Cardioprotective role of methanolic extract of bark of *Terminalia arjuna* against *in-vitro* model of myocardial ischemic-reperfusion injury

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Abstract

**Background:** The present study was designed to investigate the cardio protective role of chronic oral administration of methanolic extract of *Terminalia arjuna* bark in *in-vitro* myocardial ischemic reperfusion injury and the induction of HSP72. **Materials and Methods:** Rats, divided into three groups, and were administered with the methanolic extract of the bark powder of *Terminalia arjuna* (TAME) by oral gavage (6.75 and 9.75 mg/kg; 6 days/week for 12 weeks). Control and TAME extract treated rat hearts were subjected to *in-vitro* global ischemic reperfusion injury (5 min perfusion, 9 min noflow and 12 min reperfusion). **Results:** Oxidative stress in MIRI was evidenced by, raised levels of myocardial TBARS and depletion of endogenous myocardial antioxidants GSH, SOD and catalase. Western blot analysis showed a single band corresponding to 72 kDa in homogenates of hearts from rat treated with both the doses. In the methanolic extract of the bark powder of *Terminalia arjuna* treatment groups, both the doses had better recovery of myocardial function, with significant reduction in TBARS, and rise in SOD, GSH, catalase were observed. **Conclusion:** The results of the present study suggest that the methanolic extract of the bark powder of *Terminalia arjuna* in rat induces myocardial HSP72 and augments myocardial endogenous antioxidants, without causing any cellular injury and offers better cardioprotection against oxidative stress associated with myocardial IR injury.

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Full Text
Terminalia arjuna (TA) is a popular Indian medicinal plant with bark being used as a cardiotonic agent from time immemorial. The bark has been found to contain several bioactive compounds including saponins and flavonoids. A number of experimental and clinical studies have been conducted to explore therapeutic potential of Terminalia arjuna in cardiovascular ailments, specially in patients of coronary heart disease. Many experimental studies have reported its antioxidant, anti-ischemic, antihypertensive, and anti-hypertrophic effects, thus indicating its therapeutic potential in cardiovascular diseases in humans. Several clinical studies have reported its efficacy mostly in patients with ischemic heart disease, hypertension, and heart failure.[1],[2],[3] The cardioprotective effect of TA has been well documented in experimental animal models in which TA shows its potential effect via induction of endogenous antioxidant system.[4],[5],[6],[7],[8],[9]

Cardiovascular diseases (CVD) have become known life threatening problem for the world. The risk factors and higher mortality from CVD has been proved from the data obtained in well developed countries of Western Europe, North America, and East Asia, as well as for the vast majority of developing countries and even the large urban centers of sub-Saharan Africa.[10] In Saudi Arabia 46% of mortality due to cardiovascular disease. CVD was estimated to account almost half of the deaths in Oman and Kuwait, 49% and 46%, respectively. The rate of CVD deaths was also high in Saudi Arabia, the UAE, Bahrain, and Qatar 42%, 38%, 32%, and 23%, respectively. Furthermore, the number of deaths resulting from ischemic heart disease and hypertensive heart disease in the Middle East and North Africa region was higher.[11]

The present study was designed to evaluate the cardioprotective effect of methanolic extract of bark of TA against in-vitro model of myocardial ischemic reperfusion injury and also to find out the role of endogenous antioxidant enzymes and heat shock protein in cardioprotection of TA.

**Materials and Methods**

**Animals**

Male albino Wistar rats (120-150 g body weight) were obtained from the Institute's Experimental Animal Facility and were kept at 25°C ± 5°C in a well-ventilated animal house under 12 hour light and dark cycle. The experimental procedure was carried out with the approval of Institutional Animal Ethical Committee with approval number IAEC/3651/9/14/008.

**Plant material**

The bark of TA was collected Madurai District, Tamil Nadu, India between November–December 2012. Authentication of the bark was carried out by the Department of Pharmacognosy, K.M. College of Pharmacy (vide voucher specimen number 53). The bark was dried under shade, pulverized by mechanical grinder, passed through a 40 mesh sieve and stored in a well closed air tight container for further use.

**Preparation of TAME**

About 1000 g of dry powder was extracted with methanol by hot continuous percolation using Soxhlet apparatus. The extraction was continued for 72 hours. Subsequently, the methanol extract was filtered and concentrated to a dry mass using vacuum distillation and evaporation. A dark brownish red shiny crystal like residue of 26 g was obtained. The yield was 4.3% w/w with respect to dry powdered material.

