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Anti-apoptotic potential of gymnemic acid phospholipid complex pretreatment in Wistar rats with experimental cardiomyopathy

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Cardiomyocyte apoptosis in heart failure has been the topic of research in many recent studies. In the present investigation, the potential cardioprotective effect of gymnemic acid phospholipid complex (GPC) on myocardial apoptosis and cardiac function was studied in doxorubicin (DOX; 30 mg/kg/ip/single dose)-induced cardiomyopathy model in rats. Doxorubicin induced cardiomyopathy was evidenced by significant hemodynamic changes (increased systolic, diastolic, mean arterial pressure and heart rate), decreased heart weight to body weight ratio, increase in serum lactate dehydrogenase (LDH) and Ca^{2+} levels and decrease in myocardial Na^+/K^+ ATPase levels along with caspase-3 activation. A marked reduction in glutathione, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase and catalase levels along with increase in the levels of thiobarbituric acids (TBARS) were also observed in rat myocardium. In addition, DNA laddering observed on agarose gel electrophoresis and cardiac histopathology study further supplemented myocardial apoptosis. Pre-treatment with GPC significantly reduced DOX-induced cardiac toxicity, including improvement of hemodynamic variables and heart weight to body weight ratio, decreased serum Ca^{2+} level and LDH levels, myocardial caspase-3 levels, increased Na^+/K^+ ATPase levels and decreased myocardial TBARS levels and elevated antioxidant enzymes as compared to pathogenic control group. Further, the anti-apoptotic effect of GPC was verified by prevention of internucleosomal DNA laddering on agarose gel electrophoresis and attenuation of histopathological perturbations by doxorubicin. These observations demonstrate that GPC might serve as a cardioprotective formulation in DOX-induced cardiomyopathy in rats.

Keywords: Apoptosis, Cardiomyopathy, Doxorubicin, Gymnemic acid phospholipid complex, Oxidative stress

Cardiovascular diseases (CVD) are one of the major causes of death globally and they are estimated to be the leading cause of death in developing countries[1]. An integral part of pathogenesis of heart failure is myocyte loss. The recognition of a different genetically controlled programmed cell death phenomenon—Apoptosis—has become a major clinical interest. The presence and the role of apoptosis in CVD have been reported both in human retrospective and animal experimental studies[2,3]. Apoptosis was consistently noted in experimental models of heart failure in response to diverse forms of injurious stimuli like ischemia, ischemia-reperfusion, hypoxia, calcium excess, oxidative stress, rapid pacing, gene induction, sustained stretching, doxorubicin use, etc[4,5]. Antiapoptotic therapeutic interventions offer an appealing platform for devising ways to retard the maladaptative growth associated with cardiovascular diseases.

Doxorubicin (DOX) is one of the most effective and useful antineoplastic agent for the treatment of hematological as well as solid malignancies[6]. Cardiotoxicity of DOX is mediated by the formation of free radicals and oxidative damage to cellular components[7]. DOX is enzymatically reduced to the DOX semiquinone radical. This semiquinone radical directly transfers its electron to molecular oxygen, generating a superoxide and hydrogen peroxide[8]. Li *et al.*[9] have shown that acute doxorubicin cardiotoxicity involved cardiomyocyte apoptosis. The main cellular damage caused by reactive oxygen species (ROS) includes lipid peroxidation, protein cross-linking, and DNA fragmentation. These may lead to cardiac dysfunction, apoptosis, and development of cardiomyopathy[10]. DOX-induced

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cardiomyopathy is one of the major research tools to provide cardiotoxicity in the experimental animals[11]. Therefore, in the present study, doxorubicin was selected as cardiomyopathy model in rats. For the prevention of doxorubicin-induced cardiotoxicity, several compounds have been investigated to prevent such toxic effects with some degree of success. These include p-coumaric acid[12], garlic acid[7] erdosteine[13] and carvedilol[14]. Despite the large number of pharmacologic agents available for the treatment of cardiovascular disease, the search for additional drugs and pharmacotherapeutic approaches continues.

