



Original Research

Cardioprotective effect of Pycnogenol in ischemic-reperfusion injury (IRI) in rats

P. Xiao^{1*}, K. Zhang¹, Z. Tao¹, N. Liu¹, B. Ge²

¹Department of cardiology, Sir Run Run Hospital, Nanjing Medical University, Nanjing 211100, China

²Central laboratory, Nanjing first hospital, Nanjing Medical University, Nanjing 211100, China

Correspondence to: xiaopingxi@hotmail.com

Received November 24, 2016; Accepted October 4, 2017; Published October 31, 2017

Doi: <http://dx.doi.org/10.14715/cmb/2017.63.10.7>

Copyright: © 2017 by the C.M.B. Association. All rights reserved.

Abstract: Oxidative stress plays a critical task in the biochemical and pathological alteration linked with myocardial ischemic-reperfusion injury (IRI). This warrants identifying agents with a potential for preventing such damage in an effective way. A novel plant based product, Pycnogenol, obtained from the French maritime pine (*Pinus pinaster* ssp. *atlantica*) bark extract was known for its tremendous antioxidant potential (both *in vivo*, *in vitro*). It was able to attenuate the symptoms of immune dysfunction through restoring a cellular antioxidant status in low micronutrient-induced immune deficient mice. Consequently, the present study was deals with the determination of protective effect of Pycnogenol in ischemic-reperfusion injury (IRI) in rats via Non-recirculating Langendorff's technique. The effect of Pycnogenol on the level of various key biomarkers in the rat heart homogenate was determined, such as, myocardial thiobarbituric acid reactive substances (TBARS, a marker of lipid peroxidation), lactic dehydrogenase (LDH) (a marker of tissue injury) and effect on endogenous antioxidants, e.g., superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx). The activity of these biomarkers appreciably improved in Pycnogenol-treated group than IRI group ($P < 0.05$). The effect of Pycnogenol was further confirmed via histopathological examination of cardiac tissues, which suggests that, it considerably improved the injury related to tissue damage through suppression of edema and infiltration of neutrophil compared to IRI group. It also showed modulation of the expression of apoptotic factors, e.g. Bcl-2, bax and caspase-9 as confirmed by western blot analysis.

Key words: Cardio vascular diseases; Pycnogenol; Ischemic-reperfusion injury.

Introduction

Cardio vascular diseases (CVDs) are ranked as number one killer in the world responsible for large number of deaths than any other causes (1,2). Among the CVDs, ischemia of the heart or Ischemic heart disease (IHD) is deemed as one of the major causes particularly in the case for acute myocardial infarction responsible for unexpected fatality in the world. The IHD could be greatly contributed by moderate blood supply to the heart. It results in the apoptosis of cardiomyocyte which leads to myocardial ischemia and inexorably heart collapse. However, the resulted cardiac injury will be prevented once the normal blood supply is restored to the previous level. Various studies have found that, oxidative stress due to disparity between oxidant and antioxidant systems acts as the major apoptotic stimuli to trigger the IHDs (3-7). Therefore, to prevent the disease progression to the other parts of the affected organ and limit oxidative damage, the anti-oxidant defence system of the body needs to be boosted by supplementing with anti-oxidants (8). Due to rich content of Flavanoids, Pycnogenol, an outstanding natural anti-oxidant, traditional plants based supplements are widely used in diet to protect the heart against ischemic injury. It prevent the process of generation of reactive oxygen species (ROS) via numerous mechanisms, for instance, inhibition of xanthine oxidase, chelation of transition metal, scavenging activity and strengthening the cellular antioxidant capacity (9). Particularly in Ischemic zones, it lowers

the ROS production and improves the restoration of the blood supply. Pycnogenol, a bark extract derived from the French maritime pine (*Pinus pinaster* ssp. *atlantica*) is chiefly composed of flavan-3-ol derivatives. It includes specific blend of procyanidins, catechin and epicatechin (10). Several studies concluded its beneficial role on cardiovascular system which includes prevention of atherogenesis and formation of thrombus (11). It also improves lipid profile of the blood including reduction in plasma concentration of LDL-cholesterol and improves the level of HDL cholesterol. Moreover, it dilates the constricted arteries by stimulating the eNOS activity (12). Despite of tremendous anti-oxidant and cardioprotective potential of Pycnogenol, no study has been conducted to assess its beneficial effect in ischemic-reperfusion injury. Thus, the present study was intended to determine the protective role of Pycnogenol in ischemic-reperfusion injury (IRI) in heart of experimental rats induced by oxidative stress.

