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RESEARCH ARTICLE

GANODERMA LUCIDUM (FR.) P. KARST AMELIORATE MITOCHONDRIAL DYSFUNCTION IN DOXORUBICIN-INDUCED CARDIOTOXICITY IN RATS.

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Abstract

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Introduction:-

Doxorubicin (DOX) is an anthracycline antibiotic widely used in cancer chemotherapy. However , clinical usefulness of this antineoplastic agent is delimited by severe and cumulative cardiotoxicity. It is broadly accepted that myocardial oxidative stress and the accumulation of free radicals are involved in DOX induced cardiotoxicity (Hellmann *et al* ,1998). Mitochondria are known as the ‘power house’ of the cell. They are abundant in cardiomyocytes . More than 90% of the energy utilized by cardiomyocytes is produced by mitochondrial respiration which is tightly coupled to the tricarboxylic acid (TCA) cycle .

Mitochondria are considered as one of the major target in DOX chemotherapeutic regiments . DOX antineoplastic activity is occurred through DNA – Topoisomerases II α adduct formation (Khiati *et al* ;2014). DOX - β adduct induced cardio toxicity arises in different ways ; deleterious free radical generation by complexes with iron , redox cycling , mitochondrial dysfunction , DNA damage , alterations in protein and lipid, calcium overload etc. Mitochondrial respiration is generally recognized as the prime source of reactive oxygen species (ROS). Increased mitochondrial oxidative stress results in mitochondrial proteins damage and lipid peroxidation. ROS generation mainly occur through Complex I and III. During normal mitochondrial respiration , about 1% of oxygen is converted in superoxide radical (O₂⁻) which is further converted into hydrogen peroxide (H₂O₂) which is able to cross the membranes and react with free metal ions to form the hydroxyl radical (HO \cdot). In general, free radicals generated within the mitochondria are neutralized by their own antioxidant defence system (Tsutsui *et al* ;2008). A chronic increase of mitochondrial free radical production in the heart alters this redox balance. Among the previously mentioned reasons , DOX-induced cardiotoxicity has a strong mitochondrial component . Mitochondria from DOX-treated animals show traces of the drug, which might be due to its binding affinity for particular biomolecules within the organelle. As with nuclear DNA, DOX also forms adducts with mitochondrial DNA (mtDNA) (Minotti *et al* ; 2004), cardiomyocyte TOP II α mechanism . Goormaghtigh *et al* (1990) reported that DOX form complexes with Cardiolipin , one of the most abundant phospholipid in the inner mitochondrial membrane. Accumulated DOX in the cardiomyocyte initiate direct and delitorious side effects include : a) stimulation of ROS production, b) induction of MTP(Mitochondrial Transition Permiability) and c) inhibition of OXPHOS (oxidative phosphorylation) etc .

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Ganoderma lucidum (Fr.) P.Karst - is well known in vivid names such as Lingzhi, Reishi and Youngzhi in Asian countries and that has been used as a folk medicine to preserve human vitality and to promote longevity in traditional Chinese Medicine over 2000 years. Now it is popularly used as a nutraceutical in Western countries (Sliva *et al* ;2004). Both the fruiting body and mycelia of *G. lucidum* are reported to contain a variety of chemical components including triterpenes, polysaccharides, nucleosides, steroids, fatty acids, alkaloids, proteins, peptides, amino acids etc (Wasser, 2010). Among these polysaccharides and triterpenes are the major components. Previous investigations in our laboratory showed that *G. lucidum* occurring in south India possessed significant antioxidant, anti-inflammatory, anti-tumor, anti-nociceptive, nephroprotective, anti-aging and radioprotective properties (Lakshmi *et al* ; 2003, Smina *et al* ; 2011). Sudheesh *et al* (2010) reported that fruiting bodies of *G.lucidum* ameliorated the dysfunctions in heart mitochondrial enzymes and respiratory chain complexes in aged rats. However, the effect of *Ganoderma* on the activities of Krebs cycle dehydrogenases or enzymes complexes of electron transport chain (ETC) has not reported in DOX induced toxicity.

