DMM attenuates doxorubicin induced cardiac hypertrophy in laboratory animals

Thesis submitted in partial fulfilment of the requirement for the degree of Master of Pharmacy

Under the guidance of

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Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research as part of my work on "**DMM attenuates doxorubicin induced cardiac hypertrophy in laboratory animals".** All information in this document have been obtained and presented in accordance with academic rules and ethical conduct. I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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Signature With Date: Suchismita Patra (5.07.2023)

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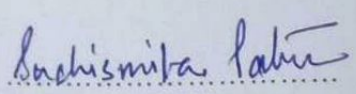

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CERTIFICATE

This is to certify that **Suchismita Patra**, Final year Masters of Pharmaceutical Technology (M.Pharm) examination student of Department of Pharmaceutical Technology, Jadavpur University, Class Roll No. **002111402036**, Registration No. **160264 of 2021-2022**, Examination Roll No. **M4PHL23024** has completed the Project work titled, **"DMM attenuates doxorubicin induced cardiac hypertrophy in laboratory animals"** under the guidance of **Prof. Sanmoy Karmakar** during his Master's Curriculum. This work has not been reported earlier anywhere and can be approved for submission in partial fulfilment of the course work.

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Dedicated to

My loving parents

Mr. Purna Chandra Patra and Late Swapna Patra

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1. INTRODUCTION

1.1 Cardiac diseases -

Cardiovascular disease (CVD) is a global health challenge affecting individuals in every corner of the world. It encompasses a range of conditions that impact the heart and blood vessels, including coronary artery disease, heart attacks, strokes, heart failure, and arrhythmias. With its prevalence and impact, CVD significantly burdens public health systems and economies worldwide.

According to the World Health Organization (WHO), CVD is the leading cause of death globally, accounting for approximately 17.9 million deaths yearly. This equates to more than 30% of all deaths worldwide. Alarmingly, CVD is not limited to developed countries but also affects low- and middle-income nations, where the burden is rapidly increasing.

1.2 Prevention is Better than Cure-

The age-old adage, "Prevention is better than cure," holds particularly true regarding cardiovascular disease (CVD). Given the widespread impact of CVD worldwide, emphasising prevention becomes crucial in reducing the burden of this disease and improving overall public health.

While treatment and cure remain vital components of managing CVD for those already affected, prevention efforts offer a more holistic and sustainable approach to combat the disease globally. Therefore, Cardioprotective medicines may be a superior option for combating CVDs.

1.3 DMM as a potential cardioprotective agent-

Unani medicine, also known as Yunani medicine, is a traditional system of medicine that originated in ancient Greece and was later adopted and developed by Islamic scholars. It is based on the principles of the Greek physician Hippocrates and the teachings of the Islamic philosopher Avicenna (Ibn Sina). Regarding cardiovascular health, Unani Medicine emphasises the importance of maintaining a healthy lifestyle, including a balanced diet, regular exercise, stress management, and adequate rest. These lifestyle modifications are considered fundamental in preventing and managing heart diseases.

In addition to lifestyle modifications, Unani medicine utilises various medicinal herbs and formulations believed to have cardioprotective properties. Dawa-ul-Misk Motadil Sada is an Unani herbal tonic. It is said to strengthen the heart, liver, and brain. However, pharmacological evaluation is required to validate these claims. So, in this study, we tried to evaluate the probable cardio-protective potential of this unani preparation.

2. Literature Review

Cardiac hypertrophy and it's molecular mechanism

Hypertrophy is derived from the Greek hyper, meaning over, and trophy, meaning growth. It is widely believed to be an adaptive response to increased work load. At the cellular level, cardiomyocyte hypertrophy is characterised by an increase in cell size, enhanced protein synthesis, and heightened organisation of the sarcomere. Heart requires more energy than any other organ of our body, to reach this enormous demand, the heart converts chemical energy stored in fatty acids and glucose into mechanical energy. If these mechanisms do not reach the demand of the heart, cardiac malfunction, mechanical failure of the heart occurs. There are numerous factors are involved in the development of cardiac hypertrophy [1]. Such as-

Activation of the Neurohumoral System

Several neurohumoral factors play a role in cardiac hypertrophy, including angiotensin II, norepinephrine, and endothelin-1. In heart failure, the pressure-baroreceptors are activated in the carotid sinus, aortic arch, and in the left ventricle. The afferent signals modify the central cardio regulatory centres to increase the circulating blood volume. Sympathetic and humoral efferent mechanisms are stimulated. This generalised sympathetic activation with concomitant parasympathetic decrease is followed by impaired heart rate variability, elevated blood pressure, and increased peripheral resistance. It results in a positive inotropic and chronotropic effect, which cause blood redistribution by peripheral vasoconstriction and central vasodilation to maintain the perfusion of vital organs. It activates the RAAS. The activation of the RAAS potentiates renin release from the kidney. The plasma renin stimulates the transformation of

the inactive dekapeptide prehormone angiotensinogen to angiotensin I by cleaving four amino acids. The octapeptide angiotensin I is then converted into angiotensin II by angiotensin-converting enzyme. Angiotensin II is a vasoactive agent, causing vasoconstriction, blood pressure elevation, myocyte hypertrophy, myocyte cell death, myocardial fibrosis, and stimulates the secretion of aldosterone from the adrenal cortex. The angiotensin II receptors are mostly found on the intraglomerular mesangial cells, causing them to contract and stimulating the adrenal cortex to release aldosterone. Angiotensin II cannot only be synthesised by the RAAS, but also through an independent pathway through a conversion by kallikrein and cathepsin G, or in the tissue through chymase activation. Chymase is primarily produced by mast cells (MCs) and is released into the extracellular interstitial space in response to inflammatory signals, tissue injury, and cellular stress under pathological conditions. Chymase has a greater ability than angiotensin I-converting enzyme (ACE) to generate angiotensin II (Ang II) and convert Ang1-12 to Ang II. Angiotensin II is a general vasoconstrictor in all arterioles with a marked effect on the renal efferent arterioles. It stimulates the release of aldosterone, induces the excretion of noradrenalin from the sympathetic nerve terminals, and inhibits the vagal tone. As a result, the intraglomerular pressure and glomerular filtration rises, which results in decreased hydrostatic and increased oncotic pressure, and therefore an induced sodium and fluid reabsorption into the peritubular capillaries. Sodium reabsorption occurs eventually due to active and passive mechanisms. Finally, the hypertrophy of other renal tubular cells through angiotensin II leads to sodium reabsorption. Angiotensin II is not only a vasoactive hormone, it has also been shown that cardiac angiotensin II has local positive inotropic, negative lusitropic effect on the heart, and increases the afterload that elevates further the energy expenditures of the heart. Angiotensin II has also a direct effect on cardiomyocytes: it promotes hypertrophy, myocyte apoptosis, and causes structural and biochemical alterations in the extracellular matrix. Angiotensin has furthermore metabolic effects, such as upregulation of tissue lipogenesis and reduction of lipolysis, and thereby causes fat mass expansion in the body. There are mainly two types of angiotensin II receptors are there. angiotensin type-1 receptor (AT1R) and the angiotensin type-2 receptor (AT2R). Vasoconstriction with vascular smooth muscle cell proliferation, cell growth, aldosterone synthesis and secretion, vasopressin secretion, and catecholamine release- all these effects are mediated by AT1R [2].

G-protein-coupled receptor (GPCR) signalling: GPCRs are involved in the transduction of signals from neurohumoral factors. Upon activation, GPCRs activate downstream signalling pathways, such as the mitogen-activated protein kinase (MAPK)

pathway and the phosphoinositide 3-kinase (PI3K)/Akt pathway. These pathways regulate gene expression, protein synthesis, and cell growth, ultimately leading to hypertrophy [3].

The MAPK pathway is a major signalling cascade involved in cardiac hypertrophy. It consists of multiple kinase modules, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Activation of these kinases leads to the phosphorylation of transcription factors, such as myocyte enhancer factor 2 (MEF2) and activator protein 1 (AP-1), which promote the expression of hypertrophic genes [4].

