The freeze-dried extracts of *Salvia coccinea* Juss. Ex Murray attenuate myocardial ischemia reperfusion injury in a global ischemia Rat model

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Abstract

Background: Ischemia reperfusion injury is the leading cause of myocardial cell death in Ischemic Heart Disease. Thus intensive research efforts are geared at discovering pharmacological approaches that prevent it. Over twenty species from the genus *Salvia* are widely applied in traditional Chinese medicine in the management of heart diseases with *Salvia miltiorrhiza* (Danshen) being a canonical example. Our study aimed to investigate the cardio-protective effects of the freeze-dried extracts of *salvia coccinea* against ischemia reperfusion injury in a rodent *in-vitro* model of global ischemia.

Methods: Forty two (42) Sprague Dawley rats were randomly assigned into five groups: positive control (Glucosamine 1000mg/kg), negative control group (Krebs Henseleit buffer), low dose test (50 mg/100ml), medium dose test (100 mg/100ml), and high dose test (200 mg/100ml).

The cardio-protective effects of the different treatments were evaluated in a global ischemia model using isolated rat hearts mounted on a Langendorff system.

Naloxone 2.2 μmol/L (μ opioid receptor blocker), and theophylline 1000 μmol/L (non-specific adenosine receptor blocker) were co-administered with 50 mg of *S.coccinea* in the mechanism of action experiments.

The following indices of cardiac function were recorded pre- and post- ischemia: left ventricular developed pressure (LVDP), heart rate, and maximum rate of contraction and relaxation. All data were expressed as Mean ± Standard Error of Mean and analyzed using one-way ANOVA and Tukey post-hoc tests. Significance was set at $p < 0.05$.

Results: The freeze-dried extracts of *S. coccinea* had significant effects on post-ischemic contractile function recovery in the early [51.4 ± 9.7% (low dose test) vs. 14.9 ± 3.3% (medium

dose test) vs. 12.7 ± 2.6% (high dose test) vs. 13.7 ± 5.7% (negative control): $p<0.05$] and late [38.6 ± 8.9% (low dose test) vs. 22.0± 7.1% (medium dose test) vs. 14.6 ± 5.8 (high dose test) vs. 12.5 ± 4.2% (negative control): $p< 0.05$]. Reperfusion phases with the highest LVDP recovery were observed at the 50 mg dosage level.

The freeze-dried extracts of *S. coccinea* had significant negative chronotropic effects on heart rate [234.0 ± 2.4 beats/min to 90.0 ± 7.0 beats/min, 50 mg vs. 102.0 ± 13.9 beats/min to 135.0 ± 25.9 beats/min, control $P<0.05$].

The cardioprotective effects of *S. coccinea* displayed an inverted U-shaped dose-response curve with low dose stimulation and high dose inhibition.

Naloxone completely abolished the LVDP recovery afforded by the freeze-dried extracts of *S. coccinea* at the 50 mg dosage level while adenosine only partly abolished the LVDP recovery (9.5 ± 3.2% (naloxone) *vs.* 15.5 ± 5.8% (adenosine): $P>0.05$).

Conclusion: The freeze-dried extracts of *S. coccinea* possessed significant cardioprotective effects which appear to be mediated by activation of the opioidergic pathway in the heart.

Key Words: Ischemia, Reperfusion Injury, Cardio-protection, Opioid Pathway, *S. coccinea*

1.0 Introduction

Cardiovascular diseases are the leading cause of death globally with a 31% annual death rate (1). Ischemic heart disease (IHD) is responsible for more than 40% of these cardiovascular deaths and thus poses a major global health problem(2).

The clinical manifestations of IHD result from subjection of the heart to ischemia-reperfusion injury which leads to myocardial injury, cardiac dysfunction, arrhythmias, heart failure and ultimately death (3).

Cardioprotection aims to prevent the death of cells during acute injury and may be achieved through myocardial or pharmacological conditioning carried out pre- or post- ischemia (4).

Salvia species are used widely in Traditional Chinese Medicine (TCM) as Danshen to manage cardiac conditions. *Salvia coccinea* has been used since the 17th century by the Chinese who refer to it as "cure all" or "sage the savior'' for the management of common maladies (5). In South America a boiled mixture is used to bathe varicosities and treat blood clots (6). The aqueous and alcohol extracts of *Salvia coccinea* contain potent phenolic antioxidants (7). The present study aimed to investigate the cardioprotective effects of the freeze-dried extract of *S. coccinea* against ischemia reperfusion injury and to determine its mode of action.

2.0 Materials and methods

2.1 Plant material and freeze dried extract preparation

The specimen of *Salvia coccinea* was harvested from Naivasha County, Kenya, and its identity confirmed by resident taxonomists of the herbarium situated at the Department of Botany, School of Biological sciences, University of Nairobi.

82 The whole plant was used in the study. The plant parts were cut into small pieces and air dried for
83 one week. The dried plant was milled using a standard mill and the resulting powder macerated in
84 cold distilled water in a ratio of 1:10 (weight: volume) with vigorous shaking for an hour. The
85 suspension obtained was filtered consecutively using cotton wool and filter paper (Whatman's™).
86 The filtrates were frozen and lyophilized and the resultant freeze-dried extracts weighed and placed
87 in sample containers. These were stored in a standard laboratory freezer (Hotpoint™). During each
88 procedure, the freeze- dried extracts were reconstituted to form the required concentrations.

89 *2.2 Experimental Animals*

90 Forty-two (42) six month old adult male Sprague Dawley rats, weighing 300-400 g were used. The
91 animals were housed in standard cages containing five rats in each. The experiments were
92 performed in the animal house at the Department of Medical Physiology, University of Nairobi.
93 The following conditions were maintained in the animal house: constant room temperature (23 +/-
94 2°C) and 30-70% relative humidity with a 12-hr. light/day cycle. The animals were provided with
95 standard rat chow pellets and tap water ad libitum. The protocol was approved by the Biosafety,
96 Animal Use and Ethics Committee, Faculty of Veterinary Medicine, University of Nairobi. Ref.
97 Number: (FVM BAUEC/2016/113). All experiments were based on internationally accepted
98 directions for the use and care of laboratory animals as stipulated by the FELASA guidelines (8).

99 *2.3 Drugs*

100 Naloxone HCL (Sanofi Ltd.), Theophylline (GlaxoSmithKline Ltd.), Glucosamine (Power Health
101 Ltd.), and Heparin (Rotex Medica Ltd.) were obtained from Dischem Pharmacy, Nairobi. Sodium
102 pentobarbital (Bayer Ltd.) was obtained from Lesukut Ltd. NaCI, KCl, KH_2PO4, $MgSO_4$,
103 $NaHCO_3$, $CaCl_2$, and glucose (LOBA Ltd.) were obtained from Chem-labs, Nairobi. All drugs
104 were freshly prepared in normal saline on the day of the experiment.

2.4 Isolated heart preparation

Each rat was anaesthetized with sodium pentobarbital 6% (40 mg/kg) intraperitoneally. There-after heparin (500 u/kg) was administered intravenously immediately the rat lost its righting reflex. The hearts were excised through a trans-abdominal incision and immediately immersed in ice-cold Krebs' Henseleit (KH) buffer solution. A plastic cannula was inserted through the aorta, clamped with a blunt artery clip and retrograde perfusion with Krebs Henseleit buffer (10 ml/min) initiated.

2.5 Measurement of isovolumic cardiac performance

A latex balloon connected to a pressure transducer was inserted into the left ventricle via the mitral valve and inflated to increase the diastolic pressure by regulating the volume of saline in it. The pressure transducer was connected to a power lab (AD Instruments, Colorado Springs, CO, USA) recording system. The following cardiac indices were then measured and averaged from twelve (12) beats: Left ventricular developed pressure (LVDP), maximum rate of contraction ($+dp/dt_{max}$), maximum rate of relaxation ($-dp/dt_{min}$), and the heart rate.