Materials and Methods

All chemicals including the pycnogenol used in the present study were of analytical grade and procured from Sigma Chemicals, USA. The study was conducted in accordance with the approval of Institutional Animal Ethical Committee for Animal Experiment.

Pharmacology

Four groups of rats were formed in random manner

containing 6 rats in each group. While 1 h prior to sacrifice, the animals were made insentient with sodium pentobarbitone [60 mg/kg i.p.] and heparinised [375 units/200 g i.p.]. The following protocol was followed for further analysis.

Induction of ischemic–reperfusion injury in isolated rat heart

Non-recirculating Langendorff's technique (Hufesco, Hungary), in constant pressure mode with a modified Krebs's Henseleits solution (KH) containing in mM: glucose 11.1; NaCl 118.5; NaHCO₃ 25; KCl 2.8; KH₂PO₄ 1.2; CaCl₂ 1.2; MgSO₄ 0.6 with a pH 7.4 was used for perfusion of hearts of the Rats after rapid excision and washing with ice-cold saline. The buffer solution equilibrated with 95% O₂ + 5% CO₂ was distributed to the aortic cannula at 37 °C under 60 mm Hg stress. After initial 10 min of equilibration period, zero-flow (ischemia) was carried for 20 min with 40 min of reflow (reperfusion). Pycnogenol (0.01% and 0.05%) was used in two concentrations in the perfusion buffer.

Classification of the animal group

The perfusion of rat hearts only for 70 min serves as Group A. In the case of Group B, the hearts of rat were subjected to 10 min of perfusion, followed by 20 min of ischemia and 40 min of reperfusion. The Pycnogenol was introduced in next two subsequent groups C and D with reperfusion with buffer containing 0.01% and 0.05 % it, respectively. In above groups, the rat hearts were subjected to 10 min of perfusion, followed by 20 min of ischemia and 40 min of reperfusion with buffer. For further biochemical estimation and histopathological studies, after finishing each experiment, the myocardial tissue was stored in liquid nitrogen and 10% buffered formalin.

Estimation of biochemical parameters

In respect of biochemical study, following parameter was assessed, such as, TBARS (Myocardial thiobarbituric acid reactive substances, a marker of lipid peroxidation) (13), LDH (lactic dehydrogenase, a marker of tissue injury) (14) and the endogenous antioxidants e.g. SOD (superoxide dismutase) (15), CAT (catalase) (16), GSH (glutathione) (17) and GPx (glutathione peroxidase) (18) in entire groups.

Preparation of homogenate of rat heart

Rat heart tissue homogenate was prepared in a ratio of 1:10, where 1 g of wet tissue was mixed with 10 times (w/v) of 0.05 M ice cold phosphate buffer (pH 7.4). The resulting mixture was homogenized in rotor stator homogenizer (ultra-turrax) and 0.2 mL of this homogenate was used for estimation of myocardial TBARS. Other biochemical parameters were estimated with the remaining part of the homogenate, thus, it was divided into two equal parts. The one part of the homogenate was used for the estimation of GSH, where, the homogenate was mixed with 10% trichloroacetic acid (1 : 1), centrifuged at 5000 ×g (4 °C, for 10 min) and resultant supernatant was used for corresponding assessment. The supernatant of the next part of the homogenate was utilized for the estimation of SOD, catalase, GPx and protein estimation (19,20).

