Journal of Medicinal Plant Research Vol. 1 (4), pp. 080-085, November 2007
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 © 2007 Academic Journals

Full Length Research Paper

Protective effect of *Picrorhiza kurroa* on antioxidant defense status in adriamycin-induced cardiomyopathy in rats

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Accepted 15, October 2007

Adriamycin, an anthracycline antibiotic, which is used in the treatment of various tumors, is known to cause severe cardiomyopathy. The present study examined the protective effects of *Picrorhiza kurroa*, an ayurvedic medicinal plant, on myocardial antioxidant defense system in adriamycin-induced cardiomyopathy in rats. Intraperitoneal administration of adriamycin (1.5 mg/kg body weight/ day, i.p. for 15 days) caused significant rise in the levels of diagnostic marker enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine phoshokinase (CPK)] in plasma and lipid peroxidation in the heart tissue of experimental rats. Concomitant decline in the level of reduced glutathione (GSH) and the activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (SOD and CAT) in the myocardial tissue were also observed. Oral administration of *P. kurroa* extract (50 mg/kg body weight/day, for a period of 15 days) significantly prevented all these adriamycin-induced adverse effects and maintained the rats at normal status. The protective effect of *P. kurroa* might be ascribable to its membrane-stabilizing property and/or antioxidant nature.

Key words: *Picrorhiza kurroa*, adriamycin, cardiomyopathy, diagnostic marker enzymes, lipid peroxidation, antioxidant status.

INTRODUCTION

Over the last 3 - 5 decades, the treatment of cancer has relied largely on the use of diverse cytotoxic chemotherapeutic agents. The exploit of chemotherapy in the treatment of cancer has opened new potential for the improvement of the quality of life of cancer patients and for the cure of disease. Regardless of its success, treatments with some of the most effective anticancer drugs can sometimes result in a number of symptoms indicating toxicity.

Adriamycin is a quinone-containing anticancer drug that is extensively used to treat a variety of human neoplastic diseases as well as an ample range of solid tumors, including breast, lung and thyroid cancer (Gianni et al., 2007). The clinical effectiveness of this drug is limited by its side effect which is cardiomyopathy or congestive heart failure in some adriamycin-treated cancer patients (Bryant et al., 2007). It induces cardiomyopathy by various mechanisms. Adriamycin-induced cardiomyopathy is generally attributed to higher free radical formation (Catala et al., 2007), impaired adrenergic stimulation (Gnanapragasam et al., 2007), altered calcium homeostasis (Kim et al., 2006), concealed mitochondrial function (Wallace, 2007), infiltration of inflammatory cells (Wang et al., 2007; Bruynzeel et al., 2007) and accumulation of fat (Abd-Ellah and Mariee, 2007). Alterations in the activities of antiperoxidative enzymes [superoxide dismutase (SOD) and catalase (CAT)] and glutathione dependent antioxidant enzymes [glutathione peroxidase (GPX) and glutathione-S-transferase (GST)] have been reported in adriamycin-induced cardiomyopathy in rats (Gnanapragasam et al., 2004; Deepa and Varalakshmi, 2003). Peroxidation of endogenous lipid is a major factor in the cardiotoxic action of adriamycin (Deepa and Varalakshmi, 2003).

Natural products have been the starting point for the discovery of many important modern drugs. This has led

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to a worldwide search for pharmacologically important substances derived from natural products. Many plants have been used for cardioprotection in the traditional Indian medicinal system. In Indian ayurvedic medicine, *Picrorhiza kurroa*, a medicinal plant, has been used to cure cardiac ailments (Kumar et al., 2001). In traditional medicine, it has also been used to cure hepatitis, abdominal pain, stomach disorders, anemia, jaundice, and for promoting bile secretion (Anandan et al., 2000). Picroside I, picroside II and kutkoside are the naturally occurring free radical scavenging principles present in the roots and rhizomes of *P. kurroa* (Rastogi et al., 2001; Russo et al., 2001). They are credited with anti-allergic, anti-anaphylactic, antidiabetic, antitumor and other beneficial properties (Rajeshkumar and Kuttan, 2001; Russo et al., 2001; Joy et al., 2000). However, the effects of *P. kurroa* on myocardial antioxidant defense system in adriamycin-induced cardiomyopathy have not yet been fully explored.