2.6 Cardioprotection experiment

The hearts were equilibrated for twenty (20) minutes and subjected to thirty (30) minutes global ischemia by halting perfusion and immersing the hearts in a KH buffer filled organ bath. The hearts were divided into five (5) groups and on reperfusion treated (Table 1).

127

128 **Table 1: Heart treatment during reperfusion**

TREATMENT STEPS **Time (minutes)**	**EXPERIMENTAL GROUPS**				
	POSITIVE CONTROL	**CONTROL**	**50 mg**	**100 mg**	**200 mg**
15	Equilibration	Equilibration	Equilibration	Equilibration	Equilibration
30	Global Ischemia	Global Ischemia	Global Ischemia	Global Ischemia	Global Ischemia
90	Reperfusion with KH buffer + Glucosamine	Reperfusion with KH buffer containing *Salvia coccinea* extract (50 mg per liter)	Reperfusion with KH buffer containing *Salvia coccinea* extract (100 mg per liter)	Reperfusion with KH buffer containing *Salvia coccinea* extract (200 mg per liter)	Reperfusion with plain KH buffer

129

130 *2.7 Mechanism of action experiments*

131 Theophylline and Naloxone, which are non-selective adenosine and opioid receptor blockers
132 respectively, were used to determine the signaling pathway of cardioprotection by *Salvia coccinea*.
133 These blockers were administered for 20 minutes at the onset of reperfusion before treatment with
134 *Salvia coccinea* extracts (Table 2).

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140 **Table 2: Heart treatment during reperfusion**

TREATMENT STEPS **Time (minutes)**	**EXPERIMENTAL GROUPS**				
	POSITIVE CONTROL	**CONTROL**	**50 mg**	**100 mg**	**200 mg**
15	Equilibration	Equilibration	Equilibration	Equilibration	Equilibration
30	Global Ischemia	Global Ischemia	Global Ischemia	Global Ischemia	Global Ischemia
90	Reperfusion with KH buffer + Glucosamine	Reperfusion with KH buffer containing *Salvia coccinea* extract (50 mg per liter)	Reperfusion with KH buffer containing *Salvia coccinea* extract (100 mg per liter)	Reperfusion with KH buffer containing *Salvia coccinea* extract (200 mg per liter)	Reperfusion with plain KH buffer

141

142 *2.9 Data and statistical analyses*

143 The experimental data were expressed as the mean +/-SEM. The data were analyzed using One-

144 way ANOVA with post- hoc statistical analysis being performed using Tukey HSD in cases of

145 significance which was set at $p<0.05$. The Kruskall Wallis non-parametric with Dunns' post- hoc
146 statistical test in cases of significance ($p<0.05$) was used to analyze the extent of the histological
147 damage. GraphPad Prism 6 suite of statistical software was used for carrying out the statistical
148 tests.

149 **3.0 Results**

150 **3.1 Effects on cardiac function**

151 *3.1.1 Baseline cardiac function*

152 There were no significant differences in LVDP between the groups during equilibration [43.8 ±
153 2.5 mmHg (positive control) vs. 40.9 ± 7.5 mmHg (negative control) vs. 51.1 ± 1.4 mmHg (low
154 dose test) vs. 54.8 ± 1.3 mmHg (medium dose test) vs. 40.6 ± 2.6 mmHg (high dose test); P=0.05].
155 The graphical representation of the equilibration LVDP values is shown in Figure 1.

156 **Figure 1. Equilibration Left Ventricular Developed Pressure (LVDP) for each group.** PC
157 (positive control), NC (Negative control).

158 *3.1.2 Effects of S. coccinea extracts on the left ventricular developed pressure (LVDP*)

159 The freeze-dried extracts of *Salvia coccinea* had significant effects on the LVDP recovery both in
160 the early [51.4 ± 9.7% (low dose test) vs. 14.9 ± 3.3% (medium dose test) vs. 12.7 ± 2.6% (high
161 dose test) vs. 13.7 ± 5.7% (negative control): $p<0.05$] and late [38.6 ± 8.9% (low dose test) vs.
162 22.0± 7.1% (medium dose test) vs. 14.6 ± 5.8 (high dose test) vs. 12.5 ± 4.2% (negative control):
163 $p< 0.05$] phases of reperfusion. Post-hoc statistical analysis using Tukey's multiple comparison
164 test showed that the low dose (50 mg) had a significantly higher cardioprotective activity in the
165 early phase compared to the medium dose (100 mg), high dose (200 mg) and negative control
166 (KH) groups ($p<0.05$). The 50 mg dose also had significant cardioprotective activity in the late

167 phase compared to the KH and 200 mg group ($p<0.01$). A graphical representation of the LVDP
168 percentage recovery is shown in Figure 2.

169

170 **Figure 2. Post ischemic Left Ventricular Developed Pressure** A) Percentage early recovery
171 after post ischemic post conditioning with *S. coccinea*. B) Percentage late recovery after post
172 ischemic post conditioning with *S. coccinea*. LVDP (Left Ventricular Developed Pressure), PC
173 (positive control), NC (Negative control). KEY: *= $P \leq 0.05$; **$P \leq 0.01$

174

175 *3.1.3 Effects of the S. coccinea extracts on the maximum rate of contraction (+dp/dt max)*

176 The freeze-dried extracts of *S.coccinea* had a significant effect on the recovery of the $+dp/dt_{max}$
177 in the early [78.2 ± 3.8% (low dose test) vs. 18.7 ± 3.9% (medium dose test) vs. 37.9 ± 8.1%
178 (negative control) vs. 41.4 ± 8.9% (high dose test): $P<0.05$] and late phases [(69.8 ± 6.9% (low
179 dose test) vs. 43.5 ± 6.6% (medium dose test) vs. 40.2 ± 14.6% (high dose test) vs. 44.8 ± 2.5%
180 (negative control): $p<0.05$] of reperfusion. Post-hoc statistical analysis using Tukey's multiple
181 comparison test showed that the extract at 50 mg dose had significant cardioprotective activity in
182 the early phase compared to the glucosamine group ($P < 0.05$) and 100 mg group ($P<0.01$). A
183 graphical representation of the $+dp/dt_{max}$ percentage recovery is shown in Figure 3.

184

185 **Figure 3. Post ischemic calculated maximum rate of contraction.** A) Equilibration values B)
186 Percentage early recovery after treatment with *s. coccinea* C) Late recovery after treatment with *s.*

187 *coccinea.* LVDP (Left Ventricular Developed Pressure), PC (positive control), NC (Negative
188 control).KEY: *=P ≤ 0.05; **=P ≤ 0.01.

189 *3.1.4 Effects of the S. coccinea extracts on the maximum rate of relaxation (-dp/dtmin)*

190 The freeze-dried extracts of *Salvia coccinea* caused a significant -dp/dt $_{min}$ recovery in the early
191 [131.6 ± 8.3 (low dose test) vs. 45.6 ± 5.3% (medium dose test) vs. 55.2 ± 13.1% (high dose test)
192 vs. 83.8 ± 11.8% (negative control): P<0.05] and late phases [149.0 ± 23.7% (low dose test) vs.
193 97.4 ± 10.8% (medium dose test) vs. 78.0 ± 31.0% (high dose test) vs. 113.6 ± 4.6% (negative
194 control): P<0.05] of reperfusion. Post-hoc statistical analysis using Tukey's multiple comparison
195 test showed that the low dose test (50 mg) possessed significant cardioprotective activity in the
196 early phase compared to the negative control (KH) group (P <0.01), the medium dose test (100
197 mg) group (P<0.001), the high dose test (200 mg) group (p<0.05) and the test positive control
198 (glucosamine) group (P<0.01). The low dose also had significant cardioprotective activity in the
199 late phase compared to the negative control (P <0.05), the high dose test (p<0.01), the medium
200 dose test (P<0.05) and the positive control (P < 0.01) A graphical representation of the -dp/dt
201 $_{min}$ percentage recovery is shown in Figure 4.