The PI3K/Akt pathway is another crucial signalling pathway involved in cardiac hypertrophy. Activation of this pathway leads to the phosphorylation and activation of Akt, which subsequently phosphorylates and inhibits the pro-apoptotic protein Bad. Akt also activates mammalian target of rapamycin (mTOR), a key regulator of protein synthesis and cell growth. Increased mTOR activity promotes hypertrophic growth [5].

Calcium signalling: Calcium is a critical regulator of cardiac muscle contraction and also plays a role in cardiac hypertrophy. Increased calcium influx and release from intracellular stores activate calcium-dependent signalling pathways, including calcium/calmodulin-dependent protein kinase II (CaMKII) and calcineurin/nuclear factor of activated T-cells (NFAT) pathway. In heart failure excitation contraction coupling can be impaired by decreased transport of calcium into the cell through L-type calcium channels. Dysfunction or lower amount of the L-type calcium channels play a central role in it; decreased cardiac L-type Ca2+ channel activity induces cardiac hypertrophy and heart failure in mice. The calcium sensitivity of Troponin C or the myofilaments can also be reduced. Thus, calcium increase in the surrounding of the troponin complex significantly attenuates excitation contraction coupling. In some forms of diastolic heart failure, the function of the sarcoplasmic ATP-dependent calcium pump is impaired. This defect delays the rate of calcium uptake by the sarcoplasmic reticulum and reduces the rate of relaxation, leading to diastolic dysfunction [6].

Doxorubicin (DOX) belongs to anthracycline family, along with epirubicin, daunorubicin, and idarubicin. Anthracyclines are well-established and highly effective anti-neoplastic agents, used to treat several adult and paediatric cancers, such as breast cancer, leukaemia, lymphomas, sarcomas, and many others. Retrospective analyses of patients treated with anthracyclines showed that many patients experienced cardiotoxicity. Anthracycline-induced cardiotoxicity (AIC) can occur as acute or chronic, and is characterised by a broad spectrum of symptoms including asymptomatic electrocardiography (ECG) changes as arrhythmia, cardiomyopathy,

pericarditis, left ventricular dysfunction, and decompensated heart failure. many researchers were trying to solve the problem of AIC pathogenesis and find effective strategies for prevention. The molecular mechanisms underlying doxorubicin-induced cardiac hypertrophy involve multiple pathways-

Dysregulation of the renin-angiotensin aldosterone system (RAAS) has been proposed to play a crucial role in development of anthracycline-induced cardiac toxicity. Various preclinical and clinical studies showed that use of different RAAS inhibitors prevents from Anthracycline Induced Cardiotoxicity. Apart from RAAS, the following factors also involved [7].

Reactive oxygen species (ROS) generation: Doxorubicin can generate reactive oxygen species, leading to oxidative stress within cardiomyocytes. ROS can cause damage to cellular components, including DNA, proteins, and lipids. The increased oxidative stress activates signalling pathways involved in cardiac hypertrophy [8].

Mitochondrial dysfunction: Doxorubicin can disrupt mitochondrial function in cardiomyocytes. It impairs mitochondrial respiratory chain complexes, leading to reduced ATP production and increased production of ROS. Mitochondrial dysfunction further contributes to oxidative stress and triggers signalling pathways involved in hypertrophic growth [9].

Calcium dysregulation: Doxorubicin can disrupt calcium homeostasis within cardiomyocytes. It impairs calcium handling proteins, such as sarcoplasmic reticulum calcium ATPase (SERCA) and ryanodine receptor (RyR), leading to increased intracellular calcium levels. Dysregulated calcium signalling activates calcium-dependent pathways associated with cardiac hypertrophy [10].

Activation of signalling pathways: Doxorubicin activates various signalling pathways involved in cardiac hypertrophy. These include the MAPK pathway (ERK, JNK, p38), PI3K/Akt pathway, and nuclear factor-kappa B (NF-κB) pathway. Activation of these pathways leads to alterations in gene expression, protein synthesis, and cell growth, promoting cardiac hypertrophy [11].

Apoptosis and cell death: Doxorubicin-induced cardiotoxicity can also result in cardiomyocyte apoptosis and cell death. Increased oxidative stress, mitochondrial dysfunction, and calcium dysregulation contribute to apoptotic signalling pathways, such as p53 and

caspases. The loss of cardiomyocytes can trigger compensatory hypertrophic responses in the remaining cells [12].

Fibrosis: Doxorubicin can stimulate cardiac fibrosis, which involves the excessive deposition of extracellular matrix components, such as collagen. Fibrosis disrupts normal cardiac tissue architecture and impairs cardiac function. It is mediated by pro-fibrotic signalling pathways, including transforming growth factor-beta (TGF-β) and connective tissue growth factor (CTGF) [13].

As per several literature, doxorubicin 2.5 mg/kg body weight i.p. in 6 equal injections alternatively for two weeks to make a total cumulative dose of 15 mg/kg body is sufficient to produce cardiotoxicity in albino rats.

The use of certain medications, such as Telmisartan and Nebivolol, has been explored as a potential cardioprotective strategy in doxorubicin-induced cardiac hypertrophy models [14].

Telmisartan is an angiotensin II receptor blocker (ARB), while Nebivolol is a beta-blocker. Both drugs have been widely used in the treatment of hypertension and have shown beneficial effects on the cardiovascular system [14].

In doxorubicin-induced cardiac hypertrophy models, the combination of Telmisartan and Nebivolol has been investigated for its potential cardioprotective effects. These studies have suggested that the combination therapy may offer several benefits:

Reduction of oxidative stress: Doxorubicin-induced cardiotoxicity is associated with increased oxidative stress, which can contribute to cardiac hypertrophy. Telmisartan has been shown to possess antioxidant properties, which can help reduce oxidative stress in the heart. Nebivolol, on the other hand, has been reported to enhance the production of nitric oxide, which can have antioxidant effects. Therefore, the combination therapy may synergistically reduce oxidative stress in the heart.

Anti-inflammatory effects: Doxorubicin-induced cardiac hypertrophy is often associated with inflammation in the heart. Both Telmisartan and Nebivolol have been shown to possess anti-inflammatory properties, which can help mitigate inflammation and potentially attenuate cardiac hypertrophy.

Modulation of signalling pathways: Telmisartan and Nebivolol may affect various signalling pathways involved in cardiac hypertrophy. Telmisartan can inhibit the angiotensin II signalling pathway, which is associated with hypertrophy and fibrosis. Nebivolol has been reported to activate the β3-adrenergic receptor, leading to the activation of protein kinase G and subsequent inhibition of cardiac hypertrophy [14].

Unani medicine and DMM

The World Health Organization (WHO) has recognized the Unani system of medicine as a form of alternative medicine used by people globally. It considers a person's entire body, mind and spirit. It sees the human body as one cohesive system made up of four basic parts with four different temperaments. The physical qualities and inherent disposition of a person are reflected in their temperament. The human body is susceptible to several ailments due to temperamental imbalances. [15]

In this article, we shall talk about DMM, a unani medicine. The main ingredients in these formulations are 26.4 mg of Berberis aristata DC (fruit) per g weight of DMM.

