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Ganoderma applanatum (Pers.) Pat. augments antitumor activity of doxorubicin and provides chemoprevention to murine tumor model

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Doxorubicin (DOX) is a chemotherapeutic drug, used widely in the treatment of a variety of solid and hematological malignancies. However, its clinical utility is markedly reduced by dose-dependent cardiotoxicity. In the present study, Dalton lymphoma Ascites (DLA) cell line was administered to create a solid tumor in a murine model. DOX (25 mg/kg body wt.) was administered intraperitoneally in overnight fasted Swiss albino mice to induce cardiac toxicity and hepatotoxicity. Thirty minutes before administering the chemotherapeutic drug, water-alcohol extract of the medicinal mushroom *Ganoderma applanatum* (GAWE) was administered to allotted groups. After five days, the extent of heart damage was analyzed by Electrocardiogram (ECG), further blood serum parameters, such as SGOT, SGPT, ALP, CK-MB, and LDH as well as antioxidants, such as GSH, GPx and tissue peroxidation by MDA level was determined for both liver and heart tissues. The mode of prevention by the mushroom extract from the damage caused by DOX at the molecular level and aberrations in tissue morphology by histopathology was also analyzed. The increase in blood serum parameters and MDA levels were significantly reduced with the GAWE administration. GAWE also upregulated heart and liver antioxidant enzymes and reduces ST, QT interval, and QRS complex, and increased heart rate as compared to DOX treated group. GAWE dose-dependently mitigated DOX-induced cellular discrepancies as evidenced by gene expression study and histological analysis. Hence, it can be concluded that the water-alcohol extract of *Ganoderma applanatum* (GAWE), is a potent candidate for adjuvant chemotherapy in cancer treatment as it effectively mitigated organ system drug-induced toxicities.

Keywords: Artist's Fungus, Cancer, Cardioprotection

Doxorubicin (DOX) is a chemotherapeutic drug originally derived from the chemical synthesis of the bacteria *Streptomyces peucetius*. It is an excellent drug used in the treatment of a variety of solid and hematological malignancies. However, its clinical utility is markedly reduced by the serious incidence of dose-dependent cardiotoxicity, leading to irreversible and degenerative cardiomyopathy and congestive heart failure[1].

The pathogenesis of DOX-induced cardiotoxicity is not fully understood, as multiple mechanisms are involved, such as cardiac oxidative stress, intracellular calcium overload, mitochondrial impairment, myofibrillar degeneration, cytokine release leading to apoptosis, and inflammation-related signaling pathway. DOX subsequently stimulates oxidative damage by increasing the pro-inflammatory mediators such as TNF-α and COX-2[2]. Oxidative stress evoked by DOX triggers the death of cardiomyocytes by both intrinsic and extrinsic apoptotic pathways. Therapeutic blockage of DOX-induced cardiac oxidative stress and programmed death is apparently a challenge that might require the identification and inhibition of numerous key players involved without compromising its anti-tumorogenic effect[3].

Various *in vivo* studies have discussed about the effective usage of plants and derived components like *Murraya koenigii*[4], *Curcuma longa*, *Ginko biloba*, and several bioactive molecules[5] together with the DOX. However, literature on the cardiopreventive role of mushrooms against DOX is very scanty. Medicinal mushrooms are now gaining wider popularity largely due to the growing number of scientific studies that confirm the traditional use of fungi in curing different types of cancer and effective against various organ toxicities. Medicinal mushrooms in combination with chemo- or radiotherapy are capable of enhancing the efficacy of these treatments by diminishing side effects and complications caused by them[6].