Gymnema sylvestre R. Br. (Asclepiadaceae) is commonly known '*Gurmar*' in Hindi for its distinctive property of temporarily destroying the taste of sweetness[15]. The plant extract has been reported to have anti-diabetic effect[16]. The anti-sweet properties of Gymnema have been attributed to a variety of compounds including a triterpene glycoside named gymnemic acid, a complex mixture of the active principles from a gymnema extract[17]. Gymnemic acids possess a wide range of medicinal properties. Ohmoria *et al*.[18] reported antioxidant activity of the aqueous extract of *G. sylvestre* against free radicals and low density lipoprotein (LDL) oxidation which is considered to be an important factor in the atherogenic progression of coronary artery disease. Galletto *et al*.[19] have investigated the antidiabetic and hypolipidemic potential of dried powdered leaves of *G. sylvestre* (GS). GS acutely did not influence the elevation of glycemia promoted by a balanced meal or by the administration of amylose or glucose. Daisy *et al*.[20] have reported normoglycemic and hypolipidemic effect of a novel dihydroxy gymnemic triacetate isolated from acetone extract of *G. sylvestre* R.Br. leaves on STZ-induced diabetic rats. The plasma glucose and insulin levels of the normal rats administered with dihydroxy gymnemic triacetate were not altered indicating its normoglycemic effect of the novel compound. Prakash *et al*.[21] have reported effect of feeding *G. sylvestre* leaves on blood glucose in beryllium nitrate treated rats. The feeding of powdered leaves of *G. sylvestre* in the diet of rats for 10 days (500 mg/rat per day, po) prior and 15 days after iv beryllium nitrate significantly protected the animals from the full fall of blood glucose seen in rats receiving beryllium nitrate alone. The feeding of the leaves for 25 days to normal rats did not alter blood glucose significantly. Chattopadhyay[22] studied the effect of water soluble fraction of alcoholic extract of *G. sylvestre* leaves (500 mg/kg, po) on glycogen content by isolated rat hemidiaphragm in normal and glucose fed hyperglycemic rats. The leaf extract by itself failed to alter the hepatic glycogen content in normal rats. In glucose fed rats, the leaf extract lowered the glycogen content of the tissue significantly and this was further lowered when both exogenous insulin and leaf extract were administered. The above studies indicated that *G. sylvestre per se* has no effect on blood glucose level in normal animals, ruling out the possibility of contribution of hypoglycemia in the present study.

The effectiveness of any herbal medicine is dependent upon delivering an effective level of the active compounds into the systemic circulation. Bioavailability of gymnemic acid is an important factor for its various pharmacological activities *in vivo*. Due to the complex structure and poor lipid solubility, permeation of the gymnemic acid to pass the biological membrane to be absorbed systematically following oral administration to produce any pharmacological effect, is poor. To overcome this limitation, development of a value added herbal formulation (gymnemic acid phospholipid complex; GPC) in combination with phospholipids has been made which has better absorption and pharmacodynamic profile. Phytosomes are advanced forms of herbal products that are better absorbed, utilized, and as a result produce better results than conventional herbal extracts[23]. In phytosome, the phyto-constituent or herbal extract is bound to phosphotidylcholine molecule, an emulsifying compound derived from soy. A phytosomal formulation is generally more bioavailable than a simple herbal extract or phytoconstituent due to its enhanced capacity to cross the lipid-rich biomembranes and finally reaching systemic circulation[24].

The aim of the present study is to examine the effect of gymnemic acid phospholipid complex (GPC), pretreatment in doxorubicin-induced cardiomyopathy model and to understand the molecular mechanism of its therapeutic effect with reference to anti-apoptotic potential, biochemical markers and antioxidant enzymes, lipid peroxidation and histopathological examination.

Materials and Methods

Drugs and chemicals—DOX was a kind gift from Dabur Research Foundation, Sahibabad, Ghaziabad, India. Caspase-3 colorimetric assay kit was purchased

from BioVision, U.S.A. LDH kit was purchased from Reckon Diagnostics Pvt. Ltd. Baroda, India and calcium content kit was purchased from Span Diagnostics Ltd. Surat, India. Soya phosphatidylcholine (Phospholipon 90H) was obtained as a gift sample from LIPOID GmbH, Germany. All the other chemicals used were of analytical grade and obtained locally. Double distilled water was used for all biochemical assays.

Plant material—Gymnema sylvestre leaves were purchased locally and authenticated by Dr. H.B. Singh, Raw Materials Herbarium & Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR, CSIR) New Delhi, India. Voucher specimens (voucher specimen no: NISCAIR/RHMD/Consult/-2008-09/980/11) were deposited in RHMD, NISCAIR, New Delhi, India.

Extraction and isolation of gymnemic acid—Gymnemic acid was isolated from *Gymnema sylvestre* as per Sinsheimer *et al*[25]. The dried leaves of the plant *G. sylvestre* were powdered mechanically with hands. Coarsely powdered leaves (1 kg) were extracted with ethanol (5 L) for 72 h. Initial defatting of the ethanolic extract of the leaves was carried out with petroleum ether (4 L) in a separating funnel. Ethanolic extract free from fatty materials was concentrated and dried in a china dish on a water bath (80°C). After that, it was dissolved in double distilled water (in the proportion of 100 mL of the above extract in 50 mL double distilled water) and filtered using Whatman filter paper (No. 44). To the filtrate 10% HCl was added to adjust the *p*H 2 which resulted in immediate precipitation of crude gymnemic acid as a green amorphous powder; the yield was 4.78% (w/w) of the dried leaves. It was allowed to stand for 30 min at room temperature. Precipitate was washed with double distilled water, collected and air dried at room temp. Because of the difficulty and tediousness of the isolation of each gymnemic acid, crude gymnemic acid isolated from *G.sylvestre* as described above was used.

Preparation of the phospholipid complex of gymnemic acid—Gymnemic acid phospholipid formulation i.e. complex of gymnemic acid with phospholipid was prepared by a novel and reproducible method[26]. The so prepared formulation was physicochemically standardized on the basis of apparent solubility study, ultra high performance liquid chromatography (UHPLC), differential scanning calorimetry (DSC), X-ray diffraction (XRD) studies, *in vitro* release study[27].