Thiobarbituric acid reactive substances (TBARS) assay

For TABRS assay, tissue homogenate (0.2 mL) was mixed with 1.5 mL of 0.8% (w/v) 2-thiobarbituric acid, 1.5 mL of 20% acetic acid and 0.2 mL of 8.1% (w/v) sodium dodecyl sulfate (SDS). The final volume was increased up to 4.0 mL with distilled water. The resulting mixture was heated at 95 °C for 60 min. After cooling, further distilled (1.0 mL) water and mixture of *n*-butanol and pyridine (15:1, v/v, 5.0 mL) were added to it. The mixture was shaken vigorously and centrifuged at 5000 ×g for 10 min and the optical density of the *n*-butanol layer was measured at 532 nm.

Histopathology of rat myocardial tissues

For histopathological analysis, the myocardial tissue of the rat was fixed in 10% buffered formalin. It was then routinely processed and implanted in paraffin. The Paraffin sections (3 μm) were cut on slides and stained with hematoxylin and eosin (H and E), periodic acid Schiff (PAS) reagent and inspected under a bright light microscope with magnification of 400x.

Western blot analysis

Briefly, the equal amounts of protein (30 μg) from the ischemic myocardium tissue samples were separated and electrotransferred onto polyvinylidene difluoride membranes (Invitrogen) that were probed with anti-Bax, anti-Bcl-2, and anti-GAPDH and respective caspase-9 antibodies diluted in blocking buffer. Each primary antibody was incubated at 4 °C overnight. After three washes with Tris-buffered saline and Tween 20 for approximately 15 min, the membranes were incubated with horse-radish peroxidase-conjugated secondary antibodies (anti-rabbit immunoglobulin G for the primary antibodies); bands were detected using an ECL system (PerkinElmer) and were quantified using the Quantity One software package (Bio-Rad Laboratories, UK). The result of the control group was defined as 100 %.

Statistical analysis

Statistical analysis were performed and the values are expressed as mean ± SE. To test the significance of biochemical data of different groups, Unpaired Student's T-test and significance was set at $P < 0.05$.

Results and discussion

The requirement of unique geographical and environmental condition has limits the availability of *P. maritime*, biological source of Pycnogenol to the Bay of Biscay in the Landes de Gascogne in France. However, the various efforts to reproduce in other geographical location have not become successful. As mentioned in US Patent (#4,698,360), the bark has been extracted with water with subsequent washing with ethyl acetate to remove water-soluble impurities to furnish Pycnogenol. This whole component consist a mixture of monomers, including catechin, epicatechin, and taxifolin and condensed flavonoids which can be classified as procyanidins/proanthocyanidins (21,22). Interestingly, it has been found that, as a dietary supplement in dosage ranging from 25mg to 250mg, it facilitate in decrease of blood pressure together with reduced level of LDL

cholesterol and increase in micro-circulation (23,24). Various experimental studies concluded the role of Pycnogenols in protection against coronary heart disease because of its rich antioxidant property (25,26). Thus, in the present study, we are prompted to elucidate the effect of Pycnogenols, in IRI of the heart of experimental rats induced by oxidative strain. For this, the heart of rat was washed with ice-cold water and then it was perfused by the non-recirculating Langendorff's technique in constant pressure mode with a modified Krebs's Henseleits solution (KH). After cycle of perfusion and reperfusion, the required concentration of Pycnogenols was pre-mixed with buffer solution and used for the study. The improvement in heart function, was determined by measuring the level of various parameters in rat heart homogenate, such as, myocardial thiobarbituric acid reactive substances (TBARS, a marker of lipid peroxidation), lactic dehydrogenase (LDH) (a marker of tissue injury) and effect on endogenous antioxidants, for instance, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx).

