rosmarinic acid towards MMP-2, STAT3, TrkB, P2Y12, and TXA2 receptors. The interaction between rosmarinic acid, fluoxetine, clopidogrel (ADP antagonist), and dabigatran (thrombin inhibitor) with abovementioned receptors are depicted in *Figure 32*. These targets are used to produce beneficial effects such as preventing psychiatric illness and cardiovascular diseases in different subjects [425, 500, 501]. Our study revealed that MMP-2, STAT3, TrkB, P2Y12, and TXA2 receptors got almost equal or less affinity with rosmarinic acid compare to fluoxetine. However, fluoxetine got more affinity with TXA2 receptors than rosmarinic acid and dabigatran treatment. Whereas, clopidogrel binds with P2Y12 with a higher binding energy than RA and fluoxetine. The binding affinity of rosmarinic acid with MMP2, STAT3, TrkB, P2Y12, and TXA2 are depicted in *Table 2*, which showed the comparable affinity of drugs with the receptors.

**Table 2.** Binding energy of RA, fluoxetine with MMP2, STAT3, TrkB and CLOP (Clopidogrel with P2Y12 and DABI (Dabigatran) with TXA2 receptors and amino acids involve between drugs and receptors.

| S./No. | Drugs | Binding energy (kcal/mol) | | | | | Total amino acid interacted | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MMP | STAT3 | TrkB | $P_2Y_{12}$ | TXA2 | MMP2 | STAT3 | TrkB | $P_2Y_{12}$ | TXA2 |
| **1.** | RA | -8.68 | -5.16 | -8.06 | -5.78 | -6.77 | 20 | 14 | 16 | 10 | 18 |
| **2.** | Fluox | -8.57 | -5.15 | -5.84 | -5.98 | -9.66 | 17 | 10 | 15 | 12 | 15 |
| **3.** | CLOP | - | - | - | -7.74 | - | - | - | - | 13 | - |
| **4.** | DABI | - | - | - | - | -6.20 | - | - | - | - | 15 |

#### 4.3.2.2 Interaction of rosmarinic acid and fluoxetine with receptors residues

For the docking study, grid was prepared around the interacting residues of all receptors. We performed the interaction of rosmarinic acid with aggregatory receptors (P2Y12 and TXA2).

In P2Y12, hydrogen group of catechol in rosmarinic acid forms conventional hydrogen bonding with AZJ1201, LYS280, and GLU281 and ketone group of rosmarinic acid forms other conventional hydrogen bonds at TYR105, GLN98, and LYS280. And benzene ring of catechol makes π-alkyl bonds with LEU284. While fluoxetine interacts with P2Y12, makes alkyl bond and π-alkyl bond with TYR32, LYS80, PHE104, and AZJ1201 at carbon surrounding fluorine group and benzene ring. Also makes the conventional hydrogen bonding with GLU281, SER288, and TYR105 at hydrogen of nitrogen group, fluorine group, and oxygen group of fluoxetine. Formation of halogen (fluorine) with LEU284 is another bonding with the receptor at fluorine group and benzene ring. In TXA2 receptor, catechol group (oxygen and hydrogen) of rosmarinic acid form conventional hydrogen bond with TRP299, CYS35, LEU78, and GLY38. The benzene ring of catechol makes π-π stacked at PHE34 and π-alkyl at ARG295. Similarly at fluorine group, fluoxetine forms conventional hydrogen bonding with GLY82 and THR81 and benzene ring also form halogen (fluorine) at LEU78 residue. Also form π-sigma with hydrogen of nitrogen group at HIS89 and π-π stacked at PHE34 and π-alkyl at ARG295.

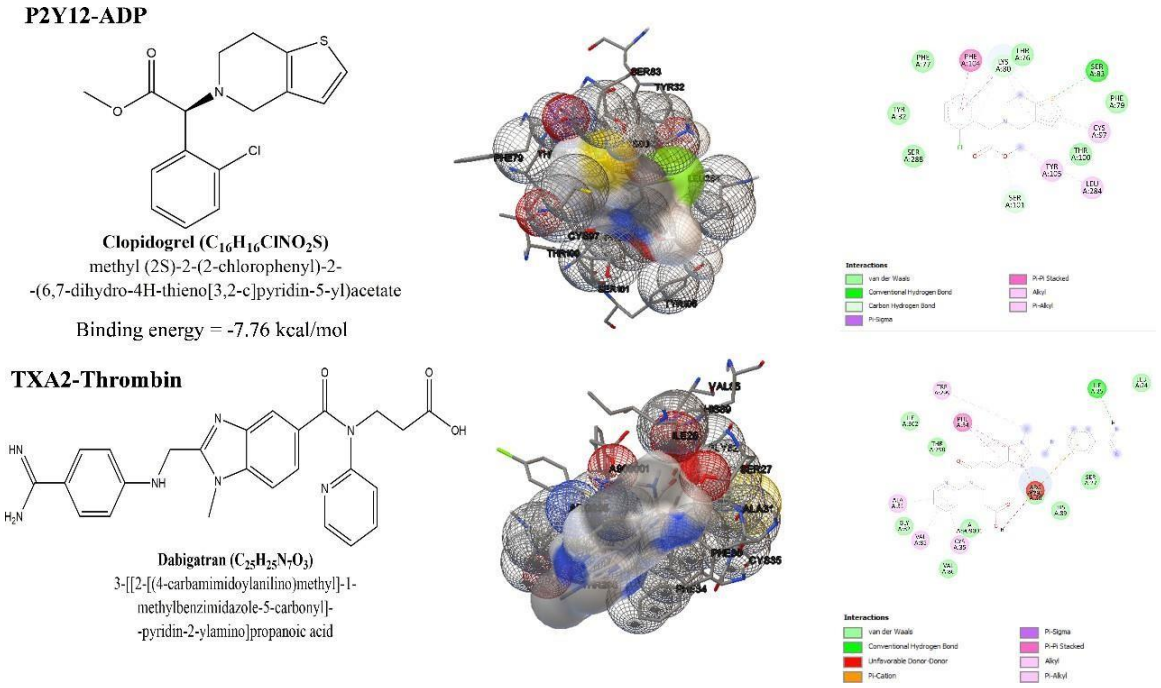



Figure 27: Computational structural comparison, 2d interaction plot, and binding features (Biovia Discovery studio and AutoDock) P2Y12 (clopidogrel), and TXA2 (Dabigatran) receptors.

Figures A and B depict the crystal structure of ligands and depict the interactions with receptors.

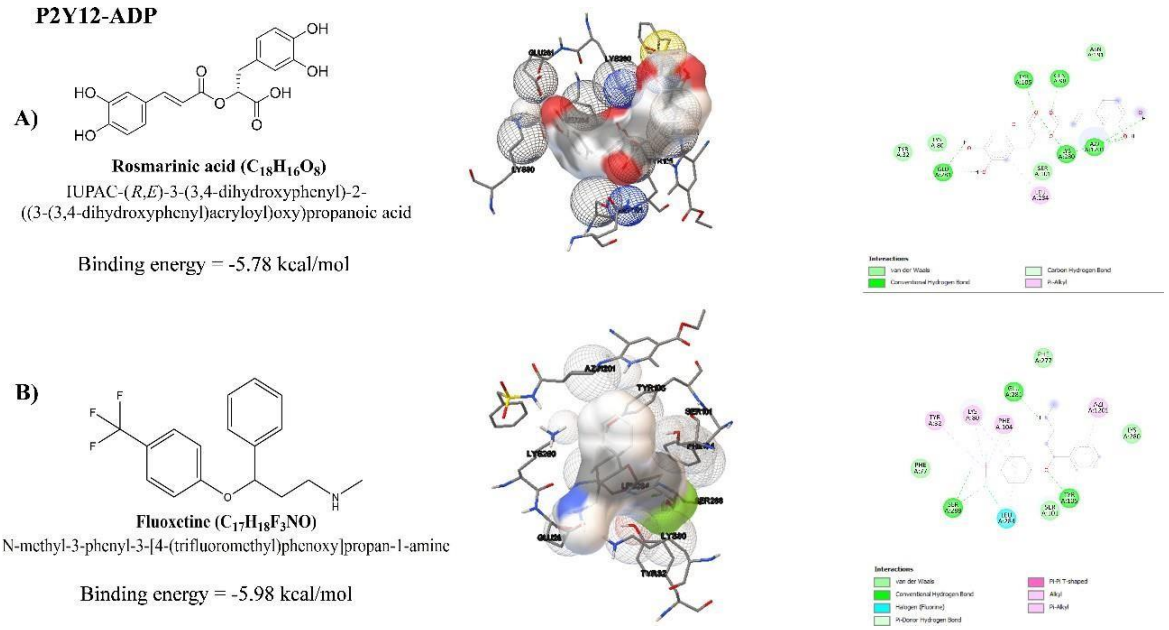



Figure 28: Computational structural comparison, 2d interaction plot, and binding features (Biovia Discovery studio and AutoDock) of rosmarinic acid and fluoxetine with P2Y12 receptor.

Figures A and B depict the crystal structure of ligands and depict the interactions with receptors.

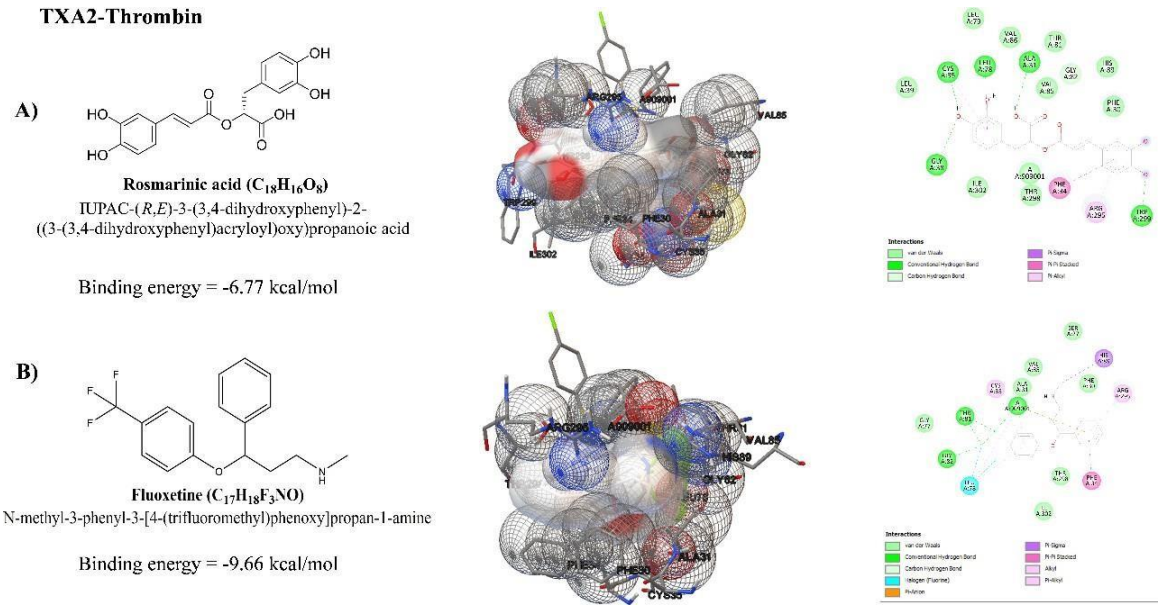



Figure 29: Computational structural comparison, 2d interaction plot, and binding features (Biovia Discovery studio and AutoDock) of rosmarinic acid and fluoxetine with TXA2 receptor.