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Since ancient times, in Oriental countries, species of *Ganoderma* commonly called the Artist's fungus or conk, are considered the king of herbs as they play a pivotal role against various types of diseases. Scientific investigations have also validated its effect against modern medicinal drug-related toxicities and have also undergone clinical trial as immuno-stimulator, glucose-lowering agent and against various types of cancer[7]. Laccate from *Ganoderma lucidum* has been exploited for its cardioprotective activity against DOX[8] while no such investigations have been carried out from its non-laccate counterpart *Ganoderma applanatum* which is founded abundantly in the Western Ghats of India— a world biodiversity hotspot in southern India. Hence, this study evaluates the therapeutic potential of water-alcohol extract of *G. applanatum* (GAWE) against DOX-induced cardio- and hepato- toxicities in tumor-bearing Swiss albino mice.

Materials and Methods

Chemicals and Reagents

Doxorubicin (Miracalus Pharma, Mumbai, India), TPTZ, ABTS, and ammonium persulfate from Sigma Chemical Company Inc., St. Louis, MO, USA. Primers were synthesized from Hysel India Pvt. Ltd., New Delhi. All other chemicals were of analytical grade and procured from reputed Indian manufacturers.

Animals

Male Swiss albino mice, 5-6 weeks old and weighing 22-25 g, were used for the study. The animals were purchased from Small Animal Breeding Section (SABS), Kerala Veterinary and Animal Sciences University Mannuthy, Kerala, India. Animals were kept for acclimatization prior to experiments under environmentally controlled conditions with 24 h *ad libitum* access to standard food and water. The experiment was carried out adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and approved by the Institutional Animal Ethics Committee (IAEC), Pushpagiri Institute of Medical Sciences and Research Centre, Thrissur, Kerala, India.

Collection and identification of mushroom

A mature basidiocarp of *Ganoderma* species was collected from a dead and unknown stump from Ranni, Pathanamthitta district of Kerala, India. The collected specimen was kept in a clean, dry and labelled polythene bag for morphological identification using the key of Moncalvo & Ryvarden[9]. The initial identification was based on the presence of characteristic double-walled basidiospores, and lack of apical (cuticular) cells, resulting in a dull upper (pileal) surface. Further identification and validation were done by Dr. Prasad Lamrood, P.G. Department and Research Centre in Botany, Ahmednagar College, Ahmednagar (affiliated to SP Pune University), Maharashtra, India.

Preparation of extracts

Ganoderma fruiting body was shadow dried, cut into small pieces, and macerated using a blender. The *Ganoderma* powder (100 g) was extracted using hot water-ethanol solvent as reported in our previous studies[10]. The total yield obtained from water-alcoholic extraction was 6.2%.

Free radical and total antioxidant activity

GAWE was subjected to ABTS scavenging effect and total antioxidant power using the FRAP method[11].

Assessment of cardio- and hepato- protective activity of GAWE

Swiss male albino mice were randomly distributed into five groups with six animals each. Animals of the first group served as normal control with access to free food and water. The solid tumor was developed in mice by the subcutaneous transfer of DLA cells ($1X10^6$) into the hind limb. The experiment began after the tumor reaches a size of 1.0 cm^3 (measured using a Vernier Caliper). The groups are as follows: Gr. I, Normal control (Untreated Tumor mice); Gr. II, GAWE control (Tumor mice + GAWE 500 mg/kg orally); Gr. III, DOX control (Tumor mice + DOX 25 mg/kg body wt. i.p.); Gr. IV & V, Experimental groups (Tumor mice + DOX 25 mg/kg body wt. + GAWE 250 or 500 mg/kg orally, respectively. Overnight fasted animals were given a single dose of DOX through i.p. mode to groups III, IV & V. GAWE (250 and 500 mg/kg body wt.) was given orally to the animals belonging to groups II, IV & V after the DOX administration and provided for five days one dose daily. On the fifth day, animals were subjected to ECG recording followed by sacrifice via cervical dislocation. The blood was collected by cardiac puncture and serum was separated for the biochemical investigations. The liver and heart were removed for various antioxidant, biochemical, and molecular analyses.

