Study on the effect of bioactive extract of morel mushroom, *Morchella esculenta* (L) Pers, in mitigating cancer chemotherapy drugs-induced cardiotoxicity

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(FACULTY OF SCIENCE)

By

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DECLARATION

I, hereby declare that thesis entitled "**Study on the effect of bioactive extract of morel mushroom, *Morchella esculenta* (L) Pers, in mitigating cancer chemotherapy drugs-induced cardiotoxicity"** is based on the original research work done by me, under the guidance of Dr. K K Janardhanan, Ph.D., FNABS, Professor at Amala Cancer Research Centre, Thrissur and co-guidance of Dr. B S Harikumaran Thampi, Ph.D., Professor, Biochemistry, University of Calicut, Thenhipalam for the partial fulfilment for the award of Doctor of Philosophy in Biochemistry. No part of this work has formed the basis for the award of any degree, diploma or other similar titles of any other university. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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This is to certify that all the corrections/suggestions recommended by the adjudicators in the Ph.D thesis of Ms. Sneha Das (U.O.No. 17535/2019/Admn, Dated, Calicut University.P.O, 16.12.2019) entitled **'Study on the effect of bioactive extract of morel mushroom, *Morchella esculenta* (L) Pers, in mitigating cancer chemotherapy drugs-induced cardiotoxicity'** have been duly incorporated and the contents in thesis and soft copy are one and the same.

Dr. K. K. Janardhanan

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1.	Name of the Research Scholar	Sneha Das	
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5.	Similar content (%) identified	Non Core **Introduction/ Theoretical overview/Review of literature/ Materials & Methods/ Methodology**	Core **Analysis/Result/Discussion / Summary/Conclusion/ Recommendations**
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Name and signature of the Supervisor

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Finally, as a tribute of love, I dedicate this thesis to my son Thrilok for sacrificing his childhood over the last four years while juggling my hectic schedule.

SNEHA DAS

DEDICATED TO MY SON

CONTENTS

ABSTRACT

Quest for bioactives that confer protection against chemotherapy induced cardio toxicity is a front- line area of cardio oncology research. Attempts have been made in recent years to find out the effects of medicinal mushroom bioactives for this purpose. Morel mushrooms have been used in traditional medicine in Asian countries to treat asthma, wound healing, cough, cold, indigestion, excessive phlegm and breathlessness. *Morchella esculenta,* commonly known as Guchhi in India is highly prized culinary morel mushroom. Investigation carried out in our laboratory have demonstrated that mycelium of *M esculenta* possessed significant antioxidant, anti-tumour and hepatoprotective activities. Experimental studies were carried out on the protective effect of bioactive extract of *M. esculenta* fruiting bodies against chemotherapy drug induced cardiotoxicity using Swiss albino mice model. The findings are reported in this thesis.

Several bioactive compounds are reported to be present in the fruiting bodies of *M. esculenta*. This might be attributed for its therapeutic properties. Methanolic extract of the fruiting bodies of *M.esculenta* was used in the current study. Chemical finger print of methanolic extract (ME) was accomplished by HPTLC analysis. Bioactive constituents were identified by LCMS analysis. The results showed a large number of bioactive compounds were present in the methanol extract of *M. esculenta.* Some of the major compounds present were 5-eicosapentaenoicacid($C_{20}H_{30}O_2$), 8-hydroxyoctadecadienoic acid($C_{18}H_{32}O_3$), 4,4-dipo-zetacarotene($C_{30}H_{44}$), CynarosideA ($C_{21}H_{32}O_{10}$). These compounds might be responsible for the observed cardio protective effect of the extract.

Despite the use of *M. esculenta* as a widely used edible mushroom worldwide including India, little is known about its safety. So, in order to find the safety, toxicity studies were conducted using Swiss Albino mice. Acute oral toxicity study was conducted following the OECD-423 guidelines. The results revealed that the mushroom does not cause any toxic effects in animals at dosages of 5000 and 2000 mg/kg b.wt. The animals were active and no changes in behavioural pattern was observed. Body weight, feed and water intake were also found normal. Thus, methanolic extract of *M. esculenta* (ME) was found safe for oral consumption. Further sub-chronic toxicity studies were carried out using ME at doses of 100, 250 and 500 mg/kg. b.wt . No toxicity was observed and all animals remained healthy throughout the study period.

Doxorubicin and Cyclophosphamide are extensively used chemotherapy drugs. These drugs show severe cardiotoxicity especially in higher doses. Several mechanisms have been proposed for the cardiotoxic effects of doxorubicin and cyclophosphamide. Oxidative stress is one of the primary mechanisms by which both of these chemotherapeutic drugs induce harmful effects. Hence, the current study was designed to find whether the antioxidant capability of ME would be capable to mitigate these negative consequences.

The cardioprotective study of ME was carried out in three stages i.e., effect on doxorubicin- induced cardiotoxicity; effect on cyclophosphamide- induced cardiotoxicity and effect on combination of cyclophosphamide and doxorubicin-induced cardiotoxicity. Doxorubicin (DOX), Cyclophosphamide(CP) and their combination (CD) are extensively used treatment options for cancer. Cardiotoxicity induced by these drugs is an impediment in their clinical use. Hence levels of cardiac injury makers were studied. The activities of Creatine kinase-MB, Lactate dehydrogenase and Troponin I levels consequent to the administration of CP, DOX and CP+DOX were studied using diagnostic kits. Depletion of endogenous antioxidant levels in myocardium was also determined by spectrophotometric assays. Nrf-2, iNOS, NF-κB and proinflammatory cytokines gene expression were determined by RT-PCR analysis. To assess the mitochondrial dysfunction, TCA cycle and electron transport chain complex enzymes activities were evaluated. Cardiac tissue damage caused by CP, DOX and CP+DOX was assessed by histopathological examination.

Cardiac injury marker levels elevated by CP, DOX and CP+DOX treatments were significantly downregulated by ME. Endogenous antioxidants such as SOD, GPx, and GSH depleted by CP, DOX and CP+DOX administration was restored to almost normal level by ME. This indicated the effect of ME to ameliorate oxidative stress caused by chemotherapy drugs DOX, CP and DOX+CP administration leading to myocardial injury. Histopathological observation supported these findings. CP, DOX and CP+DOX-induced decline of antioxidant status and expression of nuclear factor erythroid 2-related factor 2 was restored by ME. CP, DOX and CP+DOX-induced expression of NF-κB, IL-6, IL1-β, TNF-α, and iNOS was attenuated by ME (500mg/kg). Depleted levels of mitochondrial enzyme activities consequent to chemotherapy were also restored by ME.

Since bioactive extracts of morel mushrooms were found to possess profound antioxidant activity, the possible interference of these extracts with antineoplastic activity of chemotherapy drugs is often surmised. Hence another study was undertaken to evaluate the effect of doxorubicin and cyclophosphamide on solid tumour bearing mice treated with bioactive extract of *M.esculenta*. Solid tumour was induced by subcutaneous injection of Dalton's Lymphoma Ascites (DLA) cells on the right hind limbs of Swiss albino mice. Animals were administered with various concentrations of methanol extract of *M. esculenta* following tumour induction. Tumour growth (volume and mass) was measured for four weeks after tumour induction. Cardioprotective effect of methanolic extract was assessed by determining cardiac injury markers levels in serum, antioxidant status in myocardium and histopathology of heart tissue. The results showed significant cardioprotective effect of methanol extract of *M. esculenta* on tumour bearing mice The findings also suggested that methanol extract of *M. esculenta* did not delimit the therapeutic effect of doxorubicin and cyclophosphamide despite its profound antioxidant capabilities.

In silico studies using Auto dock tools were performed to determine the interaction of major bioactives of ME with KEAP-1 protein which plays a regulatory role in NRF-2 induced antioxidant defence mechanism. The results showed that the major bioactives interacted with Cys-151 residue of KEAP-1 which is found to have a pivotal role in KEAP1-NRF2 interaction.

This thesis is a compilation of the comprehensive investigation and experimental findings on the cardioprotective effect of bioactive extract of morel mushroom *Morchella esculenta* against chemotherapy induced cardiotoxicity doxorubicin and cyclophosphamide and their combination. The studies are compiled in 10 chapters: Introduction (Chapter I), Review of literature (Chapter II), Materials and Methods (Chapter III), Extraction and identification of major bioactive compounds (Chapter IV), Acute oral toxicity study of methanolic extract of *M. esculenta* fruiting body (Chapter V), Evaluation of protective effect of methanolic extract of *M. esculenta* fruiting body against doxorubicin induced cardiotoxicity(Chapter VI), Evaluation of protective effect of methanolic extract of *M. esculenta* fruiting body against cyclophosphamide induced cardiotoxicity(Chapter VII), Evaluation of protective effect of methanolic extract of *M. esculenta* fruiting body against combination treatment of

cyclophosphamide and doxorubicin induced cardiotoxicity(Chapter VIII), Evaluation of anti-tumour activity of doxorubicin, cyclophosphamide and combination of cyclophosphamide + doxorubicin vis-à-vis cardioprotective effect of *M.esculenta* (Chapter IX). *In silico* studies for evaluating interaction of bioactives with KEAP-1 (Chapter X).

Keywords: Medicinal mushrooms, *M. esculenta,* Oxidative stress, Antioxidants, Anti-tumour, Chemotherapy, Cardio protection, Mitochondrial dysfunction, Doxorubicin, Cyclophosphamide, Gene expression, Combination chemotherapy.

കീമോതെറാപ്പി മൂലമുണ്ടാകുന്ന ഹൃദയത്തിനുണ്ടാകുന്ന കേടുപാടുകൾക്കെതിരെ സംരക്ഷണം നൽകുന്ന ബയോ ആക്റ്റീവുകൾക്കായുള്ള അന്വേഷണം കാർഡിയോ ഓങ്കോളജി ഗവേഷണത്തിന്റെ ഒരു മുൻനിര മേഖലയാണ്. ആസ്ത്മ, മുറിവ് ഉണക്കൽ, ചുമ, ജലദോഷം, ദഹനക്കേട്, അമിതമായ കഫം, ശ്വാസതടസ്സം എന്നിവ ചികിത്സിക്കാൻ പരമ്പരാഗത വൈദ്യത്തിൽ 'മോറെൽ' കൂൺ ഉപയോഗിക്കുന്നു. ഇന്ത്യയിൽ പൊതുവെ ഗുച്ചി എന്നറിയപ്പെടുന്ന *മോർഷല്ല എസ്കുലെന്റ്*, വളരെ വിലമതിക്കുന്ന മോറൽ കൂൺ ആണ്. ഞങ്ങളുടെ ലബോറട്ടറിയിൽ നടത്തിയ അന്വേഷണത്തിൽ എം എസ്കുലെന്റയുടെ മൈസീലിയത്തിന് കാര്യമായ ആന്റിഓക്സിഡന്റും ഹെപ്പറ്റോപ്രൊട്ടക്റ്റീവ് പ്രവർത്തനങ്ങളും ഉണ്ടെന്ന് തെളിയിച്ചിട്ടുണ്ട്. കാൻസർ കീമോതെറാപ്പി മൂലം ഉണ്ടാകുന്ന ഹൃദയസംബന്ധമായ പ്രശ്നങ്ങൾക്കെതിരെ മോർഷെല്ല എസ്ക്യൂലെന്റയുടെ സംരക്ഷണ പാടവത്തെ കുറിച്ചാണ് ഈ പ്രബന്ധത്തിൽ പറയുന്നത്.

*മോർഷല്ല എസ്കുലെന്റ*യുടെ ഫ്രൂട്ടിങ് ബോഡിയിൽ നിരവധി ബയോ ആക്റ്റീവ് സംയുക്തങ്ങൾ ഉണ്ടെന്ന് റിപ്പോർട്ട് ചെയ്യപ്പെട്ടിട്ടുണ്ട്, ഇത് അതിന്റെ ചികിത്സാ ഗുണങ്ങൾക്ക് കാരണമാകാം. ഞങ്ങളുടെ പഠനത്തിൽ, ME യുടെ കെമിക്കൽ ഫിംഗർ പ്രിന്റ് HPTLC നടത്തി, പ്രധാന ബയോ ആക്റ്റീവ് സംയുക്തങ്ങൾ കണ്ടെത്താൻ LCMS ടെക്നിക്കുകൾ ഉപയോഗിച്ചു, കൂടാതെ *മോർഷല്ല എസ്കുലെന്റ*യുടെ മെഥനോൾ സത്തിൽ 200-ലധികം സംയുക്തങ്ങൾ ഉണ്ടെന്ന് ഫലങ്ങൾ കാണിക്കുന്നു. 5-eicosapentaenoicacid(C20H30O2), 8-hydroxyoctadecadienoic ആസിഡ്(C18H32O3), 4,4-dipo-zetacarotene(C30H44), Cynaroside A (C21H32O10) എന്നിവയാണ് നിലവിലുള്ള ചില പ്രധാന സംയുക്തങ്ങൾ, ഇവ കാർഡിയോ സംരക്ഷണത്തിന് കാരണമാകാം.

ചൈനീസ് പാചകരീതികളിൽ സാധാരണയായി ഉപയോഗിക്കുന്ന ഒരു ഭക്ഷ്യയോഗ്യമായ ഫംഗസ് ആണെങ്കിലും, അതിന്റെ സുരക്ഷയെക്കുറിച്ചോ ബയോ ആക്റ്റിവിറ്റിയെക്കുറിച്ചോ വളരെക്കുറച്ചേ അറിയൂ. അതിനാൽ, സുരക്ഷിതമായ അളവ് കണ്ടെത്തുന്നതിന് ടോക്സിസിറ്റി പഠനം നടത്തി. OECD-423 മാർഗ്ഗനിർദ്ദേശങ്ങൾ അടിസ്ഥാനമാക്കിയാണ് അക്യൂട്ട് ഓറൽ ടോക്സിസിറ്റി പഠനം നടത്തിയത്. 5000, 2000 mg/kg b.wt ഡോസേജുകളിൽ ഇത് വിഷ ഫലങ്ങളൊന്നും ഉണ്ടാക്കുന്നില്ലെന്ന് ഫലങ്ങൾ വെളിപ്പെടുത്തി. മൃഗങ്ങൾ സജീവമായിരുന്നു, പെരുമാറ്റരീതിയിൽ മാറ്റങ്ങളൊന്നും കണ്ടില്ല. ശരീരഭാരം, തീറ്റ, വെള്ളം എന്നിവയും സാധാരണ നിലയിലാണെന്ന് കണ്ടെത്തി. അങ്ങനെ, ME ഓറൽ

അഡ്മിനിസ്ട്രേഷന് സുരക്ഷിതമാണെന്ന് കണ്ടെത്തി, തുടർ പഠനങ്ങൾക്കുള്ള അളവ് 100, 250, 500 mg/kg ആയി നിശ്ചയിച്ചു. b.wt

കീമോതെറാപ്പി മരുന്നുകളായ ഡോക്സോറൂബിസിൻ, സൈക്ലോഫോസ്ഫാമൈഡ് എന്നിവയുടെ കാർഡിയോടോക്സിക് ഇഫക്റ്റുകൾക്കായി നിരവധി സംവിധാനങ്ങൾ അനുമാനിക്കപ്പെട്ടിട്ടുണ്ട്. ഈ രണ്ട് കീമോതെറാപ്പി മരുന്നുകളും ദോഷകരമായ പ്രത്യാഘാതങ്ങൾ ഉണ്ടാക്കുന്ന പ്രാഥമിക സംവിധാനങ്ങളിലൊന്നാണ് ഓക്സിഡേറ്റീവ് സ്ട്രെസ്. അതിനാൽ, ME യുടെ ആന്റിഓക്സിഡന്റ് ശേഷി ഈ നെഗറ്റീവ് പ്രത്യാഘാതങ്ങളെ ലഘൂകരിക്കുമോ എന്ന് കണ്ടെത്തുകയായിരുന്നു ഈ പഠനത്തിന്റെ ലക്ഷ്യം.

ME യുടെ കാർഡിയോപ്രൊട്ടക്റ്റീവ് പഠനം മൂന്ന് ഘട്ടങ്ങളിലായാണ് നടത്തിയത്, അതായത് ഡോക്സോറൂബിസിൻ ഇൻഡ്യൂസ്ഡ് കാർഡിയോടോക്സിസിറ്റിയിൽ മോർഷെല്ല എസ്കുലെന്റയുടെ മെഥനോൾ സത്തിന്റെ പ്രഭാവം; സൈക്ലോഫോസ്ഫാമൈഡ്-ഇൻഡ്യൂസ്ഡ് കാർഡിയോടോക്സിസിറ്റി മോർഷെല്ല എസ്കുലെന്റയുടെ മെഥനോൾ സത്തിന്റെ പ്രഭാവം, കോമ്പിനേഷൻ തെറാപ്പി (സൈക്ലോഫോസ്ഫാമൈഡ്, ഡോക്സോറൂബിസിൻ)-ഇൻഡ്യൂസ്ഡ് കാർഡിയോടോക്സിസിറ്റിയിൽ മോർഷെല്ല എസ്കുലെന്റയുടെ മെഥനോൾ സത്തിന്റെ പ്രഭാവം. ഡോക്സോറൂബിസിൻ (DOX), സൈക്ലോഫോസ്ഫാമൈഡ് എന്നിവയും അവയുടെ സംയോജനവും ക്യാൻസറിനുള്ള ഒരു ചികിത്സാ ഉപാധിയാണ്. ഈ മരുന്നുകൾ മൂലമുണ്ടാകുന്ന കാർഡിയോടോക്സിസിറ്റി അവയുടെ ക്ലിനിക്കൽ ഉപയോഗത്തിന് ഒരു തടസ്സമാണ്. CP, DOX, CP+DOX എന്നിവയുടെ അഡ്മിനിസ്ട്രേഷന്റെ ഫലമായി ക്രിയേറ്റിൻ കൈനസ്-എംബി, ലാക്റ്റേറ്റ് ഡീഹൈഡ്രജനേസ്, ട്രോപോണിൻ I ലെവൽ എന്നിവയുടെ പ്രവർത്തനങ്ങളുടെ മൂലമുണ്ടാകുന്ന വർദ്ധനവ് ഡയഗണോസ്റ്റിക് കിറ്റുകളുടെ സഹായത്തോടെ നിർണയിച്ചു. മയോകാർഡിയത്തിലെ എൻഡോജെനസ് ആന്റിഓക്സിഡന്റ് അളവ് കുറയുന്നത് പരിശോധിച്ചത് സ്പെക്ട്രോഫോട്ടോമെട്രിക് പഠനങ്ങളിലൂടെയാണ്. Nrf-2, iNOS, NF-κB, പ്രോ-ഇൻഫ്ലമേറ്ററി സൈറ്റോകൈൻസ് ജീൻ എക്സ്പ്രഷൻ എന്നിവ RT-PCR വിശകലനം വഴി നിർണ്ണയിച്ചു. മൈറ്റോകോൺഡ്രിയൽ ഡിസ്ഫംഗ്ഷൻ വിലയിരുത്തുന്നതിന്, ടിസിഎ സൈക്കിളും ഇലക്ട്രോൺ ട്രാൻസ്പോർട്ട് ചെയിൻ കോംപ്ലക്സുകളും എൻസൈമുകളുടെ പ്രവർത്തനങ്ങളും നിർണ്ണയിച്ചു. CP, DOX, CP+DOX എന്നിവ മൂലമുണ്ടാകുന്ന കാർഡിയാക് ടിഷ്യു കേടുപാടുകൾ ഹിസ്റ്റോപത്തോളജിക്കൽ പരിശോധനയിലൂടെ വിലയിരുത്തി.

ഈ മരുന്നുകൾ ഉയർത്തിയ കാർഡിയാക്ക് ഇൻജുറി മാർക്കർ ലെവലുകൾ ME ഗണ്യമായി കുറച്ചു. CP,DOX, CP+DOX അഡ്മിനിസ്ട്രേഷൻ വഴി ക്ഷയിച്ച SOD, GPx, GSH തുടങ്ങിയ എൻഡോജെനസ് ആന്റിഓക്സിഡന്റുകൾ മോർഷെല്ല എസ്ക്യൂലെന്റ ഏതാണ്ട് സാധാരണ നിലയിലേക്ക് പുനഃസ്ഥാപിച്ചു. മയോകാർഡിയൽ പരിക്കിലേക്ക് നയിക്കുന്ന കീമോതെറാപ്പി ഡ്രഗ് അഡ്മിനിസ്ട്രേഷൻ മൂലമുണ്ടാകുന്ന ഓക്സിഡേറ്റീവ് സ്ട്രെസ് കുറക്കുന്നതിനുള്ള മോർഷെല്ല എസ്കലേറ്റയുടെ പ്രഭാവത്തെ ഇത് സൂചിപ്പിക്കുന്നു. ഹിസ്റ്റോപത്തോളജിക്കൽ നിരീക്ഷണം കണ്ടെത്തലുകളെ പിന്തുണച്ചു. CP, DOX, CP+DOX-ഇൻഡ്യൂസ്ഡ് ആൻറി ഓക്സിഡന്റ് സ്റ്റാറ്റസിന്റെ കുറവും ന്യൂക്ലിയർ ഫാക്ടർ എറിത്രോയിഡ് 2-അനുബന്ധ ഫാക്ടർ 2 ന്റെ പ്രകടനവും മോർഷെല്ല എസ്ക്യൂലെന്റ പുനഃസ്ഥാപിച്ചു. NF-κB, IL-6, IL1-β, TNF-α, iNOS എന്നിവയുടെ CP,DOX, CP+DOX-ഇൻഡ്യൂസ്ഡ് എക്സ്പ്രഷൻ മോർഷെല്ല എസ്ക്യൂലെന്റ അറ്റൻവേറ്റ് ചെയ്തു. കീമോതെറാപ്പിയുടെ ഫലമായി മൈറ്റോകോൺഡ്രിയൽ എൻസൈമുകളുടെ കുറഞ്ഞ അളവും ME പുനഃസ്ഥാപിച്ചു.

മോറൽ കൂണുകളുടെ ബയോ ആക്റ്റീവ് സത്തിൽ അഗാധമായ ആന്റിഓക്സിഡന്റ് പ്രവർത്തനം ഉണ്ടെന്ന് കണ്ടെത്തിയതിനാൽ, കീമോതെറാപ്പി മരുന്നുകളുടെ ആന്റിനിയോപ്ലാസ്റ്റിക് പ്രവർത്തനത്തിൽ ഈ സത്തകൾ ഉണ്ടാകാനിടയുള്ള ഇടപെടൽ പലപ്പോഴും ഊഹിക്കപ്പെടുന്നു. അതിനാൽ മോർഷെല്ല എസ്ക്യൂലെന്റയുടെ ബയോ ആക്റ്റീവ് എക്സ്ട്രാക്റ്റ് ഉപയോഗിച്ച് ചികിത്സിക്കുന്ന സോളിഡ് ട്യൂമർ ചുമക്കുന്ന എലികളിൽ ഡോക്സോറൂബിസിൻ, സൈക്ലോഫോസ്ഫാമൈഡ് എന്നിവയുടെ സ്വാധീനം വിലയിരുത്താൻ മറ്റൊരു പഠനം നടത്തി. സ്വിസ് ആൽബിനോ എലികളുടെ വലത് പിൻകാലുകളിൽ ഡാൽട്ടന്റെ ലിംഫോമ അസൈറ്റ്സ് (ഡിഎൽഎ) കോശങ്ങൾ സബ്ക്യുട്ടേനിയസ് കുത്തിവയ്പ്പിലൂടെ സോളിഡ് ട്യൂമർ ഉണ്ടാക്കി. ട്യൂമർ ഇൻഡക്ഷനെത്തുടർന്ന് എം.എസ്കുലെന്റയുടെ വിവിധ സാന്ദ്രതയിലുള്ള മെഥനോൾ സത്തിൽ മൃഗങ്ങൾക്ക് നൽകപ്പെട്ടു. ട്യൂമർ ഇൻഡക്ഷൻ കഴിഞ്ഞ് നാലാഴ്ചത്തേക്ക് ട്യൂമർ വളർച്ച (വോളിയവും പിണ്ഡവും) അളക്കുന്നു. സെറമിലെ ഹൃദയാഘാത മാർക്കറുകളുടെ അളവ്, മയോകാർഡിയത്തിലെ ആന്റിഓക്സിഡന്റ് നില, ഹൃദയ കോശങ്ങളുടെ ഹിസ്റ്റോപാത്തോളജി എന്നിവ നിർണ്ണയിച്ചുകൊണ്ട് മെഥനോളിക് സത്തിൽ കാർഡിയോപ്രൊട്ടക്റ്റീവ് പ്രഭാവം വിലയിരുത്തി. ട്യൂമർ വഹിക്കുന്ന എലികളിൽ എം. എസ്കുലെന്റയുടെ മെഥനോൾ സത്തിൽ കാര്യമായ കാർഡിയോപ്രൊട്ടക്റ്റീവ് പ്രഭാവം കാണിക്കുന്നതായി ഫലങ്ങൾ കാണിക്കുന്നു. മാത്രവുമല്ല കീമോതെറാപ്പി

മരുന്നുകളുടെ പ്രാവീണ്യത്തിൽ ഒട്ടും തന്നെ കുറവ് മോർഷെല്ല മൂലം ഉണ്ടാവുന്നില്ല എന്നതും ഈ പഠനം തെളിയിച്ചു

കീമോതെറാപ്പി മൂലമുണ്ടാകുന്ന കാർഡിയോടോക്സിസിറ്റിക്കെതിരെ മോറെൽ മഷ്റൂം മോർഷല്ല എസ്കുലെന്റയുടെ കാർഡിയോപ്രൊട്ടക്റ്റീവ് ഫലത്തെക്കുറിച്ചുള്ള സമഗ്രമായ അന്വേഷണത്തിന്റെയും പരീക്ഷണാത്മക കണ്ടെത്തലുകളുടെയും സമാഹാരമാണ് ഈ തീസിസ്. പഠനങ്ങൾ പത്ത് അധ്യായങ്ങളായി സമാഹരിച്ചിരിക്കുന്നു: ആമുഖം (അധ്യായം I), സാഹിത്യത്തിന്റെ അവലോകനം (അധ്യായം II), മെറ്റീരിയലുകളും രീതികളും (അധ്യായം III), പ്രധാന ബയോആക്ടീവ് സംയുക്തങ്ങളുടെ വേർതിരിച്ചെടുക്കലും തിരിച്ചറിയലും (അധ്യായം IV), M. esculenta ഫ്രൂട്ടിംഗ് ബോഡിയുടെ മെത്തനോളിക് സത്തിൽ അക്യൂട്ട് ഓറൽ ടോക്സിസിറ്റി പഠനം (അധ്യായം V), ഡോക്സോറോബിസിൻ ഇൻഡ്യൂസ്ഡ് കാർഡിയോടോക്സിസിറ്റിക്കെതിരെ എം. എസ്കുലെന്റ ഫ്രൂട്ടിംഗ് ബോഡിയുടെ മെത്തനോളിക്സത്തിൽ സംരക്ഷണ ഫലത്തിന്റെ വിലയിരുത്തൽ (അധ്യായം VI), സൈക്ലോഫോസ്ഫാമൈഡ്-ഇൻഡ്യൂസ്ഡ് കാർഡിയോടോക്സിസിറ്റി ക്കെതിരെ എം. എസ്കുലെന്റ ഫ്രൂട്ടിംഗ് ബോഡിയുടെ മെത്തനോളിക് സത്തിൽ സംരക്ഷണ ഫലത്തിന്റെ വിലയിരുത്തൽ (അധ്യായം VII), സൈക്ലോഫോസ്ഫാ മൈഡിന്റെയും ഡോക്സോറൂബിസിന്റെയും സംയോജിത ചികിത്സയ്ക്കെ തിരെ എം.എസ്കുലെന്റ ഫ്രൂട്ടിംഗ് ബോഡിയുടെ മെത്തനോളിക് സത്തിൽ സംരക്ഷിത ഫലത്തെക്കുറിച്ചുള്ള വിലയിരുത്തൽ (അധ്യായം VIII), ഡോക് സോറൂബിസിൻ, സൈക്ലോഫോസ്ഫാമൈഡ്, സൈക്ലോഫോസ്ഫാമൈഡ് + ഡോക് സോറൂബിസിൻ എന്നിവയുടെ സംയോജനത്തിന്റെ ട്യൂമർ വിരുദ്ധ പ്രവർത്തന ത്തിന്റെ വിലയിരുത്തൽ (അധ്യായം IX)., മോർഷെല്ല എസ്കുലെന്റയുടെ മെത്തനോളിക് സത്തിൽ അടങ്ങിയിട്ടുള്ള പദാർത്ഥങ്ങളുടെ ഇൻ സിലിക്കോ പഠനം (അധ്യായം X).

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ABBREVIATIONS

ACEIs: Acetyl choline Esterase Inhibitors

BSA: Bovine Serum Albumin

BTB: Bric a Brac domain

CaMKII: Calmodulin kinase -II

CD: Cyclophosphamide + Doxorubicin

CK-MB: Creatine Kinase- Myoglobulin

CP: Cyclophosphamide

CPCSEA: Committee for the purpose of control and supervision of experiments on animals

CTRCD: Chemotherapy Related Cardiovascular Diseases

CVDs: Cardiovascular Diseases

DCPIP: 2,6-Dichlorophenolindophenol Sodium Salt

DEX: Dexrazoxane

DLA: Dalton's Lymphoma Ascites

DMF: Dimethyl fumarate

DMSO: Dimethyl Sulphoxide

Dox: Doxorubicin

DTNB: (5,5-dithio-bis-(2-nitrobenzoic acid)

ECG: Electrocardiogram

EDTA: Ethylene diamine tetra acetic acid

eNOS: endothelial nitric oxide synthase

FDA: Food and Drug Administration

FRAP: Ferric ion reducing antioxidant power

GPx: Glutathione peroxidase

GSH: reduced glutathione

GST: Glutathione-S-Transferase

H&E: Haematoxylin-eosin

H_2O_2: Hydrogen Peroxide

HER-2: Human Epidermal Growth Factor-2

HPTLC: High Performance Thin Layer Chromatography

IAEC: Institutional Animal Ethical Committee

ICIs: Immune checkpoint inhibitors

IL1-β: Interleukin 1-β

IL-6: Interleukin-6

iNOS: Inducible nitric oxide synthase

ITS: Internal Transcriber Sequence

KBTB: Kelch- repeat and BTB domain-containing protein

KCN: Potassium cyanide

KEAP-1: Kelch -like ECH-Associated Protein 1

KLHL: Kelch- Like protein family

LC-MS: Liquid Chromatography-Mass Spectrometry

LDH: Lactate dehydrogenase

LVD: Left Ventricular Dysfunction

Maf: Musculoaponeurotic fibrosarcoma

MDA: Malondialdehyde

ME: Methanol extract of *Morchella esculenta*

mPTP: Mitochondrial permeability transition pore

NAD: Nicotinamide adenine dinucleotide phosphate

NADH: Nicotinamide adenine dinucleotide reduced

NBT: Nitro blue tetrazolium

NF-κB: Nuclear factor Kappa B

NOXs: NADPH oxidases

Nrf-2: Nuclear factor erythroid 2-related factor 2

OECD: Organization for Economic Co-operation and Development

PDB: Protein Data Bank

PTAH: Phosphotungstic acid-haematoxylin

RAAS: Renin-Angiotensin-Aldosterone System

RCSB: Research Collaboratory Structural Bioinformatics Protein Data Bank

RICVD: Radiation Induced Cardiovascular Diseases

RIPK3: Receptor interacting protein kinase-3

ROS: Reactive Oxygen Species

RT: Radiation therapy

RT-PCR: Real Time- Polymerase Chain Reaction

SOD: Superoxide Dismutase

TBA: Thio barbituric acid

TBARS: Thio barbituric acid reactive substances

TCA: Tri carboxylic acid

TCM: Traditional Chinese Medicine

TLC: Thin Layer Chromatography

TNF-α: Tumor Necrosis Factor- α

TOP-2: Topoisomerase-2

α- LA: α- Lipoic acid

CHAPTER 1
INTRODUCTION

Introduction

In the industrialized world, cancer and cardiovascular diseases are the leading causes of morbidity and mortality. Although 99% of cancer cases are in adults, it is the most common cause of disease-related mortality in children and adolescents. The number of long-term cancer survivors is predicted to rise by roughly 30% over the next ten years due to advancements in cancer therapy, which improve patient survival. The management of severe acute and chronic toxicities resulting from chemotherapy is one of the major issues that persist in the treatment of paediatric tumours, despite advancements in the use of poly-chemotherapy and radiation. The drawbacks of clinically disrupting increasingly intensive therapy include direct and indirect cardiovascular damage (Melendez et al., 2023; Mir et al., 2023). Cardiovascular dysfunction related to cancer therapy, or Chemotherapy Related Cardiovascular Diseases (CTRCD), encompasses a range of cardiac dysfunctions, from asymptomatic cardiac damage defined only by elevated cardiac biomarker levels to heart failure with accompanying clinical symptoms. Patient comorbidities, the length of treatment, and the type of chemotherapy agent employed all vary widely in the prevalence of cardiac dysfunction linked to cancer therapies. There are three distinct forms of anthracycline-induced cardiotoxicity: acute/early-onset, chronic progressive, and late-onset. Less than 1% of patients experience acute cardiotoxicity during or just after anthracycline infusion, which presents as a transient, abrupt decline in contractility. 1.6% to 2.1% of individuals experience the early-onset chronic progressive form, which typically manifests a few months following therapy. While 1.6%–5% of patients experience late-onset chronic progressive anthracycline-cardiotoxicity at least a year after treatment ends, cardiac dysfunction can be seen in patients up to 20 years following the final cycle of medication (Meo et al., 2023).

Many anti-cancer therapies, including anthracyclines, 5-fluorouracil, cyclophosphamide, immune checkpoint inhibitors, HER-2 antagonists, tyrosine kinase inhibitors, and radiation therapy, are linked to cardiotoxicity.

1. *Anthracyclines*: Doxorubicin and idarubicin, two extensively used anthracyclines, are linked to dose-dependent cardiotoxicity in solid malignant tumours such as osteosarcoma and breast cancer, as well as hematologic

lymphoproliferative diseases such as acute lymphoblastic leukaemia and non-Hodgkin lymphoma (Triarico et al., 2022).

2. *Cyclophosphamide*

At higher dosages, cyclophosphamide can cause cardiac toxicity such as arrhythmia and fulminant myocarditis.

3. *Trastuzumab*

The monoclonal antibody trastuzumab functions by binding to the erbB-2 protein kinase receptor, which is found in cardiomyocytes and is involved in the control of several processes, including angiogenesis, cell adhesion, hypertrophy, apoptosis, and adrenergic sensitivity.

4. *Mitoxantrone*

DNA topoisomerase-II inhibitor mitoxantrone has anti-inflammatory, immunomodulatory, and antineoplastic properties. Diastolic and diastolic dysfunction in cancer patients accounts for 2% of cardiotoxicity cases, which is dose-dependent (Tetterton-Kellner et al., 2024).

Research on anthracyclines and HER2 inhibitors is the main focus of published studies on preventing chemotherapy-induced cardiotoxicity. Prior to initiating cancer chemotherapy, cardiotoxicity should be prevented. The patient's cardiovascular risk should be assessed, and the optimum course of treatment should be decided based on the findings by the cardiologist and the oncologist. First, it is advised that patients lower their cardiovascular risk by controlling their blood pressure, lowering their cholesterol, maintaining a healthy blood glucose level, eating a nutritious diet, and quitting smoking. This will help prevent chemotherapy-related cardiotoxicity. The US Food and Drug Administration (FDA) has approved dexrazoxane as the sole cardioprotective medication for anthracycline-induced cardiotoxicity. Dexrazoxane inhibits the binding of doxorubicin by altering the structure of topoisomerase IIb. This threshold may have resulted from findings that dexrazoxane can impair the antitumor efficacy of anthracyclines. Only a few clinical trials have supported the contentious use of

cardiovascular medications, such as beta-blockers, ACEIs, and angiotensin receptor blockers, to reduce cardiotoxicity (Sharalaya et al., 2018; Avila et al., 2019).

There is currently no proven treatment for anthracycline-induced cardiotoxicity; instead, efforts are primarily directed toward preventive measures, which are divided into two categories: primary measures, which aim to prevent the disease while treatment is being administered, and secondary measures, which aim to prevent the progression of symptoms (Firoozbakhsh et al., 2024). Effectiveness of natural products as a prevention of chemotherapy-induced cardiotoxicity. Traditional Chinese medicine (TCM) has a well-established track record of success in treating cancer and cardiovascular illnesses. With its potential to prevent and treat cardiotoxicity from anti-tumor medications, TCM offers several benefits, such as reduced side effects, affordability, and ease of use (Shi et al., 2024). Various study reports are available on the protective effects of fruits, vegetables, phytochemicals extracted from plants, herbs, and spices, and semi-synthetic compounds, on chemotherapy-induced cardiotoxicity.

There are unexplored medicinal resources in mushrooms that can be used to treat a variety of illnesses (Venturella et al., 2021). Various compounds, such as pyrogallol, lectins, and flavonoids, from *Agaricus bisporus* have shown anti-inflammatory and anti-aging properties. Glucans from *Auricularia auricula* possess anti-tumor properties; flammulin, FVP (flammulina polysaccharide-protein), proflamin (glycoprotein), and a prolamin (active sugar protein) from Flammulina velutipes have shown anti-tumor, antioxidant, anti-viral, and immunomodulatory effects; Ganoderic acids, ganodermanontriol, ganoderiol, polysaccharides, germanium, triterpenoids, nucleotides, and nucleosides; β-glucan from *Ganoderma lucidum* demonstrated anti-tumor, hepatoprotection, cardioprotection, and imuunomodulatory effects; Lentinan, glucan, mannoglucan, fucomannogalactan, lentin (protein), catechinflavonoids, eritadenine from *Lentinula edodes* possess immunomodulatory, antitumor, anti-inflammatory, anti-fungal, antioxidant, anti-bacterial, antifungal, antioxidant, hypolipidemic activity; Hericenones and erinacines from *Hericium erinaceus* shown neuroprotective properties; Cordycepin from *Cordyceps militaris* possess anti-depressent and hypoglycemic effect (Sahoo et al., 2022).

Morchella esculenta is an edible fungus within the Morchellaceae family. This mushroom is locally known as Guchhi. *Morchella esculenta* is well-known worldwide for its multitude of powerful medicinal ingredients that can effectively treat a wide range of human ailments (Sunil et al., 2022). Most commonly, it is found growing wild in the hilly areas in northern India (such as Uttarakhand, Himachal Pradesh, and Jammu & Kashmir). *Morchella esculenta* is a highly valued mushroom for its nutritional and medicinal benefits. *Morchella esculenta* possesses proteins, carbohydrates, polyunsaturated fatty acids, and various bioactive secondary metabolites (Badshah et al., 2021). Numerous phenolic compounds, polysaccharides, galactomannans, organic acids, tocopherols, and other phytochemical constituents are present in it (Eraslan et al., 2021). These constituents have pharmacological qualities that are beneficial to human health, such as antioxidant, antimicrobial, anti-allergenic, anti-inflammatory, antitumor, neuroprotective, and immunostimulatory qualities. This mushroom possess several ethnomedical uses. Tribal communities in the high-altitude Kashmir region, such as Gujar and Baqirwaal, use *M. esculenta* as traditional medicine to treat various ailments (Anand et al., 2023).

Various study reports are available on the antioxidant activity of *M. esculenta* in both *in vitro* and *in vivo* study models (Li et al., 2024; Chen et al., 2023). *M. esculenta* is also reported to have anti-diabetic, cytotoxic, anti-inflammatory, immunomodulatory, and hepatoprotective benefits in various experimental models (Nitha et al., 2020; Wu et al., 2023; Dissanayake et al., 2021; Li et al., 2021; Zhang et al., 2024). With the growing significance of natural product-derived cardioprotective agents, there is an urge to find substitutes that don't compromise the effectiveness of chemotherapy drugs. In our present study, an intensive investigation on the cardioprotective properties of *M. esculenta* fruiting body methanol extract was carried out and presented in this thesis.

CHAPTER 2

REVIEW OF LITERATURE

Cardio oncology: CVDs and Cancer

Modern therapeutic strategies employed in cancer treatment were successful in improving the recovery rate of cancer patients and they helped in increasing the life span for 10 years or longer (Miller et al.,2016). However, cardiovascular complexities associated with the chemotherapeutic treatment was a serious ill effect in cancer survivors indicates the side effects of chemotherapy (Ioffe et al.,2024). As a result, mortality rate of cancer survivors occurs as a result of cardiovascular diseases and not from cancer recurrence (Wang et al., 2022). Radiation therapy is employed to treat the initial stage of cancer but as the cancer advances the treatment protocol will be more specific such as chemotherapy, monoclonal antibody therapy, molecular therapy, drugs targeting signaling pathways etc. which are more likely to induce severe organ toxicities. Therefore, cardio oncology emerged as a new field of medical sciences which focus on the relation between CVDs and cancer (Dutra et al., 2024).

In developed countries more than 65% of the death rate is due to malignant diseases and CVDs which are multifactorial and both share similar risk factors such as diabetes, smoking and alcohol, age, dyslipidemia, obesity etc. (Yang et al.,2023). Various strategies can be employed to lower the risks associated with both the diseases. A person with cardiovascular risks are highly prone to the side effects by chemotherapy rather than a healthy individual (Velusamy et al., 2023). So, analyzing the preexisting cardiovascular health of an individual prior to treatment is recommended to reduce the CVD burden (Raisi-Estabragh et al., 2023). Preexisting cardiovascular diseases increases the risk associated with chemotherapy especially in case of anthracycline treatments. A prior diagnosis is essential before prescribing chemotherapeutic agents. ECG, cardiac injury marker levels, predisposing factors such as obesity, hypertension, diabetes should be considered (Abbas et al., 2023).

Cardiotoxicity associated with various cancer therapies.

1. Radiation therapy.

X-rays and Gamma rays are the most used radiations in a clinical setting. RICVD (Radiation Induced Cardiovascular Diseases), is one of the major concerns among patients suffering from cancer who have received radiation therapy (RT) (Logotheti et al., 2024). RT induces microvascular changes and causes inflammation which results

in atherosclerosis and endothelial dysfunction which eventually results in damage to pericardium and myocardium, defects in conduction system, valve damage etc (Siaravas et al., 2023). The severity of cardiac damage depends on the cumulative dose of the radiation and also the proximity of heart to the area exposed to radiation. Studies suggest that a radiation dosage above 30 Gy/m^2 is more likely to induce RICVDs, whereas dosage lower than 30 Gy/m^2 and usage of cardiac shielding decrease the pancreatitis incidence by 20%. Along with this lifestyle of the patient also plays a major role in the development of RICVDs such as smoking, diabetes, obesity, hyperlipidaemia, hypertension is some enhancing factors. Various pathophysiological factors attribute to RT induced CVDs, mainly RT induced chronic inflammation which occurs as a result of endothelium damage that leads to the release of inflammatory cytokines, monocytes etc (Vasiliadis et al., 2014). This process ultimately results in the formation of atherosclerotic plaques. In addition, oxidative stress also occurs which causes the activation of NF-kB. Persistent activation of NF-kB results in fibrotic changes which further activates inflammatory mediators (Shoukat et al., 2019).

2. **Chemotherapy.**

Chemotherapy is one of the most adopted methods for cancer treatment either as adjuvant or as a primary therapy (Angsutararux et al., 2015). But its usage is delimited by the various toxicities associated with it. The basic mechanism of chemotherapeutic drugs is to impair mitotic and metabolic functions of the cancer cells. However, in some cases they also hinder the functioning of normal cells and tissues which eventually imparts certain ill effects from mild nausea and vomiting to life threatening conditions such as heart failure. Cardiotoxicity is caused by a number of chemotherapeutic agents but anthracycline induced cardiotoxicity is the most severe (Bhagat e al.,2020). Chemotherapeutic agents including alkylating agents (cyclophosphamide, ifosfamide); antimetabolite agents (Capecitabine, 5-fluorouracil and cytarabine); antimicrotubular agents (paclitaxel, vinca alkaloids); anthracyclines and anthraquinones (doxorubicin, mitoxantrone) induces potential cardiotoxicity (Briasoulis et al., 2022). These drugs are not recommended to patients with high risk of developing cardiac complications. The severity of cardiotoxicity is also associated with the route of administration, dosage, and duration of the regimen (Chaulin et al.,2020). Anti-neoplastic agents are classified

as type I and type II drugs based on the damage they confer on cardiac tissue. Type I agents are drugs which imparts irreversible cardiac damage whereas type II agents confer reversible cardiac damage. Cardiotoxicity can be acute, which is rare, transient and dose-independent or it can be chronic which is dose dependent. Chronic toxicity is further classified in to two types, one in which the cardiotoxicity is occurs at the beginning of the treatment whereas in the other the complications begin later more than a year after the treatment (Adao et al., 2013).

3. Tumour immunotherapy.

Immune Checkpoint Inhibitors (ICIs) represent an architype revolution in oncological therapy, anchoring the immune system to identify and target cancer cells. The scope of usage of ICIs also increased as an adjuvant therapy along with other therapy strategies (Achim et al., 2022). Amidst of all the benefits there also comes the increased risk of cardiotoxicity associated with ICIs including myocarditis or acute coronary events which demands for immediate intervention with the treatment of steroids in order to combat these cardiovascular complications (Li et al., 2022). Even though the risk of cardiotoxicity consequent to ICIs are rare and reported scarcely the mortality rate associated with it is high which accounts for about 30-50% fatality. Alongside, patients underwent ICIs are more prone to cardiovascular morality and arrest and cardiogenic shock. The elevated levels of cardiac injury markers such as cardia troponin I/T, Brain Natriuretic Peptide, N-terminal pro-BNP, left ventricular impairment and cardiac inflammation which can be diagnosed by cardiac MRI or endomyocardial biopsy (Gan et al.,2022).

The pathogenesis of ICIs induced cardiomyopathy occurs as a result of the hindrance of PD-1-PD-L1 axis which associated with immune reactions. PD-L1 is expressed in cardiomyocytes which can be upregulated by various cytokines which interacts with PD-1 on the T-lymphocytes to inhibit the autoimmune response. Inflammation consequent to ICIs and coronary vasculitis induced by T cell activation contribute to the myocardial infarction after ICI treatments. Termination of ICI treatment escorted by high-dose glucocorticoids treatment are the favoured remedy in patients with ICI-mediated autoimmune myocarditis (Lobenwein et al., 2021; Brumberger et al.,2022).