Composition of Dawa ul Misk Motadil (DMM) [16]

1. Zarishk (Berberis aristata DC) Fruit 132.0 mg
2. Tabasheer Safaid (Bambusa arundinace willd.) Silicacious concretion 88 mg
3. Sandal safaid (Santalum album L.) Heart wood 88 mg
4. Sandal Surkh (Pterocarpus santalinus L.f.) Heart wood 88 mg
5. Kishneez Muqashshar (Coriandrum sativum L.) Dried seed 88 mg
6. Gule Gawzaban (Borago officinalis L.) Flower 88 mg
7. Amla (Embelica officinalis) Dried fruit 88 mg
8. Tukhm Khurfa (Portulaca oleracea L.) Dried seed 88 mg
9. Gule Surkh (Rosa damascene Herrm.) Flower 51 mg
10. Abresham Muqarraz (Bombyx mori cocoon) Cocoon 51 mg
11. Darchini (Cinnamomum zeylanicum Blume) Stem, Bark 51 mg
12. Behman Safaid (Centaurea behen L.) Root 51 mg
13. Behan Surkh (Salvia haematodes L.) Root 51 mg
14. Darunaj Aqrabi (Doronicum hookeri C.B.Clarke ex Hook.f) Rhizome 51 mg
15. Ood Hindi (Aquilaria agallocha Roxb.) Heart wood 36 mg
16. Badranjboya (Nepeta hindostana (B.Heyne ex Roth) Haines) Whole plant 36 mg

17. Mastagi (Pistacia lentiscus L.) 30 mg
18. Ushana (Usnea longissima Ach.) Thallus 30 mg
19. Dana Elaichi Khurd (Elettaria cardamomum L.) Dried seed 30 mg
20. Qand Safaid (Saccharum officinarum) Crystal 2.44 g
21. Shahad (Honey) – 1.22 g
22. Aabe Seb Shinn (Pyrus malus L.) Fruit 1.22 g
23. Zafran (Crocus sativa L.) Stigma 51 mg
24. Amber (resin) without musk - 14 mg

Summary Of Literature Review

Cardiac hypertrophy is an adaptive response to increased workload, characterized by increased cell size, enhanced protein synthesis, and sarcomere organization. Activation of the neurohumoral system, including angiotensin II, norepinephrine, and endothelin-1, plays a role in cardiac hypertrophy. The renin-angiotensin aldosterone system (RAAS) is involved, leading to vasoconstriction, myocyte hypertrophy, fibrosis, and aldosterone secretion.

GPCR signalling, particularly the MAPK and PI3K/Akt pathways, regulates gene expression, protein synthesis, and cell growth in cardiac hypertrophy. Calcium dysregulation and mitochondrial dysfunction contribute to hypertrophic growth. Doxorubicin-induced cardiac hypertrophy involves dysregulation of RAAS, reactive oxygen species (ROS) generation, mitochondrial dysfunction, calcium dysregulation, activation of signalling pathways (MAPK, PI3K/Akt, NF-κB), apoptosis, and fibrosis.

In doxorubicin-induced cardiac hypertrophy models, Telmisartan (an ARB) and Nebivolol (a beta-blocker) have been explored for their cardioprotective effects. Telmisartan has antioxidant and anti-inflammatory properties, inhibits angiotensin II signalling, and may reduce oxidative stress. Nebivolol enhances nitric oxide production, possesses anti-inflammatory effects, and activates protein kinase G to inhibit cardiac hypertrophy.

Literature Review also consists of potency of unani medicine DMM as a cardioprotective agent along with the composition of these polyherbal medicine.

3.1. Aim

To investigate if there is any cardioprotective activity of Dawa-ul-Misk-Motadil (DMM) in wistar albino rats.

3.2. Objective

1. The objective would involve assessing the extent of cardiac hypertrophy in the experiment model by comparing the cardiac hypertrophy in the doxorubicin treated group with the control and Telmisartan, Nebivolol combination treated group.
2. Evaluation of DMM effects by measuring certain cardioprotective parameters like-ECG, Corrected QT, Blood Pressure, heart weight to tail length ratio etc.
3. To study the effect of DMM on oxidative stress like MDA.
4. To evaluate whether the mitochondrial protein degradation and structural alteration are improved by DMM or not.
5. Observe the histopathological changes in different groups of rats.

4. Materials and Methods

4.1. Drugs

GLS PHARMA LTD supplied doxorubicin. Nebivolol (5mg) was purchased from Aristo Pharma Ltd, and Telmisartan (40mg) was bought from Mankind, India. DMM (batch no. MDE019) was a kind donation from The Calcutta Unani Medical College and Hospital, 8/1, Abdul Halim Lane, Kolkata, West Bengal 700016.

4.2. Chemicals

TBA reagent was supplied from HIMEDIA, while Janus Green, MDA standard, 10% TCA and Bradford reagent were purchased from Sigma-Aldrich. All the chemicals were of the analytical grade.

4.3. Animal Husbandry and Maintenance

Healthy adult male Wistar albino rats (8–9 weeks) weighing 140-180 gm were procured from West Bengal Livestock Development Corporation Limited. Buddha Park, Kalyani, Nadia-741235. FSSAI REG NO – 10012031000104, and used for the study.

The animals were kept in polypropylene cages with well-aerated stainless-steel covers. All animals were kept in a departmental animal house. Animals were kept under controlled conditions of temperature (12-hour light and dark cycle, temperature of 25 ± 2°C and 50 ± 20 % relative humidity).

The study followed the Institutional Ethical Committee (Constituted under the Guidelines Committee for Control and Supervision of Experiments on Animals).

4.4. Development of Cardiac hypertrophy in a rat model

The cardiac hypertrophy model utilised Adult Wistar rats weighing 150-180 g. The animals had free access to food and water and were kept on a 12-hour light-dark cycle. In this study, doxorubicin was administered intraperitoneally in six equal injections (each containing 2.5 mg/kg) over a period of two weeks, with a total cumulative dose of 15 mg/kg body weight.

4.5. Measurement of -

4.5.1. Blood Pressure using a Non-Invasive method

The BIOPAC—MP36 device ((Biopac Syste, Inc., USA) was used to assess systolic blood pressure in a non-invasive manner. Animals were restrained in an animal holder where a 37°C constant temperature was maintained before recording the blood pressure. A cuff was placed around the animal's tail and held in an animal restrainer. And for data augmentation, we utilised NIBP-200A software.

4.5.2. ECG

Rats were anaesthetised using Ketamine (60 mg·kg−1) and skeletal muscle relaxant xylazine (10 mg·kg−1). Using a standard lead, an ECG was taken for 5 min, immediately after anaesthesia. MP36 (BIOPAC, Goleta, CA, USA) was used to collect and analyse the ECG signals. QT and RR intervals were measured at day 0 (i.e., before the dox administration) and 28^{th} (DOX+ respective formulation). The corrected QT interval (QTc) was calculated using Bazett's formula (QT/RR^ 1/2).

4.5.3. Heart weight / Tail length ratio

In each group, heart weight/tail length ratio was measured on the day of sacrifice as a parameter of cardiac hypertrophy as described by Chowdhury et al., 2013. Body weight can be fluctuated due to aging, therefore bw cannot be a reliable reference for normalising heart weight. Heart weight normalisation by tail length appears to be authentic. Since, tail length remains constant even after maturity. Tail length was measured by using a centimetre (cm) scale. Heart weight (gm) was measured after keeping the heart in ice-cold saline (0.9% w/v) and blotting the heart with tissue paper to remove adhering liquid in folds and gaps.

4.5.4. Total mitochondrial protein

One rat was sacrificed from each group, and the heart was removed. Following removal, the hearts were immersed in ice-cold water to maintain osmolarity. After washing, the heart tissues minced into pieces and rinsed in KCL buffer. Tissue was resuspended in sucrose buffer and homogenised, then ultracentrifuged at 1000g for 10min. The supernatant was then collected, filtered and recentrifuged at 26000g for 15min using Thermo Micro-Ultracentrifuge and reconstituted in sucrose buffer after harvesting mitochondrial pellet. Finally, the total mitochondrial protein was calculated Spectroscopically using the Bradford reagent.

4.5.5. Mitochondrial Activity in Cardiomyocytes

Following mitochondrial isolation, we used a simple, time- and cost-effective Janus green (JG-B) colorimetric assay to assess mitochondrial function, activity, and toxicity. The approach relies on mitochondrial dehydrogenases to convert JG-B to diethyl safranine, which is pink and has a maximum absorption at 550 nm. For the tests involving JG-B uptake and subsequent reduction, mitochondria were incubated with JG-B before being pelleted down at 17,000 g for 5 minutes by Thermo Micro- Ultracentrifuge to eliminate excess unbound JG-B. Mitochondria bound with JG-B were then resuspended in an isolation buffer, and absorbance at 550nm was measured.