In the present study, the protective effect of ethanolic extract of *P. kurroa* [PK] in adriamycin-induced cardiomyopathy in albino rats has been examined with respect to changes in the levels of diagnostic marker enzymes, lipid peroxidation and antioxidant status.

MATERIALS AND METHODS

Chemicals

Adriamycin, epinephrine, pyruvate, cholesterol and tetramethoxy propane were obtained from M/s. Sigma Chemical Company, St. Louis. MO, USA. All the other chemicals used were of analytical grade. The dried and coarsely powdered roots and rhizomes of *P. kurroa* were extracted with 95% ethanol. The extract was filtered and concentrated to dark brownish residue. The last traces of the solvent were removed under reduced pressure (yield 8.2%).

Animals

Male wistar strain albino rats, weighing 150 – 180 g, were used for the experiment. Animals were housed individually in polypropylene cages under standard environmental conditions and allowed food (M/s Hindustan Lever Feeds, Bangalore, India) and water *ad libitum*. The experiment was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Experimental protocol

The animals were divided into four groups, comprising six rats each. Rats in group I (normal control) received standard diet and intragastrically administered with distilled water for a period of 15 days. Group II animals were orally administered with PK [50mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for a period of 15 days. In group III, animals were administered with distilled water for a period of 15 days and also intraperitoneally injected with adriamycin [1.5mg (dissolved in physiological saline)/kg body weight/ day, i.p. for 15 days] for the induction of cardiomyopathy. In group IV, the animals were pretreated with PK (50mg/kg body weight/day) and injected with adriamycin, as described in group III. Control animals (group I and group II) were injected with physiological saline alone for 15 days.

At the end of the experimental period, the rats were sacrificed and blood was collected using heparin as anticoagulant. The plasma separated was used for the determination of alanine amino transferase (ALT) (EC 2.6.1.2), aspartate amino transferase [AST] (EC 2.6.1.1), lactate dehydrogenase (LDH) (EC 1.1.1.27), and creatine phosphokinase (CPK) (EC 2.7.3.2).

The heart tissue was excised immediately and thoroughly washed with physiological saline. The heart tissue homogenates prepared in 0.1M Tris HCl buffer, pH 7.4, were used for the determination of lipid peroxides (LPO), reduced glutathione (GSH), glutathione peroxidase(GPx) (EC 1.11.1.9), glutathione-S-tranferase (GST) (EC 2.5.1.18), catalase (CAT) (EC 1.11.1.6), and superoxide dismutase (SOD) (EC 1.15.1.1).

Biochemical assays

The activity of ALT was assayed by the method of Mohur and Cook, (1957). To 1.0 ml of substrate (0.1 M phosphate buffer, pH 7.4, 0.2 M DL-alanine, 2.0 mM 2-oxoglutarate), 0.2 ml of plasma was added and incubated for 1 h at 37.8^{o}C. Then 1.0 ml of 0.02% DNPH was added and kept at room temperature for 20 min. To the control tube, sample was added after arresting the reaction with DNPH. Then 5 ml of 0.4N NaOH was added and the colour developed was read at 540 nm. The activity was expressed as μ moles of pyruvate liberated/l/h.

AST was assayed by the method of Mohur and Cook, (1957). The assay mixture containing 1.0 ml of buffered substrate (L-aspartic acid and α-ketoglutaric acid in 0.15 M phosphate buffer, pH 7.4) and 0.2 ml of plasma was incubated for 1hr at 37.8^{o}C. To the control tubes, sample was added after the reaction was arrested by the addition of 1.0 ml DNPH. The tubes were kept at room temperature for 30 min. Then 5.0 ml of 0.4N NaOH was added and the colour developed was read at 540 nm. The activity was expressed as μ moles of pyruvate liberated/l/h.