**Phytochemical analysis of TAME**

The methanolic extract of TA was subjected to preliminary phytochemical screening to identify its chemical constituents. The methods of analysis employed were those described in standard procedures.[12],[13]

**Experimental procedure**
Rats were divided into three groups and were administered the methanolic extract of TA bark 6.75 mg/kg BW p.o., and, 9.75 mg/kg BW p.o., or vehicle by gavage once a day for 90 days, at a fixed time every day. The rats had free access to standard rat chow (Hindustan Lever Ltd., India) and water ad libitum. There was no significant body weight difference in the treated rats when compared to the rats in the control group at both the beginning and end of the study period. The treated rats were not abnormally resistant to drug administration. The treatment schedule did not cause any change in food and water intake pattern. At the end of this period the rats were heparinised (375 units/200 g i.p.\[14]\) and after half an hour, they were anaesthetized and then subjected to the following protocols.

Estimation of antioxidant enzymes

Ten rats from each group were anaesthetized with anaesthetic ether and sacrificed and the hearts harvested to determine basal levels of endogenous antioxidant profile. The groups studied were:

Group BL: Vehicle treated rats
Group T1 BL: Rats treated with 6.75 mg/kg of methanol extract of TAME
Group T2 BL: Rats treated with 9.75 mg/kg of methanol extract of TAME.

At the end of each experiment cardiac tissue samples were stored in liquid nitrogen for biochemical estimations.

Production of in-vitro ischemic-reperfusion injury

Rats were anaesthetized with ether, the hearts were rapidly excised and washed in ice-cold saline and then perfused by the non-recirculating Langendorff's apparatus (AD Instruments, Australia), by using a modified Kreb's-Henseleits solution containing (in mM): Glucose 11.1; NaCl 118.5; NaHCO3 25; KCl 2.8; KH2 PO4 1.2; CaCl2 1.2; MgSO4 0.6, with a pH of 7.4. The buffer solution which was equilibrated with 95% O2 + 5% CO2 was delivered to the aortic cannula at 37°C under 65 mm Hg pressure. An initial 5 min equilibration period was followed by 9 min. of zero-flow (ischemia) and thereafter 12 min of reflow (reperfusion).\[4],[10]\) Ten rats from each group were studied for oxidant stress arising out of ischemic reperfusion injury of heart in this protocol.

The following groups were studied:

Group C: From vehicle-treated rat heart subjected to 5 min perfusion, 9 min ischemia and 12 min reperfusion
Group IR: Vehicle-treated rat heart subjected to 5 min perfusion, 9 min ischemia and 12 min reperfusion
Group T1 IR: 6.75 mg/kg of methanolic extract of TAME treated rat heart subjected to 5 min perfusion, 9 min ischemia and 12 min reperfusion
Group T2 IR: 9.75 mg/kg methanolic extract of TAME treated rat heart subjected to 5 min perfusion, 9 min ischemia and 12 min reperfusion.

At the end of each experiment cardiac tissue samples were stored in liquid nitrogen for biochemical estimations.

Biochemical parameters

Myocardial thiobarbituric acid reactive substance (TBARS) was measured by the method of Okhawa et al.,\[15]\) reduced glutathione (GSH) was measured by the method of Ellman,\[16]\) superoxide dismutase (SOD) was measured by the method of Kakkar et al.,\[17]\) catalase (CAT) was measured by the method of Aebi,\[18]\)

Isolation and characterization of Hsp72

Whole-body hyperthermia

Wistar albino male rats were anesthetized with (pentobarbital 35 mg/kg i.p.) and an intravenous catheter was inserted into the tail vein for fluid administration. After a rectal thermometer was inserted, animals were placed on a warming blanket and under heating lamps and gradually heated to 42°C for 20 minutes. They were then removed from the heating area to a cool surface and allowed to recover for 48 hours. The animals received normal saline (10-12 ml, iv) during the heating and recovery periods. Control animals were anesthetized and left at room temperature. The majority of the animals awoke, ate, drank and behaved normally. Any animal that appeared morbid after the recovery period was quickly euthanized.
To confirm that hyperthermia induces a significant increase in Hsp72, right ventricular and left ventricular (LV) samples were taken from selected animals after the hyperthermia/48-hour recovery protocol (n = 5) and from the control group (n = 5) and analyzed for Hsp72 as described below.

**Western blot protein analysis of Hsp72**

Rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and the hearts were quickly harvested and rinsed in cold saline. After removal of atrial and other connective tissues, the ventricular samples were immediately frozen in liquid nitrogen and stored at −70°C for the measurement of Hsp72 by Western blot protein analysis. The heart tissues were homogenized in 0.1 mM phosphate buffer containing 5% SDS, 1% mercaptoethanol and 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) for 4s using a polytron tissue homogenizer at 4°C. The homogenate was then strained through a 27 gauge needle followed by centrifugation at 14,000 × g for 10 min. The supernatant, representing protein lysate, was divided into small aliquots and stored at −70°C until use.

