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Protective effect of *Phyllanthus fraternus* against mitochondrial dysfunction induced by co-administration of cisplatin and cyclophosphamide

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Abstract The evolving role of mitochondria, in mediating chemotherapy-induced apoptosis motivated us for the studies described here. The combination of cisplatin and cyclophosphamide is widely used in treating various types of cancers. The purpose of our study was to understand the mechanism of the toxicity induced by the co-administration of cisplatin and cyclophosphamide, on mitochondrial bioenergetics, and to study the protective effect of prior administration of the medicinal plant extract *Phyllanthus fraternus*. Our results reveal that co-administration of cisplatin (12 mg/kg, *i.p*) and cyclophosphamide (150 mg/kg, oral) to wistar rats (100 g) significantly alters mitochondrial structure and hence function. The rate of mitochondrial respiration was decreased significantly with both NAD + and FAD-linked substrates. The respiratory control ratio, an index of membrane integrity and the P/O ratio, a measure of phosphorylating efficiency also were decreased significantly accompanied by elevation in the lipid peroxide levels in liver, kidney homogenate and liver mitochondria respectively. Also, the phospholipid content of the mitochondrial membrane, showed a significant decrease, indicating mitochondrial membrane changes. Prior administration of an aqueous extract of *P. fraternus* (100 mg/kg) to rats, showed protection on all parameters investigated. Administration of *P. fraternus* alone did not show any significant changes on mitochondrial membrane bioenergetics. Thus, we propose, that the toxic side effects of cisplatin + cyclophosphamide, are due to a chain of interconnected events, within the mitochondrial inner membrane, ultimately leading to hepatotoxicity and nephrotoxicity. Further, our work also suggests that administration of aqueous extract of *P. fraternus* can enhance the therapeutic potential of anticancer drugs by reducing drug related toxicity.

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Keywords Cisplatin · Cyclophosphamide · *Phyllanthus fraternus (P. fraternus)* · Mitochondria,RCR · P/O ratio · Free radicals · Lipid peroxidation enzyme · Succinate · Cytochrome

Abbreviations

ADP	adenosine diphosphate
BSA	bovine serum albumin
EDTA	ethylene diamine tetra acetic acid
FAD	flavin adenine dinucleotide
MDA	malonaldehyde bis dimethyl acetal
NADH	nicotinamide adenine dinucleotide (reduced)
P/O	phosphate to oxygen ratio
RCR	respiratory control ratio
ETS	electron transport chain
ROS	Reactive oxygen species

Introduction

The realization that mitochondria are at the intersection of the life and death of a cell, particularly through the

involvement of mitochondrial damage in a range of diseases, has made them a promising target for drug discovery and therapeutic interventions (Anders et al. 2006). The mitochondria are a major producer of cell energy. This vital organelle plays a central role in cellular integrity,and many cellular poisons may target it (Szewczyk and Wojtczak 2002). Cisplatin remains one of the most effective antineoplastic drugs and it is frequently used for the treatment of various malignancies (Ali and Moundhri. 2006; Somani et al. 2000). Cisplatin is used alone or in combination with other agents in the treatment of many solid tumors and metastatic cancers (including ovarian, testicular, bladder, head and neck, lung, cervical, and breast cancers) that are no longer amenable to local treatments such as surgery and/or radiotherapy. Nephrotoxicity (Hanigan and Devarajan 2003) is the most common side effect but hepatoxicity was also observed with higher doses (Ali and Moundhri 2006). The mechanisms by which cisplatin induces cytotoxicity, namely hepatotoxicity and nephrotoxicity are not completely understood. Cyclophosphamide, an alkylating agent is one of the most widely used cytostatic drugs. The main use of cyclophosphamide is together with other chemotherapy agents in the treatment of lymphomas, some forms of leukemia (Shanafelt et al. 2007) and some solid tumors (Young et al. 2006). It is a “prodrug”; it is converted in the liver to active forms that have chemotherapeutic activity. In chemotherapy, it may be used alone, but more frequently is used concurrently or sequentially with other anticancer drugs.

Although there are many therapeutic strategies including chemotherapy to treat cancer, high systemic toxicity and drug resistance limit the successful outcomes in most cases. Accordingly, several new strategies are being developed to control and treat cancer. One such approach could be a combination of effective phytochemicals with chemotherapeutic agents, which when combined, would enhance efficacy while reducing toxicity to normal tissues (Pinmai et al. 2008). The plants of the genus *Phyllanthus* (Euphorbiaceae) are widely distributed in most tropical and subtropical countries, and have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes, and hepatitis B. In recent years, the interest in the plants has increased considerably. Substantial progress on their chemistry and pharmacological properties, as well as a few clinical studies of some *Phyllanthus* species have been made. Plants belonging to the genus *Phyllanthus*, have potential beneficial therapeutic actions in the management of hepatitis *B, nefrolitiase* and in painful disorders (Calixto et al. 1998).

In the present study the protective effect of *P. fraternus* on mitochondrial dysfunction induced by co-administration of cisplatin and cyclophosphosphamide on rat liver and kidney mitochondria was studied.

Materials and methods

Animals

Albino Wistar rats weighing 120±20 g were taken from the animal house facility of University of Hyderabad and checked for proper growth for at least 8–10 days. They were fed with commercial pellet diet and tap water ad libitum.