202 **Figure 4. Post ischemic calculated maximum rate of relaxation.** A) Equilibration values B)
203 Percentage early recovery after treatment with *S. coccinea* C) percentage late recovery after
204 treatment with *S. coccinea*. LVDP (Left Ventricular Developed Pressure), PC (positive control),
205 NC (Negative control).KEY: *=P≤0.05; **=P≤0.01; ***=P≤ 0.001.

206 *3.1.5 Trends of recovery in the early and late phases of reperfusion*

207 The trend of recovery was obtained by plotting the ratios of the percentage recovery in both phases
208 for each of the cardiac indices to determine the effectiveness of *Salvia coccinea* in early and late

209 reperfusion. The low dose test group had the highest recovery rate in both phases. The graphical
210 representation of these trends is shown in figure 5, 6 and 7.

211 **Figure 5. Trends of LVDP recovery in the early and late reperfusion phases.**

212 **Figure 6. Percentage +dp/dt $_{max}$ recovery in the early and late reperfusion phases.**

213 **Figure 7. Trends of -dp/dt $_{min}$ recovery in the early and late reperfusion phases.**

214 *3.1.6 Effects of the freeze-dried extracts of S. coccinea on heart rate*

215 The freeze-dried extracts of *S.coccinea* caused a significant decrease in heart rate [234.0 ± 2.4
216 beats/min (low dose test) vs. 258.0 ± 11.5 beats/min (medium dose test) vs. 174.0 ± 32.6 beats/min
217 (high dose test)] in the early [90.0 ± 7.0 beats/min (low dose test) vs. 98.0 ± 2.0 beats/min (medium
218 dose test) vs. 107.5 ± 7.5 beats/min (high dose test): $P<0.05$] and late phases [106.0 ± 6.7 beats/min
219 (low dose test) vs. 84.0 ± 10.7 beats/min (medium dose test) vs. 117.5 ± 14.3 beats/min (high dose
220 test): $P<0.05$] of reperfusion. The heart rate in the negative and the positive control groups
221 increased from the equilibration values [150.0±13.7 beats/min (positive control) vs. 102.0 ± 13.9
222 beats/min (negative control)] in the early [146.0 ± 19.3 beats/min (positive control) vs. 135.0 ±
223 25.9 beats/min (negative control)] and late phases [175.0 ± 30.9 beats/min (positive control) vs.
224 205.0 ± 41.1 beats/min (negative control)] of reperfusion. A graphical representation of the heart
225 rates in the equilibration, and reperfusion phases is shown in Figure 8.

226 **Figure 8. Heart rate;** A) Equilibration heart rate values B) Early reperfusion phase heart rate C)
227 Late reperfusion phase heart rate. LVDP (Left Ventricular Developed Pressure), PC (positive
228 control), NC (Negative control)

229

230 **3.3 Mechanism of action experiments**

231 Co-administration of naloxone along the low dose test (50 mg) led to decreased $+dp/dt_{max}$ recovery
232 compared to treatment with 50 mg *S. coccinea* alone [0.09570±0.03294 (naloxone)
233 vs.0.3868±0.1090(low dose test): P= 0.0338]. Similarly, naloxone led to a lower $-dp/dt_{min}$
234 recovery compared to the 50 mg dose [0.7430±0.06739 (naloxone) vs.1.686±0.2693 (low dose
235 test): P= 0.0179].The graphical representation for the late recovery of the assessed indices of
236 cardiac function when blockers were used is shown in Figure 9.

237 **Figure 9. Indices of cardiac function.** A) % recovery of Left ventricular developed pressure B)
238 % recovery of maximum rate of contraction C) % recovery of maximum rate of relaxation. LVDP
239 (Left Ventricular Developed Pressure), PC (positive control), NC (Negative control) KEY: * =
240 P<0.05.

241 **3.4 Dose-response analysis**

242 The dose response curve for the cardioprotective effects of *S. coccinea* displayed a biphasic U-
243 shaped pattern. As the dose was increased, the percentage recovery decreased up to a minimum
244 point [x=153.2: y=1.0 (early phase) vs. x=173.97: y=12.49 (late phase)] beyond which the
245 percentage recovery started to increase as shown in Figure 10.

246 **Figure 10. Dose response curve showing the percentage LVDP early reperfusion phase**
247 **recovery after pharmacological post conditioning with the aqueous freeze dried extracts of**
248 ***S. coccinea* following global ischemia.**

249

250

251 **4.0 Discussion**

252 Ischemic heart disease (IHD) is among the leading causes of death and disability worldwide.
253 Myocardial infarction induced by Ischemia/reperfusion (I/R) injury is one of the most severe
254 manifestation of IHD, which makes its attenuation critical. The treatment of choice for myocardial
255 ischemic injury is prompt and efficient reperfusion which paradoxically induces further
256 cardiomyocyte damage known as reperfusion injury (9). Cardioprotection through
257 pharmacological therapy in addition to early reperfusion is needed to further reduce the infarct size
258 and reduce the mortality (10). Despite the burden caused by I/R injury there is still no effective
259 therapy for preventing reperfusion injury which necessitates the search for more potent therapies
260 (11).

261 Several salvia species are used as Danshen to treat heart diseases (5). However, the
262 cardioprotective effects of *S. coccinea* and its possible mechanism of action have not yet been
263 investigated.

264 Treatment with *S. coccinea* extract led to a significant dose-dependent recovery of the LVDP and
265 $+dp/dt_{max}$ after 30 minutes of global ischemia, with the highest recovery recorded at the low dose
266 test (50 mg). Since LVDP and $+dp/dt_{max}$ are measures of systolic function, this finding indicates
267 that *S. coccinea* has the ability to prevent the systolic dysfunction that arises during I/R injury
268 impairing the heart's ability to build enough pressure at the required rate to pump blood
269 commensurate to the body's demands (12). These findings are in concurrence with those of a study
270 that investigated the cardioprotective effects of another Salvia species: *S. miltiorhiza* (5). It is
271 noteworthy that the percentage recovery in this study was higher compared to other
272 cardioprotective herbal remedies (13).

273 The *S. coccinea* extract was shown to preserve the post-ischemic diastolic function in a dose
274 dependent manner. The hearts treated with the herb had a higher percentage -dp/dt $_{min}$ recovery
275 compared to the control group hearts. Myocardial ischemia precipitates the development of
276 diastolic dysfunction characterized by delayed relaxation, impaired filling and left ventricular
277 stiffening (14). This finding is consistent with a previous study on other salvia species which
278 preserved diastolic function (13). The finding was in contrast with other studies in which the -dp/dt
279 $_{min}$ recovered to a lower level than the equilibration value (15). Accordingly, the aqueous extract
280 of *S. coccinea* may be more potent in abating the diastolic dysfunction that arises in I/R injury.

281 The *S. coccinea* extracts led to a significant decrease in heart rate at all doses in both the early and
282 late phases of reperfusion in contrast to the control groups in which the heart rates were increased.
283 Reperfusion following ischemia induces tachycardia which initiates life threatening ventricular
284 arrhythmias (16). Low heart rates post-ischemia have thus been shown to be protective (17). These
285 previous studies suggest that the heart rate lowering effects of *S. coccinea* might have been
286 protective to the injured myocardium. This finding was however contradictory to *S. miltiorhiza*
287 and its isolated components where the heart rate remained constant with no significant decrease
288 (18).

