



## THE POTENTIAL OF INTERACTION OF METHANOLIC EXTRACT OF *AEGLE MARMELLOS* LEAF EXTRACT WITH HYDROCHLOROTHIAZIDE AGAINST ISOPROTERENOL INDUCED MYOCARDIAL DAMAGE IN RATS

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### ABSTRACT

The present study was designed to evaluate the potential interaction of methanolic extract of *Aegle marmelos* leaf extract (AMLE) with hydrochlorothiazide (HCTZ) against isoproterenol (ISO) induced myocardial damage in rats. Rats of either sex were administered hydrochlorothiazide (HCTZ, 10 mg/kg) for one week, AMLE (100 and 500 mg/kg) for three weeks, AMLE (100 and 500 mg/kg) for two week followed by hydrochlorothiazide (HCTZ, 10 mg/kg) for one week in their respective groups orally. Subsequently myocardium injury was induced by subcutaneous administration of isoproterenol (150 mg/kg) for two consecutive days. The influence of prophylactic treatment was analysed by quantification of biomarkers and antioxidants, electrocardiographic parameters and histopathological studies. The Biomarkers (LDH and CK-MB) activities were fallen in serum and raised in heart tissue where as antioxidants (SOD, Catalase) activities reduced in heart tissue of animals treated with low and high doses of AMLE; HCTZ incorporated low and high dose of AMLE as well as HCTZ compared to ISO control. High dose of AMLE+HCTZ shows the best cardioprotection and might be the best potential interactive group considering the biomarkers and antioxidant activity. Electrocardiographic parameters like heart rate, QRS duration, QT segment, RR interval, PR interval decreased significantly in the animals pretreated with low and high doses of AMLE; HCTZ incorporated low and high dose of AMLE as well as HCTZ compared to ISO control. HCTZ incorporated high and low dose of AMLE was documented as the best potential interactive groups.

**Keywords:** *Aeglemarmelos*; Antioxidants; Biomarkers; electrocardiographic parameters; Cardioprotection; Isoproterenol.

### INTRODUCTION

During the latter part of this century the practice of herbalism has become mainstream throughout the world. While such products had been used with apparent safety in traditional societies for many centuries, their introduction into western countries, where they are being combined with pharmaceutical agents, possess the possibility of potential interaction which may mimic, magnify or oppose the pharmacological effects between the two groups of substances. The possibility of herb–drug interactions is theoretically higher than drug–drug interactions, could be because synthetic drugs usually contain single chemical entities<sup>1,2</sup>.

The management of hypertension in patients with ischemic heart diseases with Diuretics, in particular hydrochlorothiazide (HCTZ) as a monotherapy demands a significant medical attention. Thiazides affect the renal tubular mechanisms of electrolyte reabsorption, modulates excretion of sodium and chloride in approximately equivalent amounts. Indirectly, the diuretic action of hydrochlorothiazide reduces plasma volume, with consequent increase in urinary potassium loss, plasma renin activity, aldosterone secretion and decrease in serum potassium<sup>3,4</sup>. With mild-to-moderate hypokalemia patients with cardiac ischemia, heart failure or left ventricular hypertrophy, may magnify the chances of cardiac arrhythmias<sup>5-7</sup>. The combination of HCTZ with ACE-I, aldosterone antagonist or angiotensin II type 1 receptor blocker is found to minimize the potassium loss characteristically induced by the thiazide component.