4.5.6. MDA concentration

ROS triggers oxidative attack on critical cell components, which has been linked to a variety of human disorders including cardiovascular disease, atherosclerosis, diabetes, and others. Thiobarbituric acid reactive substances (TBARS) are primarily low molecular weight compounds. The most common of which is Malonaldehyde (MDA), is a result of polyunsaturated fatty acid peroxidation in cells. An increase in free radicals causes MDA overproduction. Therefore, Malondialdehyde levels are frequently tested in order to assess oxidative stress and antioxidant status. We perform TBARS assay to determine the MDA concentration in rats from various groups. We first took blood from the rat and separated the plasma from it. Then the plasma is stored at -20°Celsius. We transfer 100 µL of sample (plasma) into a 1.5ml tube for the assay. After that, 200µL of ice-cold 10% TCA was added. Incubate for 5 minutes. Next, centrifuge for 5 minutes at 14,000rpm. The supernatant was

collected and mixed with the TBA reagent. Incubate for another 60 minutes at 100 degrees Celsius. This time, a colour reaction occurs when TBA combines with MDA, producing a pink colour complex. The product concentration is proportional to the colour intensity at 535nm. Finally, we use spectramax to measure the absorbance at 535nm.

4.5.7. Histopathological Staining

Following sacrifice, the heart tissue slices were preserved in 10% formalin. The specimens were processed by standard procedure and embedded in paraffin wax. The blocks were sectioned from the ventricular portion and stained according to the haematoxylin and eosin method and were examined by EVOS microscope.

4.5.8. Statistical Analysis

The mean ± standard deviation was used to represent all of the data. GraphPad Prism 9.0 was used to undertake the statistical data analysis (Graph Pad, San Diego, CA, USA). One-way ANOVA was applied to assess all of the serum parameters, and Tukey's test was used for post-hoc analysis. A statistically significant value was defined as $p < 0.05$.

5.Work Methodology

After one week of acclimatisation, the animals were separated into four groups (N = 4), each with six animals. For this experiment all groups were taken as self-controlled.

Group 1 Normal control (Untreated)

Group 2 Disease control (only Dox)

Group 3 DMM treated (DOX+DMM)

Group 4 Telmisartan and Nebivolol Combination + DOX

Group 1 served as normal control and received only food and water. Group 2 animals were treated with doxorubicin (2.5 mg/kg body weight, i.p.) in 6 equal injections alternatively for two weeks to make a total cumulative dose of 15 mg/kg body weight. Group 3 animals received DMM orally (2000 mg/kg body weight) for two weeks as a pretreatment followed by Dox

administration as in group 2. DMM was continued to the last administration of DOX. Similarly, Group 4 animals received Telmisartan (3.9mg/kg) and Nebivolol (0.5 mg/kg) combination as a pretreatment followed by Dox administration as in group 3. Changes in the body weight of rats were recorded beginning at the first injection of DOX and were completed after the injection of the last dose of DOX. All rats received standard rat chow and were allowed free access to water.

6. Results

6.1. DMM improves the decrease in body weight induced by Doxorubicin-

Figure 1 depicts the changes in body weight (bw) of rats in various groups between day 0 and day 28th. In the doxorubicin-treated group, bw of rats decreased significantly ($p<0.001$) on day 28th as compared to day 0, the reduction of bw in Dox+DMM group, was lower compared to the Dox group. There were non-significant changes in bw observed in the Telmi+ Nebi group. A modest rise in bw was observed in the normal control group.

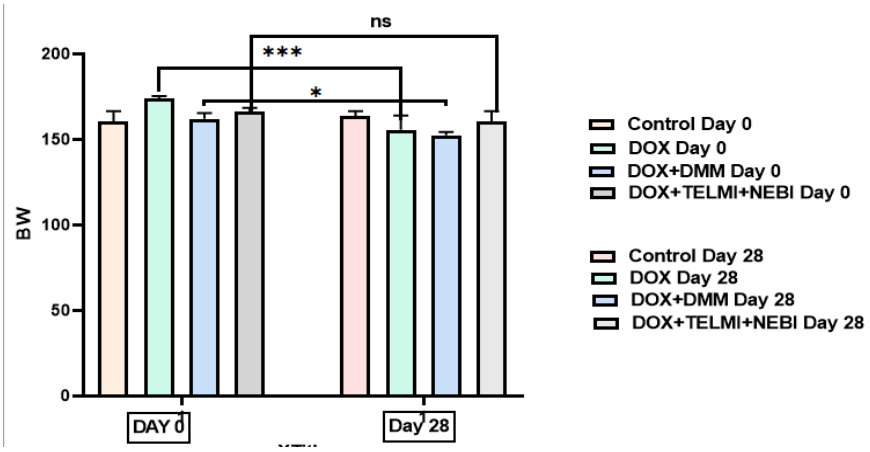


Figure 1. Represents mean ± SD of Body weight of rats of Control, Dox, DMM, Telmi+Nebi groups on day 0 and 28th. (ns- non-significant)

6.2. DMM inhibits the dox-induced reduction in Systolic blood pressure

Figure 2 represents the non-invasive systolic blood (SBP) pressure of rats in different groups on days 0 and 28. Throughout the treatment, the controls' systolic blood pressure remained rather steady at around 135 mm Hg. However, there was a substantial drop in blood pressure at day 28th in the doxorubicin group compared to the control group ($p<0.001$). When compared to the Doxorubicin group, the SBP of the DMM treated group decreased non significantly (ns). SBP decreased significantly in the Telmisartan and nebivolol treatment group ($p<0.001$) as compared to the Dox treated group.

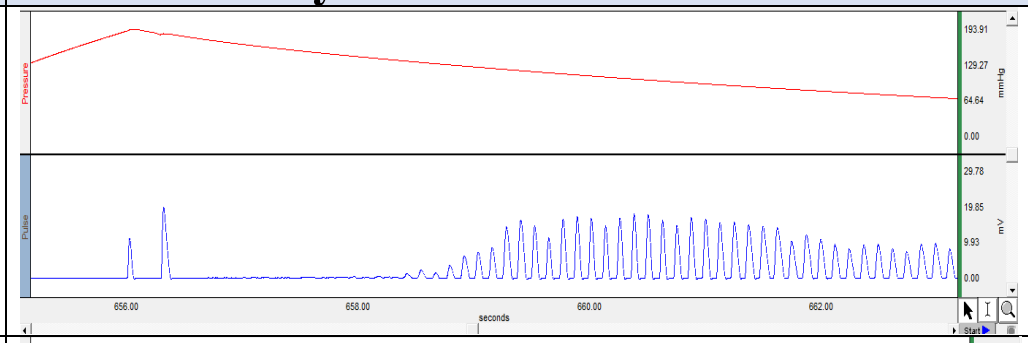
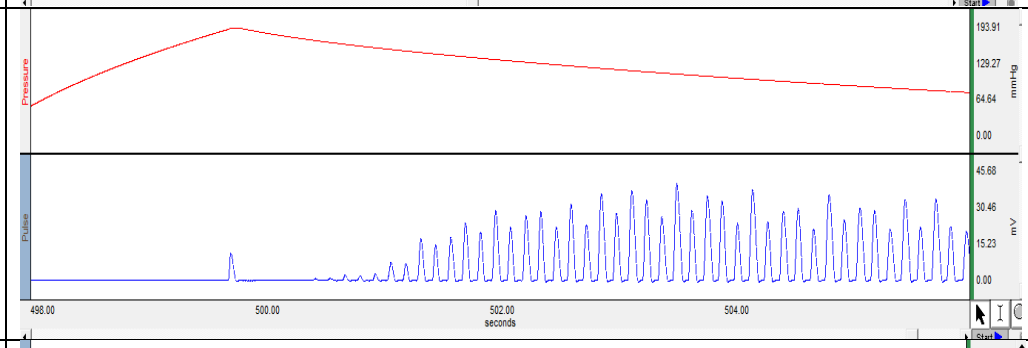
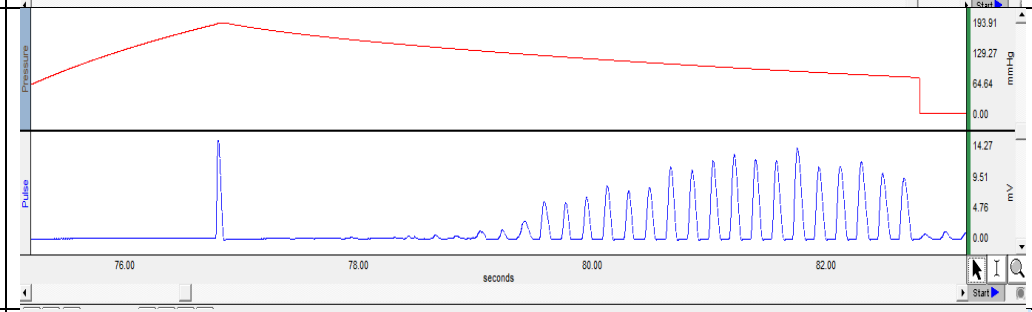
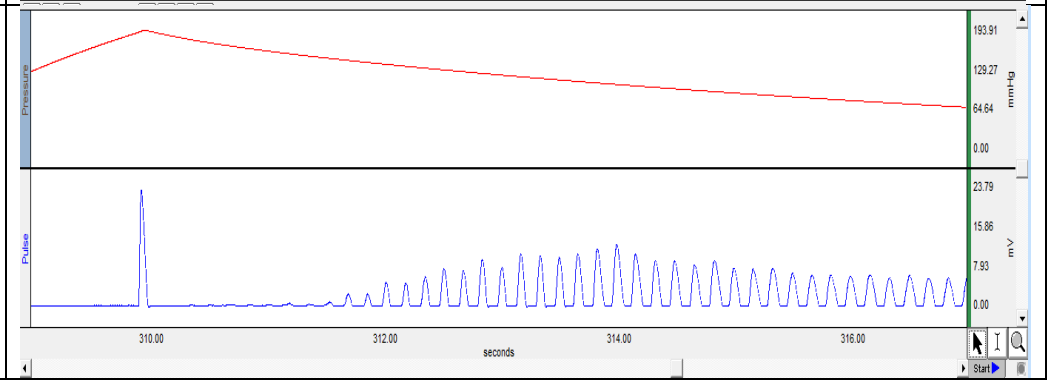
Group	Day 0
Control	
Dox	
Dox + DMM	
Dox+ Telmi+Nebi	