289 This study also sought to assess the degree of cardioprotection by *S. coccinea* in the early and late
290 phases of reperfusion. This is because most cardioprotective agents show potency only in the early
291 phase (19). The events occurring in the early and late reperfusion phases differ and the
292 cardioprotective potency of an agent in both phases depends on its ability to ameliorate the
293 injurious events in both phases (20). Lethal reperfusion injury in the early phase is due to necrosis
294 while that in the late phase is attributed to apoptosis (21). It is interesting to note that the
295 cardioprotective effects of the *S. coccinea* extract were highly maintained in the early and late

296 phases of reperfusion, a clear indication that the aqueous extracts may have the potential to prevent
297 both necrosis and apoptosis.

298 *S. coccinea* was most potent at the 50 mg dose with the 100 mg and 200 mg providing
299 cardioprotection to a lesser degree as indicated by the lower recovery of the LVDP, dp/dt_{max} and
300 dp/dt_{min} in early and late reperfusion. This finding corroborates a study that investigated the
301 cardioprotective effects of *S. miltiorhiza* where a lower dose, less than 100 mg was suggested as
302 the 100 mg dose caused stiffening of the left ventricle and consequently diastolic dysfunction (13).

303 The dose response analysis of *S.coccinea* generated an inverted dose response curve with
304 characterized by low dose inhibition and high dose stimulation of cardioprotection as opposed to
305 the monophasic S-shape favored in pharmacological analysis (22). The probable molecular
306 explanation for the inverted dose - response curve is biased agonism, a property of the ligand-
307 receptor complex where a ligand favors one signaling pathway over another. β-arrestins usually
308 associated with desensitization of the GPCRs, are involved in signaling (23) and have been shown
309 to stimulate signal transduction pathways including MAPK, PKC, PI3K (24). Biochemical data
310 shows that signaling mediated by β arrestins and G proteins have very distinct physiological
311 consequences (25).

312 Pretreatment with naloxone at the onset of reperfusion completely abolished the cardioprotective
313 effects of *S. coccinea*. This indicates that the cardioprotection by *S. coccinea* is mediated via the
314 opioid pathway. Agonist binding to the opioid receptor usually inhibits adenylyl cyclase and
315 activates phospholipase C signaling (26). Previous studies have shown that PLC signaling leads to
316 the activation of the PKC and RISK kinases which are activated by salvianolic acid A and B which
317 are phenolic compounds commonly found in salvia species (27). It is key to note that opioid

318 receptors were among the first to portray biased agonism (24), a phenomenon that was observed
319 in the dose response analysis of *S.coccinea*.

320 The synthesis and release of endogenous opioids into the circulation has been shown to increase
321 in response to stressful stimuli including ischemia. Opioids mediate organ responses to stress by
322 inducing hibernation and metabolic protection and are thus vital in enhancing the ability of the
323 heart to withstand ischemia reperfusion injury (Akil et al., 1984). Myocardial binding studies have
324 shown the presence of *kappa* and *delta* opioid receptors in ventricular myocytes. The capacity of
325 the heart to synthesize all the three opioid peptides has been extensively verified experimentally
326 (Krumins et al., 1985) (28) (29).

327 Pretreatment with theophylline partially abolished the cardio protection indicating that the
328 adenosine pathway does not work in isolation to mediate the cardioprotection by *S. coccinea*.
329 Adenosine receptors are known to interact with opioid receptors via receptor oligomerization (30).
330 Adenosine is phosphorylated to AMP by adenosine kinase which is inhibited upon opioid receptor
331 activation leading to a rise in adenosine levels (30). Consequently, it follows that, when
332 theophylline blocks the adenosine receptors, only one pathway of cardioprotection is obstructed
333 leaving the opioid pathway operational and hence the partially abolished cardioprotection.

334 Preliminary photochemistry analysis of *S.coccinea* confirmed the presence of saponins, sugars,
335 phenols, tannins, amino groups, triterpenes, alkaloids and flavonoids (31). The pharmacological
336 activities of the compounds from *S. coccinea* have not yet been fully elucidated and further studies
337 would reveal the actual constituents that are involved in mediating the cardioprotective effects as
338 observed in this present study.

339 Further work needs to be done to identify the active component of *S. coccinea* responsible for the
340 cardioprotection observed. The cardioprotection experiments should also be performed in *in-vivo*
341 models of ischemia.

342 **Conclusion**

343 The freeze-dried extracts of *S. coccinea* possessed significant activity in the prevention of ischemia
344 reperfusion injury. These actions were probably mediated by activation of the opioidergic system
345 in the heart and lowering the heart rate through activation of the opioid pathway.

346 **Competing Interests**

347 The authors(s) declare that they have no competing interests.

348

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351 **Author Contributions.**

352 PWM, FOB and NMN designed the study and analyzed the experimental data. PWM and NMN
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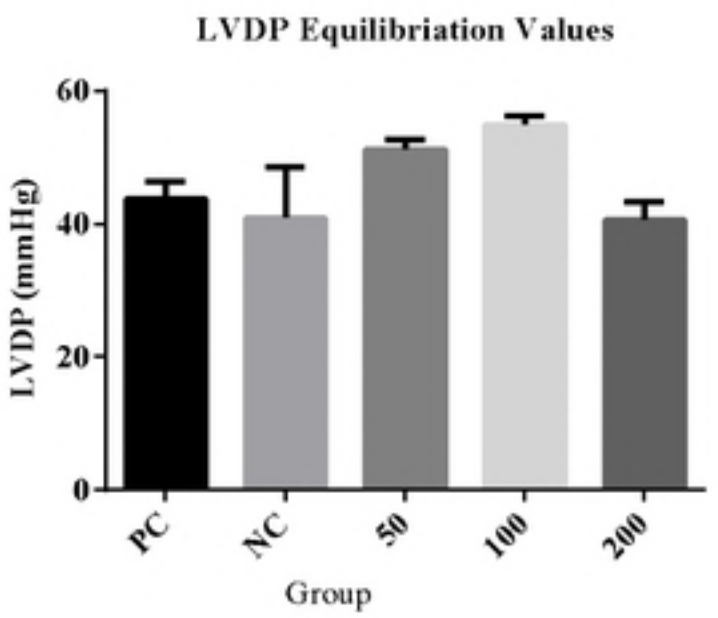