Figures A and B depict the crystal structure of ligands and depict the interactions with receptors.

The catechol group of rosmarinic acid form conventional hydrogen bond with THR144 residue of MMP2, and the ketone group of RA form conventional hydrogen bond with ALA84 and LEU83 residue of MMP2 (*Figure 30*). Catechol ring interacts *via* π-π stacked (HIS125 and HIS121), π-cation (ZN201), π-sigma (TYR143), and π-alkyl (ALA140) residues. However, catechol ring of one hydrogen bond shows unfavourable donar-donar at ALA86 residue. Similar to rosmarinic acid, fluoxetine at benzene ring shows unfavourable donar-donar at HOH312 residue. Ligand form conventional hydrogen bonds with THR144 residue at fluorine group and with GLY81 residue at nitrogen group. Fluorine group in ligand also involve to form halogen bond at LEU138 and ILE142. Benzene ring of ligand form π-sigma bond with ALA84 and ALA122 residues. Ligand also form C-H bond with PRO141 residue and Van Der Waals with TYR143 residue.

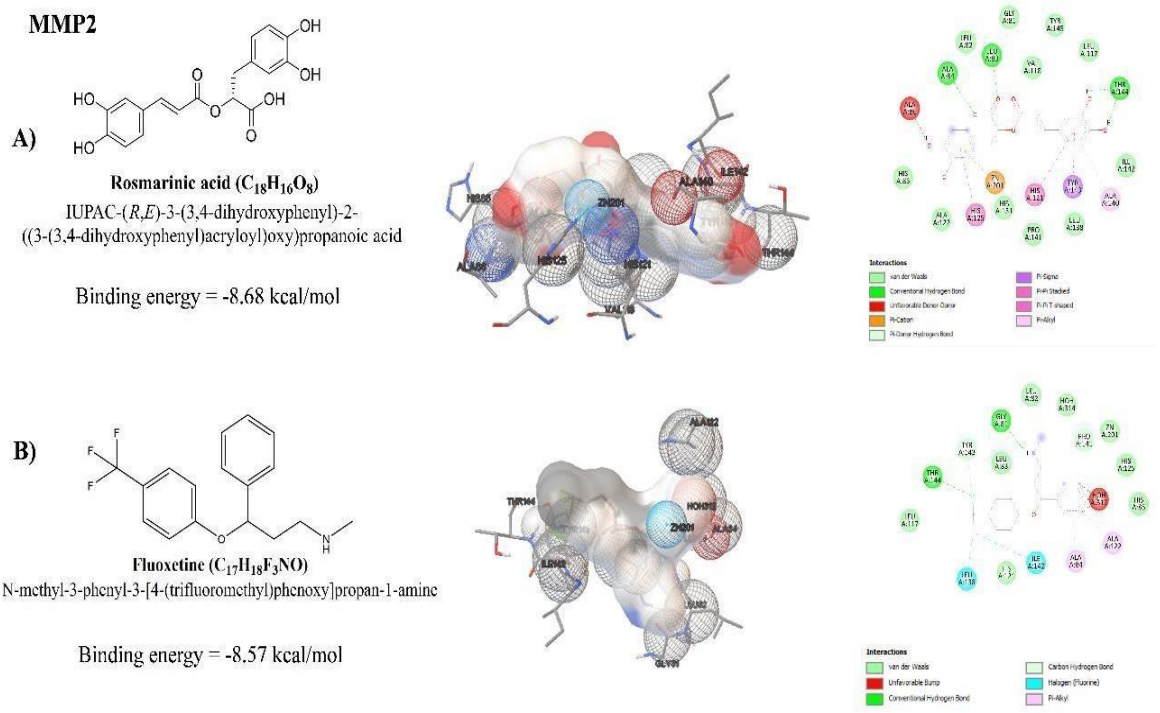



Figure 30: Computational structural comparison, 2d interaction plot, and binding features (Biovia Discovery studio and AutoDock) of rosmarinic acid and fluoxetine with MMP-2 receptor.

Figures A and B depict the crystal structure of ligands and depict the interactions with receptors.

In STAT3, catechol of rosmarinic acid form conventional hydrogen bond with ASP173 and MET200 residues and benzene ring of catechol makes π-alkyl at LEU203 residue. Whereas, fluoxetine makes π-π stacked at PHE172, π-alkyl at VAL291 in benzene ring, conventional hydrogen bonds at SER292 (oxygen group), ASP173 at nitrogen group, and GLN289 at halogen group (*Figure 31*).

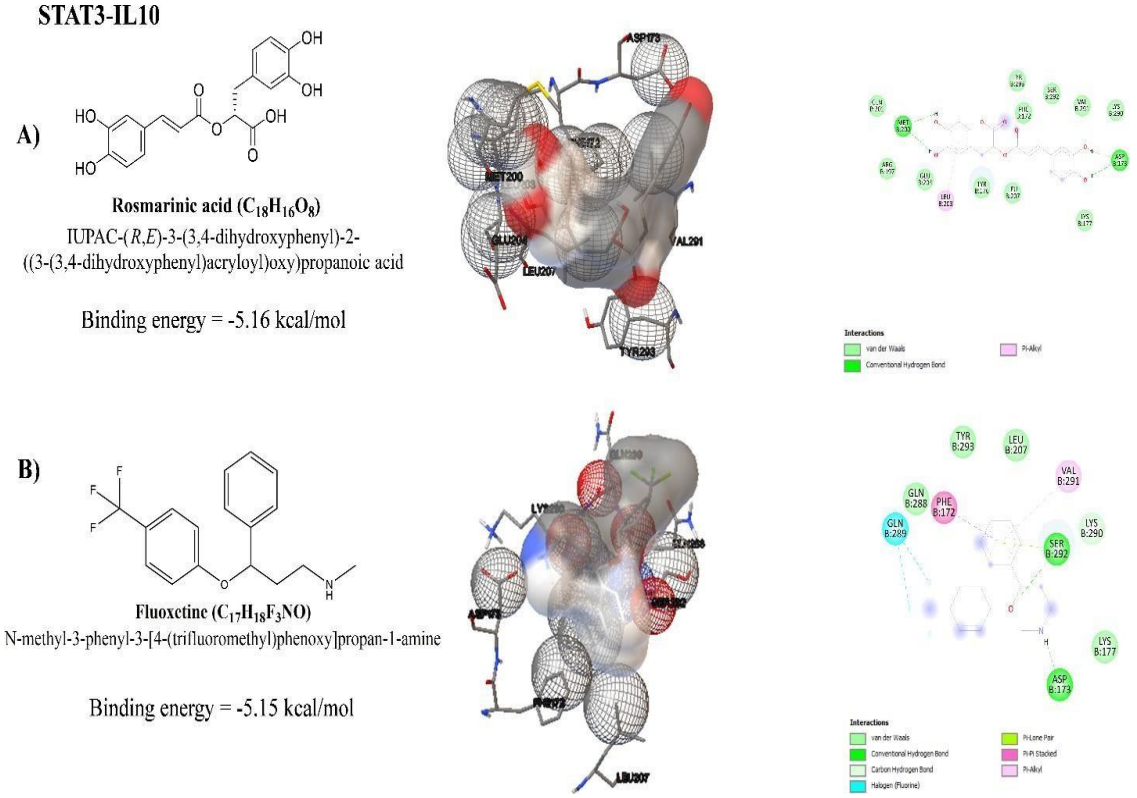



Figure 31: Computational structural comparison, 2d interaction plot, and binding features (BIOVIA Discovery studio and AutoDock) of rosmarinic acid and fluoxetine with STAT3 receptors.

Figures A and B depict the crystal structure of ligands and depict the interactions with receptors.

In TrkB interaction with rosmarinic acid, benzene ring of catechol group makes π-π stacked at TYR635, π-alkyl at LEU560, and π-sigma at MET713 residue. Further, RA forms conventional hydrogen bonds at ketone group (ASP640) and catechol group (MET636 and SER714). Similarly, benzene ring of fluoxetine makes alkyl (LEU699) and π-alkyl bond (MET713). Further, fluorine group of fluoxetine shows halogen bonding at MET636 and Van Der Waals at GLY639 residue and bonded with carbons surrounded by fluorine group make π-alkyl at TYR635 residue. With the hydrogens of nitrogen group in fluoxetine forms conventional hydrogen bonding at ASP640 and unfavourable donar-donar at TYR718 residue (*Figure 32*).

