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CARDIOPROTECTIVE EFFECTS OF SAPONIN *Momordica cymbalaria* IN ISCHEMIA REPERFUSION INJURY

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ABSTRACT

Momordica Cymbalaria has attracted steady attention in the last few years as phytomedicine possessing a wide spectrum of biological activities. In this study, saponins isolated from *Momordica cymbalaria* (SMC) was evaluated for global ischemia induced myocardial damage in male Wistar rats and hypoxia ischemia induced cardiocmyotocytes cell death *in vitro*. Male Wister albino rats were divided into six groups of six animals each and was pretreated with SMC at different doses. To evaluate the efficacy of SMC pretreatment against global ischemia induced myocardial damage, biochemical and histopathological studies were carried out. Ischemia reperfusion injury produced severe myocardial damage and depletion of antioxidant enzymes level. On the contrary, SMC pretreatment reduced myocardial damage by improvement CK-MB and lactate dehydrogenase (LDH). In addition, SMC improved the antioxidant defense system in treated animals and considerably reduced the oxidative stress induced by ischemia reperfusion IR. The reduction in oxidative stress was evident from the lipid peroxidation and enzymatic antioxidant activities. Furthermore, the increase in the levels of CK-MB and LDH in the heart tissue homogenate and the decrease of these enzymes in the perfusate was significantly reversed in the treated groups. The histopathological studies also showed that SMC pretreatment significantly minimized the damage induced by IR. Saponin of *Momordica cymbalaria* (25mg/kg) provided good cardioprotection against reperfusion induced myocardial infarction in rats compared to higher doses. Pretreatment of rat cardiomyocyte H9c2 cell line with Saponins from *Momordica cymbalaria* at 20µg/ml was more effective in protecting them from ischemic injury as compared to higher doses confirming the ex-vivo findings that saponin from *Momordica cymbalaria* provide good myocardial protection at lower doses and show toxic effects at higher doses.

Key words: Saponins, *Momordica cymbalaria*, Global ischemia, Antioxidant.

INTRODUCTION

Myocardial infarction is the leading cause of death in the world today. Ischemia reperfusion (IR) occurs in a wide range of situations including trauma, vascular reflow after contraction etc. In the ischemic heart, initial cardiac damage is prompted by diminished blood supply. However, swift restoration of normal blood supply is imperative to minimize cardiac injury. Unfortunately, reperfusion itself can lead to additional injury in the form of cardiac dysfunction, reperfusion arrhythmias, and exacerbated myocardial infarction. Oxidant stress as well as inflammation seems to play a major role for organ injury during I/R [1,2]. Increased production of reactive oxygen species (ROS) and accumulation of calcium in the cytosol and mitochondria are major causative factors of IR injury [3,4]. *Momordica Cymbalaria* has attracted steady attention in the last few years as phytomedicine possessing a wide spectrum of biological activities. It has been reported that *Momordica cymbalaria* fruits possess type 1 antidiabetic activity on both strptozotocin and alloxan induced diabeticsmodel [5,6]. Type 2 antidiabetic activity of Saponins from *Momordica cymbalaria* have been reported in Streptozotocin-Nicotinamide diabetic Mice [7]. Saponins from Momordica Cymbalaria are also reported to have antidiabetic [8,9] antiovulatory [10], antiimplantation [11], neuroprotective [12] activities. The Ethanolic Extract of roots of *Momordica cymbalaria* has been reported to have cardioprotective activity in isoproterenol induced myocardial damage [13]. Therefore in the present study the effect of Saponins of *Momordica cymbalaria* on ischemia reperfusion induced Myocardial damage is evaluated.

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MATERIAL AND METHODS

Plant Material

The informations on collection, identification, phytochemical screening and isolation of saponins fraction of Momordica cymbalaria were dircribed by us in earlier publication [12].

Experimental animals

Rats of either sex weighing 175-250 g were obtained from Indian institute of sciences, Bangalore. They were housed three per poly propylene cage under standard laboratory conditions at room temperature (25° C± 2° C) with 12 h light / dark cycle. The animals were provided with pellet chow and water ad libitum, except during experimentation. Ethical clearance was obtained from Institutional Animal Ethical Committee (IAEC) of Karnataka College of pharmacy, Bangalore.