Materials and Methods:-

Animals:-

Male Wistar rats weighing 220 ± 20 g were purchased from Small Animal Breeding Centre, Agricultural University, Mannuthy, Thrissur, Kerala, India. Animals were kept for a week under standard environmental conditions with free access to food (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. Animal experiments were carried out following the guidelines of CPCEA (Government of India) and approval of the Institutional Animal Ethics Committee (IAEC) sanction No .149/1999/CPCSEA, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India.

Chemicals :-

Doxorubicin hydrochloride (Doxowin), Wintac Pvt .Ltd. Bangalore, India was purchased from the local market. All other chemicals and reagents used in the experiments were of analytical grade.

Preparation of Extracts of *G. lucidum*:-

Sporocarps of *G. lucidum* growing on the living *Caesalpinia coriaria* Wild. trees in Thrissur District, Kerala, India were collected. The specimen was identified and the identification was confirmed by available literature.

The fruiting bodies were cut into small pieces and dried at $45-50^{\circ}\text{C}$ for 48 hrs and powdered. One hundred gram samples of powdered materials were defatted with petroleum ether using Soxhlet apparatus. The defatted material was again extracted with 70 % aqueous ethanol for 12 hrs. It was filtered through Whatman No. 1 filter paper. The solvent was evaporated at 40°C using a rotary vacuum evaporator and finally lyophilized. The yield of the extract (GLF) was 3.42 %.

Effect of *Ganoderma lucidum* on the mitochondrial dehydrogenases and respiratory complexes in the heart of Doxorubicin induced Rats:-

The animals were divided into 5 groups of six animals each and treated as follows. Group I treated with distilled water was kept as normal. Group II treated with DOX (i. p) was kept as control. Group III and IV were administered with GLF at concentrations of 250 and 500 mg/kg respectively. Group V was administered with DL – α – Lipoic acid; 100 mg/kg (dissolved in 0.5 % NaOH) and taken as standard. The DOX was administered once daily (6 mg/kg) i. p 0.75 ml for 3 alternative days (cumulative dose of 18 mg/kg) to group II, III, IV and V. The extracts were given once daily for five days prior to DOX injection to group III and IV and continued along with DOX injection for three more days. The animals were sacrificed 24 hr after last dose of treatments. Heart was immediately excised, washed and kept at -70°C for mitochondrial analyses.

Preparation of mitochondrial fraction:-

Heart tissue homogenate of rats (about 10%) was prepared in 50 mmol/l phosphate buffer ($\text{pH } 7.0$) containing 0.25 mol/l (w/v) sucrose. Homogenate was centrifuged initially at 1,000 g for 10 min at 4°C and the supernatant was subjected to 16,000g for 20 minutes at 4°C in a cooling centrifuge. The mitochondrial pellets were washed twice with phosphate buffer to remove the sucrose and suspended in 2 ml phosphate buffer. Mitochondrial fraction was frozen and thawed 3-5 times to release the enzymes (except complex IV which was extracted with 0.5% Tween 80 in phosphate buffer, v/v). The protein content was estimated in the supernatant according to the method of Lowry *et al* (1951).

Determination of the effect of *G.lucidum* on the activity of TCA cycle enzymes:-

Isocitrate dehydrogenase (ICDH) activity was estimated according to the method of Fatania *et al* (1993) and the activity was expressed as micromoles of NAD^+ reduced /min/mg protein using extinction coefficient $6.3 \text{ mM}^{-1} \text{ cm}^{-1}$. α -Ketoglutarate dehydrogenase activity (α -KGDH) activity was estimated by the method of Reed and Mukherjee (1969). The activity was expressed as μmoles of NAD^+ (reduced /min/mg protein) using extinction coefficient of $6.3 \text{ mM}^{-1} \text{ cm}^{-1}$. Succinate dehydrogenase (SDH) activity was estimated by the method of Nulton-Persson and Szweida (2001) with slight modifications. The activity of SDH was calculated using the extinction coefficient of 2,6-dichlorophenol indophenol (DCIP) ($19.1 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as μmoles of DCIP reduced /min/mg protein. Malate dehydrogenase (MDH) activity was estimated by the method of Mehler *et al* (1948) and the activity was expressed as μmoles of NADH oxidized /min/mg protein using the extinction coefficient of NADH $6.3 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of the effect of *G.lucidum* on the activity of respiratory complexes:-**Determination of Complex I**

