Chapter 2: Prophylactic administration of rosmarinic acid ameliorates CUS-associated cardiac abnormalities in Wistar rats

2.1 Introduction

Major depressive disorder is a well-known worldwide disabilities and primarily has some characteristic symptoms such as low mood, appetite change, weight gain/loss, sleep abnormality, lack of pleasure (anhedonia), and not involve in regular activities are the features of depression [212]. According to Global health exchange (GHE) data reported that 5% adults are affected from depressive disorder [213]. Whereas, cardiovascular disease (CVDs) is another disability worldwide issue, which is a group of disorder (coronary, congenital, cerebrovascular heart disease, cardiac arrhythmia, heart attack, and heart failure) of the heart and blood vessel [214]. Recent study reported that 18.5 million deaths occurred due to cardiac diseases globally [215].

Depression is a major prevalent risk factor for coronary events [216]. A recent study indicated that depression is a major comorbid condition in cardiac patients [216]. In a meta-analysis (30 prospective cohort studies), a researcher reported that depression increases the possibility of future cardiac abnormality by approximately 30% [216-218]. There is several evidence suggesting that depression and cardiac abnormalities shows bidirectional link and share numerous pathophysiological characteristics [219-221]. Chronic stress activates the HPA axis, releasing catecholamines and corticosterone [222]. These catecholamines increase pro-inflammatory cytokines, decrease heart volume, and aggravate sympathetic system leading to cardiac abnormalities [222-224]. Among catecholamines, serotonin is a common contributing factor in depression, inflammation, hypertension, and vasoconstriction that leads to cardiac abnormalities [225, 226]. Chronic stress-induced oxidative stress orchestrated by reduced anti-oxidative enzymes and increased free radicals [227] causes hyperactivation of matrix metalloproteinase (MMP-2) [228, 229]. The excess MMP-2

degrades cytoskeletal proteins such as cardiac troponin-I (cTn-I), leading to myocardial injury and cardiac dysfunction [158, 222, 230, 231].

In this study, rosmarinic acid was administered prophylactically and all the parameters related to depression and cardiac changes were measured in animals. Experimental design includes behavioral tests (forced swim test, sucrose preference test, body weight) and biochemical tests (enzyme linked immunosorbent assays for plasma corticosterone, serotonin in prefrontal cortex, plasma proinflammatory cytokines (TNF-α and IL-6), serum cTn-I, and MMP2. We also performed electrocardiography under isoflurane anesthesia *via* LabScribe software, IXTA data acquisition unit (iWorx-B3G) measured changes in P wave, T wave, RR interval, QRS complex, QT interval, PR interval, and ST segment.

Rosmarinic acid (RA) is an ester of 3,4-dihydroxy phenyl lactic acid and caffeic acid isolated from *Rosmarinus officinalis* (Lamiaceae) [232]. RA has many interesting biological activities such as anti-depressant, anti-oxidant, anti-inflammatory, antimutagenic, and antiviral activities [233, 234]. Traditionally, RA containing herbs, spices, and medicinal plants have been used extensively for their health-promoting effects [235, 236]. Earlier reports stated that chronic use of rosmarinic acid could manage depressive-like behavior [237, 238] and cardiac abnormalities [239] in separate animal models. However, the therapeutic potential of rosmarinic acid against depression associated cardiac abnormalities has not been studied yet. Therefore, the present study aimed to investigate the effects of rosmarinic acid against CUS-induced depression associated cardiac abnormalities in rats.

2.2 Materials and methods

2.2.1 Animals

Adult male Wistar rats (159–200 g) were obtained from central animal house (Institute of Medical Sciences Banaras Hindu University), Varanasi, India. Rats were acclimatized for 7 days with standard temperature, relative humidity, and light. All the experiments were performed in accordance with the principles established by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and were approved by the Institutional Animal Ethical Committee (IAEC) of the University (Banaras Hindu University, Varanasi, India) (Dean/2018/IAEC/622). All rats received proper food pellets and water during the whole experiments and behavioral tests were performed according to the experimental design.

2.2.2 Drug preparation and treatment

Rosmarinic acid (RA) was procured from Sigma Aldrich, India and for oral administration two different suspensions (25 mg/mL and 50 mg/mL) were prepared using 0.5% tween-80. The doses (25 mg/kg/day and 50 mg/kg/day) of rosmarinic acid were selected according to the earlier experimental studies [240-242].

2.2.6 Experimental design

After acclimatization, rats were divided into four groups (N = 6), and the experimental study was performed for 40 days. Group I consisted of naïve rats received food and water *ad libitum*; group II rats received chronic unpredictable stress; group III rats received chronic unpredictable stress + rosmarinic acid (25 mg/kg/day); and group IV rats received chronic unpredictable stress + rosmarinic acid (50 mg/kg/day). During

the experiments, rosmarinic acid was orally administered 30 min before induction of stress (*Figure 7*).

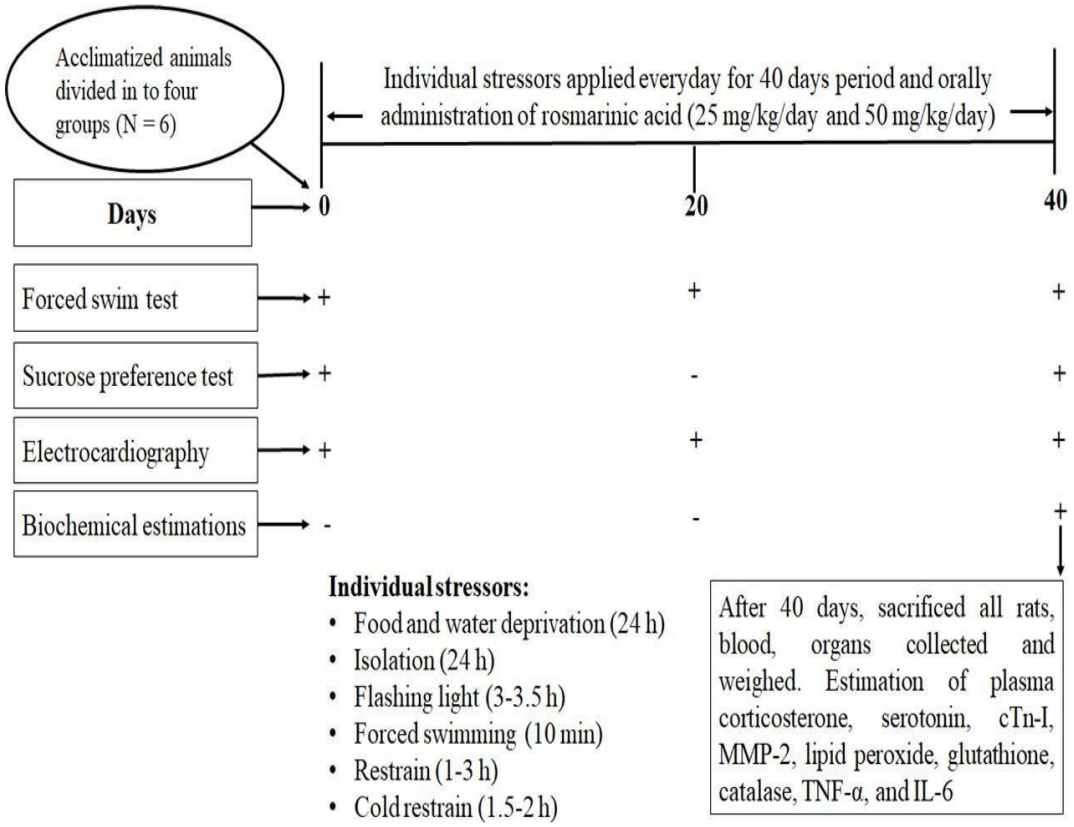

Figure 7: Schematic representation of experimental design.