Acute oral Toxicity

Acute oral toxicity for the drug was reported in our earlier publication [89].

Experimental Procedure

Animals were randomly divided into six groups (n=6) : normal control, ischemic reperfusion control (IR-control), ischemic rats pretreated with SMC at a dose of 25 mg/kg, ischemic rats pretreated with SMC at a dose of 50 mg/kg, ischemic rats pretreated with SMC at a dose of 100 mg/kg and ischemic rats pretreated with SMC at a dose of 175 mg/kg

A modified Langendorff apparatus for the isolated perfused heart was set up as described by Inamdar et al (1994) [14]. The heart was allowed to equilibrate for 10 min and then regular recordings were taken for a perfusion period of 15 min. Measurement of contractile force was done using force displacement transducer and recorded on a Student Physiograph (INCO, Mumbai, India). After the initial preischemic perfusion, heart was subjected to 15 min of global no-flow ischemia by blocking the flow of Kerbs & Henseleit solution & carbogen supply followed by 15 min of reperfusion. The heart rate and developed tension were measured during pre-ischemic and post-ischemic period and recovery (in percentage) was calculated. Lactate dehydogenase (LDH) and creatine kinase-MB (CK-MB) activity were measured in the perfusate during pre-ischemic and post-ischemic period. The heart was then homogenized to prepare heart tissue homogenate (HTH) and the activity of LDH, CK-MB was determined.

Briefly, the hearts were dissected out, washed immediately in ice-chilled physiological saline, blotted and weighed. A known weight of the heart tissue (10%) was homogenized in 0.1 M Tris-HCl buffer (pH 7.4) solutions. The homogenate was centrifuged at 3000 rpm for 5 min and the tissue homogenate sample was used for LDH and CK-MB estimations. LDH and CK-MB, SOD, CAT, GSH activities and lipids peroxidation were determined by spectrophotometric methods [15] (Schimadzu Spectrophotometer, Japan) using commercial kit supplied by Crest Biosystems, Coral Clinical Systems (Goa, India).

In vitro ischemic reperfusion Model

Ischemia reperfusion was induced in H9c2 cells by inducing hypoxia reperfusion in the culture as described by Majed [16]. Briefly, after 12 hrs serum fasting, cells were incubated with 200µM of CoCl2.6H2O for 24 hrs and cells viability was performed by the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) by mitochondrial reductase [17]. Cells were incubated with 0.01 g/ml MTT for 2 hours at 37°C. Then, 1 ml of 0.04 M HCl in isopropanol was added to each well. The converted dye was collected and the optical density was determined spectrophotometrically at λ 570 nm with background subtraction at λ 650 nm. Cell viability was calculated as a percentage of control.

Statistical analysis

Results of all above estimations have been indicated in terms of means ± SEM. Differences between the groups were statistically determined by analysis of variance, one way ANOVA with Tukey post-test. The level of significance was set at $P<0.05$.

RESULTS

Acute toxicity study of SMC

Acute toxicity result is mentioned in our earlier paper [12]. We have chosen 175, 100, 50 and 25 mg/kg doses for the present study

Effect of SMC on CK-MB and LDH activity

Pretreatment with SMC 25, 50 and 100mg/kg significantly decreased CK-MB and LDH activity in the perfusate when compared to control. The activity of CK-MB and LDH was significantly increased in the heart homogenate when compared to control. Pretreatment with SMC 175 mg/kg showed increase of CK-MB and LDH activity in the perfusate when compared to control while this activity of CK-MB and LDH was decreased in the heart homogenate.

Effect of SMC on enzymatic antioxidant activity

Ischemia reperfusion decreased significantly the activity of SOD, CAT, GSH and increased TABR level as compared to normal control. Pretreatment with SMC 25, 50, and 100 mg/kg prevented the oxidative stress by increasing significantly SOD, CAT, GSH activities and decreasing significantly TABR level when compared to IR control. However SMC 175mg/kg did not show significant difference in the values for SOD, CAT, GSH activities and TABR level when compared to IR control.