Electrocardiography (ECG)

On the fifth day, all experimental mice were subjected to ECG recording. The animals were handled gently after taking out from the cage. The animals were placed on a flat wooden platform and restrained from moving by a soft cloth band around the abdomen. The disc electrodes were attached to the pre-cleaned palmer surface of four limbs after placing

gel around the limb. Care was taken during recording, four limbs were never in contact with each other, and recording for all purposes was considered when the animals were at rest with no movement.

ECG was recorded on the student's physiograph (Inco Biodevice, Ambala) using an ECG coupler supplied with a five-pin junction box. Standard bipolar limb lead II recording was done. The chart paper was set for 50mm/s speed at normal filters applied. Parameters like RR interval, QRS interval, and QT interval were measured using calipers. The heart rate was calculated after analyzing successive 1-min duration and the mean value was considered.

Assessment of heart and liver markers enzymes and antioxidants

Blood was centrifuged at 5000 rpm for 5 min and serum was subjected to analyze cardiac markers like CK-MB and LDH and liver markers like Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP). Serum parameters were analyzed using a serum analyzer (Aggappe Ltd). All the serum parameters were analyzed using Agappe Kits. The liver and heart homogenates (10% w/v) prepared in phosphate-buffered saline (pH= 6.8-7.4) were used for antioxidant studies such as reduced glutathione (GSH), glutathione peroxidase (GPx) and lipid peroxidation (LPO). All the enzymatic assays were carried out as reported in our previous studies[10].

Gene expression studies

Total RNA was isolated from heart and liver homogenate using the acid guanidium-thiocyanate-phenol-chloroform method as mentioned in our previous study. [12] cDNA and qPCR were carried out using the protocol mentioned in our earlier report.[12] Genes were amplified from the cDNA using qPCR (AppliedBiosystems,CA, USA) with SYBR Green (Clontech, TakaraBio, USA).

Pre- and anti- apoptotic genes like BAX[13], BCL-2[13], Caspases-3 and Caspases-9[14] as amplified to determine the mode of death in the heart and liver cells, COX-2[15] and TNF-α[15] were amplified to understand the role of GAWE in inflammation and GAPDH[14] gene was used as a housekeeping gene. Each amplified sample in all wells was analyzed for homogeneity using dissociation curve analysis. The following cycling condition was used for amplification: 95°C for 10 min, 45 cycles were performed at 95°C for 15 s, 53ºC for 1min, and 72ºC for the 30 s, followed by melt curve at 60ºC for 1min and 95ºC for 15 s. Relative quantification was calculated using the comparative CT method (2_ΔΔct method: ΔΔCt method: ΔCt sample - Ct reference). Lower CT values and lower CT reflect a relatively higher amount of gene transcript[12].

Histopathological studies

After the sacrifice, a portion of the heart and liver was dissected, washed in saline, and subjected to histopathology analysis, adhering to our previous study protocol[16].

Statistical analysis

The results were presented as the mean ± SD of the studied group. Statistical analyses of the results were performed using ANOVA with Tukey–Kramer multiple comparisons tests. A level of P <0.05 was taken as statistically significant. (***P <0.001 compared to doxorubicin control; ** P <0.01 compared to doxorubicin control; ns, P <0.05 compared to doxorubicin control)

Results

Free radical and total antioxidant activity

ABTS radical scavenging activity and FRAP assay

The ABTS radical scavenging effect and ferric-reducing power of the extract were compared with the same doses of standard (BHT) and ascorbic acid (AA), respectively. The concentration-dependent scavenging and total antioxidant activity were exhibited by GAWE which is comparable to that of the standard drugs (Fig. 1 A & B).