Complex I activity was estimated by the method of Janssen *et al* (2007). Briefly $1 \mu\text{mol/l}$ antimycin A, 3 mg BSA, 2 mmol/l KCN, 5 mmol/l MgCl_2 , 65 $\mu\text{mol/l}$ ubiquinone, 80 $\mu\text{mol/l}$ DCIP and mitochondrial protein (approximately 40 μg) were mixed with phosphate buffer (25 mmol p^{H} 7.2) in a net volume of 1 ml. Absorbance at 600 nm was monitored at 15 s interval for 2 min at room temperature after the addition of NADH (0.2 mmol/l). After 2 min 1 $\mu\text{mol/l}$ rotenone was added and the absorbance was measured again for 2 min with an interval of 30 s. The linear difference in absorbance was calculated before and after the addition of rotenone. The activity was expressed as μmoles of DCIP reduced /min/mg protein (extinction coefficient of DCIP is $19.1 \text{ mM}^{-1} \text{ cm}^{-1}$).

Determination of Complex III

Decyl ubiquinol preparation: 1.3 mmol/l decylubiquinone was mixed with a few grains (approximately half volume) of sodium dithionite and vortexed vigorously. The transparent solution was centrifuged at 12,000g for 10 min. The supernatant containing decylubiquinol was used for the assay.

Complex III activity was estimated by the method of Krahenbuhl *et al* (1991). Mitochondrial protein (approximately 20 μg) was mixed with 100 $\mu\text{mol/l}$ EDTA, 2 mg BSA, 3 mmol/l sodium azide, 60 $\mu\text{mol/l}$ ferricytochrome – C, decylubiquinol and phosphate buffer (50 mmol/l, p^{H} 8) in a final volume of 1 ml. The reaction was started by the addition of decylubiquinol and monitored for 2 min at 550nm and again after the addition of 1 $\mu\text{mol/l}$ of antimycin A. The activity was calculated from the linear part of absorption time curve, which was not less than 30 s. The extinction coefficient of ferricytochrome C ($21 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for the calculation. Activity of complex III was expressed as μmoles of ferricytochrome – C reduced /min/mg protein.

Determination of Complex IV

Preparation of ferrocycytochrome C solution : 600 $\mu\text{mol/l}$ ferricytochrome – C was stirred with a few grains of sodium dithionite in 30 mmol/l phosphate buffer (p^{H} 7.4) for 10-20 min in the dark. The solution was centrifuged at 12,000g for 10 min. The transparent supernatant containing ferrocycytochrome C was used for the complex IV assay.

Complex IV activity was determined by the method of Capaldi *et al* (1995) with slight modifications. Briefly 1 ml of ferrocycytochrome C solution was mixed with approximately 10 μg of mitochondrial protein (extract in 0.5% Tween 80 in 30 mmol/l phosphate buffer, p^{H} 7.4) and phosphate buffer in net volume of 1.3 ml. The reaction was started by the addition of enzyme source and was monitored at 550nm with an interval of 15 s for 4 min. The difference in absorbance was calculated from the linear part of the absorption – time curve. complex IV activity was expressed as μmoles of ferrocycytochrome – C oxidized / min/mg protein using the extinction coefficient.

Determination of cardiac mitochondrial antioxidant status:-

The mitochondrial supernatant was used for the estimation of antioxidant status. Reduced GSH level was estimated by the method of Moron *et al.* (1979) and the malondialdehyde (MDA) level by the method of Ohkawa *et al.* (1979) using 1,1,3,3,-tetra methoxy propane as the standard and expressed as n mol of MDA formed / mg protein.

Statistical analysis:-

All data were represented as mean \pm SD. The mean values were statistically analyzed using one – way analysis of variance (ANOVA) (using the Graph Pad Instat software package). The significant differences between the groups were further analyzed by Bonferroni's t-test. p values less than 0.05 were considered as significant.

