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Calycosin reduces infarct size, oxidative stress and preserve heart function in isoproterenol-induced myocardial infarction model

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Abstract: Calycosin (CC) is a phytoestrogen, isolated from *Radix astragali* a well-known Chinese herb and used for treating various pathological conditions. The current study was projected to elucidate the cardio-preservative property of CC in isoproterenol (ISO) induced cardiac injury model (MI) in rats. Male SD rats (n=48) were equally divided into 4 groups which include normal rats (Control; n=12), ISO-MI rats (n=12) which were injected with 85 mg/kg of ISO for 2 days. ISO+CC rats (n=12) were pre and post-treated with CC (30 mg/kg). CC alone rats (n=12) were injected with only CC (30 mg/kg). Pre and post-treatment with CC after and before ISO exposure showed strong cardioprotective property through significant reduction ($p<0.05$) in the mean values of cardiac infarct size, serum cardiac markers, inflammatory markers, apoptotic markers, lipid peroxidation (oxidative stress) by improving antioxidant status as well as reversing all those histopathological changes. Based on the results, we suggest that CC might be useful against MI if consumed along with standard MI medication to lower cardiac dysfunction and its related complications. However, further studies are needed to justify the above statement.

Keywords: Calycosin, myocardial infarction, infarct size, antioxidant, apoptosis.

INTRODUCTION

Acute myocardial infarction (MI) is a major type of ischemic heart disease (IHD) with a high rate of mortality and morbidity (Mnafgui *et al*., 2016). Epidemiological studies have indicated that the incidence of MI is substantially increased in recent times. It has been estimated that every year around 7 million subjects are affected by MI (Reed *et al*., 2017). Hence, MI has a direct impact on the global economy as well as affects each individual quality of life. Therefore, the need for management or treatment of MI is of high priority. Moreover, the current medication for treating MI (antiplatelet agents, anti-thrombotic, β adrenergic/ angiotensin receptor blockers and vasodilators) is much limited as it results in various adverse effects (Roe *et al*., 2010). Considering all those above-mentioned things in mind many researchers started to focus on natural agents especially plant-derived compounds (phyto-components) to treat or manage against MI related conditions as they show less adverse effect (safe) and less expensive as well as potent cardioprotective activity with multi-protective property (Prince and Hemalatha, 2018).

Radix astragali (Astragali Radix or *Astragalus membranaceus*) is one of the famous Chinese Herbal Medicine (dried root) with various biological properties and hence extensively used in Traditional Chinese Medicine (TCM) for treating many disorders/disease conditions for several years (Sun *et al*., 2010). *Radix astragali* (dried root) has an array of phytocomponents like saponins (astragaloside), polysaccharides and isoflavonoids (Zhang *et al*., 2013). Among these phytocomponents, calycosin (CC) is a phytoestrogen, which is considered as an active component with numerous beneficial functions such as anti-diabetic, anti-inflammatory, free radical scavenging, anti-osteoarthritis, anti-tumor as well as hepatoprotective, neuroprotective and cardioprotective properties (Gao *et al*., 2014; Tang *et al*., 2010). *Radix astragali* (crude extract), were reported to lower myocardial ischemia/infarction (I/R) injury (Jin *et al*., 2014) and cerebral ischemic injury (Liu *et al*., 2013). In addition, calycosin glucoside (derivative) displayed a cardioprotective role by abolishing ischemic reperfusion injury through activating PI3K/Akt signaling pathway in the traditional ligation method in a rat model (Ren *et al*., 2016). Nevertheless, none of the researchers have conducted any experiments with calycosine alone against ISO induced MI model rats. Hence, the present animal experiment was aimed to check whether calycosine can protect myocardium after ISO-exposure in male SD rats.

MATERIALS AND METHODS

CC (98% HPLC grade) was purchased from Shanghai Tauto Biotech, Co., Ltd; Shanghai, China). Triphenyl tetrazolium chloride (TTC), xylene, paraffin and Isoproterenol hydrochloride (ISO-HCl) were bought from Sigma (MA, USA).

Healthy male albino Sprague Dawley (SD) rats weighing 210±10 g were used. All the rats were kept under normal laboratory conditions like optimum temperature (22±

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2°C) and humidity (55-65%). This experiment was approved and sanctioned by the experimental animal ethical board (EAEB) of The First Hospital of Jilin University, Jilin, China (Approval No. 3B12-2017).