RA; Rosmarinic Acid, cTn-I; Cardiac Troponin-I, MMP-2; Matrix Metalloproteinase, TNF-α; Tumor Necrosis Factor-α, IL-6; Interleukin-6, CUS; Chronic Unpredictable Stress.

2.2.3 Chronic unpredictable stress exposure

Except control group, all animals were exposed to an individual stressor (*Table 1*) for 40 days in the CUS paradigm except control group [16, 243]. Briefly, the stressors were food and water restriction (animals were restricted of both food as well as water for 24 h), flashing light (animals were exposed to a light of intensity 500 lux at the height of 50 cm from the bottom of the cage for 24 h), isolation (rat was kept in a single cage for 24 h), restraint (1-3 h), cold restraint (1.5-2 h at 4 °C), and forced swimming (rats were forced to swim for 10 or 15 min).

Table 1. Individual stressor applied during 40 days.

Days of stressor	Stressor	Duration
1	Water restriction	24 h
2	Food restriction	24 h
3	Isolation	24 h
4	Isolation	24 h
5	Isolation	24 h
6	Flashing Light	3 h
7	Food restriction	24 h
8	Forced swimming	10 min
9	Restraint	1 h
10	Water restriction	24 h
11	No stressor applied	-
12	No stressor applied	-
13	Restraint cold	2 h
14	Flashing Light	2.5 h
15	Food restriction	24 h
16	Forced swimming	15 min
17	Isolation	24 h
18	Isolation	24 h
19	Isolation	24 h
20	water restriction	24 h
21	Food restriction	24 h
22	Flashing Light	3 h
23	Restraint	2 h
24	Isolation	24 h
25	Isolation	24 h
26	Restraint cold	1.5 h
27	Forced swimming	10 min
28	Flashing Light	3.5 h
29	No stressor applied	-
30	Food restriction	24 h
31	Restraint	3 h
32	Flashing Light	2 h
33	water restriction	24 h
34	Restraint cold	2 h
35	Forced swimming	15 min
36	Isolation	24 h
37	Isolation	24 h
38	No stressor applied	-
39	Flashing Light	3 h
40	Forced swimming	10 min

2.2.4 Body weight

Body weight is an essential parameter for measuring depressive behavior in rodents as well as humans [244]. In the present study, the body weights of all the rats were recorded throughout the experiment and compared against day 0.

2.2.5 Electrocardiography

Electrocardiography (ECG) was performed for all groups on days 0, 20, and 40 after the start of experimentation. Briefly, animals were anaesthetized under isoflurane anesthesia (2% in 100% oxygen at 300 mL/min following induction in a chamber containing 3–5% isoflurane in oxygen) and body temperature was maintained at 37 ± 1 °C using a thermostat-controlled heating pad as done in our previous study [245]. ECG recording was done using LabScribe software, IXTA data acquisition unit (iWorx-B3G) connected with 3 electrodes (red, black, and green) per rat [246, 247]. The ECG parameters (P wave width, RR Interval, PR interval, QT interval, ST segment, T wave width, and QRS complex) were estimated as described previously [245, 247, 248].

2.2.7 Forced swim test

The forced swim test was conducted to measure the immobility period as described previously [249, 250]. After the trial period, on day 0, the first 5 min immobility period was recorded. After the drug administration, rats were again exposed to forced swim test and immobility period was recorded on day 20 and day 40. According to the researchers, the rat was judged immobile when it remained afloat in water, moving if the head of the rat over the water or keep its nose above the surface [251-253].

2.2.8 Sucrose preference test

Sucrose preference test was used to determine the anhedonia symptom, which is a common symptom of depression in rodents [254]. All rats were subjected to a sucrose preference test before CUS and on day 40 after CUS employment. Each rat was kept in a single isolated cage and allowed free access to 2 bottles containing 50 mL of 1% w/v sucrose solution and 50 mL of water. Then, the volume of consumed sucrose and water was recorded to estimate sucrose preference (%) = sucrose preferred (mL)/[sucrose preferred (mL) + water preferred (mL)] ×100 [16, 255].

2.2.9 Collection of tissues and tissue homogenation

After 40 days of the CUS paradigm, rats were anesthetized using CO_2, and blood was collected through retro-orbital puncture (before scarification of rats). Plasma and serum were separated from the blood by centrifugation (1500 g and 3000 g for 10 min at 4 °C, respectively) and stored at −20 ºC for estimation of corticosterone, TNF-α, IL-6, and cTn-I. After blood sample collection, animals were sacrificed by cervical dislocation and tissues (brain, heart, and adrenal glands) were collected and weighed to estimate the wet weight per 100 gm of body weight and stored at −80 ºC until assayed. Rat brain sample was used to estimate serotonin levels in the PFC of brain as described previously [250]. From each tissue sample, a 10% w/v tissue homogenate was prepared in phosphate-buffered saline (PBS) and centrifuged at 15000 g for 10 minutes to collect the supernatant for MMP-2 and oxidative stress determination.

2.2.10 Estimation of plasma corticosterone level

Corticosterone level is an important biomarker for the assessment of depressive-like behavior in rats [256-258]. The plasma concentration of corticosterone was measured using a rat corticosterone enzyme-linked immunosorbent assay kit according

to the manufacturer's instructions (DSI S.r.l., Italy). The corticosterone levels were estimated from a standard curve and values were expressed as ng/mL.

2.2.11 Estimation of serotonin level in prefrontal cortex

A reduced level of brain serotonin level has been indicated in the depressive-like behavior of rodents [250, 259]. In the present study, the serotonin levels were estimated using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Labor Diagnostika Nord GmbH & Co, Germany). The serotonin levels were estimated from a standard curve and values were expressed as ng/g of brain tissue.

2.2.12 Estimation of cTn-I and MMP-2

The levels of cardiac biomarkers cTn-I in serum and MMP-2 in the heart were estimated using enzyme-linked immunosorbent assay kits (Abcam, UK and IBL, USA, respectively) according to the manufacturer's instructions. The concentrations of cTn-I and MMP-2 were estimated from a standard curve and values were expressed as pg/mL and ng/g of tissue, respectively.

2.2.13 Estimation of oxidative stress in brain and heart tissues

Oxidative stress is a major pathological mechanism in depression and cardiac abnormalities [260]. It is well-known that lipid peroxidation is one of the biomarkers for oxidative stress [261]. Ohkawa and group specified the measurement of lipid peroxidation by quantification of the end product called malondialdehyde (MDA) [262]. The estimation of glutathione in the brain and heart was performed by the Ellman method using dithionitrobenzoic acid (DTNB) [263]. The homogenate was mixed with trichloroacetic acid (10%), centrifuged and separated supernatant was mixed with 2 mL of 0.3 M phosphate buffer (pH 8.4), 400 µL double-distilled water, and 500 µL of

DTNB. The resultant mixture was incubated for 10 min and absorbance was recorded at 412 nm by iMark microplate reader (Bio-Rad Laboratories, USA). The glutathione concentration was calculated from the standard curve and expressed in nmol/mg of protein [263, 264]. Catalase enzyme activity was estimated using a commercial kit (Sigma USA). The concentration of catalase in samples was calculated from the standard curve and expressed in µmol H_2O_2/min/mg of protein. The protein content was quantified using Lowry method as described previously [265].

2.2.14 Estimation of pro-inflammatory cytokines

Inflammation is another pathophysiological feature which links depression and associated cardiac abnormalities [266, 267]. Pro-inflammatory biomarkers (TNF-α and IL-6) in the plasma were measured using enzyme-linked immunosorbent assay kit (Ray Biotech, USA) according to the manufacturer's instructions. The concentrations of pro-inflammatory biomarkers (TNF-α and IL-6) were estimated from a standard curve and expressed as ng/mL.