LDH was assayed according to the method of King, (1965). To 1.0 ml of the buffered substrate (lithium lactate in 0.1M glycine buffer, pH 10), 0.1 ml of enzyme preparation was added and the tubes were incubated at 37.8^{o}C for 15 min. After adding 0.2 ml of NAD^{+} solution, the incubation was continued for another 15 min. The reaction was arrested by adding 0.1 ml of DNPH (2, 4-dinitrophenyl hydrazine), and the tubes were incubated for a further period of 15 min at 37.8^{o}C after which 7.0 ml of 0.4N NaOH was added and the color developed was measured at 420 nm in a Shimadzu UV-1601 spectrophotometer. Suitable aliquots of the standards were also analyzed by the same procedure. The activity of the enzyme was expressed as μmoles of pyruvate liberated/l/h.

CPK activity in plasma was determined by the method of Okinaka et al., (1961). The reaction mixture comprised of 0.05 ml of plasma, 0.1 ml of substrate, 0.1 ml of ATP solution and 0.1 ml of cysteine-hydrochloride solution. The final volume was made up to 2.0 ml with distilled water and incubated at 37.8^{o}C for 30 min. The reaction was arrested by the addition of 1.0 ml of 10% TCA (trichloroacetic acid) and the contents were subjected to centrifugation. To 0.1 ml of the supernatant, 4.3 ml distilled water and 1.0 ml ammonium molybdate were added and incubated at room temperature for 10 min. 0.4 ml of ANSA was added and the color developed was read at 640 nm after 20 min. The activity of the enzyme was expressed as μmoles of phosphorus liberated/l/h.

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al. (1979) in which the malondialdehyde (MDA) released served as the index of LPO. 1, 1, 3, 3-Tetra ethoxypropane malondialde-hyde bis (diethyl acetal) was used as standard. To 0.2 ml of

Table 1. Levels of diagnostic marker enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK)] in plasma of normal and experimental groups of rats.

Groups	Group I Control	Group II PK-treated [A]	Group III Adriamycin-administered [B]	Group IV [A+B]
ALT	129 ± 8.72 ^{a,b}	118 ± 9.25 ^{a}	315 ± 22.4^{c}	141 ± 8.59^{b}
AST	107 ± 6.94 ^{a,b}	96.5 ± 7.03 ^{a}	277 ± 19.6^{c}	118 ± 8.24^{b}
LDH	173 ± 12.3 ^{a}	205 ± 15.2 ^{b}	397 ± 35^{c}	231 ± 19.3^{d}
CPK	115 ± 7.24 ^{a}	109 ± 6.75 ^{a}	289 ± 22.4^{b}	121 ± 8.43^{c}

[A]: PK, 50 mg/kg body weight/day, orally administered for 15 days. [B]: Adriamycin, 1.5 mg/kg body weight/day, i.p. for 15 days. Results are mean ± SD for 6 animals. Values expressed: ALT, AST, and LDH, µmol pyruvate liberated $h^{-1}l^{-1}$; CPK, µmol creatine liberated $h^{-1}l^{-1}$. Values that have a different superscript letter (a,b,c,d) differ significantly with each other (P<0.05; Duncan's multiple range test).

tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBAwere added. The mixture was made up to 4.0 ml with water and then heated in a water bath at 95.8°C for 60 min using glass ball as a condenser. After cooling, 1.0 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance was measured at 532 nm. The level of lipid peroxides was expressed as nmoles of MDA formed/mg of protein.

Reduced glutathione (GSH) was estimated by the method of Ellman, (1959). 0.1 ml of tissue homogenate was precipitated with 5% TCA (trichloroacetic acid). The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.1 ml of supernatant, 2.0 ml of 0.6 mM DTNB [5, 5 dithiobis (2-nitrobenzoic acid)] reagent and 0.2 M phosphate buffer (pH 8.0) were added to make up to a final volume of 4.0 ml. The absorbance was read at 412 nm against a blank containing TCA (trichloroacetic acid) instead of sample. A series of standards treated in a similar way also run to determine the glutathione content. The amount of glutathione was expressed as nmoles/g heart tissue. 5-sulphosalicylic acid was used to prevent the oxidation of glutathione.