*Aegle marmelos* (Linn.) Correa, commonly known as bael, belongs to the family rutaceae, and is a cosmopolitan plant<sup>8</sup> distributed throughout tropical asia and africa<sup>9</sup>. The principle chemical constituents of leaves extract are tannins, skimmianin, essential oil (mainly caryophyllene, cineole, citral, citronellal, d-limonene and eugenol), sterols and triterpenoids, including lupeol, sitosterol, and amyirin, flavanoids and coumarins including aegeline, marmesin and umbelliferone<sup>10</sup>. The Bael leaves are bitter and used as a remedy for ophthalmia, ulcers, dropsy, cholera and beriberi associated with the weakness of heart<sup>11</sup>. A decoction of plant leaves and fruit is used in upper respiratory tract infections and heart ailments<sup>12</sup>. Leaves are also reported for hypoglycaemic effect<sup>13</sup>, antioxidant and anticarcinogenic properties<sup>14</sup> and can cause restoration of blood glucose and bodyweight to normal levels<sup>11</sup>. Our study previously reported the cardioprotective activity of bael leaves.

Till now there is no scientific evidence of combination therapy of *Aegle marmelos* leaves with HCTZ. Therefore the present study was designed to explore the possible interaction of *Aegle marmelos* leaf extract with HCTZ against myocardial damage induced by Isoproterenol (ISO) in rats.

### MATERIALS AND METHODS

#### Chemicals

Isoproterenol was purchased from Sigma-aldrich, U.S.A. LDH & CKMB Kits for enzyme estimation were purchased from Crest Biosystems, Coral clinical systems, Goa, India. Other chemicals used were obtained from SD fine



chemicals Ltd. (Mumbai, India). All chemicals used in the present study were of analytical grade.

### Experimental animals

Laboratory bred female Sprague-Dawley (SD) rats weighing 175-250 g were housed at  $25^{\circ} \pm 5^{\circ}\text{C}$  in a well-ventilated animal house under 12:12 h light dark cycle. Institutional Animal Ethics Committee approved the experimental protocol. The animals were maintained under standard conditions in an animal house as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

### Plant material

The shade dried leaves of *Aegle marmelos* were collected from the surroundings of Mangalore (India) and Regional Research Institute (Ay), Bangalore authenticated the leaves. The leaves were subjected cold maceration using methanol<sup>15</sup>. The extract was concentrated in water bath and stored in a desiccator until further use.

### Phytochemical estimations of the extract<sup>16,17</sup>

Methanolic extract of *Aegle marmelos* leaves (AMLE) was subjected to Qualitative analysis to investigate the presence of various phytochemical constituents like alkaloids, carbohydrates, glycosides, phytosterols, proteins, saponins, tannins and flavonoids

### Acute toxicity study

The acute oral toxicity study was performed according to the OPPTS guidelines (Office of Prevention, Pesticide and Toxic Substance) following the limit test procedure<sup>18</sup>. The animals were fasted over night prior to the experiment. Test dose of 2 g/kg and 5 g/kg were given orally to mice. Both doses were found to be safe. Hence  $1/10^{\text{th}}$  and  $1/50^{\text{th}}$  of the maximum safe dose corresponding to 500 and 100 mg/kg orally were selected as high and low doses respectively.

### Experimental protocol

The animals were divided into seven groups of eight each. Group I and Group II received saline for three weeks and termed as normal control and ISO control respectively; Group III was treated with standard Hydrochlorothiazide (HCTZ) 10 mg/kg, *p.o* for one week after two week of saline treatment; Group IV and Group V were administered AMLE 100 and 500 mg/kg orally respectively for three weeks; Group VI and Group VII were administered AMLE 100 and 500 mg/kg orally respectively for two weeks after that treated with standard Hydrochlorothiazide (HCTZ) 10 mg/kg, *p.o* for one week.

### Isoproterenol (ISO) induced myocardial necrosis in rats<sup>19</sup>

After the treatment of animals for three weeks from group II to VII according to the protocol, Isoproterenol (ISO) 150 mg/kg, *s.c* was administered for two consecutive days. Forty eight hour after the first dose of ISO administration the rats were sacrificed. Blood samples were collected by the retro orbital puncture

method. Serum was separated and biochemical markers LDH and CKMB were estimated. Heart tissue homogenate (HTH) was prepared in sucrose solution (0.25 M) and used for estimation of endogenous marker enzymes and biological antioxidants viz., superoxide dismutase (SOD)<sup>20</sup> catalase<sup>21</sup> and TBARS<sup>22,23</sup> activities.