2.2.15 Statistical analysis

Data were represented as the mean ± standard deviation (SD). Statistical analysis was performed by using GraphPad Prism version 7.03 (GraphPad Software Inc., USA). All data sets with one independent variable were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and the data sets with two independent variables were analysed using two-way ANOVA followed by Bonferroni's test. A value of $P < 0.05$ was set as statistically significant.

2.3 Results

The results obtained from different experiments in this study indicated a cardioprotective role of prophylactically administered rosmarinic acid against depression-associated cardiac abnormalities.

2.3.1 Effects of rosmarinic acid on immobility period and sucrose preference

Immobility period was significantly ($P < 0.05$) increased on day 20 (138.5 ± 10.3 versus 90.6 ± 7.8 sec, Figure 8A) and day 40 (154.5 ± 11.2 versus 93.3 ± 7.9 sec, Figure 8A) in CUS-control group compared to control group. Whereas, percentage of sucrose preference was significantly ($P < 0.05$) reduced on day 40 (31.4 ± 3.7 versus 73.2 ± 6.7 %, Figure 8B) in the CUS-control group compared to the control group. Prolonged administration of rosmarinic acid at dose 25 mg/kg (day 20, 110.3 ± 6.6 versus 138.5 ± 10.3 sec, Figure 8A; day 40, 124.1 ± 9.7 versus 154.5 ± 11.2 sec, Figure 8A) and 50 mg/kg (day 20, 95.0 ± 0.7 versus 138.5 ± 10.3 sec, Figure 8A; day 40, 113.5 ± 9.8 versus 154.5 ± 11.2 sec, Figure 8A) significantly ($P < 0.05$) decreased the immobility period as compared to CUS-control group. Whereas, in sucrose preference test, rosmarinic acid at dose 25 mg/kg (day 40, 40.4 ± 4.8 versus 31.4 ± 3.7 %; Figure 8B) and 50 mg/kg (day 40, 52.8 ± 8.1 versus 31.4 ± 3.7 %, Figure 8B) significantly ($P < 0.05$) increased the percentage of sucrose preference as compared to CUS-control group (*Figure 8*).

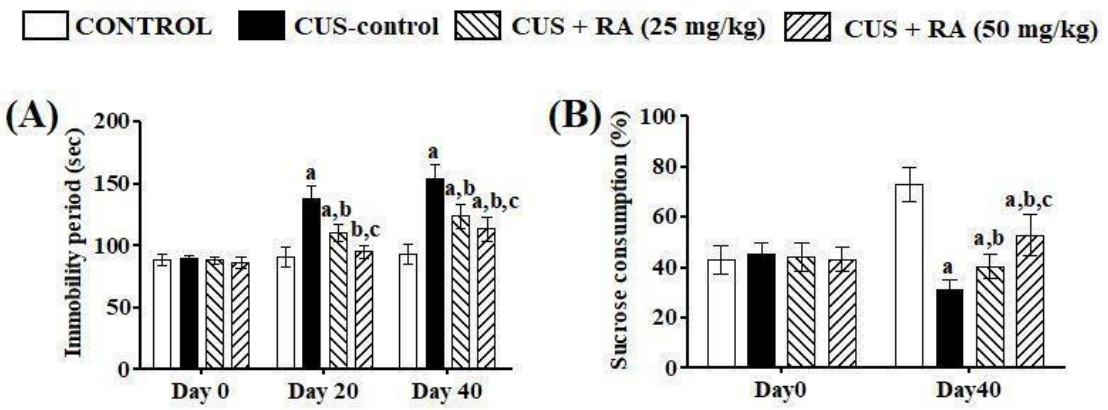

Figure 8: Effects of RA treatment on immobility period and sucrose preference.

All values represented as mean ± SD, N = 6 rats per group. [a]P < 0.05 versus control, [b]P < 0.05 versus CUS-control, [c]P < 0.05 versus CUS + RA (25 mg/kg); Repeated measure two-way ANOVA followed by Bonferroni test. RA- Rosmarinic Acid.

2.3.2 Effects of rosmarinic acid on body weight

As shown in *Figure 9*, at the beginning, the body weight of rats in each group did not show any significant (P > 0.05) difference (159.0 ± 7.9 versus 159.8 ± 6.3 g, Figure 9). But, after one week of CUS, body weight significantly (P < 0.05) decreased (147.0 ± 5.5 versus 162.7 ± 7.3 g, Figure 9) compared to the control group, and this trend continued for 40 days (78.7 ± 12.4 versus 186.0 ± 8.5 g, Figure 9). Chronic administration of rosmarinic acid 25 mg/kg (133.3 ± 4.7 versus 186.0 ± 8.5 g, Figure 9) and 50 mg/kg (181.7 ± 4.4 versus 186.0 ± 8.5 g, Figure 9), significantly (P < 0.05) prevented the loss of body weight (*Figure 9*).

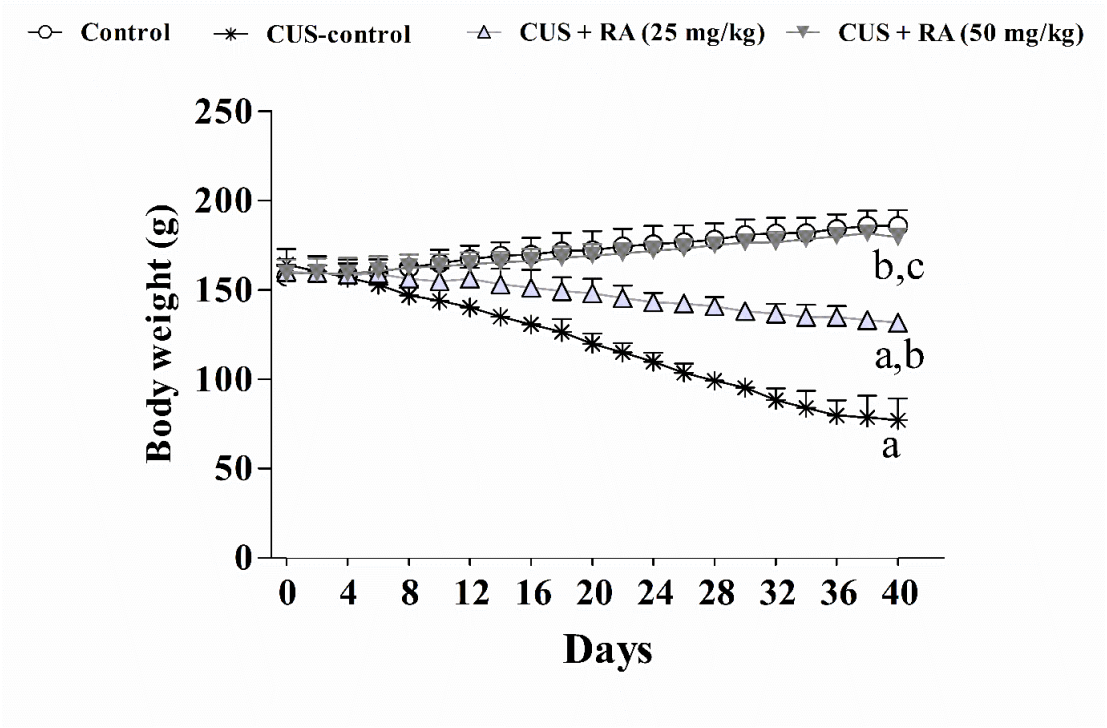

Figure 9: Effects of RA (25 and 50 mg/kg) on body weight.

