1. INTRODUCTION

Myocardial ischemia-reperfusion (IR) injury aggravates myocardial damage and chiefly oxidative stress has been shown to play an important role. Oxidative stress plays a major role in the development of IR injury through the generation of oxygen free radicals (OFR) and also depletion of endogenous antioxidants e.g. superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT).

Many plants based sources have been found to be a good source of phytochemical antioxidants and shows cardiac health improving properties by scavenging free radicals. Oxidative stress have been found during myocardial ischemia-reperfusion while antioxidants guard the heart from ischemia-reperfusion injury. Therefore, for dealing with the management of heart disease antioxidants usage, particularly natural antioxidants have aroused huge interest. 3Tea (Camellia sinensis, family Theaceae) is the most widely drunk beverage throughout the world, after water. Black tea contains more amounts of polyphenols in comparison to other tea. Tea consumption has been associated with various health benefits which are antioxidative, antimutagenic, anticarcinogenic, antibacterial, anti-diabetic, anti-inflammatory, cardio-protective properties chiefly because of tea flavanols. The present research was undertaken to compare the cardioprotective potential of black tea aqueous extract and catechin fraction of black tea by oxidative stress induced myocardial IR injury.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Black tea was collected from the source (Brooke bond, Darjeeling). All the reagents and chemicals were of analytical grade and purchased from Sigma Chemicals, USA; S.D. Fine Chemical Ltd., Mumbai, India.

2.2 Preparation of crude aqueous extract of black tea

Tea sample (500 g) was infused with freshly boiled distilled water for
10 min in a flask. The infusion was filtered through a plug of cotton wool and rapidly cooled under tap water. The extract was freeze-dried and weighed to calculate the yield.  

2.3 Preparation of Catechins enriched fraction from black tea
Black tea (500 g) was extracted with 80% ethanol and the obtained ethanolic extract further fractionalted with chloroform to obtain aqueous decaffeinated extract. The aqueous decaffeinated extract was fractionated with ethyl acetate to obtain catechin enriched fraction. The catechin enriched fraction was lyophilized and purified to obtain brownish red catechin. 5,9 Method of fractionation of catechin from black tea present in Figure I.

Figure 1. Extraction of catechin fraction from black tea.

2.4 Preliminary phytochemical screening
Qualitative Phytochemical screening of the crude aqueous extract of black tea and ethyl acetate extract (catechin fraction) was performed as per the standard procedures described in order to identify the chemical constituents. 10

2.5 Experimental animals
Male Wistar albino rats 200–250 g body weight, were obtained from the Institute’s Animal House and were kept in a well-ventilated animal house with 12 hours light and dark cycle, maintained in compliance with the Guidelines for Animal experimentation. The research protocol was approved by the Institute Animal Ethical Committee (HPI/08/60/IAEC/0050).

2.6 Experimental methods
Rats were divided into four groups (n = 6) and fed either with the crude aqueous extract of black tea in three doses 500mg/kg (BT1), 1000mg/kg (BT2), 1500mg/kg (BT3) (dose has been selected based on previous study) or vehicle (normal saline) ad libitum. Similarly for catechins enriched fraction of black tea rats were divided into four groups (n=6) and were given catechin fractions in three doses 10mg/kg (CT1), 20mg/kg (CT2), 30mg/kg (CT3) (dose has been selected based on previous study) or vehicle (normal saline) daily once a day for 30 days. After 48 hours of the last dose rats were randomly selected, heparinised 375/200gms i. p, and half an hour later rats were anesthetized with anesthetic ether as per treatment protocol.

2.7 Baseline changes in the myocardial endogenous antioxidant enzyme
The heparinized, anesthetized rats were sacrificed and hearts were harvested and stored in liquid nitrogen for the estimation of endogenous antioxidants.

The group studied for crude extract of black tea
Group BL: Vehicle (saline) treated rats.
Group BT1 BL: Rats treated with 500mg/kg of crude black tea extract in saline.
Group BT2 BL: Rats treated with 1000mg/kg of crude black tea extract in saline.
Group BT3 BL: Rats treated with 1500mg/kg of crude black tea extract in saline.