Effect of SMC on heart rate and developed tension

Pretreatment with SMC 25, 50, and 100 mg/kg showed a significant recovery of developed tension and heart rate after myocardial damage induced by ischemia reperfusion as compared to ischemia reperfusion control. However SMC 175mg/kg did not show significant difference in the recovery of developed tension and heart rate.

Effect of SMC on ischemia induced cardiomyocyte death *in vitro*

The effect of SMC to reduce ischemia induced cardiac cell death was performed by pre-incubation of SMC in various concentrations 5-50 µg/ml (Table 4). Hypoxia group was considered as control and its cell viability was 100%. Reperfusion decreased cell viability up to 78.57% as compared to hypoxia control. Pre-treatment with 5-20 µg/ml of SMC prior to hypoxia ischemia significantly prevented hypoxia ischemia induced cell death in dose dependent manner. The results showed that 20 µg/ml of SMC gave highest percentage of cell viability up to approximately 97%, while the concentration of 5 and 10 µg/ml gave the percentage of cell viability to 94.00 and 94.10% respectively. However, higher concentrations of SMC (30 and 50 µg/ml) failed to protect the H9c2 cells from hypoxia ischemia induced cell death. In contrast they showed a significant decrease in the percentage of cell viability, 77.14 and 69.32% respectively.

Fig 1. Tracing on polygraph before and after ischemic reperfusion injury and SMC treatment