Effect of Pycnogenol on thiobarbituric acid reactive substances (TBARS)

The death of cardiac myocyte in early stages of reperfusion is attributable to oxidative injury and associated higher level of lipid peroxidation. This in turn, triggers the elevated level of thiobarbituric acid reactive substances which acts as biomolecular marker for predicting the peroxidative tissue injury. Thus, limiting the generation of TBARS, would have significant ef-

fect on preventing the lipid peroxidation by limiting the generation of reactive oxygen species (ROS). As shown in Fig. 1(A1), it was suggested that, the myocardial TBARS in group A was found lower than group B. Whereas, on introduction of the test drug (Pycnogenol), the level of the TBARS has been significantly reduced in dose dependent manner, i.e group C and D. Therefore, it was corroborated that due to high antioxidant potential, Pycnogenol have sufficient ability to reduce the elevated levels of TBARS for its protective action.

Effect of Pycnogenol on myocardial LDH

The death of cardiac tissue even in small quantity could be measured significantly by quantifying the level of lactate dehydrogenase (LDH) in serum. These enzymes are present in sufficiently high content in myocardial tissue and released upon the injury. It follow a definite pattern of release, where the total LDH level rises within 24-48 hours after a heart attack, reach to highest in two to three days, and revert back to average level in roughly five to ten days. Thus, reducing the level of lactic dehydrogenase (LDH) serves as the main determinant to circumvent the coupled cellular injury. It has been found in Fig. 1(A2), the rats treated with two diverse concentration of test compound, group C and D exhibit improved level of LDH which correlates well with the protective effect of compound against ischemia-reperfusion injury in cardiac tissues.

Effect of Pycnogenol on myocardial SOD, CAT, GSH and GPx

To prevent the ischemic-reperfusion injury, myocardial tissue has its own defence mechanism, which works on generation of various enzymes, such as, SOD, CAT, GSH and GPx. These enzymes are behaved as endogenous antioxidants to counteract the action of available ROS. Therefore, agents help in augmenting the level of these enzymes would be beneficial for cardio-protective action. It was clearly exemplified in the Fig. 2 (A1-A4) that, the levels of these enzymes have been significantly elevated upon administration of Pycnogenol. Thus, it has been suggested that, the administration of Pycnogenol causes elevated levels of these natural anti-

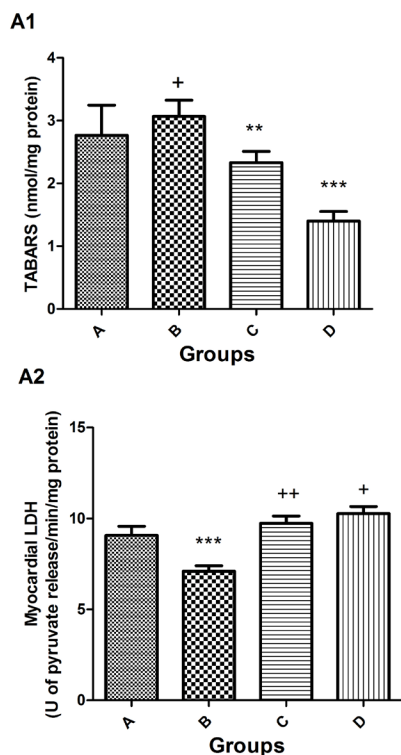


Figure 1. Effect of Pycnogenol on the level of (A1) myocardial TBARS (nmol/mg protein) and (A2) myocardial LDH (U of pyruvate release/min/mg protein) in different groups, where, A: only perfusion, B: ischemia-reperfusion injury (IRI), C: IRI+0.01% of Pycnogenol D: IRI+0.05% of Pycnogenol. No. of observation six. Values are mean \pm SE; For A1: ⁺ $P < 0.05$ vs. A; ^{**} $P < 0.01$ vs. B; ^{***} $P < 0.001$ vs. B; For A2: ^{***} $P < 0.001$ vs. A; ⁺⁺ $P < 0.001$ vs. B; ⁺ $P < 0.01$ vs. B.