### ECG Studies<sup>24</sup>

After anesthetizing the rat with a combination of ketamine hydrochloride (75mg/kg, *i.p*) and xylazine (8.0mg/kg, *i.p*), leads will be attached to the dermal layer of both the front paws and hind legs and recording will be made with the help of computerized ambulatory ECG system. Animal grouping and treatment is same as ISO model.

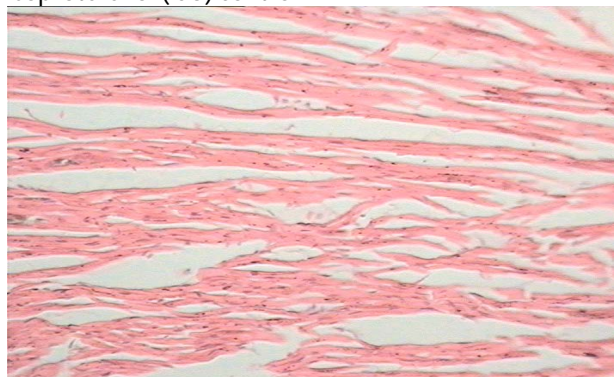
### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Statistical significance was assessed using One-way Analysis of variance (ANOVA) followed by Tukey-Karmer multiple comparison tests.  $P < 0.05$  was considered significant.

### Histological analysis

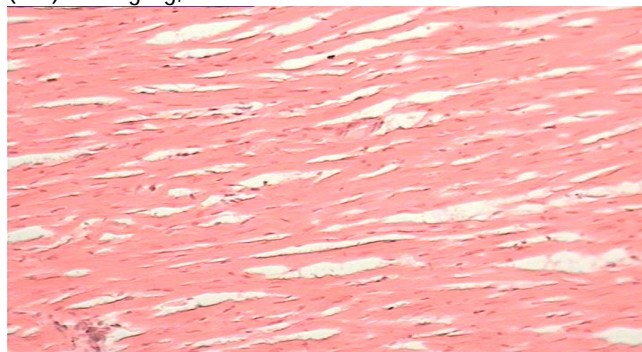
Heart sections were prepared, stained with hematoxylin and Eosin (H&E) and change in histology were observed (fig 1 to 3).

**Figure 1:** H&E ( $\times 400$ ) stained microscopic section of isoproterenol (ISO) control.



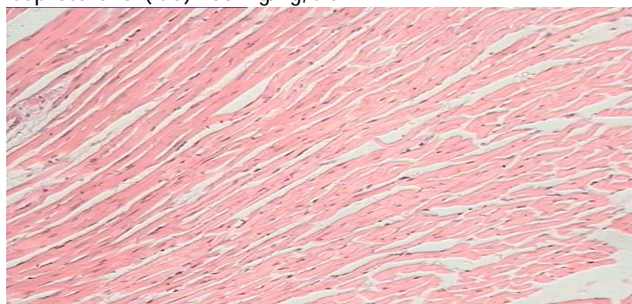
There is loss of cellular architecture, nuclear duplication and increased infiltration of leucocytes with prominent hyperchromasia.

**Figure 2:** H&E ( $\times 400$ ) stained microscopic section of heart tissue of animals pretreated with AMLE 500 mg/kg for 21 days orally and subsequently two doses of isoproterenol (ISO) 150 mg/kg, *s.c*.



Normal architecture restored with focal infiltration as evident from the figure.

**Figure 3:** H&E ( $\times 400$ ) stained microscopic section of heart tissue of animals pretreated with AMLE 500 mg/kg for 14 days orally and HCTZ 10mg/kg for 7 days and subsequently two doses of isoproterenol (ISO) 150 mg/kg, s.c.