Results:-

The administration of *G.lucidum* extract (GLF) at its highest concentration (500 mg/kg) significantly increased the activities of TCA cycle enzymes (ICDH, α -KGDH, SDH, MDH) compared to the control group (Table 1). Standard (DL- α -Lipoic Acid) also showed the similar results. But in the case of lower concentration (250 mg/kg) it was statistically non-significant from DOX control group. In the case of α -KGDH and MDH irrespective of the concentration, *G.lucidum* extract showed significant activity compared to control. *G.lucidum* at its highest concentration only possessed statistically significant activity in the case of α -KGDH and MDH enzyme assays.

Respiratory chain complexes I, III, IV in all the treated groups showed protective effect at their highest concentration (500 mg/kg) towards DOX control group (Table 2). GLF at lowest concentration (250 mg/kg) did not possess any significant protection to Complex III and IV activities.

Nevertheless, the GLF treated groups possessed remarkable protection against DOX control group in the case of lipid peroxidation (Figure 1). Similar results were observed in GSH also (Figure 2).

Discussion:-

Mitochondria have long been involved in an enormous number of cellular processes beyond its role in energy production. Cardiac cells rely upon mitochondria-generated ATP to sustain their normal homeostasis. Impairment of mitochondrial function due to anthracyclin antibiotics mainly DOX is widely studied. Due to an intricate sets of cellular and molecular mechanism of DOX induced cardiotoxicity, search to find out a compound which reduce the toxicity level at the same time maintains the antineoplastic activity of DOX is continued. Recently released FDA approved drug dexrazoxane, interferes with anti-cancer efficacy of DOX and there by increases the risk of secondary malignancies. Therefore, a compound which impart better and safer cardioprotection against DOX toxicity will clinch a great clinical attention. The results of the present study indicate that fruiting body extract of *G.lucidum* impart pronounced protection towards mitochondrial energy status of cardiomyocytes challenged with DOX.

DOX treatment causes distortion and destruction of respiratory complexes embedded in the inner mitochondrial membrane. Complex I, III and IV are the DOX sensitive sites in ETC. Along with nuclear DNA, DOX also forms adducts with mitochondrial DNA (mtDNA) and form complexes with cardiolipin, one of the most abundant phospholipid in the inner mitochondrial membrane (Minotti *et al*; 2004). The redox cycling of DOX is catalysed by oxidoreducters present in ETC. DOX undergoes one electron reduction at Complex I, catalysed by NAD(P)H reductases to yield semiquinone radical and there by generating superoxide anion and other forms of reactive oxygen species. In addition to this, experimental studies show that an exogenous NADH dehydrogenase (cytosolic) is present which is specific to cardiomyocytes the key enzyme responsible for selective cardiotoxicity of anthracyclin antibiotics (Nohl *et al*; 1998). GLF at its lowest concentration (250 mg/kg) is able to restore the electron flow of Complex I compared to DOX control. Earlier studies indicates that both the extracts of *G. lucidum* provide significant cardioprotection by validating serum cardiac marker enzymes such as CK and LDH (unpublished data). This hinges on the fact that *Ganoderma* extracts are capable to restore the alterations in oxidative phosphorylation consequent to DOX administration and thereby protecting the cardiomyocytes from DOX toxicity.

Besides, the activities of the respiratory chain complexes, Krebs cycle dehydrogenases was also declined in DOX treated control group. Earlier studies conducted by Ajith *et al* (2014) in our laboratory demonstrated the TCA cycle stimulating activity of *G.lucidum* (Sudheesh *et al*; 2010). DOX form complexes with metals like Fe^{3+} and Cu^{2+} . As the result of bio sorption of *G. lucidum*, Cu^{2+} ions are removed from DOX complexes and Ca^{2+} ions are released into the mitochondrial membrane (Muraleedharan & Venkobachar; 1994). Ca^{2+} can stimulate the TCA cycle dehydrogenases to increase the production of reduced substrate for respiratory chain complexes and further increase the rate of respiration. Our results is in agreement with this finding. Several dietary supplements, including the mitochondrial cofactor and antioxidant lipoic acid (LA), can increase the endogenous antioxidants as well as mitochondrial bioenergetics.