Preparation of the aqueous extract of *P. fraternus*

The whole plant of *P. fraternus* including roots were homogenized in water (5gm/12.5 ml) using a motor and a pestle. The homogenate was filtered through a cheese cloth. An aqueous extract equivalent to 10 mg dry powder of the plant/100 g body wt was administered daily to each rat. The dry wt content of the plant was determined after drying the plant in an oven and it was found to be 25% of the wet tissue (Sebastian and Setty. 1999).

Animal treatment

The animals were divided into four groups of six animals each.

Group 1: Rats received saline.

Group 2: Rats received single dose of cisplatin (12 mg/kg body weight, *i.p.*) and cyclophosphamide (150 mg/kg, oral). Rats were sacrificed 24 h after the administration of drugs.

Group 3: Rats received *P. fraternus* (10 mg/100 g body weight, oral).

Group 4: Rats received aqueous extract of *P. fraternus* (10 mg/100 g body weight, oral) for seven days and then received a single dose of cisplatin (12 mg/kg body weight, *i.p.*) and cyclophosphamide (150 mg/kg, oral). Rats were sacrificed 24 h after the administration of drugs.

Isolation of mitochondria

A slightly modified method of Lawrence and Davis (1986) was used for the preparation of mitochondria. Liver was homogenized followed by differential centrifugation in ice cold medium containing 220 mM d-mannitol, 70 mM sucrose, 2 mM HEPES, 0.2 mM EDTA and 0.36 mg/ml of BSA and adjusted to pH 7.4. The final pellet containing mitochondria was suspended in 3 ml of 0.25 M sucrose and the protein content was determined by Biuret method using BSA as a standard (Gornall et al. 1949). Mitochondria were used immediately for the measurement of oxidative phosphorylation and then were kept at −80 ◦ C until used for other studies.

Measurement of oxygen consumption

Polarographic determination of oxidative phosphorylation was carried out according to Estabrook (Estabrook. 1967) with a Gilson 5/6 oxygraph fitted with a Clark type of electrode.Respiration rates were measured at 25 ◦ C in a buffer (containing 50 mM sucrose, 50 mM Tris–HCl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM $MgCl_2$ chloride, pH 7.4) and 1–2 mg of freshly isolated mitochondrial protein using an oxygen electrode disc in an airtight chamber of 1 ml volume. Malate (4 mM) and glutamate (2 mM) or succinate (9 mM) was used as the substrates. Respiratory control ratio (RCR) was obtained from the ratio of ADP stimulated state-3 respiration to ADP exhausted state-4 respiration and the ADP/O = P/O ratio was calculated according to Estabrook (1967). Respiration was initiated by the addition of 9 mM sodium succinate or 2 mM glutamate plus 4 mM malate for succinate oxidase and NADH oxidase, respectively. State-3 respiration was measured by the addition of 200 and 400 nmol of ADP for succinate oxidase and NADH oxidase, respectively.

NADH Dehydrogenase: [NADH: (acceptor) Oxidoreductase, EC 1.6.5.3].

NADH dehydrogenase was measured using potassium ferricynide as electrode acceptor (King and Robert. 1967). The reaction system contained 250 mM sucrose, 30 mM Tris–HCl, 10 mM Potassium phosphate, 5 mM $MgCl_2$, 1 mM KCN, 1 mM Pottasium ferricynide, pH 7.4 and 20 μg of mitochondrial protein (frozen and thawed) in a reaction volume of 1 ml. The reaction was initiated by the addition of NADH (1.5 mM) and the rate of reduction of ferricynide was followed at 420 nm (E mM =1.0).

Succinate dehydrogenase: [Succinate :(Acceptor) oxido reductase, EC 1.3.5.1].

Succinate dehydrogenase was assayed using dichorophenol indophenol (DCPIP) as electron donor (King. 1967). The reaction system was the same as that used for the NADH dehydrogenase assay except that potassium ferricynide was substituted by 1 mM phenazine methosulfate (PMS) and 70 µM DCPIP. The rate of reduction of DCPIP was followed at 600 nm. 10 μg of mitochondrial protein was incubated with 10 µl of 0.5 M sodium succinate (pH=7.4) at room temperature for 10 min before assaying SDH activity (EmM=16.9).

Succinate- cytochrome c reductase : [succinate: Ubiquinone Oxidoreductase, EC 1.3.99.1].

The activity of Succinate-cytochrome *c* reductase was determined by the modified method of Tisdale (Tisdale 1967). The reaction was carried out in a total volume of 1 ml and consisted of buffer (10 mM Pottasium phosphate, 0.8 mM EDTA, 0.25 M Sucrose, pH 7.4) 5 mM succinate, 1 mM KCN, 2 nmoles of rotenone and 130 nmoles of ferricytochrome *c*. The reaction was initiated by adding 20 mg of mitochondrial protein. The increase in the absorbance at 550 nm was followed (EmM=19.1).

NADH-cytochrome c reductase: [NADH: Ubiquinone Oxidoreductase, EC 1.6.99.3].