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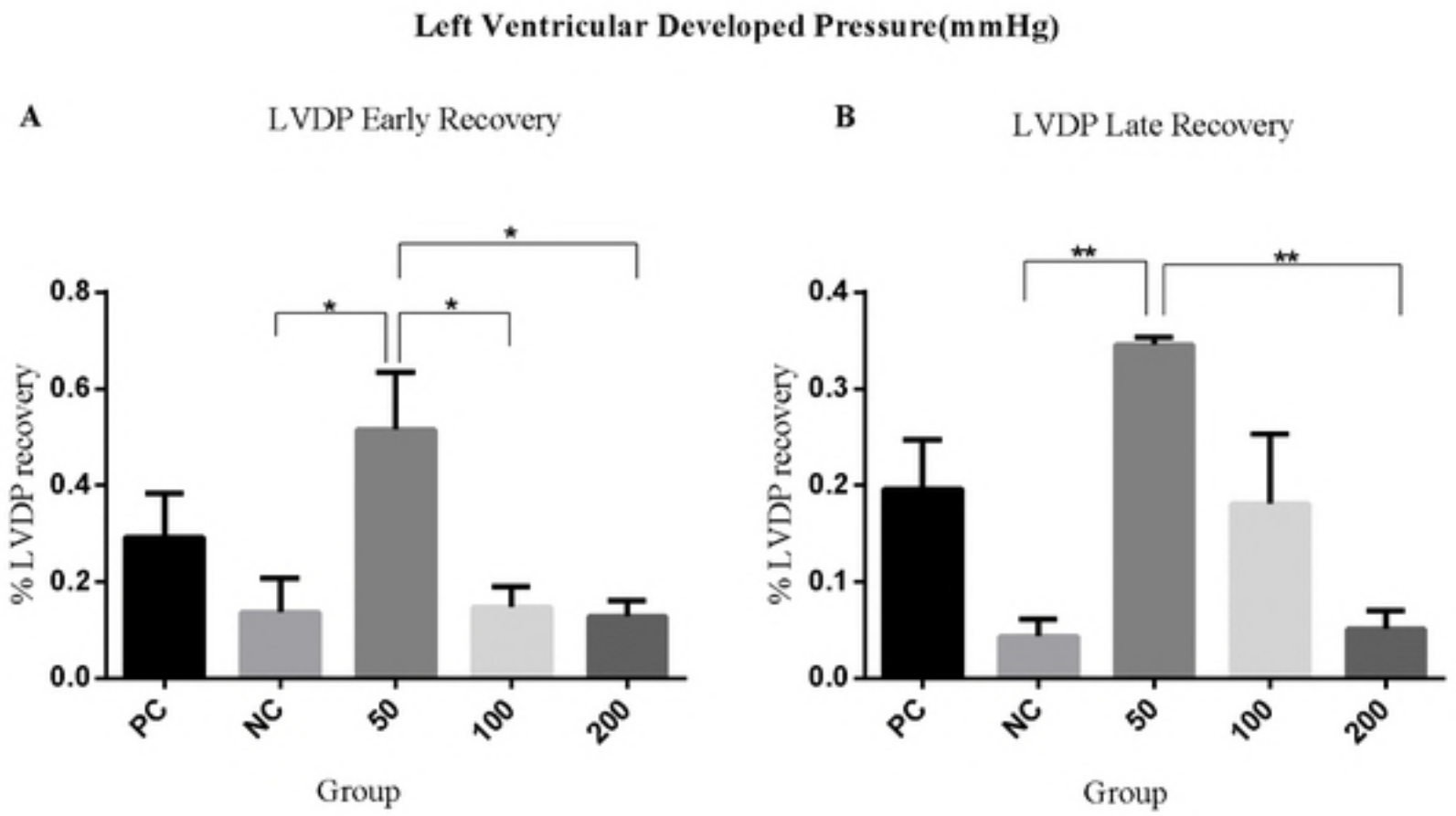

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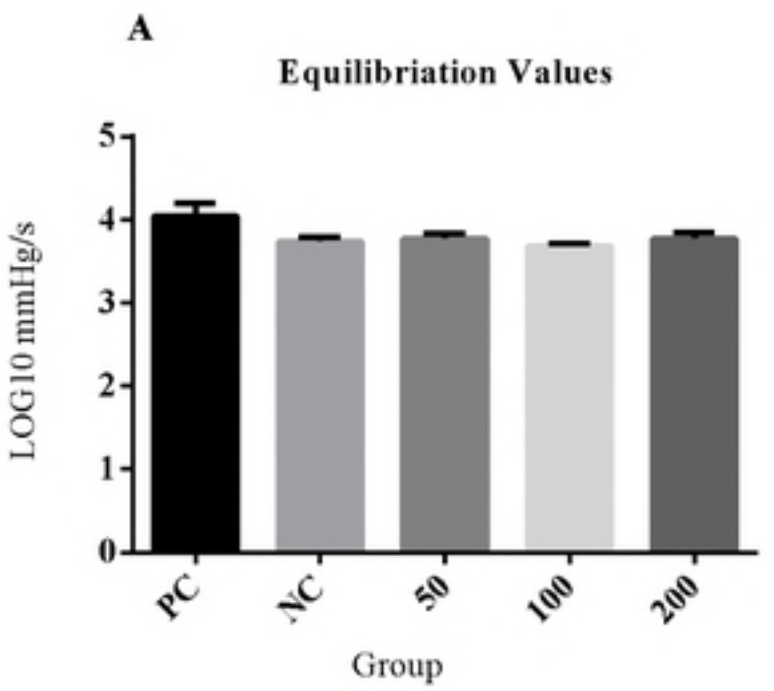
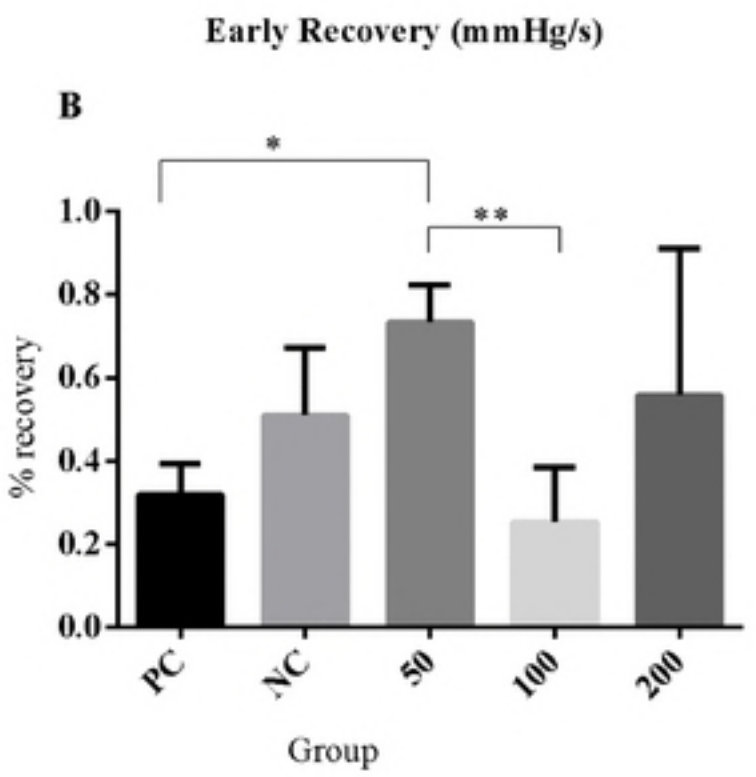
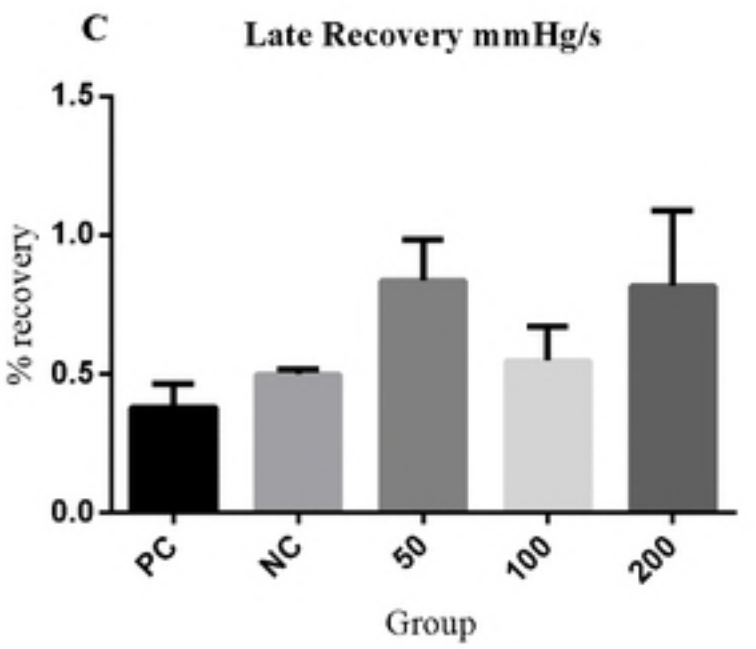
Maximum Rate of Contraction (dp/dt_{max})