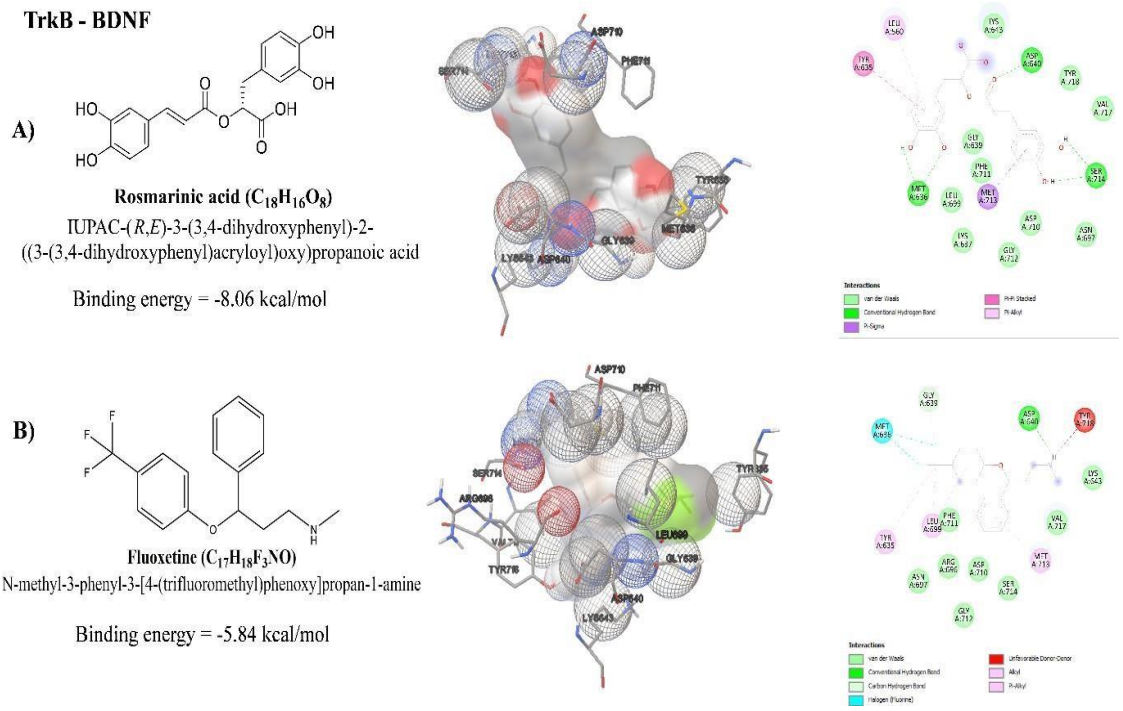



Figure 32: Computational structural comparison, 2d interaction plot, and binding features (BIOVIA Discovery studio and AutoDock) of rosmarinic acid and fluoxetine with TrkB, receptor.

Figures A and B depict the crystal structure of ligands and depict the interactions with receptors.

### 4.3.3 Rosmarinic acid decreased immobility period and anhedonia behavior

The rosmarinic acid decreased the duration of immobility period and anhedonia behavior as shown in *Figure* **33**. These tests performed to evaluate the depressive-like behavior of the rats on PND35, PND55, and PND75. The one-way ANOVA showed that MS-control, CUS-control, and MS combined CUS group had significantly higher immobility period [PND35 ($F_{7,40}$ = 33.47, $p < 0.0001$) and PND55 ($F_{7,40}$ = 47.64, $p < 0.0001$)] and anhedonia behavior [PND35 ($F_{7,40}$ = 60.29, $p < 0.05$) and PND55 ($F_{7,40}$ = 112.9, $p < 0.05$)] compared to the control group. Whereas, at PND75, isoproterenol group and MS combined CUS received isoproterenol group showed extensive increase in immobility period ($F_{8,45}$ = 62.98, $p < 0.0001$) and anhedonia behavior ($F_{8,45}$ = 92.90, $p < 0.05$) compared to the control group.

Interestingly, the rosmarinic acid (25 and 50 mg/kg) treated group showed significantly decreased immobility period and anhedonia behavior compared to MS combined CUS group (PND35 & 55) and MS combined CUS received isoproterenol (PND75) group. In contrast, fluoxetine (10 mg/kg) treatment significantly decreased immobility period and anhedonia behavior compared to rosmarinic acid, MS combined CUS, and MS combined CUS received isoproterenol group.

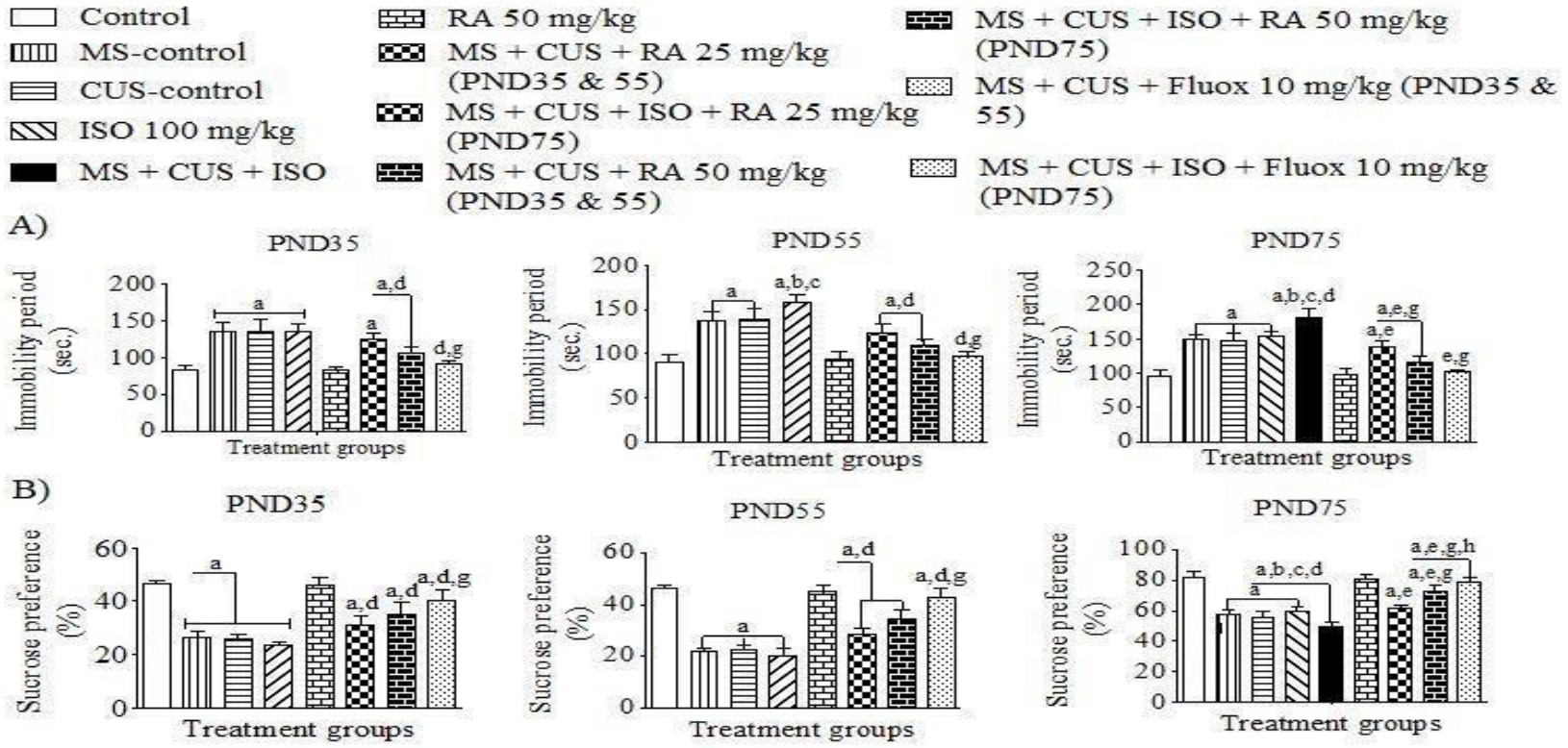



Figure 33: Rosmarinic acid and fluoxetine treatment decreased (A) immobility period (B) sucrose preference (%).

Values represented as Mean ± SD (n = 6). [a]p < 0.0001 versus control, [b]p < 0.0001 versus MS-control, [c]p < 0.0001 versus CUS-control, [d]p < 0.0001 versus ISO-control (PND75), [e]p < 0.05 versus MS + CUS + ISO (PND75), [f]p < 0.0001 versus rosmarinic acid 50 mg/kg, [g]p < 0.0001 versus MS + CUS + rosmarinic acid 25 mg/kg (PND35 & 55), [g]p < 0.0001 versus MS + CUS + ISO + rosmarinic acid 25 mg/kg (PND75), [h]p < 0.0001 versus MS + CUS + rosmarinic acid 50 mg/kg (PND35 & 55), [h]p < 0.0001 versus MS + CUS + ISO + rosmarinic acid 50 mg/kg (PND75), [i]p < 0.0001 versus MS + CUS + Fluox 10 mg/kg (PND35 & 55), [h]p < 0.0001 versus MS + CUS + ISO + Fluox 10 mg/kg (PND75).

### 4.3.4 Rosmarinic acid decreased CORT level and increased BDNF and 5-HT level

The effects of rosmarinic acid and fluoxetine on combined stress-induced alterations in plasma CORT level, BDNF level and brain serotonin level are represented in *Figure 34*. The one-way ANOVA exhibited that MS-control, CUS-control, and MS combined CUS received ISO caused significant changes ($F_{8,45} = 27.93$, $p < 0.0001$) in CORT level, ($F_{8,45} = 10.66$, $p < 0.0001$) BDNF level, and ($F_{8,45} = 157.6$, $p < 0.0001$) serotonin level. But, isoproterenol only group did not show significant ($p > 0.05$) changes in CORT level as compared to control group. However, MS combined CUS received isoproterenol group showed substantially augmented plasma CORT level, diminished BDNF level, and diminished serotonin level than MS-control, CUS-control, and ISO group. Interestingly, administration of rosmarinic acid significantly decreased the plasma CORT level, increased the BDNF level, and increased the serotonin level compared to MS combined CUS treated ISO group. However, fluoxetine significantly decreased the plasma corticosterone level and increased the BDNF and serotonin level compared to rosmarinic acid and MS combined CUS received ISO rats.