All values represented as mean ± SD, N = 6 rats per group. [a]P < 0.05 versus Control, [b]P < 0.05 versus CUS-control, [c]P < 0.05 versus CUS + RA (25 mg/kg); Repeated measure two-way ANOVA followed by Bonferroni test. RA- Rosmarinic Acid

2.3.3 Effects of rosmarinic acid on brain, heart, and adrenal gland weight

After exposure to 40 days of stress, the weight of brain (0.76 ± 0.23 versus 1.42 ± 0.15 g, Figure 10A) and heart (0.30 ± 0.04 versus 0.58 ± 0.06 g, Figure 10B) was significantly ($P < 0.05$) reduced in CUS-control group as compared to control group. Chronic administration of rosmarinic acid at dose 25 mg/kg (brain, 0.91 ± 0.24 versus 0.76 ± 0.23 g, Figure 10A; heart, 0.38 ± 0.06 versus 0.30 ± 0.04 g, Figure 10B) and 50 mg/kg (brain, 1.21 ± 0.38 versus 0.76 ± 0.23 g, Figure 10A; heart, 0.45 ± 0.08 versus 0.30 ± 0.04 g, Figure 10B) increased weight of organs significantly ($P < 0.05$) as compared to CUS-control group. In addition, significant ($P < 0.05$) adrenal hyperplasia was observed in CUS-control group as compared to control group (0.054 ± 0.01 versus 0.034 ± 0.004 g, Figure 10C). However, a significant ($P < 0.05$) decrease in adrenal hyperplasia was observed in the rosmarinic acid group at dose 25 mg/kg (0.043 ± 0.01

versus 0.054 ± 0.01 g, Figure 10C) and 50 mg/kg (0.037 ± 0.002 versus 0.054 ± 0.01 g, Figure 10C) as compared to CUS-control group (*Figure 10*).

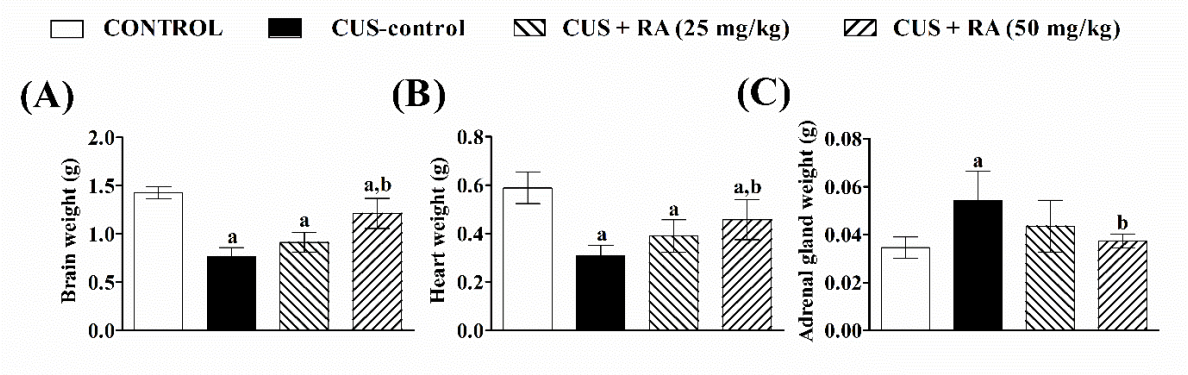

Figure 10: Effects of RA (25 and 50 mg/kg) treatment on (A) brain, (B) heart, and (C) adrenal gland weight in CUS-control group.

All values represented as mean ± SD, N = 6 rats per group. [a]P < 0.05 versus control, [b]P < 0.05 versus CUS-control, [c]P < 0.05 versus CUS + RA (25 mg/kg); Repeated measure one-way ANOVA followed by Tukey's test. RA- Rosmarinic Acid.

2.3.4 Effects of rosmarinic acid on corticosterone and serotonin levels

After continuous 40 days of CUS, the plasma corticosterone levels (168.3 ± 23.2 versus 70.6 ± 11.0 ng/mL, Figure 11A) were significantly (P < 0.05) higher and serotonin levels (135.8 ± 31.1 versus 273.3 ± 37.6 ng/g of tissue, Figure 11B) were significantly (P < 0.05) lower in CUS-control group as compared to control group. Rosmarinic acid at dose 25 mg/kg (corticosterone, 139.1 ± 18.1 versus 168.3 ± 23.2 ng/mL, Figure 11A; serotonin, 200.0 ± 41.1 versus 135.8 ± 31.1 ng/g of tissue, Figure 11B) and 50 mg/kg (corticosterone, 98.6 ± 13.8 versus 168.3 ± 23.2 ng/mL, Figure 11A; serotonin, 258.1 ± 27.5 versus 135.8 ± 31.1 ng/g of tissue, Figure 11B) showed a significant (P < 0.05) decrease in plasma corticosterone and increase in serotonin levels in prefrontal cortex compared to the CUS-control group (*Figure 11*).

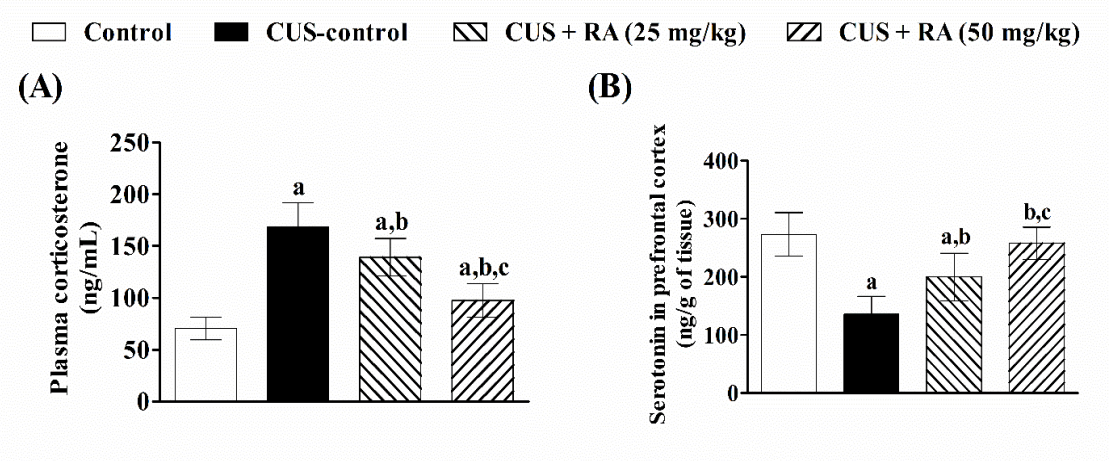

Figure 11: Effects of RA (25 and 50 mg/kg) on (A) plasma corticosterone and (B) serotonin (5-HT) in prefrontal cortex.

All values represented as mean ± SD, N = 6 rats per group. [a]P < 0.05 versus Control, [b]P < 0.05 versus CUS-control, [c]P < 0.05 versus CUS + RA (25 mg/kg); Repeated measure one-way ANOVA followed by Tukey's test. RA- Rosmarinic Acid.