Doxorubicin induced cardio toxicity

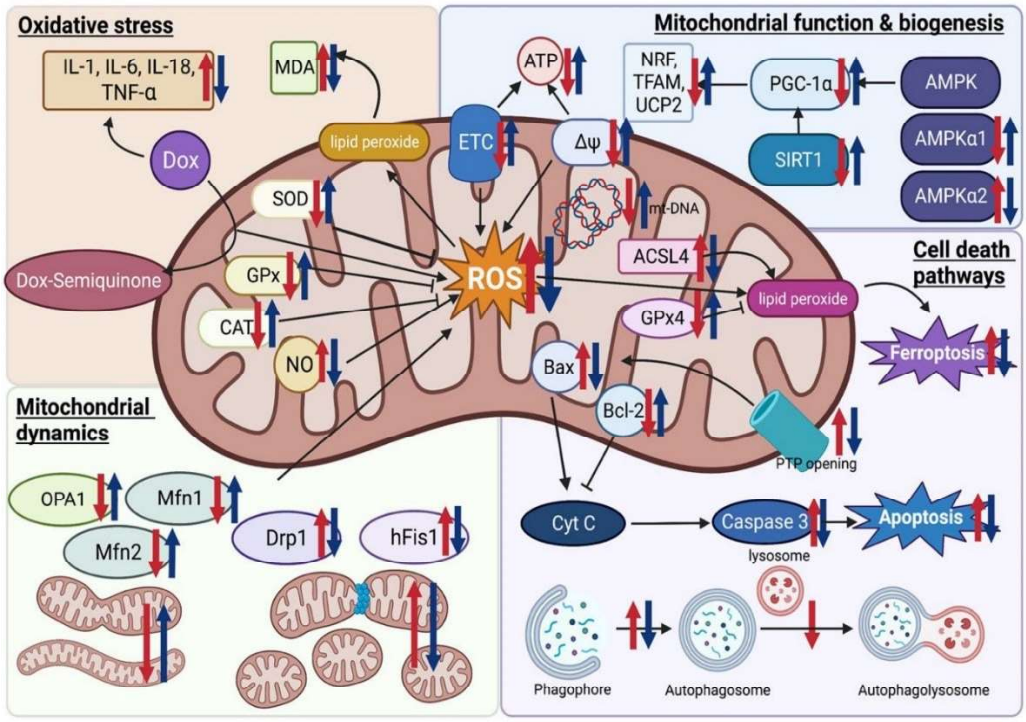

Figure.2.1. Pathways involved in doxorubicin induced cardiotoxicity (Attachaipanich et al., 2023)**.**

Anthracyclines are a class of broad-spectrum antibiotics isolated from *Streptomyces peucetius var. caesius* actinomyces which includes doxorubicin, daunorubicin, and epirubicin. They are employed in the treatment of various leukaemia and solid tumours such as breast, stomach, lungs and pancreas (Attachaipanich et al., 2023; Pournami et al.,2024). However, these therapeutic drugs are associated with dose dependent cardio toxicity which eventually leads to heart failure. A huge amount of evidence points that cardiomyopathy occurs at lower doses of anthracyclines if there are risk factors such as hypertension, arrhythmias, coronary disease as well as genetic predisposition associated with patients (Bhatia et al.,2020). Anthracycline associated cardiac injury initiates after a single dose of drug administration which is reflected in the injury marker levels. Even though, vast majority of the complications is being identified one year after completing the treatment (Cardinale et al.,2020).

There are several proposed mechanisms associated with doxorubicin induced cardiotoxicity. Oxidative stress is one of the major postulated mechanisms involved

in doxorubicin induced cardiotoxicity (Rawat et al., 2021). Doxorubicin disrupts the iron metabolism pathways which will accelerate iron accumulation and ferroptosis. This will lead to the formation of Dox-Fe complex which will cause the conversion of hydrogen peroxide to more powerful oxygen free radicals (Fang et al., 2023). Under normal physiological conditions the free radicals will be quenched by antioxidant defence mechanisms such as GSH (reduced glutathione), GPx(Glutathione Peroxidase), SOD (Super oxide Dismutase) , GST(Glutathione-S-Transferase) and Catalase. These enzymatic and non-enzymatic antioxidants aids in the quenching of the free radicals and lowers lipid peroxidation which will otherwise be detrimental to cell. Because of its ability to transport toxins across plasma membrane, GSH makes a good target for doxorubicin and thereby increasing cellular oxidative damage (Kong et al.,2022).

During doxorubicin treatment NADPH is recruited and it will act as a substrate for the production of ROS by NOXs(NADPH oxidases) and this dysregulation cause a depletion in the GSH level. This depleted levels of GSH will downregulate the expression of other antioxidants such as GPx, SOD, catalase which will again elevate the oxidative stress. Thus, Dox induced oxidative stress by chelation of iron and iron overload imparts in its cardiotoxicity (Jiang et al., 2023). Dexrazoxane is the only FDA approved cardioprotective agent which act as an iron chelator and there by conferring protection against doxorubicin induced cardiotoxicity (Hasinoff et al., 2020).

Doxorubicin induced necroptosis is another mechanism by which cardiotoxicity occurs (Kong et al., 2022). Necroptosis is another form of necrosis which involves the release of death signalling cytokines. Doxorubicin activates necroptosis through a novel pathway which along with necrosis cause more cell death than apoptosis pathway (Christidi et al.,2021). Doxorubicin elevates the expression of RIPK3 that phosphorylates calmodulin kinase II (CaMKII) which ultimately regulates the opening of the mitochondrial permeability transition pore (mPTP) leading to necroptosis and apoptosis (Chen et al., 2023). The elucidation of exact mechanism of doxorubicin induced necroptosis will aid in the development of novel agents that have the potential to block this necroptosis pathway (Robert et al.,2023). Necrostatin-1 and Kn-93 a CAMKII inhibitor are the agents which have shown

protection against doxorubicin induced necroptosis in experimental animals (Wang et al.,2023).

DNA-topoisomerase also plays a major role in DOX induced cardiotoxicity (Li et al.,2021). Recent studies reveal cytotoxicity of DOX in tumor cells is mainly due to the interaction between DOX and TOP2 levels in tumor cells (Zhang et al.,2012). TOP2α and TOP2β are the two forms of topoisomerases 2, of which TOP2α is absent in cardiomyocytes but it is over expressed in tumour cells. In tumour cells DOX forms a ternary complex with TOP2α (TOP2α-DOX-DNA) which results in DNA breakage and tumour cell death. (Lyu et al., 2007.) Bures et al., reported that TOP2β is a target for DOX induced cardiotoxicity. DOX forms ternary complex with TOP2β(TOP2β-DOX-DNA) which induce DNA double-strand breaks in cardiomyocytes, resulting in cell death. By forming ternary complex with TOP2β, DOX hindered mitochondrial biogenesis and mitochondrial function ((Bures et al., 2017).

The mechanisms involved in DOX-induced cardiotoxicity seems to be multifactorial, that involves elevated lipid peroxidation, oxidative stress, DNA/RNA damage, inhibition of autophagy, endoplasmic reticulum mediated apoptosis, and imbalance of calcium homeostasis. Although various mechanisms are shown to be involved in cardiotoxicity, the exact mechanism remains unclear (Songbo et al., 2019).

Cyclophosphamide induced cardiotoxicity

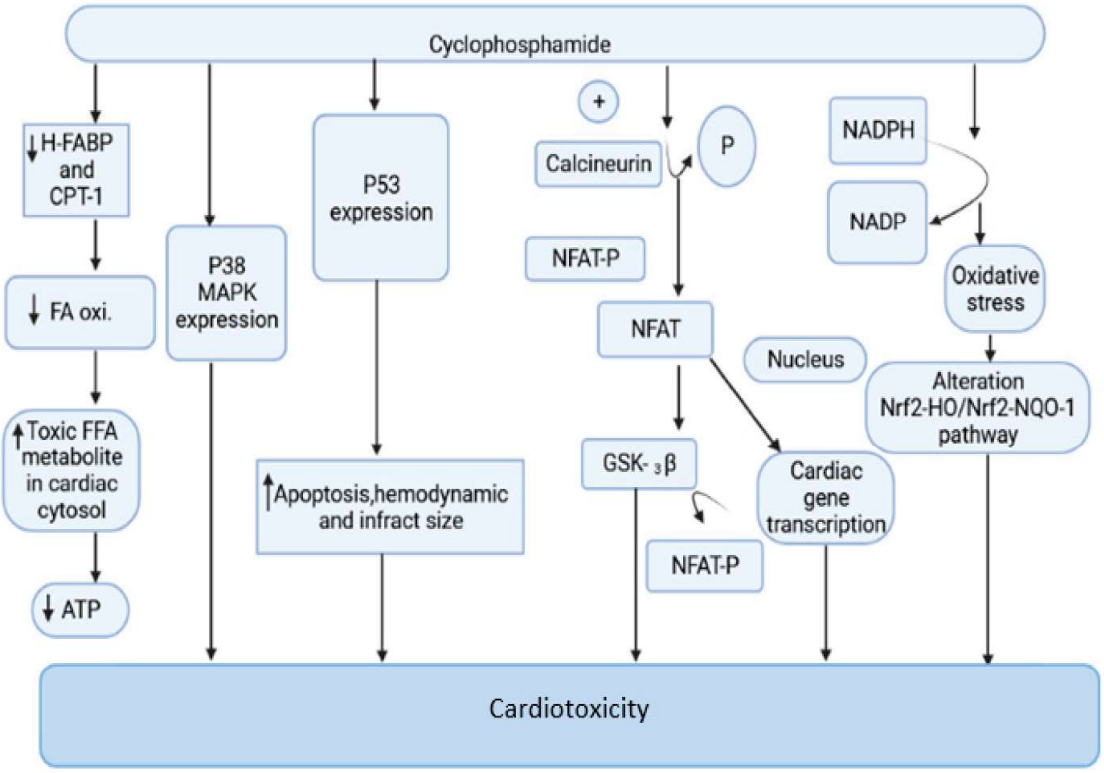

Figure.2.2. Pathway involved in cyclophosphamide induced cardiotoxicity (Adhikari et al.,2021)

Cyclophosphamide is an alkylating agent which is used as a potent immunosuppressant drug and also employed in the treatment of rheumatoid arthritis, sarcoma, myeloma, breast, lung and ovarian cancer (Ahmed et al., 2023). It is a prodrug upon administration get converted in to 4-hydrocyclophosphamide by cytochrome-P450 isozyme and coexists with aldophosphamide (tautomer) (Ayza et al., 2020). Aldophosphamide decomposes in to its active metabolites phosphoramide mustard and acrolein. The anti-tumour efficacy of the drug is imparted by phosphoramide mustard which intercalates with 7-guanine residue of DNA whereas the cardiotoxic effect is attributed by acrolein. Acrolein is an unsaturated highly reactive aldehyde to which cardiomyocytes are highly sensitive. The mechanism underlying Cyclophosphamide induced cardiotoxicity involves direct endocardial injury, followed by extravasation of toxic metabolites resulting in damage to cardiomyocytes, interstitial haemorrhage, and oedema. Acrolein interferes with the antioxidant defence mechanism and also elevates the release of

strongly active free radicals which further increase the damage level (Iqubal et al., 2021; Kamel et al., 2022).

The major implication associated with the excessive ROS generation is the damage to inner mitochondrial membrane which reduce the efficiency of cardiac mitochondria to counterbalance the ill effect of ROS. CP treatment results in the reduction of oxidative phosphorylation which causes a depletion in ATP synthesis which further damage the mitochondria (Iqubal et al., 2019). Along with this, increased expression of BAX in the mitochondrial membrane followed by CP treatment induces apoptosis. CP treatment alters calcium homeostasis which leads to the opening of mitochondrial permeability transition pore that results in the release of pro-apoptotic factors (Kohlhaas et al.,2017). Cyclophosphamide administration also results in decreased Nrf2 expression, endoplasmic reticulum stress and increase eNOS and iNOS expression (Adhikari et al., 2021).

Strategies for cardio protection

Mechanism involved in chemotherapy associated cardiotoxicity is multifaceted and various preventive strategies are targeting many of these pathways. Oxidative stress is one of the most focussed mechanisms of cardiotoxic events by chemotherapy (Cadeddu Dessalvi et al., 2021; Pondugula et al., 2022). Dexrazoxane is the first FDA approved drug which is an iron cheating agent that has been used as a protective agent against anthracycline induced cardiotoxicity. Dexrazoxane is a prodrug, upon administration get converted in to its active form by cardiomyocytes and inhibits the iron complex formation by anthracyclines, thereby reducing ROS generation. Moreover, it confers its protection by inhibiting the formation of Anthracycline-TOP2β-DNA complex and this mechanism make this drug sand out from other iron chelators (Veeder et al., 2021).

Another protective strategy against chemotherapy induced cardiotoxicity is neurohormonal blockade. Angiotensin- converting enzyme inhibitors (ACE-Is), Angiotensin II receptor blockers (ARBs), and beta-blockers alone or in combination has been employed as a preventive strategy for cardiotoxicity. Therefore, the blockade of Renin-Angiotensin-Aldosterone-System (RAAS) has proved to reduce the myocardial damage and prevention of heart failure in high-risk cardiac patients. ACE-Is such as captopril, enalapril and lisinopril are found to be effective against

chemotherapy induced cardiotoxicity which lowers ROS damage, intracellular calcium overload and interstitial fibrosis, and improves mitochondrial respiration, and cardiomyocyte metabolism (Kourek et al., 2022; Upshaw et al., 2020; Varricchi et al., 2018).

Beta-blockers are mainly used in treating patients with increased risk of heart failure with reduced ejection fraction (EF). Carvedilol, nebivolol are the last generation beta blockers and are used against anthracycline induced left ventricular dysfunction. In addition to this clinically approved drugs, natural products are emerging as an alternative cardioprotective treatment strategy (Barbosa et al., 2018). Various studies reported the cardio protective potential of curcumin, which can reduce myocardial atrophy and cardiac fibrosis. Several studies reported the cardioprotective effect of curcumin against cyclophosphamide and doxorubicin induced cardiotoxicity (Sadzuka et al., 2012; Avci et al., 2017; Chakraborthy et al., 2017). Likewise, various reports are available based on the cardioprotective nature of resveratrol, naringenin, proanthocyanin, indle-3-carbinol, apigenin, lycopene, quercetin etc., majority of them are plant flavonoids and phenols which exerts strong antioxidant effect. So, the quest for natural compounds having cardioprotective effect without hindering the anti-tumour efficacy of the chemotherapeutic drugs are underway (Sharma et al., 2018; Patel et al., 2018; Zeng et al.,2018; Wang et al., 2018; Sen et al., 2019; Colica et al., 2018; Abdelgawad et al., 2019; Salehi et al., 2019; Hajra et al., 2018; Domanska et al., 2018).

Mushrooms as food and medicine.

Mushrooms have been used as both food and medicine by humans in the past decades. Unlike medicinal mushrooms, edible mushrooms are primarily eaten as dried products or as fresh mushrooms with fruiting bodies. In contrast to their culinary versatility, medicinal mushrooms are primarily utilized in biopharmaceutical applications as powdered, loose, or liquid extract forms. They can also be eaten boiled, fried, roasted, soups, tinctures, teas, and many other cuisines (Elkhateeb et al., 2019). Folk medicine has utilized medicinal mushrooms for ages due to their biologically active chemicals that have positive health effects. These uses are widespread throughout the world. They are especially popular in Asian nations like Taiwan, Korea, China, and Japan. They are endowed with many biologically active substances, such as polysaccharides, proteins, peptides, terpenoids, polyphenols, vitamins, and mineral elements, and as a result, they

possess immunomodulatory, hypocholesterolaemia, hypoglycaemic, anti-inflammatory, and anti-cancer properties (Song et al., 2020; Elkhateeb et al., 2022).

Mushrooms are repertoires of a wide range of pharmacologically active substances. Research in the field of fungal derived bioactive substances is extensive now a days. In light of ill effects of treatment regimens, multidrug resistance etc., a constant quest for novel therapeutic compounds has become imperative. Thorough investigation and evaluation are needed for understanding the benefits of bioactive compounds obtained from mushrooms. Various study reports are available based on the therapeutic properties of medicinal mushrooms including anti-allergic, anti-arthritic, anti-asthmatic, anti-cancer, anti-inflammatory, anti-oxidant, immunomodulatory, immunostimulant, neuroprotective, hepatoprotective , nephroprotective, cardioprotective, anti-hypertensive etc (Cateni et al., 2021).

Mushrooms can be regarded as a low-calorie, low-fat food which consists of insoluble fiber, protein, vitamins, minerals and other nutrients. Major bioactive compounds in mushroom consists of phenolics, polysaccharides, tocopherols, terpenoids and phytosterols which imparts to its pharmacological properties. Various factors such as species, strain, cultivation conditions etc. determine the chemical composition of mushrooms (Barros et al., 2007; Safin et al., 2022). Mushroom bioactives can be systematically investigated and appraised, which can be extremely valuable for treating both infectious and non-infectious disorders.

Table.2.1. Therapeutic properties of mushrooms.

Sl.No.	Medicinal property	Mushroom/Bioactive compound	References
1	Anti-allergic	Chaga mushroomInotodiol, Triterpenoid	Nguyen et al., 2020
2	Anti-asthmatic	*Cordyceps sphecocephala*	Heo et al., 2010
3	Anti-cancer	*Ganoderma lucidum*Polysaccharide	Krishnakumar et al., 2023; Jones et al., 2000.
4	Anti-depressive	*Cordyceps militaris*	Lin et al., 2021
6	Anti-dermatophytic	*Lenzites quercina, Ganoderma lucidum* and *Rigidoporus ulmarius*	Ogidi et al., 2016

Sl.No.	Medicinal property	Mushroom/Bioactive compound	References
7	Anti-diabetic	*Coprinus comatus*	Husen et al., 2021
8	Anti-fertility	*Auricularia polytricha*	Agbor et al., 2020
9	Anti-fungal	*Lactarius deliciosus*	Volcao et al., 2022
10	Anti-helminthic	*Pleurotus ostreatus*	De Matos et al., 2020
11	Cytotoxic	*Ganoderma lucidum*	Khalilova et al., 2022
12	Anti-hypercholesterolemic	*Pleurotus tuberregium*	Oyetayo et al., 2020
13	Anti-inflammatory	*Psilocybe natalensis*	Nkadimeng et al., 2020
14	Anti-malarial	*Agaricus blazei*	Val et al., 2015
15	Anti-microbial	*Auricularia auricula*	Oli et al., 2020
16	Anti-oxidant	*Fomitopsis officinalis*	Altannavch et al., 2022
17	Anti-parasitic	*Trametes versicolor*	Sharma et al., 2023
18	Anti-spasmodic	Trametes hirsuta	Begum et al., 2023
19	Anti-viral	*Pleurotus ostreatus*	Lesa et al., 2022
20	Anti-cardiovascular illnesses	*Ganoderma lucidum*	Veena et al., 2022
21	Hepatoprotection	*Lentinus edodes*	Song et al., 2021
22	Immunomodulatory	*Coriolus versicolor*	Zhang et al., 2021
23	Immunostimulant	*Pleurotus florida*	Muthusamy et al., 2020
24	Insecticidal	*Pleurotus ostreatus*	Palavecino-De-La-Fuente et al., 2022
25	Larvicidal	*Pleurotus djamor*	Manimaran et al., 2021
26	Nematicidal	*Lentinula edodes*	Pineda-Alegría et al., 2021
27	Nephroprotection	*Ganoderma sp.*	Sinaeve et al., 2022
28	Neuroprotection	*Lignosus rhinocerus*	Kittimongkolsuk et al., 2021
29	Osteoprotection	*Grifola frondosa*	Xiong et al.,2024
30	Vasodilator	*Pleurotus pulmonarius*	Wistar-Kyoto et al., 2022

Table.2.2. Active ingredients in mushroom

Active ingredients	References
Glycoproteins	Kumar et al., 2015
Unsaturated fatty acids	Tel-cayan et al., 2017
Polynucleotides	Liu et al., 2016
Polysaccharides	Cheung et al., 2013; Ruthes et al., 2016
Phenolic compounds	Heleno et al., 2015
Tocopherols	Khatua et al., 2013
Ergosterols	Barreira et al., 2014
Lectins	Singh et al., 2014
Vitamins	Agrahar et al., 2005
Minerals	Wani et al., 2010
Amino acids	Wu et al., 2021

Mushrooms : Clinical significance

For hundreds of years, especially in Asian nations, medicinal mushrooms have been used as an illness remedy. It has also been utilized more recently to treat cancer and lung conditions. For almost 30 years, China and Japan have permitted the use of medicinal mushrooms to treat cancer in addition to recognized cancer therapies. These countries have an extensive proven track record of safe usage as stand-alone medications or in conjunction with chemotherapy or radiation. In Asia, about 100 different kinds of medicinal mushrooms are currently in use.

1. *Lentinula edodes* (Shiitake mushroom)

Shiitake mushrooms are employed as dietary supplements for immunomodulation, anticancer, antimicrobial, hypocholesterolemic, and hepatoprotective agent. The mushroom bioactives, are extracted either from the mushroom mycelium or the fruiting body. Bioactive compounds of shiitake mushroom possess various pharmacological effects such as:

1. Antibacterial
2. Antifungal
3. Cytostatic

4. Antioxidant
5. Anticancer
6. Immunomodulatory activity.

The major bioactive compounds in this mushroom includes:

a. Lentinan (a β-(1-3)-glucan/ polysaccharide).
b. Ergothioneine
c. Eritadenine. (Prince et al., 2023)

2. Chaga (Inonotus obliquus)

I. obliquus has been utilized as a folk remedy in Siberia, Russia, and other western nations since the 16th century. With little to no toxicity, it has been used to treat conditions like diabetes, cardiovascular disorders, and gastrointestinal cancer. The fungus was utilized as a traditional medicine in Siberia to treat liver disorders, helminth infections, and tuberculosis. Chaga focused on tinctures for the prophylaxis and treatment of stomach illnesses and cancers in North and Middle Russia. Because of the health benefits of chaga, it is also commonly consumed in Russia and Korea as tea or concentrate. Chemical characterization of *Inonotus obliquus* showed the presence of a wide range of bioactive compounds. *I. obliquus* has been shown to contain polysaccharides, polyphenols, lanostane-type triterpenoids, and inotodiol as secondary metabolites (Duru et al., 2019). These substances are thought to be the active ingredients responsible for a wide range of health-promoting properties, demonstrating its great medical worth. Moreover, it is non-toxic, well-tolerated by patients, and almost never contra-indicated for use in medicine, all of which add to its usefulness as a therapeutic agent (Ern et al., 2024).

3. ***Trametes versicolor (L.) Quél.***

Trametes versicolor, often referred to as turkey tail or tunzhi in China, has long been utilized as a "magic herb" throughout Asia, especially in China, where traditional remedies derived from this mushroom have been and continue to be used extensively to support longevity, health, and vigor. For clinical usage, the State Administration of Food and medications (SAFD) in China has currently approved at least 12 medications based on *C. versicolor*. The extract of this mushroom has two protein-bound polysaccharides that are responsible for its immunomodulatory effects: the

glycoprotein PSK (krestin), which is derived from the strain CM101 and is most commonly used in Japan, and the polysaccharide peptide (PSP), which is extracted from the deep layer cultivated mycelia of the COV-1 fungal strain and is primarily used in China (Venturella et al., 2021).

4. ***Ganoderma lucidum (Curtis) P. Karst.***

One of the most often used medicinal mushrooms in the world today is *Ganoderma lucidum,* referred to as lingzhi or reishi. It has been called the "mushroom of immortality" for a long time. Numerous pharmacological properties of *G. lucidum* are known to exist, namely cytotoxic, anti-diabetic, immunomodulatory, antihypertensive, anticancer, antimutagenic, antiaging, antibacterial, and hepatoprotective effects. These properties are mostly attributable to the two main metabolite categories found in *G. lucidum*: polysaccharides and triterpenes/triterpenoids. Triterpene compounds, which include lucidones, lucinedic acids, ganoderic acids, ganodermic acid, and ganodermic alcohols, are derived from lanosterol and have strong antitumor, antimetastatic, cytotoxic, and enzyme-inhibitory qualities (Bulam et al., 2019).

5. *Morchella esculenta* (L) Pers.

Figure.2.3. *M. esculenta* fruiting body

Morchella esculenta (L).Pers (Morchellaceae) is a highly prized culinary mushroom found in temperate regions. They are known as ‘Guchhi’ in India, it grows in natural

habitats at an altitude of 2500m-3500m as a mycorrhizal or saprobic relation with coniferous trees and hardwood. The morphological features of the fruiting body of this mushroom is similar to that of a honey comb with an upper portion which is 3-9cm long and 2-5 cm width known as pileus. The lower portion of this mushroom which is termed as stalk/stipe is hollow and 1-4 cm in length and 0.5-3 cm in thickness. The fruiting body appears as whitish to pale grey which upon maturity becomes greyish brown (Sunil et al., 2022).

Table.2.3. *Morchella esculenta*: Classification (Litchfteld et al., 2006)

Kingdom	Fungi
Phylum	Ascomycota
Class	Discomycetes
Order	Pezizales
Family	Morchellaceae
Genus	Morchella
Species	*Morchella esculenta*(L.)Pers

Table 4:- *Morchella sp.*: Active components and their pharmacological properties. (Alam et al., 2024)

Bioactive compound / Extract	Pharmacological relevance/ Bioactivity	Reference
Exopolysaccharide	Anti-diabetic	Wu et al., 2023
Mycelial extract	Anti-microbial	Badalyan et al., 2024
Polysaccharide	Hepatoprotection	Teng et al., 2023
Polysaccharide	Anti-inflammatory	Rehman et al., 2022
Fruiting body	Anti-oxidant	Taskin et al., 2021
Polysaccharide	Anti-atherosclerosis	Wang et al., 2021
Triterpenes	Anti-tumour	Wang et al., 2020
Polysaccharide	Gut microbiota modification	Rehman et al., 2022;Haq et al., 2022
Aqueous ethanolic extract	Nephroprotection	Nitha et al., 2008
Ethyl acetate extract	Arthritic edema inhibition	Ramya et al., 2022

Alkali extracted galactomannan; Polysaccharides	Immunomodulation	Zhang et al., 2022; Li et al., 2017
Crude polysaccharide; Polysaccharide from stipe of *M. esculenta*	Neuroprotection	Xiong et al., 2016; Cheng et al., 2024
Anti-aging activity	Extracellular polysaccharides	Fu et al., 2013
Anti-melanogenesis activity	Polysaccharide FMP-1	Cai et al 2018
Antiproliferative activity	Isolated compounds from fruiting body	Lee et al 2018
Anti-viral activity	Hexane and ethanol fractions from fruiting body.	Seetaha et al 2020
Anti-lipoxygenase activity	Crude extract	Bisakowski et al 2000
Proteolytic activity	Aqueous extract	Strapáč et al 2019

Various protective effects of *M. esculenta* mycelium and fruiting bodies were reported in both *in vitro* and *in vivo* study models. Chen et al., reported the antioxidant and hepatoprotective property of *M. esculenta* dextran sulfate induced liver toxicity in mice (Chen et al., 2023). Meng et al., reported the hepatoprotective effect of *M. esculenta* against alcohol induced liver injury mice model (Meng et al., 2019). Nitha et al., reported the nephroprotective activity of *M. esculenta* mycelia extract against cisplatin and gentamicin induced toxicity (Nitha et al., 2008). Hepatoprotective effect of *M. esculenta* mycelium against ethanol and CCl_4 induced chronic liver injury was reported by Nitha et al., (2013). Iqbal et al., reported the ameliorative effect of *M. esculenta* against cadmium induced reproductive toxicity (Iqbal et al., 2021).

Mushrooms as cardioprotective agents

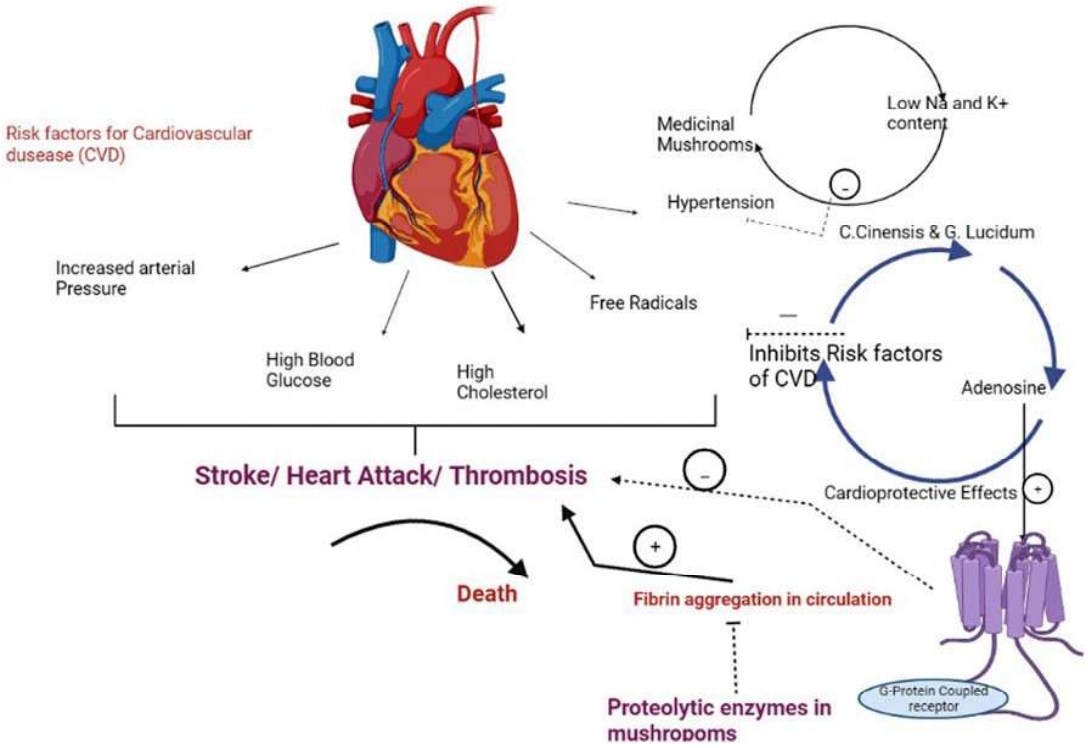

Figure.4. Role of medicinal mushrooms in prevention of cardiovascular diseases (Chugh et al., 2022).

Cardiovascular disorders (CVDs), including heart attacks and strokes, wreak havoc on the circulatory system, and heart diseases are the leading cause of death worldwide. Medicinal mushrooms have hypocholesterolaemia and hypoglycaemic properties, making them ideal as a natural, healthy diet to prevent disease and maintain cardiovascular health. Many medicinal mushrooms, including *Cordyceps sinensis* and *Ganoderma lucidum*, contain adenosine, which has long been known for its cardioprotective effects (Badalyan et al., 2019; Balandaykin et al., 2015).

According to recent studies, both endogenous and exogenous adenosines have a role in myocardial ischemia protection, which shields the heart from the negative effects of insufficient blood flow and oxygen. It binds to receptors associated to G-proteins, as well as several effector systems (Previtali et al., 2011). Hypertension, or high blood pressure, causes significant stress and impairs cardiac function. Mushrooms are beneficial dietary supplements for hypertension because they provide low sodium and high potassium levels (182-395 mg/100 g). Consuming antioxidants is an effective way to avoid the development of some CVD issues. Mushroom polysaccharides and

phenolic compounds have significant antioxidant properties. They considerably neutralize free radicals by raising the activity of oxidative enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, while also stabilizing malondialdehyde and glutathione levels (Kumaran et al., 2011).

Studies in our laboratory showed that protein-polysaccharide complex/ polysaccharides extracted from fruiting body and mycelia of *Ganoderma lucidum* possessed significant cardioprotective activity against doxorubicin induced cardiotoxicity (Veena et al., 2022). Study reports are available on the cardio protective effect of *M. esculenta* fruiting body against doxorubicin and cyclophosphamide induced cardiotoxicity by altering inflammatory gene expression pathways (Das et al., 2024). Despite of its usage in traditional medicine only scattered reports are available on the cardioprotective nature of the methanol extract of fruiting bodies of *M. esculenta.* The current study is focused on the cardioprotective effect of *M. esculenta* against chemotherapy induced cardiotoxicity and alteration in inflammatory gene expression pathway.

CHAPTER 3
MATERIALS & METHODS

Table of Contents

3.1. Materials

3.1.1 Animals

Male and female Swiss Albino mice weighing 25-30 gram obtained from Small Animal Breeding Station, Kerala Agriculture University, Mannuthy, Thrissur, Kerala, India was employed in this study. The experiments were carried out according to CPCSEA, Government of India guidelines and approval by Institutional Animal Ethic Committee (ACRC/IAEC/21(1)-P7) & (ACRC/IAEC/22(1)-P9).

3.1.2 Chemicals & Instruments

Rotenone, Antimycin-A, 2,6-dichlorophenol indophenols sodium salt (DCPIP), Trisodium citrate, Nicotinamide adenine dinucleotide phosphate (NAD), Thiamine pyrophosphate (TPP), Nicotinamide adenine dinucleotide reduced (NADH), Potassium cyanide (KCN), Oxaloacetate, α- ketoglutarate and cytochrome C were purchased from Sigma Chemical company, Saint Louis, MO, USA. Hydrogen peroxide, Chloroform, Formaldehyde, Sodium nitroprusside, sodium nitrite, EDTA, and Methanol were purchased from Merck, India Ltd, Mumbai, India. Ascorbic acid, Sodium azide, reduced glutathione (GSH), DTNB, riboflavin, Thiobarbituric acid, Vanillin, were obtained from SRL, Mumbai, India. Sodium dithionate was obtained from TCI chemicals. Doxorubicin hydrochloride (Doxowin) and Cyclophosphamide (Endoxan-N 200 mg) were purchased from the pharmacy of Amala Institute of Medical Sciences, Amala Nagar, Thrissur. DL-α-Lipoic acid was a gift from Garnett McKeen Laboratories Inc. NY, USA. CK-MB and LDH assay kit were purchased from agappe Diagnostics Limited. Cardiac Troponin-I was assayed using Vidas High Sensitivity Troponin I kit, Biomerieux. All other chemicals and reagents were of analytical reagent grade.

Table.3.1. List of instruments used for the study

S.NO.	Instrument	Company
1	Autoclave	Kemi Lab equipment, Cochin, India
2	Centrifuge	Rotek Laboratory Instruments, Kochi, India
3	Deep freezer (-20ºC)	Remi Laboratory Instruments, Mumbai, India
4	Deep freezer (-80 ºC)	Eppendorf, Germany

S.NO.	Instrument	Company
5	Electronic-weighing balance	Shimadzu, Japan
6	High speed cooling centrifuge	Remi Laboratory Instruments, Mumbai, India
7	Horizontal Laminar flow hood	Rotek Laboratory Instruments, Kochi, India
8	Hot air oven	Kemi Lab Equipment, Kochi, India
9	Microscope	Olympus BX41, Japan
10	Micro-centrifuge	Tarsons Products Private Ltd., Kolkata
11	PCR	ThermoFisher Scientific, India
12	pH meter	Elico Ltd., Hyderabad, India
13	Real time-PCR	Qiagen, Rotor Gene-Q
14	UV/Visible spectrophotometer	Systronics, PC based double beam spectrophotometer 2202
15	Rotavapor	Buchi, Switzerland
16	Vacuum Concentrator	Eppendorf, Germany
17	Vortex	Rotek Laboratory Instruments, Kochi, India

3.2. Methods

3.2.1. Cell line maintenance

DLA cell line is maintained *in vivo* in Swiss albino mice by injecting the cell line to their peritoneal cavity. The cells were collected thereafter from their right groin and 1×10^6 viable cells were used for further inoculation.

3.2.2. Experimental Material and Preparation of Methanolic Extract

Dried fruiting bodies of *M. esculenta* were procured from Organic Kashmir, a reputed mushroom dealer New Delhi. Dried mushrooms were washed thoroughly to remove dirt and any other chemicals, which was dried again at 40°C. The material was powdered, and one-hundred-gram samples were refluxed with petroleum ether (60–80 °C) for 6 h. The defatted material was then dried and extracted using methanol for 8 h in Soxhlet apparatus. The filtered extract was evaporated to dryness at 40 °C using

a rotary vacuum evaporator and the residue (ME) stored at 4 °C for further use. The species of the mushroom specimen was done using ITS sequencing method.

3.2.2.1. DNA Barcoding using universal primers of ITS.

DNA from the mushroom specimen was isolated using NucleoSpin ® Plant II Kit (Macherey-Nagel). The eluted DNA was stored at 4°C.The quality of the DNA isolated was checked using agarose gel electrophoresis. PCR Analysis was done using the primers:

ITS-1F Forward TCCGTAGGTGAACCTGCGG

ITS-4R Reverse TCCTCCGCTTATTGATATGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700,

Applied Biosystems). Sequencing was done using BigDye Terminator v3.1. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol. The cleaned-up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010).

3.2.3. Tissue homogenization

After the experimental period animals were sacrificed using CO_2 chamber as per AVMA guidelines for the euthanasia of animals. CO_2 was supplied in a precisely regulated and purified form without contaminants and adulterants. A displacement rate from 30-70 % of the chamber volume/min was recommended for rodents. CO_2 flow was maintained for at least 1 minute after respiratory arrest. Blood was drawn cardiac puncture and the serum was further used for cardiac injury marker analysis. Hearts were excised after cervical dislocation and blood was removed by repeated washing in ice cold normal saline and blotted dry using filter paper. The heart tissue was minced and homogenate was prepared in 50mM phosphate buffer pH-7.4. to obtain a 10 % homogenate(w/v) using a polytron homogenizer at 4°C. Tissue homogenate was

employed for the determination of GSH and lipid peroxidation while a part of the homogenate was centrifuge at 8000 rpm for 10 minutes to obtain the supernatant and used for determination of SOD and Catalase. The protein content was measured using Bradford reagent. The right halves of the heart were fixed in 10% formaldehyde and used for histopathological analysis.

3.2.4. Determination of cardiac injury markers

CK-MB, and LDH was analysed by spectrophotometer using diagnostic kit from Agappe Diagnostics Pvt Ltd, India. Serum Cardiac Troponin- I a cardiac specific biomarker was estimated by immuno-fluorescence assay method using Vidas High Sensitivity Troponin I kit, Biomerieux.

3.2.5. Determination of antioxidants.

3.2.5.1. Catalase activity determination

Catalase activity was determined by the method of Beers and Sizer (1952). 100 µl of tissue supernatant was mixed with 1.9 ml 0.05M Phosphate buffer pH-7.0. Reaction was started by adding 1ml of 11mM H_2O_2. The decrease in extinction of H_2O_2 was detected by spectrophotometer at 240nm. The enzyme activity was calculated by using the molar extinction coefficient of H_2O_2 (43.6 M^{-1} CM^{-1}) and expressed in micromoles of H_2O_2 decomposed /minute/milligram protein.

$$\mu\text{moles of } H_2O_2 \text{ decomposed/ minute/ milligram protein} = \frac{\Delta A/\text{min} \times 1000 \times 3}{40 \times \text{mg protein in sample}}$$

OR

(U/ mg protein)

3.2.5.2. Lipid peroxidation determination

Lipid peroxidation was determined by the method of Ohkawa et al 1979. 200 µl of tissue homogenate was suspended in 200 µl of 10% TCA and kept in ice bath for 15 minutes. Then the reaction mixture was centrifuged at 2500 rpm at 4°C for 15 min. The clear supernatant thus obtained was treated with equal amount of 0.8% TBA and kept in water bath for 30 minutes at 80°C. After cooling the reaction setup by keeping at 4°C for 5 minutes, the absorbance was measured at 532 nm. The concentration of MDA

formed was calculated from the standard graph plotted by using 1'1'3'3'-tetramethoxypropane and expressed as nmol of MDA/ mg protein.

3.2.5.3. GSH (reduced glutathione) determination

The method described by Moron et al., (1974) was employed to determine the GSH content. Briefly 100µl of tissue homogenate was mixed with 0.63 ml of 25% TCA and kept in ice for 15 minutes. After the incubation 0.3 ml of 5% TCA was further added and centrifuged for 10 minutes at 2000 rpm to obtain the protein free supernatant. 150µl of this protein free supernatant was mixed with 350µl of GSH buffer (0.2 M Phosphate buffer pH-8.0) and the reaction volume was made up to 1.5 ml by adding DTNB, followed by incubation for 5 minutes. OD values were obtained at 412nm and the concentration of GSH present in the sample was calculated from the GSH standard curve and expressed as nmol GSH/ mg protein.

3.2.5.4. SOD (Superoxide dismutase) determination

SOD activity was determined according to the method of McCord and Fridovich (1969). The reaction mixture contains 20µl of tissue supernatant, 200µl of 7mmol/l EDTA in 0.00115 ml KCN, 100µl 0.05mmol/l NBT and 2.63 ml of SOD buffer (67mM phosphate buffer, pH-7.8). Before taking the reading 50µl of 2mmol/l riboflavin was added and vortexed well. The initial reading was taken immediately at 532 nm followed by incubation for 15 minutes under incandescent light illumination. The OD was again taken after the incubation period. The volume of sample used to scavenge 50% of the generated superoxide anion was considered as 1 SOD unit activity and expressed in U/mg protein.

3.2.6. Determination of mitochondrial enzyme activities

3.2.6.1. Isolation of mitochondria

Cardiac tissue was minced to fine pieces and homogenized in isolation buffer (5mM Tris-HCl, 225mM Mannitol, 75mM Sucrose, 0.2mM EDTA). This 10% homogenate was centrifuge at 4°C for 15 minutes at 3000 rpm to remove the tissue debris. The supernatant thus obtained was centrifuged for 12500 rpm in a cooling centrifuge for 15 minutes. The pellet thus obtained was stored and the supernatant was again centrifuged

to obtain the pellet. These mitochondrial pellets were pooled and stored in 10mM ice cold hypotonic Tris-buffer pH- 7.6 at -80°C and used for further analysis.

3.2.6.2. TCA cycle enzyme activity determination

3.2.6.2.1. Malate dehydrogenase

Malate dehydrogenase enzyme activity was determined according to the method of Mehler et al., (1948). Briefly, a 3 ml reaction mixture containing 2.6 ml 0.1 M Phosphate buffer (pH-7.4), 100 µl of 0.006 M oxaloacetate and 30µl of mitochondria was prepared. The reaction was started by the addition of 200 µl 0.00375 M NADH and the absorbance was monitored at 340nm for 2 min at 30 sec intervals. The activity was expressed as micromoles of NADH oxidized/min/mg protein. Extinction coefficient of NADH : $6.3mM^{-1}\,cm^{-1}$.

3.2.6.2.2. α-ketoglutarate dehydrogenase

The method of Reed and Mukherjee (1969) was employed for determination of the enzyme activity. Briefly, the reaction mixture consisted of 300µl 10mM $MgCl_2$, 300 µl 1mM NAD, 150 µl 2mM TPP, 60 µl 0.2mM CoA, 150 µl 1mM α-ketoglutarate, 300 µl 100mM Tris-HCl. 30 µl of mitochondria fraction was added to set up the reaction. The reaction volume was made up to 1.5 ml by adding 210 µl of distilled water. The reaction was monitored at 340nm for 2 min at 30 sec intervals. The activity was expressed as micromoles of NAD^+ reduced/ min/ mg protein. Extinction coefficient of NAD^+ : $6.3mM^{-1}cm^{-1}$.

3.2.6.2.3. Isocitrate dehydrogenase

The isocitrate dehydrogenase activity was determined by using the method of Fatania et al., (1993). The method was as follows, 1.5 ml of 0.1 M phosphate buffer (pH- 7.4), 111.6 µl of 3.72 mM $MgCl_2$, 186 µl 0.62 mM $NADP^+$, 66 µl 2.25mM isocitrate and 30 µl of mitochondria fraction was mixed. The reaction volume was made up to 3 ml by adding 1.1 ml of distilled water and the reaction was monitored at 340nm for 2 min at an interval of 30 sec. The enzyme activity was expressed as micromoles of NAD^+ reduced/min/mg protein. Extinction coefficient of NAD^+ :$6.3mM^{-1}cm^{-1}$.

3.2.6.2.4. Succinate dehydrogenase

The method of Nulton-Persson and Szweda, (2001) was employed for the determination of succinate dehydrogenase activity. The reaction mixture was prepared as follows, 100 µl 0.5 M Phosphate buffer, pH-7.5, 40 µl BSA (50mg/ml), 60 µl KCN (10mM), 100 µl Sodium succinate (400mM), 290 µl DCPIP (0.015%), 30 µl mitochondria fraction. The reaction volume was made up to 3 ml by adding distilled water. The reaction was monitored after the addition of DCPIP at 600nm for 2 min at 30 sec intervals. The enzyme activity was expressed as micromoles of DCPIP reduced/ min/mg protein. The extinction coefficient of DCPIP:19.1$mM^{-1}cm^{-1}$.

3.2.7.3. Electron Transport Chain Complex determination

3.2.7.3.1. Complex I activity

Complex I was assayed by the method of Janssen et al (2007). Briefly 380 µl phosphate buffer (25mM, pH-7.2), 100 µl 20mM KCN, 100 µl 50mM $MgCl_2$, 100 µl BSA(30 mg/ml), 50 µl antimycin (20 µM in DMSO, pH adjusted by 1 N NaOH), 30 µl mitochondria fraction, 50 µl decyl ubiquinone (1300 µM in DMSO, pH 7.2), 100 µl DCPIP (800 µM) and after the addition of 100 µl 2mM NADH the absorbance was monitored at 15 s interval for 2 min at 600 nm. After the reading 10 µl of 1mM rotenone was added and mixed well followed by monitoring the absorbance as previously mentioned. The linear variation in absorbance was calculated prior to and after the addition of rotenone. The activity was expressed as micromoles of DCPIP reduced/min/mg protein. Extinction coefficient of DCPIP:19.1$mM^{-1}cm^{1}$.

3.2.7.3.2. Complex III activity

Decyl ubiquinol preparation

10mg decyl ubiquinone was mixed with 80 µl of DMSO and was used as stock. 20 µl of stock was mixed in 2 ml DMSO and 400 µl ice cold phosphate buffer (pH-8). After adding few grains of sodium dithionate vortexed thoroughly for 10 min and kept in ice for 30 min. After the incubation centrifuged at 10,000 rpm at 4°C and the clear supernatant was obtained and used for further assay.

The assay method was as follows, 100 µl EDTA (100 µM), 100 µl (20mg/ml) BSA, 100 µl (30mM) NaN_3, 100 µl Cytochrome C (600 µM), 450 µl 50mM phosphate buffer (pH-8) and 30 µl mitochondria, this reaction mixture as incubated at room temperature

for 1 minute . After the addition of 50 µl decyl ubiquinol the reaction was monitored at 550nm and monitored again after the addition of 50 µl Antimycin (20 µM). Absorption time curve, which was not less than 30s, was used to calculate the activity. The activity was expressed as micromoles of ferricytochrome-C reduced/ min/ mg protein (Krahenbuhl et al.,1991).

3.2.7.3.3 Complex IV activity

Complex IV activity was assayed using the method mentioned by Brischigliaro et al., (2022). The mitochondrial membrane was permeabilized by overnight incubation with 0.5 % Tween 80/30mmol/L Phosphate buffer; pH-7.4. 10.6 mg Cytochrome C was dissolved in 20 ml distilled water then add 50 µl of 0.1mol/L Sodium dithionate to obtain reduced cytochrome- c. 50 µl of mitochondria fraction was mixed in 1 ml of reduced cytochrome-c and the reaction was monitored at 550nm for 2 min at 30 sec interval. The difference in absorbance was calculated from the linear part of the absorption-time curve and complex IV activity was expressed as micromoles of ferrocytochrome-C oxidized/min/mg protein using the extinction coefficient : $21mM^{-1}cm^{-1}$.

3.2.8. Histopathological analysis

The cardiac tissue obtained from each animal was preserved in a 10% formalin solution for fixation. Then, a thin section of heart tissue was dehydrated and cleared using different grades of ethanol and xylene respectively in an automatic processing machine. The sections were then embedded with paraffin wax and blocked in the coronal plane. Microtome section of 4–5µm thickness of the tissue was obtained and one set of sections was stained with haematoxylin - eosin (H&E) and the slides were examined for routine histopathological features. The second set was stained with phosphotungstic acid-haematoxylin (PTAH) for observing striations and myofibrillar loss. All the slides were examined under Olympus BX41 microscope and photographed using a digital camera at a magnification of 10X.