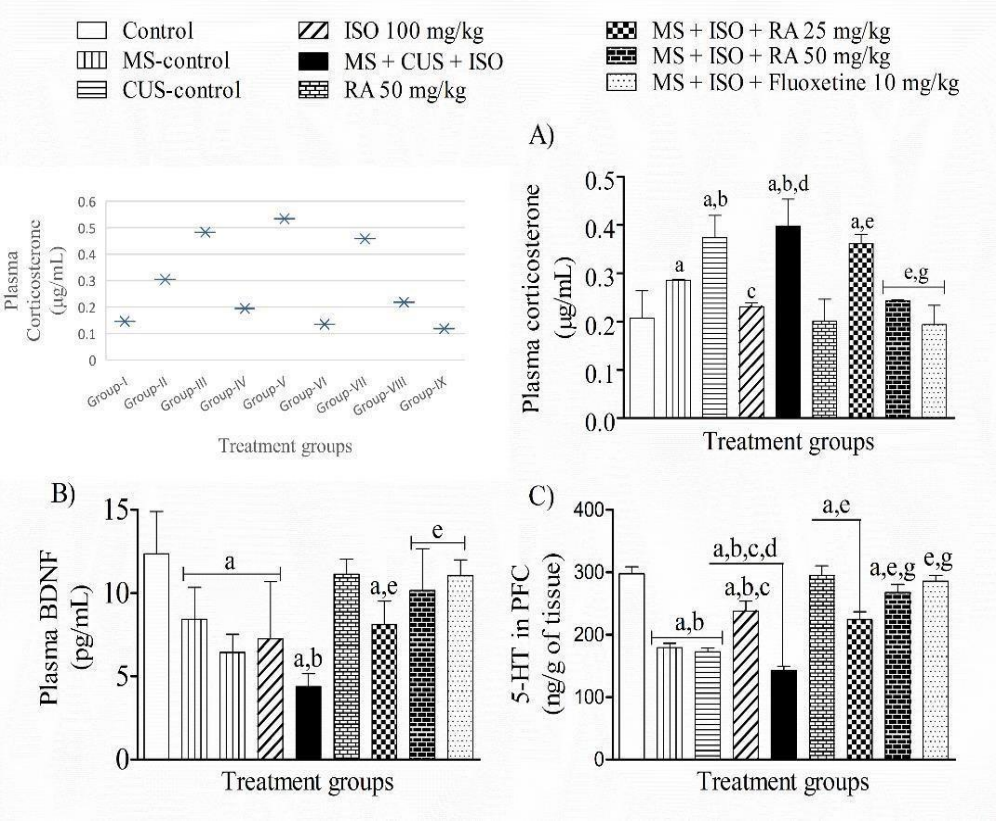



Figure 34: Rosmarinic acid (25 and 50 mg/kg) decreased A) plasma corticosterone level and increased B) plasma BDNF level and C) serotonin level in brain (prefrontal cortex). Values are represented (mean ± SD, N = 6 rats per group). [a]p < 0.0001 versus control, [b]p < 0.0001 versus MS-control, [c]p < 0.0001 versus CUS-control, [d]p < 0.0001 versus ISO-control, [e]p < 0.0001 versus MS + CUS + ISO, [f]p < 0.0001 versus rosmarinic acid 50 mg/kg, [g]p < 0.0001 versus MS + CUS + ISO + rosmarinic acid 25 mg/kg.

### *4.3.5 Rosmarinic acid increased body weight, organ weight, and heart weight index*

After exposure to MS and 40 days of CUS, the heart weight index and adrenal gland weight were significantly ($p < 0.05$) increased in MS-control and isoproterenol group as compared to the control group (*Table 3*). An exacerbation in adrenal weight and heart weight index was observed in MS combined CUS received isoproterenol group compared to MS-control, CUS-control, and isoproterenol group. Interestingly, administration of rosmarinic acid and fluoxetine significantly ($P < 0.0001$) decreased heart weight index and adrenal hyperplasia as compared to MS combined CUS received isoproterenol group.

Table 3. Effect of rosmarinic acid on body weight and organ weight

| **Parameters** | **Group I** | **Group II** | **Group III** | **Group IV** | **Group V** | **Group VI** | **Group VII** | **Group VIII** | **Group IX** |
|---|---|---|---|---|---|---|---|---|---|
| Body weight (g) | 168.17± 5.14 | 189.33 ± 5.85[a] | 165.66 ± 4.63[ab] | 173.00 ± 2.28[a,b,c] | 203.2 ± 9.15[a,b,c,d] | 167.6± 3.93 | 190.5 ± 3.33[a,e] | 178.83 ± 4.58[a,e,g] | 175.5 ± 5.35[e,g] |
| Heart weight (g) | 0.664 ± 0.050 | 1.063 ± 0.059[a] | 1.054 ± 0.046[a] | 1.228 ± 0.14[a] | 1.631 ± 0.13[a,b,c,d] | 0.685 ± 0.09 | 1.042 ± 0.06[a,e] | 0.829 ± 0.06[a,e,g] | 0.692 ± 0.04[e,g,h] |
| Heart weight index | 0.395 ± 0.034 | 0.562 ± 0.030[a] | 0.637 ± 0.036[a] | 0.711 ± 0.087[a,b] | 0.799 ± 0.05[a,b,c] | 0.409 ± 0.06 | 0.547 ± 0.04[a,e] | 0.463 ± 0.03[e] | 0.394 ± 0.02[e,g] |
| Adrenal gland weight (g) | 0.035 ± 0.004 | 0.056 ± 0.005[a] | 0.050 ± 0.012[a] | 0.048 ± 0.018[a,b] | 0.082 ± 0.003[a,b,c,d] | 0.034 ± 0.004 | 0.051 ± 0.005[ae] | 0.041± 0.011[a,e,g] | 0.037 ± 0.002[e,g] |

All values are expressed as mean ± SD (n = 6). Significantly difference from the "group-I; Control, group-II; MS, group-III; CUS, group-IV; ISO, group-V; MS + CUS + ISO, group-VI; RA 50 mg/kg, group-VII, MS + CUS + ISO + RA 25 mg/kg, group-VIII; MS + CUS + ISO + RA 50 mg/kg, group-IX; MS + CUS + ISO + Fluoxetine 10 mg/kg. HWI = heart weight (HW)/body weight (BW) [Value set as $p < 0.05$].

### 4.3.6 Rosmarinic acid inhibited platelet aggregation

$IC_{50}$ of RA against ADP- and thrombin-induced platelet aggregation are depicted in *Figure 35* and in-vitro platelet aggregation inhibitory properties of RA are depicted in *Table* **4**. $IC_{50}$ of RA were identified as 16.32 (95% CI, 13.29 to 20.04) and 22.18 (95% CI, 15.34 to 32.07) μM against ADP- and thrombin-induced platelet aggregation, respectively. One-way analysis of variance identified significant changes in aggregation among groups: ADP [$F_{5,12} = 579.7$ $p < 0.001$] and Thrombin [$F_{5,12} = 241.1$ $p < 0.0001$]. The post-hoc analysis revealed that rosmarinic acid treatment reduced the % of platelet aggregation in dose dependent manner. Rosmarinic acid 50 mg/kg, CLOP, and fluoxetine significantly decreased the platelet aggregation compared to rosmarinic acid 25 mg/kg. However, a significant difference was not observed between CLOP and fluoxetine in thrombin aggregation. These findings indicated that rosmarinic acid at 50 mg/kg could be as effective as CLOP, Dabi, and fluoxetine treatment in depression associated MI.

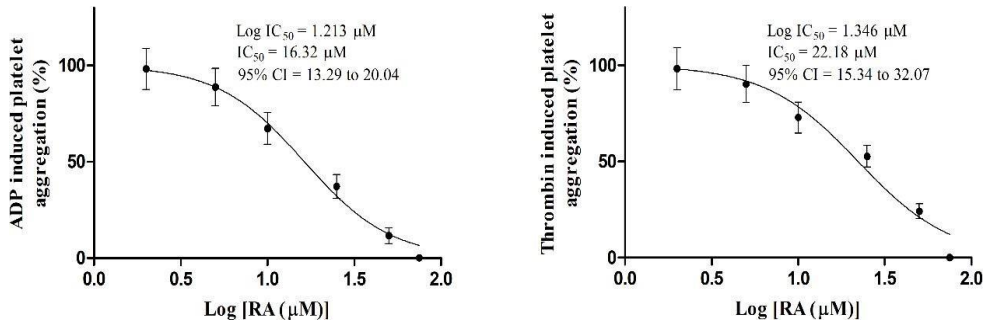



Figure 35: Inhibitory concentration ($IC_{50}$) of rosmarinic acid against ADP- and thrombin- induced platelet aggregation.

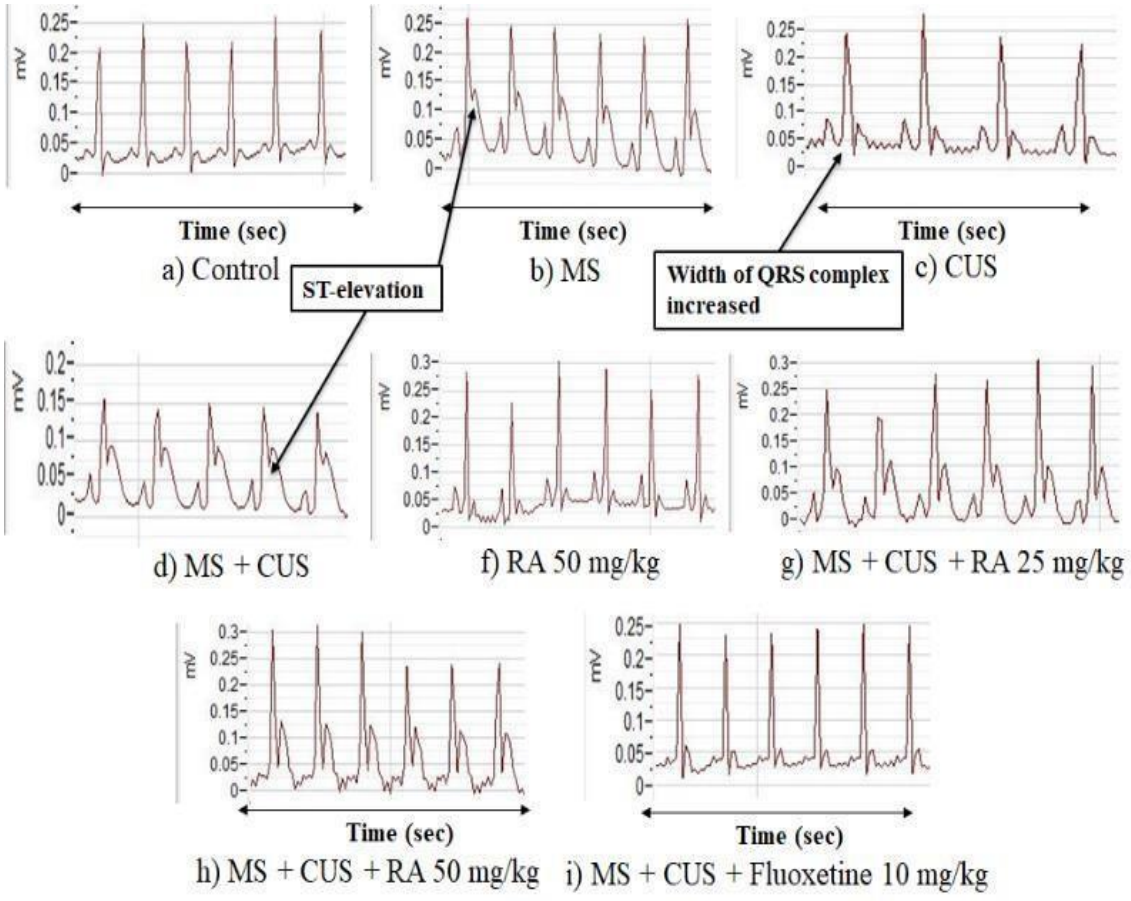
Table 4. Effect of rosmarinic acid against ADP- and thrombin-induced platelet aggregation

| **Platelet aggregation** | | | |
|---|---|---|---|
| **ADP aggregation (%)** | | **Thrombin aggregation (%)** | |
| **Group-1** (ADP-control) | 81.98% | **Group-1** (Thrombin- control) | 85.52% |
| **Group-2** (MS + CUS) | 59.38% | **Group-2** (MS + CUS) | 63.77% |
| **Group-3** (RA 25 mg/kg + ADP) | 62.89% | **Group-2** (RA 25 mg/kg + Thrombin) | 66.24% |
| **Group-4** (RA 50 mg/kg + ADP) | 30.44% | **Group-3** (RA 50 mg/kg + Thrombin) | 33.82% |
| **Group-5** (Fluox 10 mg/kg + ADP) | 24.69% | **Group-4** (Fluox 10 mg/kg + Thrombin) | 27.65% |
| **Group-6** (CLOP + ADP) | 18.84% | **Group-5** (Dabi + Thrombin) | 23.63% |