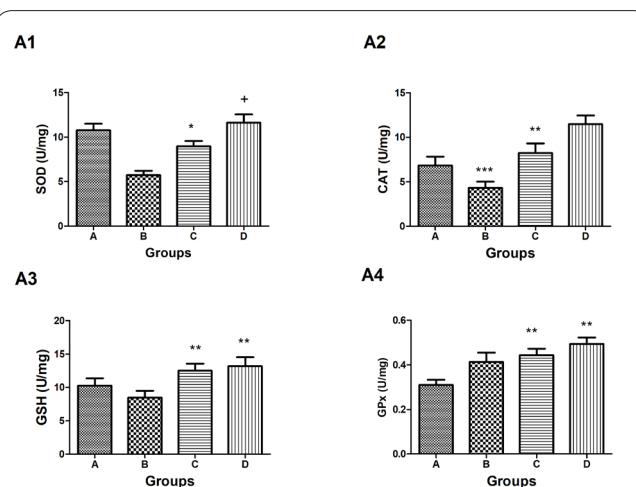


Figure 2. Effect of Pycnogenol on the levels of myocardial enzymes (in U/mg protein), A1: SOD; A2: CAT; A3: GSH; A4: GPx. Values are mean \pm SEM. No. of observation six; For A1: ^{*} $P < 0.05$ vs. A, ⁺ $P < 0.05$ vs. B; A2: ^{***} $P < 0.05$ vs. A, ^{**} $P < 0.01$ vs. B; A3: ^{**} $P < 0.05$ vs. B. A4: ^{**} $P < 0.05$ vs. B.

oxidants to shield tissue. The presence of high content of flavanoids and related compound in Pycnogenol are considered to be responsible for this action.

Histopathological study

The heart section of treated rats after Ischemic-reperfusion injury stained with haematoxylin and eosin showed degradation of fibres of the myocardial tissues. It was confirmed with the alteration in neutrophil and considerable edema as shown in Fig.3B. The introduction of Pycnogenol at 0.01 % concentration, no necrosis of the cardiac tissue was observed accompanied with edema and less neutrophil infiltration (Fig.3C). While on increasing the concentration, Pycnogenol inhibited the infiltration of neutrophil and edema (Fig.3D) as compared to the Group B.

Effect of Pycnogenol on the pro-apoptotic factor

Bcl-2 group of proteins play a vital role in apoptotic cell death. They also believed to involve as a key role in caspase activation and the regulation of apoptosis. The Bcl-2 family is further divided into two classes: one with antiapoptotic protein, such as Bcl-2 and Bcl-XL, and the other with proapoptotic protein, Bax and Bak. Our findings suggest that the protective effects of pycnogenol against I-/R-induced apoptosis were associated with the modulation of Bax and Bcl-2 expression. In addition, caspase-3 is a member of the cysteine–aspartic acid protease (caspase) family. It is the predominant caspase that plays a central role in the execution phase of cell apoptosis. We found that isolated myocardial I/R injury resulted in significantly increased caspase-9 expression compared with administration of pycnogenol before ischemia and throughout reperfusion. And, I/R increased the percentage of apoptotic cells. Such effect was significantly attenuated by adding pycnogenol. Thus, the results demonstrate that pycnogenol ameliorates I-/R-induced myocardial apoptosis.

The effect of pycnogenol was investigated on the expression of proapoptotic factor Bax and that of the