3.2.9. Analysis of gene expression

About 30mg of cardiac tissue was used to isolate RNA using RNA isolation kit (Origin Total RNA Kit, CAT.NO. ODP419). RNA (100ng) was reverse transcribed to cDNA according to the manufacture's procedure (OriginCAT.NO. ODR41). Gene expression analysis of nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear Factor kappa B (NF-κB), Interleukin-6 (IL-6), Interleukin-1 beta (IL-1β), Tumour Necrosis Factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) was done using 2X Real-time PCR Master Mix (Origin CAT No. ODQ383-M01). Quantification was done using a Qiagen Real-time PCR reaction. Rotor-Gene Q Software 2.3.5.1 was used to analyse the results. Expression of the target gene was measured and quantitated by the reference gene (18S). The 2(-Delta Delta C(T)) method was used to quantify these genes (Livak et al., 2001). The relative cycle threshold (CT) values were used to study the variations in gene expression. The PCR conditions and primer sequences utilized in this study were given in Table 1&2 respectively.

3 TABLE.3.2. PCR CONDITIONS

4 CYCLE	5 CYCLE POINT
6 HOLD	7 HOLD @ 95°C, 3MIN 0S
8 CYCLING (40 REPEATS)	9 STEP 1: HOLD @ 95°C, 45S
	10 STEP 2: HOLD @ 60°C, 35S, ACQUIRING TO CYCLING A([GREEN][1][1])
11 MELT	12 RAMP FROM 65°C TO 95°C
	13 HOLD FOR 90S ON THE 1ST STEP
	14 HOLD FOR 10S ON NEXT STEPS, MELT A([GREEN][1][1])

Table.3.3. PCR primers

Sl.NO	GENE	FORWARD PRIMER	REVERSE PRIMER	Reference
1	TNF-α	5'-GCCTCTTCTCATTCCTGCTTG-3'	5'- CTGATGAGAGGGAGGCCATT-3'	Shi et al., 2018
2	IL-6	5'- ACGGCCTTCCCTACTTCACA-3'	5'- CATTTCCACGATTTCCCAGA-3’	Terashima et al., 2018
3	IL-1β	5'TGGACCTTCCAGGATGAGGACA-3'	5'-GTTCATCTCGGAGCCTGTAGTG-3'	Xu et al., 2019
4	iNOS	5'- CACCTTGGAGTTCACCCAGT-3’	5'- ACCACTCGTACTTGGGATGC-3'	Kim et al., 2018
5	NF-κB	5’-ACGATCTGTTTCCCCTCATCT-3’	5’-TGCTTCTCTCCCCAGGAATA-3’	Dare et al., 2021
6	NRF-2	5’-GCAACTCCAGAAGGAACAGG-3’	5’-TCTCTGCCAAAAGCTGCATA-3’	Sun et al., 2014
7	KEAP 1	5’- AACTCGGCAGAATGTTACTACCC -3’	5’- CTACGAAAGTCCAGGTCTCTGTCTC -3’	Guifang et al., 2018
8	18S	5’-AAGTTCGACCGTCTTCTCAGC-3’	5’-GTTGATTAAGTCCCTGCCCTTTG-3’	Teh et al., 2019

3.2.10. Statistical Analysis

All experimental data were statistically analysed with Graph Pad prism software and expressed as mean ± SD. The analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett's test and $P<0.05$ was considered significant.

CHAPTER 4

Extraction and identification of major bioactive compounds

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4.1. Introduction

M. esculenta is a highly valued wild edible mushroom and is considered important for its medicinal properties (Li et al., 2023). It is widely distributed in the hilly areas of Korea, Japan, and Europe (Teng et al., 2023). In India it is found in the high-altitude areas of Himalayas especially in Jammu and Kashmir. In northern India *Morchella* species are known as Gucchi. Various bioactive compounds such as polysaccharides, terpenes, phenolic compounds, proteins, protein hydrolysates are attributed for its medicinal potential (Talie et al., 2024). Recent studies reported the anti-inflammatory, antitumor, antioxidant, and antimicrobial activities of this mushroom (Zhang et al.,2023). Several studies were carried out on the bioactivities of polysaccharides isolated from *M. esculenta* fruiting bodies. Majority of works were devoted to the mycelia of *M. esculenta*. Studies were carried out on the isolation of major bioactive compounds present in the methanolic extract of *M. esculenta* fruiting bodies and analysed by HPTLC and LC-MS techniques. The results are presented in this chapter.

4.2. Fruiting body collection

Dried fruiting bodies of *M. esculenta* were procured from a reputed mushroom dealer, Organic Kashmir, New Delhi. The identification was accomplished by ITS sequencing. Method was mentioned in section 3.2.2.1.

4.3. Preparation of extracts of *Morchella esculenta* fruiting body.

Dried mushrooms were washed thoroughly to remove dirt and any contaminants, which is dried again at 40^{o}C. Refer section.3.2.2.

4.4. HPTLC analysis of methanolic extract of *Morchella esculenta* fruiting body.

Analysis was carried out using HPTLC system (CAMAG, Switzerland). The sample was dissolved in chloroform and 4 microlitre of this solution was applied to a silica gel 60 F_{254} TLC plate - (E. Merck, Germany) (5 cm x 10 cm) using Linomat V sample applicator. The plate was developed in a twin trough glass chamber using the mobile phase chloroform-methanol 8:2. After the development, the plate was sprayed with anisaldehyde-sulphuric acid reagent and heated at 110^{o}C for 10 min. The plate was scanned at 580 nm using TLC Scanner 3 equipped with WinCats software.

4.5. Liquid chromatography-mass spectrometry (LC-MS) analysis of *Morchella esculenta* fruiting body.

LC-MS analysis of ME for the determination of bioactive compounds in the extract was carried out at Sophisticated Analytical Instrument Facility, IIT-Mumbai, using TOF/Q-TOF Mass Spectrometer. Extract was prepared in methanol. Mass spectrometry analysis was carried out in positive and negative ionization mode with mass/charge ratio from 120 to 1200 (m/z). Gas chromatography was maintained at 250˚C with gas flow of 13 psi/min. Separation was done in column of Eclipse Plus C18 150 x 2.1mm-5 MICRON. The sample injection volume was 8 µL and elution was done using a solvent gradient of acetonitrile–water (95:5) with flow rate of 0.3 mL/min for 35 min.

4.6. Results

4.6.1. HPTLC profile of methanolic extract of *Morchella esculenta* ME.

The HPTLC profile depicting the chemical finger print of ME is presented in **Figure.4.1.A.** The chromatogram showed 14 peaks of Rf 0.01, 0.05, 0.10, 0.15, 0.20, 0.28, 0.33, 0.38, 0.42, 0.48, 0.52, 0.65, 0.75, 0.80. Major peaks were Rf 0.05 and 0.65. The figures of derivatized plates are shown in **Figure.4.1.B**.

4.6.2. LC-MS analysis of methanolic extract of *Morchella esculenta* ME.

A number of compounds were identified using HR-LCMS/Q-TOF analysis in both negative and positive ionization mode (**Figure.4.2.A&B).** The major compounds identified in negative ionization mode were Diepomuricanin A (RT: 19.387, $C_{35}H_{62}O_4$, MW: 546.472); Imperialine (RT: 18.859,$C_{27}H_{43}NO_3$, MW: 429.33); Petroselinic acid (RT: 18.573, $C_{18}H_{34}O_2$, MW: 282.2601); Solacapaine (RT:17.687, $C_{27}H_{48}N_2O_2$, MW: 432.376); Eicosapentaenoic acid-d5 (RT: 15.485, $C_{20}H_{25}D_5O_2$, MW: 307.2549); 9-HOTE (RT: 14.981,$C_{18}H_{30}O_3$, M.W: 294.2226) while Muricoreacin (RT:20.636, $C_{35}H_{64}O_9$, M.W: 628.4601), Dihydromethoxylycopene (RT:20.174, $C_{41}H_{60}O$, M.W: 568.4616); D8'-Merulinic acid A (RT:19.778, $C_{24}H_{38}O_4$, MW: 390.2711); Uvaricin (RT: 18.967, $C_{39}H_{68}O_7$, M.W: 648.4865); γ-Tocopheryl quinone (RT: 18.674, $C_{28}H_{48}O_3$, M.W: 432.3531); Luffariellolide (RT: 15.721, $C_{25}H_{38}O_3$, M.W: 386.2856); 4,4'-Diapo-zeta-carotene (RT: 13.569, $C_{30}H_{44}$, M.W: 404.3485); Cynaroside A (RT: 12.201, $C_{21}H_{32}O_{10}$, M.W: 444.1983) were identified as major compound in positive

ionization mode. Structures of the compounds identified from LC-MS data are shown in **Figure.4.2.C**

4.7.Discussion

Morchella esculenta is an excellently edible mushroom which is widely used in traditional medicine in Asian countries. The major bioactive compounds found in this mushroom include polysaccharides, phenolics, tocopherols and ergosterols. Morels have both nutritional and medicinal value. Attention is given to phytochemical compositions of different morel species in recent years. In this chapter the HPTLC and LC-MS profiling of chemical components of methanol extract of *M.esculenta* is presented. A number of compounds were identified by HR-LCMS/Q-TOF analysis in both positive and negative ionization mode.

The LC-MS analysis of MEME revealed the bioactive compounds present in the methanolic extract are known to possess anti-inflammatory, anti-oxidant and cardioprotective properties. Imperialine, one of the identified compounds is reported to show anti-inflammatory activity by suppressing the production of pro-inflammatory cytokines TNF-alpha and Interleukin-1 beta (Wu et al., 2015). These proinflammatory cytokines are regarded as potential markers for heart failure. Increased levels of these markers are an indication of cardiac inflammation which show crucial role in CP induced cardiotoxicity. Anti-inflammatory activity of Imperialine which inhibits the Nf-kb phosphorylation might be considered as a cardioprotective bioactive present in ME (Sirwi et al., 2022). Eicosapentaenoic acid was found as one of the major peaks in LC-MS spectra. Previous reports have demonstrated the cardioprotective property of this compound (Andelova et al., 2022). Likewise, luffariellolide and Uvaricin are reported to have anti-inflammatory, Nrf-2 activation, antioxidant activities. Presence of these bioactives compounds in the extract might be responsible for the cardioprotective effect of ME (Albizati et al., 1987; Popoola et al., 2021).

4.8.Figures and Tables

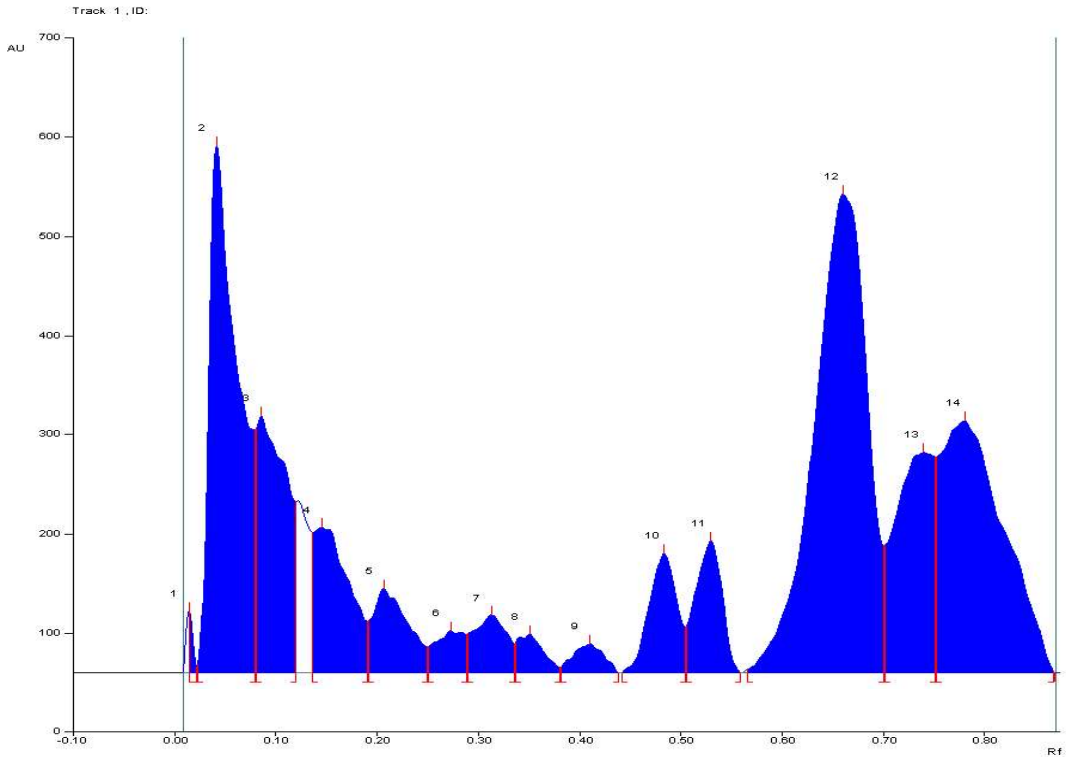

Figure.4.1.A. HPTLC chemical finger print of ME.

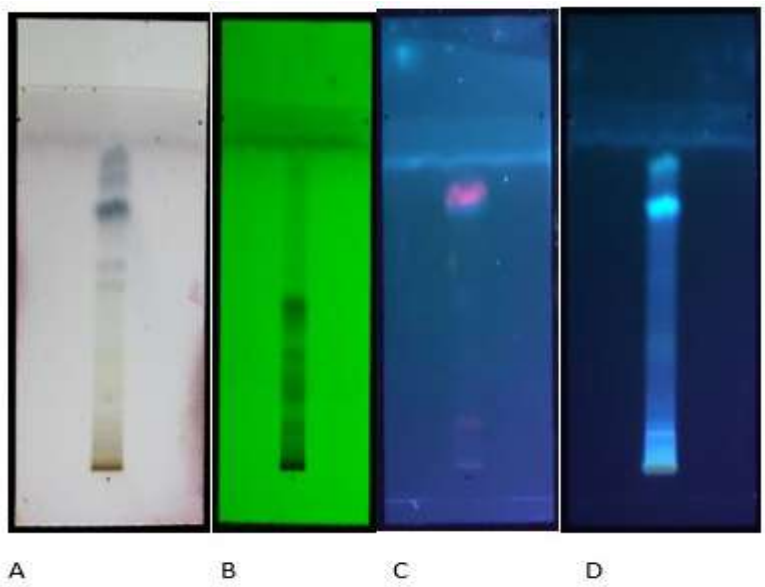

Figure.4.1.B. HPTLC plates A: Derivatized plate in white light; **B:** Developed plate at UV 254 nm; **C:** Derivatized plate under UV 366nm; **D:** Developed plate at UV 366 nm.

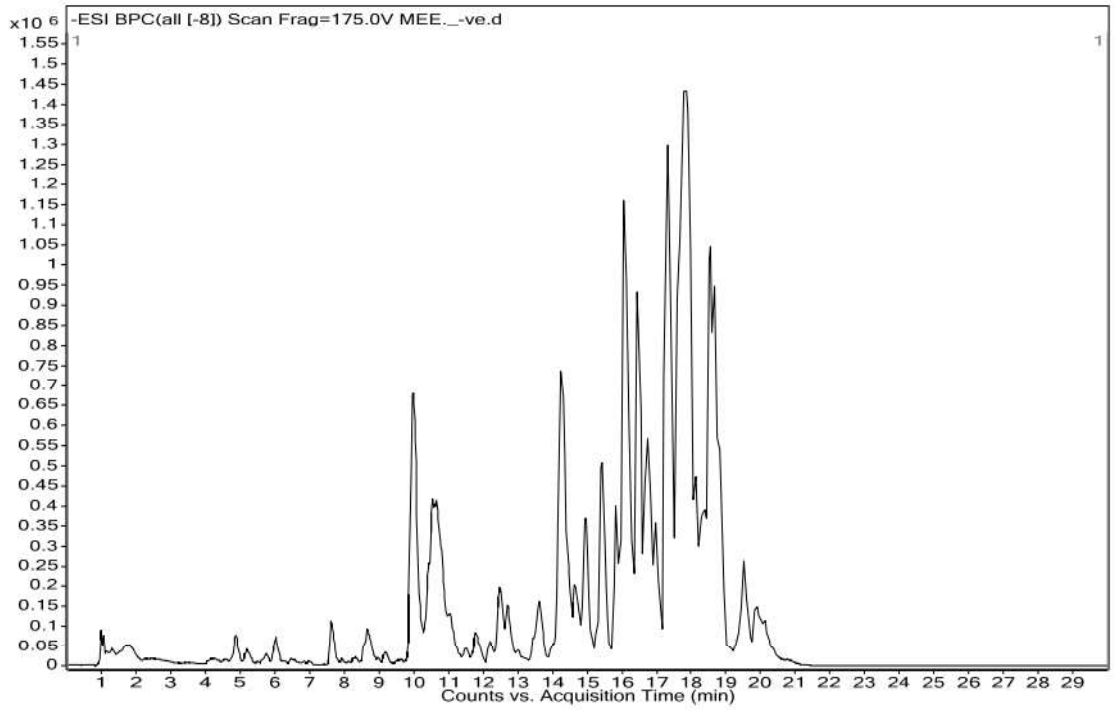

Figure.4.2.A. LC-MS spectra of ME in negative ionization mode.

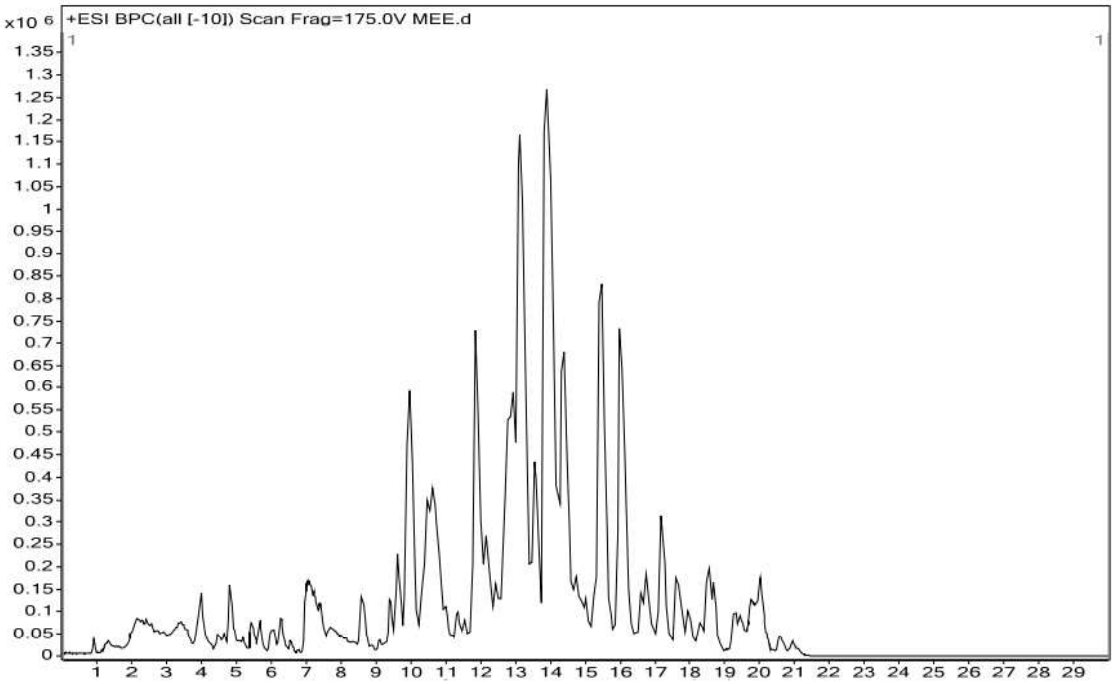

Figure.4.2.B. LC-MS spectra of ME in positive ionization mode.

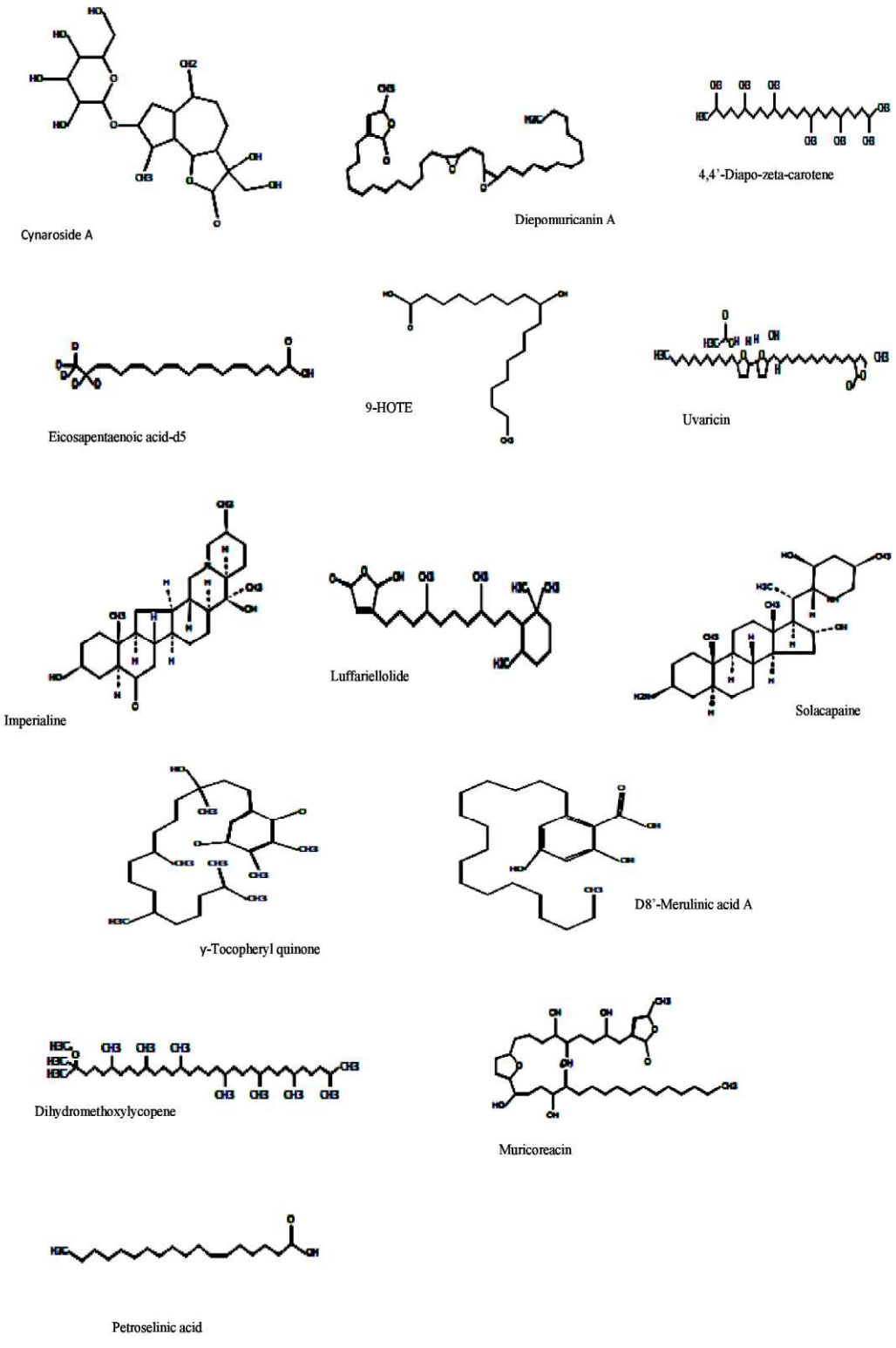

Figure.4.2.C. Structures of compounds identified in ME by LC-MS analysis data.

CHAPTER 5

Oral Toxicity Studies

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5.1. Introduction

Assessment of toxicity is necessary to determine the safety of drugs. The objective of the experiments mentioned in this chapter was to determine the toxic effects of methanolic extract of *M. esculenta* prior to its therapeutic usage. *M. esculenta* is an edible mushroom which is valued for its unique flavour and efficient health promoting properties. Even though, it is an edible mushroom and widely used in Chinese cuisines, there is inadequate knowledge about its safety. So, it was essential to find out the toxicity of methanolic extract of *M. esculenta* to determine the safe dosage levels, prior to experimenting it as a health promoting agent. In this study acute and sub-acute toxicity analysis was carried out to evaluate the safety and potential risk associated with its usage. Acute toxicity test involved the administration of a high dose of the drug as a single dose and evaluate to its toxic symptoms if any. The parameters studied were mortality, morbidity, behavioural, biochemical and histopathological parameters. The study results are mentioned in this chapter.

5.2.Materials and Methods

5.2.1. Preparation of extract

Methanolic extract of *M esculenta* was prepared by the method mentioned in **Section.3.2.2**

5.2.2. Animals

Male and female Swiss albino mice weighing 25±2 g was employed in this study.

5.2.3. Toxicity assessment

Toxicity of methanolic extract of *M esculenta* (ME) was determined by using acute and subacute toxicity models. The experimental methods were conducted based on OECD guidelines- 423.

5.2.3.1. Acute oral toxicity.

Acute toxicity studies were conducted as per OECD – 423 guidelines for testing the safety dose for further studies.

Female Swiss Albino mice weighing 25±2 gram was employed for the study. 3 animals were randomly selected to administer the extract. They were observed for 7 days to

ascertain their normal feed, water intake and other behavioural patterns before drug administration. After the observation period animals were orally administered with the extract (5000mg/kg b.wt and 2000mg/kg b.wt) on day one as a single dose. Animals were observed daily for 14 days to check mortality, morbidity, salivation, diarrhoea, lethargy, coma etc . The study was completed in 21 days. Animals were sacrificed at the end of the experimental period and vital organs were collected for histopathological observation.

5.3. Results

5.3.1. Acute oral toxicity of ME.

Acute toxicity study revealed that methanolic extract of *M esculenta* did not show any ill effect on experimental animals at the dosages of 2000 and 5000 mg/kg b.wt. The animals showed no mortality and morbidity during the entire experimental period. Paradoxically the treated animals were alert, sound and active as normal animals. No changes were observed during the cage site for lacrimation, salivation, changes in colour of skin/fur and respiration rate. The body weight was noted on 0th and 14 th day (post dosing) of the experimental animals individually. There were no significant changes observed in the body weight of the animals. Feed and water intake were also monitored and found normal throughout the experimental period.

5.4.Discussion

The acute toxicity study conducted using Swiss Albino mice at doses of 2000 and 5000 mg/kg. b.wt. showed no abnormalities in the experimental animals. No mortality or abnormal behavioural pattern throughout the study duration were found. Thus, this study demonstrates that methanolic extract of *M. esculenta* is non-toxic and safe.

CHAPTER 6

Evaluation Of Protective Effect Methanol Extract of Morchella Esculenta Against Doxorubicin Induced Cardiotoxicity

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6.1 Introduction

Doxorubicin is an anthracycline chemotherapy agent, which is highly efficient and used against a wide variety of cancers either alone or in combination with other drugs (Kciuk et al., 2023). However, the efficiency is delimited by the acute and chronic ill effects imparted by this drug, especially the dose dependent cardiotoxicity in the form of congestive heart failure and cardiomyopathy (Liu et al., 2020). The mechanism of Doxorubicin induced cardiotoxicity is not understood clearly which makes it more complicated. However, oxidative stress is considered as the major reason for cardiotoxicity (Rawat et al., 2021). Cardiotoxic effects of Doxorubicin comprises of ROS generation, endothelial damage, endoplasmic reticulum stress, mitochondrial dysfunction, cardiac muscle damage, imbalance of calcium homeostasis etc (Shi et al., 2023). In order to prevent these toxic effects, the cumulative dose is limited to 450-600 mg/m^2 however, it does not lower the chance for late onset of complications. A large amount of evidence points those subclinical changes in the left ventricular ejection fraction (LVEF) even in low dose treatment regimen (200-300 mg/m^2) (Todorova et al., 2020; Zeng at al.,2019). These ill effects necessitate an adjuvant treatment regimen for lowering the toxic complications.

Dexrazoxane is the only FDA approved drug which acts by reducing ROS generation by chelating iron(Benjamin et al.,2021). Dexrazoxane is administered intravenously, 30 minutes prior to Doxorubicin treatment at a dosage ratio of 10:1 (DEX: DOX). But its use is associated with complications such as myelotoxicity and secondary malignancy development etc. (Hasinoff et al.,2020). Especially in pediatric patients the long-term use of DEX is of serious concern. So, the quest for natural products with less or no harmful effects is one of the first line area of research. Various studies reported the cardioprotective properties of natural compounds such as dioscin, thymoquinone, resveratrol etc. against Doxorubicin induced cardiotoxicity (Karabulut et al., 2021; Zhang et al., 2019; Yu et al., 2018; Zhao et al., 2018). Amidst of these plant-based compounds medicinal mushrooms are of great interest as they contain number of antioxidant compounds that possess potent antioxidant activity.

Current study was carried out on the cardioprotective effect of an edible mushroom *Morchella esculenta* also known as Gucchi in India. The bioactive extract of this mushroom possess enormous pharmacologically relevant properties such as

immunomodulation, hepatoprotection, antidiabetic, antiviral and antitumor effect. The objective of the current study was to evaluate the cardioprotective effect of methanolic extract of *Morchella esculenta* (ME) fruiting bodies on Doxorubicin induced cardiotoxicity.

6.2. MATERIALS AND METHODS

6.2.1. Chemicals

Doxorubicin was purchased from the pharmacy of Amala Institute of Medical Sciences, India (EnDoxan-N) and CK-MB, LDH kits from Agappe Diagnostics India Limited.

6.2.2. Experimental Material and Preparation of Methanolic Extract

Dried fruiting bodies of *M. esculenta* were procured from Organic Kashmir, mushroom dealer New Delhi. Dried mushrooms were washed thoroughly to remove dirt and any other chemicals and dried again at 40°C. The material was powdered, and one-hundred-gram samples were refluxed with petroleum ether (60–80 °C) for 6 h. The defatted material was dried and then extracted using methanol for 8 h in Soxhlet apparatus. The filtered extract was evaporated to dryness at 40 °C using a rotary vacuum evaporator and the residue (ME) stored at 4 °C till further use. Identification of the mushroom was accomplished by ITS sequencing.

6.2.3.*In Vivo* Studies

Cardioprotective activity of ME against Dox induced cardio toxicity was determined using Male Swiss Albino mice weighing 25-30 gram obtained from Small Animal Breeding Station, Kerala Agriculture University, Mannuthy, Thrissur, Kerala, India. The experiments were carried out according to guidelines of CPCSEA, Government of India and approval of Institutional Animal Ethic Committee (ACRC/IAEC/22(1)-P7).

Animals were divided into six groups containing 6 animals in each group. The treatment protocol was as follows: Group I served as the control group treated with drinking water for the whole experimental period; Group II animals were injected with three doses of Dox (6 mg/kg b.w.) i.p. a (cumulative dose of 18 mg/kg b.w.). The treatment groups III, IV, V and VI were orally administered with ME 100, 250, 500 and standard α-lipoic acid 100 mg/kg b.w. respectively for the entire experiment period. All groups except Group I was administered Dox (i.p.) on 8^{th}, 10^{th} and 12^{th} day of the

experiment. Twenty-four hours after the last dose of treatment, animals were weighed and euthanized in a CO_2 chamber. The blood was collected by cardiac puncture immediately after cervical dislocation and serum collected by centrifugation at 3500 rpm for 10 minutes in a cooling centrifuge. The clear supernatant thus obtained was used for the determination of cardiac injury markers such as CK-MB, LDH, and Cardiac troponin-I. Hearts were removed and weighed. Right halves of hearts were rinsed with PBS and stored in -80℃ which was further used for estimation of oxidative stress markers, mitochondrial electron transport chain complexes and left halves were preserved in 10% formalin for histopathological evaluation (Das et al., 2022; Sandamali et al., 2020).

6.2.3.1. Effect on body weight and heart weight of animals

The body weight was measured for each experimental animal on the initial day of the experiment. These measurements were again taken on the 7 th day before Dox treatment and on the 13th day after last dose of Dox treatment. Body weight and heart weight ratio was calculated and analysed at the end of the experiment.

6.2.3.2. Determination of Cardiac Injury Marker levels.

CK-MB, and LDH was spectrophotometrically analysed using diagnostic kit obtained from Agappe Diagnostics Pvt Ltd, India. Serum Cardiac Troponin- I a cardiac specific biomarker was estimated by immuno-fluorescence assay method using Vidas High Sensitivity Troponin I kit, Biomerieux.

6.2.3.3. Effect on antioxidant status in cardiac tissue

A 10% cardiac tissue homogenate was prepared in 50mM phosphate buffer (pH 7.4) containing 1mM EDTA. A part of the homogenate was transferred to 0.5 ml vials and used for estimation of lipid peroxidation by TBARS method (Ohkawa et al., 1979) and GSH by Moron et al., (1979) activities. The supernatant was obtained by centrifuging at 5000 rpm for 15 minutes in a cooling centrifuge and further used for determining catalase (Beers et al., 1952), SOD (McCord et al.,1969) and GPx (Hafemann et al., 1974). Details of methods are mentioned in section 3.2.5.

6.2.3.4. Isolation of mitochondria

Mitochondria isolation was done based on differential centrifugation according to the method described by Ajith et al., with slight modifications. The details of the method are described in section 3.2.6.1.

6.2.3.5 TCA cycle enzyme activity estimation

Succinate dehydrogenase enzyme was assayed based on method described by Sudheesh et al.,(2009). TCA cycle enzymes were determined by the method described in the section 3.2.6.2.

6.2.3.6. Estimation of Mitochondrial electron transport chain complex activity.

6.2.3.6.1. NADH Dehydrogenase (Complex I)

NADH dehydrogenase activity was assayed based on the method of Janssen et al.,2007, with slight modifications. Refer section 3.2.7.3.1 for the detailed method.

6.2.3.6.2. Estimation of Q-cytochrome C Oxido-reductase (Complex III)

Complex III was estimated based on the method mentioned by Krahenbuhl et al., 1991 with slight modifications. Refer section 3.2.7.3.2 for the assay method.

6.2.3.6.3. Cytochrome C Oxidase (Complex IV)

Complex IV was estimated based on the method mentioned by Brischigliaro et al., 2022, with slight modification. The assay was done as described in the section 3.2.7.3.3.

6.2.3.7. Histopathological analysis of Cardiac Tissue

Histopathological analysis of cardiac tissue was done by the method described in section 3.2.8.

6.2.3.8. Gene expression analysis

Gene expression analyses of Nf-κB p65, IL-6,IL-1β, TNF-α, iNOS, NRF-2 and KEAP-1 were done according to the methods described in section 3.2.9.

6.2.4. Statistical Analysis

All experimental data were statistically analysed with Graph Pad prism software and expressed as mean ± SD. The analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett's test and $P<0.05$ was considered significant.

6.3 RESULTS

6.3.1. Effect of ME on body weight and heart weight of animals.

Animals treated with ME showed a marked increase in body weight after 7 days of treatment prior to Dox administration. There was a reduction in the body weight of animals treated with Dox alone in a significant manner whereas the ME treatment reduced the weight loss to a great extent . Heart weight was high in Dox treated animals which might be due to cardiac inflammation. The heart appeared to be swollen and pale. The heart to body weight ratio was found to be higher than that of other groups in Dox treated animals while the ratio was restored to normal level in other groups significantly (**Table.6.1**).

6.3.2. Effect of ME on Cardiac injury marker levels.

Administration of Dox up- regulated the levels of serum cardiac injury markers, creatine kinase (CK-MB) and lactate dehydrogenase (LDH). The animals treated with ME alleviated the levels of CK-MB and LDH significantly (**Figure. 6.1.A, 6.1.B**). Similarly cardiac troponin I level was also found to be increased by the administration with DOX and the treatment with ME down regulated its level (**Figure.6.1.C**).

6.3.3. Effect of ME on antioxidant system in cardiac tissue.

The Dox administration resulted in decline of antioxidant levels in the myocardium. Treatment by ME enhanced the activities of antioxidants significantly in the myocardium. This was evident from the levels of GPx, GSH, SOD and Catalase in the heart tissue (**Figure.6.2.A-D**) Dox treatment increased lipid peroxidation which was reflected from the levels of TBARS. However, treatment with ME effectively reduced TBARS indicating the decline of lipid peroxidation **(Figure.6.2.E).**

6.3.4. Effect of ME on TCA cycle enzymes and ETC complex in cardiac mitochondria

The effect of ME treatment on TCA cycle enzyme levels and ETC complex of cardiac mitochondria are presented in **Table.6.2 & 6.3.** respectively. From the results it was evident that Dox administration resulted in a drastic decline in the mitochondrial enzyme levels whereas ME was successful in improving the Krebs' cycle enzymes activity and ETC activity in a dose dependent manner. Especially in high dose of ME almost restored the dehydrogenase level back to normal. ME at doses of 250 and 500 mg/kg b.wt. showed a statistically significant increase in mitochondrial dehydrogenases activity. ME treatment at a higher dose resulted in 10.3-, 10.5-, 4.2- and 2.9-fold increase in Malate dehydrogenase, α-ketoglutarate dehydrogenase, Isocitrate dehydrogenase and Succinate dehydrogenase activities compared to the decline caused by Dox alone treatment. Likewise, higher dose treatment of ME along with Dox resulted in 2.75-, 2.2- and 5.76- fold increase in Complex I, III and IV levels than Dox alone treated group. These results indicated protection imparted to mitochondrial functions by ME treatment.

6.3.5. Histopathological changes in cardiac tissue.

Histopathological examination showed that Dox administration caused severe cardiac tissue damage. The haematoxylin-eosin staining revealed the tissue damage caused by Dox administration. Dox administration caused cardiac inflammation, myofibrillar loss, nuclear pyknosis and cytoplasmic vacuolization. PTAH staining revealed the loss of striations in cardiac tissue by Dox administration. The treatment with ME restored the normal architecture of cardiac tissue in a dose dependent manner (**Figure.6.3., Figure.6.4.).**

6.3.6. Impacts of ME against Dox-induced alteration in cardiac mRNA gene expression.

Dox treatment declined the expression levels of NRF2 and elevated the expression of KEAP-1 which was reversed by the treatment with ME at different dosages. On the contrary, the gene expression levels of NF-κB and pro-inflammatory cytokines such as IL-6, IL-1B, TNF-α and iNOS were upregulated by Dox treatment which was alleviated

by ME treatment in cardiac tissue of the experimental animals. The results are presented in **Figure.6.5.(a-g).**

6.4. DISCUSSION

Doxorubicin is one of the most widely used anticancer drugs either alone or in combination for chemotherapy (Elfadadny et al., 2023). Dose depended cardiotoxicity is the major limiting factor of its clinical use. Various established mechanisms of Dox toxicity are oxidative stress, mitochondrial dysfunction, ER stress, ferroptosis, apoptosis etc (Kong et al., 2022). Dox administration results in release of cardiac injury marker enzymes and troponin I (Abdulkarem et al., 2021). Since the regenerative capacity of cardiomyocytes is low, they are more vulnerable to side effects imparted by chemotherapeutic drugs (Hermann et al.,2020). However definitive mechanism of cardiotoxicity induced by Dox remains unclear. Various prevention strategies are currently blooming in order to lower the toxic effects of chemotherapeutic drugs without hampering their anti-cancer efficacy (Dubey et al., 2021).

Cardiotoxicity of Dox is exerted via various mechanisms and major role has been demonstrated to the increased ROS generation and consequent oxidative stress. The present study emphasizes on the amelioration of oxidative stress as a protective strategy against Dox induced cardiotoxicity by methanolic extract of morel mushroom (ME). *M.esculenta* is known for its immense therapeutic potential. Previous studies in our lab revealed the anti-cancer, anti-inflammatory, anti-oxidant, hepatoprotective and nephroprotective activities of morels (Nitha et al., 2010;Nitha et al., 2013;Nitha et a., 2007;Nitha et al., 2017).

Currently there is an unprecedented drive to use herbal medicinal preparations for treatment and the drive is powered by cost effective therapeutic promise and safety. Over the years of laboratory data have shown that medicinal herbs may have therapeutic value that can intervene with several risk factors in cardiovascular treatment. The current study reveals *in vivo* cardioprotective effect of morel mushroom , *M. esculenta* . *In vivo* studies showed that administration of Dox caused leakage of cardiac injury markers to the serum which was attenuated by the treatment with ME. CK-MB, LDH and Cardiac Troponin- I which are cardiac specific markers found to be released into the blood consequent to cardiac damage (Mert et al., 2023). The levels of cardiac injury

markers reveal the severity of damage caused by Dox to cardiomyocytes. Our experimental results indicate that ME efficiently protected the myocardium by down regulating the leakage of injury markers to the blood stream. The current experimental results indicate that bioactive extract of morel mushroom, *M.esculenta* is capable to alleviate oxidative stress mediated cardiotoxicity induced by Dox. Since *M.esculenta* is an excellently edible mushroom, the finding suggests the potential therapeutic use of morel mushroom to ameliorate cardiotoxicity induced by Doxorubicin.

6.5. Figures and Tables

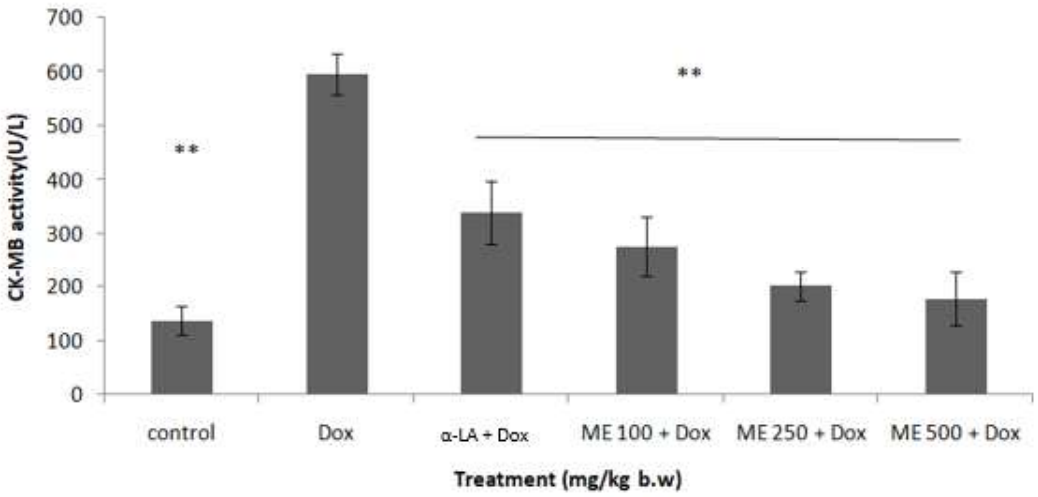


Figure.6.1.A CK-MB activity: values are the mean ± S.D; (n=6). ** *P*< 0.01 significantly different from DOX control group (Dunnett's test) .

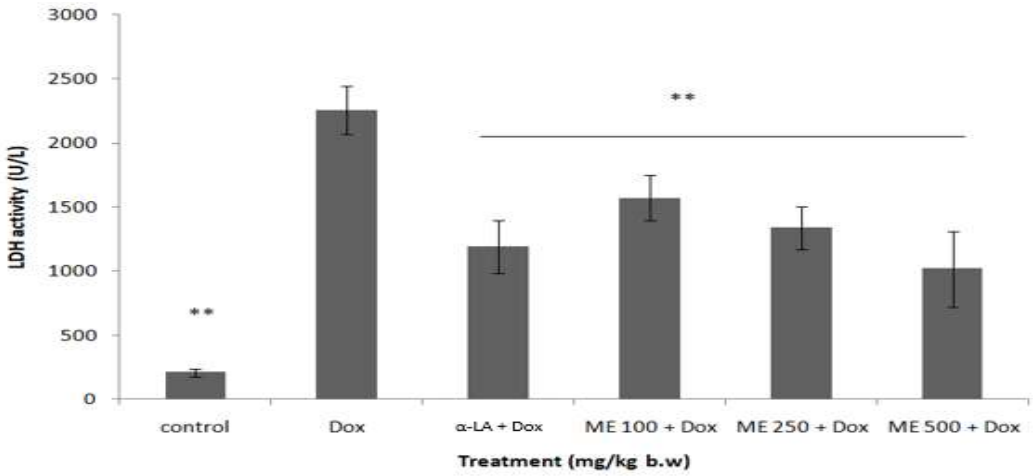


Figure.6.1.B LDH activity: values are the mean ± S.D; (n=6). ** *P*< 0.01 significantly different from DOX control group (Dunnett's test) .

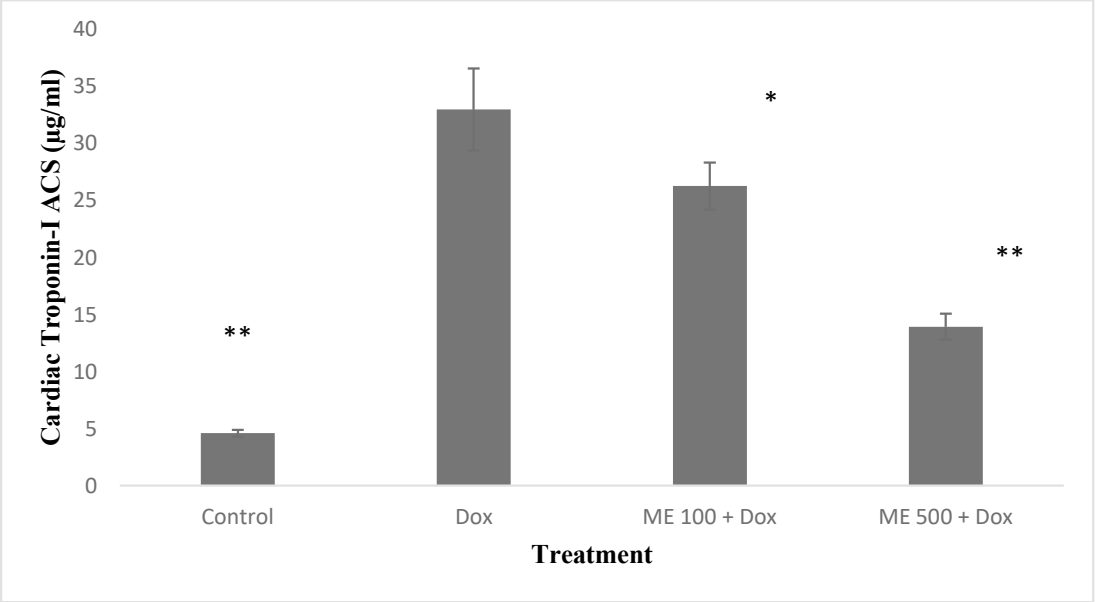


Figure.6.1.C. Cardiac Troponin-I: values are the mean ± S.D; (n=6). ** *P*< 0.01& * P<0.05 significantly different from DOX control group (Dunnett's test) .