Experimental design

Overall 48 healthy male SD rats were used for this study and were housed at an animal house (Jilin University, Jilin, China) for 2 weeks. Then the rats were equally divided into 4 groups as control rats (n=12), which were administered only with saline for all 30 days. Whereas, ISO-MI (n=12) group rats were injected intraperitoneal (i.p) with 85 mg/kg of ISO only on 15th and 16th day. Meanwhile, ISO+CC rats (n=12) were pretreated with CC (30 mg/kg via i.p) for 14 continuous days and followed by ISO induction (as indicated above) and again treated (post-treatment) with CC (30mg/kg via i.p) for another 14 days. Finally, the CC alone drug control rats (n=12) were also injected (i.p) only with CC (30mg/kg via i.p) for 30 days.

Blood and cardiac tissue processing

After 30 days of the experiment (14+2+14 days), all the animals (rats) were overnight fasted on the 31st day morning all the animals were sacrificed under 50mg/kg of pentobarbital sodium and the whole blood sample was collected and serum sample was separated after centrifugation procedure. Cardiac tissues were removed immediately and a part of heart tissue was fixed in 4% paraformaldehyde for histological examination. Remaining heart samples were chopped and homogenized and centrifuged and the resultant supernatant was used for further analysis.

TTC staining to determine infarct size/volume

Cardiac infarct size/volume was quantified using a TTC stain. In short, the cardiac slice of 2 mm thickness was prepared and incubated with 1% TTC stain for 40 min at 37°C and fixing with 4% paraformaldehyde and pictured (digital camera). The infarct size of cardiac tissue was measured from the picture using Image J Software (Ver 2.8) from NIH (MD, USA).

Antioxidant and lipid peroxidation products

The cardiac enzymic antioxidants activities like catalase (CAT) and superoxide dismutase (SOD) as well as the cardiac lipid peroxidation level (malondialdehyde; MDA) was measured by commercial kit purchased from Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd., (Beijing, China), respectively in accordance with the supplier's procedure.

Cardiac markers

The levels of various serum cardiac markers like creatine kinase isoform (muscle/Brain; CK-MB), cardiac troponin T (cTn T), cardiac troponin I (cTn I) and lactate dehydrogenase (LDL) was measured using rat specific ELISA kit supplied by CUSABIO Technology LLC (TX, USA) and MyBioSource (CA, USA) respectively based on manufacturers instruction.

Inflammatory markers and Apoptotic markers

The cell fractionation kit was bought from Abcam (Cambridge, UK) to separate the nuclear and cytosolic fraction from the cardiac tissue homogenate (ultracentrifugation technique). The values of the nuclear factor kappa B p65 subunit (NF-κB p65) was determined by the NF-κB p65 ELISA kit from My Bio Source (CA, USA). While, the concentrations of interleukins six (IL-6), interleukins 1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) were assessed using commercial specific ELISA kit from Cayman Chemical (MI, USA). The cardiac caspase 3 and 9 (apoptotic markers) are measured using an enzymatic ELISA multiplex activity kit purchased from Abcam (Cambridge, UK) in accordance with the supplier's protocol.

Histomorphological changes

A portion of the cardiac section (tissue) was fixed in 4% paraformaldehyde and embedded with paraffin wax and was sectioned using ultra-microtome (4-5µm thickness) and mounted on a microscopic slide and followed by staining with hematoxylin and Eosin stain (H & E). The stained section (mounted in the slide) were examined for any histomorphological alterations using a computerized digital optical microscope attached with Olympus Digital camera (Olympus Co, Tokyo, Japan).

STATISTICAL ANALYSIS

Data are shown as the mean ± standard deviation (SD). The p-value (significant value) between experimental groups were expressed as either $^{**}p<0.01$, $^{*}p<0.05$ (Con Vs ISO) or $^{\$\$}p<0.01$, $^{\$}p<0.05$ (ISO Vs CC+ISO). The p-value was determined using SPSS software (Ver 21) IBM, Co., (NY, USA) using one-way ANOVA followed by Dunnett's multi-comparison test.