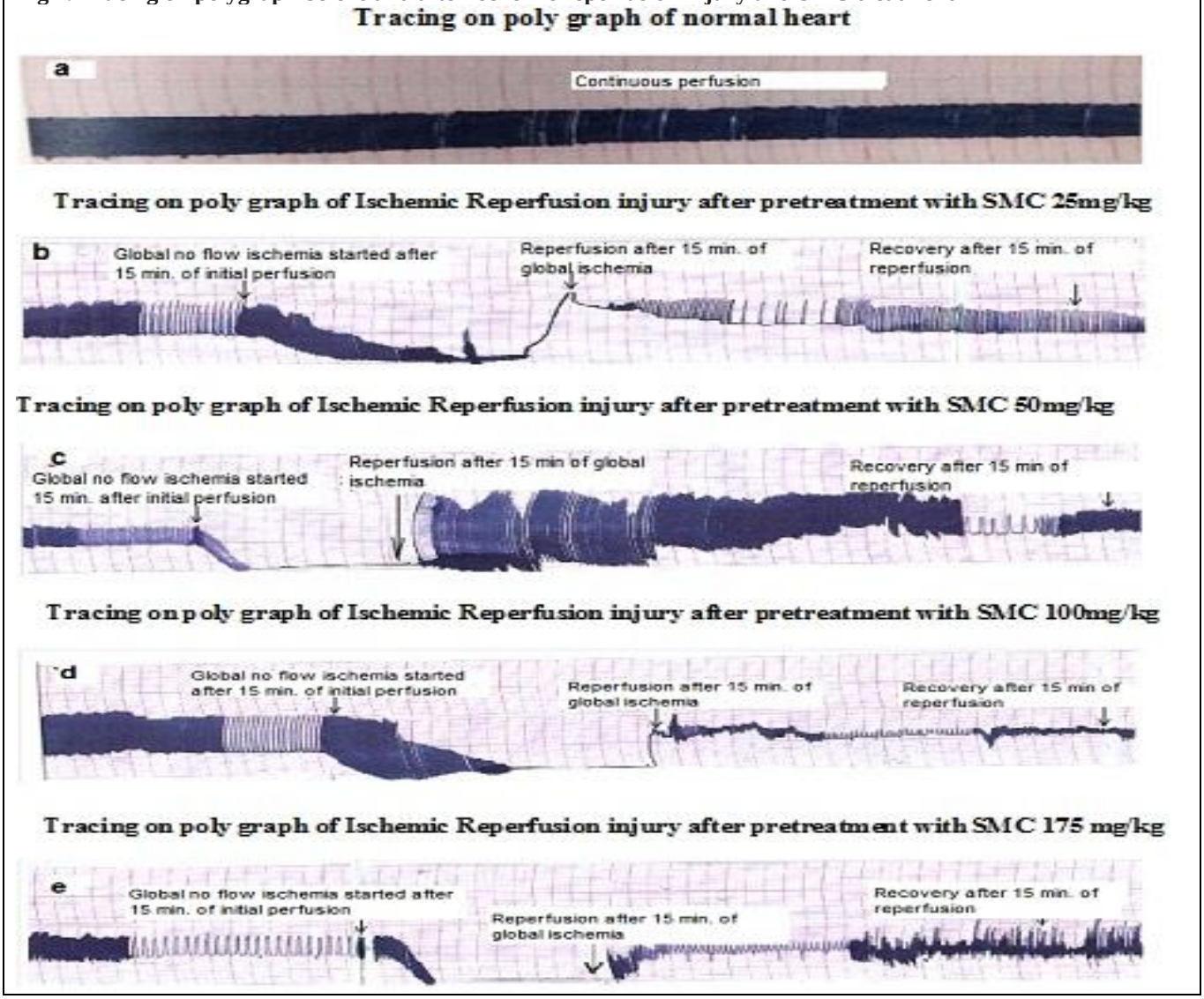

Table 1. Effect of SMC on CK-MB and LDH activity in the perfusate and heart homogenate.

Groups	CK-MB Activity		LDH Activity	
	Perfusate (unit/lit)	HTH (unit/gm)	Perfusate (unit/lit)	HTH (unit/gm)
C-IR control	41.69±0.49	22.66±0.4729	119.6±1.534	87.55±1.509
SMC-25mg/kg	12.34±0.45***	84.61±0.6749***	22.71±0.7524***	154.4±1.141***
SMC-50mg/kg	16.97±0.55***	79.71±0.8479***	27.60±0.6775***	144.7±1.831***
SMC-100mg/kg	27.93±0.39***	52.56±0.4608***	48.34±0.6979***	115.1±1.015***
SMC-175mg/kg	54.37±0.61*	20.60±0.3120	121.7±0.7737	82.960.4361

Values expressed as mean ± SEM, n=6 ***P<0.001 when compared to to IR control

Table 2. Effect of SMC on enzymatic antioxidant activity

Treatments	SOD (Unit/mg protein)	Catalase (Unit/mg protein)	GSH (Unit/mg protein)	TABR (Units/mg protein)
Normal control	5.19±0.12	8.09± 0.05	89.17± 1.28	24.05± 0.59
IR control	1.90± 0.04	2.198± 0.02	15.58± 0.51	81.77± 0.36***
MC-25mg/kg	5.51± 0.24***	7.81± 0.07***	83.11± 0.64***	13.87± 0.31***
MC-50mg/kg	5.09± 0.14***	6.97± 0.04***	73.08± 0.51***	20.31± 0.30***
MC-100mg/kg	3.03± 0.03***	5.778± 0.04***	49.27± 0.65***	26.45± 0.40***
MC-175mg/kg	2.69± 0.08	3.07± 0.03	21.04± 1.04	62.05± 0.32

Values expressed as mean ± SEM, n=6***P<0.001 when compared to IR control

Table 3. Percentage recovery of heart rate and developed tension in rats pretreated with SMC

Groups	Percentage Recovery	
	Developed Tension	Heart Rate
C-IR	22.00±0.93	29.50±3.84
SMC-25mg/kg	90.23±1.27***	91.13±2.17***
SMC-50mg/kg	82.83±4.27***	85.53±7.17***
SMC-100mg/kg	52.83±4.61*	71.33±1.91***
SMC-175mg/kg	34.50±6.78*	36.00±7.34*

Values expressed as mean ± SEM, n=6 *P<0.05, , ***P<0.001 when compared to IR control

Table 4. Effect of SMC on H9c2 cells viability

	Hypoxia Control	Reperfusion Control	SMC 5 µg/ml	SMC 10 µg/ml	SMC 20 µg/ml	SMC 30 µg/ml	SMC 50 µg/ml
% viable	100	78.57	94.00	94.10	97.07	77.14	69.32

DISCUSSION

Myocardial damage was induced using ischemia reperfusion (IR) model. The IR was induced following no flow global ischemia [18], where sudden occlusion of physiological salt solution results in immediate biochemical alterations. The increase in intracellular Na+ serves to drive Ca^{2+} intracellularly via $Na+/Ca^{2+}$ exchange that results in irreversible damage to myocardium at the end of 15 min global ischemia [19].