ADP; Adenosine diphosphate, MS; Maternal Separation, CUS; Chronic Unpredictable Stress, RA; Rosmarinic acid, CLOP; Clopidogrel, Dabi; Dabigatran, Fluox; Fluoxetine.

### 4.3.7 Rosmarinic acid alleviated cardiac abnormalities

Typical ECG traces of animals from respective group are shown in *Figure 36*. ECG examination indicated that MS-control, ISO, and MS combined CUS treated ISO caused significant ($P < 0.05$) increase in ST elevation and CUS-control increased QRS complex compared to control rats on PND35 and PND75. All values are depicted in *Table 5* and *Table 6*. Interestingly, rosmarinic acid showed significant ($P < 0.05$) decrease in ST elevation and QRS complex compared to the MS combined CUS treated isoproterenol group. The other ECG parameters were insignificantly ($P > 0.05$) changed in MS-control, CUS-control and MS combined CUS group than control group.



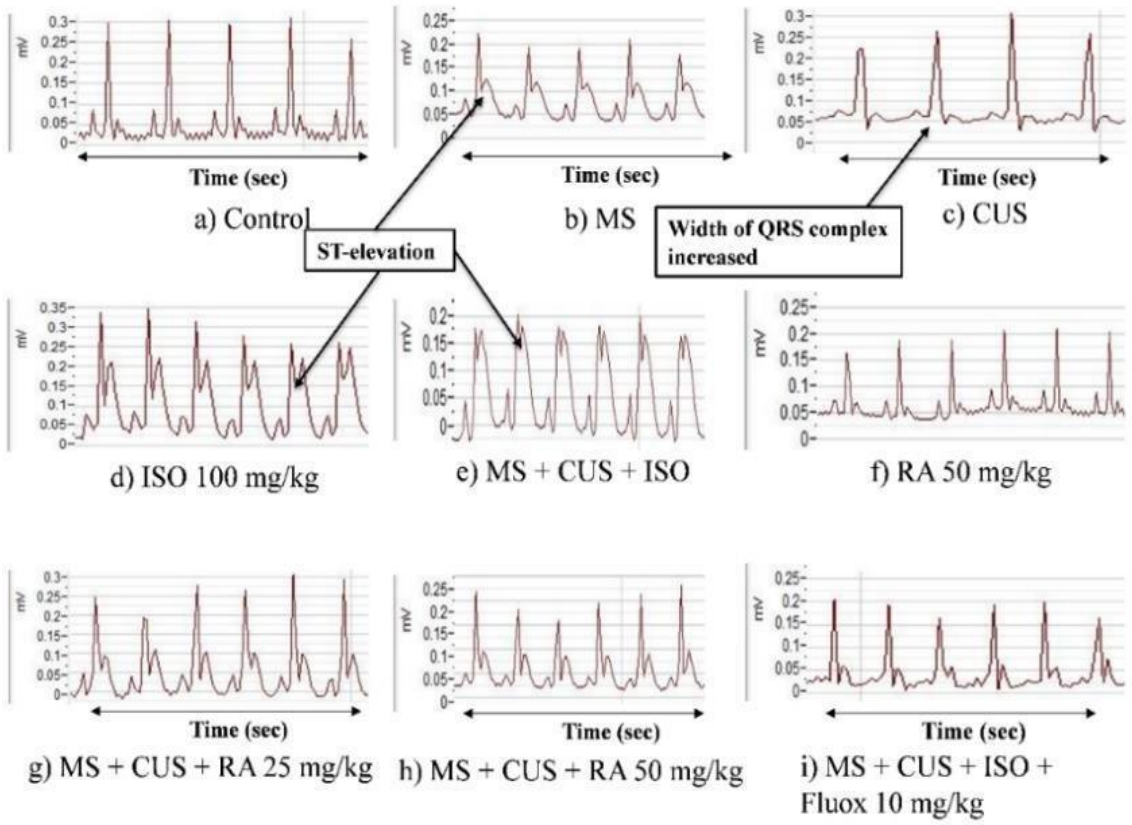



Figure 36: Effects of rosmarinic acid on combined MS and CUS-induced variation in ECG parameters.

Illustrative ECG recordings: ECG tracing shows increase in QRS complex and ST-elevation in MS-control, ISO control, and MS combined CUS received ISO control rats reversed by rosmarinic acid at PND35 and PND75.

Table 5. Rosmarinic acid on ECG parameters in stress induced cardiac abnormalities at PND35.

| ECG parameters | Group-1 | Group-2 | Group-3 | Group-4 | Group-5 | Group-6 | Group-7 | Group-8 |
|---|---|---|---|---|---|---|---|---|
| T-wave (ms) | 24.16 ± 2.78 | 23.66 ± 2.87 | 24.66 ± 2.33 | 25.00 ± 1.41 | 24.33 ± 1.21 | 24.00 ± 1.67 | 24.50 ± 1.64 | 23.8 ± 0.98 |
| QRS complex (ms) | 20.16 ± 2.56 | 21.83 ± 1.83 | 35.50 ± 3.39[a,b] | 37.50 ± 4.18[a,b] | 21.33 ± 2.94 | 31.00 ± 3.22[a,c,d] | 25.83 ± 2.78[a,d,f] | 21.66 ± 2.58[d,f,g] |
| ST-segment (mV) | 0.0137 ± 0.002 | 0.088 ± 0.007[a] | 0.0132 ± 0.002 | 0.075 ± 0.003[a,c] | 0.013 ± 0.002 | 0.053 ± 0.003[a,b] | 0.030 ± 0.002[a,d,f] | 0.012 ± 0.001[d,f,g] |
| PR-interval (ms) | 45.50 ± 3.67 | 45.16 ± 3.86 | 46.50 ± 3.78 | 44.66 ± 4.03 | 44.83 ± 2.4 | 44.33 ± 4.76 | 45.66 ± 3.32 | 46.33 ± 4.27 |
| RR-interval (ms) | 150.66 ± 6.34 | 149.66 ± 5.24 | 150.50 ± 4.50 | 151.16 ± 4.07 | 150.66 ± 4.13 | 150.16 ± 4.16 | 151.00 ± 5.79 | 151.33 ± 4.32 |
| QT-interval (ms) | 62.50 ± 5.32 | 62.33 ± 4.54 | 63.66 ± 3.67 | 63.16 ± 5.03 | 63.83 ± 5.56 | 62.66 ± 3.98 | 62.00 ± 6.54 | 61.50 ± 3.67 |
| P-wave (ms) | 18.66 ± 1.03 | 18.83 ± 1.32 | 19.83 ± 1.83 | 19.00 ± 1.78 | 18.50 ± 0.837 | 18.66 ± 1.50 | 18.50 ± 1.04 | 19.16 ± 0.98 |

All values are expressed as mean ± SD (n = 6). [a]$p < 0.01$ vs G-1 (control), [b]$p < 0.01$ vs G-2 (MS), [c]$p < 0.01$ vs G-3 (CUS), [d]$p < 0.01$ vs G-4 (MS + CUS), [e]$p < 0.01$ vs G-5 (rosmarinic acid 50 mg/kg), [f]$p < 0.01$ vs G-6 (MS + CUS + rosmarinic acid 25 mg/kg), [g]$p < 0.01$ vs G-7 (MS + CUS + rosmarinic acid 50 mg/kg), [h]$p < 0.01$ vs G-6 (MS + CUS + fluoxetine 10 mg/kg).

**Table 6.** Rosmarinic acid on ECG parameters in stress and ISO induced cardiac abnormalities at PND75

| ECG parameters | Group-1 | Group-2 | Group-3 | Group-4 | Group-5 | Group-6 | Group-7 | Group-8 | Group-9 |
|---|---|---|---|---|---|---|---|---|---|
| T-wave (ms) | 24.50 ± 2.94 | 23.80 ± 3.06 | 24.00 ± 2.36 | 25.66 ± 2.87 | 24.16 ± 2.04 | 24.33 ± 2.06 | 25.00 ± 2.09 | 24.66 ± 2.16 | 23.16 ± 1.94 |
| QRS complex (ms) | 20.50 ± 2.66 | 24.16 ± 2.78 | 37.50 ± 4.18[a,b] | 44.16 ± 1.49[a,b,c] | 29.66 ± 3.26[a,c,d] | 20.66 ± 2.65 | 34.00 ± 3.52[a,e] | 24.66 ± 2.58[a,e,g] | 21.66 ± 2.88[e,g] |
| ST-segment (mV) | 0.012 ± 0.002 | 0.107 ± 0.005[a] | 0.014 ± 0.002[b] | 0.143± 0.016[a,b,c] | 0.165 ± 0.009[a,b,c] | 0.013 ± 0.001 | 0.066 ± 0.005[a,e] | 0.036 ± 0.002[e] | 0.024 ± 0.001[e] |
| PR-interval (ms) | 45.83 ± 3.97 | 46.00 ± 4.28 | 45.16 ± 2.85 | 45.33 ± 4.08 | 43.83 ± 4.02 | 44.50 ± 3.20 | 45.66 ± 4.08 | 45.83 ± 4.91 | 44.00 ± 2.96 |
| RR-interval (ms) | 156.5 ± 5.00 | 155.8 ± 5.77 | 156.7 ± 4.41 | 155.1 ± 5.30 | 155.5 ± 5.99 | 156.0 ± 4.89 | 157.5 ± 3.20 | 154.7 ± 4.32 | 155.0 ± 3.84 |
| QT-interval (ms) | 63.00 ± 4.69 | 63.50 ± 4.23 | 62.16 ± 5.67 | 63.33 ± 3.93 | 63.16 ± 4.26 | 61.33 ± 4.27 | 64.00 ± 5.01 | 62.00 ± 4.33 | 63.50 ± 4.63 |
| P-wave (ms) | 19.83 ± 1.32 | 19.33 ± 1.63 | 19.50 ± 1.51 | 18.00 ± 0.894 | 19.66 ± 1.96 | 18.50 ± 1.64 | 19.16 ± 1.47 | 20.00 ± 1.09 | 19.00 ± 2.09 |

All values are expressed as mean ± SD (n = 6). [a]$p < 0.01$ vs G-1 (control), [b]$p < 0.01$ vs G-2 (MS), [c]$p < 0.01$ vs G-3 (CUS), [d]$p < 0.01$ vs G-4 (ISO), [e]$p < 0.01$ vs G-5 (MS + CUS + ISO) [f]$p < 0.01$ vs G-6 (rosmarinic acid 50 mg/kg), [g]$p < 0.01$ vs G-7 (MS + CUS + ISO + rosmarinic acid 25 mg/kg).