Electrocardiography (ECG)

DOX control displayed aberrations in ST, QT interval, and QRS complex (Fig. 2B) as compared to

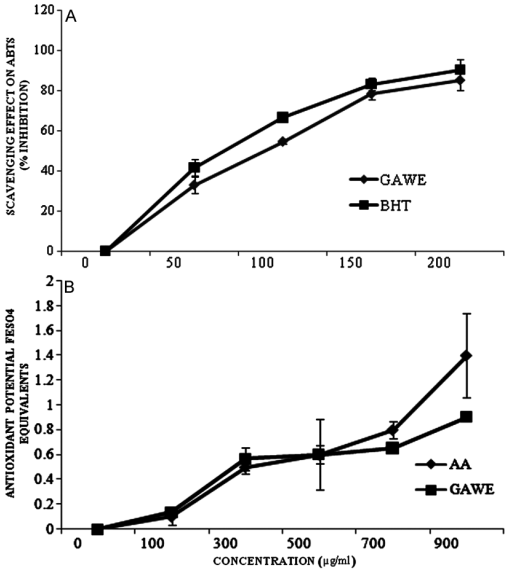

Fig. 1 — (A) ABTS radical scavenging activity; (B) Total antioxidant activity by FRAP assay. [The results were presented as mean ±SD of the studied group]

normal control (Fig. 2A). GAWE at both concentrations reduces ST, QT interval and QRS complex and increased heart rate as compared to DOX treated group as shown in Fig. 2 C & D and Table 1.

Effect of GAWE on Heart and Liver markers enzymes

Evaluation of heart serum markers

The administration of DOX exerts a significant increase in serum LDH and CK-MB levels as compared to that of the control (Table 2). However, administration of GAWE together with DOX at both concentrations significantly reduced LDH and CK-MB levels as compared to DOX drug control (***P <0.001). GAWE control group showed no significant variation from than control.

Evaluation of liver serum markers

Administration of DOX also exerts a significant increase in serum SGOT, SGPT, and ALP levels as compared to that of normal control mice (Table 2). However, administration of GAWE together with DOX at 250 mg/kg body wt. significantly (P <0.001) reduce SGOT, SGPT and ALP levels as compared to DOX drug control while GAWE at 500 mg/kg body wt. restored the serum SGOT, SGPT and ALP levels near the control. No significant variation was observed in GAWE control to normal control.

Effect of GAWE on antioxidant levels of cardiac and hepatic tissues

A considerable elevation in lipid peroxidation with decreased GSH and GPx levels in cardiac and hepatic tissues was observed in DOX drug control. While aberrations observed due to the DOX treatment were significantly (P <0.001) regressed by the administration of GAWE at both concentrations group as depicted in Table-3.

Gene expression studies

Inflammatory markers and apoptotic markers in heart and liver tissue

The mRNA levels of inflammatory markers COX-2 and TNF-α, and apoptotic markers such as Bax, Bcl-2, caspase-9 and caspase-3 are shown in Fig. 3 A-F. Our findings revealed that mRNA levels of COX-2 and TNF-α were increased in DOX-treated mice which

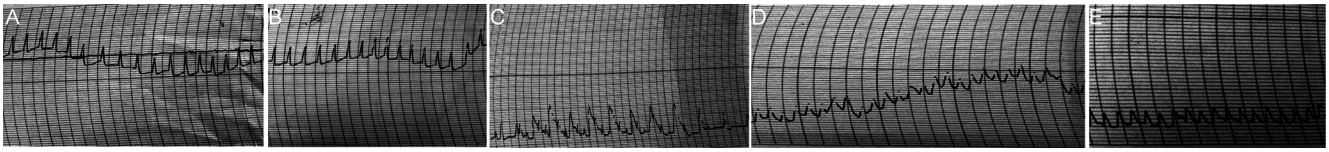

Fig. 2 — Electrocardiogram (ECG): (A) Tumor control (B) GAWE control (C) DOX control; and (D & E) DOX + 250 or 500 mg/kg body wt. GAWE, respectively