The group studied for the catechin fraction of black tea
Group BL: Vehicle (water) treated rats.
Group CT1 BL: Rats treated with 10mg/kg of catechin fraction in water.
Group CT2 BL: Rats treated with 20mg/kg of catechin fraction in water.
Group CT3 BL: Rats treated with 30mg/kg of catechin fraction in water.

2.8 Production of in-vitro myocardial ischemic-reperfusion injury
Rats were anaesthetized with ether, hearts rapidly excised and washed in ice – cold saline and then perfused by the non-recirculating Langendorff’s technique (AD instruments, Australia), using a modified Kreb’s Henseleits solution pH 7.4 containing (in mM): glucose 11.1; NaCl 118.5; NaHCO3 25; KCl 2.8; KHPO4 1.2; CaCl2 1.2; MgSO4 0.6, with a pH of 7.4. The buffer solution equilibrated with 95% O2 + 5% CO2 was delivered to the aortic cannula at 37°C less than 65 mm Hg pressure. An initial 5 min equilibration period was followed by 9 min. of zero flow (ischemia) and thereafter 12 min of re-flow (reperfusion) in order to produce IR injury. 11-13

Six rats from each group were subjected to oxidant stress arising out of IR injury of heart in this protocol and the following groups were studied.

For crude aqueous extract of black tea
Group BL: Vehicle (saline) treated rats.
Group C: vehicle- treated rat hearts subjected to 26 min. flow.
Group I: Vehicle-treated rat hearts subjected to 5 min perfusion + 9 min ischemia.
Group IR: vehicle- treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.
Group BT1 IR: 500mg/kg treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.
Group BT2 IR: 1000mg/kg treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.
Myocardial reduced glutathione GSH was measured at 532 nm. Data are expressed as nmol of TBARS/gm wet weight.

For the catechin enriched extract of black tea
Group BL: Vehicle (saline) treated rats
Group C: vehicle- treated rat hearts subjected to 26 min. flow
Group I: Vehicle-treated rat hearts subjected to 5 min perfusion + 9 min ischemia
Group IR: vehicle- treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.
Group CT1 IR: 10mg/kg treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.
Group CT2 IR: 20mg/kg treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.
Group CT3 IR: 30mg/kg treated rat hearts subjected to 5 min flow + 9 min. no-flow + 12 min. reflow.

At the end of each experiment, cardiac tissue samples were stored in a frozen condition in nitrogen container for biochemical estimations.

2.9 Biochemical parameters
In the heart homogenate the following biochemical parameters were studied:

Myocardial thiobarbituric acid reactive substances TBARS
Myocardial thiobarbituric acid reactive substances (TBARS) were determined by a modified version of the method described by Okhawa et al. (1979). First homogenization of the rat hearts was done in 10% trichloroacetic acid (TCA) at 4°C. Then in a test tube homogenate (0.2ml) was pipetted out, followed by the addition of 0.2ml of 8.1% sodium dodecyl sulfate (SDS), 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% thiobarbituric acid (TBA). Boiling of the tubes was done for 60min at 95°C and then was cooled on ice. In the tubes double distilled water (1.0ml) and 5.0ml of n-butanol–pyridine (15:1 v/v) mixture were added and centrifuged at 4000 x g for 10min. The absorbance of the organic layer was measured at 560 nm. Data are expressed as units per mg protein.

Myocardial reduced glutathione GSH
Myocardial reduced glutathione (GSH) was estimated by the method of Ellman et al. (1959). The rat hearts were homogenized with 10% TCA and centrifuged at 3000 x g for 10 min. The reaction mixture constituted of 0.1mL of supernatant, 2.0ml of 0.3M phosphate buffer (pH- 8.4), 0.4 ml of double-distilled water and 0.5ml of 5, 5 dithio bis 2-nitrobenzoic acid (DTNB). Incubation of reaction mixture was done for 10 minutes and then absorbance was measured at 412 nm. Data are expressed as mole per gram wet weight.