Experimental animals—Male Wistar albino rats weighing 200-250 g were maintained under standard laboratory conditions at 25°±2°C, 50±15% RH and normal photoperiod (12:12 h L:D cycle) for 7 days Commercial pellet diet (Nav Maharastra Chakan Oil Mills Ltd., New Delhi, India) and water was provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee of Hamdard University, New Delhi, which is registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India (Registration no. 173/CPCSEA, January 28, 2000).

Study design—Rats (40) were randomly divided into following 5 groups of 8 each: (i) Group-I (vehicle control rats; vehicle control), rats were treated with 0.5% carboxy methyl cellulose (CMC) in normal saline (2 ml/kg/po/day) for 30 days, (ii) Group-II (pathogenic control rats; DOX), rats were treated with 0.5% CMC in normal saline (2 ml/kg/po/day) for 30 days+doxorubicin (30 mg/kg, single ip injection) on 31st day, (iii) Group III (GPC 50), rats were treated with gymnemic acid phospholipid complex (50 mg/kg/po/day) for 30 days+doxorubicin (30 mg/kg, single ip injection) on 31st day, (iv) Group IV (GPC 100), rats were treated with gymnemic acid phospholipid complex (100 mg/kg/po/day) for 30 days+doxorubicin (30 mg/kg, single ip injection) on 31st day and (v) Group-V (phospholipid *per se*), rats were treated with phospholipid (Phospholipon 90H) (200 mg/kg/po/day) for 30 days. The dose of doxorubicin (30 mg/kg/single dose) was selected based on earlier study in which doxorubicin has shown significant acute-cardiotoxicity in rats[7]. Similarly, GPC doses (50 and 100 mg/kg/po) were based on an equivalent doses of gymnemic acid reported in rats[28,29]. The dose of phospholipid *per se* group was taken as an inert vehicle used for the preparation of gymnemic acid phospholipid formulation to see whether it has any effect *per se* in doxorubicin-induced cardiomyopathy in rats. The corresponding dose of 200 mg/kg was used that is used in the preparation of gymnemic acid phospholipid formulation highest dose (100 mg/kg).

Rats were kept on overnight fasting with water *ad libitum*. Hemodynamic measurements were carried out using tail cuff method on CODA non invasive blood pressure measurement instrument (Kent

Scientific, USA) on 32nd day and after that the blood was collected from the retro-orbital plexus of overnight fasted rats and serum separated for biochemical estimations. The rats were then sacrificed by cervical dislocation under light ether anesthesia. The hearts were weighed and homogenized for biochemical estimations in heart tissue.

Hemodynamic measurements—Every rat in each group was given training in the restrainer for 15 min every day at least 10-15 days prior to the day of measurement of the hemodynamic parameters (systolic, diastolic, mean blood pressures and heart rate). Hemodynamic measurements were carried out using tail cuff method on CODA non invasive blood pressure recorder instrument (Kent Scientific, USA).

Estimation of heart: body weight ratio—In each group, rats were taken for the estimation of heart weight: body weight ratio. Body weight was measured on the day of sacrifice. Heart weight was measured after keeping the heart in cold saline and squeezing out the blood.

Biochemical estimations—In serum, lactate dehydrogenase (LDH)[30] and serum Ca^{2+} were estimated[31]. A 10% homogenate of heart tissue in phosphate buffer (*p*H 7.4) was used for the assay of the malondialdehyde[32], and Na^+/K^+ ATPase[33,34]. The post mitochondrial superanatent (PMS) was used for the assay of reduced glutathione (GSH) content[35], activities of glutathione peroxidase (GPx)[36], glutathione reductase (GR)[37], glutathione-S-transferase (GST)[38], catalase (CAT)[39], superoxide dismutase (SOD)[40] and protein estimation[41].

Caspase-3 activity—Caspase-3 activity was measured using Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, USA) as per Gurtu *et al.*[42] and the activity was calculated as units/mg/protein/h.

Agarose gel electrophoresis—Apoptosis was evaluated by examining the characteristic pattern of DNA ladder generated in apoptotic myocardium using gel electrophoresis. Agarose gel electrophoresis study was carried out at Central Instrumentation Facility (CIF), Faculty of Science, Hamdard University, New Delhi. Myocardial samples were homogenized in solution containing 50 mmol/L Tris-HCl (*p*H 8.0), 100 mmol/L EDTA, 100 mmol/L NaCl, and 1% SDS. The tissue was digested with 5 µl of proteinase K (stock soln 20 mg/mL; Sigma Chemical Co.) at 56º C for 2 h and incubated with RNase A (1 µL/mL stock soln 1mg/mL; Sigma Chemical Co.) at 37° C for 1 h. After that, phenol/choloroform extraction was performed twice. The tissues were precipitated and centrifuged at 10, 000 rpm for 10 min. Supernatants containing DNA were precipitated with isopropanol (0.6 volume) and the resulting DNA pellets after centrifugation were washed with chilled 75% ethanol and dissolved in tris HCl and EDTA solution (TE solution; 10 mmol/L Tris HCl [*p*H 8.0], 1 mmol/L EDTA). DNA samples (5 µl) were subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide. DNA laddering, an indicator of tissue apoptotic nucleosomal DNA fragmentation was visualized and photographed under ultraviolet transilluminator (UVITEC Gel Doc systems, UVItec Limited, UK).