2.3.5 Effects of rosmarinic acid on cardiac abnormalities

Electrocardiography (ECG) is a primary diagnostic technique of heart in clinical settings [268, 269]. ECG is used for identifying the cardiac abnormalities through different ECG parameters such as (A) P wave width, (B) T wave width, (C) QRS complex, (D) RR interval, (E) QT interval, (F) ST segment, and (G) PR interval. Representative ECG tracings of rats from each group depicts in *Figure 12*. Decreased in T wave width (day 20, 15.3 ± 0.7 versus 26.6 ± 0.7 ms, Figure 12B; day 40, 12.6 ± 1.7 versus 26.6 ± 0.7 ms, Figure 12B) and increase in QRS complex (day 20, 31.6 ± 1.2 versus 19.9 ± 1.9 ms, Figure 12C; Day 40, 36.3 ± 1.5 versus 22.6 ± 2.1 ms, Figure 12C) in stressed rats compared to control rats. Interestingly, rosmarinic acid at dose 25 mg/kg (T wave width, day 20, 20.6 ± 0.5 versus 15.3 ± 0.7 ms, Figure 12B; T wave width, day 40, 18.3 ± 1.7 versus 12.6 ± 1.7 ms, Figure 12B; QRS complex, day 20, 27.6 ± 2.2 versus 31.6 ± 1.2 ms, Figure 12C; QRS complex, day 40, 29.6 ± 1.7 versus 36.3 ± 1.5 ms, Figure 12C) and 50 mg/kg (T wave width, day 20, 23.6 ± 1.0 versus 15.3 ± 0.7 ms, Figure 12B; T wave width, day 40, 24.3 ± 0.7 versus 12.6 ± 1.7 ms, Figure 12B; QRS

complex, day 20, 22.0 ± 2.0 versus 31.6 ± 1.2 ms, Figure 12C; QRS complex, day 40, 24.6 ± 2.1 versus 36.3 ± 1.5 ms, Figure 12C) showed significant ($P < 0.05$) increase in T wave width and decrease in QRS complex as compared to the CUS-control group. In the other parameters of ECG such as P wave width (Day 20, 17.5 ± 1.08 versus 17.3 ± 0.61 ms, Figure 12A; Day 40, 17.2 ± 1.0 versus 17.5 ± 1.3 ms, Figure 12A), RR interval (Day 20, 166.3 ± 1.7 versus 162.6 ± 1.7 ms, Figure 12D; Day 40, 162.3 ± 1.7 versus 163.3 ± 1.7 ms, Figure 12D), QT interval (Day 20, 64.6 ± 1.5 versus 64.0 ± 1.0 ms, Figure 12E; Day 40, 63.6 ± 1.1 versus 64.6 ± 1.5 ms, Figure 12E), ST segment (Day 20, 0.01 ± 0.001 versus 0.01 ± 0.001 mV, Figure 12F; Day 40, 0.01 ± 0.001 versus 0.01 ± 0.001 mV, Figure 12F), and PR interval (Day 20, 43.3 ± 1.7 versus 44.6 ± 1.7 ms, Figure 12G; Day 40, 44.3 ± 0.7 versus 43.3 ± 0.7 ms, Figure 12G) showed insignificant ($P > 0.05$) changes in CUS-control group compared to control group.

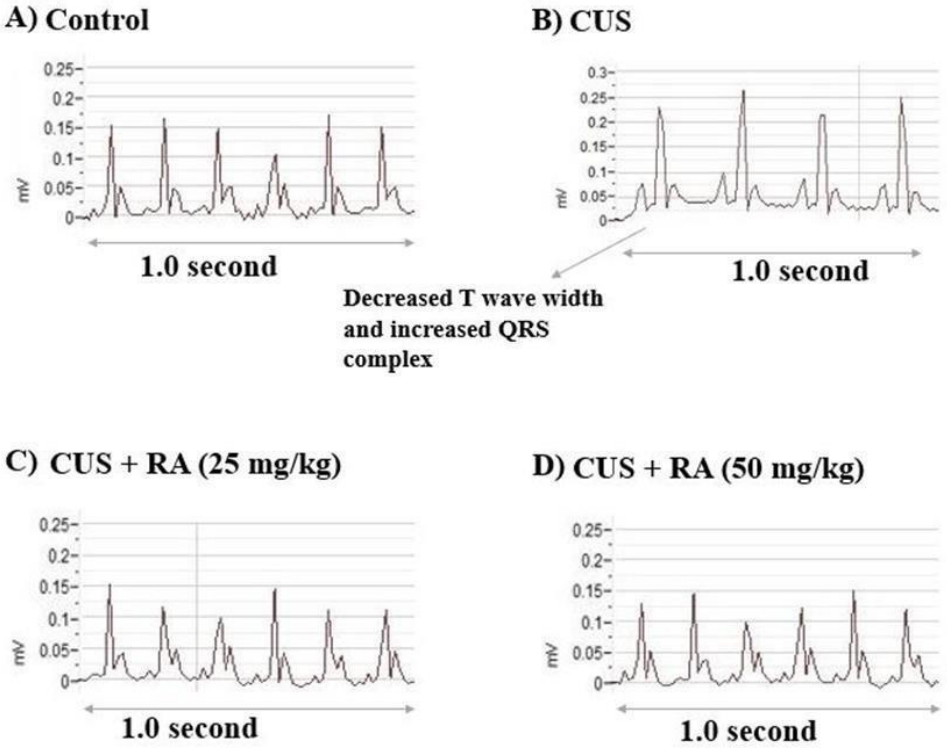

Figure 12: Effects of RA (25 and 50 mg/kg) pretreatment on CUS-induced alteration in ECG pattern.

Representative ECG recordings: (A) control; (B) CUS-control; (C) and (D) CUS + RA 25 mg/kg and 50 mg/kg, respectively. ECG tracing shows decrease in T wave width and increase in QRS complex of CUS control group prevented by rosmarinic acid at 25 and 50 mg/kg.

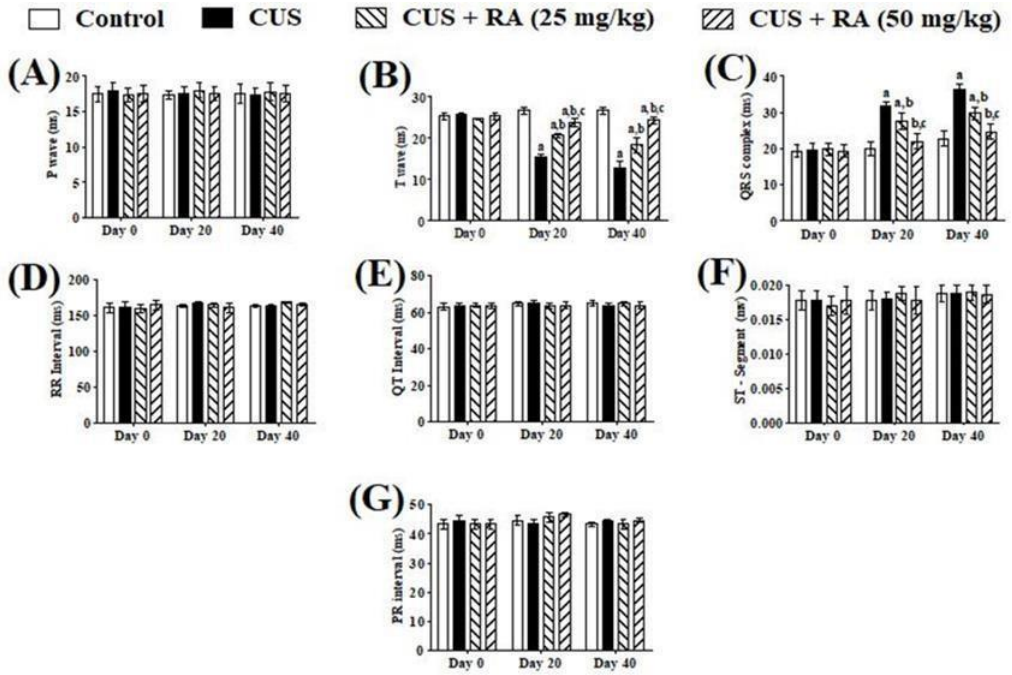

Figure 13: ECG analysis revealed that CUS causes significant ($P < 0.05$) decrease in (B) T wave width and (C) increased QRS complex.

2.3.6 Effects of rosmarinic acid on cardiac biomarkers in serum and heart tissue

After continuous 40 days of CUS, serum cTn-I levels (33.5 ± 4.2 versus 14.7 ± 1.72 pg/mL, Figure 14A) and MMP-2 level in heart (49.2 ± 3.0 versus 10.6 ± 0.4 ng/g of tissue, Figure 14B) were significantly ($P < 0.05$) higher in CUS-control group as compared to control group. Rosmarinic acid at dose 25 mg/kg (cTn-I, 25.6 ± 2.6 versus 33.5 ± 4.2 pg/mL, Figure 14A; MMP-2, 33.9 ± 1.8 versus 49.2 ± 3.0 ng/g of tissue, Figure 14B) and 50 mg/kg (cTn-I, 14.9 ± 3.1 versus 33.5 ± 4.2 pg/mL, Figure 14A; MMP-2, 14.2 ± 1.1 versus 49.2 ± 3.0 ng/g of tissue, Figure 14B) showed a significant ($P < 0.05$) decrease in levels of cTn-I and MMP-2 compared to the CUS-control group (*Figure 14*).