NADH-cytochrome *c* reductase was determined by modified method of Hatefi and Reiske (Hatefi and Reiske. 1967). The reaction mixture consisted of (1.0 M potassium phosphate- HCl buffer). 1 M NaN_3, 1 mM EDTA, 1% deoxycholate, pH 8.0 and 1% ferricytochrome *c*. 20 µg of mitochondrial suspension was taken and the reaction was initiated by the addition of 10 mM NADH. After 15 s incubation at 30° C, the reaction was followed for 1 min by recording the increase in absorbance of cytochrome *c* at 550 nm. The activity of NADH-cytochrome *c* was deduced from increasing rate of absorbance (EmM=19.1).

Cytochrome c oxidase (EC 1.9.3.1).

The enzyme was assayed by following the decrease in the absorbance of ferrocytochrome *c* at 550 nm (Cooperstein and Liazarow 1951). The reaction was initiated by addition of 1 mg protein (EmM=19.1).

Preparation of reduced cytochrome *c*: 17 mg of Cytochrome *c* was dissolved in 20 ml of 30 mM Potassium Phosphate buffer, pH 7.4. It was then reduced by addition of small amounts of sodium dithionate (Yonetani 1967). Excess sodium dithionate was removed by dialysis against 30 mM phosphate buffer, pH 7.4 for 10–20 h with three to four changes of buffer.

Assay of lipid peroxides by Thiobarbituric acid reaction.

Lipid peroxide level was determined in liver and kidney homogenates and mitochondria (Ohkawa et al. 1979). A 10% homogenate was prepared in 1.15% KCl using a Potter Elevehjem homogenizer. Mitochondria were washed with 1.15% KCl and suspended in the same medium. Protein estimation was done by the Biuret method. To 5 mg protein, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5 with NaOH) and 1.5 ml of 0.67% (w/v) aqueous solution of thiobarbituric acid were added. The total volume was made up to 4.0 ml with distilled water and the tubes were heated in a water bath at 95 ^{0} C for 60 min using a marble as condenser. A blank was also run simultaneously and tetramethoxy propane was used as an external standard. After cooling, 1 ml of distilled water and 5 ml of n-butanol were added, vortexed and then centrifuged at 4000 rpm for 10 min at room temp. The absorbance of the organic layer was measured at 535 nm. The level of lipid peroxides are expressed as nmoles/100 mg protein.

Separation of mitochondrial phospholipids

Thin layer chromatography was used for the separation of phospholipids, Mitochondrial lipid were extracted by the procedure of Bligh and Dyer (Bligh and Dyer 1959). About 6–8 mg of mitochondria was used. The phospholipids were separated using Chloroform: methanol: water

(65:25:4). Inorganic phosphorus was estimated (Fiske and Subba Row 1925). It is expressed as phospholipid phosphorus/gm tissue.

Chemicals

Cisplatin [*cis*-Diamminedichloroplatinum (II)] and cyclophosphamide were purchased from local medical stores. ADP, NADH, bovine serum albumin (BSA), Hepes [N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)], EDTA (ethylenediaminetetraacetic acid) and cytochrome *c*, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sucrose and mannitol were purchased from MERK (Darmstadt, Germany). All the other chemicals were of research grade. Commercially reconstituted cisplatin solution was used. Cyclophosphamide solution was prepared by dissolving tablets in sterile double distilled water. Solutions were prepared with double distilled water.

Statistical analysis

All values were expressed as mean ± S.D. Statistical significance was calculated using students *t* test and $P<0.05$ was considered to be significant.

Results

The effect of co-administration of cisplatin and cyclophosphamide on the oxidative stress and the protective effect of an aqueous extract of *P. fraternus* on allyl alcohol-induced toxicity were studied. Results of all the parameters in this study were expressed relative to a control, which was taken as 100.

The effect of co- administration of cisplatin and cyclophosphamide was studied on rat liver and kidney mitochondria as a function of time. The results showed that the effects are significant at 24 h. Thus all the experiments using cisplatin and cyclophosphamide combination were carried out at 24 h after the administration of drugs. The effect of co-administration of cisplatin and cyclophosphamide on the oxidative stress and the protective effect of an aqueous extract of *P. fraternus* on the toxicity induced due to co-administration of cisplatin and cyclophosphamide were studied.

Externally added NADH cannot penetrate the tightly coupled mitochondria. So, glutamate and malate were used to reduce the NAD+ pool in the matrix, which is then oxidized by the respiratory chain. This NADH oxidase gives information on the ability of transfer of electrons through all three sites of the electron transport chain. There was 40%, 32% and 38% decrease in state 3, RCR and P/O ratio using glutamate+ malate (NADH oxidation) as substrate (Fig. 1). Administration of *P. fraternus* to rats for a period of 7 days before the co-administration of cisplatin and cyclophosphamide relieved the inhibition on state 3, RCR and P/O ratio's by 90%, 89% and 78% respectively (Fig. 1). While, the state3, RCR and P/O ratios were decreased by 44%, 33% and 30% respectively with the administration of anticancer drugs. Prior administration of *P. fraternus* could protect succinate oxidase enzyme from inhibtion to the extent of 75%, 100% and 100% respectively on state 3, RCR and P/O ratios (Fig. 2). Thus it may be concluded that administration of prior administration of *P. fraternus* extract can relieve the oxidative stress caused due to the combination chemotherapy with cisplatin and cyclophosphamide.