Table-1 — Effect of GAWE on ECG changes in DOX-induced Swiss albino mice

Groups	Heart rate (beats/min)	PR interval (ms)	QT interval (ms)	QRS complex duration (ms)
I (Control)	590	20	60	24
II (GAWE control)	600	22	55	24
III (DOX control @25 mg/kg)	400	30	90	30
IV (DOX+GAWE @250 mg/kg)	550	27	78	29
V (DOX+GAWE @500 mg/kg)	610	24	65	27

[A level of P <0.05 was taken as statistically significant. ***P <0.001, **P <0.01 and ns, P <0.05 compared to Gr. II, respectively]

Table-2 — Effect of GAWE on serum markers for assessing cardiotoxicity and hepatotoxicity in DOX-induced Swiss albino mice

Groups	sGOT (IU/l)	sGPT (IU/l)	ALP (KA unit)	CK-MB (U/L)	LDH (U/L)
I (Control)	59.88±5.14	50.58±4.01	124.16±5.89	63.21±5.11	388.4±4.30
II (GAWE control)	56.07±4.5	46.13±4.38	239.19±7.37	56.75±4.26	367.1±.32
III (DOX control @25 mg/kg)	95.16±2.07	120.53±6.7	323.38±5.11	87.74±7.21	500.91±6.15
IV (DOX+GAWE @250 mg/kg)	75.74±5.68***	100.62±3.43***	183.82±4.53***	79.74±0.91ns	475.9±5.08***
V (DOX+GAWE @500 mg/kg)	60.5±4.38***	65.96±5.39***	136.01±0.57***	63.21±5.11***	406.3±5.31***

[A level of P <0.05 was taken as statistically significant. ***P <0.001, **P <0.01 and ns, P <0.05 compared to Gr. II, respectively]

Table 3 — Effect of GAWE on antioxidants status and lipid peroxidation in cardiac/liver tissue of DOX-induced Swiss albino mice

Groups	GSH (nmol/mg protein)		GPx (Unit/mg protein)		MDA (nmol/mg protein)	
	Cardiac	Liver	Cardiac	Liver	Cardiac	Liver
I (Control)	95.48±3.8	52.78±4.33	90.72±4.28	31.12±2.13	1.17±0.43	1.02±0.37
II (GAWE control)	83.84±3.6	58.5±4.29	96.07±4.92	30.82±4.37	1.45±0.03	1.23±0.11
III (DOX control @25 mg/kg)	61.56±4.1	31.45±2.7	80.65±5.06	18.9±1.29	1.57±0.2	3.59±0.04
IV (DOX+GAWE @250 mg/kg)	45.34±3.9	43.27±3.81^{ns}	69.79±2.5	24.69±1.32*	3.50±0.22	2.03±0.27***
V (DOX+GAWE @500 mg/kg)	58.52±4.54***	97.1±4.36***	80.05±2.41***	32.09±0.85***	2.12±0.66**	1.62±0.26***

[A level of P <0.05 was taken as statistically significant. ***P <0.001, **P <0.01 and ns, P <0.05 compared to Gr. II, respectively]

were significantly (P <0.001) reduced by the concomitant administration of GAWE at both doses.

We also observed that the intrinsic mode of apoptosis is induced by DOX in both heart and hepatic tissues followed by a caspase-dependent mitochondrial pathway. mRNA level of BAX was increased in both tissues of the DOX control group. However, treatment with GAWE significantly (P <0.001) reduced BAX levels in DOX + GAWE treated groups at both concentrations (Fig. 3C). DOX induction down-regulated the expression of the mRNA level of anti-apoptotic gene BCL-2 whereas GAWE at both concentrations maintained BCL-2 expression near to normal and GAWE control (Fig. 3D). An increase in BAX ultimately activates caspases. An increased mRNA level of caspase-9 and caspase-3 in cardiac tissues was observed in DOX control mice. Treatment with GAWE at both doses reversed the increased mRNA expressions of caspase-9 and caspase-3 in the liver and heart in a dose-dependent manner (Fig. 3 E & F). GAWE control group showed a similar pattern of relative fold change to that of the untreated control.