Normal architecture restored with focal infiltration as evident from the figure.

## RESULTS

### Preliminary phytochemical investigation

The preliminary phytochemical investigation of the AMLE extract showed the presence of alkaloids, carbohydrates, flavonoids, cardiac glycosides, proteins, saponins, tannins and terpenoids. The percentage yield of AMLE was found to be 17%.

### Effect on hemodynamic parameters

#### Effect on LDH activity (Table 1)

The LDH activity of AMLE-100, AMLE-500, AMLE-100+HCTZ and AMLE-500+PRO was compared with normal (NC), ISO and HCTZ. ISO treated group shows a very significant ( $p < 0.01$ ) increase in the LDH activity in serum compare to normal group. In LDH activity in heart tissue homogenate ISO group shows extremely significant decrease ( $p < 0.001$ ) and HCTZ, AMLE-100 group shows a very significant ( $p < 0.01$ ) decrease while SANE-100+PRO group shows just significant ( $p > 0.05$ ) decrease compared to normal control. There was an extremely significant decrease ( $p < 0.001$ ) in LDH activity in AMLE-500+HCTZ, AMLE-100+HCTZ and a very significant ( $p < 0.01$ ) decrease in HCTZ, AMLE-100 and AMLE-500 treated groups compare with the ISO group. The LDH levels in IU/L in serum decrease were found to just significant ( $p > 0.05$ ) in

AMLE-500+HCTZ group compare with HCTZ treated group.

#### Effect on CK-MB activity (Table 1)

There was an extremely significant increase ( $p < 0.001$ ) in CK-MB activity in serum of ISO treated group when compared to the normal control (NC). Comparing all the treatment groups viz. HCTZ, AMLE-100, AMLE-500, AMLE-100+PRO and AMLE-500+PRO with the ISO group an extremely significant decrease ( $p < 0.001$ ) in CK-MB activity in serum was observed.

In heart tissue homogenate, there was an extremely significant ( $p < 0.001$ ) decrease in the CK-MB activity in the ISO, and AMLE-100 treated group. An extremely significant ( $p < 0.001$ ) increase in the level of CK-MB in IU/L, in the homogenate in AMLE-100, AMLE-500, AMLE-100+HCTZ, AMLE-500+HCTZ groups compared to ISO group was noticed and a very significant ( $p < 0.01$ ) increase in CK-MB in AMLE-500+HCTZ treated group compare with HCTZ treated group.

#### Effect on SOD and Catalase (Table 2)

The SOD and Catalase activity were estimated in the heart tissue homogenate. When ISO, HCTZ, AMLE-100, AMLE-500, AMLE-100+HCTZ and AMLE-500+ HCTZ was compared with the normal control group, the antioxidant activity was found to show an extremely significant ( $p < 0.001$ ) decrease. On comparing HCTZ, AMLE-500, AMLE-100+ HCTZ, AMLE-500+ HCTZ with ISO, an extremely significant ( $p < 0.001$ ) decrease was observed.

#### Effect on TBARS (Table 2)

TBARS levels increased extreme significantly ( $p < 0.001$ ) in ISO, and AMLE-100 when compared to control group. In AMLE-500, AMLE-100+HCTZ and AMLE-500+HCTZ shows an extremely significant decrease ( $p < 0.001$ ) was observed as compared to ISO group. There was an extremely significant decrease ( $p < 0.001$ ) was observed in AMLE-500+ HCTZ and there is a very significant ( $p < 0.01$ ) decrease in AMLE-100+ HCTZ pre treated group compare with the HCTZ group

**Table 1:** Effects on LDH and CKMB level in serum and heart tissue homogenate against Isoproterenol induced myocardial infarction