Histopathological studies—Animals were sacrificed on the day of withdrawal of blood, hearts were removed, washed immediately with saline and then fixed in 10% formalin and were embedded in paraffin, sections cut at 5 µm thickness and stained with hematoxylin and eosin (H & E). These sections were then examined under a light microscope for histoarchitectural changes. The sections were evaluated by histopathologist without prior knowledge of the treatments given to the animals. Sections were examined and cardiomyocytes exhibiting cytoplasmic vacuolization and/or myofibrillar loss were considered as damaged. The grading system was as follows: Grade 0 = no damage or focal myofibrillary loss; Grade 1 = focal myofibrillary loss in less than 5% cells; Grade 2 = focal myofibrillary loss in less than 10% cells; Grade 3 = focal myofibrillary loss and diffuse cell damage in above 10% cells.

Statistical analysis—The data are expressed as mean±SE. Statistical differences between means were determined by one-way analysis of variance (ANOVA), followed by Dunnett's-test. The values of $P<0.05$ were considered as significant.

Results

Effect of GPC on hemodynamic variables—Pathogenic control group rats showed a significant increase in arterial pressures and heart rate (Table 1). Thirty days pre-treatment of GPC (50 and 100 mg/kg) decreased all the parameters as compared to pathogenic treated group. Administration of phospholipid *per se* alone (200 mg/kg/day) did not evoke any significant changes in hemodynamic parameters ($P>0.05$).

Heart weight/body weight ratio—Gross size of heart from Gr.II was comparatively smaller than the vehicle control group and the GPC treated (50 mg/kg

and 100 mg/kg) groups and phospholipid *per se* group. After 24 h of a single injection of doxorubicin (30 mg/kg), there was a 10 % decrease in heart weight /body weight ratio in doxorubicin treated pathogenic group as compared with vehicle control rats, but these changes were not statistically significant. There was no significant difference in the heart weight: body weight ratio in Gr. V when compared to Gr I.

Effect of GPC on DOX-induced changes in various biochemical parameters—Intraperitoneal administration of DOX caused cardiotoxicity in all rats. Serum LDH and Ca^{2+} levels were significantly increased (3-folds and 1.4-folds) as compared with Gr. I. Pretreatment of the animals with GPC (50 and 100 mg/kg, po) before single injection of DOX reduced significantly (P<0.01) the increased levels of serum LDH and Ca^{2+} as compared to pathogenic control rats. Similarly, myocardial tissue thiobarbituric acid reactive substances (TBARS) and caspase-3 were significantly (P<0.01) raised in Gr. II as compared to Gr. I. GPC pre-treatment dose-dependently decreased the augmented TBARS and caspase-3 levels s compared to Gr. II. Myocardial level of Na^+/K^+ ATPase enzyme was significantly lowered in doxorubicin-treated Gr. II as compared to Gr.I. GPC (50 and 100 mg/kg/day) i.e. Gr. III and IV counteracted the deleterious effect of DOX by increasing the content of Na^+/K^+ ATPase. Phospholipid *per se* did not have any significant effect on above parameters (P>0.05) (Table 2).

Myocardial antioxidants—Pretreatment with GPC on various anti-oxidant parameters (reduced GSH, GPx, GR, GST, SOD and CAT enzymes) in doxorubicin-induced cardiomyopathy in rats is presented in Table 3.

Agarose gel electrophoresis—Doxorubicin-induced apoptosis was further confirmed by agarose gel electrophoresis (Fig. 1). DNA laddering with the lowest band below 200 bps, indicative of apoptotic inter-nucleosomal DNA fragmentation, was observed 24 h after DOX treatment. Ladders were not detected in Gr. I, III, IV and V.

Histopathological observations—Normal cardiac architecture with normal myofibrillar structure (grade 0) with striations and branched appearance and maintained myofibrillar structure integrity and intact sarcomeres were seen in photomicrographs of animals in Gr. I and V (Fig. 2a and e). Marked tissue injury with myofibril loss and focal cytoplasmic vacuolization in about 15% of cells (grade 3), disorganization and degeneration of myocardium with loss of striation and mild infiltration of cells was seen in Gr. II (Fig. 2b). Photomicrograph of Gr. III (Fig. 2c) displayed slight myofibrillar degeneration in less than 10% cells (grade 2) with slightly separated

Table 1— Effect of gymnemic acid phospholipid complex (GPC) pretreatment on hemodynamic variables in doxorubic in-induced cardiomyopathy in Wistar rats
[Values are mean ± SE from 8 animals each]