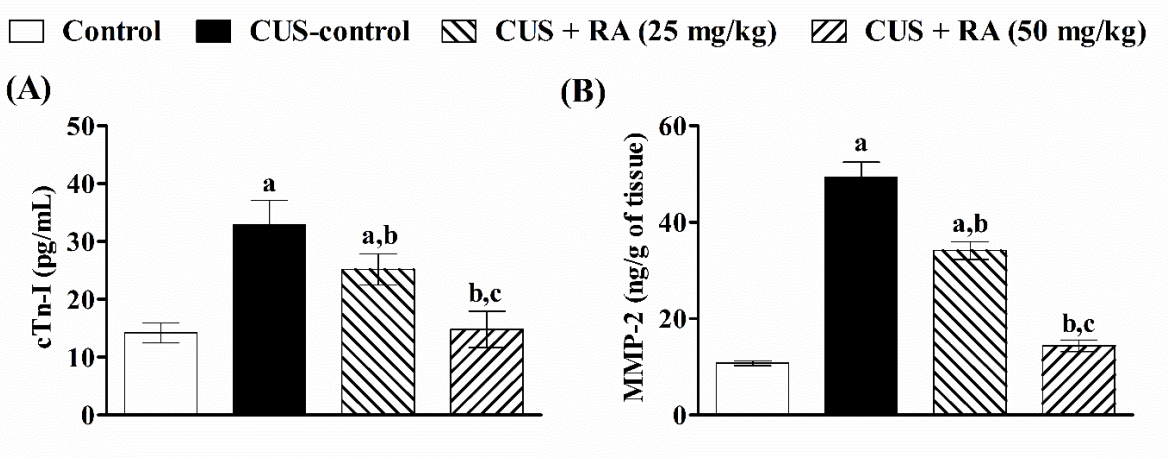

Figure 14: Effects of RA (25 and 50 mg/kg) on (A) serum cTn-I and (B) MMP-2 in heart tissue.

All values represented as mean ± SD, N = 6 rats per group. [a]$P < 0.05$ versus Control, [b]$P < 0.05$ versus CUS-control, [c]$P < 0.05$ versus CUS + RA (25 mg/kg); Repeated measure one-way ANOVA followed by Tukey's test. RA- Rosmarinic Acid.

2.3.7 Effects of rosmarinic acid on oxidative stress in brain and heart

2.3.7.1 Effects of rosmarinic acid on lipid peroxidation

In comparison to control group, CUS-control rats showed significantly ($P < 0.05$) increased levels of lipid peroxide, evidenced by increased malondialdehyde (MDA)

content in brain (14.3 ± 1.0 versus 5.4 ± 0.8 nmol MDA/mg protein, Figure 15A) and heart (16.2 ± 1.1 versus 6.0 ± 0.9 nmol MDA/mg protein, Figure 15B). Rosmarinic acid at dose 25 mg/kg (brain, 10.2 ± 1.1 versus 14.3 ± 1.0 nmol MDA/mg protein, Figure 15A; heart, 11.1 ± 1.1 versus 16.2 ± 1.1 nmol MDA/mg protein, Figure 15B) and 50 mg/kg (brain, 7.2 ± 0.8 versus 14.3 ± 1.0 nmol MDA/mg protein, Figure 15A; heart, 8.5 ± 0.8 versus 16.2 ± 1.1 nmol MDA/mg protein, Figure 15B) showed significant ($P < 0.05$) decrease in lipid peroxide levels comparison to CUS-control group (*Figure 15*).

2.3.7.2 Effects of rosmarinic acid on glutathione level

Oxidative stress was assessed by estimating glutathione levels in (C) brain and (D) heart. The glutathione levels in brain (0.09 ± 0.04 versus 0.9 ± 0.1 nmol/mg of protein, Figure 15C) and heart (0.03 ± 0.03 versus 0.8 ± 0.1 nmol/mg of protein, Figure 15D) were significantly ($P < 0.05$) lower in CUS-control rats compared to control group. However, chronic administration of rosmarinic acid at dose 25 mg/kg (brain, 0.4 ± 0.1 versus 0.09 ± 0.04 nmol/mg of protein, Figure 15C; heart, 0.6 ± 0.05 versus 0.03 ± 0.03 nmol/mg of protein, Figure 15D) and 50 mg/kg (brain, 0.7 ± 0.06 versus 0.09 ± 0.04 nmol/mg of protein, Figure 15C; heart; 0.7 ± 0.1 versus 0.03 ± 0.03 nmol/mg of protein, Figure 15D) significantly ($P < 0.05$) improved the levels of glutathione in brain and heart (*Figure 15*).

2.3.7.3 Effects of rosmarinic acid on catalase activity

We assessed oxidative stress by estimating the content of catalase in the brain and heart. In comparison to the control group, the CUS-control rats showed significantly ($P < 0.05$) lower levels of catalase in the brain (18.7 ± 3.4 versus 39.7 ± 6.4 µmol H_2O_2/min/mg of protein, Figure 15E) and heart (28.6 ± 2.8 versus 49.6 ± 5.8 µmol H_2O_2/min/mg of protein Figure 15F). On the other hand, rosmarinic acid at dose 25

mg/kg (brain, 27.1 ± 6.8 versus 18.7 ± 3.4 µmol H_2O_2/min/mg of protein, Figure 15E; heart, 37.0 ± 3.5 versus 28.6 ± 2.8 µmol H_2O_2/min/mg of protein, Figure 15F) and 50 mg/kg (brain, 35.3 ± 3.1 versus 18.7 ± 3.4 µmol H_2O_2/min/mg of protein, Figure 15E; heart, 45.3 ± 4.1 versus 28.6 ± 2.8 µmol H_2O_2/min/mg of protein, Figure 15F) caused a significant ($P < 0.05$) increase in the contents of catalase compared to CUS-control group (*Figure 15*)