GPx was assayed by the method of Paglia and Valentine (1967). The reaction mixture consisted of 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of reduced glutathione, 0.4 ml of 0.4 M phosphate buffer pH 7.0, and 0.2 ml of tissue homogenate and was incubated at 37.8°C for 10 min. The reaction was arrested by the addition of 0.5 ml of 10% TCA (trichloroacetic acid) and the tubes were centrifuged at 2000 rpm. To the supernatant 3.0 ml of 0.3 mM disodium hydrogen phosphate and 1.0 ml of 0.04 % DTNB were added and the colour developed was read at 420 nm immediately. The activity of GPx was expressed as µmoles of glutathione oxidized/min/mg protein.

GST was assayed by the method of Habig et al. (1974). To 0.1 ml of homogenate, 1.0 ml of 0.3M phosphate buffer pH 6.5, 1.7 ml of water and 0.1 ml of 30 mM CDNB (1-chloro-2, 4-dinitrobenzene) were added. After incubation at 37.8°C for 15 min, 0.1 ml of GSH was added and change in OD was read at 340 nm for 3 min at an interval of 30 sec. Reaction mixture without the enzyme was used as blank. The glutathione-S-transferase activity was expressed as units/min/mg protein.

CAT was assayed by the method of Takahara et al. (1960). To 1.2 ml of 50 mM phosphate buffer pH 7.0, 0.2 ml of the tissue homogenate was added and reaction was started by the addition of 1.0 ml of 30 mM H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 30 s intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as µmoles of H_2O_2 decomposed/min/mg protein.

SOD was assayed by the method of Misra and Fridovich, (1972). 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine (freshly prepared) and the increase in absorbance at 480 nm was measured in a Shimadzu UV-1601 spectrophotometer. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation.

Statistical analysis

Results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Duncan's multiple comparisons test using a statistical package program (SPSS 10.0 for Windows).

RESULTS

Table 1 depicts the levels of diagnostic marker enzymes (ALT, AST, LDH and CPK) in plasma of normal and experimental groups of rats. There were significant (p<0.05) increases noted in the levels of diagnostic marker enzymes in plasma of Group III adriamycin-injected rats as compared to that of Group I control rats. Oral administration of PK significantly (p<0.05) prevented the adriamycin-induced release of these marker enzymes into plasma in group IV animals as compared to group III rats.

Table 2 and Table 3 depict the levels of lipid peroxidation and the activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (CAT and SOD) in the heart tissue of normal and experimental groups of rats. Intraperitoneal administration of adriamycin caused significant (p<0.05) elevation in the level of lipid peroxidation in the heart tissue of group III rats as compared to that of group I normal rats. This was paralleled by a decline in the level of reduced glutathione (Figure 1) and the activities of glutathione-dependent antioxidant enzymes and antiperoxidative enzymes. Oral pretreatment with PK significantly (p<0.05) counteracted all these adriamycin-induced adverse effects and maintained the myocardial antioxidant defense system at a status comparable to that of control animals

Table 2. Level of lipid peroxidation (LPO) in the presence of promoters (2mM) ascorbic acid, ferrous sulphate ($FeSO_4$) and *tert*-butyl hydroperoxide (*t*-BH) in the heart tissue of normal and experimental groups of rats.

Groups	Group I Control	Group II PK-treated [A]	Group III Adriamycin administered [B]	Group IV [A+B]
Basal	0.94 ± 0.08 ^{a,b}	0.86 ± 0.08 ^{a}	1.99 ± 0.11^{c}	1.03 ± 0.08^{b}
Ascorbic acid	3.18 ± 0.25 ^{a}	2.79 ± 0.12 ^{b}	5.45 ± 0.29^{c}	3.57 ± 0.21^{d}
$FeSO_4$	4.71± 0.39 ^{a}	4.35 ± 0.41 ^{a}	6.93 ± 0.52^{b}	4.77 ± 0.35^{a}
***t*-BH**	6.18 ± 0.43 ^{a}	6.03 ± 0.38 ^{a}	8.85 ± 0.69^{b}	6.84 ± 0.51^{c}

[A]: PK, 50 mg/kg body weight/day, orally administered for 15 days.**[B]:** Adriamycin,1.5 mg/kg body weight/ day, i.p. for 15 days. Results are mean ± SD for 6 animals. Values expressed: LPO, nmol MDA released mg^{-1} protein. Values expressed: LPO, nmol MDA released mg^{-1} protein. Values that have a different superscript letter (a,b,c,d) differ significantly with each other (P<0.05; Duncan's multiple range test).