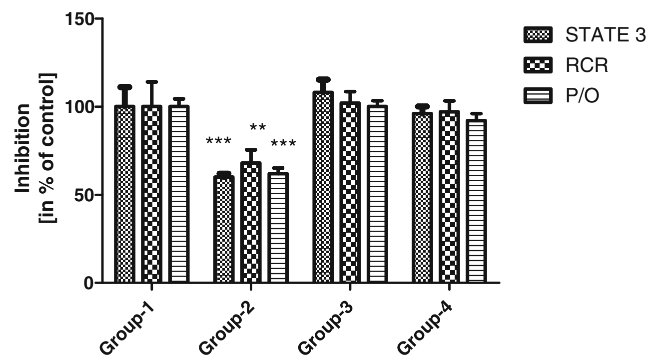

Fig. 1 Protective effect of *P. fraternus* on inhibition induced by co-administration of cisplatin and cyclophosphamide on NADH oxidase. All the values are expressed relative to the control, which is taken as 100. The control value for State -3 ADP respiration, RCR and P/O ratio was 72±7.9, 4.32±0.60 and 2.90±0.13 respectively. Values are Mean±SD for at least 13 animals. $*p<0.05$, $**p<0.01$ and $***p<0.001$

The activities of NADH dehydrogenase and succinate dehydrogenase was increased in liver (35% and 60%) and kidney (27% and 30%) mitochondria when compared to the control group. Increased activity of NADH dehydrogenase and succinate dehydrogenase was brought down by 86%, 89% and 64% and 46% respectively in liver and kidney mitochondria (Figs. 3 and 4).

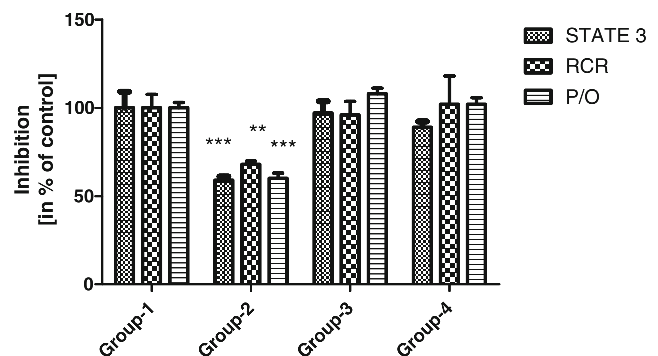

Fig. 2 Protective effect of *P. fraternus* on inhibition induced by co-administration of cisplatin and cyclophosphamide on succinate oxidase. All the values are expressed relative to the control, which is taken as 100. The control value for State 3 ADP respiration, RCR and P/O ratio was 120±11, 4.1±0.31 and 1.85±0.056 respectively. Values are Mean±SD for at least 13 animals. $*p<0.05$, $**p<0.01$ and $***p<0.001$

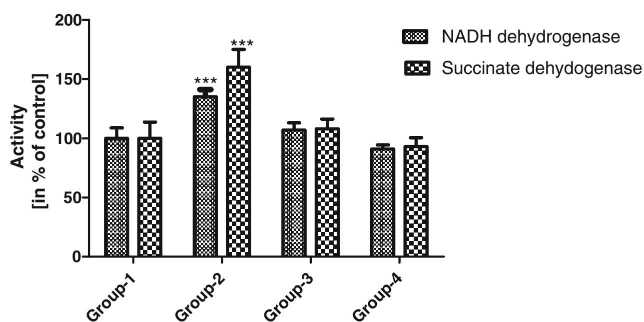

Fig. 3 Protective effect of *P. fraternus* against the effects induced by the co-administration of cisplatin and cyclophosphamide on NADH dehydrogenase and succinate dehydrogenase activity of liver mitochondria. Analytical and experimental conditions are described in Materials and Methods. All the values are expressed relative to the control, which is taken as 100. The control values of NADH Dehydrogenase and succinate dehydrogenase are 2750±243 and 146±20 respectively. Values are Mean±SD for at least 9 animals. **p*<0.05, ***p*<0.01 and ****p*<0.001

Individual specific activity of Complex-I (rotenone-sensitive NADH-Ubiquinone oxidoreductase, EC 1.6.99.3) II (succinte-ubiquinone oxidoreductase, EC1.3.99.1), IV (cytochrome *c* oxidase EC 1.9.3.1), which constitute the enzyme complexes of the mitochondrial electron transport chain (ETC) were assayed to estimate the toxicity of the anticancer drugs (cispaltin and cyclophosphamide) and the protection provided by prior administration of *P. fraternus*. Activities of NADH- cytochrome *c* reductase, succinate-cytochrome *c* reductase and cytochrome *c* oxidase showed inhibition by 34%, 50%, 53% in liver and by 26%, 46%, 54% respectively in kidney mitochondria (Figs. 5 and 6). Prior administration of *P. fraternus* resulted in protection over the inhibition caused by 100%, 81%, 82% and 83%,