RESULTS

Impact of CC on infarct size

Fig. 1 illustrates the efficacy of CC on infarct size (aortic) in ISO induced and control rats. An exponential increase ($p<0.01$) in the volume of infarct size were observed in MI model rats- (ISO injected) than saline-treated control rats. Nevertheless, rats induced with ISO and treated with CC (ISO+CC; $p<0.01$) showed a considerable reduction in the levels of cardiac infarct size as compared to ISO-exposed rats.

Impact of CC on MDA and antioxidants

Table 1 exemplifies the impact of CC on MDA (lipid peroxidation) and antioxidants (cardiac homogenate) in ISO induced and control rats. Animals administered with ISO showed marked decrease ($p<0.01$) in CAT and SOD activities along with inclined values of MDA.

Comparison with MI induced animal (ISO-induced), the CC supplemented animal showed increased SOD and CAT activities along with declined MDA levels (p<0.01).

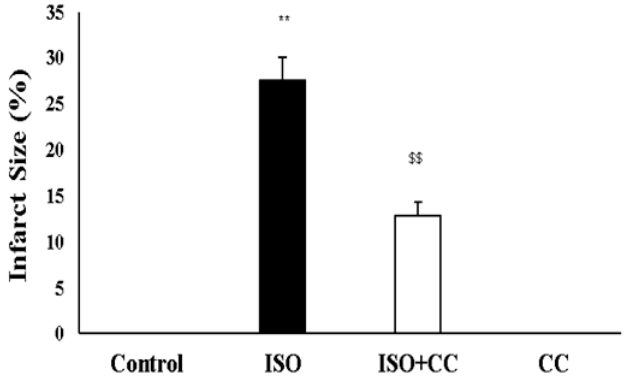

Fig. 1: represent the efficacy of calycosin (CC) on cardiac infarct size in control and ISO induced rats. Data are shown as the mean ± standard deviation (SD). The p-value was represented as **p<0.01 for comparison between control Vs ISO group; $p<0.05, $$p<0.01 for comparison between ISO Vs ISO+CC group. CC: Calycosin, ISO: Isoproterenol.

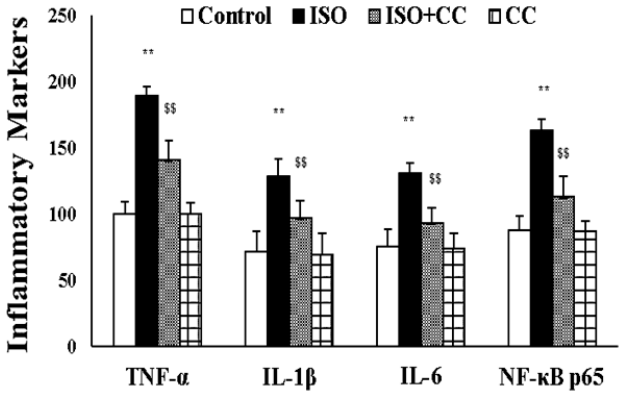

Fig. 2: represent the efficacy of calycosin (CC) on cardiac inflammatory markers in control and ISO induced rats. Data are shown as the mean ± standard deviation (SD). The p-value was represented as **p<0.01 for comparison between control Vs ISO group; $p<0.05, $$p<0.01 for comparison between ISO Vs ISO+CC group. TNF-α: tumor necrosis factor-alpha; IL-1β/6: Interleukins 1beta/6; NF-κB: Nuclear factor-kappa B; CC: Calycosin, ISO: Isoproterenol.

Impact of CC on Cardiac markers

As indicated in table 2, the mean values of various serum cardiac diagnostic markers like LDL, CK-MB, cTn I and cTn T was dramatically escalated in the serum sample of ISO-exposed rats (p<0.01) than control rats. Nonetheless, rats injected with CC (p<0.01) for 28 days (pre and pro treatment), could significantly decrease those serum cardiac markers to a near-normal level as similar to the control group.