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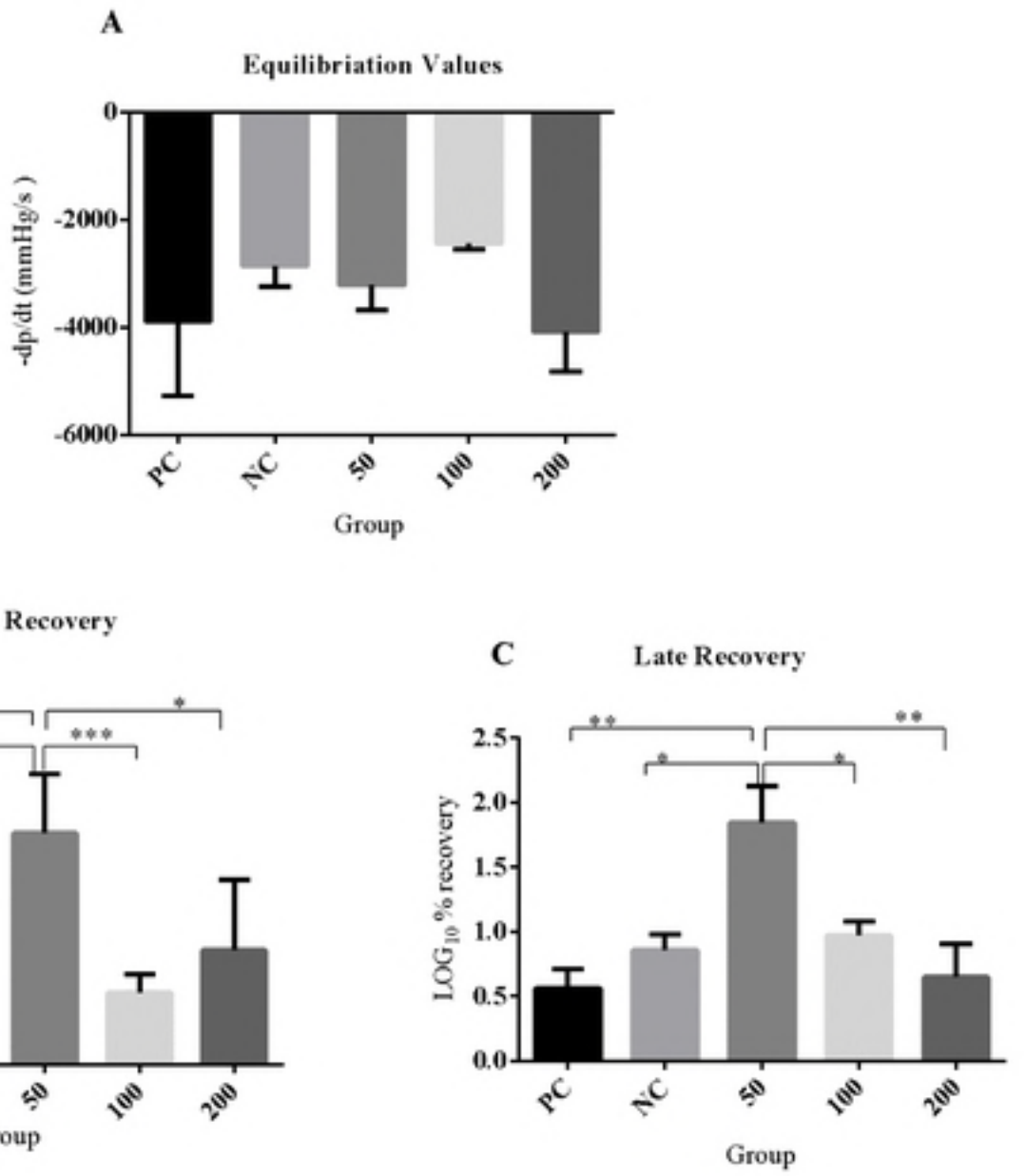
MAXIMUM RATE OF RELAXATION (mmHg/s)



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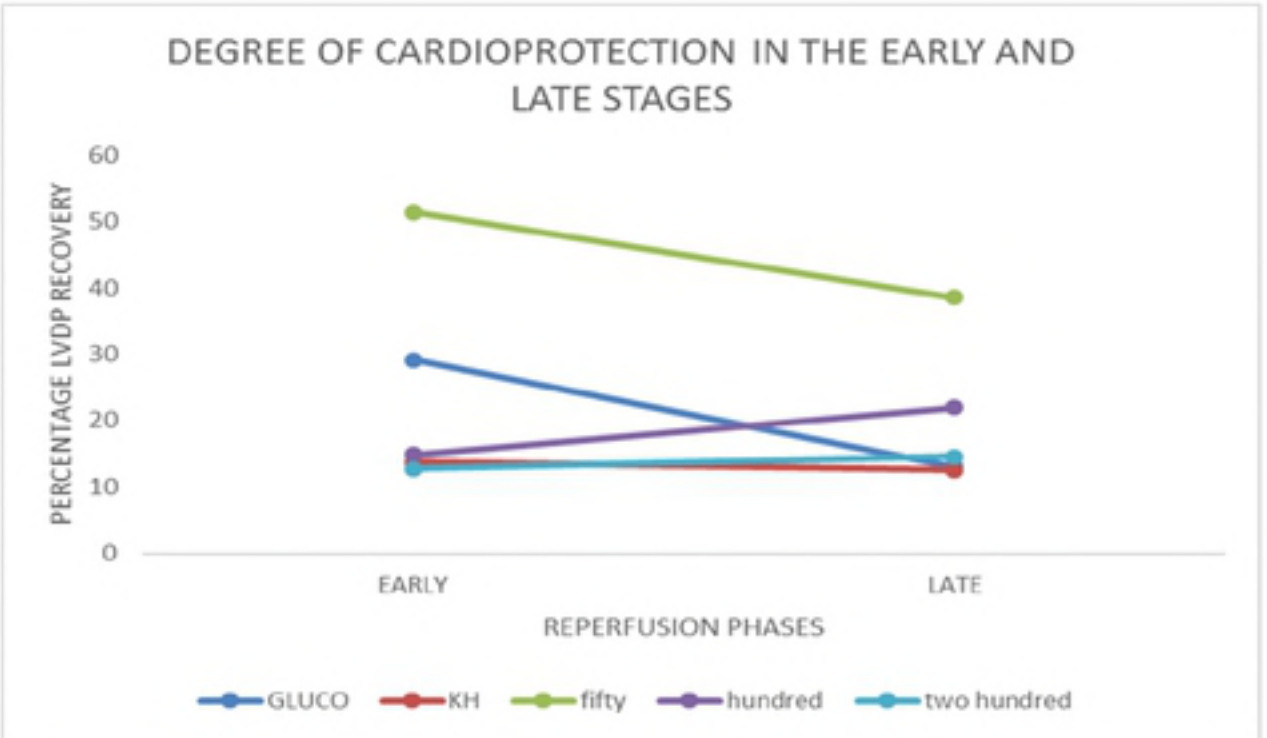