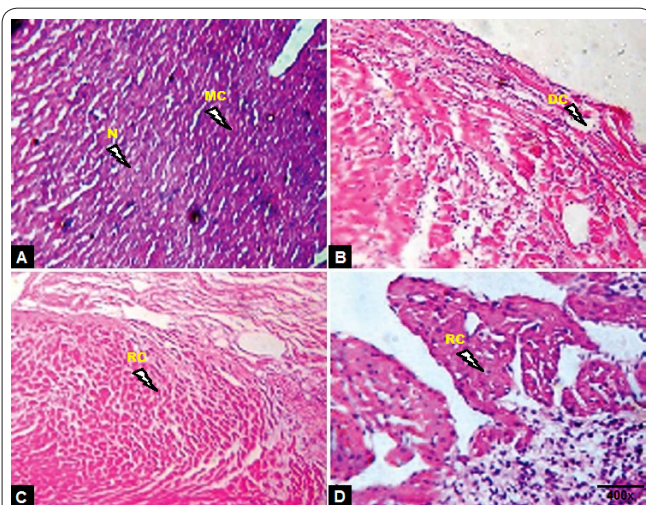


Figure 3. Tissues of rats under bright light micrograph ($\times 400$), rat heart was subjected to A) perfusion for 70 min only. B) ischemia of 20 min and 40 min of reperfusion injury. C) 20 min of ischemia and reperfusion for 40 min with 0.01% of Pycnogenol. D) 20 min ischemia and reperfusion for 40 min with 0.05% of Pycnogenol. Where, N: Normal cells, RC: Regenerative Cells, MC: Myonecrotic cells and DC: Degenerative cells.

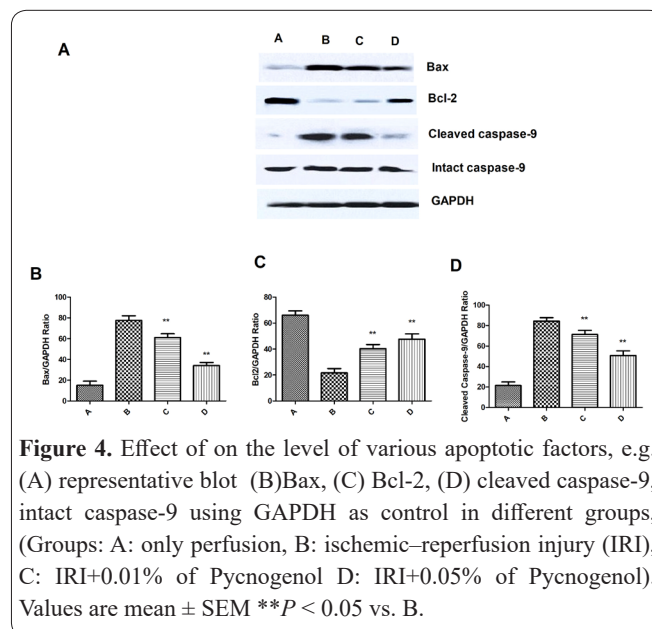


Figure 4. Effect of on the level of various apoptotic factors, e.g. (A) representative blot (B)Bax, (C) Bcl-2, (D) cleaved caspase-9, intact caspase-9 using GAPDH as control in different groups, (Groups: A: only perfusion, B: ischemic–reperfusion injury (IRI), C: IRI+0.01% of Pycnogenol D: IRI+0.05% of Pycnogenol). Values are mean \pm SEM ** $P < 0.05$ vs. B.

antiapoptotic factor Bcl-2 as specific markers of cell apoptosis via western blot analysis. Results as shown in Fig. 4 suggested that, animals belongs to the I/R group showed significant increase in the Bax expression and reduced Bcl-2 expression as compared with control ($p < 0.01$).

As a concluding remark, it has been found that, considerably improved the injury related to tissue damage through suppression of edema and infiltration of neutrophil compared to IRI group. It also showed modulation of the expression of apoptotic factors, e.g. Bcl-2, bax and caspase-9 as confirmed by western blot analysis.

Interest conflict

The authors declare no conflict of interest.

Author's contribution

Xiao P. performed design of experiment, Zhang K. performed animal experiment, Tao Z. performed TBARS assay, Liu N. performed histopathology of rat myocardial tissues and Ge B. performed western blot analysis.