GROUPS	CKMB ACTIVITY		LDH ACTIVITY	
	Serum (unit/lit)	HTH (unit/gm)	Serum (unit/lit)	HTH (unit/gm)
NORMAL CONTROL	12 $\pm$ 1.4	211 $\pm$ 20	325 $\pm$ 16	17.2 $\pm$ 2.1
ISO CONTROL	93 $\pm$ 2.2 ***	35 $\pm$ 10 ***	644 $\pm$ 3.1 **	3.4 $\pm$ 0.7 ***
HCTZ	20 $\pm$ 5.2 ***	97 $\pm$ 1.6 ****	397 $\pm$ 40 **	8.2 $\pm$ 0.6 **
AMLE-100	20 $\pm$ 3.6 ***	138 $\pm$ 2.1 ****	417 $\pm$ 18 **	6.8 $\pm$ 1.2 **
AMLE-500	17 $\pm$ 0.5 ***	168 $\pm$ 4.8 ****	457 $\pm$ 43 *	8.2 $\pm$ 1.5 **
AMLE-100 + HCTZ	17 $\pm$ 1.2 ***	135 $\pm$ 12 ****	497 $\pm$ 59 *	9.5 $\pm$ 2.3 *
AMLE-500+ HCTZ	14 $\pm$ 3.3 ***	164 $\pm$ 7.1 ****	199 $\pm$ 14 ****	11.5 $\pm$ 1.3

Statistical analysis: one way ANOVA followed by Tukey multiple comparisons' test. All values are mean  $\pm$  SEM, n=6,  $p < 0.1$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  when compared to normal control group, \*  $p < 0.1$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to isoproterenol group and \*  $p < 0.1$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to Hydrochlorothiazide. AMLE-100 - *Aegle marmelos* leaf extract 100mg/kg (30 days treatment, *p.o.*), AMLE - 500 - *Aegle marmelos* leaf extract - 500mg/kg (30 days treatment, *p.o.*), AMLE -100+HCTZ- *Aegle marmelos* leaf extract 100mg/kg (30 days treatment, *p.o.*) and Hydrochlorothiazide 10mg/kg *p.o.* was added during the last week of treatment. AMLE -500+HCTZ- *Aegle marmelos* leaf extract 500mg/kg (30 days treatment, *p.o.*) and Hydrochlorothiazide 10mg/kg *p.o.* was added during the last week of treatment





**Table 2:** Effects on SOD, Catalase and TBARS in Heart tissue homogenate against Isoproterenol induced myocardial infarction.

Group	HEART TISSUE HOMOGENATE		
	SOD (unit/mgprot)	CATALASE (unit/mg prot)	TBARS (mmol/gm tissue)
NORMAL CONTROL	16.4±0.15	7.56±0.04	73.5±5.0
ISO CONTROL	5.1±0.07 ***	1.04±0.01***	25.1±2.2***
HCTZ	7.9±0.26 ****	3.73±0.28****	32.7±2.2***
AMLE-100	6.1±0.37 ***	1.62±0.02***	35.8±2.2***
AMLE-500	7.6±0.31 ****	3.28±0.04****	58.66±0.3***
AMLE-100 + HCTZ	7.7±0.05 ****	3.77±0.21****	63.33±1.4****
AMLE-500 + HCTZ	9.2±0.07 ****	4.36±0.33****	70.5±1.4****

Statistical analysis: one way ANOVA followed by Tukey multiple comparisons' test. All values are mean±SEM, n=6, p<0.1, \*\* p<0.01, \*\*\* p<0.001 when compared to normal control group, \*p<0.1, \*\*p<0.01, \*\*\*p<0.001 compared to isoproterenol group and \*p<0.1, \*\* p<0.01, \*\*\* p<0.001 compared to Hydrochlorothiazide. AMLE-100 - *Aegle marmelos* leaf extract 100mg/kg (30 days treatment, p.o), AMLE - 500 - *Aegle marmelos* leaf extract - 500mg/kg (30 days treatment, p.o), AMLE -100+HCTZ- *Aegle marmelos* leaf extract 100mg/kg (30 days treatment, p.o) and Hydrochlorothiazide 10mg/kg p.o was added during the last week of treatment. AMLE -500+HCTZ- *Aegle marmelos* leaf extract 500mg/kg (30 days treatment, p.o) and Hydrochlorothiazide 10mg/kg p.o was added during the last week of treatment.