Table 3. Level of reduced glutathione and the activities of glutathione peroxidase (GPx), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) in the heart tissue of normal and experimental groups of rats

Groups	Group I Control	Group II PK-treated [A]	Group III Adriamycin-administered [B]	Group IV [A+B]
Glutathione-dependent antioxidant enzymes				
GPx	4.72 ± 0.27 ^{a}	4.38 ± 0.24 ^{b}	2.63 ± 0.11^{c}	4.16 ± 0.21^{b}
GST	1576 ± 118 ^{a}	1538 ± 134 ^{a}	942 ± 65.4^{b}	1482 ± 123^{a}
Antiperoxidative enzymes				
CAT	12.4 ± 0.67 ^{a}	11.7 ± 0.59 ^{b}	5.42 ± 0.23^{c}	10.7 ± 0.84^{b}
SOD	5.38 ± 0.29 ^{a}	5.25 ± 0.22 ^{b}	2.69 ± 0.12^{c}	5.10 ± 0.24^{b}

[A]: PK, 50 mg/kg body weight/day, orally administered for 15 days. **[B]:** Adriamycin, 1.5 mg/kg body weight/ day, i.p. for 15 days. Results are mean ± SD for 6 animals. Values expressed: GPx, nmol GSH oxidized min^{-1} mg^{-1} protein; GST, µmol 1-chloro-2, 4-dinitrobenzene conjugate formed min^{-1} mg^{-1} protein; CAT, nmol H_2O_2 decomposed min^{-1} mg^{-1} protein; SOD, one unit of the SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation. Values that have a different superscript letter (a, b, c) differ significantly with each other (P<0.05; Duncan's multiple range test).

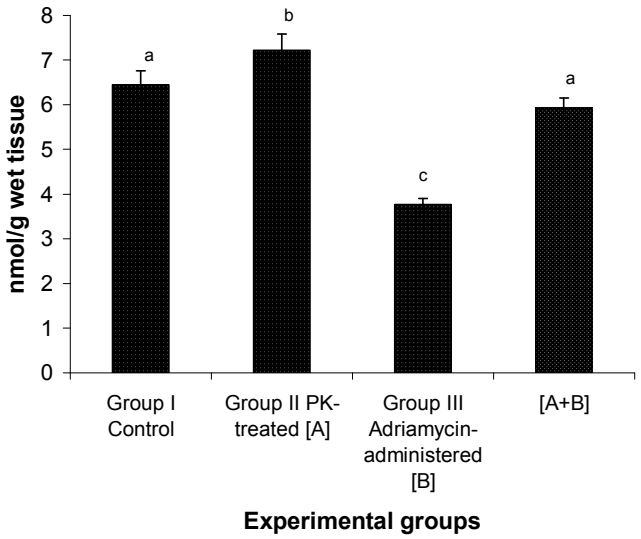

Figure 1. Level of reduced glutathione [GSH] in the heart tissue of normal and experimental groups of rats. Results are mean± SD for 6 animals. Values expressed: GSH, nmol/g wet tissue. Values that have a different superscript letter (a, b, c) differ significantly with each other (P<0.05; Duncan's multiple range test).

The group II normal rats receiving PK alone did not show any significant change when compared with normal rats, indicating that it does not have any adverse effects.

DISCUSSION

Adriamycin is a antitumor antibiotic used for the treatment of a variety of soft and solid human malignancies. However, treatment is usually complicated by its acute and chronic side effects. One of the major chronic side effects is the development of cardiomyopathy and ultimately congestive heart failure. Increased oxidative stress and antioxidant discrepancy have been suggested to play a major part in adriamycin-induced cardiomyopathy (Subashini et al., 2006). The focus of the current study was to investigate the effects of *P. kurroa* for its antioxidant, antilipidemic and membrane-stabilizing properties during adriamycin-induced cardiomyopathy.