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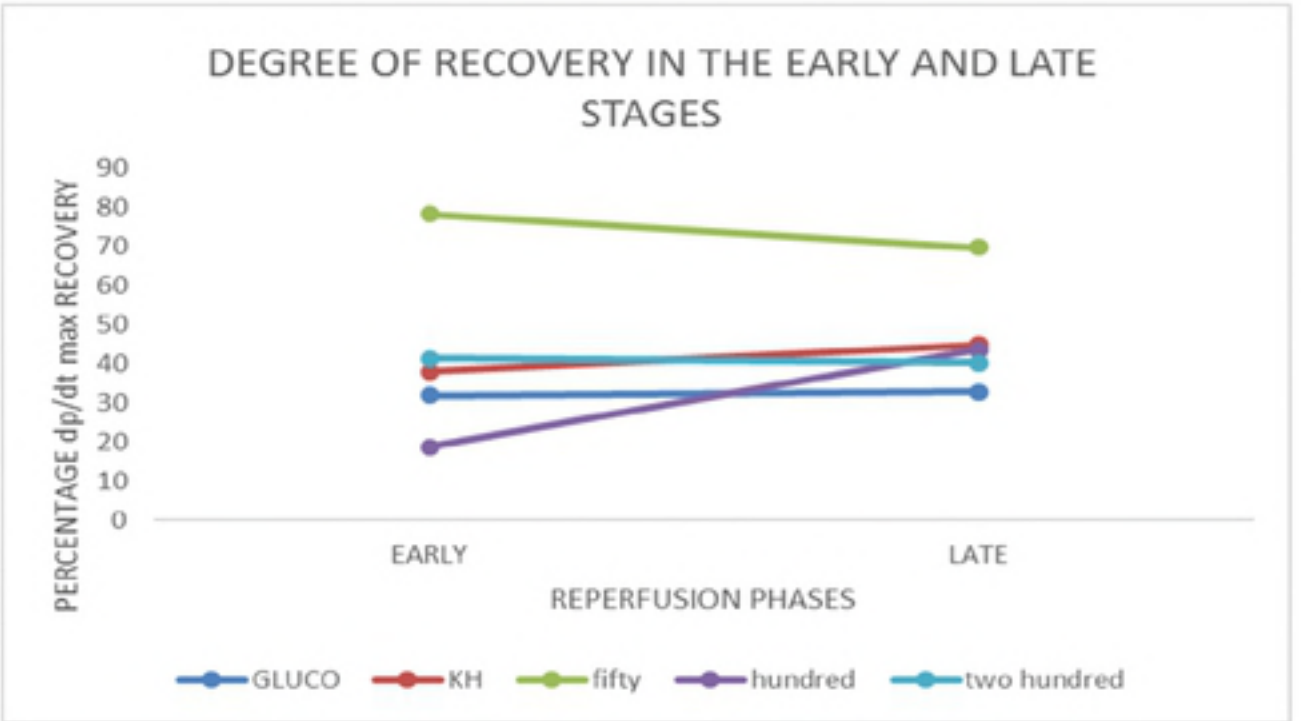

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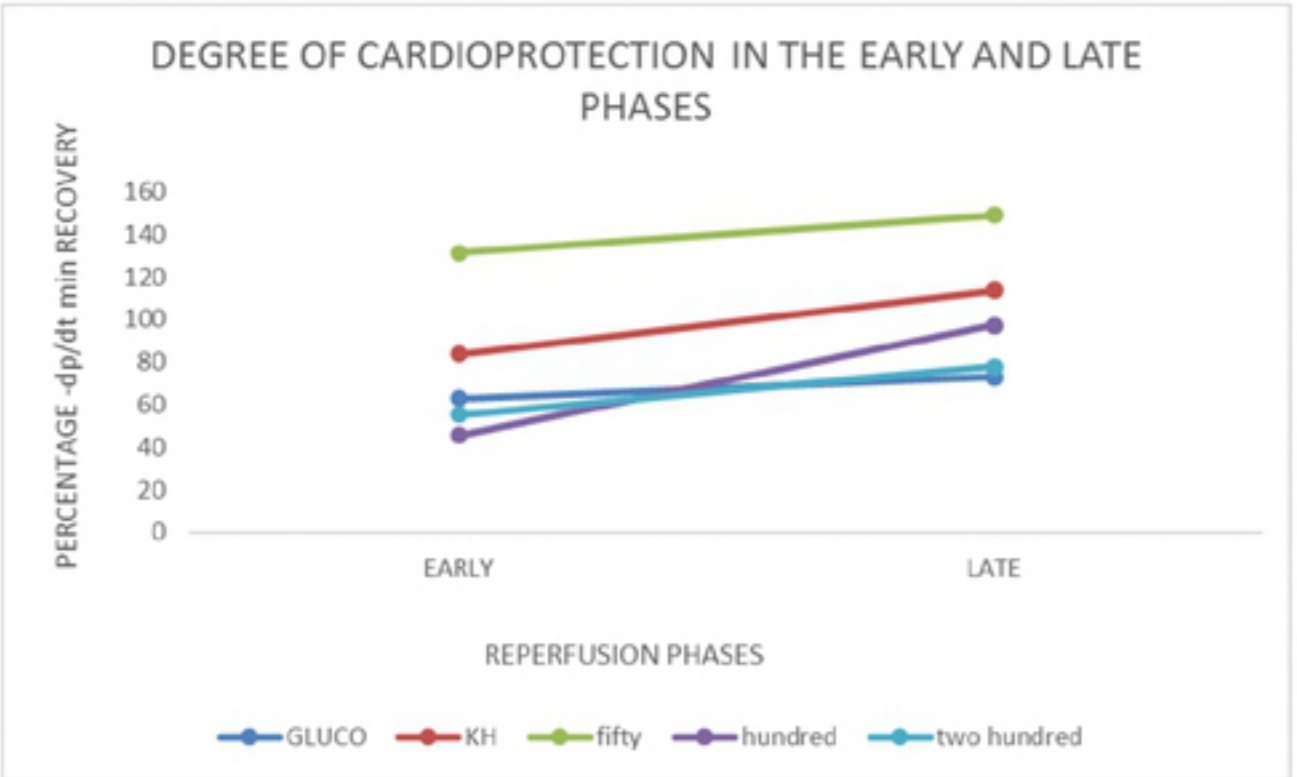

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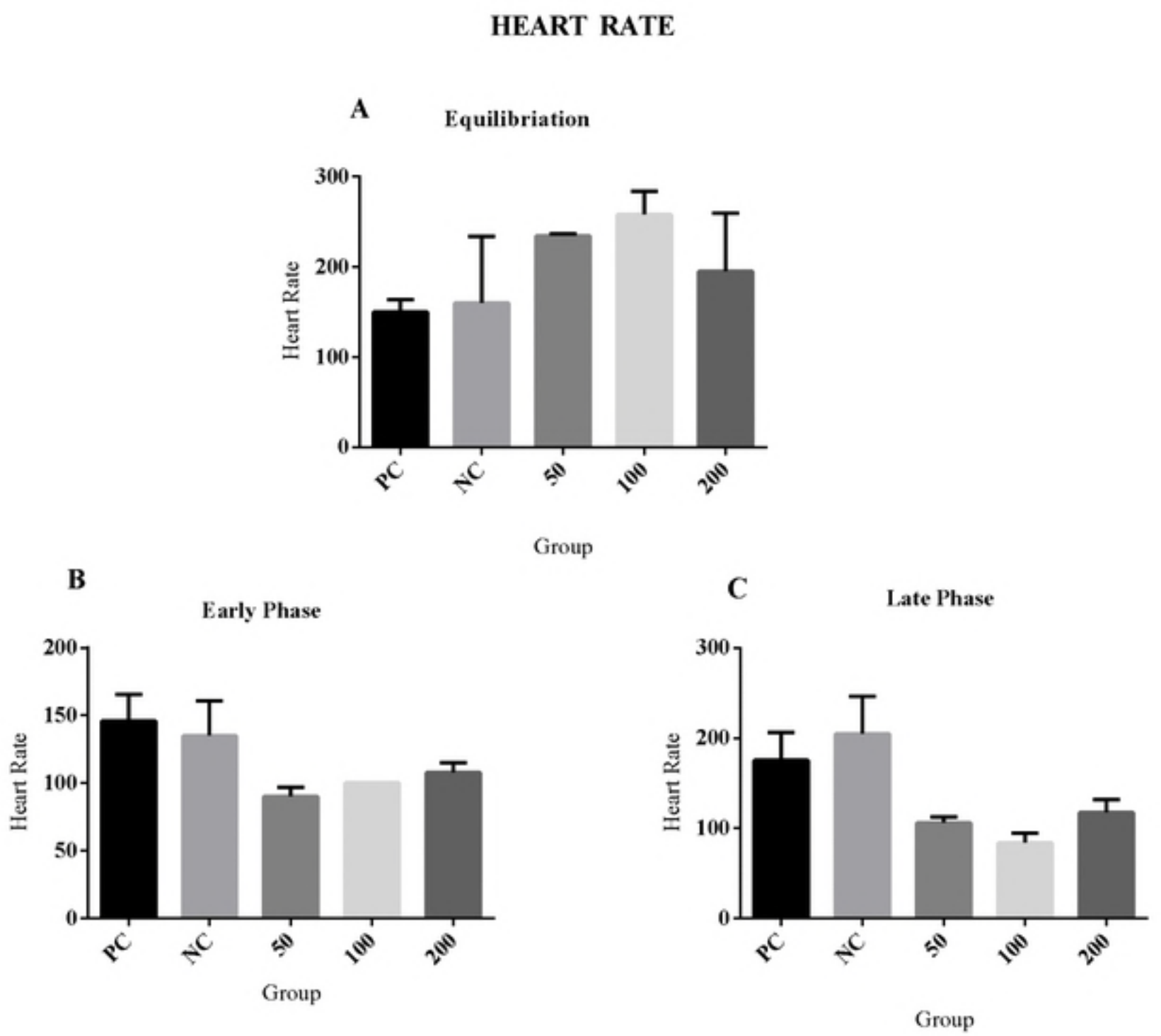