Impact of CC on Inflammatory and apoptotic markers

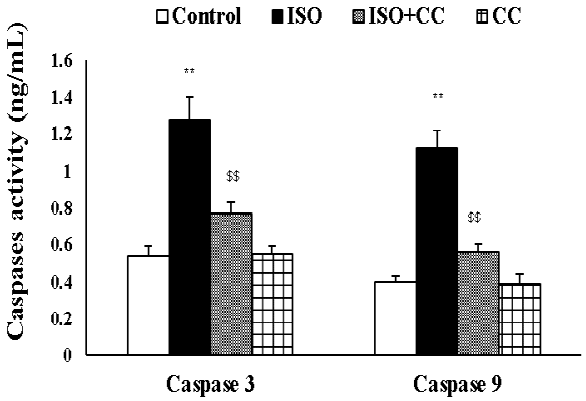

Fig. 3: represent the efficacy of calycosin (CC) on cardiac apoptotic markers in control and ISO induced rats. Data are shown as the mean ± standard deviation (SD). The p-value was represented as **p<0.01 for comparison between control Vs ISO group; $p<0.05, $$p<0.01 for comparison between ISO Vs ISO+CC group. CC: Calycosin, ISO: Isoproterenol.

Impact of CC on Histopathological assessment

Fig. 4 illustrates the efficacy of CC on cardiac histomorphological changes in ISO induced and control rats. The control rats (4A) and CC alone rats (4D) cardiac slides display normal cardiac structure (myofibril) without any inflammatory or apoptotic changes (pathological changes). The ISO-induced rats cardiac section (4B) shows disorientated myofibrillar arrangement and edema with large inflammatory cells infiltration and apoptotic/necrotic changes. However, the cardiac slide of CC treated rats (4C) before and after ISO-induction display mild inflammatory infiltration and minimal edematic or necrotic/apoptotic changes.

DISCUSSION

An ample amount of studies inferred that injection of isoproterenol (synthetic catecholamine) at supramaximal dose could induce MI like cardiac damage by increasing oxidative stress (imbalance free radical and antioxidants), inflammatory response and apoptotic cascade (Raish *et al*., 2017; Hassan *et al*., 2015). Therefore, ISO induced

Table 1: Efficacy of calycosin (CC) on cardiac antioxidants and lipid peroxidation products in control and ISO induced rats

Parameters	Control	ISO	ISO+CC	CC
SOD (U/mg pro)	5.43 ± 0.30	3.18 ± 0.27**	4.71 ± 0.41$^{\$\$}$	5.40 ± 0.40
CAT (U/mg pro)	13.65 ± 1.20	7.85 ± 0.85**	11.92 ± 1.00$^{\$\$}$	13.40 ± 1.15
MDA (nmol/mg pro)	0.61 ± 0.04	1.32 ± 0.12**	1.08 ± 0.09$^{\$\$}$	0.60 ± 0.05

Data are shown as the mean ± standard deviation (SD). The p-value was represented as **p<0.01 for comparison between control Vs ISO group; $^{\$}$p<0.05, $^{\$\$}$p<0.01 for comparison between ISO Vs ISO+CC group. SOD: Superoxide dismutase, CAT: Catalase; MDA: Malondialdehyde, Pro: protein, CC: Calycosin, ISO: Isoproterenol.

Table 2: Efficacy of calycosin (CC) on serum cardiac markers in control and ISO induced rats

Parameters	Control	ISO	ISO+CC	CC
CK-MB (IU/L)	75.10 ± 9.00	141.35 ± 10.70**	98.40 ± 7.30$^{\$\$}$	72.90 ± 7.00
LDH (IU/L)	82.40 ± 6.55	151.50 ± 14.79**	104.22 ± 11.25$^{\$\$}$	81.90 ± 9.40
cTn T (ng/mL)	0.54 ± 0.42	1.10 ± 0.10**	0.74 ± 0.07$^{\$\$}$	0.52 ± 0.30
cTn I (ng/mL)	0.30 ± 0.02	1.05 ± 0.10**	0.59 ± 0.05$^{\$\$}$	0.32 ± 0.04

Data are shown as the mean ± standard deviation (SD). The p-value was represented as **p<0.01 for comparison between control Vs ISO group; $^{\$}$p<0.05, $^{\$\$}$p<0.01 for comparison between ISO Vs ISO+CC group. CC: Calycosin, ISO: Isoproterenol, cTn T: Cardiac troponin T, cTn I: Cardiac troponin I, CK-MB: Creatine kinase Isoform (muscle/Brain), LDL: Lactate dehydrogenase.

MI model in rats is considered as one of the reliable and easy non-invasive method to check the cardio-protective property (especially against MI) of any drugs (Khan *et al*. 2018; Randhawa *et al*., 2013). The outcome of the current study clearly demonstrates the cardioprotective activity of CC by the significant reduction in the levels of cardiac infarct size, inflammatory markers, serum cardiac markers, apoptotic markers, lipid peroxidation (oxidative stress) as well as lowered histopathological changes.