Figure 2(A) systolic blood pressure on day 0

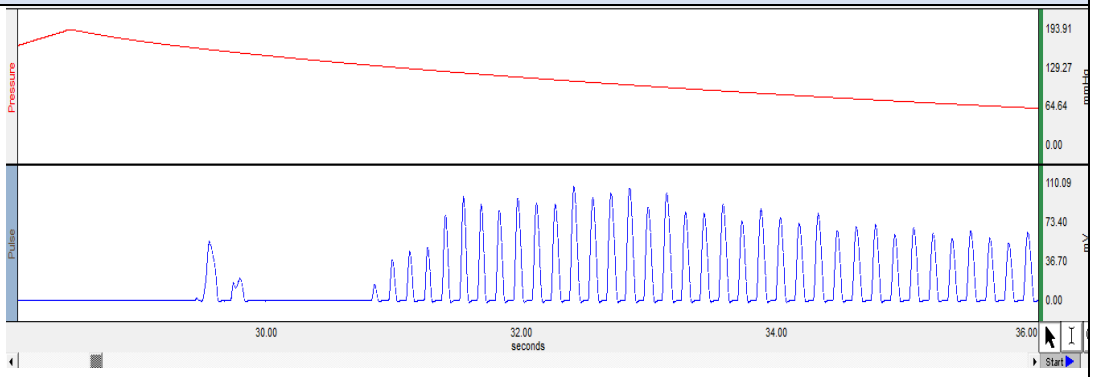
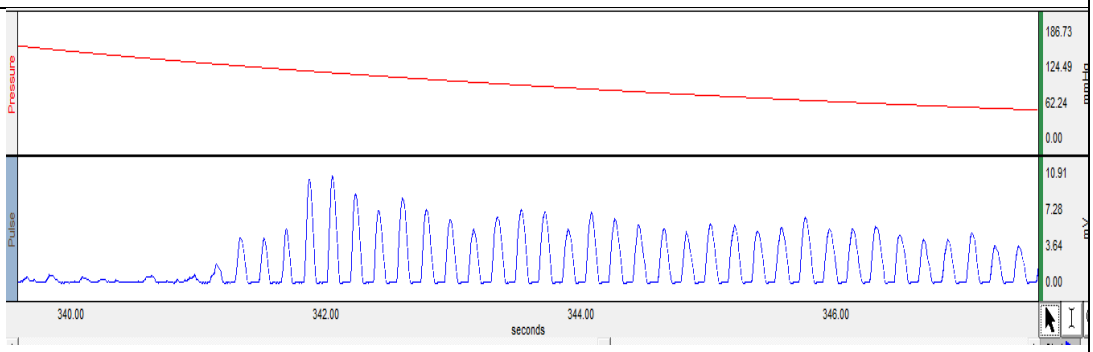
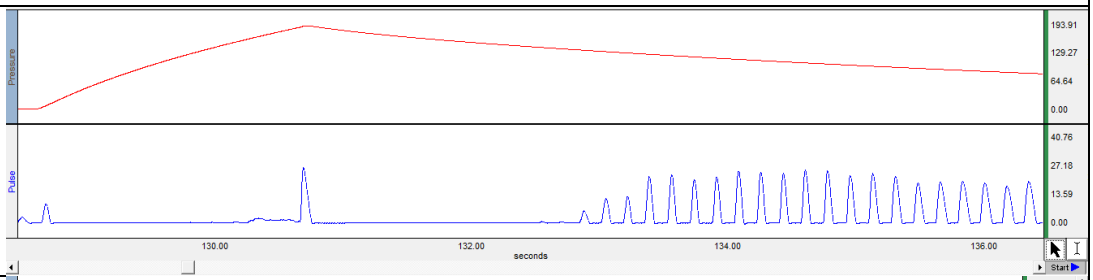
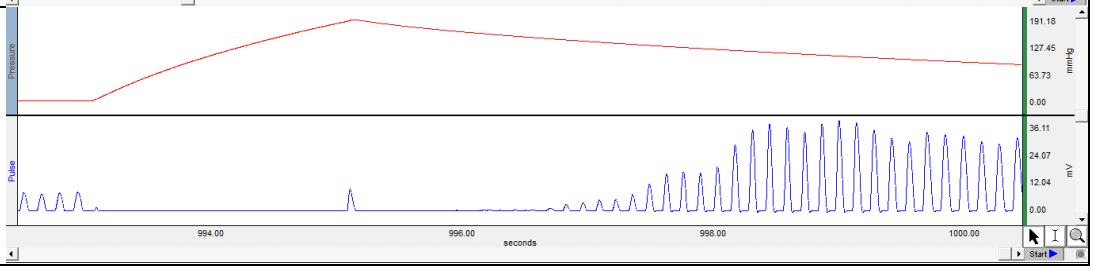
Group	Day 28th
Control	
Dox	
Dox + DMM	
Dox+ Telmi+Nebi	