Myocardial SOD
Superoxide dismutase (SOD) levels in the hearts were determined by the method of McCord & Firdovich method (1969) and modified by Kakkar et al., 1984. The sample (0.6ml) was added to sodium pyrophosphate buffer (pH-8.3), followed by the addition of 0.1ml of 186 M phenazine methosulfate, 0.3ml of 300mM nitroblue tetrazolium and 0.2ml of 780M NADH. For 90 seconds the reaction mixture was incubated at 30°C and then the reaction was stopped by adding 1.0ml of acetic acid, further 4.0ml of n-butanol was added and then the reaction mixture was centrifuged at 3000 x g for 10min. The absorbance of the organic layer was measured at 340 nm. Data are expressed as units per mg protein.

Myocardial catalase
Catalase was estimated by the method described by Aebi and Bergmeyer (1974). The sample was added to a 3.0-ml cuvette containing 1.95ml of 50mM phosphate buffer (pH 7.0). Then after adding 1.0ml of 30mM hydrogen peroxide, changes in absorbance were followed for 30 s at 240nm at an interval of 15 s. Catalase levels are expressed as units per mg protein.

3. RESULTS

3.1 Preliminary phytochemical screening
A dark brownish red shiny crystal like residue of 2.0% w/w yield was obtained for catechin fraction. Preliminary phytochemical screening for the black tea crude extract and catechin fraction was performed. Crude extract showed positive test for alkaloids, glycosides, phenolic compounds, tannins whereas catechin fraction showed the presence of phenolic compounds and flavonoids group, catechins, tannins.

3.2 Biochemical parameters
The results of biochemical parameters are shown in Table 1 and 2.

3.2.1 Myocardial TBARS
In a crude black tea extract treated groups, a significant increase in basal levels of myocardial TBARS on administration of black tea, at a dose of 500 mg/kg (BT1 BL) was observed. While in the, 1000mg/kg and 1500 mg/kg groups, the baseline TBARS was not significantly altered. In in vitro ischemic reperfusion injury of the treated groups, there was a significant decrease in TBARS in BT1 IR (500mg/kg) and BT2 IR (1000mg/kg) groups.

Administration of catechin fraction shows, no change in basal levels of myocardial TBARS was seen. In in vitro IR injury of the catechins fraction pre-treated groups there was a significant decrease in myocardial TBARS levels with all the three doses.

3.2.2 Myocardial GSH
In group treated with crude black tea extract a significant rise in basal levels of GSH was observed in all groups. In in vitro ischemic reperfusion injury of the treated groups a significant rise in levels of GSH was observed in all groups.
**Table 1. Observation table for the Myocardial TBARS, GSH, SOD and catalase in crude aqueous extract (black tea) pre-treated groups.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS nmole/g wet wt</th>
<th>Parameters</th>
<th>GSH µg/g wet wt</th>
<th>SOD I.U/mg protein</th>
<th>Catalase I.U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>45.94 ± 1.57</td>
<td></td>
<td>327.08 ± 7.4</td>
<td>3.8 ± 0.1</td>
<td>45.0 ± 7.0</td>
</tr>
<tr>
<td>C</td>
<td>49.10 ± 1.9</td>
<td></td>
<td>337.65 ± 4.6</td>
<td>3.2 ± 0.1</td>
<td>44.5 ± 2.9</td>
</tr>
<tr>
<td>I</td>
<td>48.8 ± 1.31</td>
<td></td>
<td>323.55 ± 6.06</td>
<td>1.8 ± 0.3’</td>
<td>25.1 ± 6.1’</td>
</tr>
<tr>
<td>IR</td>
<td>59.73 ± 1.62’’’’’</td>
<td></td>
<td>279.41 ± 6.05’</td>
<td>1.7 ± 0.2’</td>
<td>33.4 ± 0.9’</td>
</tr>
<tr>
<td>BT1BL</td>
<td>64.64 ± 7.88</td>
<td></td>
<td>450.82 ± 24.37’</td>
<td>6.5± 0.6’</td>
<td>35.6 ± 0.18’</td>
</tr>
<tr>
<td>BT1IR</td>
<td>44.18 ± 6.41</td>
<td></td>
<td>363.64 ± 16.54’</td>
<td>8.2 ± 0.5’</td>
<td>32.4 ± 0.43’</td>
</tr>
<tr>
<td>BT2BL</td>
<td>95.55 ± 16.09’’’</td>
<td></td>
<td>571.28 ± 80.05</td>
<td>18.8 ± 0.4’</td>
<td>94.6 ± 0.13</td>
</tr>
<tr>
<td>BT2IR</td>
<td>74.28 ± 14.06’’’</td>
<td></td>
<td>544.77 ± 123.05’</td>
<td>15.9 ± 0.1’</td>
<td>82.4± 8.1</td>
</tr>
<tr>
<td>BT3BL</td>
<td>61.52 ± 5.17’’</td>
<td></td>
<td>406.77 ± 38.26</td>
<td>15.4 ± 0.7’</td>
<td>69.6 ± 6.6’</td>
</tr>
<tr>
<td>BT3IR</td>
<td>71.51 ± 4.63’’</td>
<td></td>
<td>401.89 ± 36.35’</td>
<td>13.7 ± 0.1’</td>
<td>70.8 ± 4.2’</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM (n=6) ***p < 0.001 vs BL & I **p < 0.01 vs IR & BL *p < 0.05 vs IR & BL (One way ANOVA)