References

- Deaton C, Froelicher ES, Wu LH, Ho C, Shishani K, Jaarsma T. The global burden of cardiovascular disease. *Eur J Cardiovasc Nurs* 2011; 2: S5-13.
- Thomas AG. Reducing The Growing Burden Of Cardiovascular Disease In The Developing World. *Health Aff (Millwood)* 2007; 26: 13–24.
- Zucchi R, Ghelardoni S, Evangelista S. Biochemical basis of ischemic heart injury and of cardioprotective interventions. *Curr Med Chem* 2007; 14: 1619–37.
- Jain AK, Mehra NK, Swarnakar NK. Role of antioxidants for the treatment of cardiovascular diseases: Challenges and opportunities. *Curr Pharm Des* 2015; 21: 4441-55.
- Victor VM, Rocha M. Targeting antioxidants to mitochondria: a potential new therapeutic strategy for cardiovascular diseases. *Curr Pharm Des* 2007; 13: 845-63.
- Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now?. *J Lab Clin Med* 1992; 119: 598–620.
- Buja LM, Entman ML. Modes of myocardial cell injury and cell death in ischemic heart disease. *Circulation* 1998; 98:1355–57.

8. Hearse DJ. Prospects for antioxidant therapy in cardiovascular medicine. *Am J Med* 1991; 91:118S–21S.
9. Watson R. Pycnogenol and cardiovascular health. Evidence-based integrative medicine. 2003; 1: 27-32.
10. Watson RR. Reduction of cardiovascular disease risk factors by French maritime Pine bark extract. *Cardiovascular Rev. Rep.* 1999; 20: 326-29.
11. Schoonees A, Visser J, Musekiwa A, Volmink J. Pycnogenol (extract of French maritime pine bark) for the treatment of chronic disorders. *Cochrane Database Syst. Rev* 2012; 4: CD008294.
12. Packer L, Rimbach G, Virgili F. Antioxidant activity and Biological properties of a Procyanidin-rich extract from pine (*Pinus Maritima*) bark, Pycnogenol. *Free Radic Biol Med.* 1999; 27: 704-24.
13. Okhawa H, Oohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351–58.
14. Molders P, Hogberg J, Omenivs S. Isolation and use of liver cells. *Methods Enzymol* 1978; 52: 60–71.
15. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys.* 1984; 21: 130–32.
16. Aebi H, Bergmeyer HU. *Methods of Enzymatic Analysis*. 2nd ed. Chemic Academic Press Inc, Verlag 1974; 2: 673-85.
17. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959; 82: 70–7.
18. Wendel A. Glutathione peroxidase. *Methods Enzymol.* 1981; 77: 325–33.
19. Jiankang L, Rei E, Hideaki K, Akitane M. Antioxidant action of Guilingji in the brain of rats with FeCl₃-induced epilepsy. *Free Radic Biol Med* 1990; 9: 451–54.
20. Annadora JB, Michel B. Oxygen free radicals in rat limbic structure after kainate-induced seizures. *Free Radic Biol Med* 1995; 18: 993–1002.
21. Kim SM, Kang SW, Jeon JS, Um BH. A comparison of Pycnogenol® and bark extracts from *Pinus thunbergii* and *Pinus densiflora*: Extractability, antioxidant activity and proanthocyanidin composition. *Journal of Medicinal Plants Research* 2012; 6(14): 2839-49.
22. Peng Z, Wei B, Lau HS. Pycnogenol inhibits tumor necrosis factor- α -induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. *Cellular and Molecular Life Sciences CMLS* 2000; 57(5): 834-41.
23. Zibadi S, Rohdewald PJ, Park D, Watson RR. Reduction of cardiovascular risk factors in subjects with type 2 diabetes by Pycnogenol supplementation. *Nutr. Res* 2008; 28: 315–20.
24. Belcaro G, Cornelli U, Luzzi R, Cesarone MR, Dugall M, Feragalli B, et al. Pycnogenol?? supplementation improves health risk factors in subjects with metabolic syndrome. *Phyther. Res.* 2013; 27: 1572–8.
25. Iravani S, Zolfaghari B.. Pharmaceutical and nutraceutical effects of *Pinus pinaster* bark extract. *Res Pharm Sci* 2011; 6(1): 1–11.
26. Lavie CJ, Milani RV. Cardiac Rehabilitation and Exercise Training in Secondary Coronary Heart Disease Prevention, *Prog. Cardiovasc. Dis.* 2011; 53: 397–403.