Figure 2(B) Systolic blood pressure on day 28th

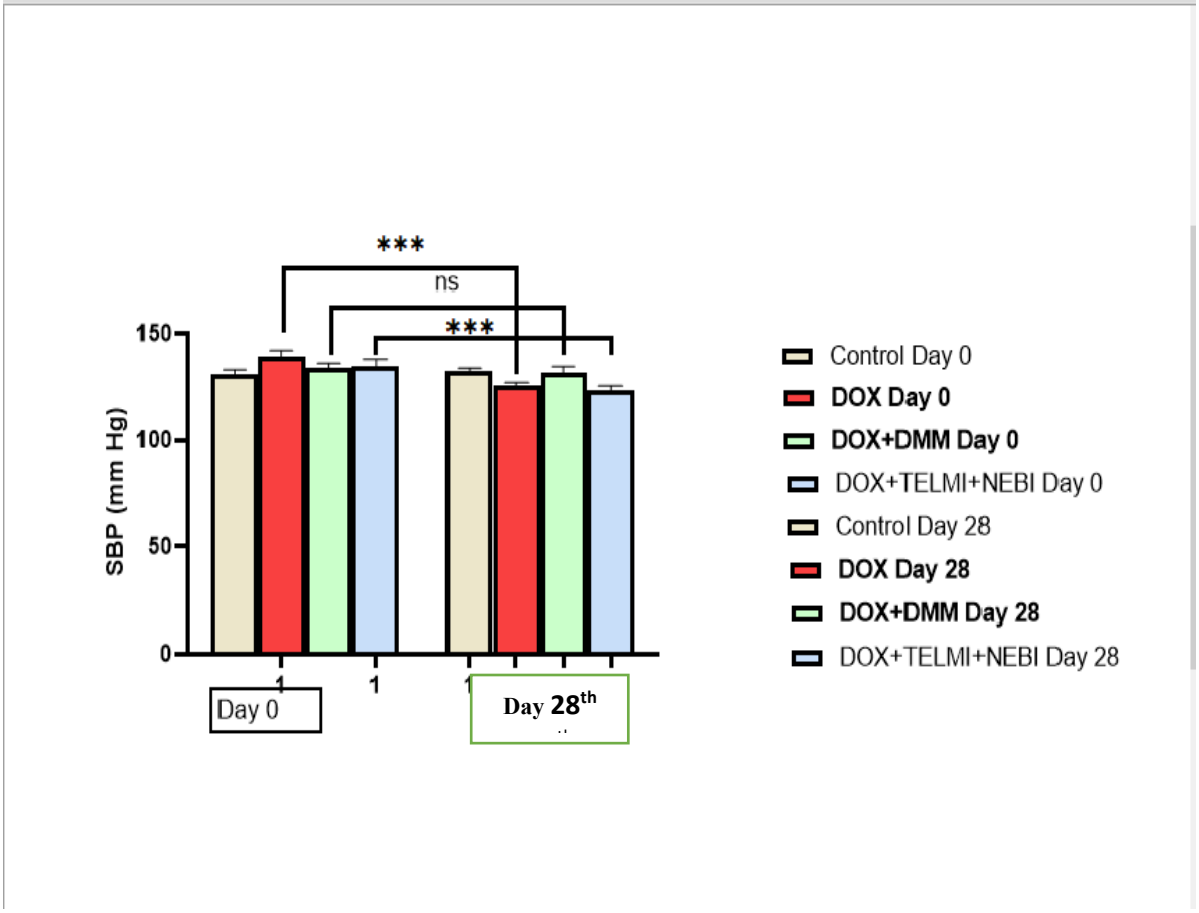


Figure 2(C). Represents mean ± SD of e non-invasive systolic blood pressure of rats of Control, Dox, DMM, Telmi+Nebi groups on days 0 and 28th. (ns-non-significant), (*p<0.001)**

6.3. DMM attenuates the dox-induced QTc prolongation-

The QTc in the DOX group was significantly prolonged (p<0.001, fig 3C) compared to the non-significantly prolonged QTc in the control group. QTc was significantly less prolonged (p<0,01) in both Dox+DMM and Telmi+Nebi treated groups compared to the Dox treated group.

Figure 3- ECG

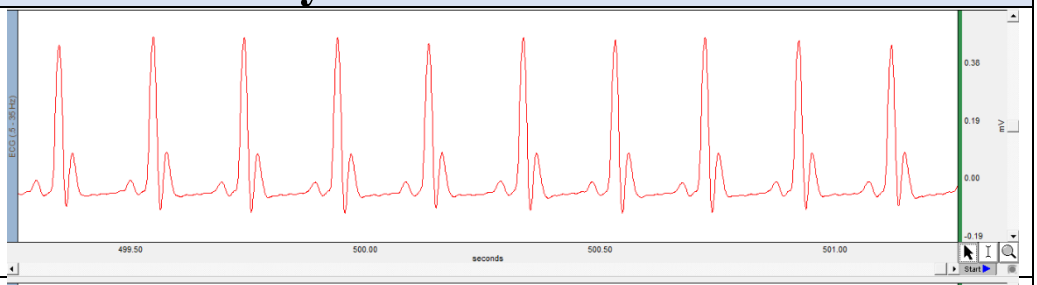
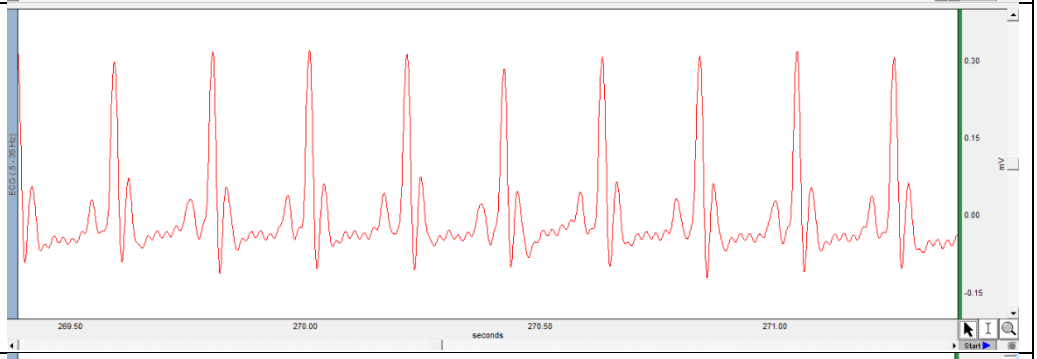
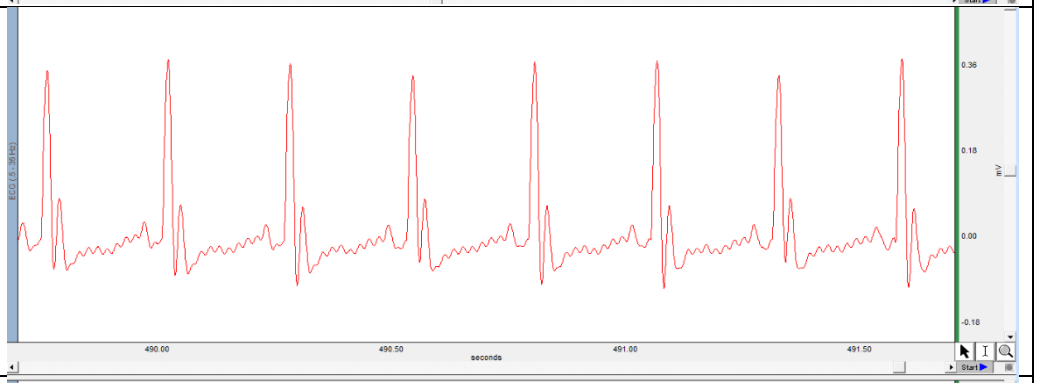
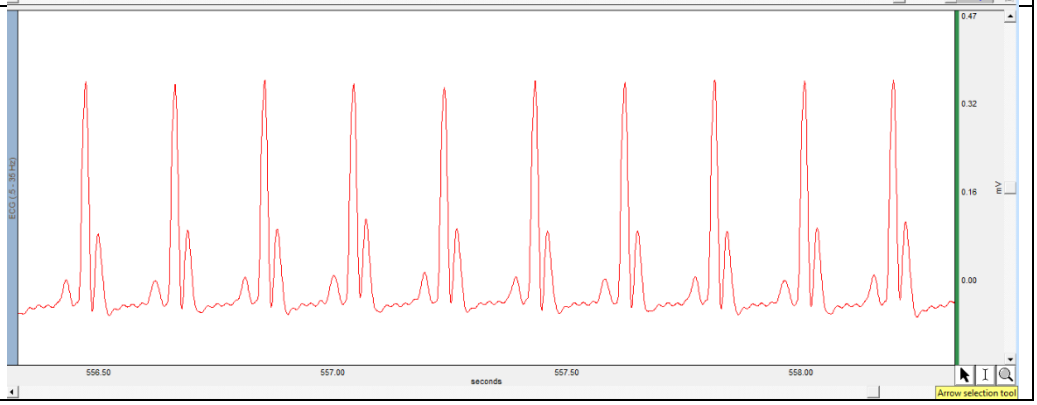
Group	Day 0
Control	
Dox	
Dox + DMM	
Dox+ Telmi+Nebi	