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**Table 2 Observation table for the Myocardial TBARS, GSH, SOD and catalase in catechins fraction (black tea) pre-treated groups.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS nmole/g wet wt</th>
<th>Parameters</th>
<th>GSH µg/g wet wt</th>
<th>SOD I.U/mg protein</th>
<th>Catalase I.U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>45.94 ± 1.57</td>
<td></td>
<td>327.08 ± 7.4</td>
<td>3.4 ± 0.1</td>
<td>43.0 ± 7.0</td>
</tr>
<tr>
<td>C</td>
<td>49.10 ± 1.9</td>
<td></td>
<td>337.65 ± 4.6</td>
<td>3.2 ± 0.1</td>
<td>44.5 ± 2.9</td>
</tr>
<tr>
<td>I</td>
<td>48.8 ± 1.31</td>
<td></td>
<td>323.55 ± 6.06</td>
<td>1.8 ± 0.3’</td>
<td>25.1 ± 6.1’</td>
</tr>
<tr>
<td>IR</td>
<td>59.73 ± 1.62’’’’’</td>
<td></td>
<td>279.41 ± 6.05’</td>
<td>1.8 ± 0.3’</td>
<td>33.4 ± 0.9’</td>
</tr>
<tr>
<td>T1BL</td>
<td>58.4 ± 3.9’’</td>
<td></td>
<td>849.6 ± 18.0’</td>
<td>11.7 ± 1.0’</td>
<td>62.4 ± 3.3’</td>
</tr>
<tr>
<td>T1IR</td>
<td>52.1 ± 3.8’’</td>
<td></td>
<td>436.2 ± 16.5’</td>
<td>8.4 ± 0.9’</td>
<td>43.5 ± 3.3’</td>
</tr>
<tr>
<td>T2BL</td>
<td>48.3 ± 5.7</td>
<td></td>
<td>1059.5 ± 18.1’</td>
<td>18.2 ± 1.1’</td>
<td>47.0 ± 1.8</td>
</tr>
<tr>
<td>T2IR</td>
<td>53.8 ± 3.4’’</td>
<td></td>
<td>519.4 ± 12.1’</td>
<td>9.0 ± 0.7’</td>
<td>40.3 ± 5.9</td>
</tr>
<tr>
<td>T3BL</td>
<td>54.2 ± 3.3</td>
<td></td>
<td>636.6 ± 8.7’</td>
<td>10.3 ± 0.6’</td>
<td>66.3 ± 6.3’</td>
</tr>
<tr>
<td>T3IR</td>
<td>55.2 ± 4.6</td>
<td></td>
<td>463.0 ± 3.9’</td>
<td>8.2 ± 0.7’</td>
<td>50.8 ± 4.8’</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ±SEM (n=6) *p < 0.001 vs BL ?p < 0.001 vs IR (One way ANOVA)

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Administration of catechin fraction shows a significant increase in the levels of antioxidants GSH in T1 BL and T2 BL groups was seen. In both CT2IR and CT3IR groups, a significant rise in the levels of GSH was observed, and they showed a better recovery profile than the T1IR groups subjected to in vitro IR injury.

### 3.2.3 Myocardial SOD

In crude black tea extract treated group a significant rise in basal levels of SOD was observed in all groups. In group BT2 IR, there was no significant increase in SOD levels.