Elevated cardiac infarct size is one of the major pathophysiological events that occur during MI condition and hence infarct size measurement is an important criterion to assess the anti-MI property of any drug (Panda *et al*., 2014). Rats injected with ISO, showed larger or increased infarct size than control rats, due to ISO induced oxidative stress and myocardial dysfunction (lack of blood supply). Similar kind of results was also reported by Chen and his co-workers (2015). However, rats treated with CC considerably reduced the infarct size and thereby showcasing its cardioprotective property. Previously, Ren and others (2016), indicated that treatment with calycosin glucoside could considerably reduce the myocardial infarct size.

As mentioned before oxidative stress (excess free radical/ROS generation) and inflammatory response are the major pathophysiological event contributes to ISO induced MI. Also, studies have indicated that human MI is also strongly associated with free radical generation (Heusch and Gersh, 2016; Burke and Virmani, 2007). Therefore, we examined the cardiac oxidative status by checking antioxidants like SOD and CAT as well as lipid peroxidation products like MDA in cardiac tissue. Results showed that ISO induction enhanced ROS production and thus raise the MDA production by lowering antioxidant activities (SOD/CAT). But, upon treatment with CC for 28 days (pre and post-treatment), considerably lowered free radical production and thus MDA levels as well as improve antioxidant activity. The above data showed that CC possess anti-lipid peroxidation and antioxidant activities and thus protect myocardium from ROS induced damage. Likewise, Liu and his co-workers (2016), demonstrated that calycosin can suppress oxidative stress by lowering ROS production and thus protect myocardium from injury or damage. Due to excessive free radical generation after ISO induction the concentration of serum cardiac markers (cTn T, cTn I, CK-MB, LDH) were considerably escalated (ROS damage myocytes and release marker enzyme into the blood). Nevertheless, treatment with CC substantially decreased the mean values of serum cardiac markers due to its anti-lipid peroxidation and free radical scavenging activities (Guo *et al*., 2012).

A growing body of evidence indicated that inflammation and oxidative stress are interconnected and directly involved in MI (Reed *et al*. 2017; Heusch and Gersh, 2016). The levels of inflammatory markers (IL-6, IL-1β, TNF-α, and NF-κB p65) were markedly increased in ISO-induced rats, but rats treated with CC could greatly attenuate those inflammatory markers due to its potent anti-inflammatory and antioxidant activities (Su *et al*., 2016). Many researchers have confirmed that ISO-induction could enhance apoptosis by modulating caspase cascade especially caspase 3 and 9 (Othman *et al*., 2017; Prince and Roy, 2013). To endorse the above state, in this study the levels of caspase 3 and 9 were significantly