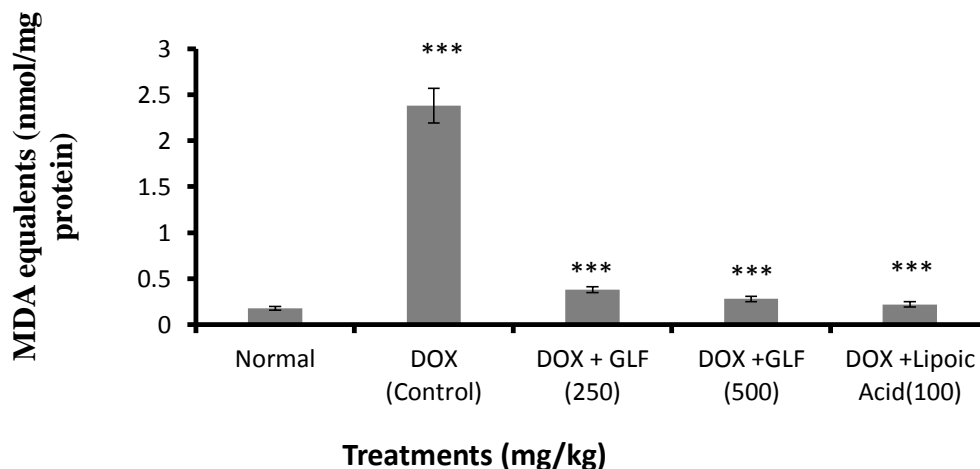
The deleterious action of DOX mainly affects the peroxidation of mitochondrial membrane lipids. Peroxidation of membrane phospholipids associated with a decrease in membrane fluidity which correlates with GSH content in the membrane there by reduced activity of TCA cycle enzymes. Our results reveal that pre-treatment with *G.lucidum* extracts significantly ameliorated DOX-induced cardiac oxidative damage by down regulating the generation of

Thiobarbituric Acid Reacting Substances (TBARS) and augmenting GSH content. These results are in agreement with previous reports showing inhibitory activity of *G. lucidum* against membrane damage (Sudheesh *et al* ;2009).

Conclusion:-

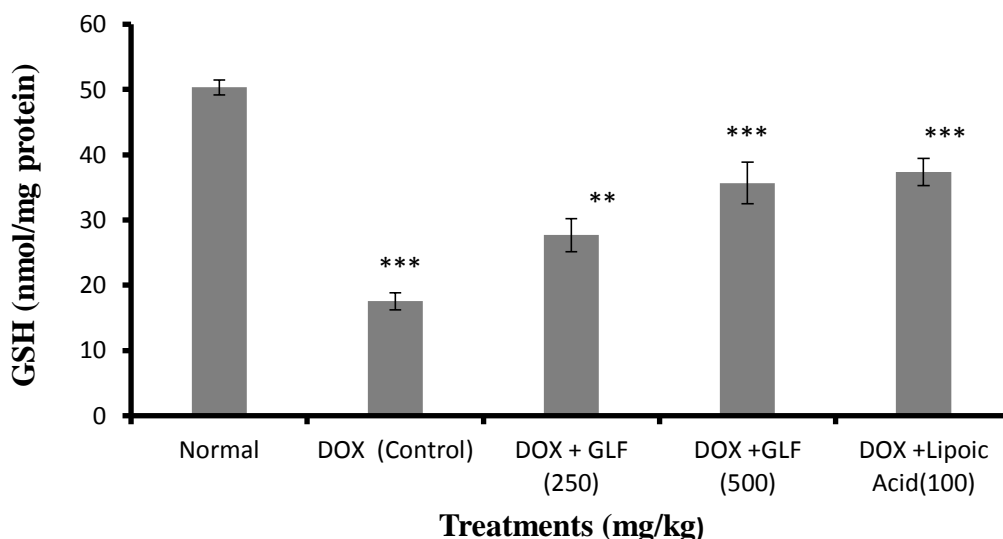
In conclusion the results of the study indicate that aqueous ethanolic extract of *G.lucidum* is capable to protect the cardiomyocytes from DOX induced toxicity which hampered the TCA cycle and respiratory chain complexes in the mitochondria. Amelioration of toxicity by *Ganoderma* occurs in different ways: acting as a strong antioxidant, free radical scavenger, Topoisomerase II β binding inhibitor etc. However the exact mechanism is still unknown and deserves further investigation.