Significant (p<0.05) rise observed in the levels of diagnostic marker enzymes (ALT, AST, LDH and CPK) in plasma of group III rats compared to group I normal rats (Table 1), is indicative of adriamycin-induced necrotic lesions in myocardial membrane. This current observation is in agreement with earlier reported studies (Gnanapragasam et al., 2004; Deepa and Varalakshmi, 2003), which verified that the amount of diagnostic marker enzymes present in plasma is directly correlated with the necrotic lesions present in the myocardium. Since the enzymes are localized only intracellularly in tissues such as the heart, increase in the activities of these enzymes in the plasma is an indicator of myocardial damage.

In the present study, the oral administration of PK resu-

lted in considerable ($p<0.05$) diminution in the levels of these diagnostic marker enzymes in plasma of group IV animals as compared to group III rats, indicating cytoprotective action of PK. It probably did so by its membrane stabilizing property (Upadhyay et al., 2001). Reports by Anandan et al. (1999) pointed out that oral supplementation of PK offered hepatoprotective action against D-galactosamine-induced hepatitis by restraining the necrotic damage-mediated discharge of diagnostic marker enzymes into the systemic circulation. Previously Kumar et al. (2001) reported that administration of ethanol extract of *P. kurroa* sheltered cellular membranes against isoprenaline-induced ischemic insult by its membrane-stabilizing and antioxidant properties.

Lipid peroxidation of membranes is recognized to play an important role in the pathogenesis of adriamycin-induced cardiomyopathy (Daosukho et al., 2007). Lipid peroxidation is a progression of oxidative deterioration of cell membranes regulated by the availability of substrate in the form of polyunsaturated fatty acids, the ease of use of inducers such as free radicals and excited state molecules to instigate propagation, the antioxidant defense status of environment, and the physical status of the membrane lipids (Farvin et al., 2004). In the present study, there was a significant elevation noticed in the levels of lipid peroxides (Table 2) with a simultaneous decline in the level of reduced glutathione (Figure 1) and the activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (Table 3) in the heart tissue of Group III rats as compared to that of group I animals. The oxidation of polyunsaturated fatty acids in biological membranes may cause impairment of membrane function, and increase of non-specific permeability to ions leading to disruption of membrane structure. Lipid peroxidation reaction has been associated with distorted membrane structure and enzyme inactivation (Kumar and Anandan, 2007), as observed in the present study (Tables 2 and 3). The present observations are in accordance with preceding reported studies (Daosukho et al., 2007; Gnanapragasam et al., 2004), which showed that myocardial antioxidant defense system was operating at a lower rate despite higher level of oxidative stress in adriamycin-induced cardiomyopathy condition.

In the present investigation, the group IV rats pre-treated with PK experienced reduced level of lipid peroxidation as compared to group III rats. Also the level of GSH and the activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes were comparatively higher for this group. It appears therefore that PK protects against adriamycin-induced lipid peroxidation and has antioxidant property. *In vivo* adriamycin undergoes a two-electron reaction to form alkylating quinone and methides, which in turn endure redox cycling to engender highly reactive oxygen species (Kalyanaraman et al., 1991). The unpaired electron present in the hydroxyl free radical generated during adriamycin-induced myocardial toxicity might have been captivated and dismuted by the electrophilic substances such as Picroside I, Picroside II and Kutkoside, which are present in rich quantities in the roots and rhizomes of *P. kurroa* (Anandan and Devaki, 1999).

In conclusion, the results of present study indicated the cardioprotective effect of *P. kurroa* against adriamycin-induced cardiomyopathy. The overall protective action of *P. kurroa* extract was probably due to its membrane stabilizing action, or to a counteraction of free radicals by its antioxidant nature, or to its ability to maintain the normal status activities of free radical enzymes and the level of reduced glutathione, which protect the myocardial membrane against peroxidative damage by decreasing lipid peroxidation and strengthening the myocardial membrane.

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