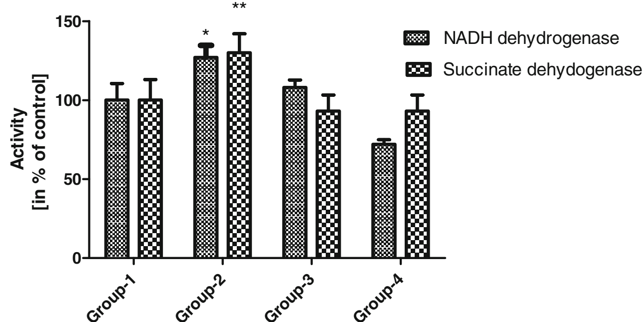

Fig. 4 Protective effect of *P. fraternus* against the effects induced by the co-administration of cisplatin and cyclophosphamide on NADH dehydrogenase and succinate dehydrogenase activity of kidney mitochondria. Analytical and experimental conditions are described in Materials and Methods. All the values are expressed relative to the control, which is taken as 100. The control values of NADH dehyrogenase and succinate dehydrogenase are 2030±214 and 107±14 respectively in kidney mitochondria. Values are Mean±SD for at least 6 animals. **p*<0.05, ***p*<0.01 and ****p*<0.001

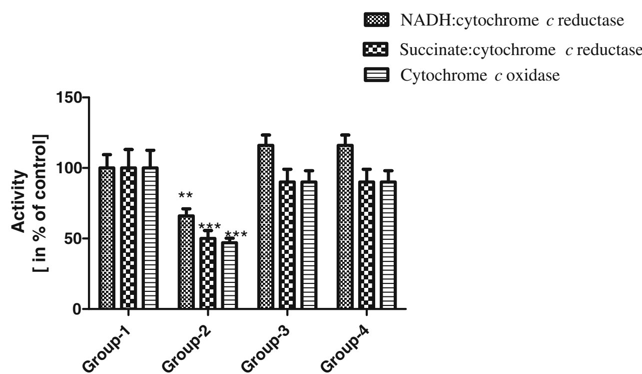

Fig. 5 Protective effect of *P. fraternus* against the effects induced by the co-administration of cisplatin and cyclophosphamide on NADH: cytochrome c reductase, succinate:cytochrome c reductase and cytochrome c oxidase of liver mitochondria. Analytical and experimental conditions are described in Materials and Methods. All the values are expressed relative to the control, which is taken as 100. The control values of NADH: cytochrome c reductase, succinate:cytochrome c reductase and cytochrome c oxidase 254±24, 49±6.4 and 1138±134. respectively in liver mitochondria. Values are Mean±SD for at least 6 animals. **p*<0.05, ***p*<0.01 and ****p*<0.001

60% and 80% respectively in liver and kidney mitochondria (Figs. 5 and 6).

Lipid peroxidation is a well-defined mechanism of cellular damage in both animals and plants that occurs in vivo during aging, disease states or drug assaults. Lipid peroxides are markers of oxidative stress. Effect of the co-administration of anticancer drugs cisplatin and cyclophosphamide with and without the prior administration of the aqueous extract of *P. fraternus* on lipid peroxide level was tested in liver homogenate, kidney homogenate and liver mitochondria. In the

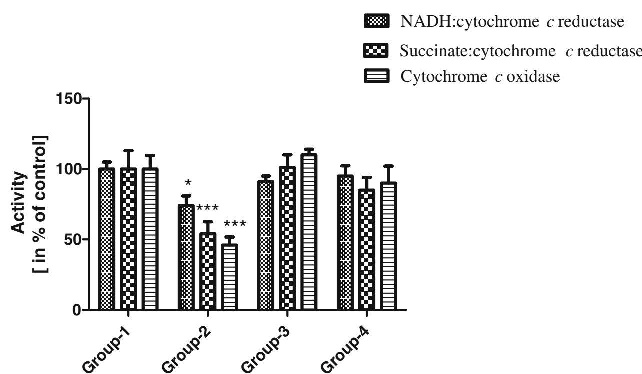

Fig. 6 Protective effect of *P. fraternus* against the effects induced by the by co-administration of cisplatin and cyclophosphamide on NADH: cytochrome c reductase, succinate:cytochrome c reductase and cytochrome c oxidase of kidney mitochondria. Analytical and experimental conditions are described in Materials and Methods. All the values are expressed relative to the control, which is taken as 100. The control values of NADH: cytochrome c reductase, succinate:cytochrome c reductase and cytochrome c oxidase 262±24, 33±4.04 and 884±86 respectively in liver mitochondria. Values are Mean±SD for at least 8 animals. **p*<0.05, ***p*<0.01 and ****p*<0.001

homogenate and mitochondria of liver the lipid peroxide level is significantly increased (44% and 35%, respectively). The same effect was observed in the kidney homogenate (36%). Prior administration of the aqueous extract of *P. fraternus* offered, complete protection, thus maintaining the levels of lipid peroxide to normal (Fig. 7)

Co-Administration of cisplatin and cyclophosphamide resulted in (32%, 31%, 31%) decrease in the content of phosphotidylcholine, phosphotidylethanolamine and cardiolipin content respectively, whereas the total phospholipid content showed a 13% decrease, when compared to control (Fig. 8). Prior adminstration of *P. fraternus* resulted in complete protection to the mitochondrial membrane from damage. Administration of *P. fraternus* alone did not show any significant changes on mitochondrial membrane bioenergetics.