Groups	Systolic arterial pressure (mm Hg)	Diastolic arterial pressure (mm Hg)	Mean arterial pressure (mm Hg)	Heart rate (beats per min)
Gr.I (vehicle control)	127.83±6.86	96±4.65	106.16±5.00	421.83±15.77
Gr.II (pathogenic control)	192.83±17.65**	151.16±17.45*	164.83±16.96**	614±67.83**
Gr.III (GPC 50)	126.80±1.31$^{\#\#}$	93.00±2.18$^{\#\#}$	110.76±1.43$^{\#\#}$	462.18±11.57$^{\#\#}$
Gr.IV (GPC 100)	124.82±1.70$^{\#\#}$	89.62±2.66$^{\#\#}$	102.42±3.16$^{\#\#}$	438.83±13.83$^{\#\#}$
Gr.V (phospholipid *per se*)	123.16±3.84^{ns}	94.90±3.05^{ns}	107.83±3.74^{ns}	444.60±6.04^{ns}

P values: *< 0.05; ** < 0.01 when compared to Gr.I, $^{\#\#}$<0.01 when compared to Gr.II. ns = non significant ANOVA followed by Dunnett' s test.

Table 2—Effect of gymnemic acid phospholipid complex (GPC) pretreatment on various biochemical parameters in doxorubic in-induced cardiomyopathy in rats
[Values are mean ± SE from 8 animals each]

Groups	LDH level (IU/L)	Ca^{2+} level (mg/dL)	TBARS (nmol MDA/mg protein)	Sodium Potassium ATPase (Pi liberated/min/mg tissue)	Caspase 3 (nano mol/hr/mg protein)
Gr.I (vehicle control)	59.92±6.68	9.67±0.28	1.07±0.05	0.297±0.01	62.34±9.28
Gr.II (pathogenic control)	177.66±11.06**	13.74±0.24**	1.54±0.14**	0.178±0.007**	218.98±49.56**
Gr.III (GPC 50)	86.51±17.71$^{\#\#}$	11.13±0.36$^{\#\#}$	1.14±0.17$^{\#}$	0.269±0.12$^{\#\#}$	120.44±19.10$^{\#}$
Gr.IV (GPC 100)	81.27±7.65$^{\#\#}$	10.67±0.44$^{\#\#}$	1.10±0.09$^{\#\#}$	0.279±0.006$^{\#\#}$	87.86±24.09$^{\#\#}$
Gr.V (phospholipid *per se*)	59.08±8.32^{ns}	9.28±0.52^{ns}	1.04±0.01^{ns}	0.303±0.01^{ns}	43.10± 3.57^{ns}

P values: *< 0.05; ** < 0.01 when compared to Gr.I, $^{\#\#}$<0.01 when compared to Gr.II. ns = non significant ANOVA followed by Dunnett' s test.

Table 3— Effect of gymnemic acid phospholipid complex (GPC) pretreatment on heart tissue reduced GSH, GPx, GR, GST, SOD and CAT levels in doxorubicin-induced cardiomyopathy in rats
[Values are mean ± SE from 8 animals each]

Groups	Reduced GSH (nano moles of GSH/g tissue)	Glutathione Peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione Reductase (nmol NADPH oxidized/min/mg protein)	Glutathione-S-transferase (nmol CDNB conjugate formed min/mg protein)	SOD (U/mg protein)	CAT (nmol H_2O_2/mg protein)
Gr.I (vehicle control)	6.13±0.55	196.06±23.20	72.39±4.02	46.95±2.36	9.44 ±0.17	48.51±4.04
Gr.II (pathogenic control)	3.49±0.31**	134.8±15.7*	42.83±6.17**	29.98±1.90**	7.25±0.21*	22.32±1.96**
Gr.III (GPC 50)	5.34±0.62$^{\#\#}$	184.95±12.34$^{\#}$	63.80±13.58$^{\#}$	39.70±3.46$^{\#}$	7.84±0.37$^{\#}$	33.10±4.5$^{\#}$
Gr.IV (GPC 100)	5.84±0.65$^{\#\#}$	188.17±15.86$^{\#}$	69.63±11.56$^{\#\#}$	42.35±2.57$^{\#\#}$	8.27±0.80$^{\#}$	38.95±5.02$^{\#\#}$
Gr.V (phospholipid *per se*)	6.29±0.58^{ns}	199.12±7.90^{ns}	72.23±11.40^{ns}	47.99±4.18^{ns}	9.06±0.23^{ns}	48.06±5.87^{ns}

P values: *< 0.05; ** < 0.01 when compared to Gr.I, $^{\#}$<0.05; $^{\#\#}$<0.01 when compared to Gr.II. ns = non significant ANOVA followed by Dunnett' s test.

myofibrils and infiltrative cells. Heart of rats of Gr. IV exhibited organized myofibril arrangement (grade 0), no infiltrative cells present with very few areas of edematous changes (Fig. 2d).

Discussion

Heart failure is the final common pathway of diverse etiologies that are characterized by impaired systolic and/or diastolic function with high morbidity and mortality[43]. Cardiomyocyte apoptosis has been documented in many cardiovascular pathologies, including myocardial infarction, ischemia/reperfusion, end-stage heart failure and doxorubicin-induced cardiomyopathy. Many apoptotic stimuli in the heart have been recognized, theses include reactive oxygen species (ROS), calcium ion overload, mitochondrial dysfunction and loss of cardiomyocyte survival factors[44]. Caspases are the generally accepted executioners of apoptosis significant in executing cardiac myocyte death[45].