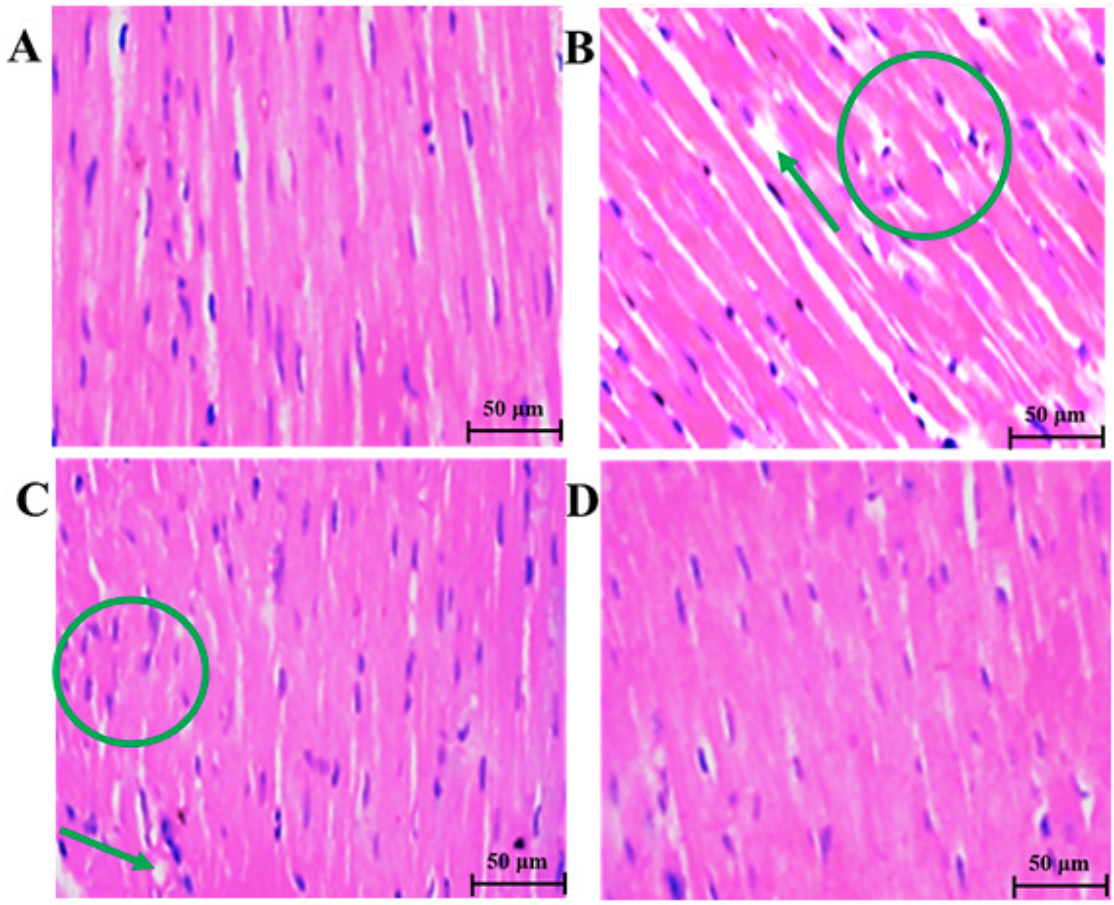

Fig. 4: illustrate the effect of calycosin (CC) on cardiac histomorphological changes under a light microscope (400 ×). The cardiac slides of control rats (4A) and CC alone rats (4D) showed normal cardiac structure with a distinct cardiac myofibrillar arrangement without any inflammatory or apoptotic changes. However, the cardiac section of ISO-induced rats (4B) shows disoriented myofibrillar arrangement and edema (arrow mark) with large inflammatory cells infiltration (circle) and apoptotic as well as necrotic changes. The cardiac slide of CC treated rats (4C) before ISO-induced display mild inflammatory infiltration (circle) and lesser edematic (arrow mark) or necrotic/apoptotic changes. Scale bar: 50 µm.

increased in ISO-exposed rats. Rats supplemented with calycosin exhibit potent anti-apoptotic property by abolishing caspase 3 and 9 activity and thus showcasing its anti-MI property (cardioprotective protective). Similarly, administration with calycosin glucoside significantly downregulates the protein expression of caspase 3 and 9 in I/R induced rat model and thus preserving cardiomyocyte from apoptosis (Ren *et al*., 2016).

Finally, the histopathological changes were examined to cross-check all the biochemical changes. The ISO-induced rats (cardiac section) displays abnormal cardiac architecture with disoriented myofibrillar arrangement and edema as well as large inflammatory cells infiltration and apoptotic/necrotic changes. However, the CC treated rats showed only mild inflammatory infiltration and minimal edematic or necrotic/apoptotic changes with normal myofibrillar arrangements. Cheng and his colleagues (2015), indicated that administration with calycosin and gallic acid together reduced neutrophil infiltration (inflammation) and protect myofibers in isoproterenol-induced MI model. The above outcome confirmed that CC preserves cardiac function by abolishing histopathological changes because of its antioxidant, anti-lipid peroxidation, anti-inflammatory, and anti-apoptotic activities. This study has some limitations, as we did not focus on the underpinning mechanism for its cardioprotective activity by checking various signaling pathways (AktPI3K or MAPK) and its impact on the ER receptor (since CC is a phytoestrogen).

CONCLUSION

Overall, both pre and post-treatment with CC after and before ISO exposure display potent cardioprotective property through significantly reducing the levels of cardiac infarct size, inflammatory markers, serum cardiac

markers, apoptotic markers, lipid peroxidation (oxidative stress) and by improving antioxidant status as well as reversing all those histopathological changes. Nevertheless, further studies are needed to justify the above results and also the in-depth mechanism need to be explored.

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