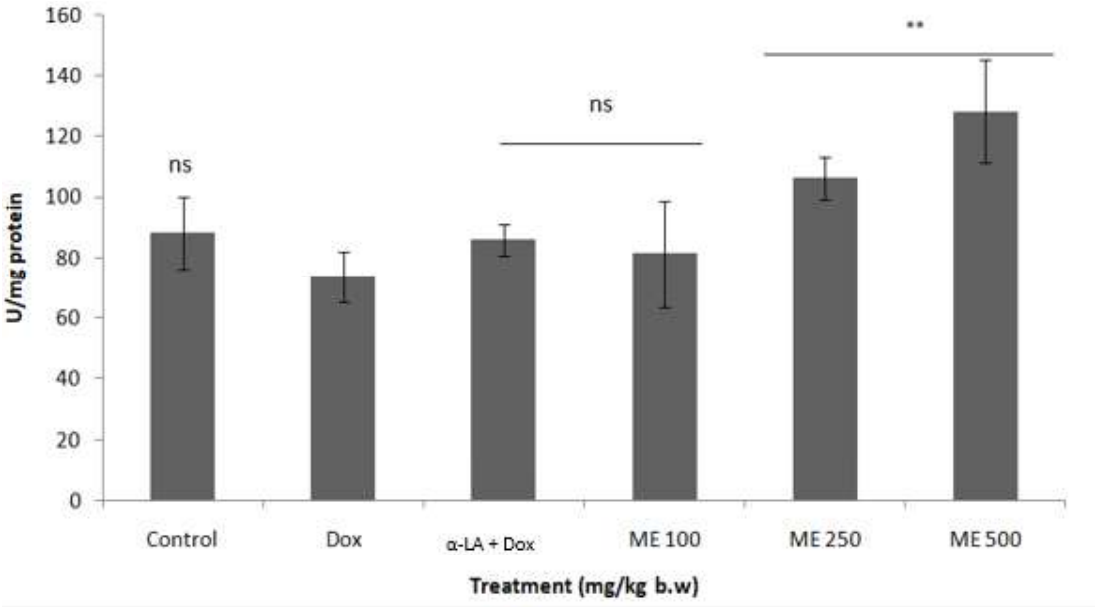


Figure.6.2.A. GPx activity. All values expressed as Mean ± SD (n=6).*P<0.05; **P<0.01;ns P>0.05 compared to DOX administered animals (Dunnett's test).

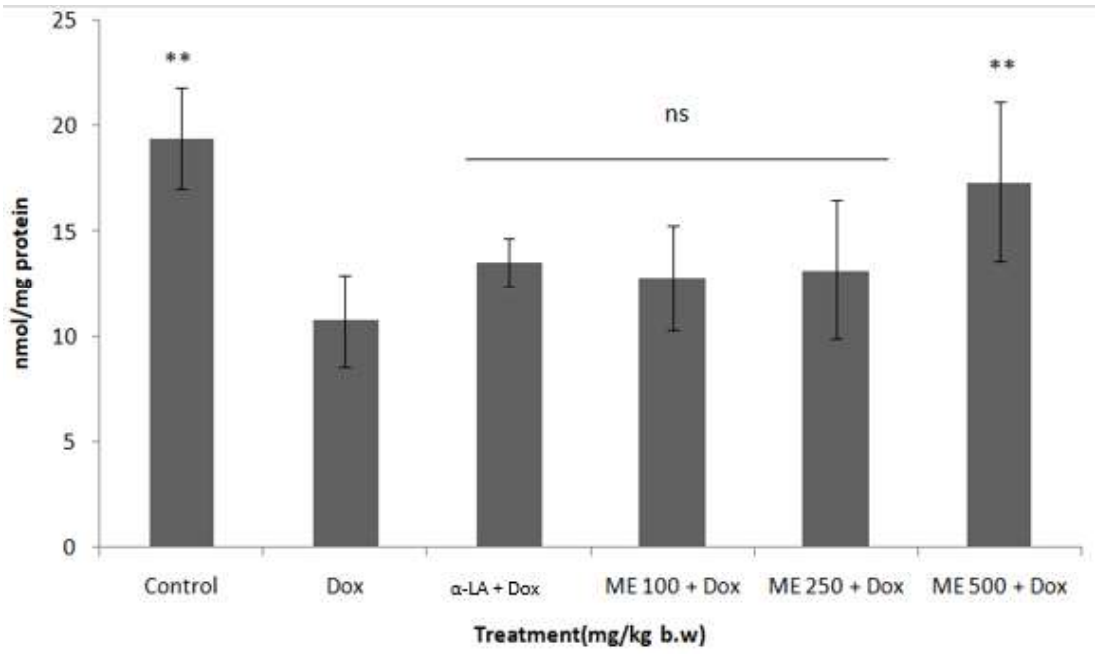


Figure.6.2.B. GSH content. All values expressed as Mean ± SD (n=6).*P<0.05; **P<0.01;ns P>0.05 compared to DOX administered animals (Dunnett's test).

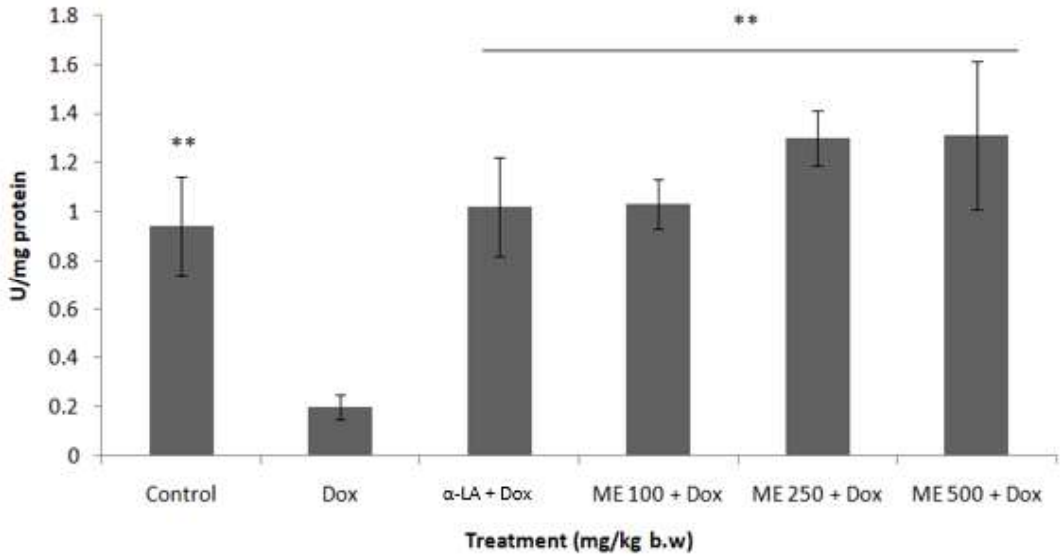


Figure.6.2.C. SOD activity. All values expressed as Mean ± SD (n=6).*P<0.05; **P<0.01;ns P>0.05 compared to DOX administered animals (Dunnett's test).

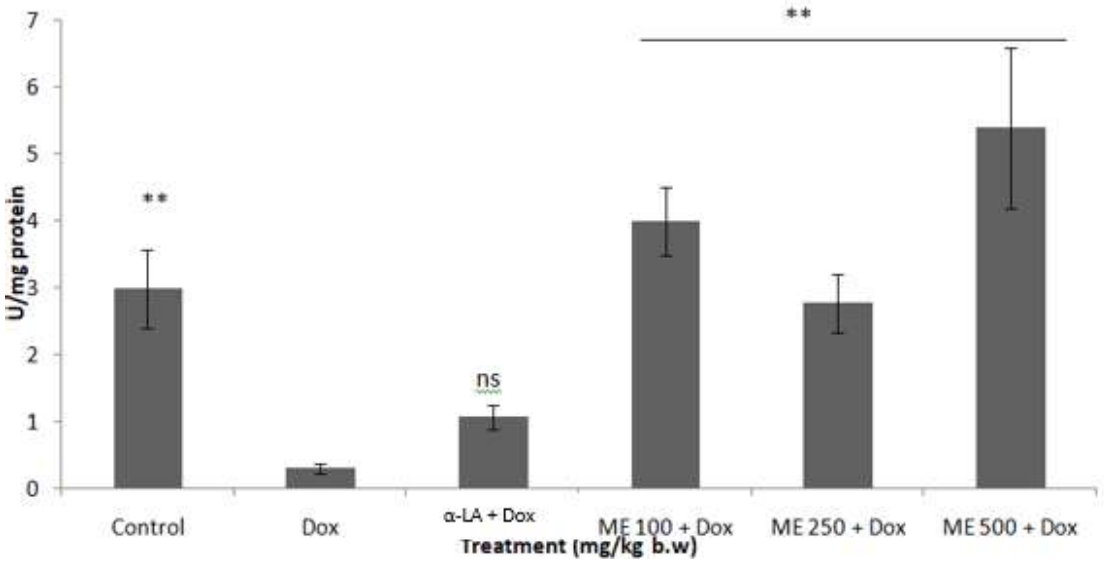


Figure.6.2.D. Catalase activity. All values expressed as Mean ± SD (n=6).*P<0.05; **P<0.01;ns P>0.05 compared to DOX administered animals (Dunnett's test).

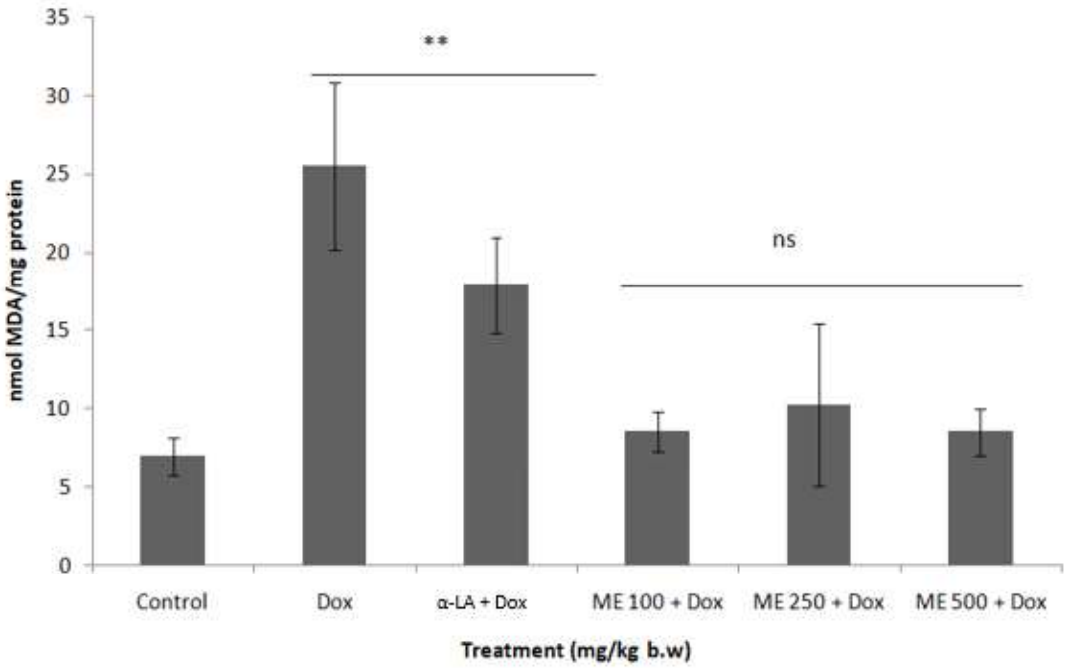


Figure.6.2.E. Lipid peroxidation. All values expressed as Mean ± SD(n=6).*P<0.05; **P<0.01;ns P>0.05 compared to control untreated animals (Dunnett's test).

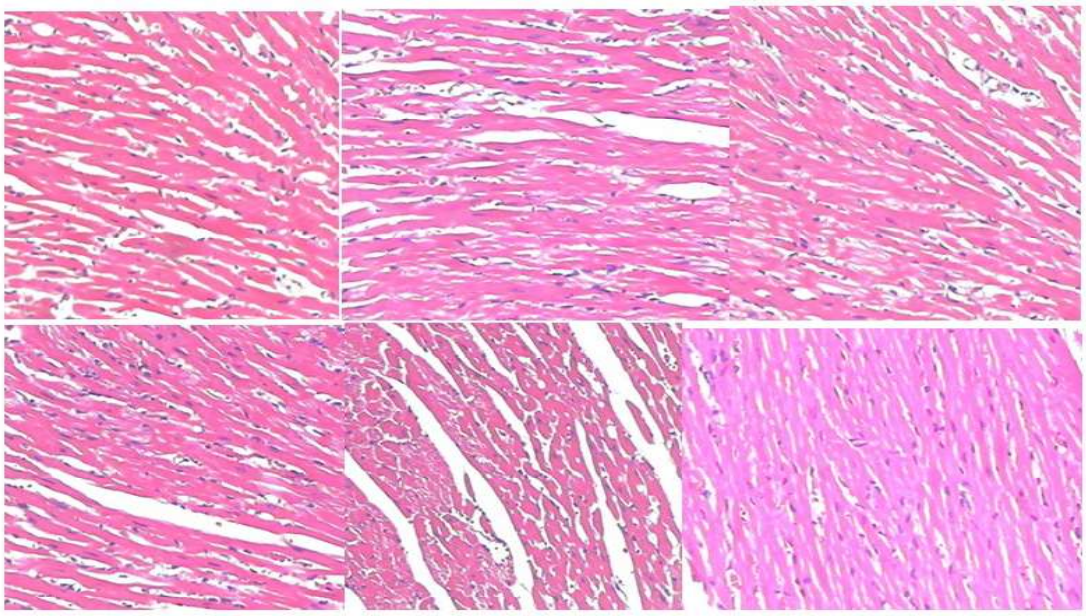


Figure.6.3. A. Histopathological image showing normal architecture of cardiac tissue obtained from untreated-animals.

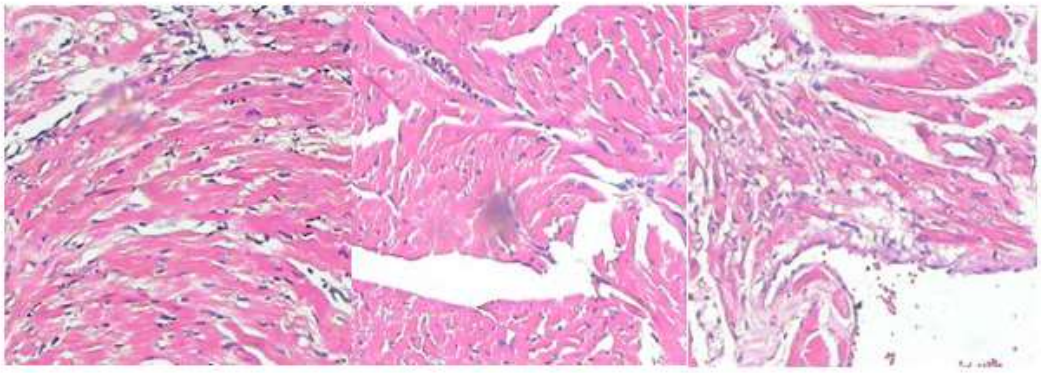


Figure.6.3. B. Histopathological image of cardiac tissue obtained from Dox treated animals showing, internal haemorrhage, Cardiac hypertrophy, wavy myofibrils, nuclear infiltration, pyknotic nuclei and myoplasm degeneration.

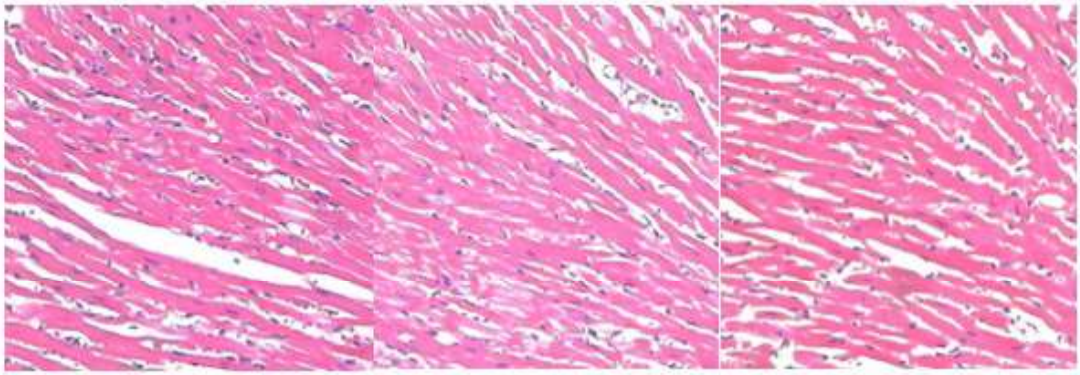


Figure.6.3. C. Cardiac tissue from animals treated with Dox along with ME at a concentration of 100 mg/kg. b.w.

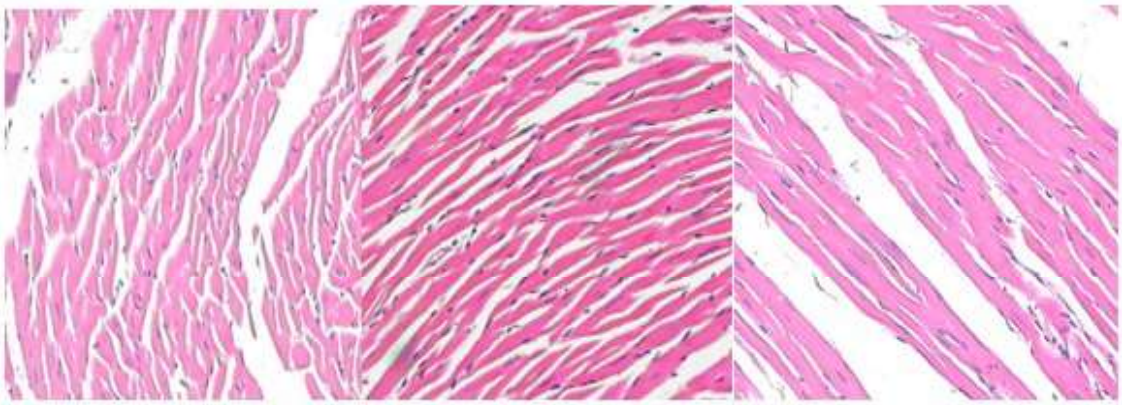


Figure.6.3. D. Cardiac tissue from animals treated with Dox along with ME at a concentration of 250 mg/kg. b.w.

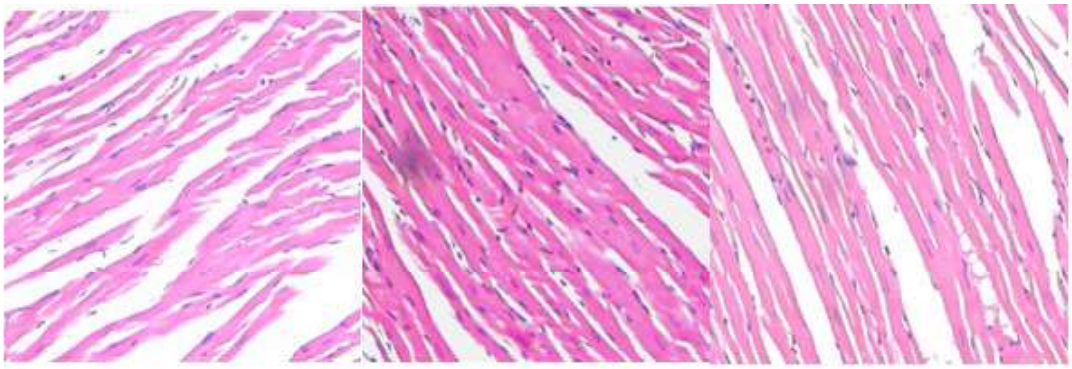


Figure.6.3. E. Cardiac tissue from animals treated with Dox along with ME at a concentration of 500 mg/kg.b.w.

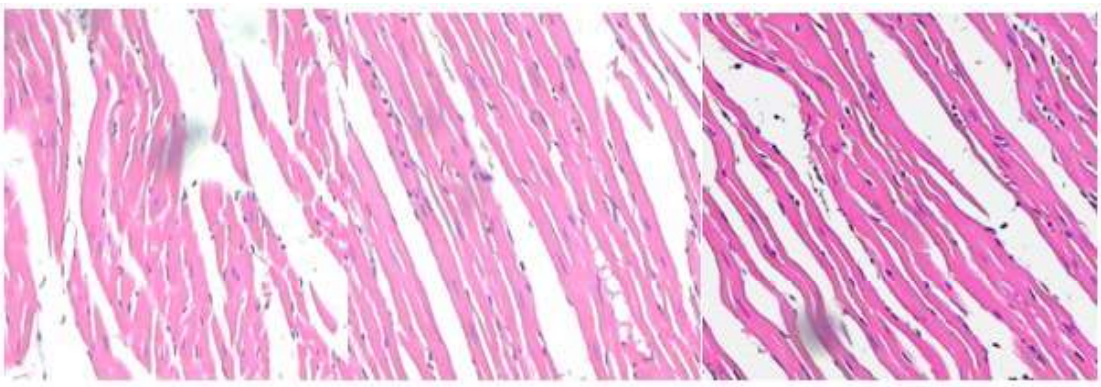


Figure.6.3. F. Cardiac tissue from animals treated with Dox along with DL-α-Lipoic acid at a concentration of 100 mg/kg.b.w.

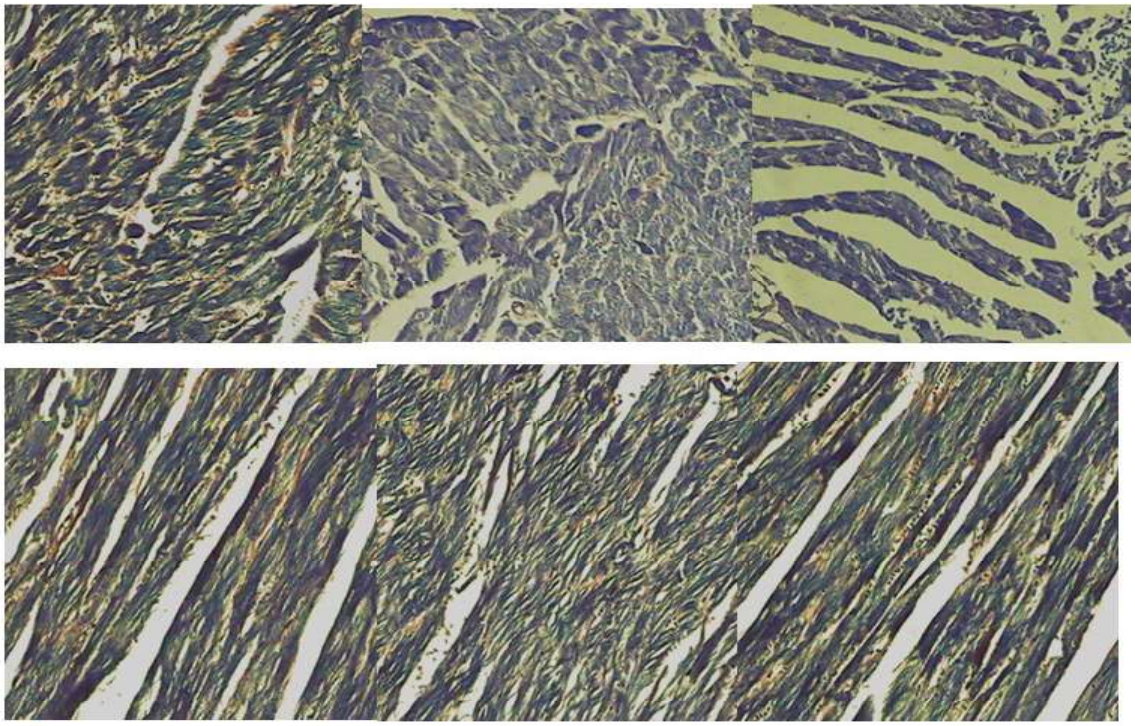


Figure.6.4.A. PTAH-stained images of cardiac tissue from untreated control animals.

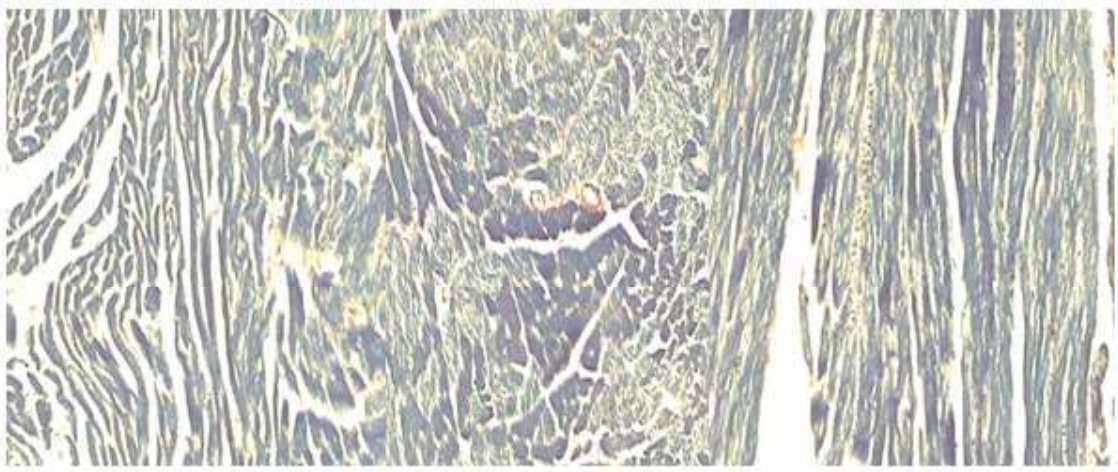


Figure.6.4.B. Cardiac tissue images from Dox treated animals.

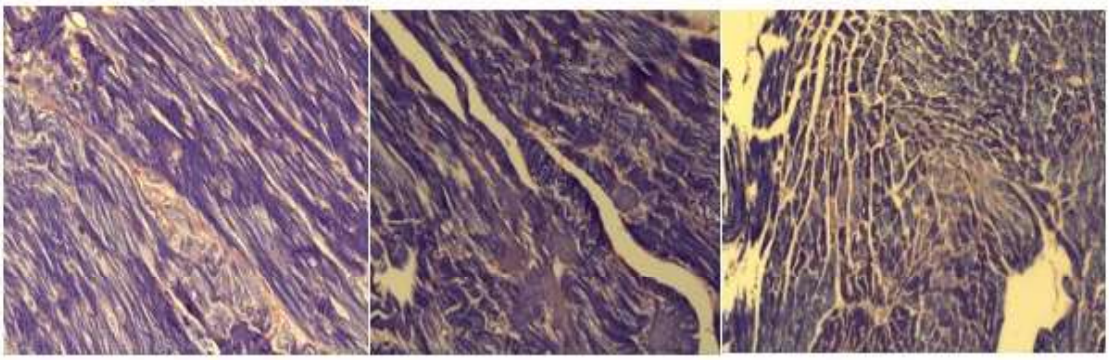


Figure.6.4.C. PTAH-stained images of ME 100mg/kg + Dox treated animals

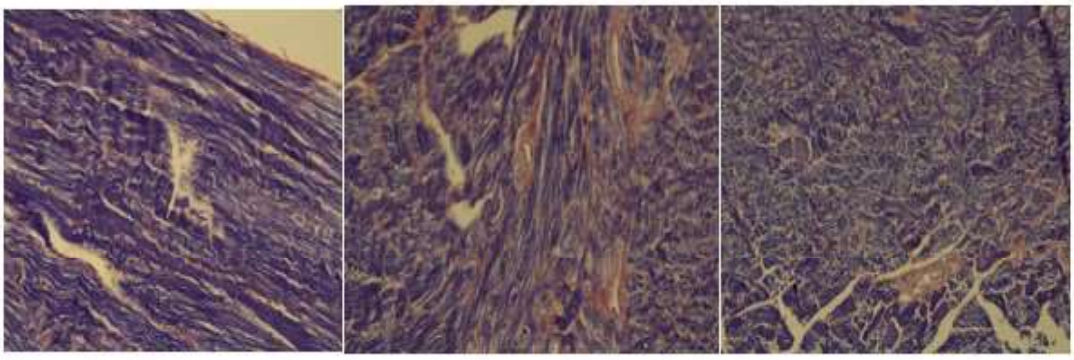


Figure.6.4.D. PTAH-stained images of cardiac tissue obtained from ME 250 mg/kg + Dox treated animals

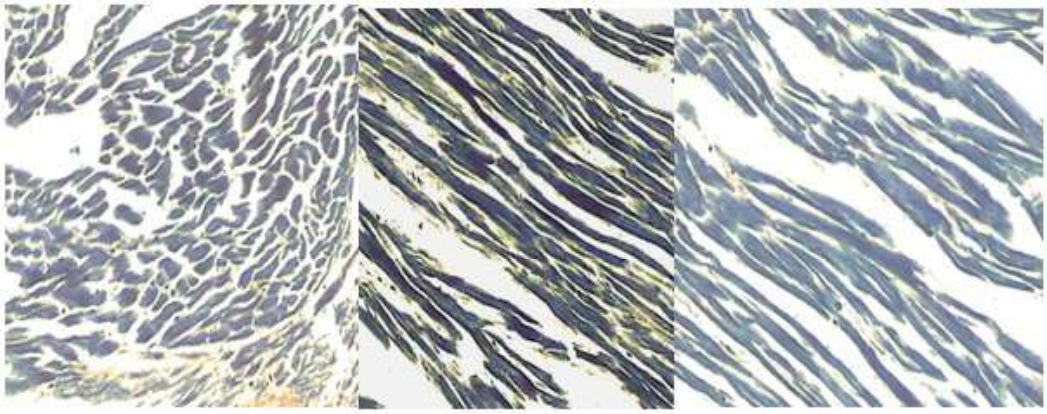


Figure.6.4.E. PTAH-stained images of cardiac tissue obtained from ME 500 mg/kg + DOX treated animals.

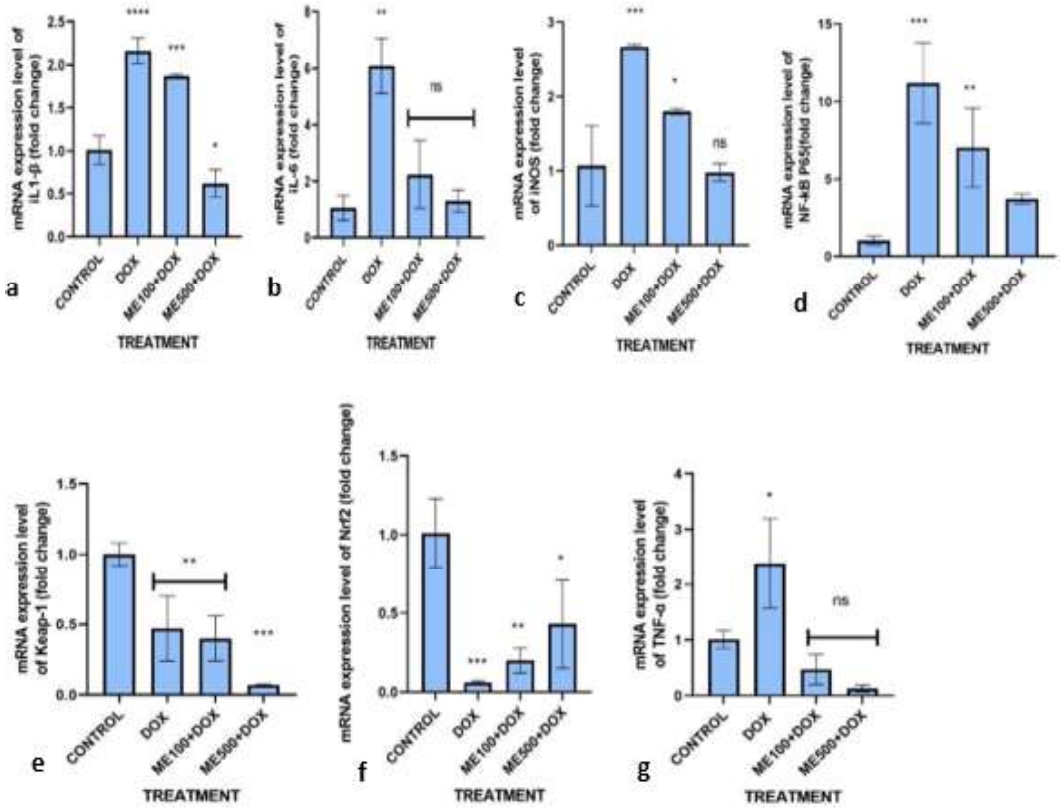


Figure.6.5.(a-g). Analysis of gene expression. All values expressed as Mean ± SD(n=6). **** P<0.0001;*** P<0.001; **P<0.01;ns P>0.05 compared to normal untreated animals (Dunnett's test).

Tables

Table.6.1.Body weight, Heart weight and Relative heart weight of animals. All values are expressed as Mean±SD **** P<0.0001; ns P<0.05; *** P<0.003; ** P<0.0019; * P <0.0393 F.B.W : Final Body Weight, H.W: Heart Weight, %Relative heart weight= (H.W/B.W)*100.

GROUP	F.B.W	Δ weight before Dox treatment	Δ weight after Dox treatment	H.W	% Relative heart weight
Normal	35±5.9	2.4±0.21	3.675±1.3	0.142±0.03	0.4±0.04
Dox	26±5.0	3.2±0.82	-4.66±1.6	0.173±0.02**	0.6±0.04**
α-LA + Dox	28±6	2.1±0.58	-4.14±2.5	0.118±0.02**	0.42±0.02**
ME 100 + Dox	28±3.24	4.8±1.4	3.3±0.3	0.125±0.016**	0.48±0.08*
ME 250 + Dox	31.75±2.4	4.25±1.07	3±1.5	0.117±0.03**	0.41±0.04**
Me 500 + Dox	27.1±10	4.65±0.96	3.50±0.65	0.129±0.028**	0.47±0.09*

Table.6.2. Effect of ME on TCA cycle enzyme levels.

Values are the mean ± SD, n = 6, **** P<0.0001,***P=0.0001, ** P=0.0098, *P<0.01and ns P >0.05 (Dunnett test) with respect to Dox alone treated groups : Isocitrate dehydrogenase (ICDH)—µmoles of NAD^+ reduced/min/mg protein; a-ketoglutarate dehydrogenase (a-KGDH)— µmoles of NAD^+ reduced/min/mg protein; succinate dehydrogenase (SDH)— µmoles of DCIP reduced/min/mg protein; malate dehydrogenase (MDH)— µmoles of NADH oxidized/min/mg protein

Group	MDH	SDH	ICDH	AKDH
Control	1293.45±452.6****	69.5±19.3****	95.9±8.4***	48.3±17.1***
Dox	152.7±48.34	18.5±4.9	30.2±2.9	2.92±0.94
ME100+Dox	1244.8±330.5****	$31.44±6.2^{ns}$	$57±13^{ns}$	$21±6.27^{ns}$
ME250+Dox	2147.6±289.78****	55.05±5.8****	123±58***	76.2±15.8****
ME500+Dox	1575.6±340.7****	54.7±6.3****	129.1±11.5****	106.9±20.9****
α-LA + Dox	$153±30^{ns}$	34.3±4.3*	$65.9±7.5^{ns}$	43±21***

Table.6.3. Effect of ME on Electron Transport Chain Complex.

Values are the mean ± SD, n = 6, **** P<0.0001,***P=0.0001, ** P=0.0098, *P<0.01and ns P >0.05 (Dunnett test) with respect to Dox alone treated groups. Units: Complex I—µmoles of DCIP reduced/min/mg protein; Complex III— µmoles of ferricytochrome C reduced/min/mg protein; Complex IV—µmoles of ferro cytochrome C oxidized/min/mg protein.

Group	C1	C3	C4
Control	29.7±6.6****	10.09±3.05****	1371±195.6**
Dox	2.4±0.7	3.66±1.003	585.6±160.9
ME100+Dox	5.3±0.58^{ns}	2.17±1.1^{ns}	680.2±97.8^{ns}
ME250+Dox	12.6±3****	7.2±1.4**	1248±409**
ME500+Dox	14.6±1.6****	8.2±2.4***	3376±580****
α-LA + Dox	7.6±2.7*	2.37±0.87^{ns}	709±275^{ns}

CHAPTER 7

Evaluation Of Protective Effect Methanol Extract of Morchella Esculenta Against Cyclophosphamide Induced Cardiotoxicity

Table of Contents

7.1. **INTRODUCTION**

The advancement in therapeutic intervention of neoplastic diseases has effectively reduced the morbidity and mortality, yet toxicities associated with chemotherapy remain a serious problem. Phenomenal progresses in chemotherapy helped to increase the survival rate of cancer patients (Miller et al., 2019). Cyclophosphamide (CP) is one of the front-line chemotherapeutic drugs currently in use. Drug-induced cardiotoxicity associated with CP regimens has been a hindrance in its intensive clinical use. Cyclophosphamide is an oxazaphospharine nitrogen mustard alkylating drug used as anti-neoplastic and immunosuppressive agent. It is a prodrug and upon administration it gets converted to its active metabolites phosphoramide mustard and acrolein (Iqubal et al., 2021; Elrashidy et al., 2021). The anti-neoplastic efficacy of CP is imparted by phosphoramide mustard, which interferes with DNA and thus contribute to immunosuppressive and cytotoxic properties. The treatment regimens of CP demand for high dosages which is often associated with multiple organ toxicity (Wang et al., 2021). Various reports suggest that the toxic metabolite, acrolein is responsible for cardiotoxicity induced during chemotherapy. Acrolein interferes with the antioxidant defense system leading to massive generation of free radicals resulting in oxidative stress.

High dose treatments with CP causes cardiac injury within 10 days leading to congestive heart failure, arrhythmia, myopericarditis, cardiac tamponade etc. (Iqubal et al., 2019). Clinical studies revealed that long term use of CP caused decline of antioxidant defense resulting in oxidative stress. Declined antioxidant defense in turn causes massive release of ROS that cause damage to biomolecules leading to cellular damage (El-Agamy et al., 2017). ROS generation is a key factor involved in CP induced cardiomyopathy (Abd El Ghafar et al., 2021; Ali et al., 2023). Therefore, mechanisms that target prevention of oxidative stress would be a promising therapeutic approach to attenuate drug induced cardiomyopathy. Dexrazoxane is the only FDA approved cardioprotective agent used as an adjuvant in doxorubicin treatment. So far, no permitted adjuvant is available to attenuate CP induced cardiotoxicity. Various studies suggest that natural products having high antioxidant capacity might provide protective effect against CP induced cardiac damage (Singh et al., 2023).

Mushrooms have immense therapeutic potential and their bioactives have been stated to possess significant antioxidant activity. Morel mushroom, *Morchella esculenta* also known as 'Guchhi' in India is an edible mushroom (Teng et al., 2023; Chen et al., 2023; Haq et al., 2023; Li et al.,2023). Previous studies in our laboratory found anti-tumor, anti-inflammatory, anti-oxidant, hepatoprotective activities of *Morchella* bioactives (Nitha et al., 2008;Nitha et al., 2013; Nitha et al.,2020). Studies were carried out to evaluate the efficacy of methanol extract of *M. esculenta* (ME) to attenuate cyclophosphamide induced cardiotoxicity. The findings are presented in this chapter.

7.2. MATERIALS AND METHODS

7.2.1. Chemicals

Cyclophosphamide was purchased from the pharmacy of Amala Institute of Medical Sciences, India (Endoxan-N) and CK-MB, LDH assay kits from Agappe Diagnostics India Limited.

7.2.2 Experimental material and preparation of methanolic extract of *M. esculenta* fruiting body.

Dried fruiting bodies of *M. esculenta* were procured from Organic Kashmir, a reputed mushroom dealer from New Delhi. The dried mushrooms were washed thoroughly to remove dirt and any other chemicals, which is dried again at 40^{o}C. The material was powdered, and one-hundred-gram samples were refluxed with petroleum ether (60–80 °C) for 6 h. The defatted material was then dried and extracted using methanol for 8 h in Soxhlet apparatus. The filtered extract is evaporated to dryness at 40 °C using a rotary vacuum evaporator and the residue (ME) thus obtained was stored at 4 °C for further use.

7.2.3.*In Vivo* Studies

Cardioprotective activity of ME against CP induced cardio toxicity was determined using male Swiss Albino mice weighing 25-30 gram obtained from Small Animal Breeding Station, Kerala Agriculture University, Mannuthy, Thrissur, Kerala, India was employed in this study. The experiments were carried out following the guidelines

of CPCSEA, Government of India and approval by Institutional Animal Ethic Committee (ACRC/IAEC/21(1)-P7).

Animals were divided into six groups containing 6 animals in each group. The treatment protocol was as follows: Group I served as the control group treated with drinking water for the whole experimental period; Group II animals were injected with three doses of CP (66.6 mg/kg b.w.) i.p. a cumulative dose of 200 mg/kg b.w. The treatment groups III, IV, V and VI were orally administered with ME 100, 250, 500 and standard α-lipoic acid 100 mg/kg b.w. respectively for the entire experiment period. All groups except Group I was administered CP (i.p.) on 8^{th}, 10^{th} and 12^{th} day of the experiment. Twenty-four hours after the last dose of treatment, animals were weighed and euthanized in a CO_2 chamber. The blood was collected by cardiac puncture immediately after cervical dislocation and serum collected by centrifugation at 3500 rpm for 10 minutes in a cooling centrifuge. The clear supernatant thus obtained was used for the determination of cardiac injury markers such as CK-MB, LDH, and Cardiac troponin-I. Hearts were removed and weighed. Right halves of hearts were rinsed with PBS and stored in -80℃ which was further used for estimation of oxidative stress markers, mitochondrial electron transport chain complexes and left halves were preserved in 10% formalin for histopathological evaluation (Sandamali et al., 2020).

7.2.3.1. Effect on body weight and heart weight of animals.

The body weight of each experimental animal on the initial day of the experiment. These measurements were again taken on the 7 th day before CP treatment and on the 13^{th} day after last dose of CP treatment. Body weight and heart weight ratio was calculated and analysed at the end of the experiment **(Table.7.1)**.

7.2.3.2. Effect on cardiac Injury Marker levels.

CK-MB, and LDH was spectrophotometrically analysed using diagnostic kits of Agappe Diagnostics Pvt Ltd, India. Serum Cardiac Troponin- I a cardiac specific biomarker was estimated by immuno-fluorescence assay method using Vidas High Sensitivity Troponin I kit, Biomerieux.

7.2.3.3. Effect on antioxidant status in cardiac tissue.

A 10% cardiac tissue homogenate was prepared in 50mM phosphate buffer (pH 7.4) containing 1mM EDTA. A part of the homogenate was transferred to 0.5 ml vials and used for estimation of activities of lipid peroxidation by TBARS method (Ohkawa et al., 1979) and GSH by the method of Moron et al., (1979). The supernatant was obtained by centrifuging at 5000 rpm for 15 minutes in a cooling centrifuge and further used for determining the activities of catalase (Beers et al., 1952), SOD (McCord et al.,1969) and GPx (Hafemann et al., 1974). Details of methods are mentioned in section 3.2.5.

7.2.3.4. Isolation of mitochondria

Mitochondria isolation was done based on differential centrifugation method described by Ajith et al., with slight modifications (Ajith et al., 2009). The method is described in section 3.2.6.1.

7.2.3.5 TCA cycle enzyme activity estimation.

Succinate dehydrogenase enzyme was assayed based on method described by Sudheesh et al.,(Sudheesh et al., 2009). TCA cycle enzymes determination methods are described in the section 3.2.6.2.

7.2.3.6. Estimation of Mitochondrial electron transport chain complex activity.

7.2.3.6.1. NADH dehydrogenase (Complex I)

NADH dehydrogenase activity was assayed based on the method of Janssen et al.,(2007), with slight modifications. Refer section 3.2.7.3.1 for the detailed method.

7.2.3.6.2. Estimation of Q-cytochrome c oxidoreductase (Complex III)

Complex III was estimated based on the method mentioned by Krahenbuhl et al., (1991) with slight modifications. Refer section 3.2.7.3.2 for the assay method.

7.2.3.6.3. Cytochrome C oxidase (Complex IV)

Complex IV was estimated based on the method mentioned by Brischigliaro et al., (2022), with slight modification. The assay was done as described in the section 3.2.7.3.3.

7.2.3.7. Histopathological analysis of cardiac Tissue

Histopathology observation of cardiac tissue was done by method described in section 3.2.8.

7.2.3.8 Gene expression analysis

Gene expression analysis of Nf-κB p65, IL-6,IL-1β, TNF-α, iNOS, NRF-2 and KEAP-1 was done according to the method described in section 3.2.9.

7.2.4. Statistical Analysis

All experimental data were statistically analysed with Graph Pad prism software and expressed as mean ± SD. The analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett's test and $P<0.05$ was considered significant.

7.3 RESULTS

7.3.1. Effect of ME on body weight and heart weight of animals.

Animals treated with ME showed a marked increase in body weight after 7 days of treatment prior to CP administration. There was a reduction in the body weight of animals treated with CP alone significantly whereas the ME treatment reduced the weight loss to a greater extend . Heart weight was high in CP treated animals which might be due to cardiac inflammation. The heart was appeared to be swollen and pale. The heart to body weight ratio was found to be higher than that of other groups in CP treated animals while the ratio was restored to normal level in other groups significantly **(Table.7.1).**

7.3.2. Effect of ME on Cardiac injury marker levels.

Administration of CP elevated levels of serum cardiac injury markers, CK-MB and LDH. The animals treated with ME caused significant decline of CK-MB and LDH levels **(Figure.7.1. A&B)**. Administration of CP resulted in elevated cardiac Troponin I level and the treatment with ME caused appreciable reduction which indicated the decline of severity of cardiac tissue damage**(Figure.7.1.C)**.

7.3.3. Effect of ME on antioxidant system in cardiac tissue.

The CP administration resulted in deterioration of antioxidant levels in the cardiac tissue. Treatment with ME boosted the activities of antioxidants significantly in the myocardium. This was evident from the levels of GPx, GSH, SOD and Catalase in the heart tissue **(Figure.7.2.A, B, C&D)**. CP treatment increased lipid peroxidation which was evident from the increased levels of TBARS. However, lipid peroxidation was effectively lowered by ME treatment **(Figure.7.2.E)**.

7.3.4. Effect of ME on activities of TCA cycle enzymes and ETC complex in cardiac mitochondria.

The effect of ME treatment on TCA cycle enzyme levels and ETC complex of cardiac mitochondria are presented in **Table.7.2 & 7.3** respectively. From the results it was evident that CP administration resulted in a drastic decline in the mitochondrial enzyme levels whereas ME was successful in improving the Krebs' cycle enzymes activities and ETC activity in a dose dependent manner. Especially in high dose of ME almost restored the dehydrogenase level back to normal. ME at doses of 250 and 500 mg/kg b.wt. showed a statistically significant increase in mitochondrial dehydrogenases activity. ME treatment at a higher dose along with CP resulted in 4.1,6.5-,3.05- and 1.8-fold increase in Malate dehydrogenase, α-ketoglutarate dehydrogenase, Isocitrate dehydrogenase and Succinate dehydrogenase activity compared to CP alone treated groups. Likewise, higher dosage treatment of ME along with CP showed 5.2,3.6- and 1.89-fold increase in Complex I, III and IV levels than CP alone treated group. These results were indicative of mitochondrial protection imparted by ME treatment.

7.3.5. Histopathological changes in cardiac tissue.

Histopathology examination revealed that CP administration caused severe cardiomyopathy. The tissues stained with haematoxylin-eosin revealed damage caused by CP administration as evidenced by cardiac inflammation, myofibrillar loss, nuclear pyknosis and cytoplasmic vacuolization**(Figure.7.3.)**. PTAH staining revealed the presence of collagen (red stain) in cardiac tissues of animals administered with CP **(Figure.7.4.).** The treatment with ME restored the normal architecture of cardiomyocytes in a dose dependent manner.