Doxorubicin (DOX) is a well known inducer of cardiomyopathy in rats[46]. DOX-induced cardiac injury results from summation and feedback of diverse effects, i.e., increased oxidative stress, increased membrane lipid peroxidation, inhibition of protein synthesis, abnormality in calcium handling, and impairment of mitochondrial enzymatic activity, in which apoptosis is primarily involved[47]. Prevention of apoptosis may be beneficial for treating cardiomyopathies.

Current treatment regimens require multiple medications to control symptoms and risk factors. Not only is heart disease life-threatening, its treatment is burdensome and expensive for patients. Herbal therapy may be an alternative strategy for the prevention and treatment of heart disease since herbal

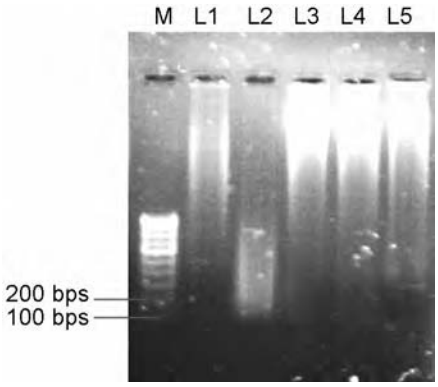

Fig. 1—Effect of GPC pretreatment on DNA fragmentation detected by agarose gel electrophoresis. Lane M = 100 bps ladder, L1 = Gr. I (vehicle control group), L2 = Gr. II (pathogenic control group), L3 = Gr. III (GPC 50 (50 mg/kg) treated group), L4 = Gr. IV (GPC 100 (100 mg/kg) treated group), L5 = Gr. V (phospholipid *per se* group)

medicines have been an essential component of oriental medicines, which existed for over many years.

Gymnema sylvestre used in the Ayurvedic system of medicine for the treatment of diabetes mellitus has been known from antiquity also to have an antisaccharin taste effect. Various hypoglycemic principles of *G. sylvestre* isolated from the saponin fraction of the plant are referred as gymnemosides and gymnemic acids[48]. However, there is no report of gymnemic acid in the prevention of doxorubicin-induced myocardial injury.

In the present study, doxorubicin produced a significant cardiomyopathy in doxorubicin-induced acute model (30 mg/kg/ip/single dose) in Wistar rats as evidenced by cardiac dysfunction, decreased heart weight to body weight ratio, increased serum Ca^{2+} and LDH levels, altered endogenous cardiac antioxidant defense system, increased cardiac lipid peroxide