Figure- 3(A) ECG on day 0

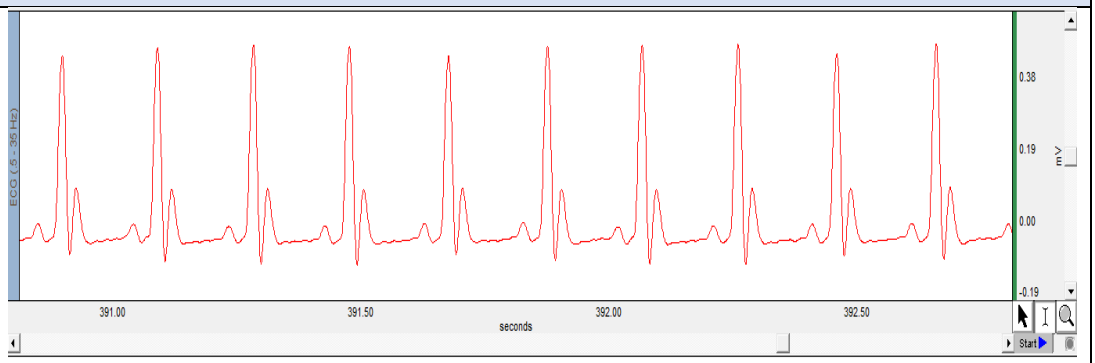
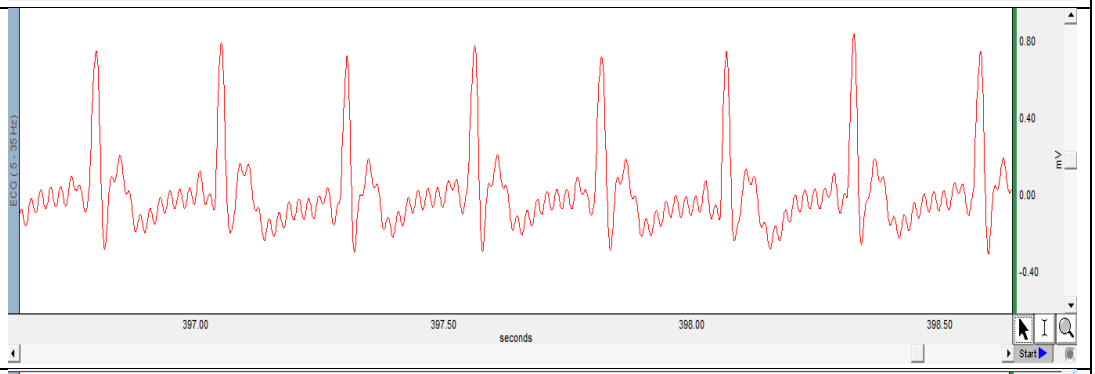
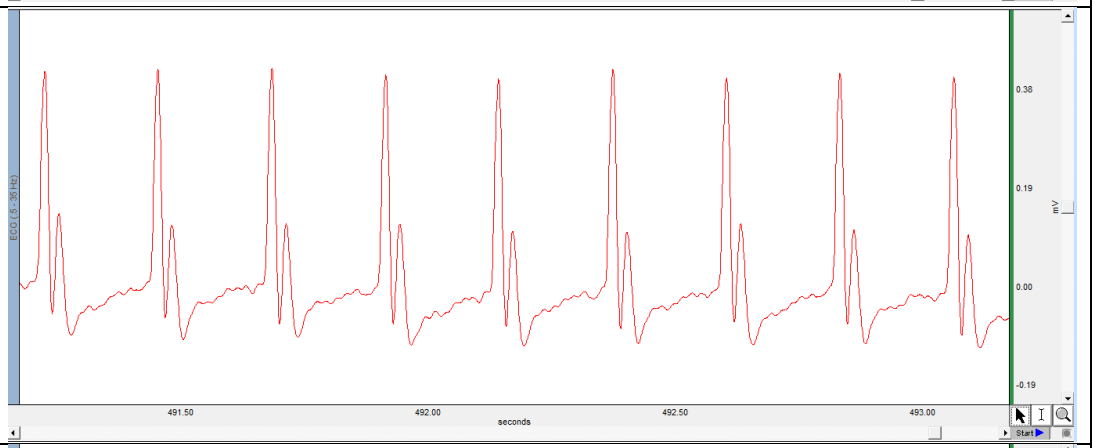
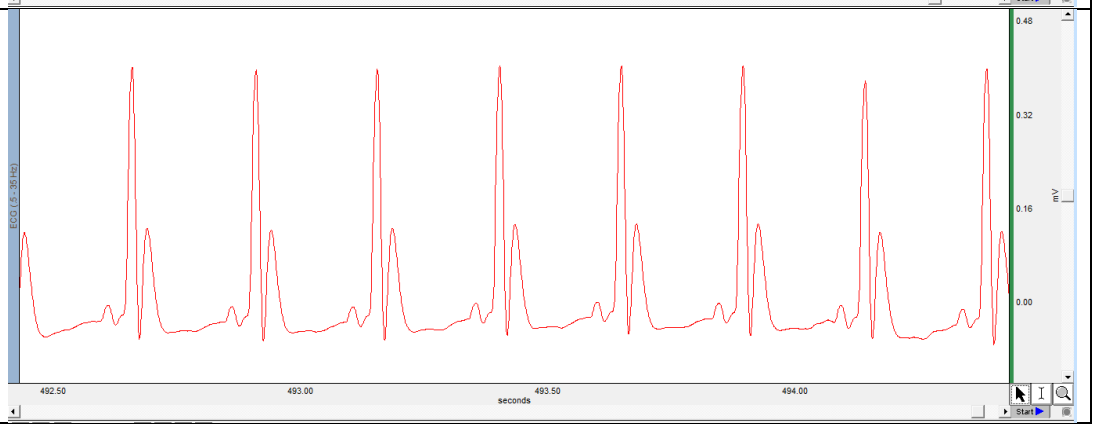
Group	Day 28th
Control	
Dox	
Dox + DMM	
Dox+ Telmi+Nebi	