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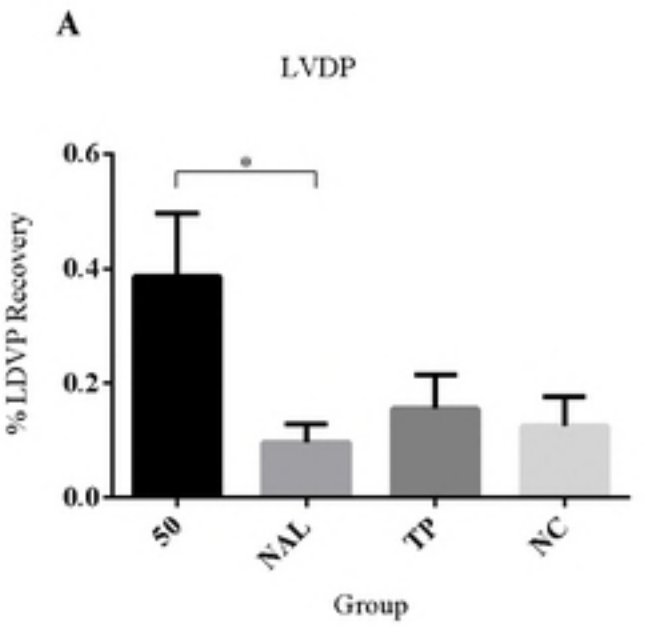
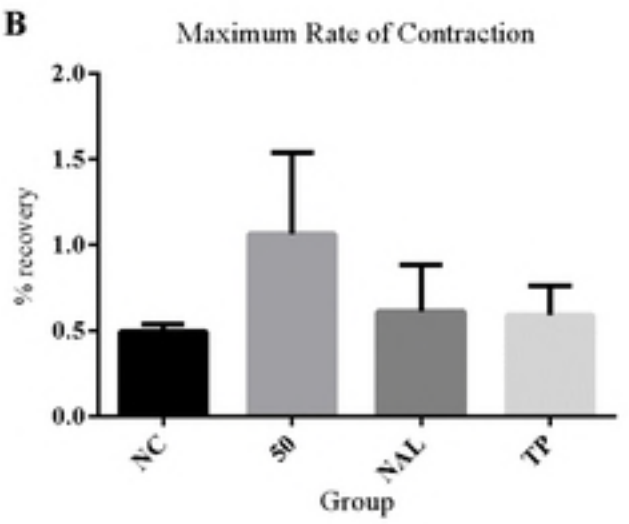
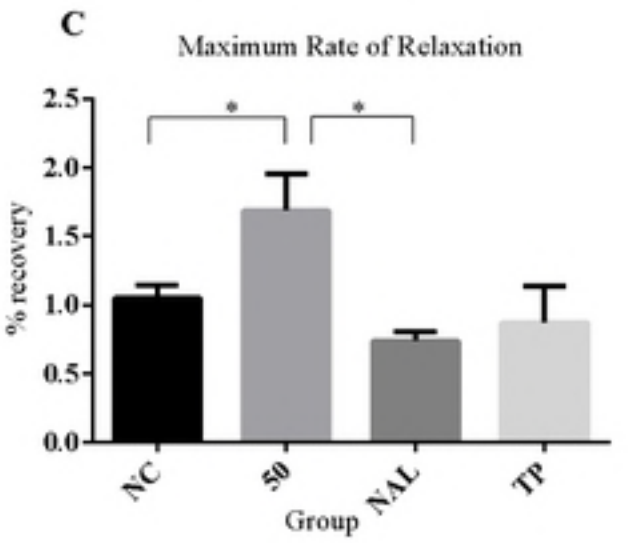
RECOVERY AFTER BLOCKER TREATMENT







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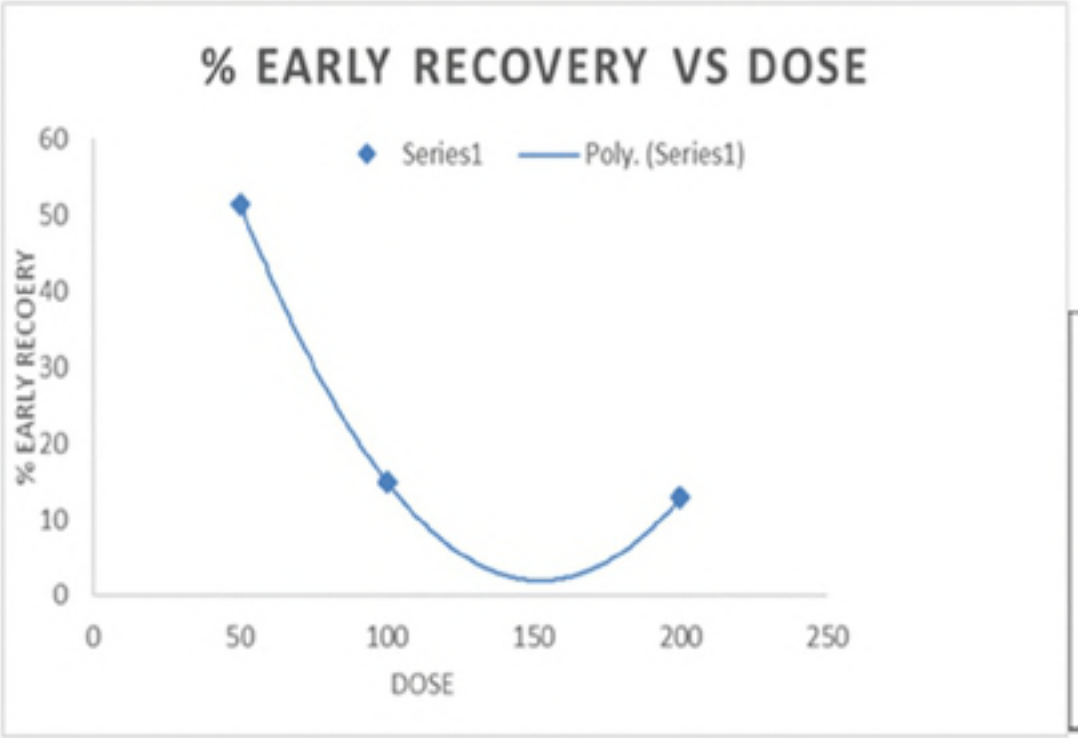

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