### Effect on electrocardiographic parameters

The parameters like heart rate, QRS interval, QT interval RR interval and PR interval were interpreted from electrocardiograph of the animals. These four parameters of AMLE-100, AMLE-500, AMLE-100+HCTZ and AMLE-500+HCTZ were compared with normal and isoproterenol treated group.

**Heart rate (Table 3):** Electrocardiography revealed that there was an extremely significant (p<0.001) increase in the number of beats per minute in the animals treated with isoproterenol compared to the normal group. Comparing the other treatment groups with isoproterenol, it was found that there was an extremely significant (p<0.001) decrease in heart rate of AMLE-100, AMLE-500, AMLE-500+HCTZ treated group while a very significant (p<0.01) decrease in HCTZ, AMLE-100+HCTZ treatment groups.

**QRS interval (Figure 4):** There was an extremely significant (p<0.001) increase in the QRS interval in the groups treated with ISO as compared to normal control group. There was an extremely significant (p<0.001) decrease in the QRS interval in the groups treated with AMLE-100 + HCTZ and AMLE-500+HCTZ, very significantly (p<0.01) decrease in group AMLE-100, and significant (p>0.05) decrease in group treated with HCTZ and AMLE-100 as compared to isoproterenol treated group. However, for the groups treated with AMLE-100+HCTZ and AMLE-500+HCTZ there was a just significant (p>0.05) decline in QRS interval when compared to HCTZ.

**QT segment (Figure 4):** There was no significant change in the length of the QT segment when HCTZ, AMLE-100, AMLE-500, AMLE-100+HCTZ, AMLE-500+HCTZ were compared with normal control however isoproterenol treated group showed an extremely significant (p<0.001) increase in the QT segment when compared to normal control. All the treatment groups were when compared with isoproterenol, it was found that there was a very

significant (p<0.01) decrease in the length of QT segment for the HCTZ, AMLE-500, and AMLE-500+HCTZ treated groups and a just significant (p<0.01) decrease in AMLE-100+ HCTZ. The change in the length of the QT segment was found to be non significant when the treatment groups were compared with HCTZ group.

**P-R interval (Figure 5):** The PR interval was found to show an extremely significant (p<0.001) increase in ISO treated, and just significant (p>0.05) increase in HCTZ treated group when compared to the normal control. On comparing AMLE-100, AMLE-500, AMLE-100+HCTZ and AMLE-500+HCTZ with isoproterenol treated group, P-R interval showed an extremely significant (p<0.001) decrease while there was a significant decrease (p<0.01) in AMLE-100+HCTZ. However there was extremely significant (p<0.001) increase in the length or P-R interval when compared to HCTZ.

**RR interval (Figure 5):** The RR interval was found to show a very significant (p<0.01) increase in ISO treated and AMLE-100 treated group when compared to the normal control. RR interval showed a very significant (p<0.01) decrease when compared with AMLE-500 + HCTZ treated group.

## DISCUSSION

Large doses of Isoproterenol, which is a synthetic catecholamine and  $\beta$ -adrenoceptor agonist, is reported for myocardial damage<sup>25</sup>. Probable mechanisms which can worsen the condition including relative hypoxia, coronary microcirculatory disturbances and catecholamine oxidation products have been postulated, however intracellular calcium load is now the most established trigger cause of catecholamine cardiac toxicity<sup>26</sup>. Isoproterenol administration through subcutaneous route develops increase in calcium uptake and energy consumption leading to cell death<sup>27</sup>. Different biomarker enzymes elevation in serum is due to the