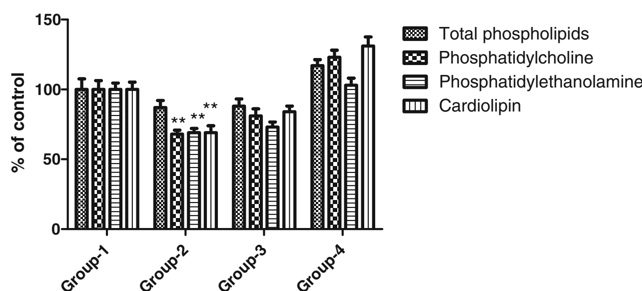

Fig. 8 Protective effect of *P. fraternus* against the effects induced by the co-administration of cisplatin and cyclophosphamide on the phispholipid composition of liver mitochondria. Analytical and experimental conditions are described in Materials and Methods. All the values are expressed relative to the control, which is taken as 100. The control values of are 24±1.8, 32±2.0, 26±1.2 and 36±1.0 for total phospholipids, phosphatidylcholine,phosphatidylethanolamine and cardiolipin respectively.Values are Mean±SD for at least 10 animals. **p*<0.05, ***p*<0.01 and ****p*<0.001

Discussion

The present study was planned to investigate (a) The mechanism of toxicity induced due to co-administration of anticancer drugs's cisplatin and cyclophosphamide on liver and kidney mitochondrial bioenergetics ultimately leading to hepatotoxicity and nephrotoxicity. (b) The extent of protection offered by prior administration of medicinal plant extract *P. fraternus* to rats followed by the treatment with anticancer drugs namely cisplatin and cyclophosphamide.

The mitochondrial respiration is tightly coupled to oxidative phosphorylation in intact and normal tissue (Tzagoloff and Mayers 1986). Mitochondrial respiration can be measured using an oxygraph apparatus (oxygen electrode) in which mitochondria when incubated in an isotonic medium containing substrate and phosphate, the addition of ADP causes a sudden burst of oxygen uptake as the ADP is converted into ATP. The actively respiring state is referred to as "state 3" respiration, while the slower rate after all the ADP has been phosphorylated to form ATP is referred to as "state 4". The ratio of state 3 rate to state 4 is called the respiratory control ratio (RCR) and indicates the tightness of the coupling between respiration and phosphorylation. Typical RCR values range from 3 to 10, varying with the substrate and the quality of the preparation. Another parameter defining the ability of mitochondria to face an ATP depletion is the ratio of ADP used (equivalent as mole of ATP synthesized) to consumed oxygen atom (P/O). Co-administration of cisplatin (12 mg/kg, *i.p*) and cyclophosphamide (150 mg/kg, oral) to rats significantly altered mitochondrial membrane integrity resulting in a decreased state 3, RCR and P/O ratios resulting in loss of the ions that cause uncoupling of mitochondria. A decrease of RCR and P/O indicates a loss of the coordination between the activity of the oxidative chain (complex I to IV) and the ATPase (complex V). In mitochondria the respiratory chain is stochiometrically related to that of ATP synthesis (Pozzan et al. 1979). If the substrate is oxidized at all three sites of the electron transport chain, three ATP/atom of oxygen are generated (NADH Oxidation). On the other hand if it is oxidized in two sites (site 2 plus site 3), only two ATP/atom of oxygen (succinate oxidation) are generated and oxidation of ferrocytochrome *c* involves only one site (site 3) and thus generates only one ATP/atom of oxygen. The observed decrease in the P/O ratios with the co-administration of cisplatin and cyclophosphamide indicates that most of the energy that is liberated by substrate oxidation could not be utilized for phosphorylation, resulting in loss of energy in the form of heat which would stimulate the body to lose heat in the form of sweating. The low P/O ratios will also affect the vital reactions in the cell. This appears to be the possible mechanism for the observed side effects in chemotherapy such as fatigue, fever, nausea etc. The observed decrease in P/O may

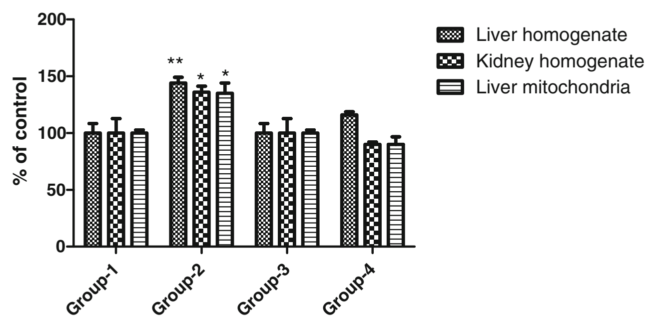

Fig. 7 Protective effect of *P. fraternus* against the effects induced by the by co-administration of Cisplatin and Cyclophosphamide on lipid peroxide levels.. Analytical and experimental conditions are described in Materials and Methods. All the values are expressed relative to the control, which is taken as 100. The control values of are 139±11.7, 149±19 and 76±2.1 for liver homogenate, Kidney homogenate and liver homogenate respectively. Values are Mean±SD for at least 8 animals. **p*<0.05, ***p*<0.01 and ****p*<0.001

be due to site electron leak in the respiratory chain. This type of electron leak can lead to univalent electron transfer to oxygen at a site other than cytochrome aa3 resulting in free radical generation and leading to ATP depletion through an uncoupling effect on oxidative phosphorylation. Previous studies on cisplatin have reported oxidative damage as the mechanism of cisplatin-induced renal cell death in vitro as well as in vivo (Kameyama and Gemba. 1991; Zhang and Lindup 1994; Sugihara et al. 1987; Gemba et al. 1988). However, these studies could not establish the relationship between mitochondrial dysfunction and oxidative damage.