7.3.6. Impacts of ME on CP-induced alteration in cardiac mRNA gene expression.

CP treatment lowered the expression levels of NRF2 and elevated the expression of KEAP-1 which was reversed by the treatment with ME at different dosages. On the contrary, the gene expression levels of NF-κB and pro-inflammatory cytokines such as IL-6, IL-1B, TNF-α and iNOS were upregulated by CP treatment which was alleviated by ME treatment in cardiac tissue of the experimental animals. The results are presented in **Figure.7.5.(a-g).**

7.4. Discussion

Cyclophosphamide is a widely used anticancer drug alone or in combination therapy of various chemotherapeutic regimens. However, the treatment regimen demands for the high dosage of CP. Even though the exact mechanism of CP induced cardiac injury is not well understood, studies suggests that the toxic metabolites of CP lead to oxidative stress promote endothelial capillary damage resulting in the release of proteins, erythrocytes, and toxic metabolites. However, early detection of CP induced cardiac damage is possible through the detection of cardiac injury markers (Katayama et al., 2009; Swamy et al., 2013; Ayza et al, 2020; Bhatt et al., 2017).

Current study indicates that there was an increase in body weight of experimental animals prior to CP administration. The gain in body weight was halted in all the animals except the untreated and ME 500 mg/kg b.w treated groups of animals after CP treatment. The administration of ME at high dose was effective in reducing the weight loss caused by CP administration significantly. Similarly, the heart / body weight ratio was higher in CP treated animals and this was restored to normal level by ME treatment. The increase in heart to body weight ratio might be due to the increase in heart weight as a result of cardiac hypertrophy, cardiac inflammation, widened interstititum, and leukocyte infiltration. These results are consistent with the histopathological observations.

The serum cardiac injury biomarkers CK-MB, LDH, and Cardiac troponin-I levels are elevated by CP treatment which indicates cardiac necrosis (Selvakumar et al., 2005). This might have forced the release of cardiac injury markers consequent to damage of endothelium of myocardium. This also might have resulted in ROS generation leading to loss of myocardial membrane integrity (Pimenta et al., 2024). Cardiac troponin I is

considered as a reliable marker of acute myocardial damage (Selvakumar et al., 2005). The serum collected from animals treated with different concentrations of ME showed a marked decrease in the cardiac troponin I levels which indicated the reduced severity of the cardiac damage caused by CP treatment. Similarly, CK-MB and LDH levels which were increased by CP treatment were also lowered by ME indicating its protective effect against CP induced cardiac injury.

CP downregulated SOD, CAT, GPx and GSH and ME could restore these antioxidant levels in a dose dependent manner. Most of the antioxidant enzymes that are involved in balancing the oxidative stress are regulated by NRF2/KEAP1signalling (Sorice et al., 2023). Previous studies by Li et al.(2022) reported that CP- induced lowering of NRF2/KEAP1signalling and antioxidant defence system. ME up-regulated the expression of NRF2 which was lowered consequent to CP treatment. The declined CAT, SOD and GPx activities along with GSH level, augmented the myocardial injury. The depleted activity of SOD caused accumulation of superoxide anion radicals which induced damages to bio-molecules (Wang et al., 2018). The lowered activity of CAT and GPx resulted in elevation of H_2O_2 and hydroxyl radicals. GSH, a non-enzymatic antioxidant with reducing property due to its reactive - SH group (Mukwevho et al., 2014), was depleted in CP treated animals. This might be due to the direct conjugation of CP and its metabolites with GSH (Selvakumar et al., 2005).

CP treatment enhanced lipid peroxidation level as evident from the elevated levels of MDA. ME could effectively protect the antioxidant status and thus lowered the MDA level. The protective effect might be attributed to the bioactives present in ME (Acay., 2021). A decline in the antioxidant defence mechanism will lead to ROS accumulation in mitochondria (Kim et al., 2021) which in turn overload calcium to the mitochondria resulting in loss of mitochondrial membrane potential and decreased ATP generation (Wang et al., 2023).Furthermore, mitochondrial dysfunction can also be attributed to CP-induced opening of pores in inner mitochondrial membrane (Iqubal et al., 2021). CP treatment alleviated the mitochondrial electron transport chain complexes and TCA cycle enzyme activities(Sudharsan et al., 2006; Iqubal et al.,2019).ME treatment successfully combated the mitochondrial damage.

Cardiac inflammation was found associated with CP- induced cardiotoxicity (Iqubal et al., 2019, AL Shaima et al., 2023). NF-κB is a redox-sensitive transcription

factor that regulates the gene expression of proteins COX-2, iNOS and pro-inflammatory cytokines such as IL-6, IL1-β and TNF-α (Refaie et al., 2022). Therefore, NF-κB activation causes an up-regulation of redox sensitive and fibrotic genes that causes inflammation and apoptosis (Baig et al., 2022). In this study, CP treatment elevated the gene expression level of NF-κB p65 which was lowered by ME treatment. The expression of other pro-inflammatory cytokines elevated from CP treatment was also lowered in ME treated animals. The levels of iNOS and COX-2 were also elevated in CP treated group. Nitrative stress associated with ROS generation was previously demonstrated in the impairment of mitochondrial homeostasis (Taha et al., 2023, Yan et al., 2023).

Cardiac hypertrophy and loss of cardiac tissue architecture was found in the CP alone treated animals. Wavy myofibrils associated with increased hydrostatic pressure of interstitial oedema was observed in CP control group(Eichbaum, 1975). This change was absent in normal as well as ME treated groups. PTAH-stained images showed high amount of collagen in CP alone treated control group, indicating cardiac fibrosis. ME treatment at a concentration of 500 mg/kg b. w. was highly effective in restoring the architecture of cardiomyocytes.

The findings presented in this chapter is in accordance with previous study reports which was supported by biochemical analysis and histopathological observations (Das et al., 2022). Being an excellently edible mushroom, the findings suggest the potential therapeutic use of *Morchella esculenta* and its bioactive extract to attenuate cardiotoxicity induced by cyclophosphamide chemotherapy.

Figures & Tables

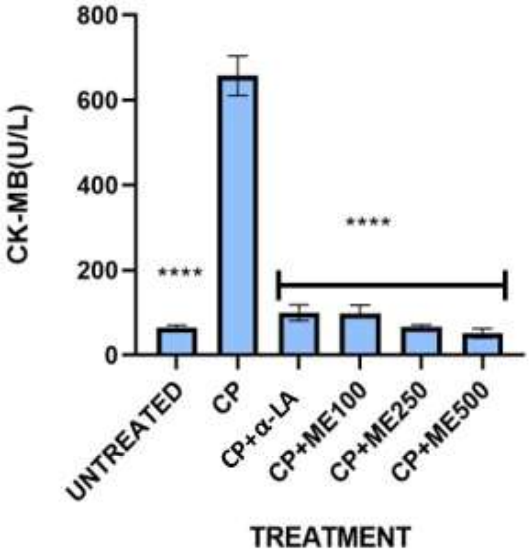


Figure.7.1. A CK-MB activity: Values are the mean ± S.D; (n=6).**** P<0.0001;** *P*< 0.01 significantly and ^{ns}P>0.05 non significantly different from CP control group (Dunnett's test) .

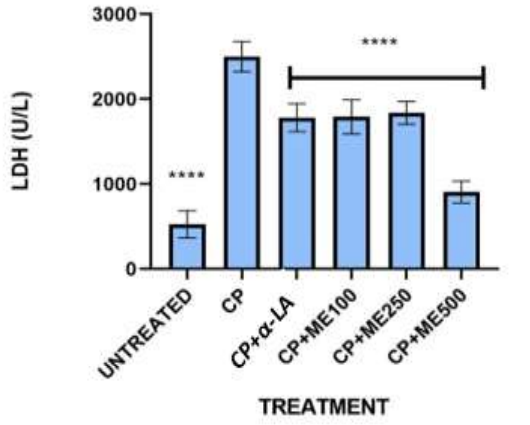


Figure.7.1. B LDH activity: Values are the mean ± S.D; (n=6).**** P<0.0001;** *P*< 0.01 significantly and ^{ns}P>0.05 non significantly different from CP control group (Dunnett's test) .

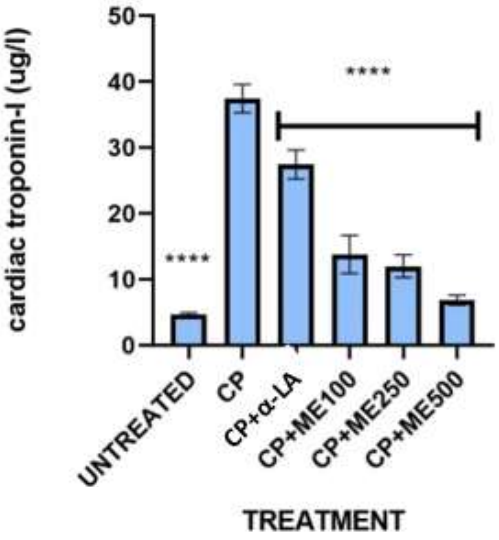


Figure.7.1. C. Cardiac Troponin-I: Values are the mean ± S.D; (n=6).**** P<0.0001;** *P*< 0.01 significantly and ^{ns}*P*>0.05 non significantly different from CP control group (Dunnett's test).

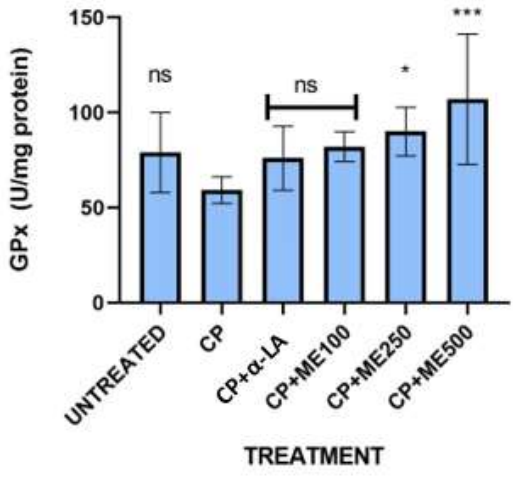


Figure.7.2. A. GPx activity. All values expressed as Mean ± SD (n=6).**** P<0.0001;** *P*< 0.01 significantly and ^{ns}*P*>0.05 non significantly different from CP control group (Dunnett's test) .

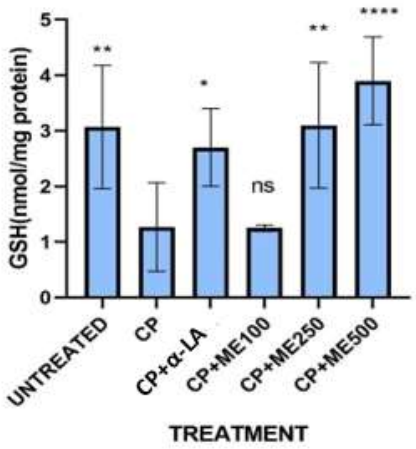


Figure.7.2. B. GSH content. All values expressed as Mean ± SD (n=6). **** P<0.0001; ** *P*< 0.01 significantly and ^{ns}*P*>0.05 non significantly different from CP control group (Dunnett's test) .

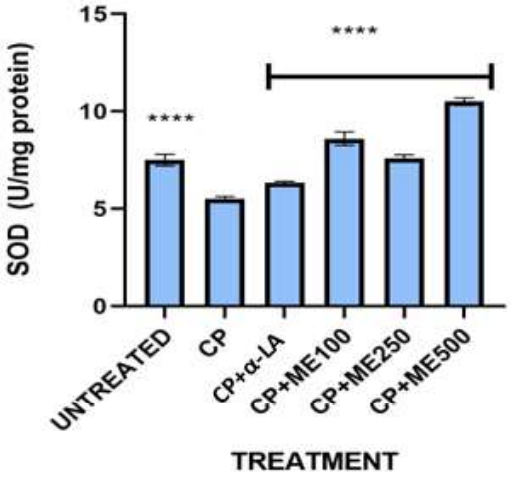


Figure.7.2. C. SOD activity. All values expressed as Mean ± SD (n=6). **** P<0.0001; ** *P*< 0.01 significantly and ^{ns}*P*>0.05 non significantly different from CP control group (Dunnett's test) .

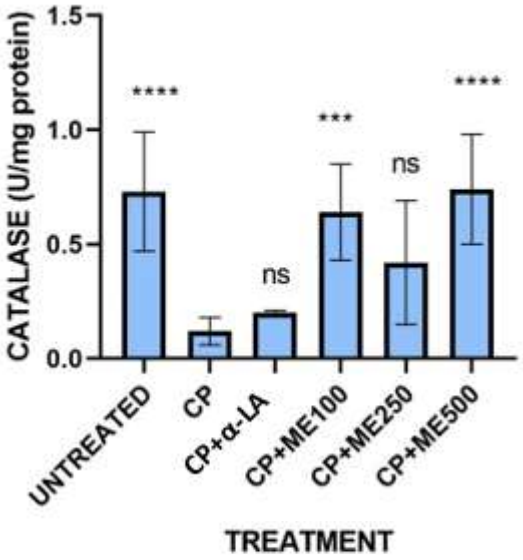


Figure.7.2. D. Catalase activity. All values expressed as Mean ± SD (n=6).**** P<0.0001;** *P*< 0.01 significantly and ^{ns}*P*>0.05 non significantly different from CP control group (Dunnett's test) .

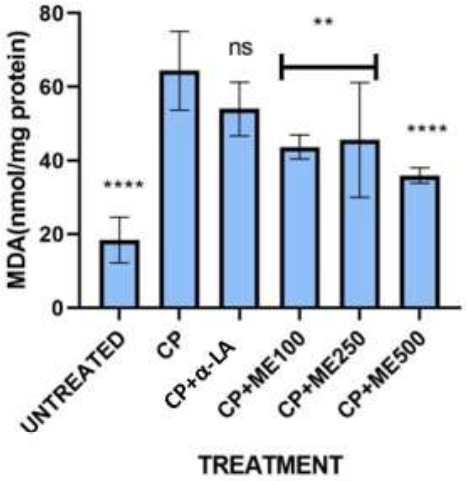


Figure.7.2. E. Lipid peroxidation. All values expressed as Mean ± SD(n=6).**** P<0.0001;** *P*< 0.01 significantly and ^{ns}*P*>0.05 non significantly different from CP control group (Dunnett's test) .

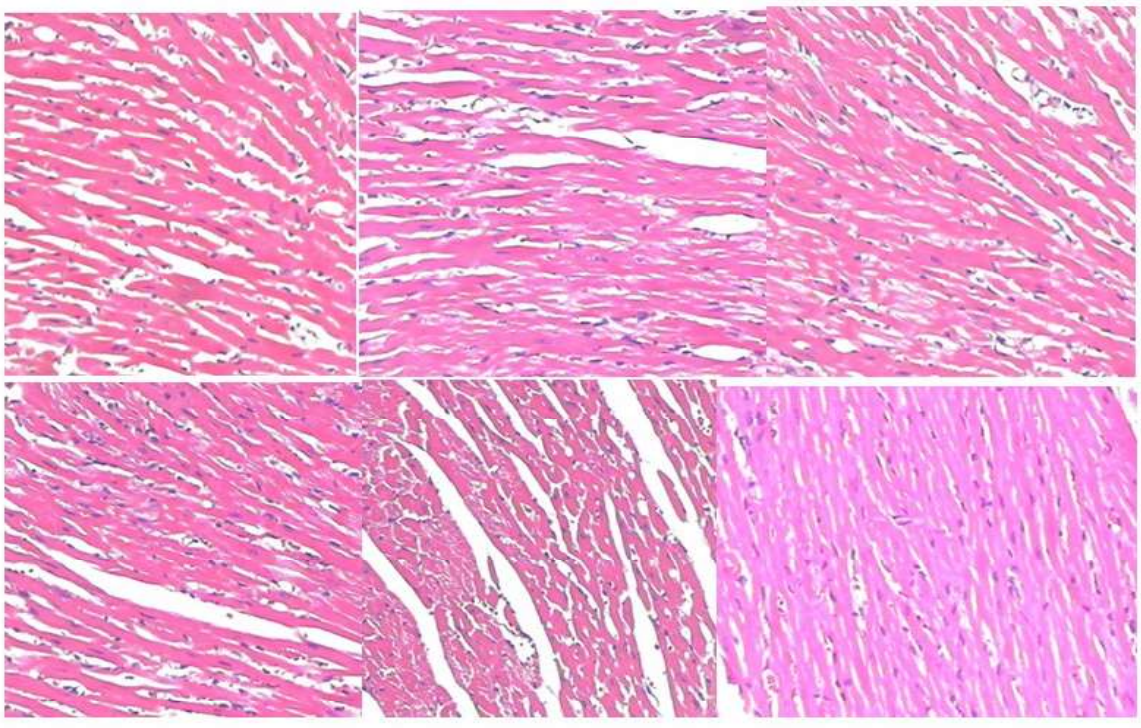


Figure.7.3. A. Histopathological image showing normal architecture of cardiac tissue obtained from untreated-animals.

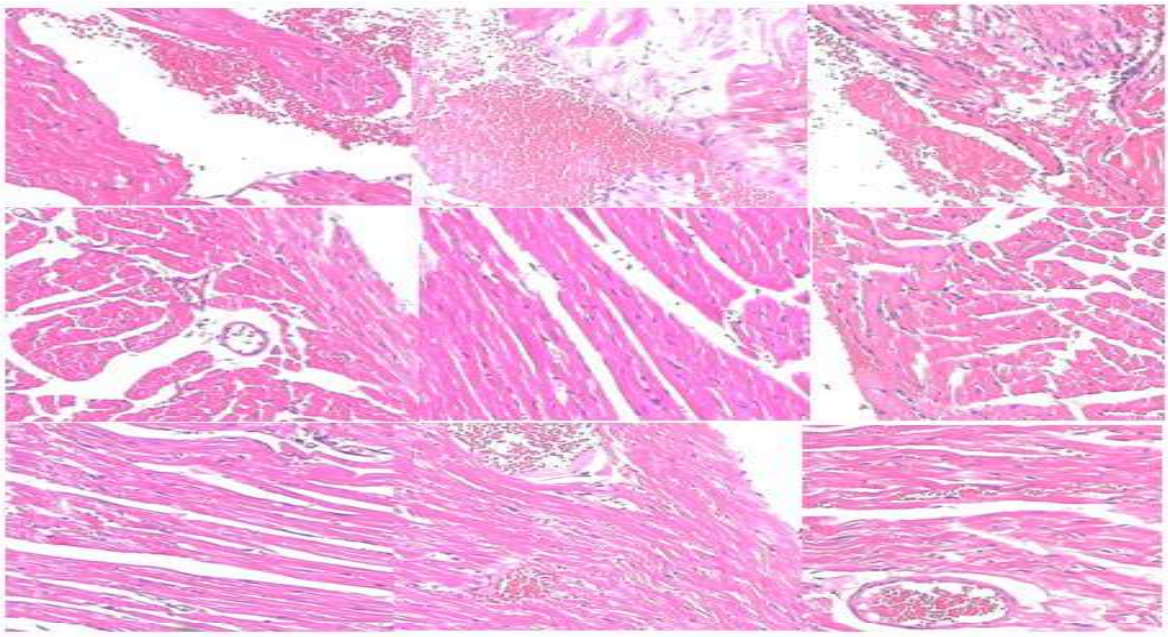


Figure.7.3. B. Histopathological image of cardiac tissue obtained from CP treated animals showing, internal haemorrhage, Cardiac hypertrophy, wavy myofibrils, nuclear infiltration, pyknotic nuclei and myoplasm degeneration.

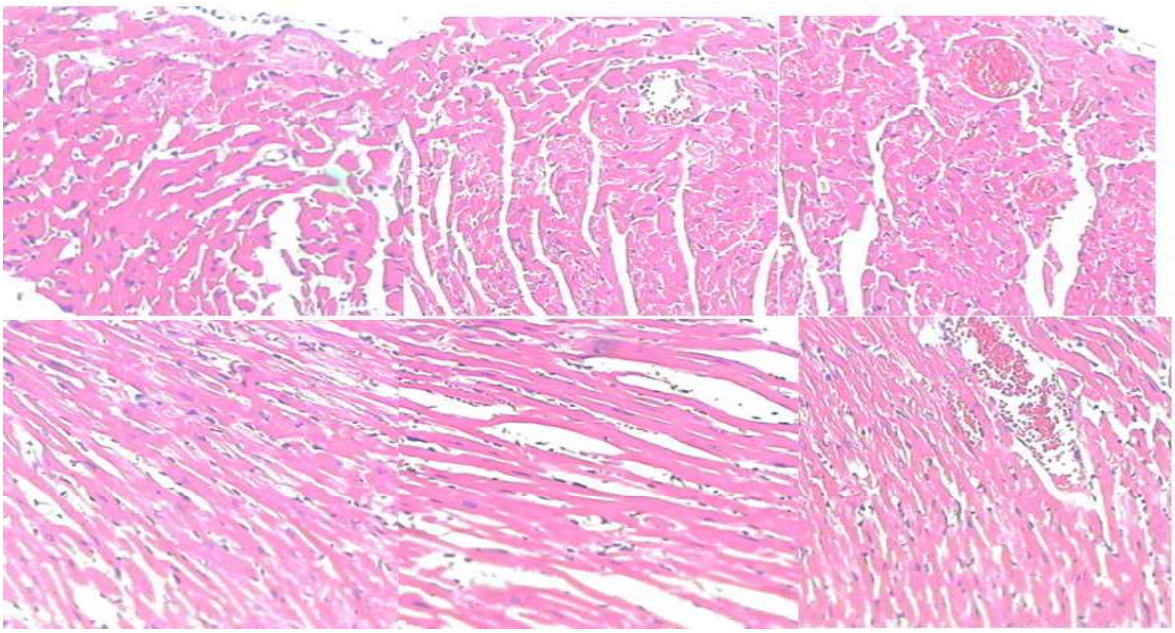


Figure.7.3. C. Cardiac tissue from animals treated with CP along with ME at a concentration of 100 mg/kg. b.w.

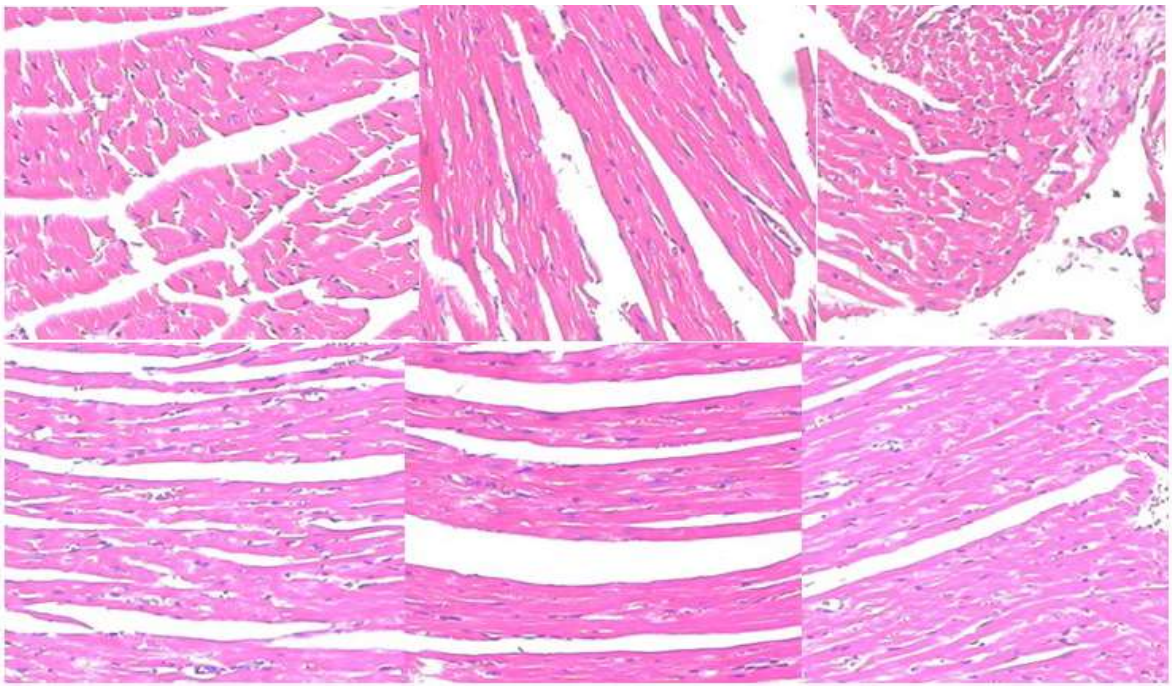


Figure.7.3. D. Cardiac tissue from animals treated with CP along with ME at a concentration of 250 mg/kg. b.w.

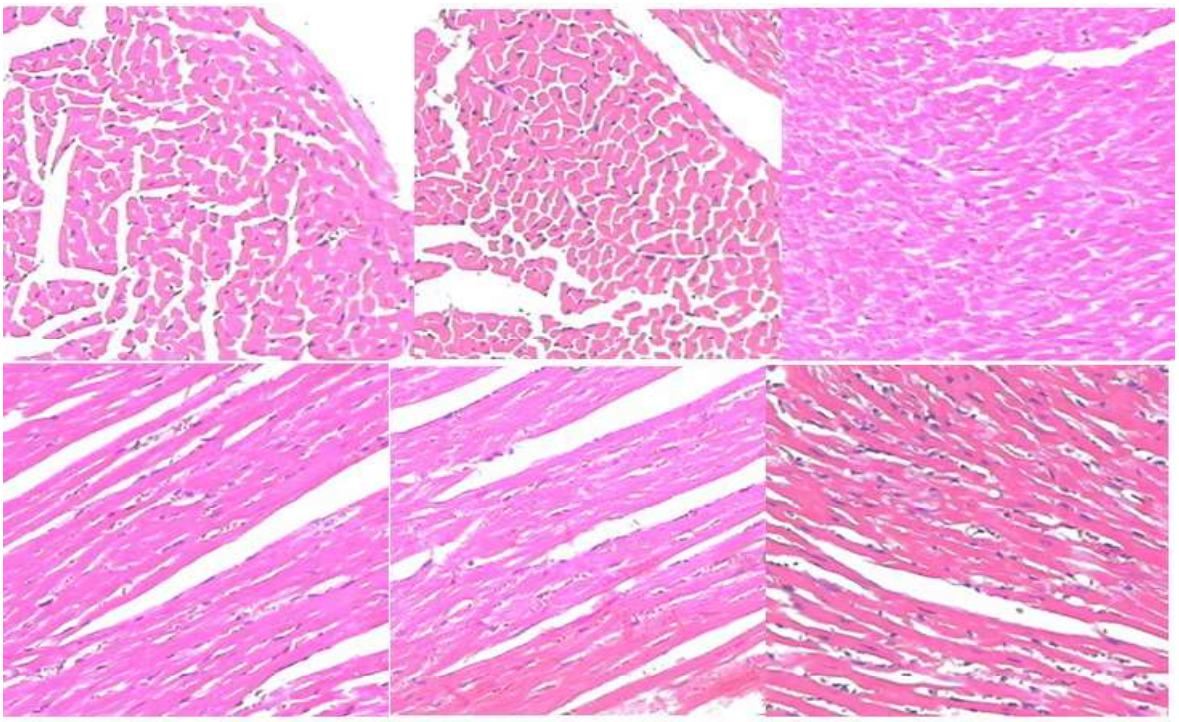


Figure.7.3. E. Cardiac tissue from animals treated with CP along with ME at a concentration of 500 mg/kg.b.w.

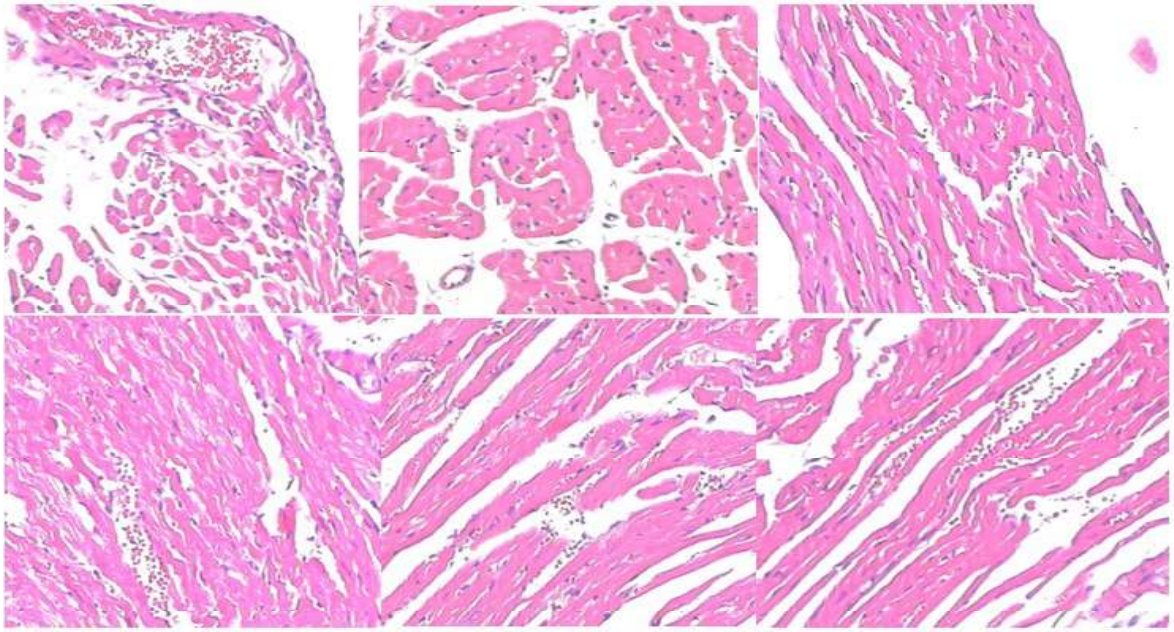


Figure.7.3. F. Cardiac tissue from animals treated with CP along with DL-α-Lipoic acid at a concentration of 100 mg/kg.b.w.

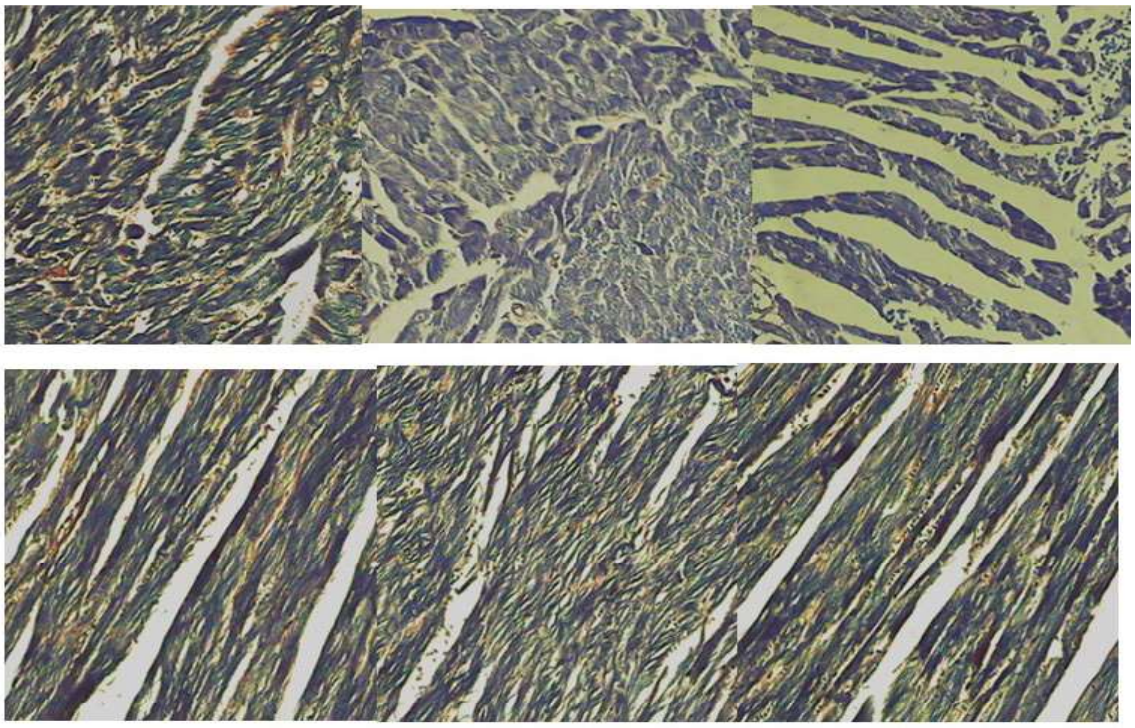


Figure.7.4.A. PTAH-stained images of cardiac tissue from untreated control animals.

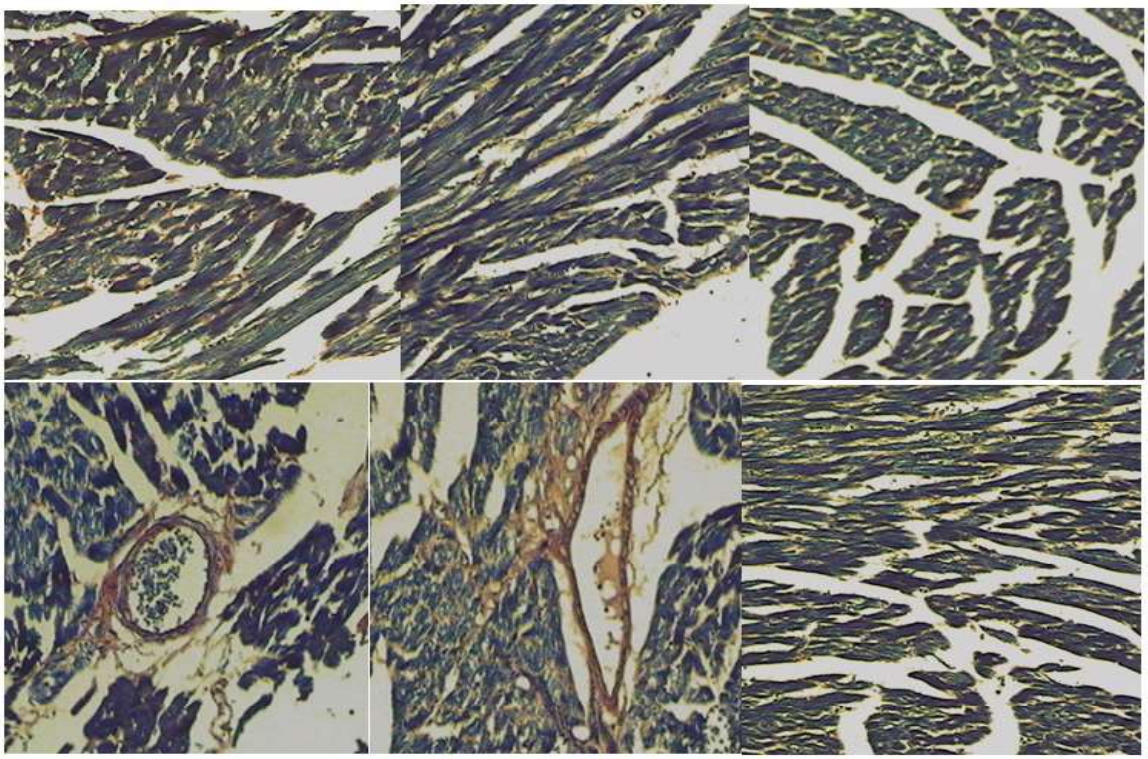


Figure.7.4.B. Cardiac tissue images from CP treated animals.

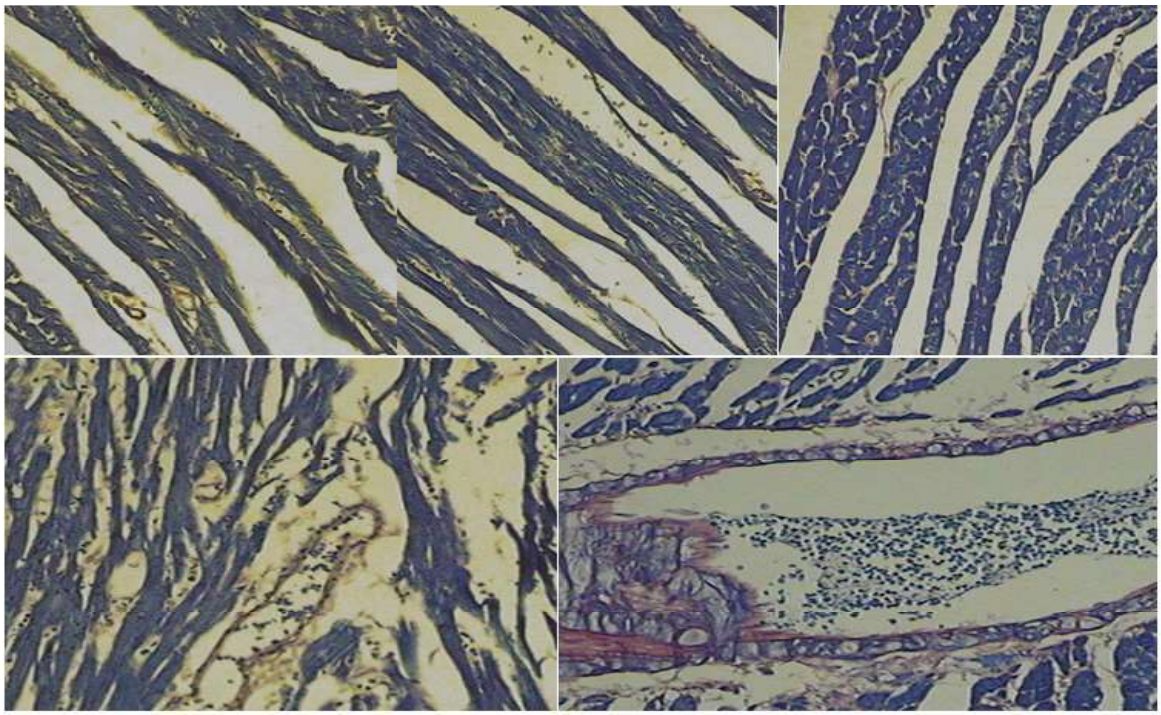


Figure.7. 4.C PTAH-stained images of ME 100mg/kg + CP treated animals

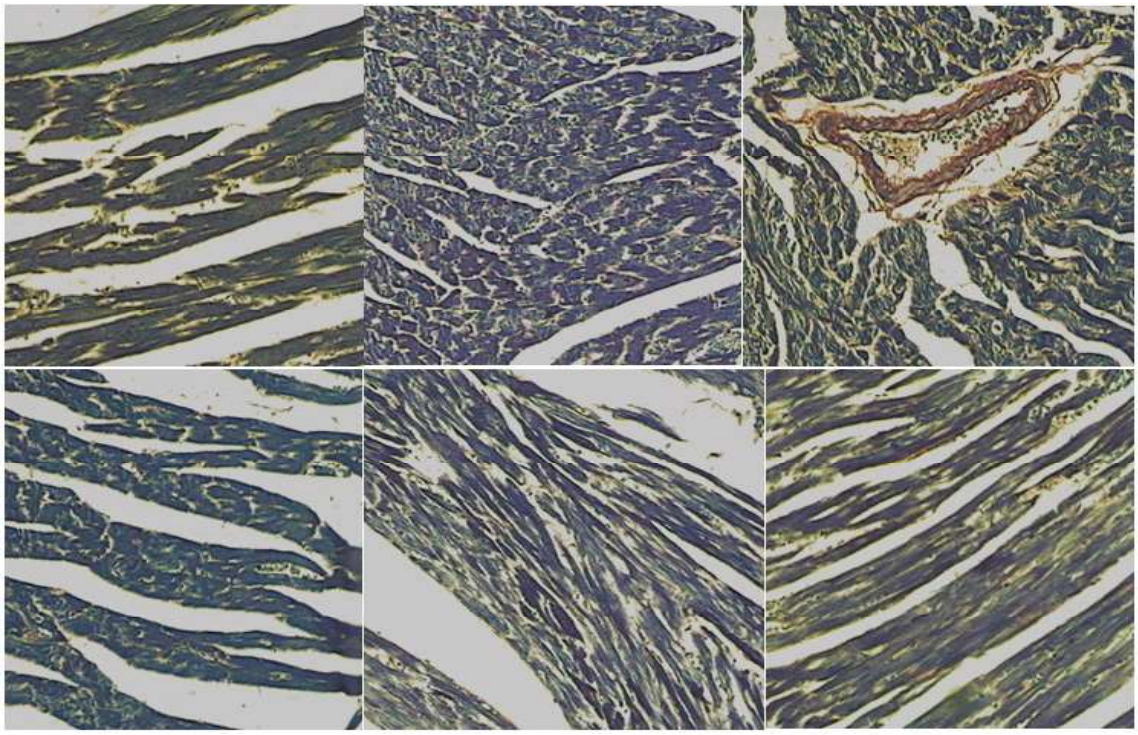


Figure.7.4.D. PTAH-stained images of cardiac tissue obtained from ME 250 mg/kg + CP treated animals

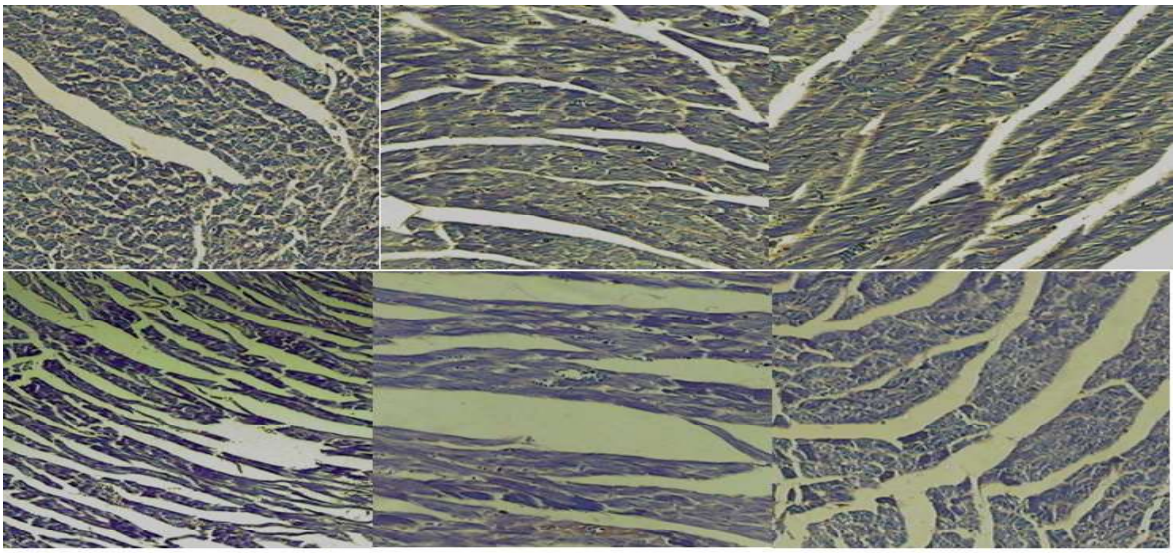


Figure.7.4.E. PTAH-stained images of cardiac tissue obtained from ME 500 mg/kg + CP treated animals.

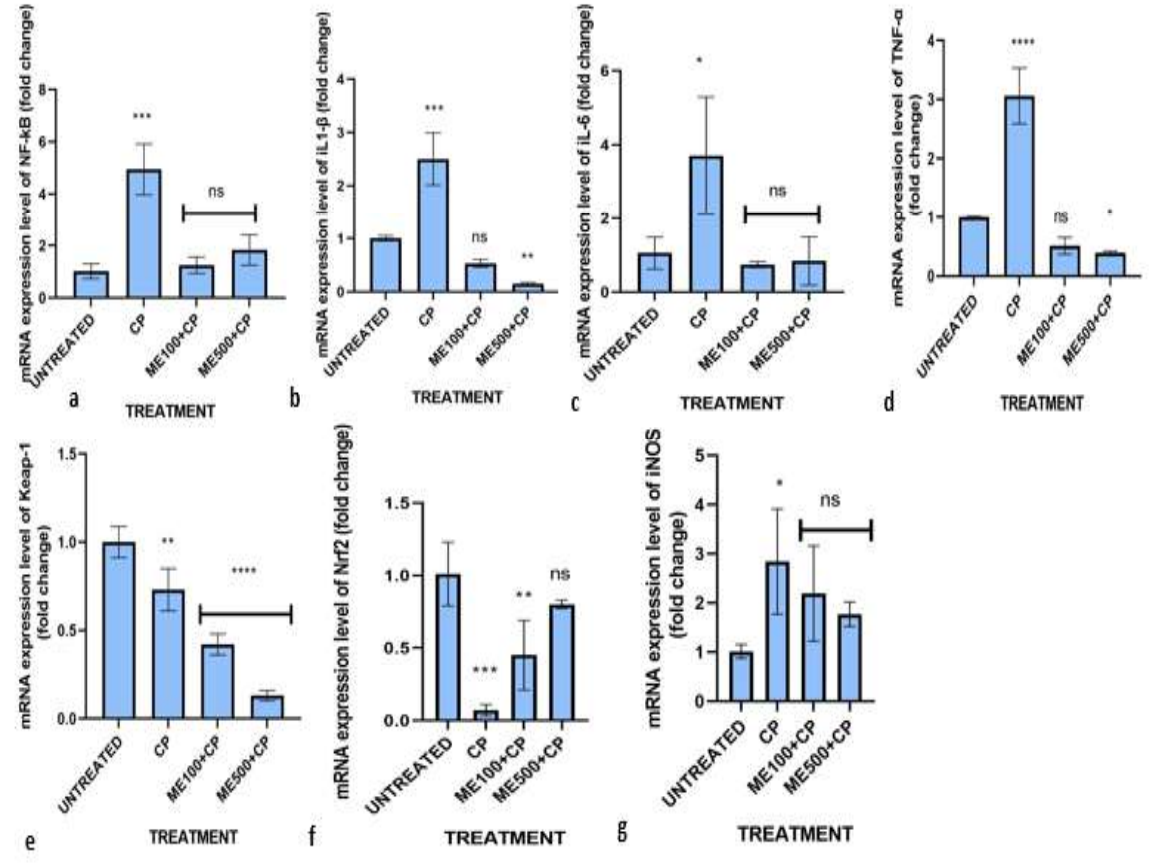


Figure.7.5.(a-g). Analysis of gene expression. All values expressed as Mean ± SD(n=6). **** P<0.0001; **P<0.01; *** P<0.001; ns P>0.05 compared to normal untreated animals (Dunnett's test).