Histology of heart and liver tissue

Induction of cardiotoxicity and hepatotoxicity in our study by DOX was further assessed using hematoxylin and eosin-stained sections of the respective tissues. Heart tissue from the DOX control [Fig. 4A(ii)] groups revealed hyalinization of the muscle fiber with congested blood vessels, interstitial edema, and cytoplasmic vacuole formation as compared to the control group [Fig. 4A(i)]. Myocardial

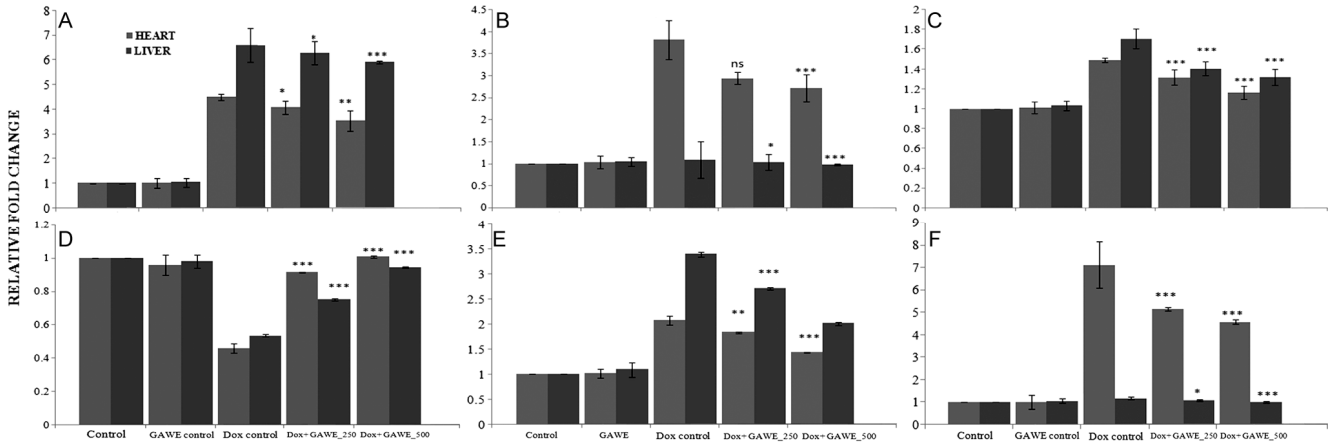

Fig. 3 — mRNA expression of (A) COX-2; (B) TNF-α; (C) BAX; (D) BCL-2; (E & F) Caspase-9 and caspase-3 in heart and liver of treated and non-treated mice. [Value of P <0.05 was taken as statistically significant. (***P <0.001, **P <0.01 and ns P <0.05 compared to doxorubicin control)