NADH dehydrogenase introduces electrons to the electron transport chain at site 1. In the present study the activity of NADH dehydrogenase activity was stimulated (Fig. 3). The observed stimulation may be due to a decreased phospholipid content (Fig. 8) as it is possible that changes in the phospholipid composition may influence the movement of electrons from NADH to ubiquinone resulting in stimulation of activity (Yu et al. 1978). An understanding of the lipid-protein interactions within Complex-I and their influence on the kinetics of electron flow needs to be investigated further.

Succinate dehydrogenase is a membrane-bound component of the respiratory chain of aerobic organisms. It couples the reduction of ubiquinone (Q) to the oxidation of succinate and is, as such, a Krebs cycle as well as a respiratory chain enzyme. This duality suggests a potentially important role in the control of energy metabolism, a reason why the enzyme's regulation is important. In the present study the activity of succinate dehydrogenase increased significantly. Activation of succinate dehydrogenase was reported on the beef heart enzyme by Kearney (Kearney 1957). He reported that there are two observations that a process of activation may involve; The first of these is a very high energy of activation of the process, which suggests that one or more relatively strong bonds, or several weaker bonds, in the enzyme are altered in the transition to the activated form. The second observation is the change in the absorption spectrum which occurs simultaneously with the increase in enzymatic activity. While the spectral change does not permit definite localization of the groups involved in the transformation, it seems probable that it occurs somewhere in the vicinity of the iron-protein bonds, which appear to be responsible for the majority of the color of the enzyme over the entire spectral range affected by the activation. Our study on the effects of the co- administration of cisplatin and cyclophosphamide to rats observed stimulation in the activity of succinate dehydrogenase which may be due to alteration of strong bonds or weak bonds with in the enzyme as reported by Kearney (Kearney 1957). We propose that the stimulation in the activity of succinate dehydrogenase may be due to increased permeability of the mitochondrial membrane due to administration of cisplatin and cyclophosphamide as cisplatin induced permeability is already reported (Custódio et al. 2009). This increased permeability may result in substrate activation of succinate dehydrogenase as reported by (Levrat and Louisot 1994). The exact mechanism of stimulation needs to be investigated further and appears to play an important role biochemically in drug induced hepatotoxicity and nephrotoxicity.

In the present study on one hand we have observed stimulation of NADH dehydrogenase and succinate dehydrogenase and on the other hand, inhibition in activities of Complex-I (rotenone-sensitive NADH-Ubiquinone oxidoreductase, EC 1.6.99.3), II (succinte-ubiquinone oxidoreductase, EC1.3.5.1) and IV (cytochrome *c* oxidase EC 1.9.3.1). These findings can be further correlated to the observed decrease in the rate of respiration (Fig. 1) induced by the co-administration of cisplatin and cyclophosphamide. The cytochromes contain iron which is highly vulnerable to reactive oxygen species. Inhibition in the activity of cytochrome *c* oxidase indicates that the transfer of electrons through complex IV is hampered (Fig. 5). The observed inhibition can be attributed to altered phospholipid concentration, as peroxidation induced on the molecular species phosphatidylcholine and phosphatidylethanolamine containing only unsaturated fatty acids was accompanied by losses in enzyme activities of NADH- cytochrome *c* reductase and succinate- cytochrome *c* reductase (Santiago et al. 1977).

There was a significant increase in the free radicals in liver homogenate and kidney homogenate respectively with the co-administration of cisplatin and cyclophosphamide (Results not shown). This finding is in agreement with previous reports where cisplatin and cyclophosphamide when, administered individually, resulted in the production of reactive oxygen species leading to the depletion of the mitochondrial antioxidant defense system in liver (Santos et al. 2007; Stankiewicz et al. 2002).

These free radicals would lead to mitochondrial dysfunction (Sangeeta et al. 1990; Hanaki et al. 1990 and Sugiyama et al. 1989). This appears to be the likely mechanism of inhibition in the activities of the respiratory chain enzymes like NADH-cytochrome *c* reductase and succinate- cytochrome *c* reductase, as free radicals can induce metal catalysed oxidation within the active centers of the respiratory chain complexes initiating a chain reaction involving the protein peroxides within the enzyme complexes, finally leading to inactivation of proteins (Simpson et al. 1992; Davis et al. 1995).