Tables

Table.7.1. Body weight, Heart weight and Relative heart weight of animals. All values are expressed as Mean±S.D **** P<0.0001; ns P<0.05; *** P<0.003; ** P<0.0019; * P <0.0393

F.B.W : Final Body Weight, H.W: Heart Weight, %Relative heart weight= (H.W/B.W)*100

GROUP	F.B.W	Δ weight before CP treatment	Δ weight after CP treatment	H.W	% Relative heart weight
Normal	31±1.9	2.425±0.43	(+)2.82±0.56	0.126±0.008	0.45±0.03
CP	25.3±4.3	3.58±0.47	(-)3.11±1.4	0.142±0.005	0.57±0.11
ME 100 + CP	23.42±4.62	1.28±0.88	(-)2.26±1.15	0.109±0.004	0.39±0.13
ME 250 + CP	26.5±1.08	4.52±1.29	(-)1.9±0.82	0.109±0.001	0.41±0.016
ME 500 + CP	28.95±1.1	3.65±0.77	(+)3.6±1.19	0.115±0.002	0.41±0.021
α-LA + CP	26.7±0.7	0.65±0.36	(-)2.6±1.41	0.08±0.01	0.3±0.045

Table.7.2. Effect of ME on TCA cycle enzyme levels

Values are the mean ± SD, n = 6, **** P<0.0001,***P=0.0001, ** P=0.0098, *P<0.01and ns P >0.05 (Dunnett test) with respect to CP alone treated groups : Isocitrate dehydrogenase (ICDH)—µmoles of NAD^+ reduced/min/mg protein; a-ketoglutarate dehydrogenase (a-KGDH)— µmoles of NAD^+ reduced/min/mg protein; succinate dehydrogenase (SDH)— µmoles of DCIP reduced/min/mg protein; malate dehydrogenase (MDH)— µmoles of NADH oxidized/min/mg protein

GROUP	MDH	AKDH	ICDH	SDH
CONTROL	1293.45±452.6*	48.3±17.1****	95.9±8.4****	69.5±19.3****
CP	408.7±218	11.15±4.3	34.7±9.2	30.1±3.7
ME 100 + CP	$597±80^{ns}$	$17.6±9^{ns}$	113.5±4.9****	$30.5±4.8^{ns}$
ME 250 + CP	$965±411^{ns}$	34.7±8.9**	65.6±9.7****	$43.3±5.69^{ns}$
ME 500 + CP	1686±929***	73±19****	106.12±12****	56.5±7.9***
α-LA + CP	$334.98±88^{ns}$	$29±12^{ns}$	129±6.1****	34.3±4.3**

Table.7.3. Effect of ME on Electron Transport Chain Complex.

Values are the mean ± SD, n = 6, **** P<0.0001,***P=0.0001, ** P=0.0098, *P<0.01and ns P >0.05 (Dunnett test) with respect to CP alone treated groups. Units: Complex I—µmoles of DCIP reduced/min/mg protein; Complex III— µmoles of ferricytochrome C reduced/min/mg protein; Complex IV—µmoles of ferro cytochrome C oxidized/min/mg protein

GROUP	Complex I	Complex III	Complex IV
CONTROL	29.7±6.6****	10.09±3.05****	1371±195.6^{ns}
CP	6.27±2.8	1.7±0.5	1024±246
ME 100 + CP	14.9±3.7^{ns}	4.9±0.66**	642±35^{ns}
ME 250 + CP	20.7±7.3**	2.8±0.2^{ns}	1618±258*
ME 500 + CP	33.1±9.8****	6.14±1.12***	1939±524***
α-LA + CP	7.9±0.59^{ns}	5.05±1.7**	1153±433^{ns}

CHAPTER 8

Evaluation of Protective Effect Methanol Extract of Morchella Esculenta Against Combination Treatment of Doxorubicin and Cyclophosphamide Induced Cardiotoxicity

Table of Contents

8.1. Introduction

Cancer is an important public health concern and it is anticipated to be the leading cause of death in the 21 st century (Leiter et al.,2023). Therefore, quest for novel and specific chemotherapeutic agents and the identification of specific targets are emerging fields in oncology research. Even though several drugs are effective when administered alone, combination chemotherapy with combination of one or more drugs are found to have enhanced response rates and longer remissions (Anand et al., 2023). This may be attributed to the additive or synergistic action of the drugs and which probably lowers the tendency of cancerous cells to become drug resistant. This combination therapy is mainly employed in the treatment of breast cancer (Jin et al.,2023). Cyclophosphamide, Docetaxel, Doxorubicin Hydrochloride, 5-Fluorouracil, Gemcitabine Hydrochloride, Methotrexate, etc., are the FDA approved chemotherapeutic agents currently used against breast cancer (Chaurasia et al.,2023). Doxorubicin- Cyclophosphamide combination along with Paclitaxel is widely used for the treatment of breast cancer (Zenjanab et al.,2024).

Doxorubicin and Cyclophosphamide elevate the release of reactive oxygen species which exhaust the antioxidant defence mechanism ultimately leading to cell death (Zhou et al., 2023). Due to the low levels of antioxidants and high mitochondrial levels cardiac tissue is more prone to the oxidative stress mediated toxicity (Jomova et al., 2023). So, compounds that can alleviate oxidative stress induced cardiomyopathy is of interest now a days. Extensive studies are under progress for the mitigation of ill effects imparted by these chemotherapeutic agents. Several reports are available on the use of natural compounds which can be used as adjuvant therapy as cardio protectant without compromising the chemotherapeutic benefits (Marino et al.,2023).

Current study focuses on the cardioprotective activity of methanolic extract of *Morchella esculenta,* an edible wild mushroom. *M esculenta* is widely used in traditional Chinese medicine for decades (Li et al.,2023). Previous studies reported the antioxidant, immunomodulatory, hepatoprotective, cardioprotective, anti-inflammatory and anti-tumour activities of the bioactive extracts of *Morchella esculenta* (Nitha et al., 2010;Nitha et al., 2013;Nitha et a., 2007;Nitha et al., 2017). Major objective of the current study is to exmine the cardioprotective effect of methanolic extract of *Morchella esculenta* (ME) against combination treatment of

doxorubicin(Dox) and cyclophosphamide (CP) using Swiss Albino mice model and the results are presented in this chapter.

8.2. MATERIALS AND METHODS

8.2.1. Chemicals

Doxorubicin and Cyclophosphamide were purchased from the pharmacy of Amala Institute of Medical Sciences, India and CK-MB, LDH assay kits were obtained from Agappe Diagnostics India Limited. All other chemicals and reagents used were of analytical grade.

8.2.2. Experimental material and preparation of methanolic extract of *M. esculenta* fruiting body.

Dried fruiting bodies of *M. esculenta* were procured from Organic Kashmir, a reputed mushroom dealer New Delhi. Refer section 3.2.2.

8.2.3. *In Vivo* Studies

Cardioprotective activity of ME against combination treatment of Dox and CP induced cardio toxicity were determined in Male Swiss Albino mice weighing 25-30 gram obtained from Small Animal Breeding Station, Kerala Agriculture University, Mannuthy, Thrissur, Kerala, India was employed in this study. The experiments were carried out following CPCSEA, Government of India guidelines and approval by Institutional Animal Ethic Committee (ACRC/IAEC/21(1)-P7).

Animals were divided into six groups containing 6 animals in each group. The treatment protocol was as follows: Group I served as the control group treated with drinking water for the whole experimental period; Group II animals were injected with three doses of CP(50mg/kg b.wt) + DOX (3.33 mg/kg b.w.) i.p. on the 8^{th}, 10^{th} and 12^{th} day of the experiment. The treatment groups III, IV, V and VI were orally administered with ME 100, 250, 500 and standard α-lipoic acid 100 mg/kg b.w. respectively for the entire experiment period. The experimental period was of 12 days, animals except Group I was administered Dox + CP (i.p.) on 8^{th}, 10^{th} and 12^{th} day of the experiment. Twenty-four hours after the last doe of treatment, animals were weighed and euthanized in a CO_2 chamber. The blood was collected by cardiac puncture immediately after cervical dislocation and serum collected by centrifugation at 3500 rpm for 10 minutes

in a cooling centrifuge. The clear supernatant thus obtained was used for the determination of cardiac injury markers such as CK-MB, LDH, and Cardiac troponin-I. Hearts were removed and weighed. Right halves of hearts were rinsed with PBS and stored at -80°C which was further used for estimation of oxidative stress markers, mitochondrial electron transport chain complexes and left halves were preserved in 10% formalin for histopathological evaluation (Das et al., 2022; Sandamali et al., 2020).

8.2.3.1. Effect on body weight and heart weight of animals.

The body weight was measured for each experimental animal on the initial day of the experiment. These measurements were again taken on the 7 th day before Dox and CP treatment and on the 13th day after Dox and CP treatment. Body weight and heart weight ratio was calculated and analysed at the end of the experiment **(Table.1)**.

8.2.3.2. Effect on Cardiac Injury Marker levels.

CK-MB, and LDH was spectrophotometrically analysed using diagnostic kit from Agappe Diagnostics Pvt Ltd, India. Serum Cardiac Troponin- I, a cardiac specific biomarker was estimated by immuno-fluorescence assay method using Vidas High Sensitivity Troponin I kit, Biomerieux.

8.2.3.3. Effect on antioxidant status in cardiac tissue.

A 10% cardiac tissue homogenate was prepared in 50mM phosphate buffer (pH 7.4) containing 1mM EDTA. A part of the homogenate was transferred to 0.5 ml vials and used for estimation of lipid peroxidation by TBARS method (Ohkawa et al., 1979) and GSH by Moron et al., (1979) activities. The supernatant was obtained by centrifuging at 5000 rpm for 15 minutes in a cooling centrifuge and further used for determining activities of catalase (Beers et al., 1952), SOD (McCord et al.,1969) and GPx (Hafemann et al., 1974). Detailed methods are presented in section 3.2.5.

8.2.3.4. Isolation of mitochondria

Mitochondrial isolation was done based on differential centrifugation according to the method described by Ajith et al., with slight modifications (Ajith et al., 2009). The method is described in section 3.2.6.1.

8.2.3.5. TCA cycle enzyme activity estimation

Succinate dehydrogenase enzyme was assayed according to the method described by Sudheesh et al.,(Sudheesh et al., 2009). TCA cycle enzymes were determined by methods described in the section 3.2.6.2.

8.2.3.6. Estimation of mitochondrial Electron Transport Chain Complex activity

8.2.3.6.1. NADH Dehydrogenase (Complex I)

NADH dehydrogenase activity was assayed by the method of Janssen et al.,(2007), with slight modifications. Refer section 3.2.7.3.1 for the detailed method.

8.2.3.6.2. Estimation of Q-cytochrome C Oxido-reductase (Complex III)

Complex III was estimated based on the method described by Krahenbuhl et al., (1991) with slight modifications. Refer section 3.2.7.3.2 for the assay method.

8.2.3.6.3. Cytochrome C Oxidase (Complex IV)

Complex IV was estimated based on the method described by Brischigliaro et al., (2022), with slight modification. The assay was done as described in the section 3.2.7.3.3.

8.2.3.7. Histopathology Examination of Cardiac Tissue

Histopathological analysis of cardiac tissue was done by method described in section 3.2.8.

8.2.3.8 Gene expression analysis

Gene expression analysis of Nf-κB p65, IL-6,IL-1β, TNF-α, iNOS, NRF-2 and KEAP-1 was done according to the method described in section 3.2.9.

8.2.4. Statistical Analysis

All experimental data were statistically analysed with Graph Pad prism software and expressed as mean ± SD. The analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett's test and $P<0.05$ was considered significant.

8.3 RESULTS

8.3.1. Effect of ME on body weight and heart weight of animals.

The body weight of animals treated with ME showed a marked increase after 7 days of treatment prior to CP+DOX administration. There was a reduction in the body weight of animals treated with CP +DOX alone in a significant manner whereas the ME treatment reduced the weight loss to a greater extend . Heart weight was higher than normal in CP+DOX treated animals which might be due to cardiac inflammation. The heart was appeared to be swollen and pale. The heart to body weight ratio was found to be higher than that of other groups in CP +DOX treated animals while the ratio was restored to normal level in other groups significantly (**Table.8.1**).

8.3.2. Effect of ME on cardiac injury marker levels.

Elevated levels of serum cardiac injury markers, CK-MB and LDH was observed in animals administered with CP+DOX. The animals treated with ME caused significant decline of CK-MB and LDH levels **(Fig.8.1. A&B)**. Administration of CP+DOX resulted in elevated cardiac Troponin I level and the treatment with ME caused appreciable reduction which indicated the decline of severity of cardiac tissue damage**(Fig.8.1.C)**.

8.3.3. Effect of ME on antioxidant system in cardiac tissue.

The CP+DOX treatment resulted in deterioration of antioxidant levels in the cardiac tissue. Treatment with ME enhanced the activities of antioxidants significantly in the myocardial tissue. This was evident from the levels of GPx, GSH, SOD and Catalase in the heart tissue **(Fig.8.2.A, B, C&D)**. CP+DOX treatment increased lipid peroxidation which was evident from the increased levels of TBARS. However, lipid peroxidation was effectively lowered by ME treatment **(Fig.8.2.E)**.

8.3.4. Effect of ME on activities of TCA cycle enzymes and ETC complex in cardiac mitochondria.

The effect of ME treatment on TCA cycle enzyme levels and ETC complex of cardiac mitochondria are presented in **Table.8.2 & 8.3** respectively. From the results it is evident that CP+DOX administration resulted in a drastic decline in the mitochondrial enzyme levels whereas ME was successful in improving the Krebs' cycle activity and

ETC activity in a dose dependent manner. Especially in high dose of ME it almost restored the dehydrogenase level back to normal. ME at doses of 250 and 500 mg/kg b.wt. showed a statistically significant increase in mitochondrial dehydrogenases activity. ME treatment at a higher dose along with CP+DOX resulted in 4.1,6.5-,3.05- and 1.8-fold increase in Malate dehydrogenase, α-ketoglutarate dehydrogenase, Isocitrate dehydrogenase and Succinate dehydrogenase activity compared to CP+DOX alone treated groups. Likewise, higher dosage treatment of ME along with CP 5.2,3.6- and 1.89-fold increase in Complex I, III and IV levels than CP+DOX alone treated group. These results are indicative of mitochondrial protection imparted by ME treatment.

8.3.5. Histopathological changes in cardiac tissue

Histopathology examination revealed that CP+DOX administration caused severe cardiomyopathy. The tissues stained with haematoxylin-eosin revealed damage caused by CP+DOX administration as evidenced by cardiac inflammation, myofibrillar loss, nuclear pyknosis and cytoplasmic vacuolization**(Fig.8.3.A)**. PTAH staining revealed the presence of collagen (red stain) in cardiac tissues of animals administered with CP+DOX **(Fig.8.4.B).** The treatment with ME restored the normal architecture of cardiomyocytes in a dose dependent manner.

8.3.6 Impacts of ME against CP+DOX-induced alteration in cardiac mRNA gene expression.

CP+DOX treatment lowered the expression levels of NRF2 and elevated the expression of KEAP-1 which was reversed by the treatment with ME at different dosages. On the contrary, the gene expression levels of NF-κB and pro-inflammatory cytokines such as IL-6, IL-1B, TNF-α and iNOS were upregulated by combination treatment of CP+DOX, which was alleviated by ME treatment in cardiac tissue of the experimental animals. The results are presented in **Fig.8.5.(a-g).**

8.4. Discussion

Doxorubicin and cyclophosphamide are frontline chemotherapeutic agents and are often used in the treatment of triple negative breast cancer as AC (Adriamycin and Cyclophosphamide) regimen. This combination regimen raises a major concern of cardiotoxic evets associated with it. The cardiotoxicity of both the drugs are mainly

attributed to the excessive free radical generation. Since cardiac cells have least antioxidant protection rather than other organs, they are highly prone to the ill effects imparted by these agents. The current experimental results reveal the cardioprotective effect of methanol extract of *M. esculenta* against combination chemotherapy of Dox and CP.

The current study shows that the body weight of experimental animals' increased before the CP+DOX regimen. The administration of ME at high dose was effective in reducing the weight loss caused by CP+DOX significantly. Similarly, the heart / body weight ratio was higher in CP+DOX treated animals and this was restored to normal level by ME treatment. The increased heart weight resulting from cardiac hypertrophy, cardiac inflammation, expanded interstititum, and leukocyte infiltration could be the cause of the increased heart to body weight ratio. These results are consistent with the histopathological observations.

In this study we observed an elevation in the cardiac injury markers such as cardiac troponin-I, CK-MB and LDH which are released to the blood stream consequent to myocardial damage. Nevertheless, ME was able to reduce these signs of cardiac injury, which may be interpreted as a sign that the chemotherapy drugs induced heart muscle damage was shielded by ME. Likewise, the indigenous antioxidant defence system was unsuccessful in balancing the free radical generation caused by the combination chemotherapy regimen (CP+DOX) thus the observed depletion in the antioxidant levels. This was combated by ME treatment as evident from the successful restoration of antioxidants such as GPx, GSH, Catalase and SOD. Lipid peroxidation levels were also lowered by ME treatment. This was supported by the gene expression analysis of NRF2/KEAP-1 signalling. CP+DOX treatment caused an elevation in KEAP-1 gene expression and lowering of NRF-2 gene expression. ME treatment thus restored the antioxidant levels in cardiac tissue which was otherwise downregulated by CP+DOX treatment as evident from gene expression pattern .

Gene expression of pro-inflammatory genes such as NF-KB, IL-6, IL1-β and TNF-α was found to be elevated in CP+DOX treated groups which was lowered by ME treatment. This elevated expression of pro-inflammatory markers in cardiac tissue might be the reason of observed cardiac inflammation. However, ME successfully combated all the ill effects induced by the combination chemotherapeutic regimen.

iNOS gene expression was increased in CP+DOX treatment which might be one of the reasons of depleted levels of mitochondrial TCA cycle enzymes and ETC complex in cardiac tissue mitochondria. ME significantly restored the cardiac mitochondrial enzyme activity.

The experimental findings presented in this chapter reveal the profound cardioprotective effect of ME against combination chemotherapy using CP+DOX treatment in animal model. The findings also reveal the potential therapeutic use of bioactive extract of *M. esculenta* in chemotherapy using drug combinations of cyclophosphamide and doxorubicin.

Figures & Tables

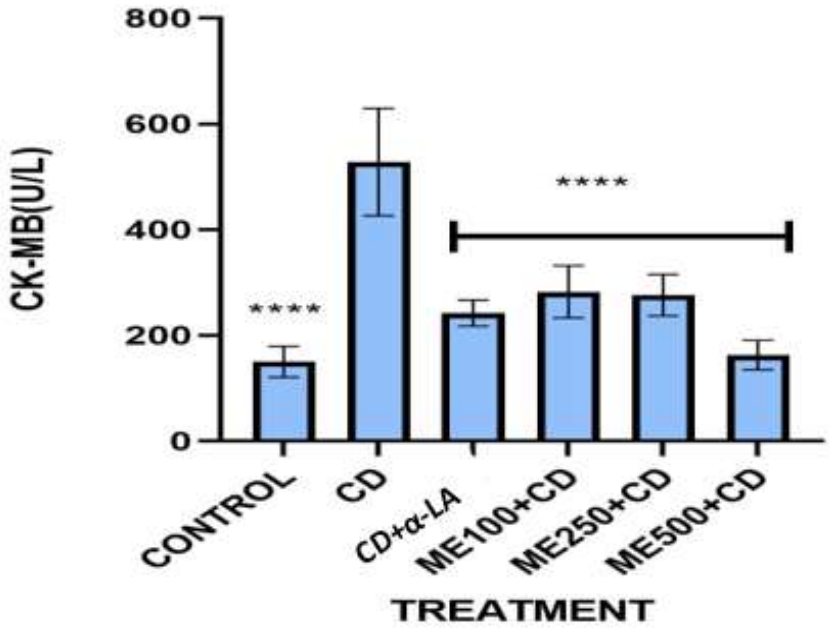


Figure.8.1. A CK-MB activity: values are the mean ± S.D; (n=6). ** $P<$ 0.01 significantly and $^{ns}P>0.05$ non significantly different from CP+DOX control group (Dunnett's test). CD: CP+DOX.

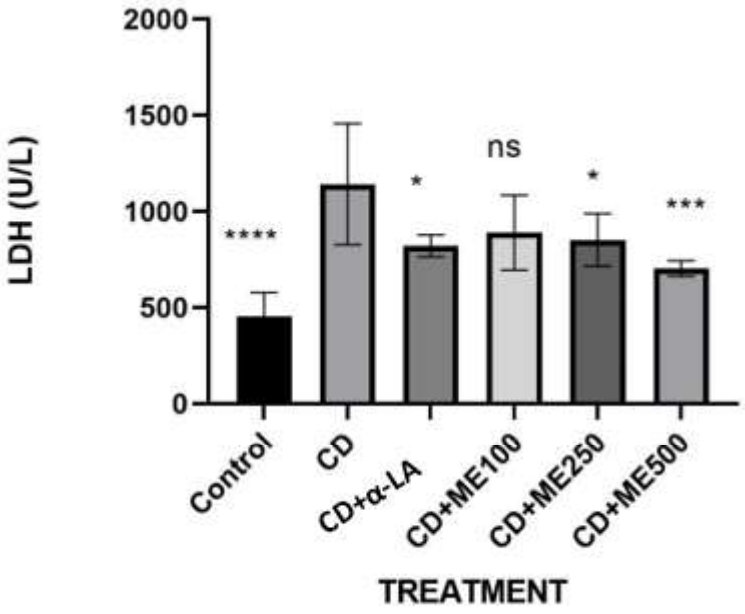


Figure.8.1. B LDH activity: values are the mean ± S.D; (n=6). ** *P*< 0.01 significantly and nsP>0.05 non significantly different from CP+DOX control group (Dunnett's test). CD: CP+DOX.

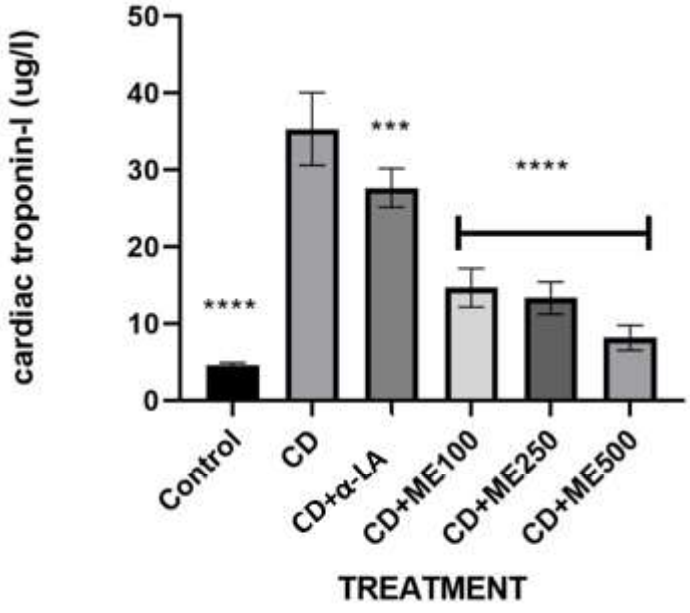


Figure.8.1. C. Cardiac Troponin-I: values are the mean ± S.D; (n=6). ** *P*< 0.01& * P<0.05 significantly different from CP+DOX control group (Dunnett's test). CD:CP+DOX.

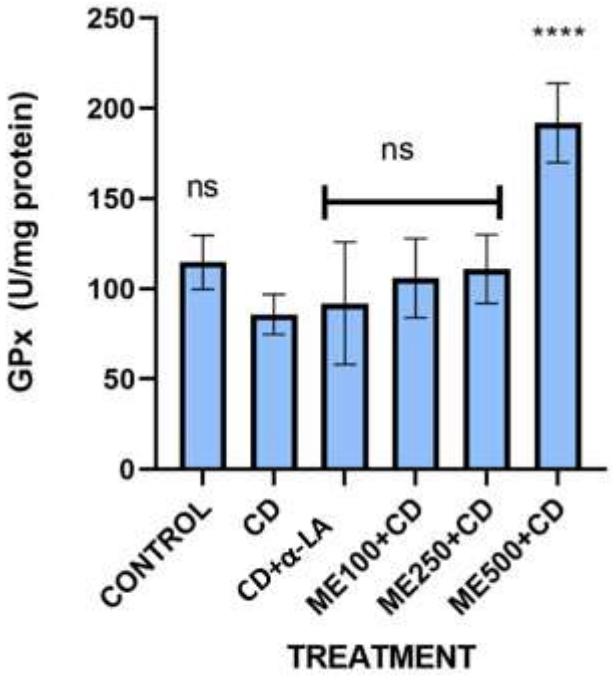


Figure.8.2. A. GPx activity. All values expressed as Mean ± SD (n=6). *P<0.05; **P<0.01; ns P>0.05 compared to CP+DOX administered animals (Dunnett's test). CD:CP+DOX.

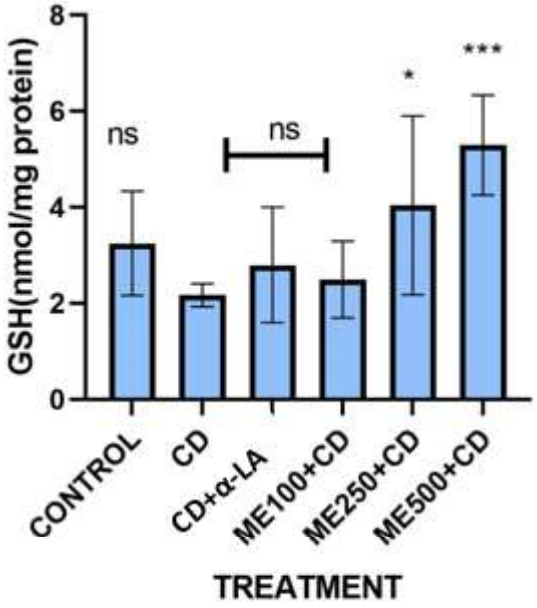


Figure.8.2. B. GSH content. All values expressed as Mean ± SD (n=6). *P<0.05; **P<0.01; ns P>0.05 compared to CP+DOX administered animals (Dunnett's test). CD: CP+DOX.

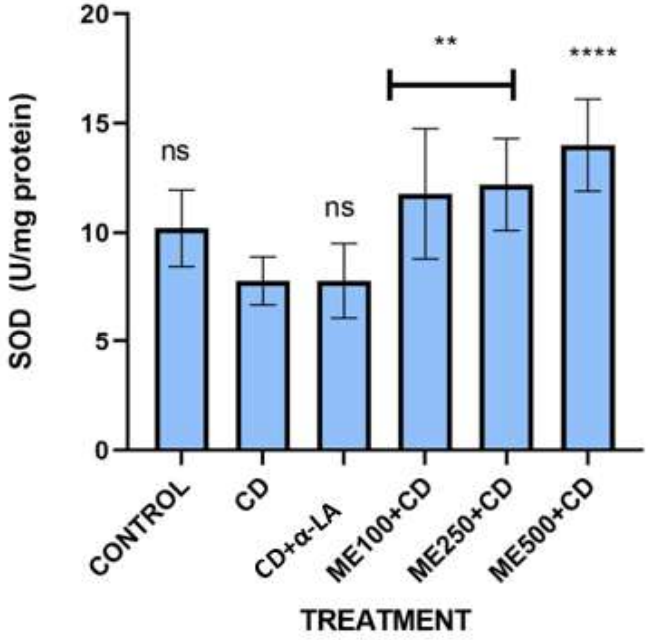


Figure.8.2. C. SOD activity. All values expressed as Mean ± SD (n=6). *P<0.05; **P<0.01; ns P>0.05 compared to CP+DOX administered animals (Dunnett's test). CD:CP+DOX.

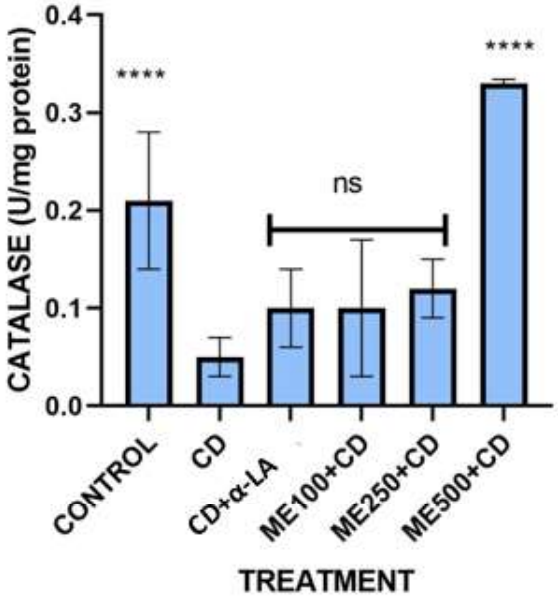


Figure.8.2. D. Catalase activity. All values expressed as Mean ± SD (n=6). *P<0.05; **P<0.01; ns P>0.05 compared to CP+DOX administered animals (Dunnett's test). CD: CP+DOX.

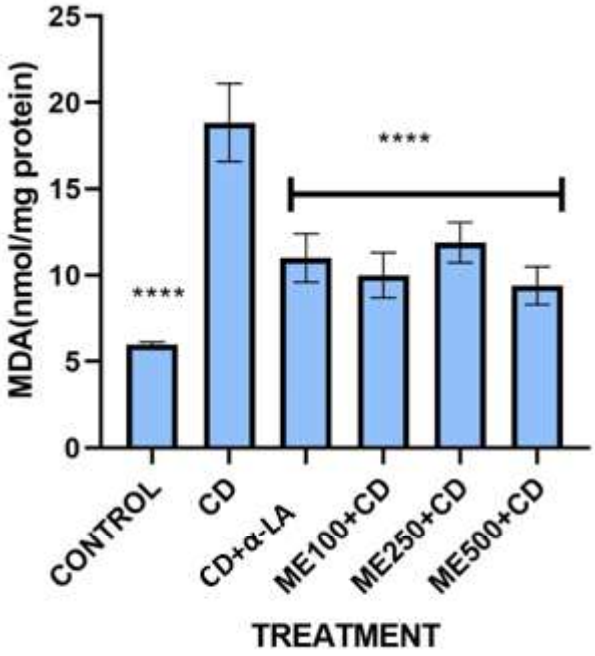


Figure.8.2. E. Lipid peroxidation. All values expressed as Mean ± SD(n=6). **P<0.01; **** P<0.0001; ns P>0.05 compared to CP+DOX administered animals (Dunnett's test). CD: CP+DOX.

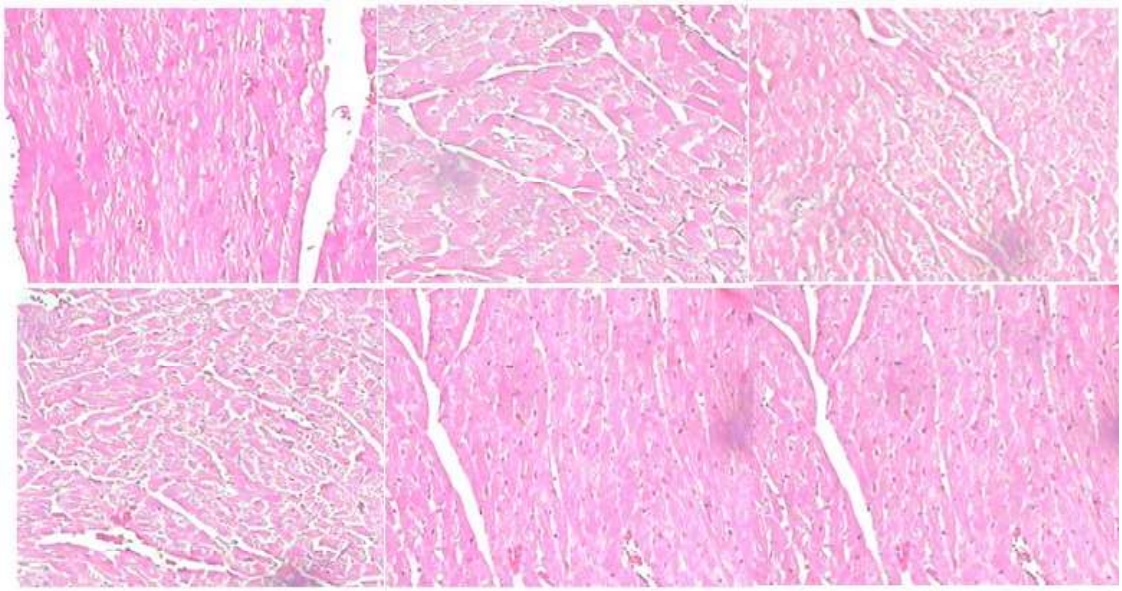


Fig.8.3. A. Histopathological image showing normal architecture of cardiac tissue obtained from untreated-animals.

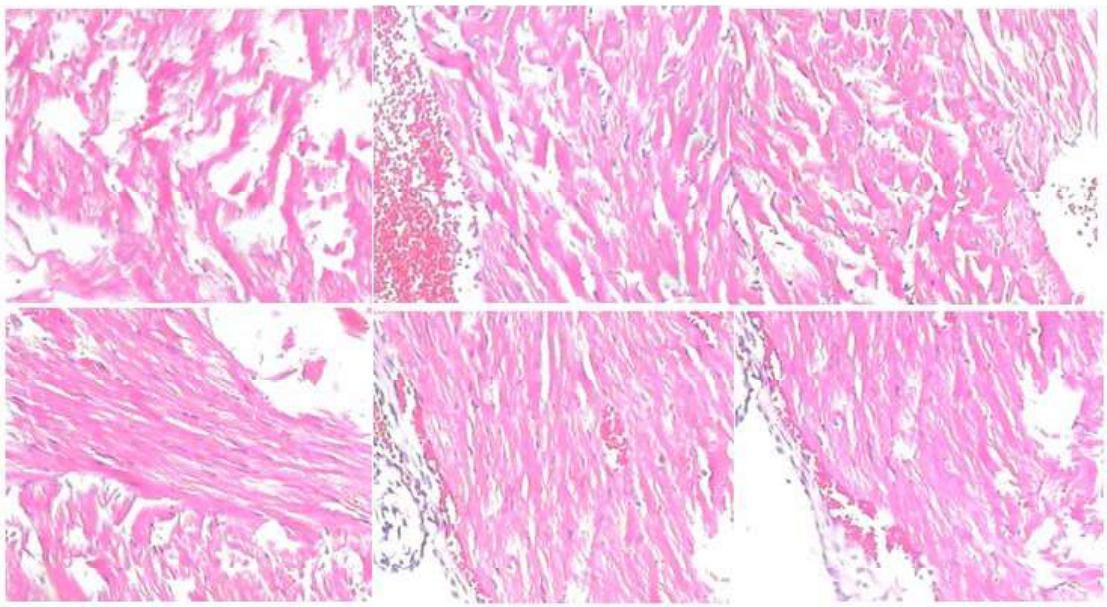


Fig.8.3. B. Histopathological image of cardiac tissue obtained from CP+DOX treated animals showing, internal haemorrhage, Cardiac hypertrophy, wavy myofibrils, nuclear infiltration, pyknotic nuclei and myoplasm degeneration.

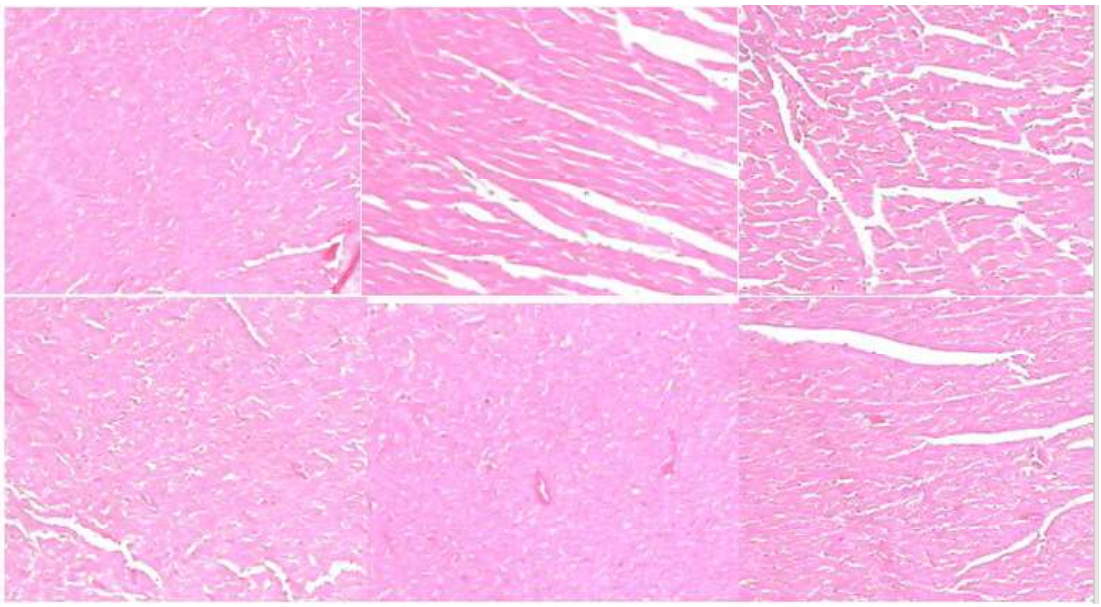


Fig.8.3. C. Cardiac tissue from animals treated with CP+DOX along with ME at a concentration of 100 mg/kg. b.w.

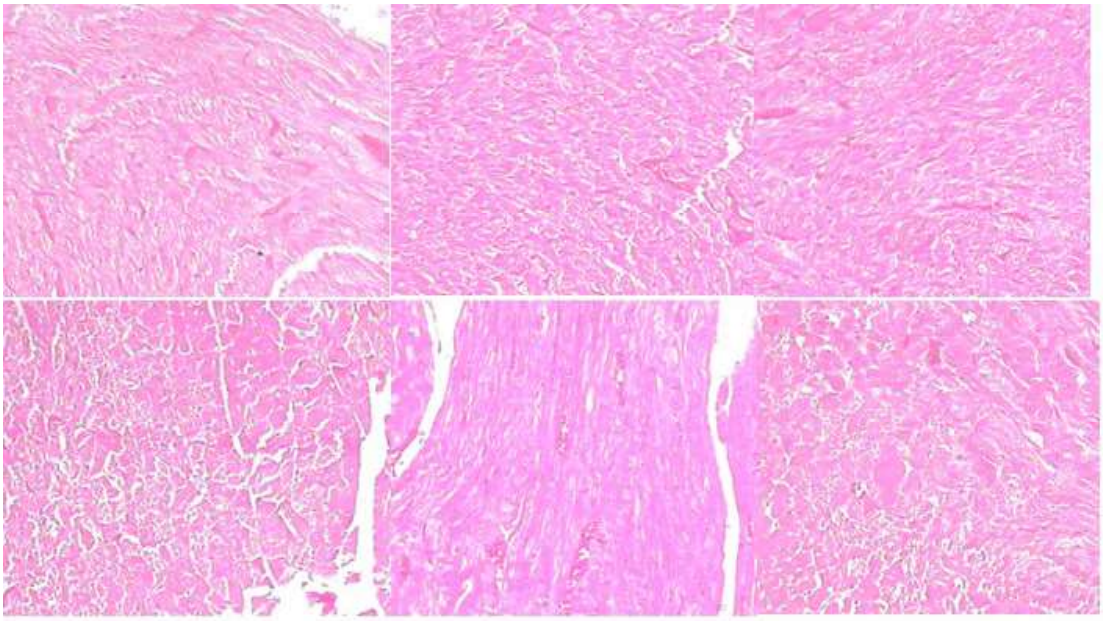


Fig.8.3. D. Cardiac tissue from animals treated with CP+DOX along with ME at a concentration of 250 mg/kg. b.w.

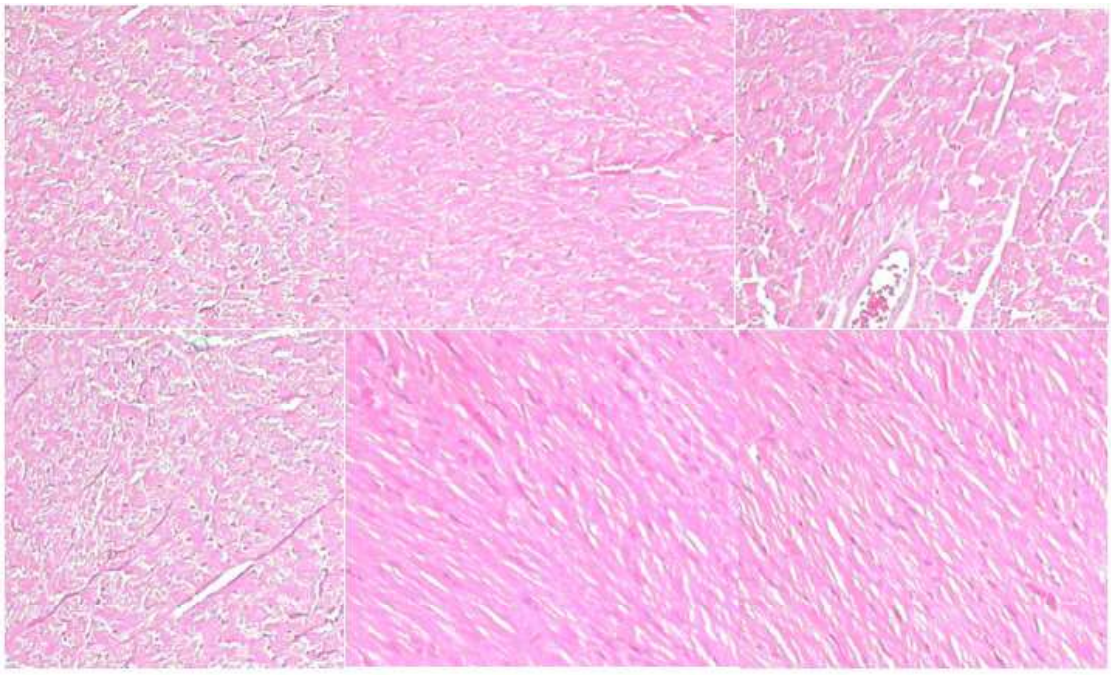


Fig.8.3. E. Cardiac tissue from animals treated with CP+DOX along with ME at a concentration of 500 mg/kg.b.w.

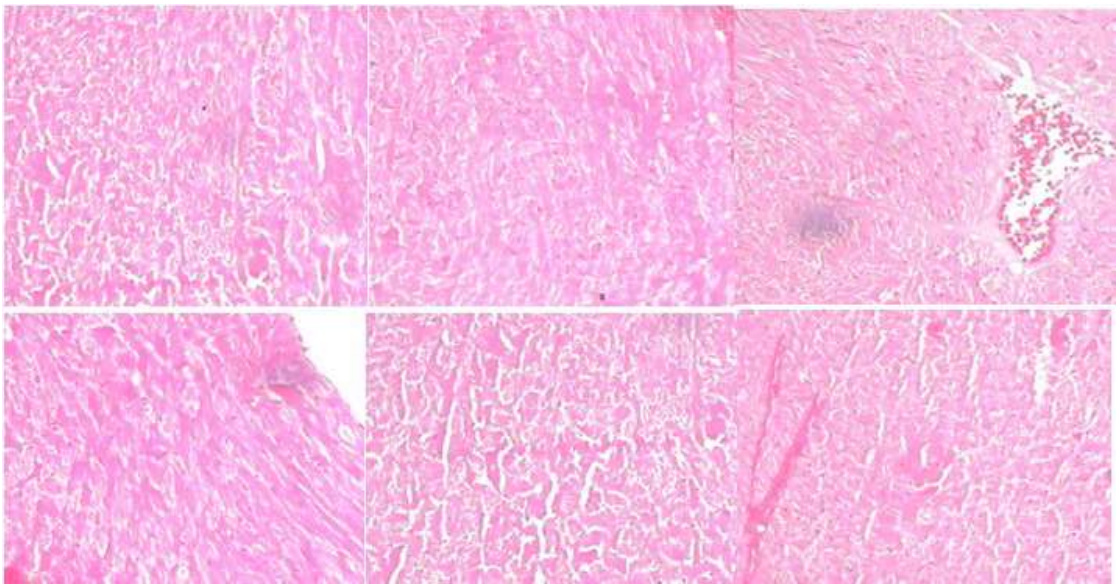


Fig.8.3. F. Cardiac tissue from animals treated with CP+DOX along with DL-α-Lipoic acid at a concentration of 100 mg/kg.b.w.

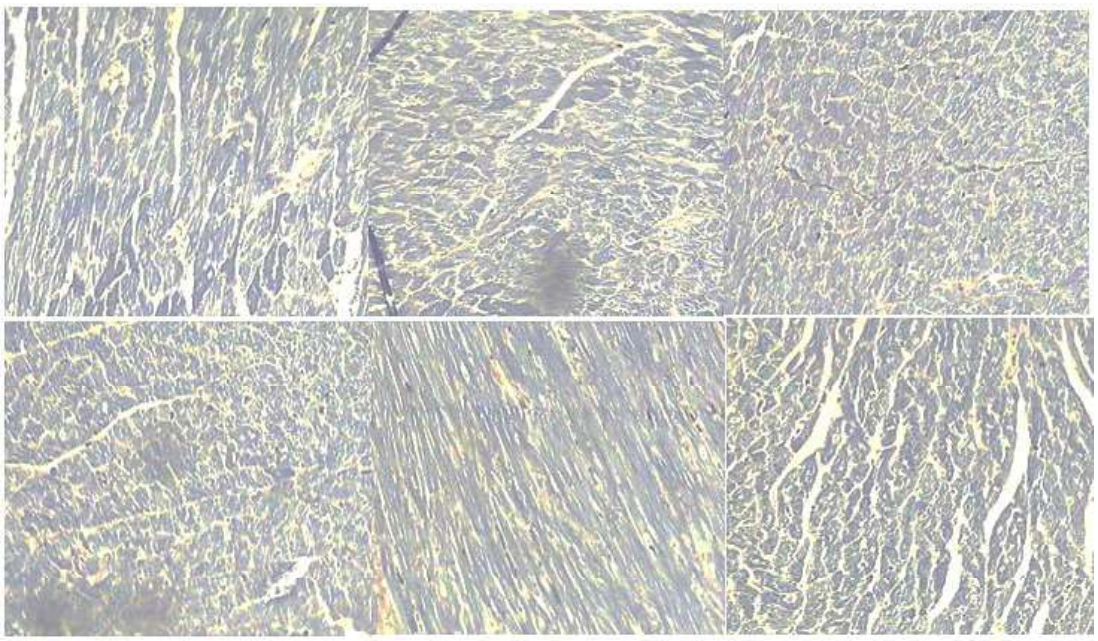


Fig.8.4.A. PTAH-stained images of cardiac tissue from untreated control animals.

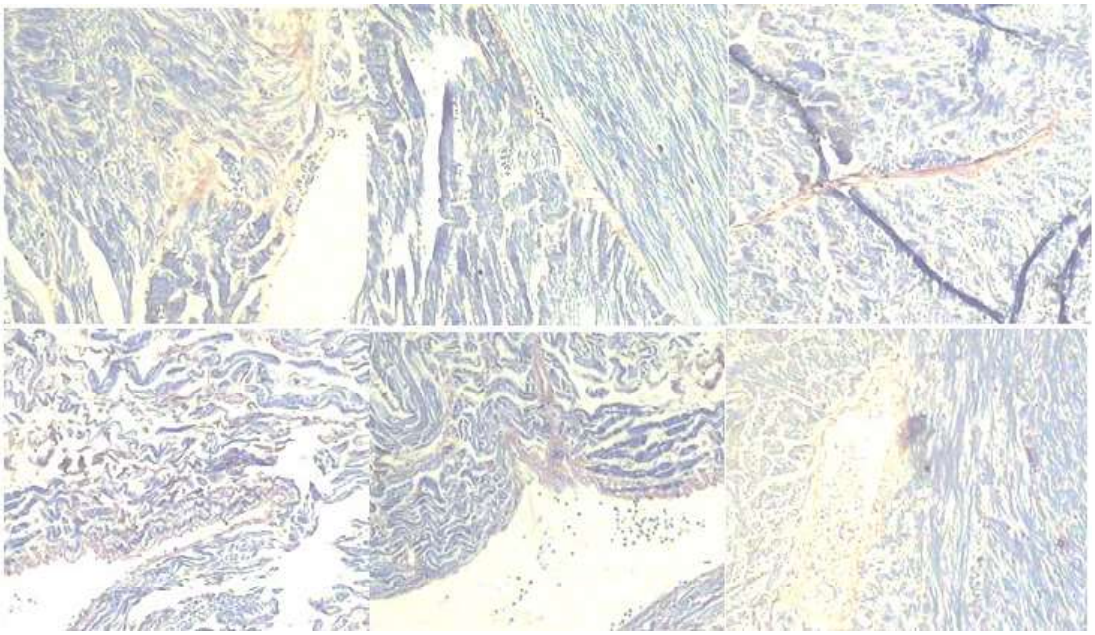


Fig.8.4.B. Cardiac tissue images from CP+DOX treated animals.