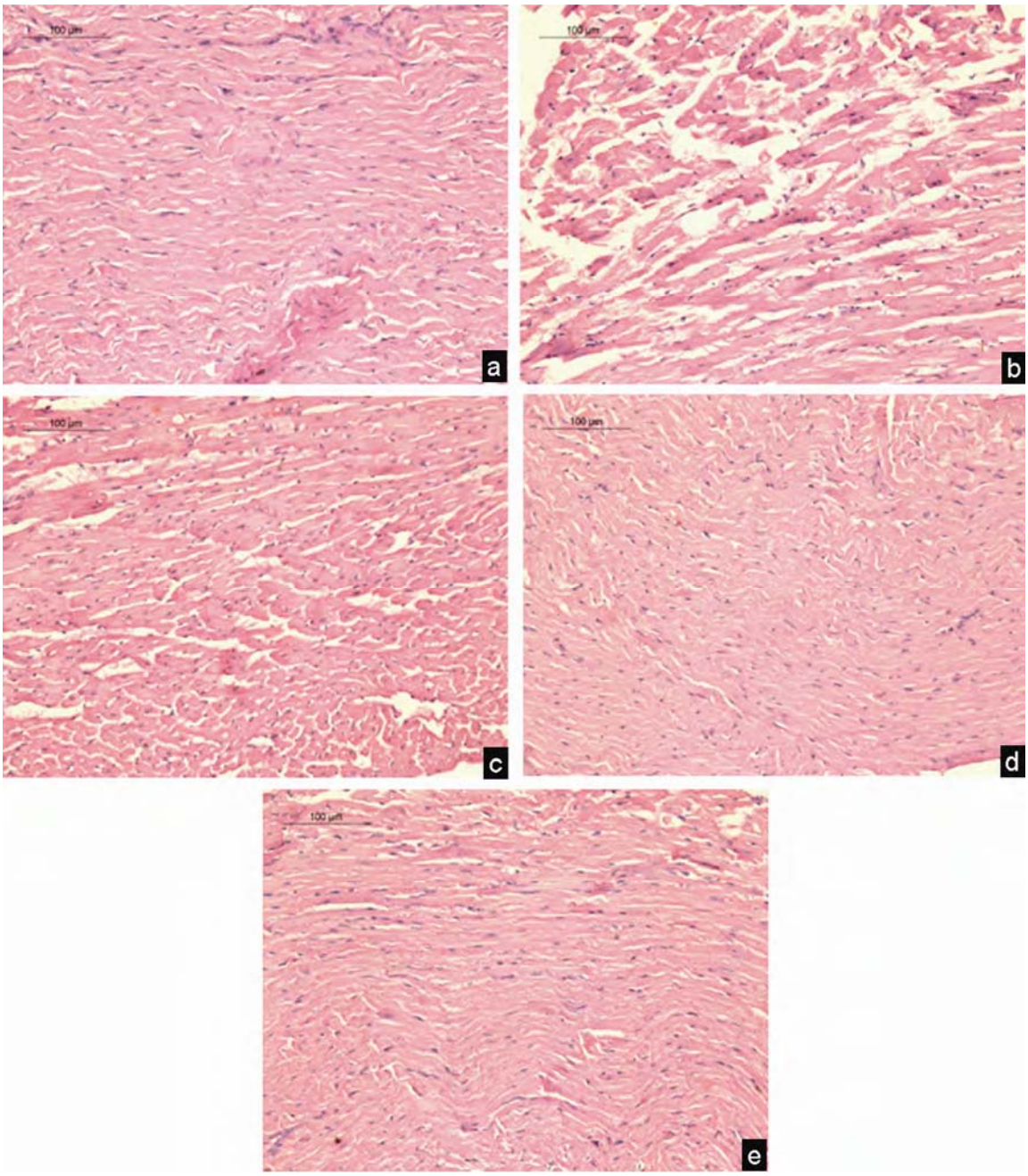

Fig. 2—Photomicrograph of rat heart from (a) Gr. I showing normal myocardium morphology and normal myofibrillar structure, (b) Gr. II showing marked tissue injury with myofibril loss and focal cytoplasmic vacuolization and mild infiltration of cells, (c) Gr. III showing slight disorganization myofibrils and myofibrillary loss in less than 5% cells and separated myocytes at few places, (d) Gr. IV showing regular myofibril arrangement and better-preserved appearance of cardiac muscle fibers with very few infiltrative cells, and (e) Gr. V showing regular cell distribution and normal myocardium (H&E; Scale bar=100 μm).

levels and cardiac caspase-3 levels and decreased cardiac Na^+/K^+ ATPase levels. The results were further supplemented by DNA agarose-gel electrophoresis and histopathological studies of cardiac tissue, which are in agreement with Mukherjee *et al.*[7], in which significant acute-cardiotoxicity was reported in rats 24 h after DOX treatment i.e. 30 mg/kg/ip/single dose. In the present study, pretreatment with gymnemic acid phospholipid complex (GPC) at a dose of 50 and 100 mg/kg efficiently suppressed cardiac injury induced by DOX. The protective effect of GPC against DOX-induced cardiovascular damage is primarily due its prominent anti-apoptotic activity.

Hemodynamic dysfunction provides some clues about the performance of the heart and properties of myocardial contractility. It is known that DOX affects hemodynamic variables[13]. In consistent with these findings, in the present study, DOX administration caused an increase in systolic, diastolic, mean BP and HR as compared to the normal healthy control rats. Accelerated myocardial apoptosis and catecholamine release following doxorubicin treatment may be responsible for such hemodynamic elevations. The present results suggest that gymnemic acid phospholipid complex (50 and 100 mg/kg/day) offers protection to the myocardium by attenuating the hemodynamic dysfunction through maintaining the hemodynamic parameters to near normal status in doxorubicin-treated rats. Another effect of DOX cardiomyopathy is characterized by decreased heart to body weight ratio[49]. DOX administration caused decrease in heart to body weight ratio, indicating loss of myofibrils and cytoplasmic vacuolization in myocytesc due to apoptosis. This decrease was prevented by GPC pre-treatment (50 mg/kg and 100 mg/kg) indicating that GPC pre-treatment improved the whole body condition of rat.

Cardiac tissue injury induced by DOX in rats was indicated by the elevated level of the marker enzyme such as serum LDH. In the present study, marked elevation (3 folds-increase) in the activities of LDH in the serum of doxorubicin intoxicated rats was observed (P<0.01) which is consistent with Gnanapragasam *et al*.[50] who reported 1.5 folds-increase in serum LDH level after a cumulative dose of doxorubicin 15 mg/kg. The rise in serum LDH level suggests an increased leakage of this enzyme from mitochondria into systemic circulation after DOX treatment. By treating the rats with GPC (50 and 100 mg/kg), LDH enzyme activity was decreased to a level near to that of vehicle control group. This suggests the cardioprotective effect of GPC against DOX toxicity.