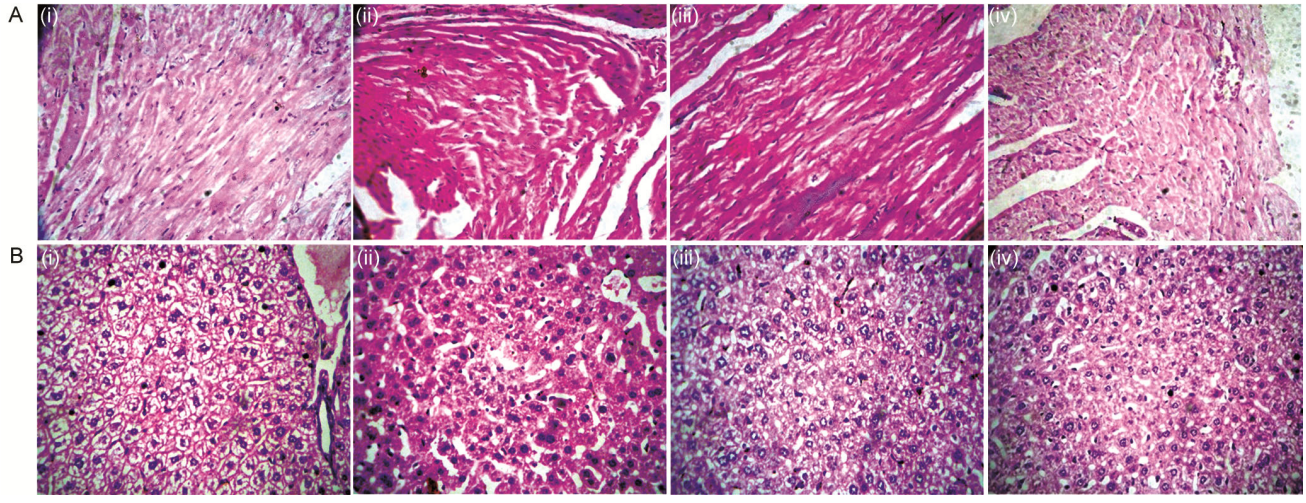

Fig. 4 — Histopathological analysis of (A) Cardiac; and (B) Liver tissue of animals treated with DOX and administrated with the GAWE: (i) Control; (ii) DOX control; (iii & iv) DOX + 250 or 500 mg/kg body wt. GAWE, respectively

lesions and the rest of the alterations in the histology of the heart were significantly reduced in the DOX + GAWE (250 and 500 mg/kg body wt.) treated groups[(Fig. 4A (iii & iv)].

Liver tissue of the control untreated group [Fig. 4B(i)] shows a normal architecture of hepatic cells with intact polygonal structure with glycogen storage content, While in DOX treated group [Fig. 4B (ii)] the hepatocytes were anucleated with decreased glycogen storage and pericentral necrosis. On the other hand, in the DOX + GAWE treated groups at both concentrations [Fig. 4B (iii & iv)] displayed intact glycogen storage with no inflammatory cells, and vacuolated cytoplasm with prominent large nuclei.

Discussion

Despite the remarkable development in chemo-therapeutic treatments against certain types of cancer; still persists a list of hurdles which ranges from adverse drug-induced toxicities to relapse of disease after a brief disease-free interval[17]. Being one of the most effective chemotherapeutic agents; DOX administration often leads to severe dose-dependent cardio and hepatotoxicity mediated through oxidative injury[18]. Various strategies are implemented to alleviate this drug-induced toxicity, such as dose optimization, synthesis, and use of analogs or combined therapy with adjuvants having antioxidant properties to reduce oxidative stress without interfering with antitumor properties[19]. The anti-oxidant dexrazoxane is an FDA-approved adjuvant to be used in conjunction with DOX to attenuate cardiotoxicity. However, the usefulness of this drug is often limited[20]. Our results have shown that intra-peritoneal administration of DOX (25 mg/kg body wt.) produced signs of cardiomyopathy with hepatomegaly[21]. This observation was confirmed by increased creatine kinase isoenzyme (CK-MB) and lactate dehydrogenase activities, the most specific and highly sensitive serum markers for myocardial cell injury. These results are consistent with the earlier reports[22]. Further, there is an increase in serum parameters like ALP, SGOT, and SGPT, sensitive indicators of liver damage in DOX-treated groups. Our results are consistent with earlier reports[23].

Mushrooms belonging to higher basidiomycetes have been shown to mitigate organ toxicity induced by modern medicines[24]. Similar observations were obtained here, GAWE at both concentrations was able to normalize the elevated level of these enzymes dose-dependently. Antioxidant enzymes like GSH and GPx offer protection against oxidative tissue damage. The present results coordinate and support the postulated mechanisms of DOX toxicity like radical formation with elevated lipid peroxidation[25], depletion of reduced glutathione[26], and decrease GPx levels in various tissues like the heart and liver[27]. In the present study the antioxidant GSH and GPx were significantly reduced in cardiac and hepatic tissues of DOX-administered mice as compared to normal control mice but administration of GAWE together with DOX at both concentrations restored the antioxidant level. Our results are supported by earlier studies where triterpenes from *G. lucidum* restored the DOX-induced organ toxicity[28].