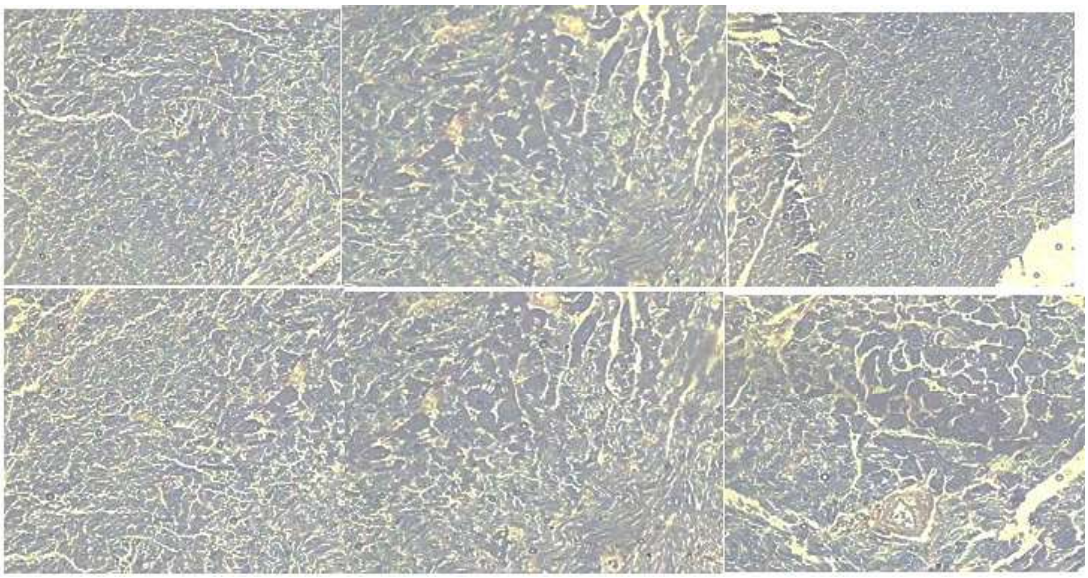


Fig.8.4.C. PTAH-stained images of ME 100mg/kg + CP+DOX treated animals

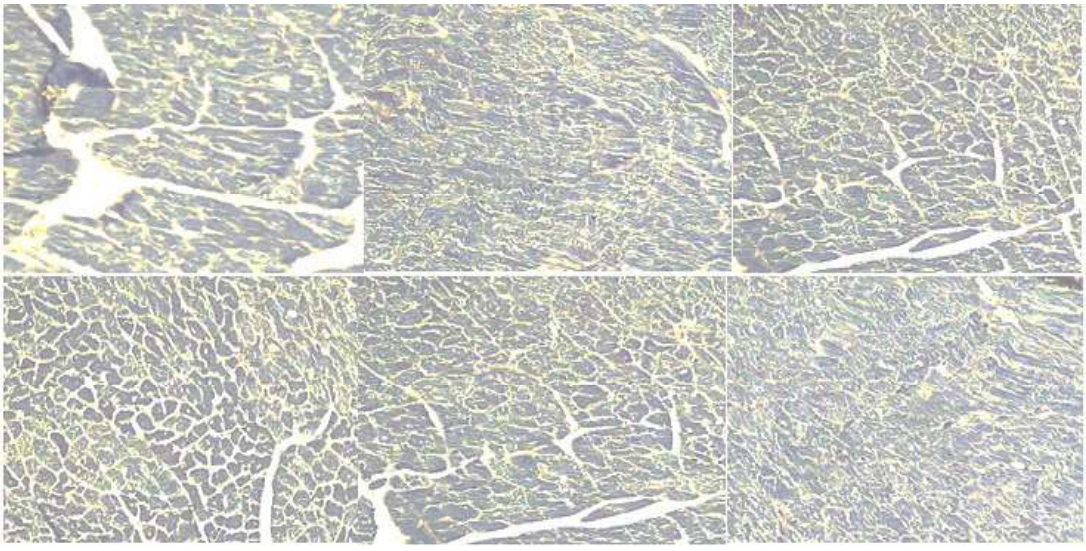


Fig.8.4.D. PTAH-stained images of cardiac tissue obtained from ME 250 mg/kg + CP+DOX treated animals

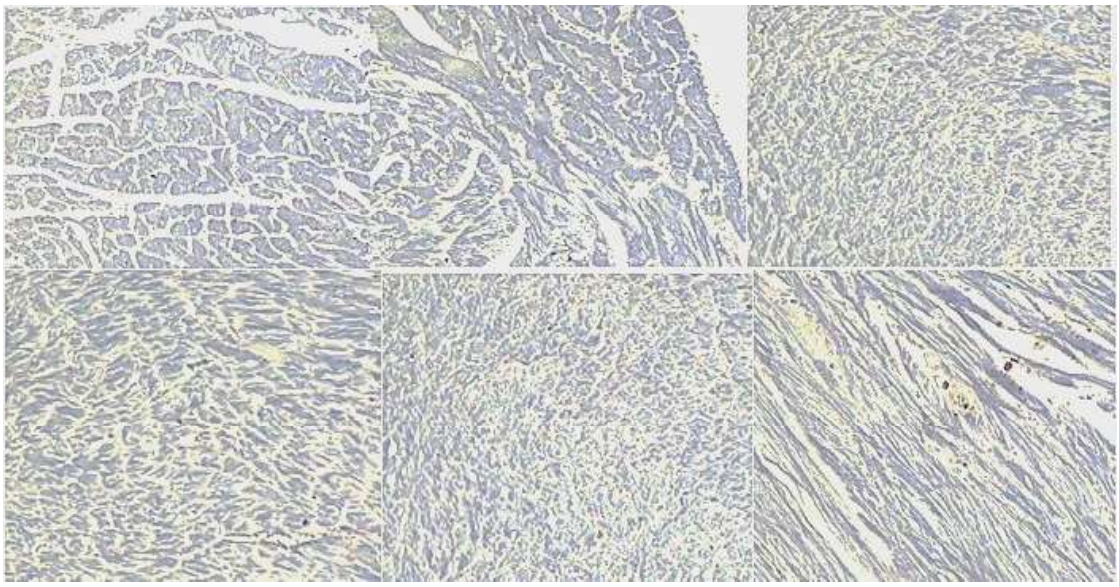


Fig.8.4.E. PTAH-stained images of cardiac tissue obtained from ME 500 mg/kg + CP+DOX treated animals.

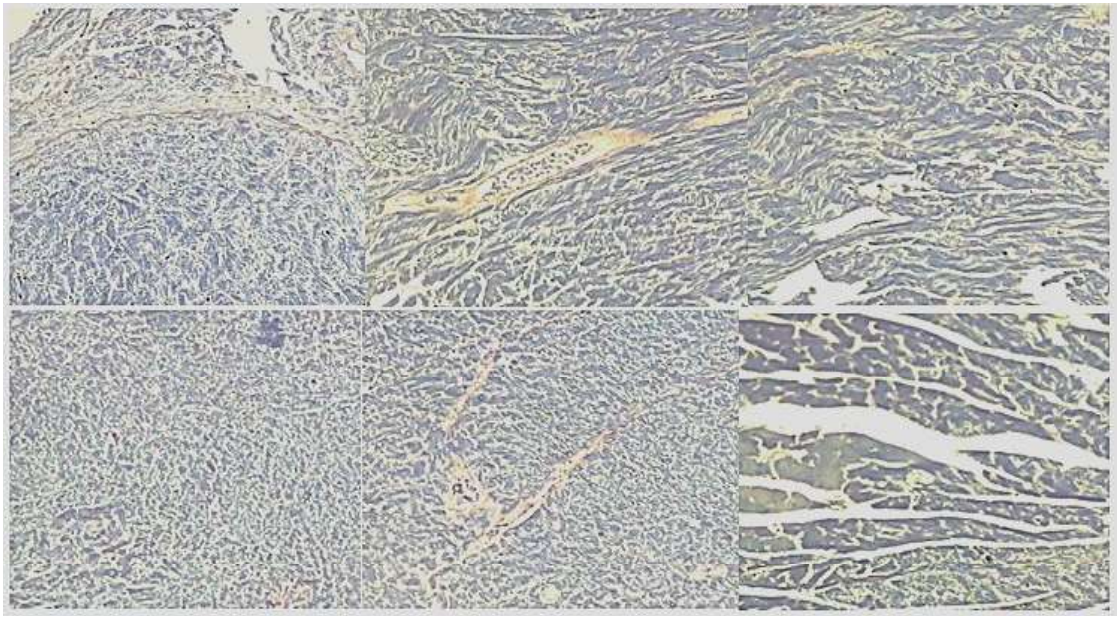


Fig.8.4.F. PTAH-stained images of cardiac tissue obtained from α-Lipoic acid 100 mg/kg + CP+DOX treated animals.

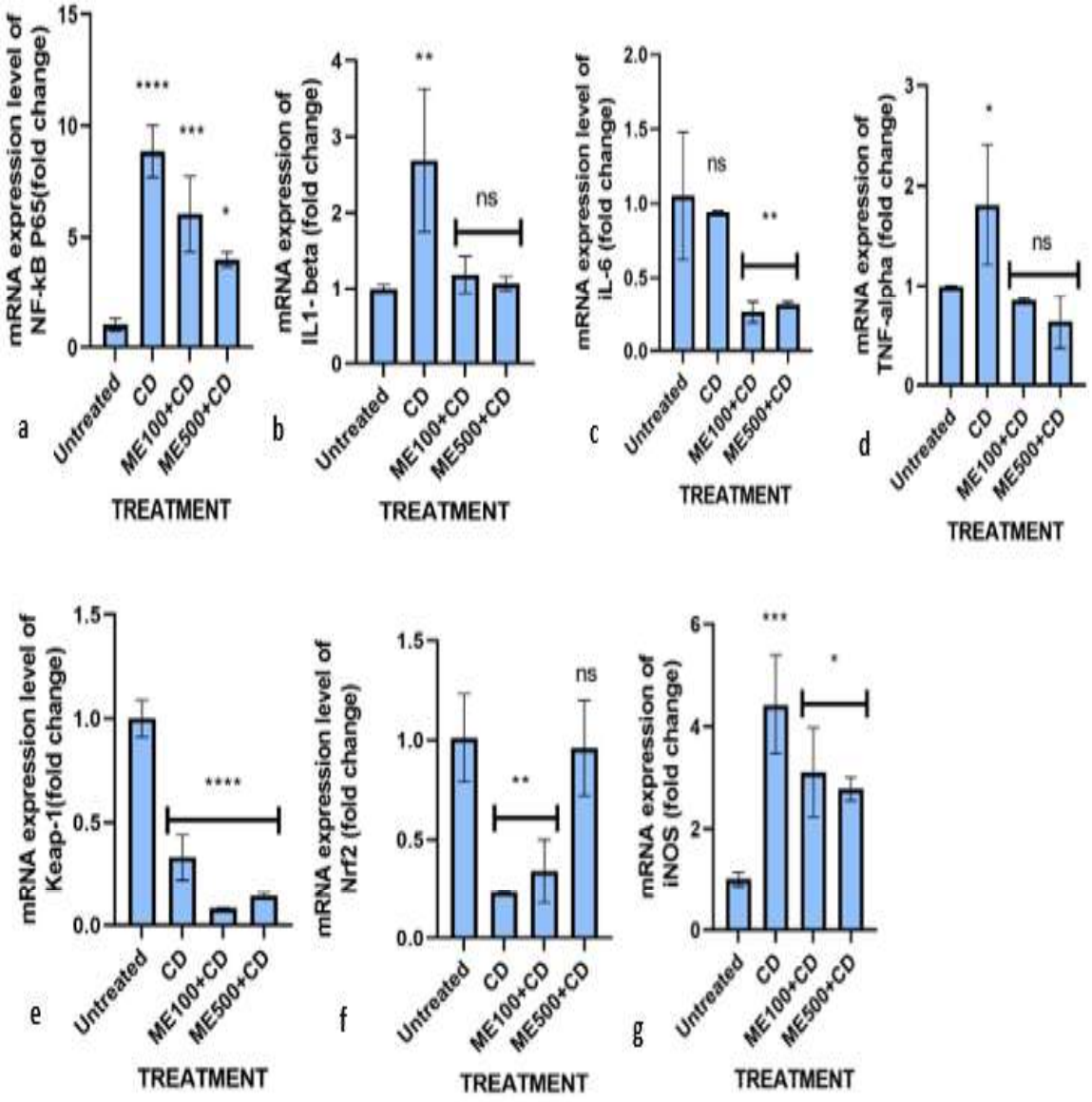


Fig.8.5.(a-g). Analysis of gene expression. All values expressed as Mean ± SD(n=6). **** P<0.0001; **P<0.01; *** P<0.001; ns P>0.05 compared to normal untreated animals (Dunnett's test). CD: CP+DOX

Tables

Table.8.1. Body weight, Heart weight and Relative heart weight of animals. All values are expressed as Mean±S.D **** P<0.0001; ns P<0.05; *** P<0.003; ** P<0.0019; * P <0.0393

F.B.W : Final Body Weight, H.W: Heart Weight, %Relative heart weight= (H.W/B.W)*100, CD: CP+DOX.

GROUP	F.B.W	Δ weight before CP+DOX treatment	Δ weight after CP+DOX treatment	H.W	% H.W/B.W
Normal	32.2±1.23	2.125±1.3	(+)3.57±0.5	0.123±0.003*	0.388±0.003****
CD	24.91±4.15	2.416±1.43	(-)3.66±1.7	0.145±0.025	0.59±0.11
ME 100 + CD	24.18±2.59	1.2±0.44	(-)3.02±0.97	0.099±0.012****	0.35±0.10****
ME 250 + CD	26.17±1.17	3.85±0.92	(-)2.17±0.12	0.112±0.004***	0.43±0.038***
ME 500 + CD	29.45±0.52	1.95±0.17	(+)3±0.6	0.116±0.001***	0.39±0.008****
α-LA + CD	25.97±0.974	2.5±0.5	(-)3.75±1.18	0.09±0.003****	0.35±0.03****

Table.8.2. TCA cycle enzyme levels in cardiac mitochondria.

Values are the mean ± SD, n = 6, **** P<0.0001,***P=0.0001, ** P=0.0098, *P<0.01and ns P >0.05 (Dunnett test) with respect to CP+DOX treated groups : Isocitrate dehydrogenase (ICDH)—µmoles of NAD^+ reduced/min/mg protein; a-ketoglutarate dehydrogenase (a-KGDH)— µmoles of NAD^+ reduced/min/mg protein; succinate dehydrogenase (SDH)— µmoles of DCIP reduced/min/mg protein; malate dehydrogenase (MDH)— µmoles of NADH oxidized/min/mg protein. CD:CP+DOX.

GROUP	MDH	AKDH	ICDH	SDH
Normal	1293.45±452.6****	48.3±17.1***	95.9±8.4****	69.5±19.3****
CD	189.8±64.9	14.6±4.2	19.5±2.5	19±5.9
ME 100 + CD	580.2±160.4*	14.9±4^{ns}	21±6.7^{ns}	47.8±2.5***
ME 250 + CD	515.7±153^{ns}	80.84±18.3****	106±28****	79.9±8.82****
ME 500 + CD	1273.9±175****	95.4±15.6****	149.4±13.8****	53.4±10.1****
α-LA + CD	260±42^{ns}	18±1.17^{ns}	108.4±20.5****	47.9±2.9***

Table.8.3. Effect of ME on Electron Transport Chain Complex.

Values are the mean ± SD, n = 6, **** P<0.0001,***P=0.0001, ** P=0.0098, *P<0.01and ns P >0.05 (Dunnett test) with respect to CP+DOX treated groups. Units: Complex I—µmoles of DCIP reduced/min/mg protein; Complex III— µmoles of ferricytochrome C reduced/min/mg protein; Complex IV—µmoles of ferro cytochrome C oxidized/min/mg protein. CD: CP+DOX.

GROUP	Complex I	Complex III	Complex IV
Normal	29.7±6.6****	10.09±3.05****	1371±195.6****
CD	9.67±2.88	1.66±0.6	64.9±20.9
ME 100 + CD	4.3±1.9ns	3.5±0.25^{ns}	273±94^{ns}
ME 250 + CD	12.2±1.6^{ns}	3.5±0.3^{ns}	373±76**
ME 500 + CD	35.07±6.8****	10.28±2.6****	1236.3±289.5****
α-LA + CD	7.3±3.3^{ns}	1.12±0.12^{ns}	346.9±48*

CHAPTER 9

EVALUATION OF ANTITUMOR EFFECT OF DOXORUBICIN, CYCLOPHOSPHAMIDE AND THEIR COMBINATION vis-à-vis CARDIO PROTECTIVE EFFECT OF METHANOL EXTRACT OF Morchella esculenta

Table of contents

9.1 Introduction

Chemotherapy induced cardiotoxicity is one of the bottlenecks in clinical settings as it causes severe complications in patients under treatment (Florescu et al., 2019). Cardiotoxicity is a major concern in chemotherapy because of the increased risk of mortality and morbidity associated with it (Adhikari et al., 2021). Doxorubicin and cyclophosphamide are front line chemotherapeutic drugs which are used either as a single candidate or in combination therapy. Both these drugs are known to cause various cardiac complications including left ventricular dysfunction, heart failure, arrythmia, myocarditis at their treatment dosages (Pondugula et al., 2022). Cardiotoxic effects may range from acute/subacute cardiotoxicity including abnormal ECG, arrythmias, LVD to chronic progressive cardiotoxicity which involves myocarditis, congestive heart failure etc (Iqubal et al., 2019). Anthracycline induced cardiotoxicity has been reported during early 1970s and various studies reported the toxic nature of doxorubicin. The greatest determining factor of anthracycline induced cardiotoxicity is the dosage of application, 550mg/m^2 cumulative dose showed highest prevalence of heart failure (Mallouppas . et al.,2021). Likewise, cyclophosphamide induced cardiotoxicity is also dose dependent, administration at a dose of 150mg/kg body weight or above can lead to irreversible cardiotoxicity. Various mechanisms were proposed on the pathophysiology of these drugs and the emphasized mechanism is the induction of oxidative stress and endothelial capillary damage which take a toll on myocardium.

Cardioprotective agents are given a paramount importance in order to improve the quality of life in cancer patients. Cardio protection strategies are categorized in to primary and secondary. Identifying the risk factors and their proper management falls under the primary protection strategy. Numerous risk factors are associated with the cardiotoxicity which comprises of smoking, age, lack of exercise, obesity, high dosage regimen of chemotherapeutic drugs, cardiovascular complications, diabetes mellitus etc. A thorough assessment of the cardiac health by reviewing the cardiac injury markers and imaging evaluation is recommended (Omland et al., 2022). Cardioprotective medical therapy for the past two decades include the use of beta-blockers, angiotensin converting enzyme inhibitors, statins, dexrazoxane and physical

exercise. (Upshaw .2020). Quest for natural products as cardioprotective agents is an area of interest in cardio-oncology.

Various studies are reported on the cardioprotective property of medicinal mushrooms such as *Ganoderma sp., Cordyceps sp.,* etc (Chugh et al., 2022). Adequate information on the cardioprotective effect of morel mushroom *Morchella sp.,* against drug induced cardiotoxicity s not available. Previous studies in our lab using H9C2-cardio myoblast cell lines found the cardioprotective effect of methanolic extract of *Morchella esculenta* against doxorubicin induced cardiotoxicity(Das et al.,2022). Current study aimed to find out cardioprotective property of methanolic extract of *Morchella esculenta* against doxorubicin, cyclophosphamide, and combination of doxorubicin and cyclophosphamide in DLA induced solid tumour bearing Swiss Albino mice model.

9.2.Materials and methods

9.2.1. Chemicals

Doxorubicin and cyclophosphamide were purchased from Amala Institute of Medical Sciences, Kerala, India. CK-MB and LDH diagnostics kits were purchased from agape diagnostics. All other chemicals used were of the highest analytical grade possible.

9.2.2. Animal Experiment

Seventy-eight male Swiss albino mice weighing approximately 25g purchased from Small Animal Breeding Station, Kerala Agricultural University, Thrissur, Kerala, India, were used for the study. They were housed in well-ventilated cages with controlled conditions of light and humidity (photoperiod: 12 h artificial light and 12 h darkness, temperature: 25°C-27°C, humidity: 65–67 %) for two weeks for acclimatization. The experiments were carried out following the guidelines of CPCSEA, Government of India and approval by Institutional Animal Ethic Committee (ACRC/IAEC/22(1)-P9).

9.2.3.Tumour Induction

DLA cells were obtained from the culture maintained *in vivo* in peritoneal cavity of Swiss albino mice in our research centre. Cells were aspirated, washed with phosphate-buffered saline (pH-7.4) to remove cell debris and dead cells. Cell viability was estimated by trypan blue assay. Solid tumour was induced by injecting 1 million DLA

cells (1×10^6 cells) subcutaneously into the right hind limbs of all animals except in the normal group of animals. Animals were then grouped as follows into 13 groups containing 6 animals in each group:

Group 1: Normal animals without tumour induction or treatment

Group 2: DLA cells injected control animals

Group 3: DLA-induced tumour treated with DOX (cumulative dose of 18 mg/kg body weight)

Group 4: DLA-induced tumour treated with CP (cumulative dose of 200 mg/kg body weight)

Group 5: DLA- induced tumour treated with DOX + CP (cumulative dose of 10mg/kg and 150 mg/kg body weight respectively)

Group 6: DLA-induced tumour treated with ME (100mg/kg body weight)

Group 7: DLA-induced tumour treated with ME (500mg/kg body weight)

Group 8: DLA-induced tumour treated with ME (100 mg/kg) + DOX (cumulative dose 18 mg/kg body weight)

Group 9: DLA-induced tumour treated with ME (100 mg/kg) + CP (cumulative dose 200 mg/kg body weight)

Group 10: DLA-induced tumour treated with ME (100 mg/kg) + DOX + CP (cumulative dose 10mg/kg and 150 mg/kg body weight respectively)

Group 11: DLA-induced tumour treated with ME (500 mg/kg) + DOX (cumulative dose 18 mg/kg body weight)

Group 12: DLA-induced tumour treated with ME (500 mg/kg) + CP (cumulative dose 200 mg/kg body weight)

Group 13: DLA-induced tumour treated with ME (500 mg/kg) + DOX+ CP (cumulative dose 10mg/kg and 150 mg/kg body weight respectively).

The day of transplantation of DLA cells was considered as day 0. Animals in all groups except group 1 and 2 were administered with ME from day 8 to18 of the experiment after tumour development. Group 3, 4 and 5 were injected with DOX (6mg/kg b.wt/animal/day), CP (66.6 mg/kg b.wt/animal/day) and combination

treatment of DOX (3.3mg/kg b.wt/animal/day) and CP (50mg/kg b.wt/animal/day) on day 8, 10 and 12 as mentioned. Group 6 and 7 were administered with ME at dosages 100 and 500 mg/kg. b.wt. respectively orally for 10 days after the tumour development. Group 8, 9 and 10 received combination treatment of ME 100mg/kg b.wt along with chemotherapy as mentioned above. Group 11,12 and 13 were treated with ME 500mg/kg b.wt along with chemotherapy as described previously. Horizontal and vertical diameters of the tumour were measured using vernier callipers on 7, 14, 21 and 28thday of the experimental duration. Tumour volume was calculated using the following formula:

Tumour volume = $\frac{4\,\pi r1^2 r2}{3}$

Where ‘r1 ’ and ‘r2 ’ represent the major and minor diameter, respectively. Animals were sacrificed on 29 th day of the experimental period. Blood was collected by cardiac puncture and serum was obtained by centrifugation and used for assay of cardiac injury marker. Heart tissue was homogenized in 40mM Tris-HCl buffer and used for biochemical analysis. A section from the left half of the heart was transferred to 10% formalin and used for histopathology observation(Das et al., 2022).

9.2.4.Assay for Cardiac Injury Markers

Cardiac injury markers are released consequent to myocardial injury and this can be used as an index for assessing cardiac damage. Cardiac injury markers, CK-MB and LDH were estimated using Agape Diagnostics kits following the methods described by the manufacture.

9.2.5.Determination of Antioxidant Status in Cardiac Tissue

Antioxidant status in the cardiac tissue was evaluated by determination of activities of catalase, GSH, SOD and lipid peroxidation following methods described previously(Das et al., 2024).

9.2.6. Histopathology Observation of Cardiac Tissue

The cardiac tissue obtained from each animal was preserved in a 10% formalin solution. Thin sections of heart tissue were cut, dehydrated and cleared using different grades of ethanol and xylene respectively in an automatic processing facility. The sections were

then embedded in paraffin wax blocks. Microtome sections of 4-5µm thickness of the tissue were obtained and sections were stained with haematoxylin - eosin (H&E) and the slides were examined for routine histopathological features(Das et al., 2022). All the slides were examined under Olympus BX41 microscope and photographed using a digital camera at a magnification of 10X.

9.2.7.Statistical Analysis

All experimental data were statistically analysed with Graph Pad prism software and expressed as mean ± SD. The analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett's test and $P<0.05$ was considered significant.

9.3.RESULTS

9.3.1.Effect of ME on relative body and heart weight upon administration of chemotherapeutic drugs

The body weights of the animals were found drastically reduced after the administration of cyclophosphamide and doxorubicin, whereas the weight loss of animals was not significant when treated with ME along with CP or DOX (**Figure.9.1).** In DLA solid tumour bearing mice without drug treatment, there was a steady increase in the body weight. The relative heart weight was high in CP, DOX and CP + DOX treated group of animals compared to normal mice whereas no significant difference in the heart to body weight ratio was observed between ME treated groups and normal animals (**Table.9.1).**

9.3.2.Effect of Cardiac injury marker levels in tumour bearing animals administered with ME and chemotherapeutic drugs

Levels of cardiac injury markers (CK-MB and LDH) in the serum were assessed in different experimental groups to evaluate the cardioprotective effect of ME on tumour bearing animals administered with chemotherapeutic drugs. Compared to normal mice LDH levels were elevated in chemotherapeutic drugs treated groups of animals. However, in animals administered with ME along with chemotherapeutic drugs the levels of cardiac injury markers were relatively low. This indicated the protective effect conferred by ME on tumour bearing animals against chemotherapy drug induced cardiotoxicity **(Figure.9.2.A&B).**

9.3.3.Effect on oxidative stress induced by chemotherapeutic drugs on tumour bearing animals treated with ME

The activities of antioxidant such as catalase, GSH and SOD were found to be reduced following administration of CP and DOX while lipid peroxidation was enhanced. These deleterious effects on antioxidant status in the myocardium caused by CP and DOX was down regulated by the treatment with ME in a dose dependent manner **(Figure.9.3.A-D).**

9.3.4.Effect on tumour growth on mice administered with chemotherapeutic drugs and ME

The tumour volume was found to be increasing up to day 14 in all the groups of animals and after 14 days a significant reduction in tumour volume was noted in drug administered groups of animals. ME alone treated groups also showed a marked reduction in the tumour volume. ME administration did not delimit antitumor effect of doxorubicin or cyclophosphamide treatment either alone or in combination. ME at high doses was successful in reducing the tumour volume significantly. DOX, CP and CP+DOX also reduced the tumour volume significantly however it was accompanied by weight loss of animals. The treatment with ME alone or in combination with CP, DOX and CP+DOX was effective in lowering the tumour burden without loss of body weight **(Figure.9.4.).**

9.3.5. Histopathology observation of cardiac tissue of tumour bearing mice treated with chemotherapeutic drugs and ME

Histopathology observation of cardiac tissue of animals revealed that severe damage was inflicted to the cardiac myocytes by CP, DOX and their combination treatments. The treatment with ME at different concentrations ameliorated the damages induced by chemotherapeutic drugs on cardiac tissue without affecting their antitumor effect **(Figure.9.5.A-L).**

9.4. Discussion

Chemotherapy induced myocardial damage is one of the hurdles in cancer treatment and it is the limiting factor determining the efficacy of the treatment (Pushparaji et al., 2021). Cyclophosphamide and doxorubicin are the major front-line drugs used either as single agents or in combination chemotherapy regimen (Rej et al., 2023). Both these

drugs are reported to induce multiple organ toxicity . Therefore, the versatility of these chemotherapeutic agents is diminished by the organ toxicity associated with the treatment regimen. Both these drugs are known to exert their ill effects by generating reactive oxygen species and thereby creating an oxidative stress environment which will eventually cause cellular damage.

Dexrazoxane is the only FDA approved cardioprotective agent that confers protection against doxorubicin induced cardiotoxicity, but recent studies have shown that it causes secondary malignancy development in cancer patients (Hoeger et al., 2020). In this context the relevance of plant-based products and natural compounds which can mitigate the toxic effect is envisaged. A number of reports are available on plant based natural products having high antioxidant capability which are capable to reduce the oxidative stress load and provide protection against chemotherapy induced cardio toxicity (Cadeddu Dessalvi et al., 2021, Ikram et al., 2021, Sahiner et al., 2022).

Current study is focussed on the cardioprotective nature of methanolic extract of *Morchella esculenta* against chemotherapy induced cardiotoxicity in DLA tumour bearing mice. Previous studies in our laboratory have demonstrated the anti-tumour, and hepatoprotective property of Morel mushrooms (Nitha et al., 2020 & Ramya et al., 2022). Cardioprotective nature of medicinal mushroom *Ganoderma lucidum* against doxorubicin induced cardiotoxicity in Swiss Albino mice model was also reported from our lab. (Veena et al.,2022). In the current study, the cardioprotective effect of *M. esculenta* against treatment of cyclophosphamide and doxorubicin, either alone or in combination on solid tumour bearing mice was observed by us.

In vivo antioxidant activities were enhanced by treatment with *M. esculenta* extract which is evident from the lowering of lipid peroxidation and enhanced activities of SOD, Catalase and GPx. The elevated levels of cardiac injury markers consequent to chemotherapy is also found to be lowered by the extract which also supports our hypothesis. Histopathological images were in concordance with the above conclusion that the normal architecture of the cardiac tissue in chemotherapy treated animals were disturbed which was restored to considerable extent by the treatment with *M. esculenta* extract.

Current experimental results show that the cardioprotective property of ME does not hinder the chemotherapeutic potential of doxorubicin or cyclophosphamide either alone or in combination as evident from the inhibition of tumour growth by these drugs even after the administration of ME. The anti-tumour potential of the chemotherapeutic agents was not compromised with the co-treatment with ME. This indicated that bioactive extract of *M. esculenta* did not delimit the antineoplastic effect chemotherapeutic drugs. The findings envisage the potential use of *M.esculenta* bioactive extract in clinical settings for prevention of chemotherapy induced cardiotoxicity.

Figures

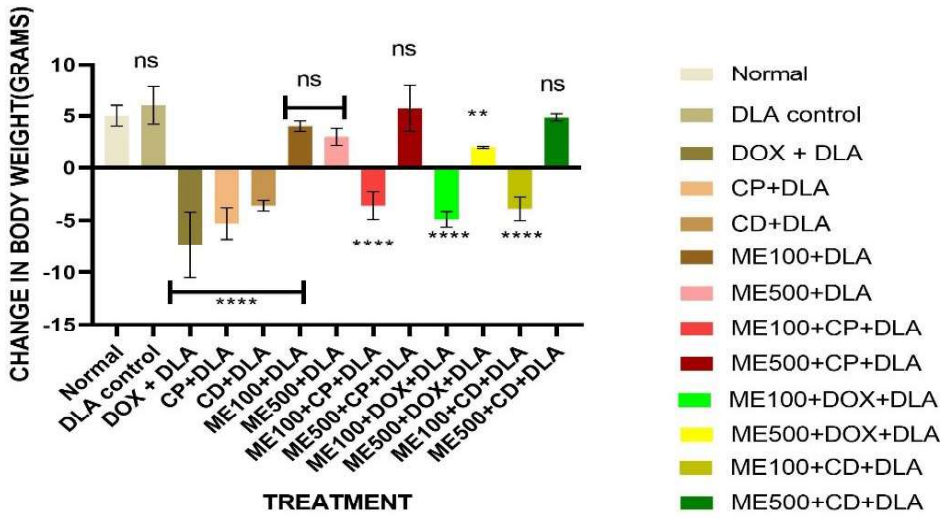


Figure.9.1. Change in body weight of animals administered with ME and chemotherapeutic drugs. Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05

DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

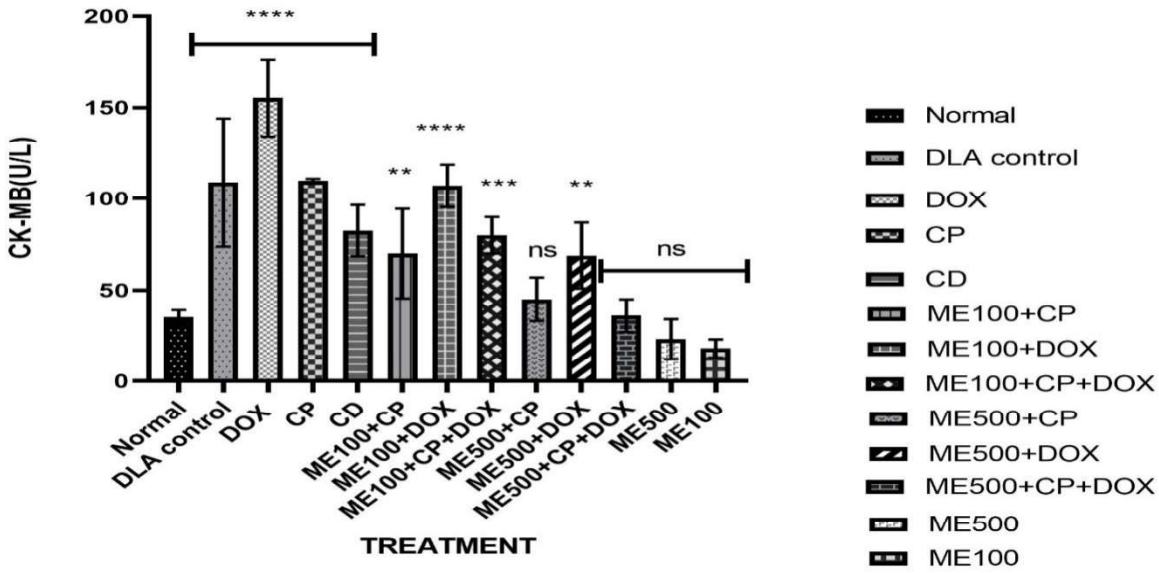


Figure.9.2.A. CK-MB levels in serum of animals administered with ME and chemotherapeutic drugs.

Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05.

DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

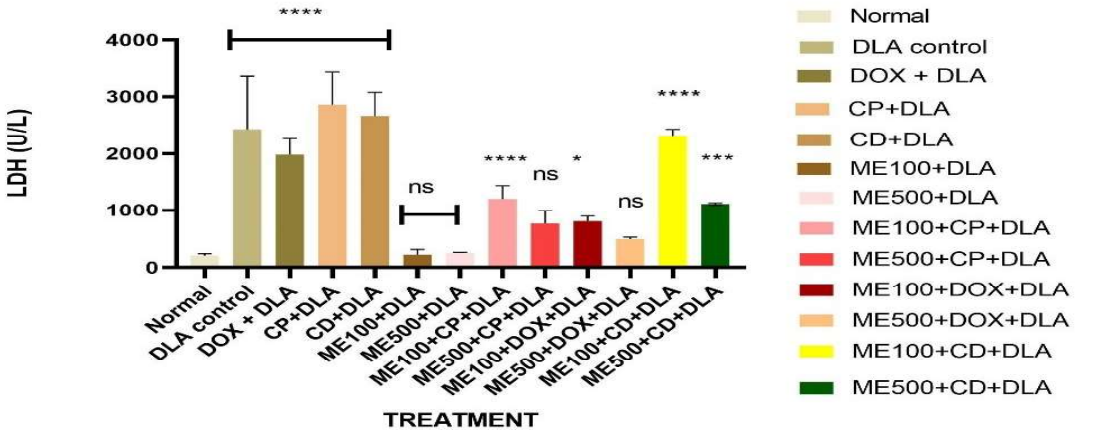


Figure.9.2.B. LDH levels in serum of animals administered with ME and chemotherapeutic drugs.

Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05.
DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

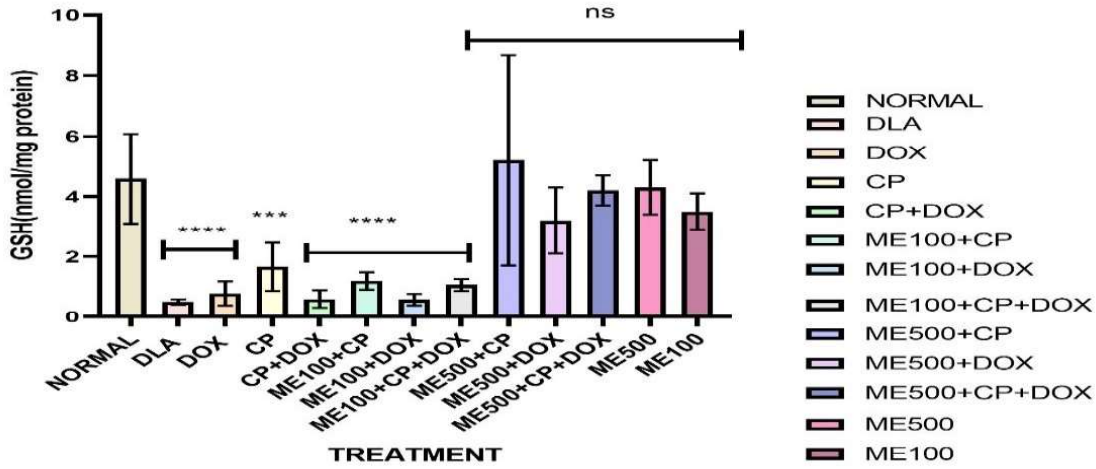


Figure.9.3.A. GSH levels in cardiac tissue homogenate of animals administered with ME and chemotherapeutic drugs.

Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05.
DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

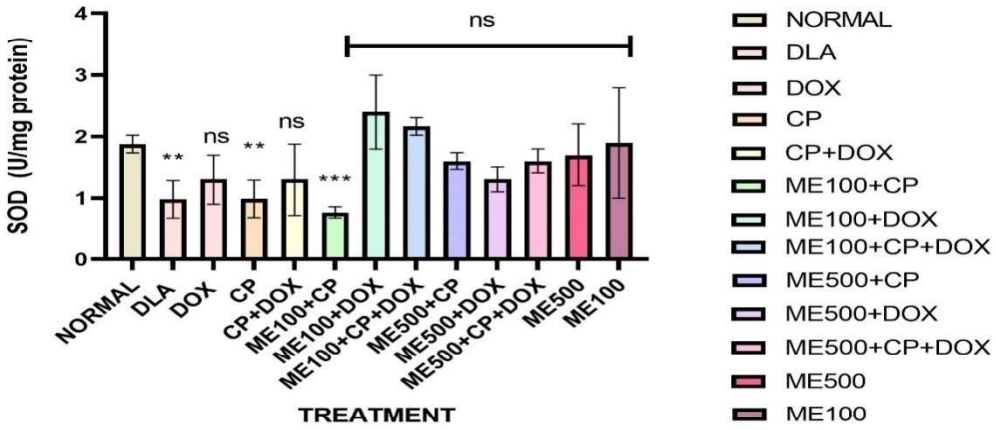


Figure.9.3.B. SOD activity in cardiac tissue homogenate of animals administered with ME and chemo drugs.

Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05.
DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

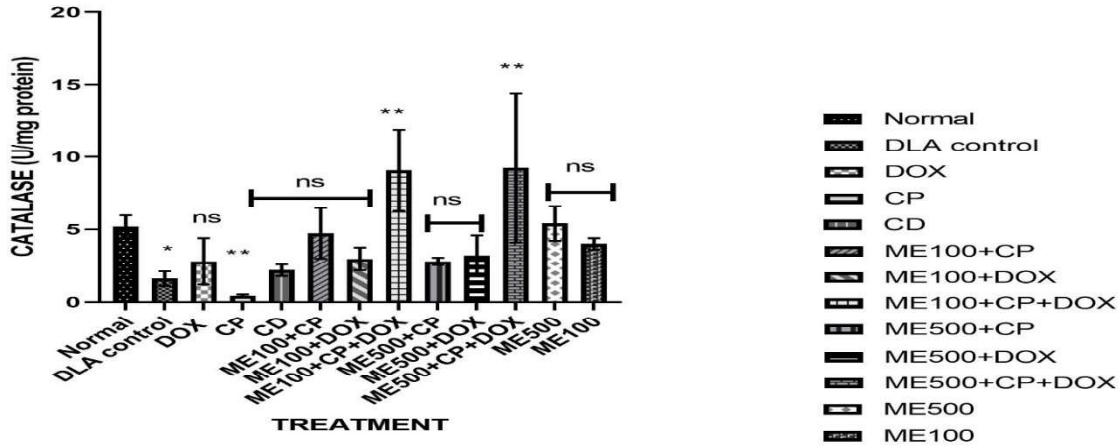


Figure.9.3.C. Catalase activity in cardiac tissue homogenate of animals administered with ME and chemotherapeutic drugs.

Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05.
DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

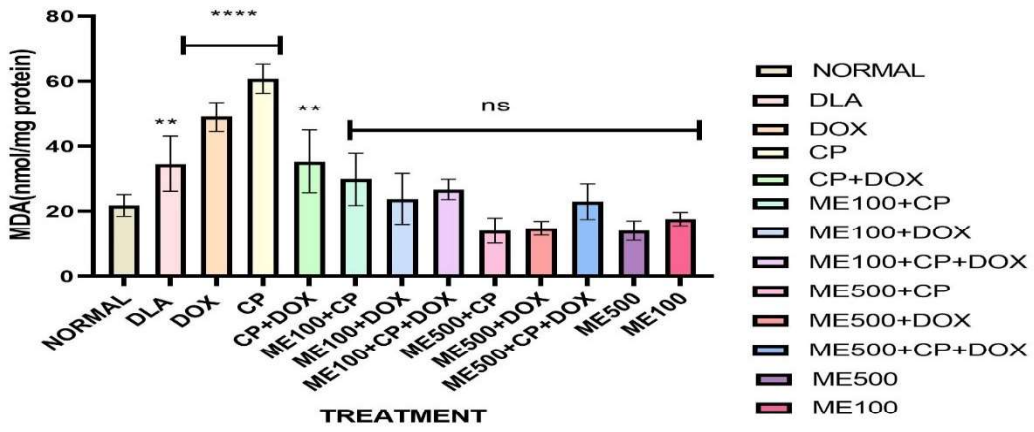


Figure.9.3.D. MDA levels in cardiac tissue homogenate of animals administered with ME and chemotherapeutic drugs.

Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05.
DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

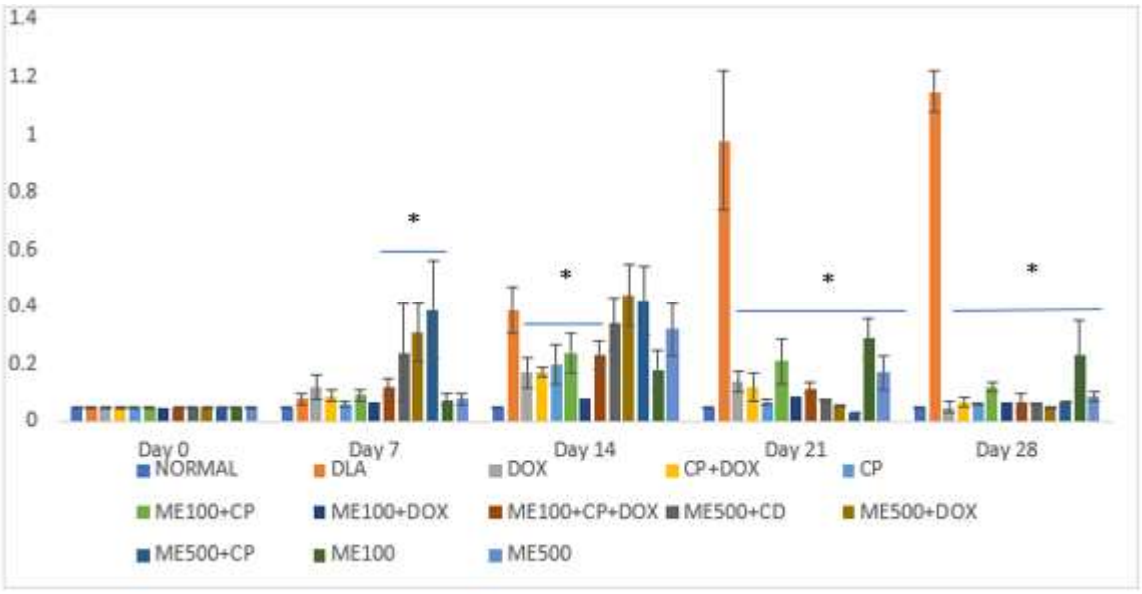


Figure.9.4. Effect of ME on tumour growth (volume) in different periods after the administration of DOX, CP and CP+DOX .