### 4.3.8 Rosmarinic acid decreased cardiac biomarkers

One-way ANOVA showed that MS-control, CUS-control, ISO, and MS combined CUS received ISO group significantly changed the CKMB level ($F_{8,45}$ = 120.4, $p < 0.0001$), LDH level ($F_{8,45}$ = 154.9, $p < 0.0001$), cTn-I level ($F_{8,45}$ = 140.0, $p < 0.0001$), AST level ($F_{8,45}$ = 59.08, $p < 0.0001$), ALT level ($F_{8,45}$ = 75.47, $p < 0.0001$), and MMP-2 level ($F_{8,45}$ = 245.4, $p < 0.0001$) (*Figure 37*). Interestingly, rosmarinic acid treatment at 25 and 50 mg/kg significantly decreased the CKMB, LDH, cTn-I, AST, ALT, and MMP-2 levels compared to diseased groups. In contrast, fluoxetine treatment at 10 mg/kg substantially decreased the CKMB, LDH, cTn-I, AST, ALT, and MMP-2 levels compared to rosmarinic acid and MS combined CUS received ISO group (*Figure 37*).

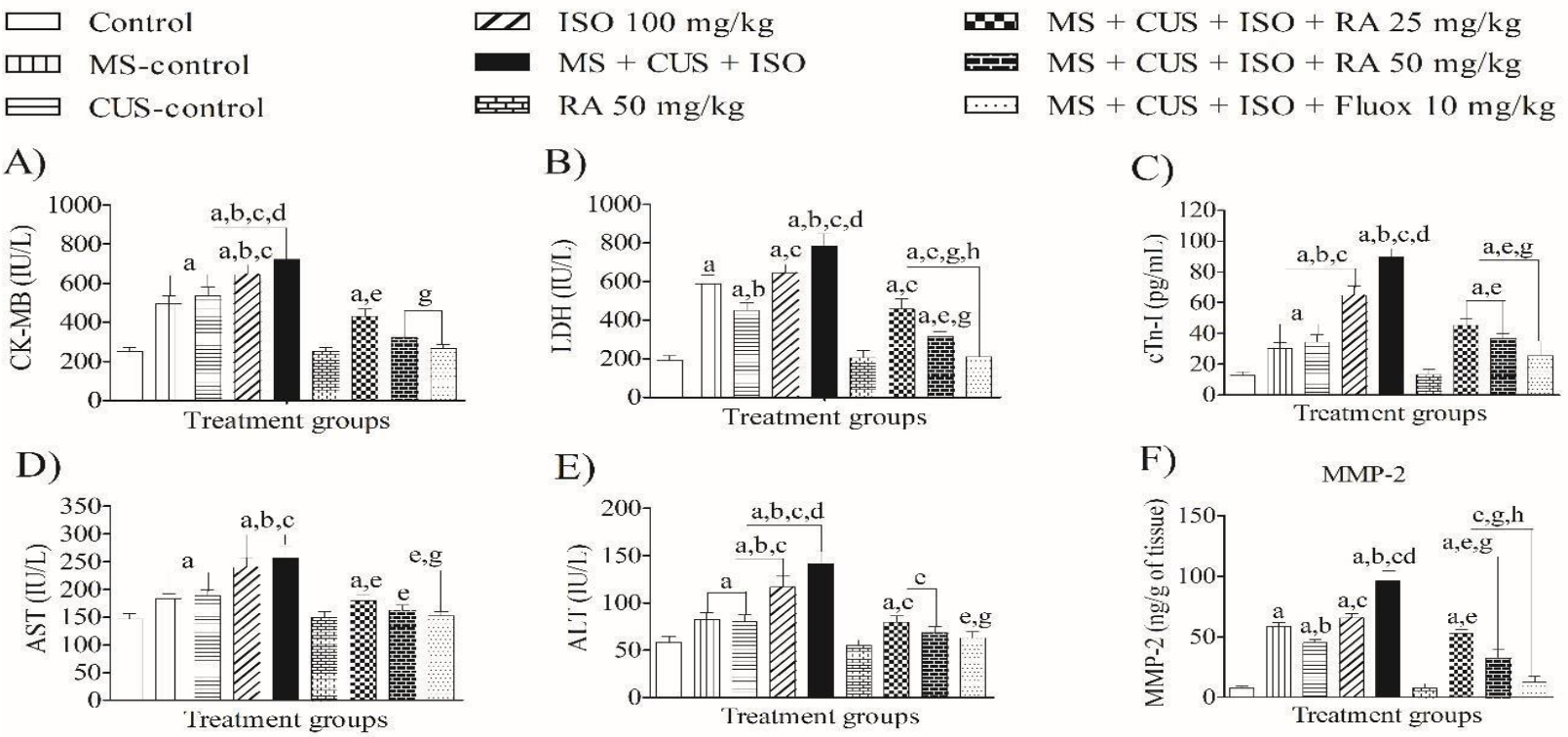



Figure 37: Rosmarinic acid treatment decreased (A) CKMB, (B) LDH, (C) cTn-I, (D) AST, and (E) ALT levels.

Values are represented (mean ± SD, N = 6 rats per group). [a]p < 0.0001 versus control, [b]p < 0.0001 versus MS-control, [c]p < 0.0001 versus CUS-control, [d]p < 0.0001 versus ISO-control, [e]p < 0.0001 versus MS + CUS + ISO, [f]p < 0.0001 versus RA 50 mg/kg, [g]p < 0.0001 versus MS + CUS + ISO + rosmarinic acid (25 mg/kg); [h]p < 0.0001 versus MS + CUS + ISO + rosmarinic acid (50 mg/kg).

### 4.3.9 Rosmarinic acid decreased SOD activity and increased MDA and nitrite level

In comparison to control group, MS-control, CUS-control, ISO, and MS combined CUS received ISO group showed significantly decreased SOD activity [brain ($F_{8,45} = 180.9$, $p < 0.0001$) and heart ($F_{8,45} = 71.80$, $p < 0.0001$)] and increased lipid peroxidation evidenced by augmented MDA level [brain ($F_{8,45} = 394.7$, $p < 0.0001$) and heart ($F_{8,45} = 371.2$, $p < 0.0001$) and nitrite level [brain ($F_{8,45} = 56.32$, $p < 0.0001$) and heart ($F_{8,45} = 114.0$, $p < 0.0001$)]. Rosmarinic acid treatments (25 and 50 mg/kg) significantly increased SOD activity while decrease in MDA and nitrite levels comparison to MS combined CUS received isoproterenol group. Similar results wre observed with fluoxetine treatment (*Figure 38*).