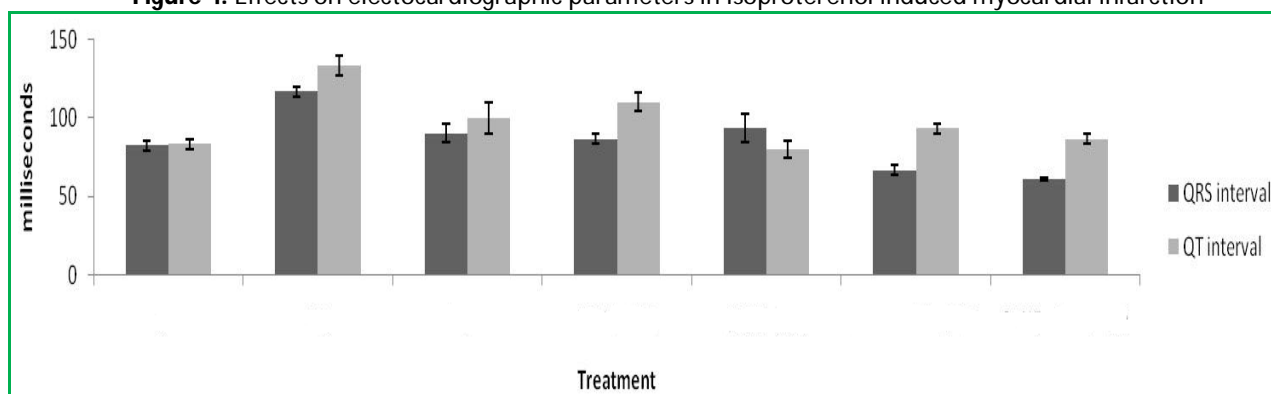
leakage of enzymes from the heart as a result of isoproterenol-induced necrosis<sup>28</sup>. Isoproterenol generates free radicals through its auto-oxidation. It has been suggested that the oxidative products of catecholamines produce changes in the myocardium by stimulating lipid

peroxidation and responsible for irreversible damage to the myocardial membrane. This alters membrane permeability, leading to the loss of function and integrity of myocardial membranes<sup>29</sup>.

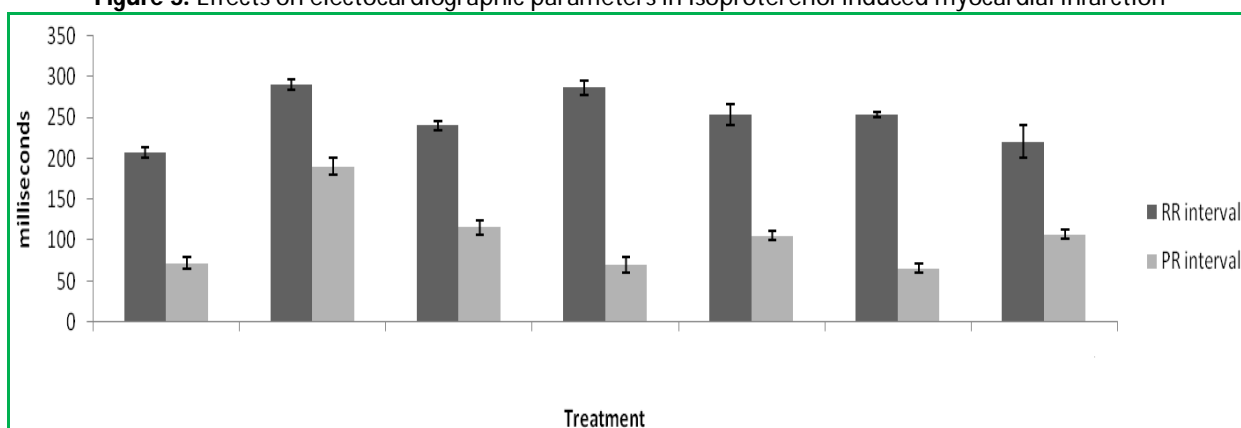
**Table 3:** Effects on electrocardiographic parameters in Isoproterenol induced myocardial infarction