The values are expressed as ± SD of three separate experiments. *p<0.05 Significant compared to DLA control group on the respective days.
DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

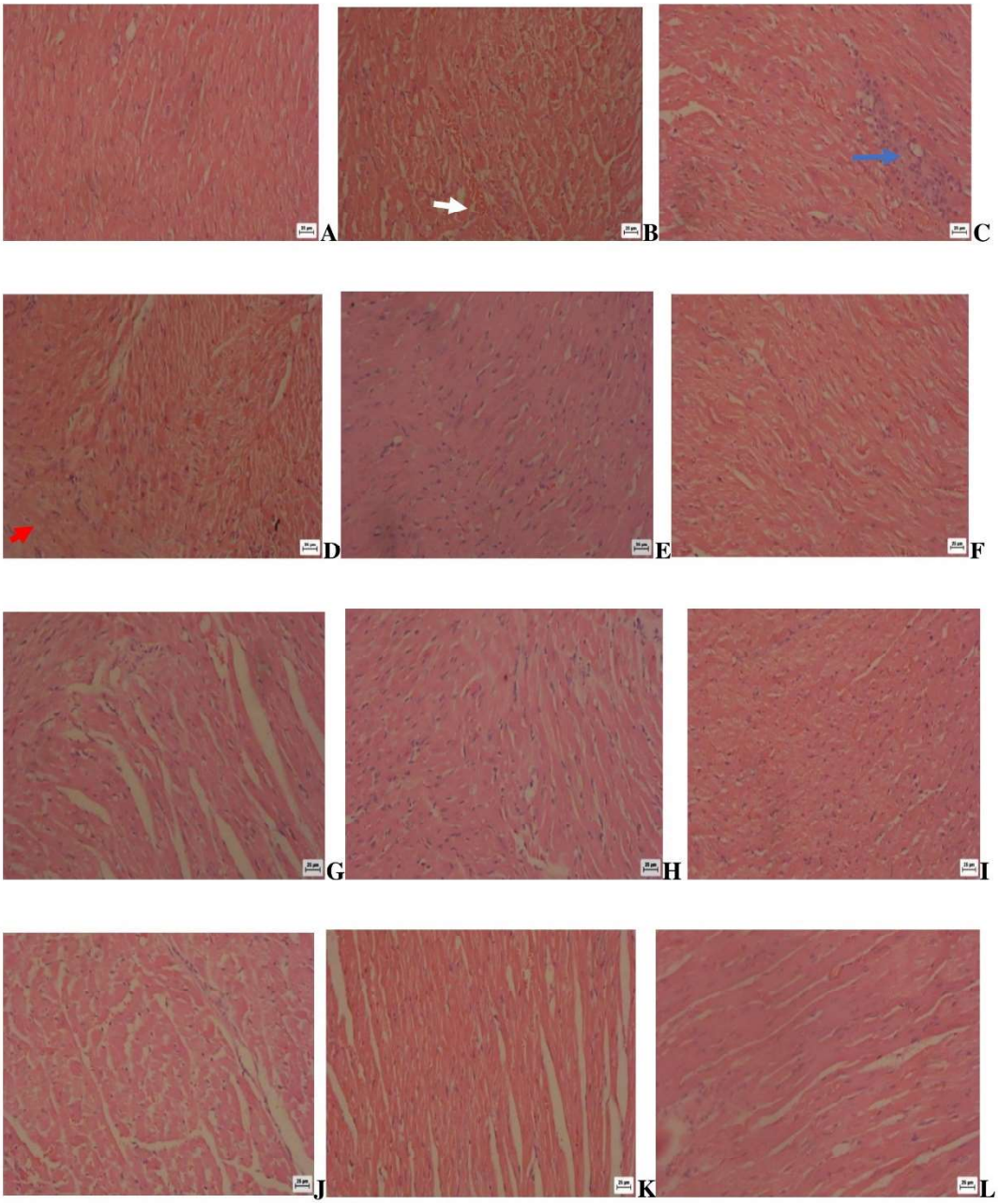


Figure.9.5. Histopathology images of cardiac tissue of animals administered with ME and chemo drugs.

A: DLA treated animal**; B, C and D:** Cardiac tissue of Dox, CP and CP+DOX treated animal respectively, marked by nuclear pyknosis and cytoplasmic degeneration(red arrow), nuclear infiltration(blue arrow) Internal haemorrhage(White arrow)**. E,F,G**: Cardiac tissue of ME 100 mg/kg b.wt along with DOX, CP and CP+DOX treated animals showing reduction in the pyknotic nuclei and internal haemorrhage. **H, I, J**: Cardiac tissue of ME 500mg/kg b.wt along with DOX, CP, CP+DOX treated animal

showing cardiac architecture similar to that of normal cardiac tissue; **K and L** represents the cardiac tissue of ME 100 and ME500 treated animals. DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

Table

Table.9.1. Final body weight, heart weight and relative heart weight. Values are expressed as Mean±S.D, n=6. **** P<0.0001; ***P=0.0001;**P<0.001;*P<0.01;ns P>0.05. DLA: Dalton lymphoma ascites; CP: Cyclophosphamide; DOX: Doxorubicin; CP+DOX: Cyclophosphamide + Doxorubicin; ME: Methanol extract of *M. esculenta*.

Group	Body weight(B.W)	Heart Weight(H.W)	H.W/B.W
NORMAL	31±1.9	0.113±0.015	0.004±.0002
DLA	38.3±5.5	0.16±0.04****	0.004±0.0008^{ns}
CP	27±5.03	0.139±0.011^{ns}	0.0052±0.0007**
DOX	24.75±0.5	0.134±0.007^{ns}	0.0054±0.0002**
CD	33±2.8	0.165±0.018****	0.0057±0.0009****
ME100+CP	23.3±2.8	0.105±0.008^{ns}	0.004±0.0002^{ns}
ME100+DOX	27.3±4.5	0.12±0.023^{ns}	0.004±0.001^{ns}
ME100+CD	24.25±4.9	0.115±0.018^{ns}	0.004±0.0007^{ns}
ME500+CP	35±1	0.111±0.009^{ns}	0.0031±0.0001^{ns}
ME500+DOX	34.3±0.57	0.13±0.009^{ns}	0.003±0.0003*
ME500+CD	28±4.5	0.143±0.016*	0.005±0.0004*
ME 500	27.25±2.6	0.116±0.003^{ns}	0.0043±0.0004^{ns}
ME100	31.3±1.2	0.119±0.003^{ns}	0.0037±0.0002^{ns}

CHAPTER 10

In Silico Studies for Evaluating Interaction of Bioactives with Keap-1

Table of contents

10.1. Introduction

Enhanced awareness of the toxicity of synthetic drugs has created a hesitation among the people to use synthetic chemical medications because of their negative effects. As a result, traditional herbal remedies are becoming increasingly popular due to their naturalness, environmental friendliness, and lack of adverse effects. Drug discovery is a critical component in the development of novel drug molecules with a variety of moiety attachments, which results in the management of numerous diseases. One of the most often utilized methods for drug discovery is molecular docking. With docking, one may discover new therapeutically relevant molecules by targeting the molecule and predicting the target–ligand interactions as well as different conformation of ligand at various positions. The prediction signifies the effectiveness of the molecule or the developed molecule having different affinity with target (Sahu et al., 2024).

Wild mushrooms have gathered exceptionally high level of interest in the fields of medicine, and food in recent years due to their exceptional nutritional and therapeutic properties. Mushrooms have been recognized as a nutritious source and have the potential to enhance health by promoting significant effects on immunity. According to several researchers, mushrooms contain compounds that possess anti-inflammatory, antioxidant, antimicrobial and anticancer properties (Janardhanan et al, 2020,Ejaz et al., 2024).

This chapter describes the molecular docking analysis of most abundant compounds present in the methanol extract of *M. esculenta* based on LC-MS analysis.

10.2. Materials and Methods.

10.2.1. Experimental Material and Preparation of Methanolic Extract

Dried fruiting bodies of *M. esculenta* were procured from Organic Kashmir, a reputed mushroom dealer New Delhi. The methanolic extract (ME) was prepared by the previously described method. Refer section 3.2.2.

10.2.2. Liquid chromatography-mass spectrometry (LC-MS) analysis of ME

LC-MS analysis of ME was carried out by the previously described method . Refer section 4.5.

10.2.3. Ligand selection.

LC-MS data was analysed for choosing the most abundant compound present in ME. Thereafter literature survey was done for further selection of compounds based on their bioactivity such as anti-oxidant, anti-inflammatory and cardio protection.

10.2.4. Drug-Likeness and Toxicity Prediction

The drug-likeness was predicted using Lipinski's rule of five, which establishes the consistency of medications that are taken orally. The Swiss ADME web tool predictor was used to screen the chosen chemicals (Solacapine, Gamma-tocopheryl quinone, Imperialine, Petroselinic acid, and Diepomuricanin A) in this investigation. The number of hydrogen donors, acceptors, and rotatable bonds is provided by the Swiss ADME predictor.

10.2.5. Molecular docking analysis

From the biochemical and gene expression analysis it was clear that ME was efficient in increasing antioxidant enzyme levels. Therefore, molecular docking was done to analyse the interaction energies of Solacapine, Gamma-tocopheryl quinone, Imperialine, Diepomuricanin A and Petroselinic acid with Keap-1 (PDB ID: 5GIT). The 3D structure of Keap-1 was downloaded from RCSB PDB (Research Collaboratory Structural Bioinformatics Protein Data Bank). Ligand structures were downloaded in .sdf format from PubChem and converted into .pdbqt file using Open babel: The Open-Source Chemistry Toolbox . Docking was performed using the software Autodock Tools Ver.1.5.6 and the optimization of receptor was completed by deleting water molecule, add kollmann charges, add polar hydrogen and arranging the grid box. The grid parameters are as follows

Number of grid points (x, y, z): 61, 69, 63

Grid size (x, y, z): 22.500000, 25.500000, 23.250000A

Grid spacing: 0.375000A. Discovery Studio 2024 client software was used to visualize the interactions and creation of images (Ismail et al., 2020;Abukhalil et al., 2021)

10.2.6. MD simulation analysis

MD simulations were done to predict how well these compounds bind to its target protein.MD simulations were performed with GROMACS using the CHARMM36 force field. Energy minimization was carried out using the steepest descent algorithm with a convergence threshold of 1000 kJ mol^{-1} nm^{-1} to resolve steric clashes. Post-Simulation analyses included root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), hydrogen bonding patterns, PCA Analysis. Binding free energies were evaluated using MMPBSA and MMGBSA methods for five key ligands: Gammatocopheryl_quinone, Solacapine, Imperialine, Petroselinic_acid, and Diepomuricanin_A. To monitor conformational and structural changes of the backbone atoms of the Keap-1 and protein-ligand (Gammatocopheryl_ quinone, Solacapine, Imperialine, Petroselinic_acid, and Diepomuricanin_A) complexes were carried out by RMSD analysis. The Root Mean Square Fluctuation (RMSF) is used for analyzing local changes along with the protein chain residues and analyzing changes in the ligand atom position at a specific temperature and pressure. FEL refers to the potential energy of a protein-ligand complex as a function of the positions and orientations of the protein and ligand atoms. FEL can be used to predict the most likely binding modes and affinities of a protein's ligand and identify potential binding sites on the protein.

10.3. Results

10.3.1. Drug-Likeness and Toxicity Predictions

SWISS ADME software was used for predicting drug likeness. Lipinski's rule of five, which determines the consistency of orally active drugs, was employed to predict the toxicity and drug-likeness of major compounds identified in the ME. Lipinski's rule of five states that a drug molecule generally does not violate more than one of the following five rules: molecular mass < 500 Da, high lipophilicity (expressed as LogP< than 5), < 5 hydrogen bond donors, < 10 hydrogen bond acceptors, and molar refractivity between 40 and 130. 5 compounds were finally chosen for molecular docking studies.

As displayed in **Table 10.1.** Gamma tocopheryl quinone, Imperialine, Petroselinic acid, Diepomuricanin A and Solacapine did not violate any rules, suggesting that they could be suitable for oral administration.

10.3.2. Molecular docking analysis.

Drug discovery frequently uses molecular docking studies to comprehend gene pathways and drug–receptor interactions. The cardioprotective effect of ME might be attributed to its antioxidant capacity which was proved by *in vitro* and *in vivo* studies. Gene expression analysis results implies that ME administration is causing an elevation in Nrf2 levels and the biochemical assays resulted in an enhanced expression of antioxidant enzymes such as SOD, GSH, GPx and Catalase. Therefore, *in silico* studies to determine the Keap1-Nrf-2 regulation was done. Keap-1 is a cysteine rich- protein which is a negative regulator of Nrf-2. The substrate adaptor function of KEAP1 is compromised by chemical alteration of its sensor cysteine(s), which results in NRF2 build up and increased transcription of NRF2-dependent genes. The upstream regulatory regions of these genes contain antioxidant response elements (AREs), where NRF2 binds as a heterodimer with a small Maf transcription factor.

In this study Gamma tocopheryl quinone, Imperialine, Petroselinic acid, Diepomuricanin A and Solacapine showed lowest binding energies -7.53, -7.95, -6.06, -5.71 and -7.01 kcal/mol respectively towards the targeted KEAP-1 protein resulted in highest binding affinities. Based on the result from Discovery Studio Visualizer 4.1 client these ligands show a bond formation with the cys-151 residue which is the most active cysteine residue of KEAP-1. The results are shown in **Figure.10.1.**

10.3.3. MD simulation analysis.

10.3.3.1. Binding Free Energy Analysis

Binding free energies were evaluated using MMPBSA and MMGBSA methods for five key ligands: Gammatocopheryl_quinone, Solacapine, Imperialine, Petroselinic_acid, and Diepomuricanin_A. Among these, Gammatocopheryl_quinone exhibited the strongest binding stability, driven primarily by van der Waals forces and electrostatic interactions. Poisson-Boltzmann (PB) calculations revealed significant polar solvation energy effects, indicating differences in solvent accessibility compared to Generalized Born (GB) results. Solacapine demonstrated moderate binding stability, with

hydrophobic residues contributing predominantly, while PB calculations indicated higher solvation penalties. Imperialine displayed balanced contributions from polar and nonpolar interactions, supported by hydrogen bonding networks. Petroselinic_acid exhibited stability driven largely by van der Waals interactions, with moderate electrostatic contributions. Finally, Diepomuricanin_A showed moderate binding stability with distinct contributions from aromatic residues, suggesting mixed electrostatic and van der Waals forces. The results are given in Table **10.1 & 10.2.**

10.3.3.2. Dynamic Behaviour

RMSD profiles indicated structural equilibration within 5–10 ns, with sustained stability throughout the simulation. Solacapine exhibited slightly elevated deviations, reflecting flexible binding **(Figure.10.2. A).** RMSF analysis identified loop regions with higher flexibility, highlighting potential roles in dynamic ligand recognition **(Figure.10.2.B).** PCA analysis showed compact clustering for stable ligands like Gammatocopheryl_quinone, while Solacapine exhibited broader dispersion, consistent with higher RMSD values **(Figure.10.3).** DCCM analysis results are shown in **Figure. 10.4.** Free energy landscape (FEL) analysis revealed deeper wells for Gamma tocopheryl_quinone, reflecting rigid binding and reduced conformational entropy, while Solacapine and Diepomuricanin_A occupied shallower wells, suggesting flexible dynamics and multiple binding modes. Results are represented in **Figure. 10.5.**

10.4. Discussion

Molecular docking of the ligands Solocapine, Gamma-tocopheryl quinone, Petroselinic acid and Imperialine with KEAP-1 BTB domain were studied and summarized in this chapter. KEAP1 is a cysteine rich protein which is the negative regulator of NRF2. At homeostatic conditions KEAP1 mediates the ubiquitination and proteasomal degradation of NRF2 (Dinkova-Kostova et al., 2017).

Keap1 belongs to the BTB-Kelch protein family. Although it still goes by the gene name KEAP1, it is also referred to as KLHL19 in accordance with the protein family's naming scheme. KLHL and KBTB proteins are the two subtypes of BTB-Kelch proteins. Typically, KLHL proteins have a C-terminal Kelch domain composed of 5e6 Kelch motif repeats, a BACK domain, and an N-terminal Broad complex, Tramtrack,

and Bric a brac (BTB) domain. The N-terminal BTB domain and the C-terminal Kelch domain, which is composed of two to four Kelch repetitions, make up KBTB proteins. Sometimes they have a BACK domain as well (Dinkova-Kostova 2017).

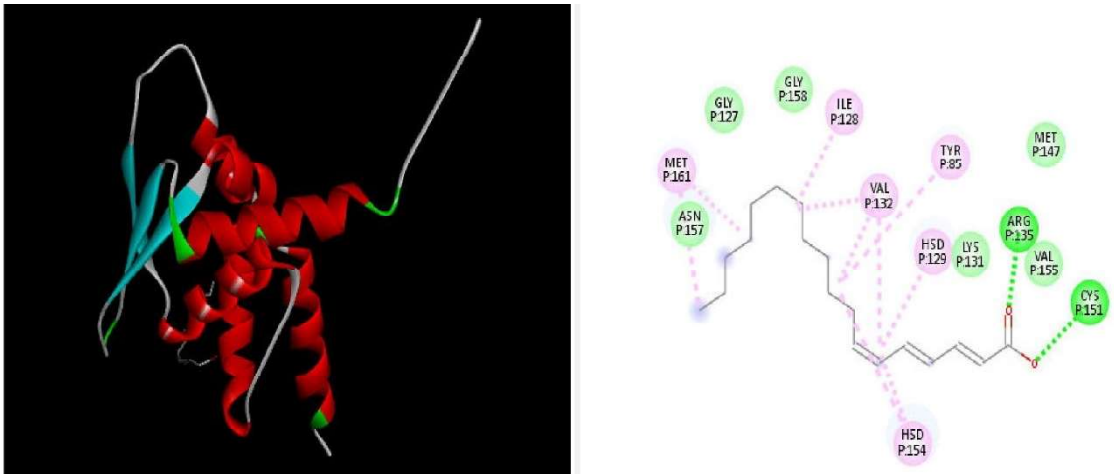
The complex's dimeric structure is essential to its operation. One molecule of the substrate, Nrf2, is engaged by two molecules of Keap1. The ETGE and DLG motifs are the two motifs in the Nrf2 protein that each of the Kelch domains interacts . By binding to Keap1, the central helix is positioned in the center of the complex, ready for the attachment of ubiquitins from the activated E2 enzymes on each side. These two motifs are located at either side of a core lysine-rich a-helix. It is believed that a suggested "hinge-and-latch" mechanism connects Nrf2 to Keap1. A number of extremely reactive Cys residues that function as cellular redox status sensors are found in KEAP1, including Cys151 at the BTB domain, Cys226, Cys273, and Cys288 at the IVR domain, and Cys613 at the Kelch domain.

Numerous substances classified as NRF2 inducers are undergoing preclinical and clinical investigation due to NRF2 pathway's significant influence on numerous physiological processes and its potential as a therapeutic target in various disorders. According to previously established processes, the majority of these are electrophilic compounds that can combine with KEAP1-Cys residues to trigger NRF2 nuclear accumulation. One well-known example is DMF, which is authorized to treat relapsing-remitting multiple sclerosis. Monomethyl fumarate (MMF), a metabolite generated from DMF, easily interacts with Cys151 at the KEAP1 BTB domain to activate the NRF2 pathway.
Sulforaphane and cyanoenone triterpenoids, which are also undergoing clinical testing, are significant instances of electrophilic NRF2 inducers. Remarkably, cyclic cyanoenes have demonstrated a reversible covalent bond with KEAP1, suggesting a marginally distinct mode of action with possible benefits such the lack of long-term target modification (Crisman et al.,2023).

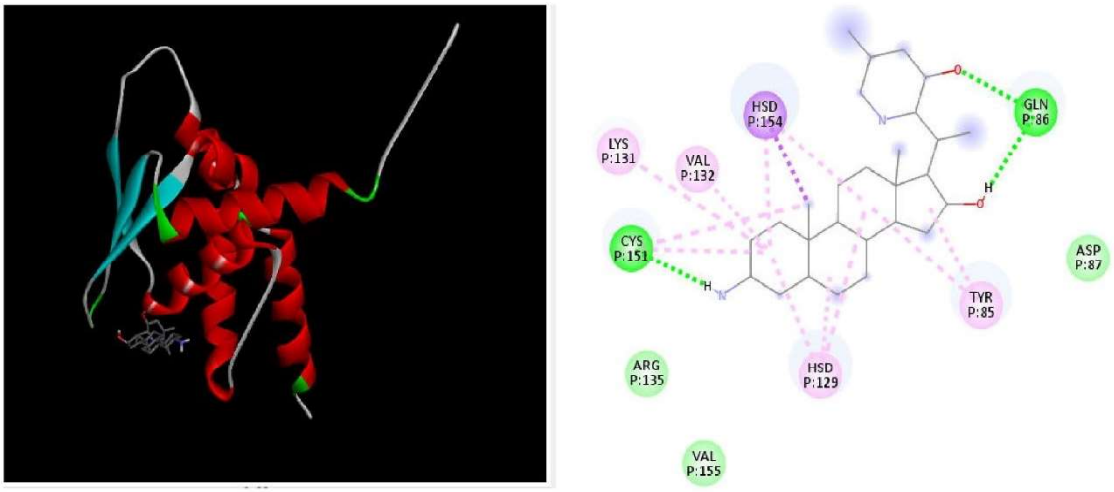
These results underscore the importance of ligand-receptor interactions in drug design. Gammatocopheryl_quinone emerged as a highly stable candidate for applications requiring strong receptor interactions. Solacapaine displayed greater flexibility, which may suit adaptive binding scenarios. Imperialine, Petroselinic_acid, and Diepomuricanin_A demonstrated balanced and moderate interactions, respectively,

highlighting their potential for diverse pharmacological applications. The complementary PB and GB analyses emphasized the solvent-dependent nature of binding affinities, providing valuable insights for future optimization efforts. The ligands Solocapine, Imperialine, Petroselinic Acid, and Gamma-tocopheryl Quinone have found good binding affinities with CYS-151 in this investigation. Further research inputs on ME and its chemical constituents would open up their unique cardioprotective characteristics beneficial for clinical application and innovation in cardio oncology.

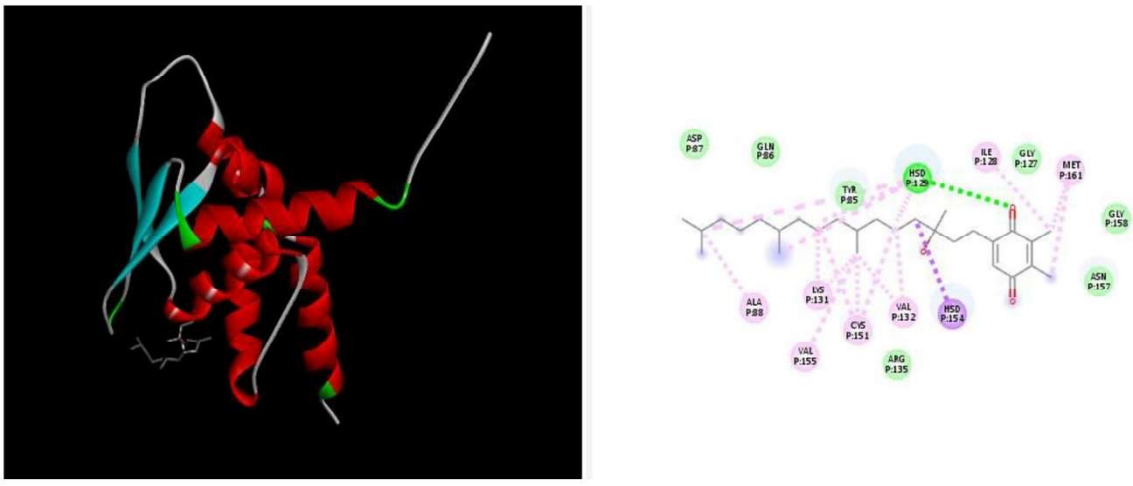
Figures



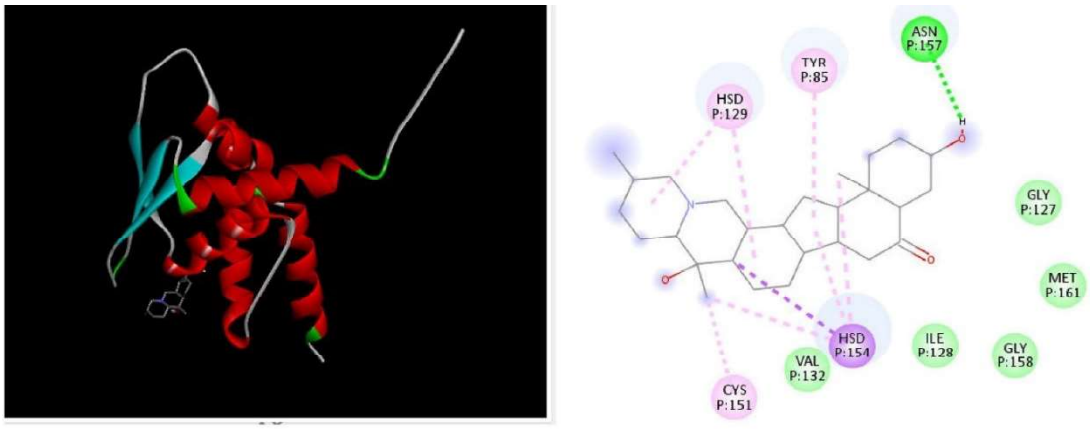
10.1.A. Molecular interaction between Keap-1 and Petroselinic acid.



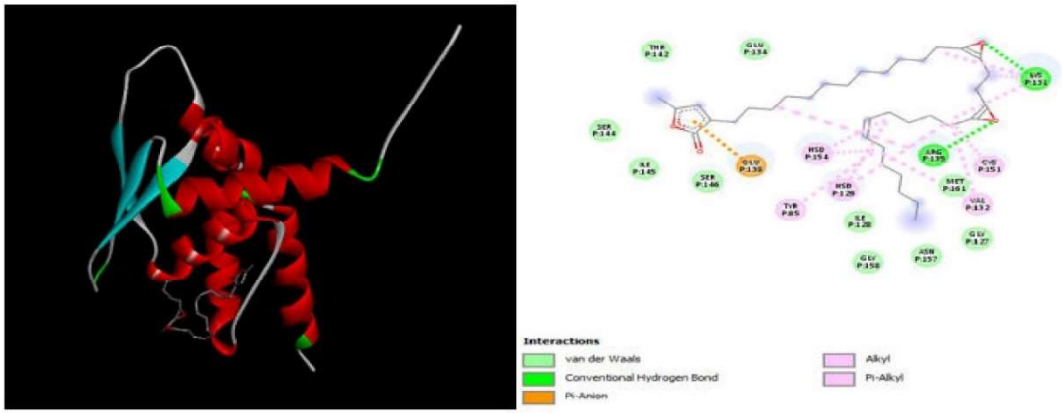
10.1.B. Molecular interaction between Keap-1 and Solacapine.



10.1.C. Molecular interaction between Keap-1 and gamma tocopheryl quinone.



10.1.D. Molecular interaction between Keap-1 and Imperialine.



10.1.E. Molecular interaction between Keap-1 and Diepomuricanin A.

Figure.10.1. Molecular docking and interaction pattern analysis of the compounds with KEAP1.

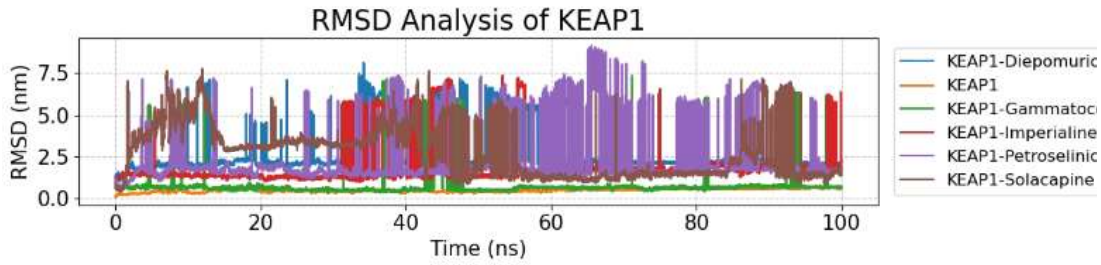


Figure.10.2. A. RMSD analysis of KEAP-1

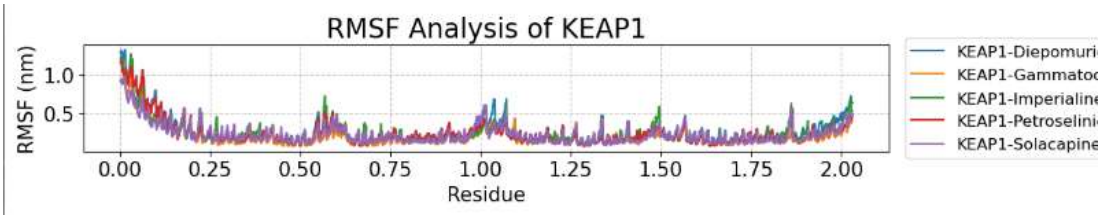


Figure.10.2.B. RMSF analysis of KEAP-1

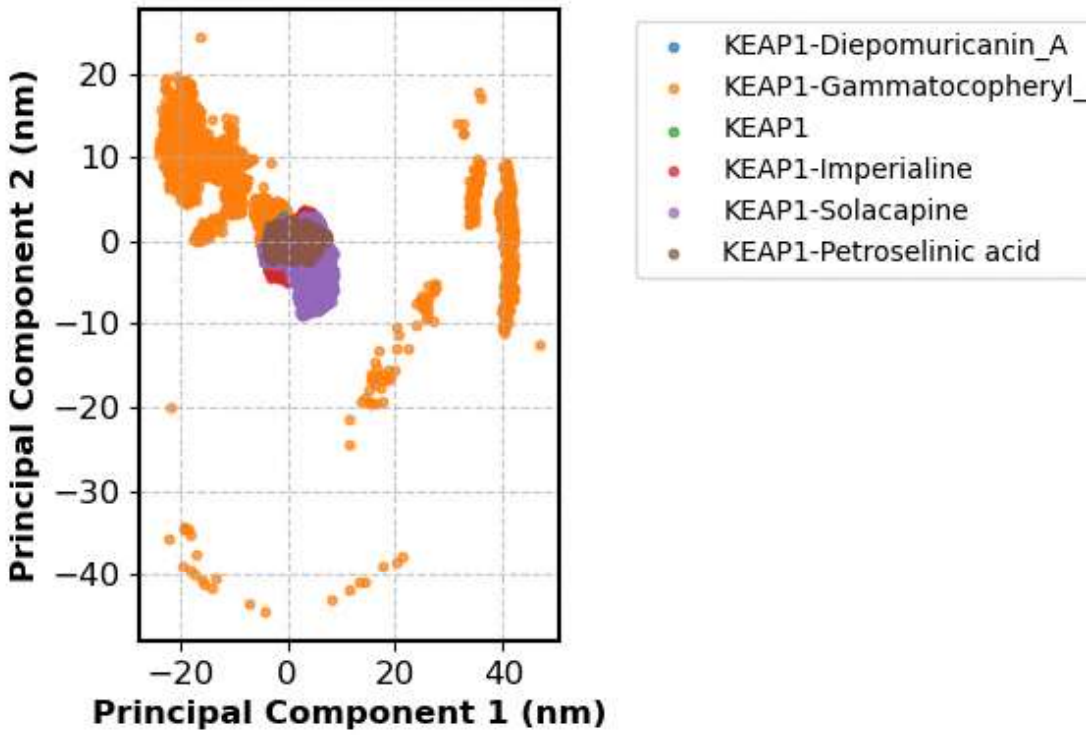
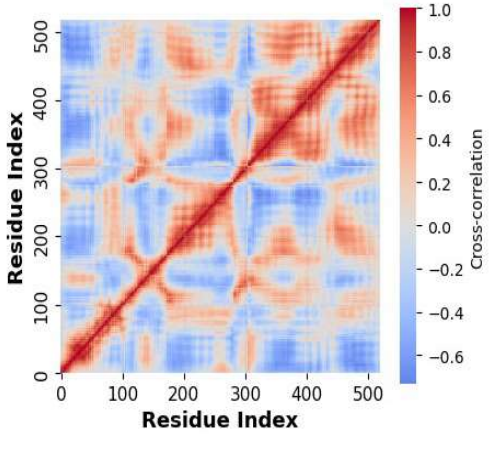
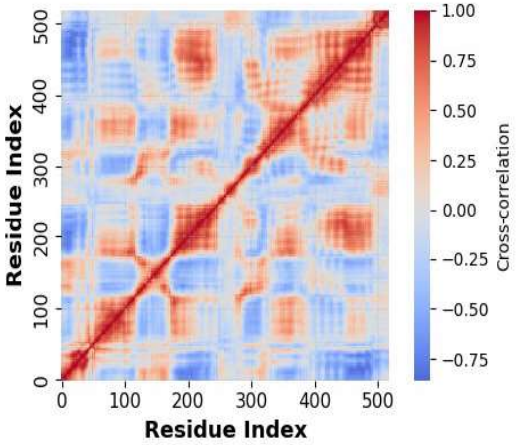


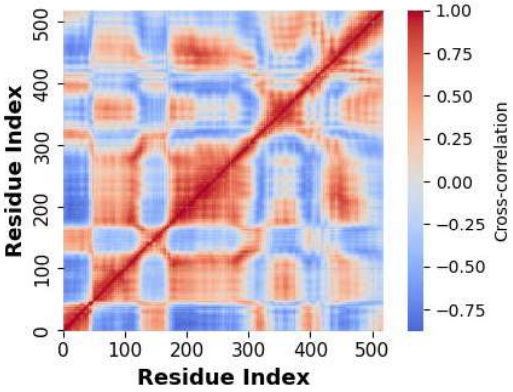
Figure.10.3. PCA analysis.



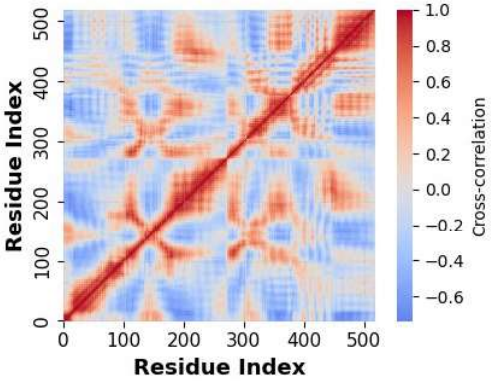
Gammatocopheryl_quinone



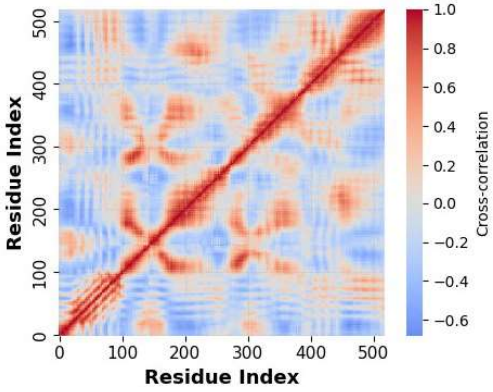
Diepomuricanin_A



Petroselinic acid

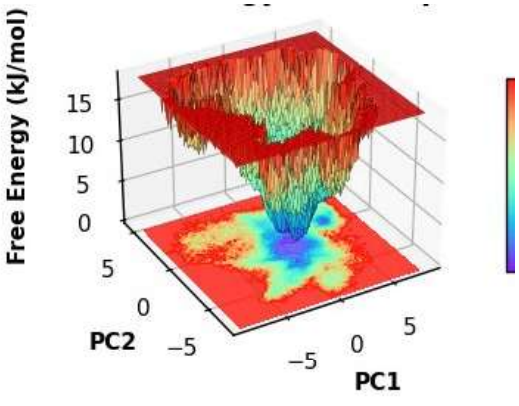


Imperialine

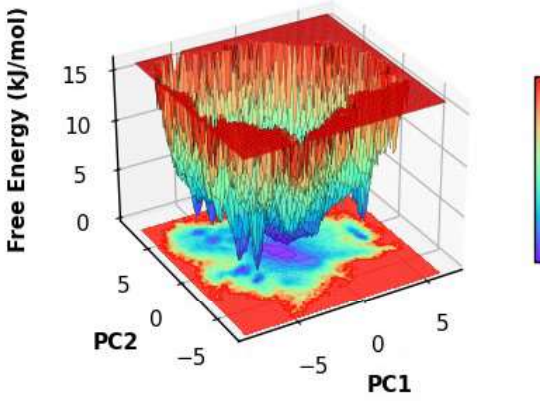


Solacapine

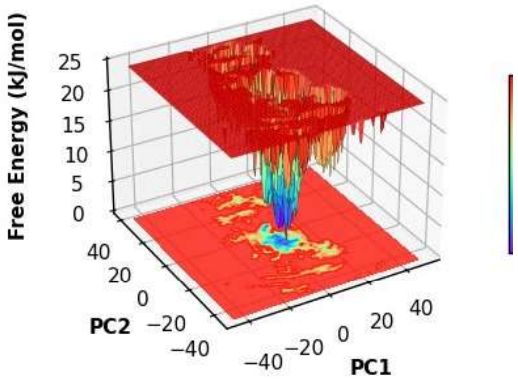
Figure.10.4. DCCM analysis of KEAP-1



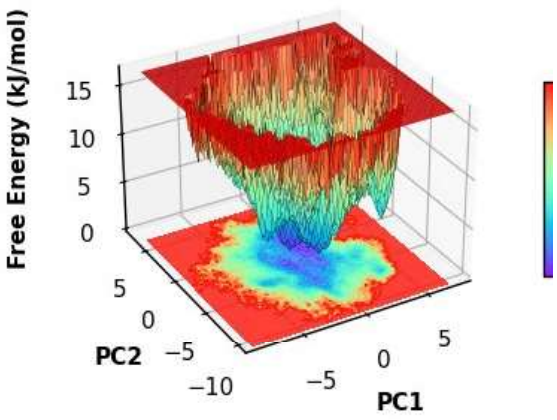
KEAP1



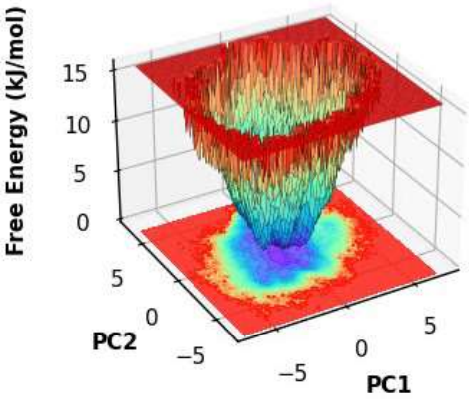
Diepomuricanin_A with KEAP1



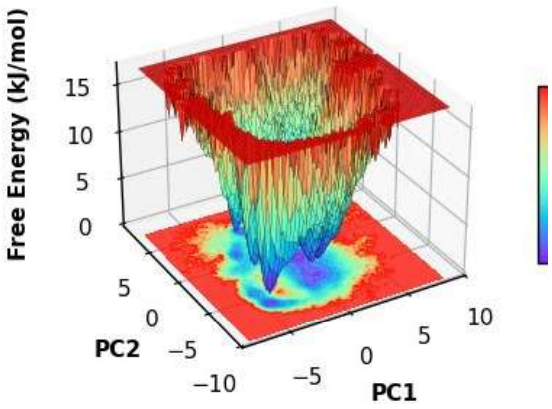
Gammatocopheryl_quinone with KEAP1



Imperialine with KEAP1



Solacapine with KEAP1



Petroselinic acid with KEAP1

Figure.10.5. FEL analysis of KEAP-1.

Table.10.1. Lipinski's rule of five assessed by Swiss ADME web tool.

Compound	Molecular weight	Hydrogen bond donor	Hydrogen bond acceptor	Log P	Molecular refractivity
Solacapine	432.68g/mol	4	4	4.02	132.56
Gamma-tocopheryl quinone	432.68g/mol	1	3	4.43	135.26
Imperialine	429.64g/mol	2	4	3.76	128.33
Petroselinic acid	282.46g/mol	1	2	4.16	89.94
Diepomuricanin A	546.86g/mol	0	4	5.90	167

Table 10.2. MMPBSA(Molecular Mechanics Poisson-Boltzmann Surface Area) MM-GBSA(Molecular Mechanics-Generalized Born Surface Area)

	Gammatocopheryl_ quinone	Solacapine	Imperialine	Petroselinic_ acid	Diepomuricanin _A
ΔVdW	-129.37 ± 10.84	-36.28 ± 29.79	-84.81 ± 13.35	-93.55 ± 12.09	-93.76 ± 33.26
ΔEele	-74.68 ± 12.84	-20.04 ± 33.47	-56.19 ± 17.03	-32.09 ± 26.82	-21.38 ± 17.07
ΔGgas	-204.05 ± 15.27	-56.32 ± 51.55	-141.00 ± 20.63	-125.60 ± 29.62	-115.14 ± 43.22
ΔGPB	131.08 ± 9.46	42.80 ± 38.12	83.22 ± 11.38	69.12 ± 20.17	74.22 ± 29.71
ΔGNPOLAR	-15.73 ± 1.09	-5.90 ± 4.48	-10.17 ± 1.00	-12.84 ± 1.00	-12.76 ± 4.27
ΔGsol	115.35 ± 9.41	36.94 ± 34.35	73.05 ± 10.92	56.27 ± 19.50	61.46 ± 26.11
ΔGbind	-88.70 ± 11.25	-19.37 ± 19.25	-67.95 ± 14.60	-69.33 ± 15.61	-53.68 ± 21.34

Table.10.3. MM-GBSA(Molecular Mechanics-Generalized Born Surface Area)

	Gammatocopheryl_quinone	Solacapine	Imperialine	Petroselinic_acid	Diepomuricanin_A
ΔVdW	-129.37 ± 10.84	-36.28 ± 29.79	-84.81 ± 13.35	-93.55 ± 12.09	-93.76 ± 33.26
ΔEele	-74.68 ± 12.84	-20.04 ± 33.47	-56.19 ± 17.03	-32.09 ± 26.82	-21.38 ± 17.07
ΔGgas	-204.05 ± 15.27	-56.32 ± 51.55	-141.00 ± 20.63	-125.60 ± 29.62	-115.14 ± 43.22
ΔGGB	112.88 ± 8.45	43.47 ± 36.57	70.63 ± 9.75	57.86 ± 17.66	60.21 ± 24.81
ΔGSURF	-20.79 ± 1.46	-5.69 ± 4.69	-12.43 ± 1.30	-17.03 ± 2.05	-15.31 ± 5.36
ΔGsol	92.09 ± 8.16	37.78 ± 32.68	58.20 ± 9.29	40.84 ± 16.48	44.89 ± 20.59
ΔGbind	-111.96 ± 11.25	-18.54 ± 21.21	-82.80 ± 16.48	-84.81 ± 16.15	-70.25 ± 25.86

SUMMARY & CONCLUSION

Despite tremendous advances in cancer diagnosis and treatment, chemotherapy-induced cardiotoxicity remains a key problem in oncology. Various chemotherapeutic agents are available in the market. Doxorubicin ad cyclophosphamide are major drugs used alone or in combination in various treatment regimens. Doxorubicin and cyclophosphamide are chemotherapy medicines commonly used to treat breast cancer. Doxorubicin, commonly known as Adriamycin, is a medication that breaks DNA at any stage during the cell cycle, preventing cancer cells from growing and dividing. Cyclophosphamide, widely known as Cytoxan, destroys DNA in cancer cells during their resting phase, preventing them from replicating. The combination of doxorubicin and cyclophosphamide is referred to as AC. It is most usually used to treat early-stage breast cancer, although it can also be used to treat recurrent breast cancer. AC is usually administered after surgery; however, it can also be given before surgery.

The combination treatment of doxorubicin and cyclophosphamide, which is used to treat numerous diseases including leukaemia, lymphoma, and breast cancer, has been demonstrated to cause cardiac and haematological damage. Dexrazoxane is the only FDA approved cardioprotective agent used clinically but its usage is delimited due to secondary malignancy development. Therefore, quest for natural products with minimum or no side effects are under research and various reports are available.

Current investigation focussed on the cardioprotective effect of a wild edible mushroom *Morchella esculenta* on chemotherapy induced cardiotoxicity. Cardioprotective effect of *M. esculenta* was studied in doxorubicin, cyclophosphamide and their combination therapy induced cardiotoxicity in male Swiss albino mice. *M. esculenta* also known as 'Guchhi' is a highly prized edible mushroom with potent medicinal properties. It is widely used in Chinese traditional medicine. Previous studies in our laboratory reported the hepatoprotection, anti-inflammation and anti-cancer property of *Morchella sp.*

In this study we used the dried fruiting body of *M. esculenta* as the starting study material. Species identification was done by ITS sequencing and verified that the sample material was *M.esculenta.* Further investigations were carried out to find out the cardioprotective effect of methanolic extract of *M. esculenta* fruiting body (ME). HPTLC and LCMS analysis were carried out to identify the major bioactives present in ME. Various compounds such as Imperialine, Luffariellolide, Cynaroside A,

Muricoreacin, Petroselinic acid etc., were found to be present in ME. Antioxidant assays DPPH, FRAP, and ABTS proved the *in vitro* antioxidant potential of ME.

Protective effect of ME against doxorubicin, cyclophosphamide and their combination were studies. Cardiac injury markers such as CK-MB, LDH, and Cardiac troponin-I levels elevated subsequent to chemotherapy administration were significantly lowered by the oral administration of ME. Since, oxidative stress is regarded as one of the major mechanisms involved in chemotherapy induced cardiotoxicity antioxidant levels were estimated in the cardiac tissue of experimental animals. The results showed that depleted antioxidant levels such as reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and elevated lipid peroxidation by product malondialdehyde (MDA) in doxorubicin and cyclophosphamide treated animals. However, ME successfully restored the antioxidant levels and lowered lipid peroxidation. Cardiac tissue histopathology showed alterations in the normal cardiac architecture such as cardiac hypertrophy, nuclear infiltration and myoplasm degeneration. The administration of ME ameliorated these effects raised consequent to chemotherapy treatment and restored the normal cardiac architecture.

Studies were carried out to find out the effect of ME on cardiac mitochondria. Doxorubicin and cyclophosphamide resulted a decline in the levels of TCA cycle enzymes and electron transport chain complexes (I,III and IV). ME successfully combated this mitochondrial dysfunction caused by chemotherapy. To analyse the gene expression patterns of NF-κB, NRF-2, KEAP-1, IL-6, IL1-β, TNF-α and iNOS which are involved in oxidative stress and inflammation we further carried out Real time PCR technique. The results revealed that ME upregulated NRF-2 expression which was lowered by doxorubicin and cyclophosphamide treatment. The gene expression levels of NF-κB, KEAP-1, IL-6, IL1-β, TNF-α and iNOS were elevated in doxorubicin and cyclophosphamide treated animals which was reversed by ME administration. From these studies we observed that doxorubicin and cyclophosphamide usage either alone or in combination causes cardiotoxicity and ME efficiently downregulated the ill effects caused by chemotherapy.

Another concern in this cardio protection regimen was that whether the administration of ME along with chemotherapy lower the anticancer efficacy of the chemotherapeutic

agents. In order to resolve this, we further investigated the cardio protective effect of ME vis-à-vis anti-tumour efficacy of doxorubicin and cyclophosphamide using DLA induced solid tumour model. The results showed that ME provided cardio protection in tumor bearing mice without hindering the anti-tumour efficacy of doxorubicin and cyclophosphamide.

The results of current study indicate that ME is successful in ameliorating doxorubicin and cyclophosphamide and their combination treatment induced cardiotoxicity. The most probable cardioprotective mechanism of ME might be their potential to reduce oxidative stress induced damage and cardiac mitochondrial protection. Further studies are needed to find out the pathways involved in the cardio protection mechanism.

RECOMMENDATIONS

In this work, we showed that the edible fungus *Morchella esculenta,* which is used medicinally, has excellent cardioprotective properties against the cardiotoxicity caused by cancer chemotherapy. The cardioprotective effect of methanol extract of the fruiting body of *M. esculenta*, which includes over 100 bioactive compounds. This investigation did not successfully isolate the pure compounds exhibiting biological action. Furthermore, *in silico* studies have demonstrated that certain bioactive compounds in the methanol extract of *M. esculenta* have the potential to be developed as a pharmacologically active drug, and MD simulation studies conducted in this study have demonstrated that these compounds have the ability to bind with the Keap-1 receptor. Therefore, more research should be done to determine these substances' biological potential. Further studies on the various pathways involved in the cardioprotective property of the compounds and their receptor mediated mechanisms hold a promise to the development of a potential cardioprotective agent and will be a boon to the field of cardio oncology.

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LIST OF PUBLICATIONS

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