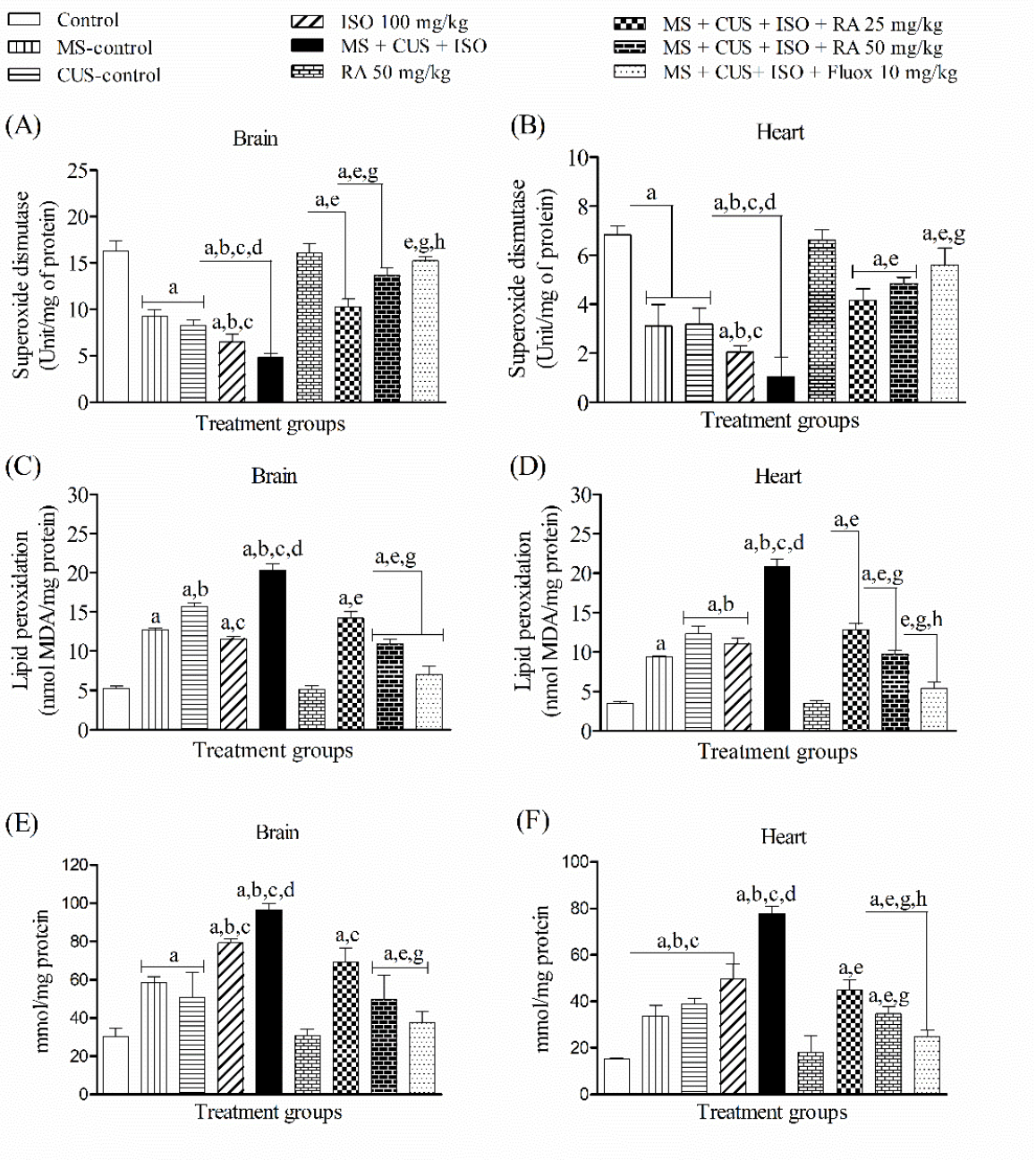
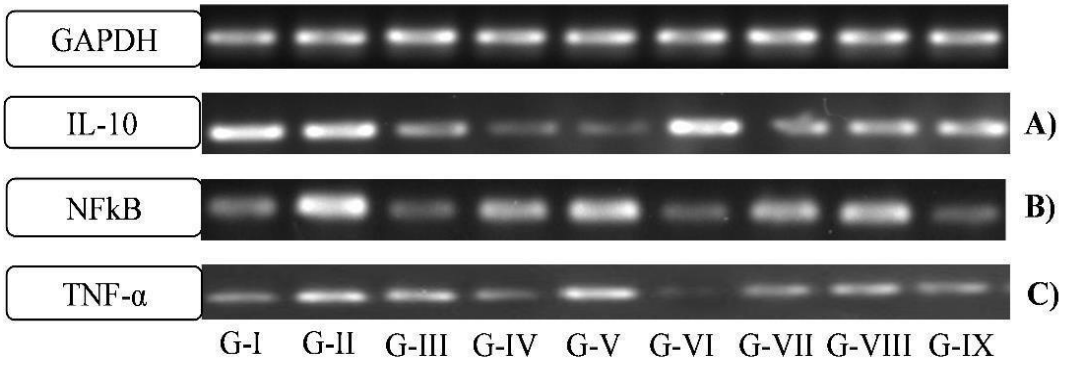



Figure 38: Rosmarinic acid (25 and 50 mg/kg) treatment on SOD activity, lipid peroxidation (LPO), and nitrite level in (A) brain and (B) heart tissue.

Values represented (mean ± SD, N = 6 rats per group). ap < 0.0001 versus control, bp < 0.0001 versus MS-control, cp < 0.0001 versus CUS-control, dp < 0.0001 versus ISO-control, ep < 0.0001 versus MS + CUS + ISO, fp < 0.0001 versus rosmarinic acid 50 mg/kg, gp < 0.0001 versus MS + CUS + ISO + rosmarinic acid (25 mg/kg); hp < 0.0001 versus MS + CUS + ISO + rosmarinic acid (50 mg/kg).

### 4.3.10 Rosmarinic acid increased anti-inflammatory and decreased pro inflammatory cytokine levels

We estimated the effect of rosmarinic acid (25 and 50 mg/kg) treatment on plasma levels of anti-inflammatory biomarker IL-10, NF-kB, and TNF-α (*Figure 39*). The one-way ANOVA showed significant ($p < 0.0001$) variations among the groups [IL-10; ($F_{8,45} = 10.13$, $p < 0.0001$), [NF-kB; ($F_{8,45} = 15.68$, $p < 0.0001$), and TNF-α; ($F_{8,45} = 29.83$, $p < 0.0001$)]. Interestingly, rosmarinic acid at dose 50 mg/kg and fluoxetine 10 mg/kg significantly ($P < 0.05$) increased the levels of IL-10 and decreased the levels of NF-kB and TNF- α in MS combined CUS received ISO group rats.



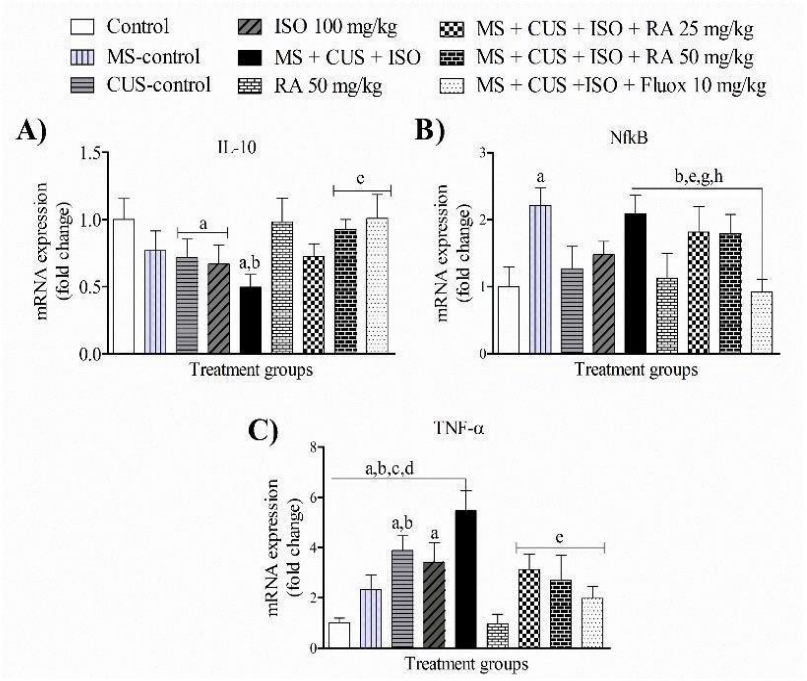



Figure 39: Rosmarinic acid (25 and 50 mg/kg) treatments reversed the expressions of cytokines A) IL-10, B) NFkB, and C) TNF-α.

[a]p < 0.05 versus control, [b]p < 0.05 versus MS-control, [c]p < 0.05 versus CUS-control, [d]p < 0.05 versus ISO-control, [e]p < 0.05 versus MS + CUS + ISO (100 mg/kg). G-I Control, G-II Maternal separation (MS), G-III CUS, G-IV Isoproterenol (ISO) 100 mg/kg, G-V MS + CUS + ISO, G-VI Rosmarinic acid (RA) 50 mg/kg, G-VII MS + ISO + RA 25 mg/kg, G-VIII MS + ISO + RA 50 mg/kg, G-IX MS + ISO + Fluoxetine 10 mg/kg. Values represented (mean ± SD, N=6 rats per group).

### 4.3.11 Histopathology

The rosmarinic acid treatment significantly reversed the MS-control, CUS-control, isoproterenol, and MS combined CUS treated ISO induced changes in heart. The histopathological changes in experimental group are presented in *Figure 40*. The control group and only rosmarinic acid 50 mg/kg treated group showed normal cardiac muscle fibres and without any tissue infarction. However, tissues from the MS treated, CUS treated, and ISO treated rats exhibited separation of myofibrillar in heart. Interestingly, rosmarinic acid (25 and 50 mg/kg) treated diseased group revealed near control cardiac muscle fibres in the heart.

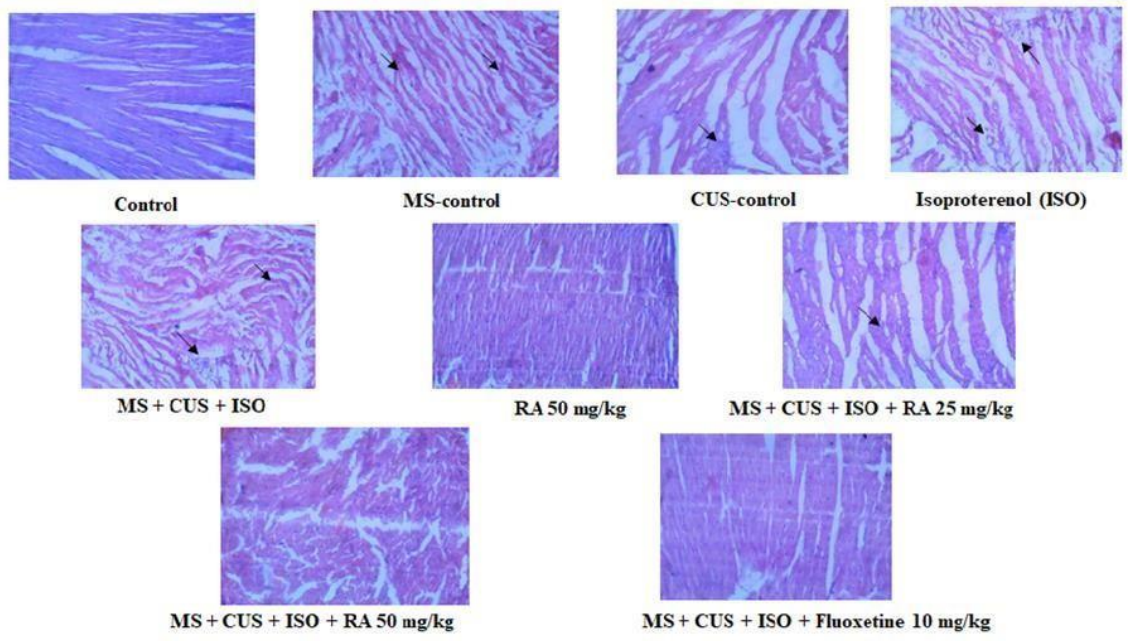


Figure 40: Rosmarinic acid (25 and 50 mg/kg) treatments in heart tissue experimental groups.

A) Control and F) Rosmarinic acid (50 mg/kg) group showing normal histology of myocardial section and clear transverse striations in heart. B) MS, C) CUS, D) ISO, and E) MS + CUS + ISO groups showing separations of cardiac myofibrillar in heart; G) MS + CUS + ISO + rosmarinic acid 25 mg/kg and H) MS + CUS + ISO + rosmarinic acid 50 mg/kg group showing improvement on myocardial necrosis; I) MS + CUS + ISO + Fluoxetine 10 mg/kg showing clear transverse striations with normal myocardial arrangement.