Many apoptotic stimuli in the heart have been recognized, including oxidative stress, calcium overload, mitochondrial dysfunction, proapoptotic factors such as TNF-α, and loss of cardiomyocyte survival factors[44,51]. ROS may be the principal mediators of cardiomyocyte dysfunction in various cardiac pathologies causing apoptosis[52]. In the present study, DOX-treated animals showed an elevation in myocardial TBARS and depletion in myocardial GSH and endogenous antioxidant enzymes (GPx, GR, G-S-T, SOD and CAT) as compared to vehicle control group, which are important indices of oxidative damage. Decrease in the activities of these enzymes could be due to increased reactive oxygen free radicals, which can themselves reduce the activity of these enzymes[53]. The present results corroborate well with findings of Siveski-Iliskovic[54] and Mukherjee *et al*.[7] who demonstrated the involvement of oxidative stress and lipid peroxidation in doxorubicin induced cardiomyopathy. GPC exhibited significant antioxidant activity as it restored GSH levels and anti-oxidant enzymes activity and reduced lipid peroxidation compared to pathogenic control group.

Altered homeostasis of intracellular calcium within the myocyte may be alternate hypothesis to explain doxorubicin-related cardiotoxicity[55]. It implicates the disruption of mitochondrial calcium homeostasis, intracellular calcium overload and myocyte death[56]. In addition, increased intracellular calcium is associated with mitochondrial calcium accumulation and activation of caspases, which initiate apoptotic cell death[57]. This is consistent with the present findings where doxorubicin induced an elevation in serum calcium concentration significantly as compared to normal healthy control (P<0.01). GPC pretreatment lowered this increase in serum Ca^{2+} level.

K^+ is the predominant intracellular cation. Evidence suggests that K^+ efflux lead to a decrease in intracellular K^+ which may be a critical driver of apoptosis[58]. Doxorubicin damages cell membrane as evident from significant (1.6-folds) decrease in levels of membrane bound enzymes like $Na^{+/}K^+$ ATPase as compared to vehicle control (P<0.01). The present results are in agreement with the findings of Shah *et al*.[59] who also reported a 1.5-folds decrease in Na^+/K^+ ATPase level in the doxorubicin (10 mg/kg/iv/single dose) treated rats as compared to normal healthy rats. The decrease of ATPases on doxorubicin administration indicates enhanced lipid peroxidation by free radicals. Since this membrane bound enzymes are 'SH' group containing enzymes, so are lipid dependant. Oral administration of GPC (50 and 100 mg/kg) along with doxorubicin intoxicated rats, dose dependently maintained the concentration of Na^+/K^+ ATPase level at near control levels.

Caspases are crucial mediators of apoptosis. The increased production of ROS activates the Bcl-2 family proteins, the mitochondrial pathway, finally

activating caspases, the executionars of apoptotic pathway, leading to cellular hypertrophy, apoptosis and eventual heart dysfunction. In the present results, a 3.5-folds increase in caspase-3 levels in heart tissues of doxorubicin treated rats was observed, which corroborates well with the findings of Chularojmontri *et al*.[60] who reported 2.27-folds increase in caspase-3 level in doxorubicin-induced cardiotoxicity. Several studies indicate that caspases promote cardiac myocyte apoptosis. The cleaved, activated forms of caspase-3 were detected in explanted hearts in one study of human cardiomyopathy[61], and in another study of explanted hearts from patients with dilated cardiomyopathy[62]. GPC administration before doxorubicin treatment inhibited the apoptosis induced by doxorubicin treatment, by reducing the increased cardiac caspase-3 levels in a dose dependent manner.

Specific DNA fragmentation at nucleosomal units is one of the most characteristic biochemical features of apoptosis[63]. Apoptotic DNA fragmentation can be detected by using molecular biologic assays such as DNA gel electrophoresis. In the present study, apoptotic death of myocardial cells was demonstrated in DOX-treated rats by DNA agarose-gel electrophoresis method. Inter nucleosomal DNA fragmentation in doxorubicin-treated rats (i.e. Gr. II) was confirmed by the consistent observation of characteristic DNA ladders on agarose gel by agarose gel electrophoresis. Treatment with GPC (50 and 100 mg/kg) significantly decreased the appearance of DNA laddering in the left ventricular region of rat heart.

Histopathological findings of heart suggest that GPC pretreated groups attenuates the DOX-induced loss of myofibrils and vacuolization of the cytoplasm and showed no evidence of apoptotic damage. This further reconfirmed the protective action of GPC. Myocardium of phospholipid *per se* treated group showed normal cardiac fibers without any pathological changes. This indicates that phospholipid does not possess any adverse effects under normal conditions.

The present study put forward for the first time the antiapoptotic potential of gymnemic acid phospholipid treatment in doxorubicin-induced cardiotoxicity in rats and suggests that gymnemic acid have protective prophylactic effects against doxorubicin-induced cardiotoxicity and may have potential as a cardioprotective agent. In the light of these findings, present study supports the hypothesis of potential beneficial effects of gymnemic acid phospholipid complex on rat heart against experimental cardiomyopathy and apoptosis as evidenced by improved cardiac function, augmented enzymatic and morphologic biomarkers. The present study provides a lead for further exploring other mechanisms contributing to the cardioprotective effect of GPC. Whether the conclusions drawn on the basis of the current data can be extrapolated to clinical setting, remains to be defined by well controlled studies in-patients. Nonetheless, the results of the present study are rather encouraging, because they could unravel a new therapeutic approach for the prevention of cardiac diseases.

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