Effect on electrocardiographic parameters and haemodynamic findings in rats			
Group	Heart rate (Beats/min)	Body weight (gm)	Hw/Bw
NORMAL CONTROL	180±20.00	213.33±3.33	1.9±0.05
ISO CONTROL	340±20.00***	193.3±1.60**	2.08±0.03
HCTZ	240±20.00**	210±5.00	2.4±0.01
AMLE-100	200±20.00***	206.6±3.30	2.9±0.06****
AMLE-500	200±20.00***	205.00±5.00	2.9±0.02****
AMLE-100 + HCTZ	240±10.00**	190±2.80**	2.5±0.08****
AMLE-500 + HCTZ	160±20.00***	181.6±6.00****	2.2±0.12*

**Figure 4:** Effects on electrocardiographic parameters in Isoproterenol induced myocardial infarction



**Figure 5:** Effects on electrocardiographic parameters in Isoproterenol induced myocardial infarction



As discussed above ISO induces free radical formation (TBARS) and reduction in antioxidant activities such as superoxide dismutase (SOD) and Catalase and also reduction in marker enzyme such as LDH and CKMB in HTH. Pretreatment of animals with low dose of AMLE (100 mg/kg, *p.o.*) produces no significant changes in antioxidant activities compare with the ISO group. High dose of AMLE (500 mg/kg, *p.o.*), AMLE-100+HCTZ, AMLE-500+HCTZ causes significant level of elevation in SOD

activities with simultaneous increase in CAT and TBARS activity, increased SOD activity may lead to intracellular accumulation of H<sub>2</sub>O<sub>2</sub> with detrimental effects<sup>30</sup>. Hence simultaneous rise in SOD and CAT scavenge free radicals more effectively. In this paper we are documenting that AMLE-500+HCTZ treated group is significantly beneficial in TBARS and SOD activity compare with the PRO treated group and the AMLE-100+HCTZ pretreated group

significantly effective in SOD, Catalase and TBRS activity compare with AMLE-100 group.

It has been known that biochemical markers are tissue specific and leak from the damaged tissue. Damage to the membrane induced by the ISO causes release of enzymes in the serum and deficiency of enzymes in HTH reflects the damage to the myocardium. The entire treated group AMLE-100, AMLE-500, AMLE-100+HCTZ, AMLE-500+HCTZ decreases serum CKMB and both the doses causes increase in CKMB activities in HTH. AMLE-100, AMLE-500, AMLE-100+HCTZ AMLE-500+HCTZ treated group demonstrated significant level of decrease in serum LDH activity.

Isoproterenol, which is a sympathomimetic drug causes sympathetic hyperactivity accompanied by vagal hypo activity which causes severe myocardial damage<sup>25</sup>. The electrocardiographic parameters which we studied revealed that ISO treated group causes the significant level of elevation in heart rate, QRS duration, QT segment, RR interval, PR interval. The high and low dose of AMLE, HCTZ incorporated high and low dose of AMLE shows significant level of decrease in all the haemodynamic parameter compare with the ISO treated group. Furthermore the pre treated groups AMLE high dose+HCTZ and AMLE low dose+HCTZ shows significant level of decrease in QRS duration and PR interval compare with the PRO treated group.

From our study it may be concluded that the high dose of AMLE (500 mg/kg), low dose of AMLE (100 mg/kg), high dose of AMLE (500 mg/kg)+HCTZ, low dose of AMLE (100 mg/kg)+HCTZ possess good cardio protective activity against Isoproterenol (ISO) induced myocardial necrosis in rats and HCTZ incorporated both high and low dose is found to be effective compare with the single administration of high and low dose. AMLE-500+HCTZ is found to effective compare with the HCTZ treated group so the combination of high dose of AMLE + HCTZ is established as the most effective interactive group.

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