Protein concentration was measured using the BIORAD protein assay based on the Bradford dye binding procedure with bovine serum albumin as standard.[19] At the time of analysis, samples were thawed and volumes were pipetted to allow loading of approximately 100 µg of total protein per lane on a slab gel. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 1 mm thick, 12.5% acrylamide gels. After electrophoresis the proteins on the gel were transferred to nitrocellulose membranes by electroelution. Protein transfer was confirmed by employing pre-stained molecular weight markers (Biorad Laboratories). Following transfer, the membrane was blocked with non-fat dry milk, and the nitrocellulose membranes were incubated with a goat monoclonal antibody cross-reacting to the Hsp72 (Santa Cruz Biotechnology USA) at a dilution of 1:1000. The secondary antibody was conjugated with anti-goat IgG (Santa Cruz Biotechnology USA) used at 1:200 dilution and subsequently the membrane was incubated with avidin-biotin HRP complex signal was detected using DAB (Diaminobenzidine). The membrane was developed, and densitometry was performed to quantify the levels of Hsp72 (Biorad Gel Doc system, USA).

**Statistical analysis**

All values were expressed as mean ± SEM and n = 10 in each group for biochemical parameters and n = 5 in each group for western blot analysis. One way ANOVA was applied to test for significance of biochemical data of the different groups. Significance was set at P ≤ 0.05.

**Results**

**Base line changes**

The results obtained in the different groups of extract treated rats are presented in the [Table 1].

**Myocardial TBARS**

Myocardial baseline TBARS were significantly high (P ≤ 0.001) in T1BL and T2BL treated groups (200.8 ± 5.5 n mol/g wet wt. and 198.4 ± 2.8 n mol/g wet wt.) (P ≤ 0.001) respectively in comparison to BL group (47.2 ± 1.5 n mol/g wet wt.).

**Myocardial GSH**

Myocardial baseline GSH were significantly increased (P ≤ 0.001) in T1BL and T2BL groups (378.5 ± 2.3 µg/g wet wt. and 395.1 ± 5.3 µg/g wet wt.) (P ≤ 0.001) respectively in comparison to BL group (324.3 ± 6.8 µg/g wet wt.).

**Myocardial SOD**

Myocardial baseline SOD were significantly (P ≤ 0.01) increased in T1BL and T2BL groups (9.1 ± 0.6 units/mg protein and 9.0 ± 0.3) (P ≤ 0.001) respectively in comparison to BL group (2.9 ± 0.7 units/mg protein).
Myocardial CAT

Myocardial baseline CAT were significantly (P ≤ 0.001) increased in T1BL and T2BL groups (145.9 ± 2.4 units/mg protein and 134.5 ± 1.3 units/mg protein) (P ≤ 0.001) in comparison to BL group (43.0 ± 7.0 units/mg protein).

Ischemic reperfusion injury changes

The results obtained in the different groups subjected to in-vitro ischemic reperfusion injury are presented in the [Table 1].

Myocardial TBARS

Myocardial TBARS in the group IR (57.9 ± 3.0 n mol/g wet wt) was significantly (P ≤ 0.001) higher than that in group C (46.6 ± 1.2 n mol/g wet wt). In the T1IR group myocardial TBARS was significantly lower and in T2IR group it is increased (P ≤ 0.001) (49.6 ± 3.4 n mol/g wet wt and 79.7 ± 4.7 n mol/g wet wt) in comparison to IR.

Myocardial GSH

Myocardial GSH was significantly (P ≤ 0.001) lower in group IR (337.9 ± 2.5 µg/g wet wt) in comparison to that of group C (430.1 ± 2.53 µg/g wet wt). In the T1IR group, myocardial GSH was not changed (289.1 ± 4.5 µg/g wet wt) significantly. In the T2IR group myocardial GSH was significantly (P ≤ 0.001) raised (308.5 ± 8.7 µg/g wet wt) in comparison to group IR.

Myocardial SOD

Myocardial SOD was significantly lower (P ≤ 0.001) in group IR (2.6 ± 0.2 units/mg protein) than that in group C (3.21 ± 0.07 units/mg protein). Myocardial SOD levels were significantly (P ≤ 0.001) higher in the both T1IR and T2IR groups (3.9 ± 0.6 units/mg protein and 4.8 ± 0.8 units/mg protein) in comparison to group IR.