## 4.4 Discussion

The aim of the present study was to measure the severity of MS + CUS (dual stress model) induced depressive-like behavior which may further lead to cardiac aberrations in rats. In results, independent MS, chronic unpredictable stress, and ISO significantly increased the depression and cardiac abnormalities in animals. While, MS combined CUS received ISO aggravated the disease condition than independent stressors. This was supported by an increase in immobility period, anhedonia behavior, body weight, heart weight index, adrenal hyperplasia, plasma corticosterone level, levels of cardiac biomarkers (CKMB, LDH, cTn-I, AST, ALT, and MMP-2), lipid peroxidation, nitrite level, and ST-elevation. Whereas, a decrease in BDNF level, serotonin level, SOD activity, and anti-inflammatory cytokine IL-10 level was evident. Interestingly, rosmarinic acid administration was able to normalize all the abnormal behavioural and biochemical parameters related to depression and cardiac disease.

Through network pharmacology, we have identified 10 genes (TNF, STAT3, JUN, IL1B, CASP3, PTGS2, CXCL8, FOS, CREB1, NFKB1) in which TNF signalling pathway plays a major contribution in MI, depression, and rosmarinic acid. TNF plays an important role in increasing the heart abnormalities and depression (Chu et al., 2012, Xu et al., 2020). Rosmarinic acid has been shown to produce anti-inflammatory activity

*via* targeting TNF and NF-kB signalling pathway (Han et al., 2017). Further, the identified targets were validated through docking study. All the drug targets showed good affinity towards their respective ligands. The findings from the network pharmacology and docking correlates well with the results reported in Chapter 1 and 2.

This is the first study to check the cardiac abnormalities using two combined stress model (MS and CUS) in Wistar rats. Now-a-days, living with trauma (maternal stress and impulsive stress such as unpredictable stress) experience is a new normal for human beings worldwide. The increased immobility period and changes in body weight exhibits typical indices of depressive-like behavior [272-274]. Similarly, decreased sucrose preference (anhedonia symptoms) is a core feature of depressive-like behavior suggesting decreased responsiveness to rewarding stimuli in animal models. The combined stress (MS and CUS) model may perhaps induce enduring behavioral trouble and signs of clinical depression suitable for assessing antidepressant effects. Data from the literature states that the model of depressive-like behavior may cause other behavioral abnormalities in animals [502]. In our study, behavioral data (modulation of immobility period, anhedonia behavior, and body weight) suggested that rosmarinic acid produce an antidepressant-like outcome that may standstill further cardiac issues *via* improving behavioral abnormalities.

Chronic stress-like MS and CUS exposure may alter feeding behavior which may lead to loss/gain of body weight and augment adrenal gland weight in experimental animals [282, 284, 503, 504]. In chronic stress condition, hypersecretion of ACTH (adrenocorticotropic hormone) from pituitary gland leads to unnecessary stimulation of adrenal gland causing hyperplasia [286, 287]. In our study, with changes in body weight, we also observed the adrenal hyperplasia in stressed model group (MS-control,

CUS-control, ISO, and MS combined CUS received ISO) rats compared with control rats. Interestingly, long-term rosmarinic acid administration reversed weight changes may be by improving (immobility time and anhedonia symptoms) behavioural issues.

In depressed condition, alteration in molecular biomarkers (corticosterone, BDNF, and 5-HT) are evident in body, which is managed by anti-depressant agents [289, 290]. It has been reported that MS or CUS paradigms may cause excess amount of corticosterone release by hyperactivation of HPA-axis [287, 291, 292]. In line with literature, we detected significantly higher levels of corticosterone in stressed (MS-control, CUS-control, and ISO group) rats. ISO binds with β-hypothalamic receptor which also increases the glucocorticoid level (Axelrod and Reisine, 1984). In addition, we estimated serotonin level in PFC part of brain, which involved in the depressive condition [295]. Some reports stated that chronic oxidative stress and cytokines (inflammation) reduce PFC size causing dysfunction in secretion of neurotransmitters [296-298]. In addition, serotonin dysfunction plays a vital role in the induction of cardiac abnormalities *via* blood vessels constriction and inflammation [225, 226, 299]. Previous studies reported that RA inhibit the synthesis of indoleamine 2,3-dioxygenase (IDO, an enzyme responsible for metabolism of tryptophan amino acid or kynurenine pathway) and activate the enzyme tryptophan hydroxylase to increase the serotonin synthesis [505, 506]. Further, research reports stated that upregulation of serotonergic system *via* rosmarinic acid treatment could be due to enhancement in antioxidative system and inhibition of monoamine oxidase-A (MAO-A) enzyme involved in the metabolism of serotonin in the brain [300, 303-305]. In line with earlier reports, in the present study, rats in the rosmarinic acid group showed significantly increased serotonin level relative to all stressed rats. In adults, BDNF maintain the integrity of the vascular system *via* neural signals, cardiac afferent fibres, and antioxidant system to protect

ischemic heart injuries. In our studies, sharp decrease in BDNF level was observed in stressed rats. However, rosmarinic acid administration increased the plasma BDNF levels. In stressed rats, reduced BDNF level leads to behavioural anomalies indicated by increased immobility period, anhedonia behavior, and body weight.

Platelet aggregation is a major contributing factor in depression associated cardiac abnormalities. Because, platelet serotonin shows opposite role than brain serotonin. In blood, serotonin causes platelet activation leading to platelet aggregation which is one of the major causes of myocardial damage. Platelet dysfunction in blood including increased platelet aggregation *via* secretion of serotonin provides clue for vulnerability of depressed individuals towards cardiac abnormalities. Earlier reports showed that long-term fluoxetine administration reduces blood serotonin and platelet level exerting an inhibitory effect on platelet activation [507]. Fluoxetine not only affect neuronal serotonin but also modulate peripheral serotonin to protect from MI [508]. Our *in-silico* and *in-vitro* results indicated that rosmarinic acid and fluoxetine significantly ameliorated and inhibited ADP - and thrombin -induced platelet aggregation. Therefore, rosmarinic acid could be a potential therapeutic option to treat depression associated MI.

For survival of patients, an early detection of illnesses as well as initial treatment as early as possible is important [309]. For the detection of cardiac deviations, ECG is the major preclinical or clinical test [310]. In the present study, we observed that MS and CUS caused cardiac abnormalities indicated by ST-elevation and increased QRS complex (ventricular depolarization). The ST-segment elevation is the characteristic feature of myocardial infarction whereas; increased width of QRS complex indicates abnormalities in the conduction system (slow ventricular depolarization) leading to

ventricular hypertrophy and hyperkalaemia [313, 314]. Our findings revealed that MS and CUS mainly affect ST segment and QRS complex of the ECG. Additionally, cardiac biomarkers (cTn-I, CKMB, LDH, AST, ALT, and MMP2) are important for diagnosis of cardiac abnormalities [315]. Therefore, in this study, blood (plasma and serum) and heart tissue were used to measure the cardiac biomarkers. The MMP2 is a proteolytic enzyme degrades troponin complexes in cardiac tissue causing increase in cTn-I level in the bloodstream [158]. Oxidative stress is a major factor for abnormal levels of cTn-I and MMP-2 enzyme [316] in MS and CUS rats, reversed by rosmarinic acid due to higher antioxidant and anti-inflammatory activity.

In chronic stress, the release of a massive amount of ROS/RNS or free radicals leads to cellular damage due to imbalance in oxidative and antioxidative system [317, 318]. Lipid peroxidation is a major biomarker associated with increased depressive signs *via* oxidative stress [261]. Similarly, SOD is an anti-oxidant enzyme that converts free radicals into hydrogen peroxides, water, and molecular oxygen [65, 323, 325]. In this study, SOD activity, lipid peroxidation, and nitrite levels were altered in the stressed group (MS-control, CUS-control and ISO) than the control group. Whereas, rosmarinic acid significantly increased SOD activity, decreased lipid peroxidation, and decreased nitrite level in tissues. Consequently, rosmarinic acid exhibited a defensive effect towards the MS and CUS-induced oxidative damage in the tissues. Chronic stress causes elevation of inflammation *via* overactivation of sympathetic nervous system and cell mediated acquired immunity immune dysfunctions which involved in the development of comorbidities such as psychiatric disorders and cardiac anomalies [326]. During exposure of stress, neuroendocrine factors lead to dysfunction of anti-inflammatory cytokine [327]. The anti-inflammatory cytokine (IL-10) in lower-level has been linked to depression associated cardiac anomalies [70, 328, 329]. In this study,

stressed group (MS-control, CUS-control, ISO, and MS combined CUS treated ISO) rats showed significantly decreased IL-10 level than control group. Our results reassert the role of inflammation in the pathogenesis of depression associated cardiac anomalies. Remarkably, rosmarinic acid administration significantly increased the level of anti-inflammatory cytokine (IL-10) relative to the stressed rats. These aforementioned outcomes revealed that rosmarinic acid has exhibited defensive and promising therapeutic properties against MI comorbid depression in rats.

## 4.5 Conclusion

The present study revealed a defensive role of rosmarinic acid treatment *via* abrogation of MS combined CUS-induced increase in behavioral dysfunctions such as immobility period, sucrose preference, and body weight. Further, rosmarinic acid decreased the adrenal hyperplasia, plasma corticosterone levels, pro-inflammatory cytokine level, and cardiac biomarkers in stressed rats. Whereas, treatment with rosmarinic acid increased serotonin level, BDNF level and increased SOD activity. Moreover, rosmarinic acid modulated the gene expressions of IL-10, NF-kB, and TNF-α in stress rats. Furthermore, rosmarinic acid treatment modulated the irregularities in ECG parameters such as ST elevation and increased QRS complex. In addition, network pharmacology and docking study further validated our study findings *via in-silico* method. By using network pharmacology, we found the common target pathways such as TNF and NF-kB signalling pathway between MI and depression. Further, the docking study revealed that RA binds with the targeted receptors (MMP2, STAT3, TrkB, $P_2Y_{12}$, and TXA2 receptors) with good binding energy. In addition, we found that rosmarinic acid had good binding affinity with the receptors as compare to the reference drug. The protective effects of rosmarinic acid against MS combined CUS induced cardiac changes was additionally supported by decrease in lipid peroxidation, nitrite level, and increased SOD activity. Interestingly, the current findings suggest that rosmarinic acid is a good treatment choice against dual model (MS combined CUS) aggravating cardiac abnormalities.