Apart from the significant increase in free radicals the level of lipid peroxides also increase in the liver homogenate, liver mitochondria and kidney homogenate of rats treated with cisplatin and cyclophosphamide, and this can be related to the toxic potential of the combination of cisplatin and cyclophosphamide as is the case for another anticancer drug doxorubicin (Ogawa et al. 1987). Increased lipid peroxide levels may lead to hepatocellular injury

(Pompella et al. 1991). As enhanced levels of lipid peroxides were found in both liver and kidney homogenates it can be concluded that oxidative stress leading to increased free radical production and thus higher lipidperoxide levels is the mechanism of hepatocellular or nephrotoxic injury/toxicity. The observed decrease in the content of phosphotidylcholine, phosphotidylethanolamine and cardiolipin content will in turn result in the enzymatic degradation of proteins of mitochondria (Kozel'tsev et al. 1980) leading to altered membrane bioenergetics ultimately leading to hepatotoxicity and nephrotoxicity. Studies with cisplatin alone results are in conformation with earlier studies on cisplatin mediated toxicity. These have shown that cisplatin caused liver injury by promoting mitochondrial structural and functional damage, depleting the antioxidant defense system with consequent redox status alteration, oxidation of mitochondrial proteins and lipids, including cardiolipin, and finally cellular death by apoptosis (Martins et al. 2008).

Although there are many therapeutic strategies including chemotherapy to treat cancer, high systemic toxicity and drug resistance limit the successful outcomes in most cases. Accordingly, several new strategies are being developed to control and treat cancer. One such approach could be a combination of effective phytochemicals with chemotherapeutic agents, which when combined, would enhance efficacy while reducing toxicity to normal tissues. The plants of the genus *Phyllanthus* have long been used in folk medicine to treat, among others, kidney and urinary bladder disturbances, intestinal infections, diabetes, and hepatitis B. An excellent review on the medicinal value of the plants belonging to this genus is that of Calixto and his group (Calixto et al. 1998). In recent years, the interest in the plants of the genus *Phyllanthus* has increased considerably, especially regarding their therapeutic potential for the management of many diseases. Several reasons contribute to this, such as: (1) their greater distribution in many tropical and subtropical countries, (2) the great number of species in this genus, (3) their broad therapeutic use in folk medicine, and (4) the greater diversity of secondary metabolites present in such plants. Clinical studies carried out with *P. niruri* and *P. amarus* revealed that their extracts are well-tolerated in human beings with no evidence of side effects. To date, reported clinical studies on *Phyllanthus* plants have focused their potential clinical utility on the treatment of hepatitis B and nephrolitiase (Calixto et al. 1998).

Our results demonstrates that administration of *P. fraternus* (100 mg/kg) prior to administration cisplatin and cyclophospamide protected mitochondria against free radical damage induced by the drugs, which resulted in significant protection from the inhibition observed with glutamate or as substrate. Increased activity of NADH deydrogenase and succinate deydrogenase were brought down . The protection over inhibition was seen more on liver mitochondria compared to kidney mitochondria. The inhibition on NADH-cytocrome *c* reductase, Succinate- cytocrome *c* reductase and cytochrome *c* oxidase was relieved by 100%, 81% and 82% respectively in liver mitochondria and 83%,60% and 80% respectively in kidney mitochondria. Prior administration of *P. fraternus* to rats completely protected increased levels of lipid peroxides and decreased content of phospholipid composition. These results are in agreement with the earlier reports demonstrating the protective effect of *P. fraternus* against CCl_4 (Padma and Setty. 1997, Manjrekar et al. 2008) and chronic alchoholism (Sebastian and Setty 1999). Nimsulide (Chatterjee and Sil 2006) allyl alcohol-induced oxidative stress in liver mitochondria (Sailaja and Setty. 2006) induced oxidative stress. Although further investigations are required to understand the exact mechanism of action, the protein fraction of *P. niruri* was shown to protect liver tissues against oxidative stress in mice, probably acting by increasing antioxidative defense (Bhattacharjee and Sil 2006). Therefore, studies relating to chemical characteristics and structural properties of the bioactive phytochemicals found in *P. fraternus* are very useful for further research on this plant as phytochemical studies carried out on these plants isolate and characterize classes of compounds, including alkaloids, flavonoids, lignans, phenols, and terpenes. These seem to be mainly responsible for the pharmacological actions reported in relation to these plants. Most of these compounds were found to interact with most key enzymes, such as aldose reductase, angiotensin converting enzyme, mitrochondrial ATPase, both cylo and lipooxygenases, phospholipase A2, tyrosine kinase, reverse transcriptase, and phosphodiesterases. Finally, the clinical use of these plants as phytomedicinals will depend on the establishment of sensitive analytical methodologies necessary for standardization of the numerous secondary metabolites existing in such herbals preparations.

In the light of all these aspects, the present studies suggest that that prior administration of an aqueous extract of *P. fraternus* has the potential to reduce toxic side effects related to mitochondrial dysfunction due to co-administration of cisplatin and cyclophosphamide. In order to reduce the renal and hepatic damage induced by cisplatin, antioxidant therapy targeted to mitochondria was suggested, to ameliorate both the toxicities simultaneously (Martins et al. 2008). The toxic effects of cyclophosphamide was related to its lipid peroxidation induction potential of cyclophosphamide. Furthermore, the antiperoxidative effect of ascorbic acid was suggested as a reason to prevent cyclophosphamide induced lipid peroxidation (Supratim et al. 2005).

Our findings shows are that the toxic side effects induced by administration of cisplatin and cyclophospamide on normal

cells can be ameliorated with prior administration of aqueous extract of *P. fraternus* and can play a crucial role in reducing dose dependent drug induced toxicity. Further investigations are needed to determine the phytochemicals responsible for this protective effect.

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