Myocardial CAT

Myocardial CAT was significantly (P ≤ 0.001) lower (33.4 ± 1.1 units/mg protein) in group IR, when compared to that in group C (44.3 ± 2.9 units/mg protein). Myocardial CAT were significantly higher (P ≤ 0.001) in the T1IR and T2IR groups (63.8 ± 1.4 units/mg protein and 67.3 ± 1.9 units/mg protein) in comparison to IR.

Western blot analysis

Western blot analysis showed a single band corresponding to 72 kDa in homogenates of hearts from rats treated with both the doses and in rats that were subjected to whole body hyperthermia. Hsp72 expression was undetectable in vehicle treated rats, which were not fed with the extract. Densitometric scanning results showed a significant (P ≤ 0.001) increase in the expression of Hsp72 in rats of T1 (3236.8 ± 81.5), and T2 (4172.75 ± 55.18) groups. Heat-shocked rats were taken as positive controls, which showed comparable expressions (1751.75 ± 169.4) of Hsp72. Even 50 µg of total protein was enough to detect the signal. Detection of signals for Hsp72 was increased with increasing amounts of protein [Figure 1].

Discussion

Myocardial ischemia caused by ischemic heart disease (IHD) is a major cause of death globally. Currently, reperfusion therapy is the most effective way of treating acute myocardial infarction. However, myocardial ischemia/reperfusion injury (MIRI) is an important factor affecting reperfusion treatment.[20] IHD is one of the common cardiovascular diseases, and has a high incidence. Basic research and clinical treatments for cardiovascular diseases are in continuous development and great progress has been made. Currently, treatment for IHD mainly lies in thrombolysis, intervention and surgical bypass. Unfortunately, IRI is a problem regardless of the therapy used. IRI can be aggravated when the blood supply is recovering. Therefore, the prevention and treatment of IRI is a critical issue that needs to be
resolved.[21]

In-vitro MIRI in rat heart model was used to evaluate the antioxidant and cardioprotective effect of TAME. In the present study MIRI is associated with oxidative stress, as evidenced by increased myocardial TBARS level as oxidative stress marker and depletion of myocardial endogenous antioxidant system in IR group.

In the present study, the oral chronic administration of TA at two different doses significantly decreases the myocardial TBARS and hence the concomitant increase in the levels of endogenous antioxidants (GSH, SOD and catalase). Elevated levels of myocardial TBARS is indicative of increase in the oxidative stress during ischemia which might be due to stimulation of the self-protective mechanism via increasing the level of myocardial endogenous antioxidant systems.[10],[22],[23],[24]

Enhancement of basal endogenous antioxidant by any therapeutic alternative is presently the focus of great scientific interest [24],[25] as it is anticipated to produce better protection against oxidative stress than any exogenously administered antioxidants. In the present study, there was a large increase in myocardial GSH, SOD and catalase activities in group T2, which shows better activity augmented by TA. It is particularly important that both SOD and catalase were increased, since it has been shown that an increase in cellular SOD without a concomitant rise in catalase is more harmful by favouring the formation of H2O2.[26],[27] In this respect, TA may be a particularly useful agent, as it could enhance myocardial endogenous antioxidants, without producing any cytotoxic effects.

The TAME extract has significantly protected the myocardium from the oxidative stress damage via reduction of TBARS and enhancement of endogenous antioxidant enzymes.

The results of the present study showed enhanced expression of HSP72 with both the doses of methanolic extract of TA. However, in the T2BL group a significantly high increase in HSP expression was observed in comparison to T1BL group. In group-V expression of the HSP was detectable. The HSP72 expression was observed in both T1IR and T2IR groups which were also significantly more in comparison to that of control group. In this study, we have shown that the free radical scavenging ability was observed in both T1BL and T2BL groups. Better recovery profile was seen in the T1IR and T2IR treated groups subjected to in-vitro IR injury. Although the mechanism of the cytoprotective effect of HSP is not clearly known, it has been reported that it enhances the myocardial endogenous free radical scavenging ability by increasing cardiac catalase activity. It might be possible that reduced expression of HSP in T1BL treated group resulted in reduced protection. However, the cause for the relatively lower HSP expression is not immediately clear from the present study. In this respect, the present study shows for the first time that the bark of Terminalia arjuna is particularly useful, as it could enhance myocardial endogenous antioxidants and HSP72 expression without producing any cytotoxic effects.

**Conclusion**

These results demonstrate that pretreatment with both the doses of Terminalia arjuna methanol extract can provide post-ischemic myocardial preservation. The induction and expression of HSP72 suggest an important role for the Terminalia arjuna methanol extract in myocardial cell protection after regional myocardial IR injury.

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Nil.

Conflicts of interest

There are no conflicts of interest.

**References**

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