Ischemic reperfusion injury in the present study was also associated with increased oxidative stress, as evidenced by increase in myocardial TBARS and depletion of myocardial endogenous antioxidants enzymes such as SOD, Catalase, GSH and hence leakage of CK-MB, LDH endogenous biological markers. Similar observations were made earlier [20-23], using similar models. Increased oxidative stress may be responsible for such myocyte injuries.

It was reported by us earlier that pretreatment with ethanolic extract of *Momardica cymbalaria* at 250 and 500 mg/kg prevented alterations in the oxidative stress markers like Lipid Peroxidase (LPO), Glutathione (GSH), Catalase (CAT), and Superoxide Dismutase (SOD) caused by isoproterenol (60 mg/kg s.c, 2days)- induced myocardial infarction in rats. The protective effect was confirmed by histological findings and was more prominent at 500 mg/kg [13].

The major mediators of reperfusion injury are oxygen radicals, calcium loading, and neutrophils. The burst of oxygen radicals on reperfusion is produced by myocytes. The oxygen radicals promote lipid peroxidation and membrane damage, which leads to calcium loading [24]. Lipid peroxidation triggers loss of membrane integrity, necrosis and cell death [25]. In the present study, TABR level in myocardium was found to be significantly ($p < 0.001$) higher in IR control group when compared with normal control group, while pretreatment with different doses of Saponins of *Momordica cymbalaria* (25,50,100mg/kg; po/30days) decreased significantly the level of TABR in dose-dependent manner. 25mg/kg was found to be more effective, whereas 175mg/kg showed high TABR level in myocardium. SOD, CAT, GSH also demonstrated similar activity confirming antioxidant effect of the drug. Similar observations were made earlier by other studies, using similar models [26,27].

Ischemic reperfusion in the present study increases the CK-MB and LDH activities in the perfusate and decreased the same in heart tissue homogenate. Pretreatment with saponin of *Momordica cymbalaria* (25, 50,100 mg/kg) significantly ($p < 0.001$) reduced the levels of perfusate CK-MB and LDH and significantly ($p < 0.001$) increased tissue CK-MB and LDH activity dose dependently. 25mg/kg was found to be more effective, whereas 175mg/kg was not effective [28].

When myocardial cells, containing CK-MB and LDH, are damaged or destroyed due to deficient oxygen supply or glucose, the cell membrane becomes permeable or may rupture, which results in the leakage of enzymes. This accounts for the decreased activities of these enzymes in heart and increased activities of these enzymes in perfusate of rats with myocardial ischemia [22,23]. Among the four doses of saponins of *Momordica cymbalaria* only 25mg/kg dose was found to be more effective as compared to the individual treatments.

The increase in functional parameters like developed tension and heart rate at the end of 15 min reperfusion is an indication of good recovery from global

ischemia [18]. Similar observation made earlier by other studies, using similar models [29].

In the present study pretreatment with SMC showed significant recovery of developed tension and heart rate in dose-dependent manner. Maximum recovery was seen in group with SMC at dose of 25mg/kg whereas the dose of 175 mg/kg showed toxic effect, as indicated by poor recovery from ischemia reperfusion injury.

Pretreatment of rat cardiomyocyte H9c2 cell line with SMC at the dose of 20µg/ml was more effective in protecting them from ischemic injury. At dose of 30 and 50µg/ml it showed a toxic effect to cardiomyocyte H9c2 cell line. These findings are consistent with the ex-vivo data, that Saponins of *Momordica cymbalaria* are effective on ischemia reperfusion injury at lower doses and have toxic effect on cardiac cells at higher doses. Kumphune *et al* (2012) reported the cardioprotective of *Aquilaria Crassna* using similar model [30].

CONCLUSION

Ischemia reperfusion model demonstrated the efficacy of saponins of *Momordica cymbalaria* (25mg/kg) in protecting the heart against ischemia reperfusion injury whereas, higher dose were cardiotoxic. The antioxidant property may be responsible for protecting the heart against global ischemia.

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