Administration of catechin fraction shows significant increase in the levels of antioxidants SOD in T1 BL and T2 BL groups. A significant rise in SOD level was observed in both CT2IR and CT3IR groups.

### 3.2.4 Myocardial Catalase

Crude black tea extract: A significant rise in basal levels of catalase was observed in all groups. In group BT2 IR, there was no significant increase catalase level.

Administration of catechin fraction shows a significant increase in the levels of catalase was observed only in the T2 BL group. A significant rise in SOD level was observed in both CT2IR and CT3IR groups.

### 4. DISCUSSION

In our study, the antioxidant activity of black tea in in vitro model of myocardial ischemic-reperfusion injury was evaluated. Interestingly, there was a significant increase in basal levels of myocardial TBARS on administration of black tea, at a dose of 500 mg/kg (BT1 BL). It is generally accepted that oxygen free radicals are key mediators of myocardial oxidative stress-induced injury. While in the, 1000mg/kg and 1500 mg/kg groups, the baseline TBARS was not significantly altered. The reason why a lower dose of 500 mg/kg caused a significant TBARS accumulation is not forthcoming from the present study. Also, significant rise in baseline GSH, SOD and Catalase was noted in black tea treated groups. These are clear indications that oral administration of black tea can augment the endogenous cardiac antioxidant defense system, along with an apparent lipid peroxidation, occurring during this phase. The exact mechanism of this effect is not clear and needs further investigation.

In in vitro ischemic reperfusion injury of the treated groups, a significant decrease in TBARS in BT1 IR (500mg/kg) and BT2 IR (1000mg/kg) groups was seen. A significant rise in levels of GSH was observed...
in all groups. In group BT2 IR, there was no significant increase in SOD and catalase levels. It has been proven that if cellular SOD increases without an associated increase in catalase cause more harm by favouring the production of $H_2O_2$. In the present study, treatment with catechin fraction showed that there was no change in basal levels of myocardial TBARS, while there was a significant increase in the levels of antioxidants (GSH and SOD) in CT1 BL and CT2 BL groups. A significant increase in the levels of catalase was observed only in the CT2 BL group. Similarly, catechin pre-treated rats showed no significant changes in the specific activities of antioxidant enzymes. The increased activities of endogenous antioxidant enzyme levels should protect the myocardium when subjected to oxidative stress. In the present study, reperfusion of the ischemic myocardium was reported to cause depression in the biochemical recovery (antioxidant status), as reported earlier. The widely reported oxidant stress induced by IR injury was evidenced by enhanced lipid peroxidation and deterioration of endogenous antioxidant status (SOD, catalase and glutathione).

In in vitro IR injury of the catechins fraction pre-treated groups there was a significant decrease in myocardial TBARS levels with all the three doses. In both CT2IR and CT3IR groups, a significant rise in the levels of GSH, SOD and catalase were observed, and they showed a better recovery profile than the CT1IR group subjected to in vitro IR injury. This indicates that this dosage withstands the oxidative stress associated with in vitro IR injury. On the other hand, in CT1IR group, the GSH and SOD show significant protection but not the catalase. Previous studies have shown that changes in tissue antioxidant enzyme activities in response to internal or external stimuli, do not always parallel each other. The exact stimulus for the altered activity of the catalase is not known; however during oxidation stress radical formation is increased which they may act as a signal.

5. CONCLUSION

The results from pharmacological activities demonstrate that pre-treatment with the Black tea crude extract at the dose of both 500 & 1000 mg/kg and administration of catechin at a dose of 20 mg/kg has a positive, dose-dependent variable effect on the antioxidant status of the heart. Thus catechins fraction from black tea can provide post-ischemic myocardial preservation through endogenous antioxidant potential mechanism. The CT2IR treated group offered better Cardioprotection when subjected to IR injury. There was also no certain pattern of change in the level of TBARS and the endogenous antioxidants. Therefore these varying actions appear to be enzyme-specific. Further study is required to explore mechanisms causing these changes in antioxidant enzyme expression due to oxidant stress and drug administration also, it will be more fruitful if isolation of the compounds is done which are responsible for the particular enzyme action.

Declaration of Conflicting Interests

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REFERENCES


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