Fig 1:- Effect of *G. lucidum* on Lipid Peroxidation



Values are the mean \pm S.D; n=6 animals. *** P < 0.001 significantly different from Doxorubicin control (Bonferroni test). DOX: Doxorubicin, GLF: *G.lucidum* extract

Fig 2:- Effect of *G. lucidum* on the levels of GSH activity



Values are the mean \pm S.D; n=6 animals. *** P < 0.001 significantly different from Doxorubicin control (Bonferroni test). DOX: Doxorubicin, GLF: *G.lucidum* extract

Table 1:- Effect of aqueous ethanolic extract of *G. lucidum* on TCA cycle enzymes

Groups	reatments (mg/kg)	ICDH	α -KGDH	SDH	MDH
Normal	-	1308.25 \pm 98.39	621.24 \pm 35.78	186.91 \pm 12.07	3403.56 \pm 208.53
Doxorubicin (Control)	Cumulative dose of 18 mg/kg(3 doses of 6 mg/kg)	617.02 \pm 41.22***	200.4 \pm 15.0***	87.61 \pm 0.42***	337.69 \pm 11.34***
<i>G.lucidum</i> (GLF)	250	971.74 \pm 35.73*	392.58 \pm 11.31***	123.08 \pm 3.15*	1944.85 \pm 106.28***
	500	1272.04 \pm 21.16***	503.87 \pm 17.11***	159.74 \pm 10.27***	2792.05 \pm 76.30***
DL- α -Lipoic Acid	100	1194.24 \pm 22.16***	588.06 \pm 24.78***	159.74 \pm 10.27***	2983.59 \pm 146.93***

Values are the mean \pm S.D; (n=6), *** P < 0.001 and ** P < 0.01 significantly different from Doxorubicin control Group (Bonferroni test).Units : Isocitrate dehydrogenase (ICDH)- μ moles of NAD⁺ reduced /min/mg protein ; α -ketoglutarate dehydrogenase (α -KGDH)- μ moles of NAD⁺ reduced /min/mg ; succinate dehydrogenase (SDH) - μ moles of DCIP reduced /min/mg ; malate dehydrogenase (MDH)- μ moles of NADH oxidized /min/mg protein.

Table 2:- Effect of aqueous ethanolic extract of *G. lucidum* on the respiratory complex

Groups	Treatments (mg/kg)	Complex I	Complex III	Complex IV
Normal	-	40.08 \pm 1.61	12.02 \pm 1.08	141.31 \pm 9.26
Doxorubicin (Control)	Cumulative dose of 18 mg/kg(3 doses of 6 mg/kg)	9.42 \pm 1.93***	3.77 \pm 0.16***A	24.06 \pm 0.73***
<i>G.lucidum</i> (GLF)	250	25.53 \pm 2.67***	6.51 \pm 0.38 ^{ns}	45.55 \pm 4.28 ^{ns}
	500	37.12 \pm 2.33***	8.97 \pm 0.71**	76.84 \pm 4.78***
DL- α -Lipoic Acid	100	39.51 \pm 3.35***	10.18 \pm 0.86***	63.95 \pm 7.75**

Values are the mean \pm S.D; (n=6), *** P < 0.001, ** P < 0.01 significantly and P < 0.05^{ns} non –significantly different from Doxorubicin control Group (Bonferroni test).Units : Complex I - μ moles of DCIP reduced /min/mg ; Complex III - μ moles of ferricytochrome C reduced /min/mg ; Complex IV - μ moles of ferrocycytochrome C oxidized /min/mg.

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