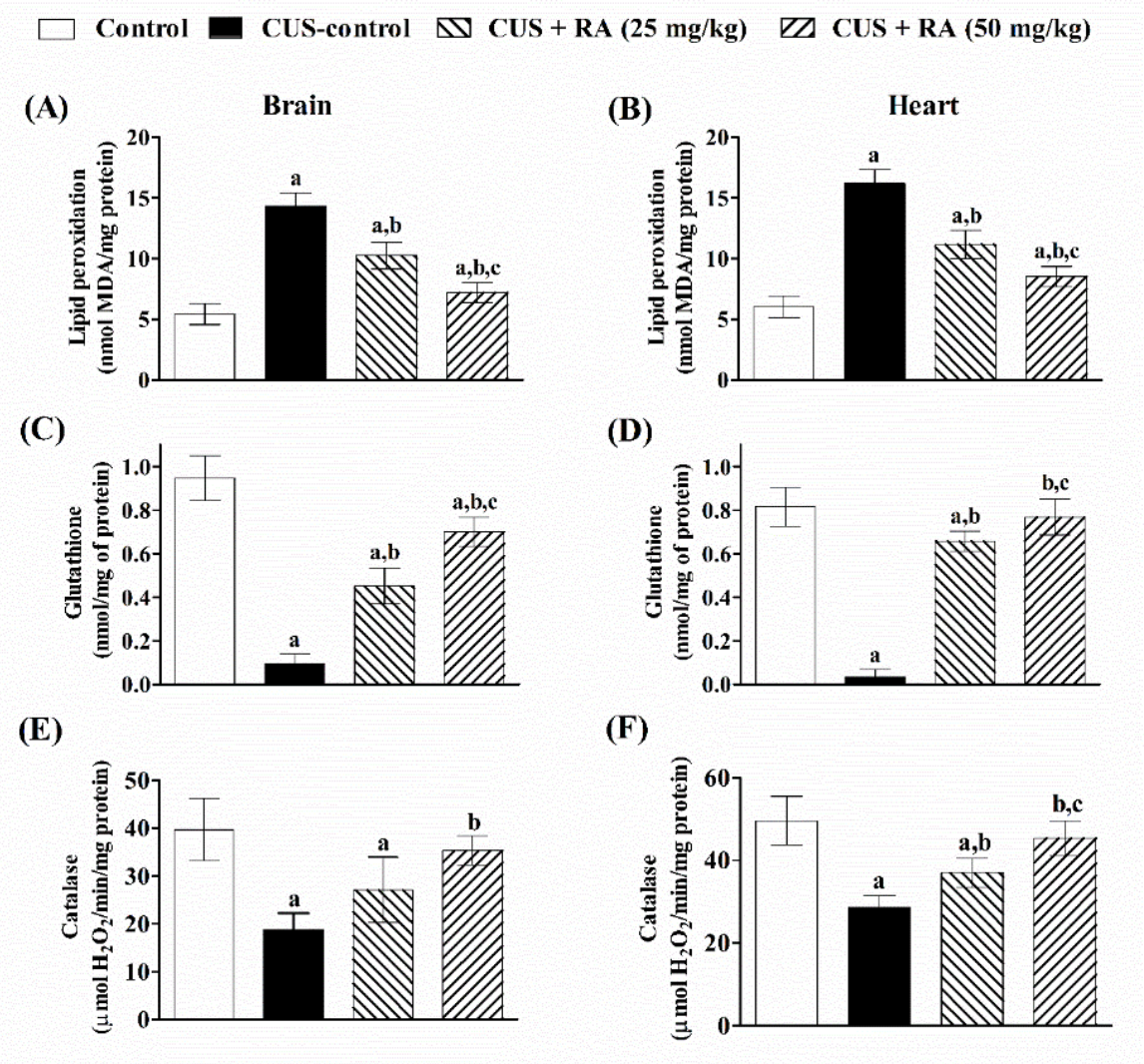

Figure 15: Effects of RA (25 mg/kg and 50 mg/kg) on lipid peroxidation level (LPO) in (A) brain and (B) heart, glutathione (GSH) level in (C) brain and (D) heart, and catalase activity (CAT) in (E) brain and (F) heart.

All values represented as mean ± SD, N = 6 rats per group. [a]$P < 0.05$ versus Control, [b]$P < 0.05$ versus CUS-control, [c]$P < 0.05$ versus CUS + RA (25 mg/kg); Repeated measure one-way ANOVA followed by Tukey's test. RA- Rosmarinic Acid.

2.3.8 Effects of rosmarinic acid on pro-inflammatory cytokines

We evaluated the effect of the rosmarinic acid (25 and 50 mg/kg) on the plasma levels of pro-inflammatory biomarkers such as TNF-α and IL-6. The results depicted that significant ($P < 0.05$) increase in levels of TNF-α (312.5 ± 16.8 versus 102.5 ± 17.1 ng/mL, Figure 16A) and IL-6 (50.3 ± 3.9 versus 14.06 ± 1.7 ng/mL, Figure 16B) were observed in CUS-control group than control group. Interestingly, rosmarinic acid at dose 25 mg/kg (TNF-α; 262.1 ± 21.8 versus 312.5 ± 16.8 ng/mL; Figure 16A), (IL-6; 40.5 ± 3.1 versus 50.3 ± 3.9 ng/mL; Figure 16B), and 50 mg/kg (TNF-α; 148.1 ± 19.3 versus 312.5 ± 16.8 ng/mL; Figure 16A), (IL-6; 22.7 ± 4.05 versus 50.3 ± 3.9 ng/mL; Figure 16B) significantly ($P < 0.05$) lowered the levels of TNF-α and IL-6 compared to CUS-control group (*Figure 16*).

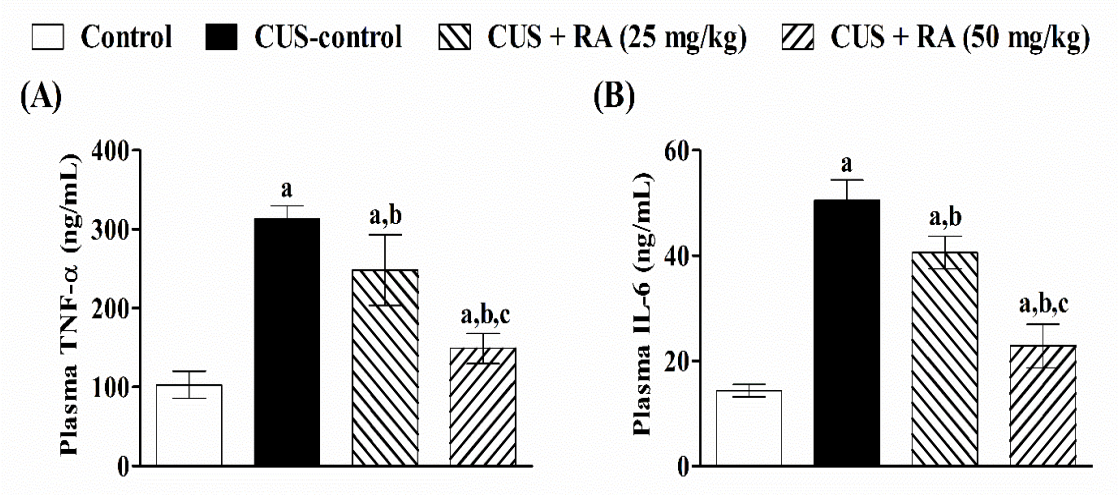

Figure 16: Effects of RA (25 and 50 mg/kg) on plasma pro-inflammatory cytokines (A) TNF-α and (B) IL-6.

All values represented as mean ± SD, N=6 rats per group. [a]$P < 0.05$ versus Control, [b]$P < 0.05$ versus CUS-control, [c]$P < 0.05$ versus CUS + RA (25 mg/kg); Repeated measure one-way ANOVA followed by Tukey's test. RA- Rosmarinic Acid.

2.4 Discussion

In 21st century, living with chronic stress exposure is normal for human beings worldwide. Several studies reported that chronic exposure to stress results in onset and relapse of depression [270, 271]. In animal models, the characteristic manifestations of depressive-like behavior are detected by increased immobility period in forced swim test [272, 273] and increased anhedonia in sucrose preference test [221, 274]. The CUS model could induce long-term behavioral disturbance and symptoms of clinical depression [275, 276], and it has been used in evaluating the efficacy of anti-depressant drugs through a series of behavioral tests [272, 273]. Similarly, in the present study, long-term prophylactic administration of rosmarinic acid (25 and 50 mg/kg) prevented the CUS-induced increase in immobility period and decrease in sucrose preference. Taken together, behavioral data suggested that rosmarinic acid pre-treatment produced an antidepressant-like effect that may halt further triggering of cardiac abnormalities.

According to DSM-V, body weight gain/loss is one of the significant symptoms of depression [277] [278, 279]. Several studies reported that chronic stress exposure might alter feeding behavior leading to weight loss in experimental animals [280-284]. Similarly, our results stated that body weight decreased in the CUS-control group compared to the control group though they received the equivalent feeding and growing conditions, indicating depressive-like behavior. In addition, a great body of literature reported a decrease in weight of brain and heart but an increase in the adrenal gland [243, 280, 284, 285]. In chronic stress conditions, hypersecretion of ACTH from the pituitary gland causes excessive stimulation of the adrenal gland leading to hyperplasia [286, 287]. In this study, wet weights of the brain and heart were reduced, and adrenal hyperplasia was detected in CUS-control compared with the control group. Similarly as

previous study [288], rosmarinic acid (25 and 50 mg/kg) reversed the decreased body weight and remarkably prevented the tissue weight loss and adrenal hyperplasia induced by the CUS model.