This result can also be corroborated with the *in vitro* antioxidant potential of GAWE as depicted by FRAP and ABTS analysis which clearly shows GAWE as an enriched source of antioxidants similar to their laccate counterpart *G.lucidum*[29].

Treatment with DOX resulted, in not only altered serum and antioxidant levels of the heart tissue but also showed a reduction of cardiac rate, depression of ST segment, and prolongation of ST, QT intervals and QRS complex. Similar changes in ECG tracing have been reported by other studies[30]. The ECG tracings collected from the group treated with GAWE at both concentrations concomitantly with DOX restores ECG changes towards normalcy.

The exact mechanisms of DOX-induced cardiac and hepatic toxicity still remain unclear although various mechanisms such as oxidative stress, inhibition of DNA and protein synthesis, myofibrillar degeneration, cardiomyocyte apoptosis via caspase, mitochondrial DNA damage, etc. have been postulated[31]. Among those diverse mechanisms, most evidence designates the involvement of free radicals generated from the metabolism of DOX[32]. Several studies show that DOX administration induces inflammatory effects by increasing pro-inflammatory cytokines like TNF-α and COX-2 in cardiomyocytes and the administration of antioxidants having anti-inflammatory activity reduce DOX-induced cardiotoxicity[33]. In the present study, DOX toxicity significantly increased TNF-α and COX-2 expressions in both heart and liver tissues reflecting amplified inflammatory responses. On the contrary, GAWE administration significantly reduced the expression of COX-2 and TNF-α in both tissues. Thus, GAWE acts as an anti-inflammatory agent

against DOX-induced toxicities. Apparently, for over a decade, apoptosis has been implicated as one of the pathways involved in DOX-induced cardiotoxicity. Upregulation of BAX and downregulation of BCL-2[34] have been observed in most DOX-induced cardiotoxicity cases. Blockage of BAX by expressing BCL-2 diminishes mitochondrial injury and DOX-induced apoptosis[35]. In the present study, the mRNA level of BAX has increased with a decrease in BCL-2 level in the cardiac and hepatic tissues of the DOX control group as compared to the normal control group. The expression levels of these genes were reversed in both tissues of GAWE-administered groups in a dose-dependent manner. Activation of caspase-3 and caspase-9[36] plays an important role in the execution of apoptosis in DOX-induced cardiomyopathy. Inhibition of caspases can be a therapeutic approach that will be able to address the toxicity associated with DOX. In both tissues, DOX toxicity significantly increases caspase-3 and caspase-9 while GAWE administration abrogates this state. However, further in-depth studies are required to validate traditional knowledge on *Ganoderma* to translate into clinical research for a better understanding of its alleviating ability towards drug-induced toxicities.

Conclusion

The results of the above study have demonstrated the potential of water-alcohol of the medicinal mushroom *Ganoderma applanatum* (GAWE) as an effective adjuvant therapy to mitigate the dose-dependent cardiotoxicity of Doxorubicin (DOX) in cancer treatment. Adjuvant administration of GAWE with DOX significantly reduced the increase in blood serum parameters and MDA levels, upregulated antioxidant enzymes in the heart and liver, and improved heart function by reducing ST, QT interval, and QRS complex. It also mitigated DOX-induced cellular discrepancies and tissue damage at the molecular and histological levels. GAWE can be considered a safe and effective adjuvant therapy for reducing drug-induced toxicities in organ systems during cancer treatment. Further studies must be conducted to explore the various pathways by which GAWE exhibits organ system protective activity.

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Conflict of Interest

Authors declare no competing interests.

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