In depressed conditions, alteration in molecular biomarkers such as corticosterone and monoamines are evident in both humans and rodents, which is reversed by anti-depressant agents [289, 290]. It has been reported that the CUS paradigms cause hyperactivation of the HPA axis to release an excess amount of ACTH leading to hypersecretion of corticosterone (considered as an adaptive feedback mechanism) leads to a cascade of endocrine events [287, 291, 292]. In line with the literature, we observed higher levels of plasma corticosterone in CUS-control rats. Whereas, long-term prophylactic administration of rosmarinic acid (25 and 50 mg/kg) decreased plasma corticosterone levels relative to the CUS-control group, similar to results obtained using mitragynine and fluoxetine [293]. Sufficient studies are available which suggest the link between the HPA axis and monoamines level in depression [294]. Hyperactivity of the HPA axis causes suppression of glucocorticoid expression and feedback regulation, which inhibits the enzyme tryptophan hydroxylase leading to decrease in serotonin synthesis. Therefore, along with plasma corticosterone level, we estimated serotonin levels in the prefrontal cortex region of the brain, which is believed to be the major brain region involved in clinical depression [295]. It has been reported that neuronal damage due to chronic oxidative stress and inflammation leads to a reduction in prefrontal cortex size and dysfunction in the production of neurotransmitters in depressed patients [296-298]. There are many pieces of evidence that brain serotonin dysfunction plays an essential role in developing cardiac abnormalities *via* vasoconstriction and inflammation [225, 226, 299]. In the present study, rosmarinic acid (25 and 50 mg/kg) increased serotonin level relative to the CUS-control group. The

observed upregulation of the serotonergic system in the brain [300, 301] due to prophylactic rosmarinic acid treatment could be attributed to the activation of antioxidative systems [302, 303] and inhibition of monoamine oxidase-A enzyme responsible for serotonin metabolism in the brain [304-306]. Furthermore, the neuroprotective mechanisms are supported by a desired pharmacokinetic profile of rosmarinic acid that describes its entry into the brain through the blood-brain barrier [307].

Early studies reported that depression is a predisposing factor for cardiac comorbidity with coronary heart disease patients [308]**.** For survival of patients, early treatment is essential, which needs early detection of diseases [309]. ECG is the first and foremost pre-clinical or clinical test for the detection of cardiac abnormalities [309, 310]. A lower width and pointed T-wave represents occlusion in the coronary artery and hyperkalaemia [311], which are the common causes of cardiac arrhythmia [312]. In contrast, an increase in the QRS complex indicates a slow ventricular depolarization due to dysfunction in the conduction system, which in turn leads to ventricular hypertrophy and hyperkalaemia in heart [313, 314]. In our finding, the result of ECG revealed that CUS primarily affect T wave width (ventricular repolarization) and QRS complex (ventricular depolarization) of the heart. Furthermore, cardiac biomarkers are essential for diagnosis of cardiac abnormalities such as heart failure, congestive heart disease, coronary events, and ischemic heart diseases [315]. In this study, we measured the cardiac biomarkers cTn-I and MMP-2 in serum and heart tissue, respectively. Pathologically, MMP-2 acts as a proteolytic enzyme and degrades troponin complexes in heart leading to an increase in cTn-I concentration in the bloodstream [158]. Both cTn-I and MMP-2 levels were increased due to oxidative stress [316] in CUS rats, which was reversed by rosmarinic acid treatment, possibly due to antioxidant activity.

Therefore, these results suggested that the key mechanism of behind the defensive effect of rosmarinic acid against CUS inducing cardiac abnormalities can be due to its high free-radical scavenging and anti-oxidative properties.

In chronic unpredictable stress conditions, the release of an enormous amount of ROS/RNS leads to oxidative stress and cellular damage due to the imbalance between the oxidative and anti-oxidative systems [317]. The imbalance between the oxidative and anti-oxidative systems of the body further leads to overproduction of free radicals or reactive oxygen species leading to major depressive disorder and cardiac abnormalities [318]. A previous study reported that lipid peroxidation is one of the major biomarkers for oxidative stress, which is associated with increased depressive signs [261]. Rosmarinic acid is a potent radical scavenger molecule because it defence against oxidative ROS/RNS [319, 320]. An earlier research reported that rosmarinic acid is a natural antioxidant in preventing lipid peroxidation or against oxidative stress-inducing alteration in the lipid membrane, which showed a negative correlation between rosmarinic acid and (MDA) lipid peroxidation [321, 322]. In this study, rats with the CUS-control group increased lipid peroxidation levels compared to the control group. Whereas rosmarinic acid (25 and 50 mg/kg) reduced the level of lipid peroxidation in tissues. In addition to lipid peroxidation, anti-oxidant enzymes (glutathione and catalase) play a major role in the body *via* conversion of free radicals and catalysing the reduction of free radicals into hydrogen peroxides, and H_2O and molecular oxygen [323-325]. In our study, glutathione and catalase were both reduced in the CUS-control group as compared to the control group. Whereas, rosmarinic acid (25 and 50 mg/kg) increased the level of endogenous anti-oxidant system (glutathione and catalase) in tissues. Therefore, rosmarinic acid exhibited a protective effect towards the CUS-induced oxidative damage in the brain and heart.

Chronic unpredictable stress causes immune dysfunctions that lead to activation of inflammation involved in the development of depression and associated cardiac abnormalities [326]. During chronic stress exposure, some neuroendocrine factors impair cytokines functions [327]. The pro-inflammatory cytokines (TNF-α and IL-6) in excess level are linked to depression and cardiac diseases *via* binding to several receptors [70, 328, 329]. Rosmarinic acid attributed interesting anti-inflammatory properties *via* reductions of TNF-α, IL-8, IL-6, IL-1β, NG-kβ, interferon-1γ, IL-12, and Toll-like receptor-2 signalling pathways [330-333]. In this study, the CUS-control group increased TNF-α and IL-6 compared to the control group, which reasserts the role of inflammation in the pathogenesis of CUS-induced depression and associated cardiac abnormalities. Interestingly, long-term administration of rosmarinic acid (25 and 50 mg/kg) decreased levels of pro-inflammatory cytokines (TNF-α and IL-6) relative to the CUS-control group. Taken together, all these results stated that rosmarinic acid has protective and promising therapeutic effects against CUS-induced depressive-like behavior and associated cardiac abnormalities *via* serotonergic, oxidative, and inflammatory pathways in Wistar rats. This study was primarily designed to focus on the impact of stressors in the adulthood rather than childhood. In this regard, further studies incorporating stressor in the childhood period are required to understand the impact of childhood depression on cardiac abnormalities in the later life.

2.5 Conclusion

In conclusion, results obtained from this study revealed for the first time a defensive role of prophylactic action of rosmarinic acid treatment (25 and 50 mg/kg) against depression associated cardiac abnormalities. Rosmarinic acid administration abrogated CUS-induced decrease in serotonin level, increase in corticosterone level,

pro-inflammatory cytokine level, oxidative stress, cardiac troponin-I, matrix mettaloportienase-2, adrenal hyperplasia, and abnormalities in ECG parameters. The protective effects of rosmarinic acid against CUS-induced depressive-like behavior were further evidenced by the decrease in immobility period and anhedonia symptoms. Overall, the current findings suggest that rosmarinic acid could be a promising prophylactic treatment option against depression associated cardiac abnormalities.