Figure 3(B) ECG on day 28th

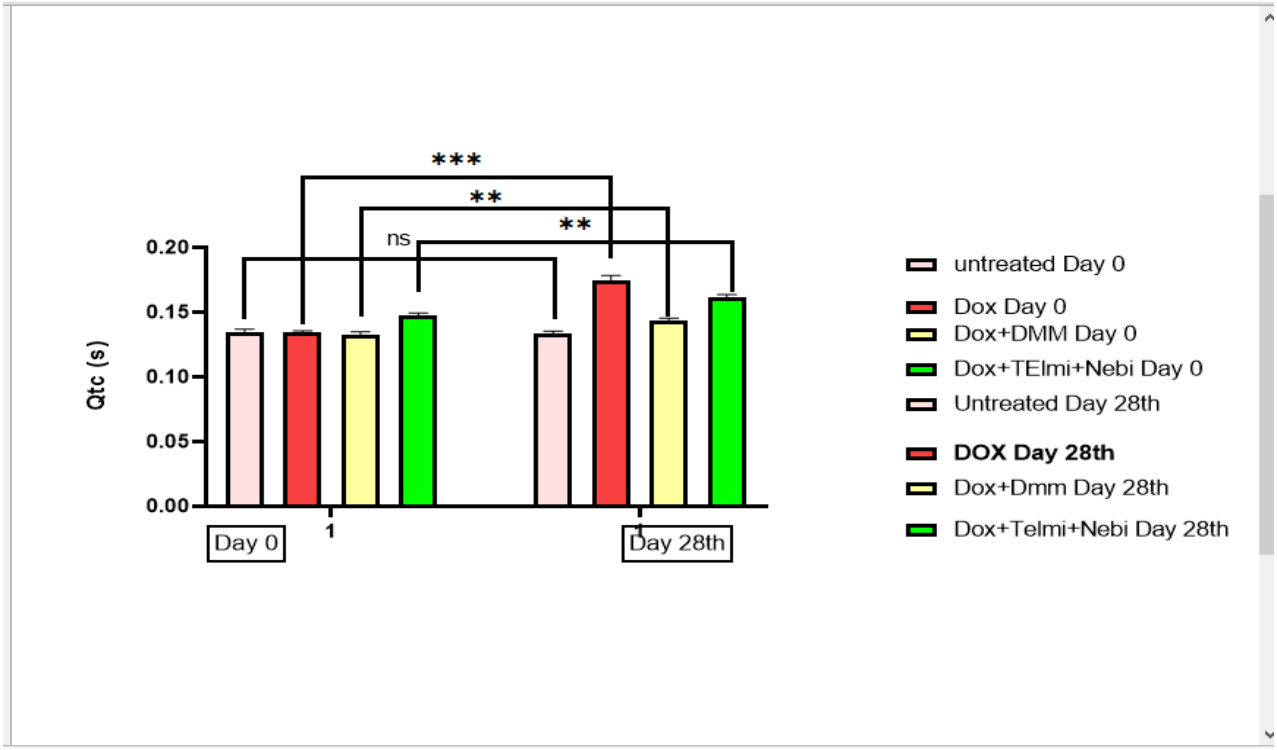


Figure 2(C). Represents mean ± SD of corrected QT of rats of Control, Dox, DMM, Telmi+Nebi groups on days 0 and 28th. (ns-non-significant), (*p<0.001, p<0.01)**

6.4. Heart weight and the ratio of heart weight to tail length-

The effect of Dox on heart weight is shown in Figure no 4. Compared to the normal control group, the heart weight to tail length ratio significantly increased ($p<0.001$) in the doxorubicin-treated group. In contrast, this ratio was significantly decreased in both DMM and Telmi+ Nebi groups compared to the dox group. ($p<0.001$)

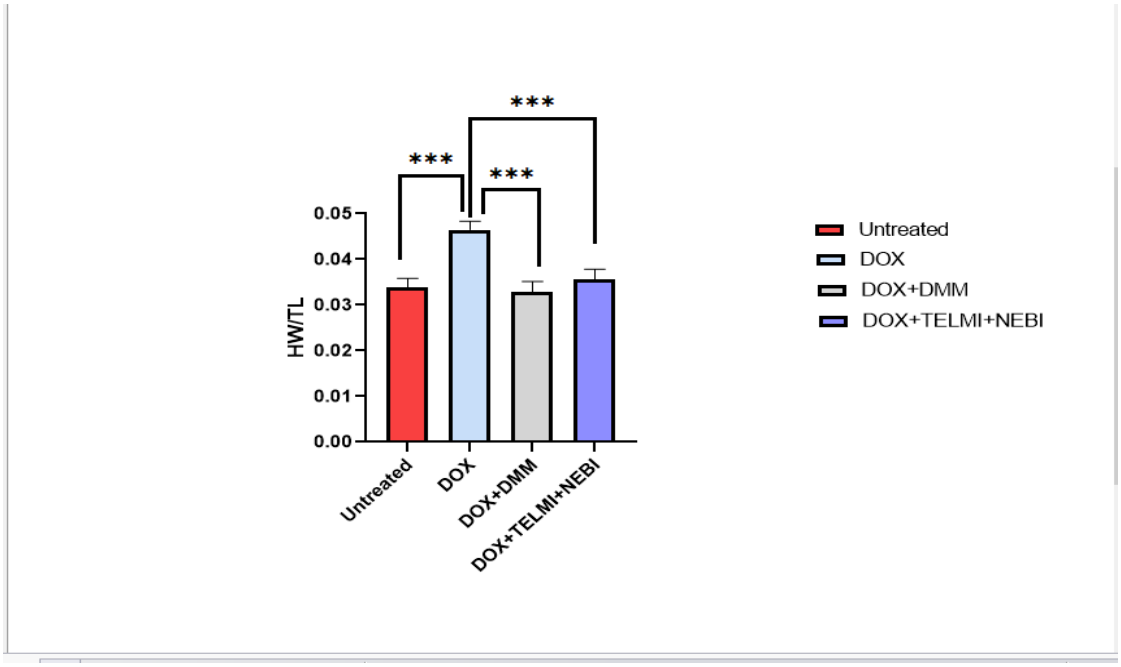


Figure 4- Represents mean ± SD of Heart weight vs tail length ratio of the rats of Control, Dox, DMM, Telmi+Nebi groups on day 0 and 28th. (*p< 0.001)**

6.5. Measurement of oxidative stress via measuring MDA level

MDA level of each group was measured at day 0 and 28th. Compared to the control group, serum MDA levels were noticeably increased in the DOX group. (P<0.001), (Pic-5). while the MDA level was decreased in both Dox+Dmm (Fig. 5, P < 0.01). and Telmi+Nebi group (Fig. 5, P < 0.05). compared to the DOX group (Fig. 5, P < 0.05).

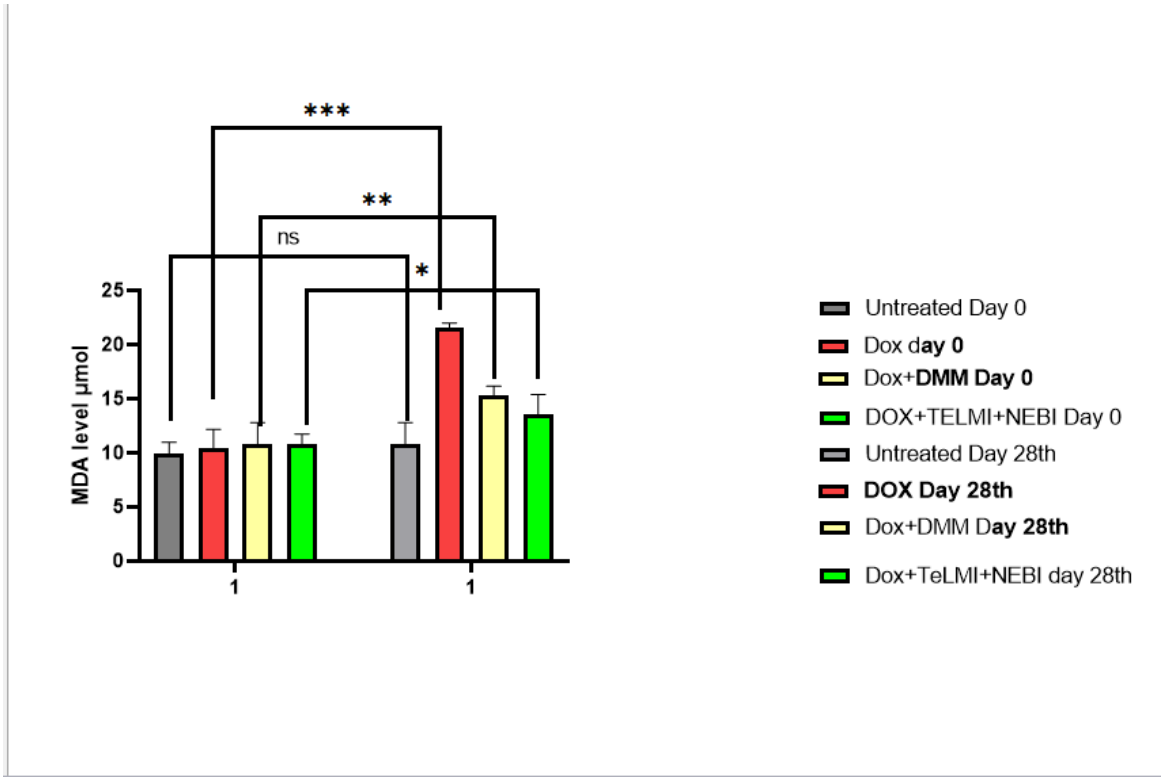


Figure 5- Represents mean ± SD of MDA level in µmol MDA equivalent of the rats of Control, Dox, Dox+DMM, Telmi+Nebi groups. (*p< 0.001, ns-non-significant)**

6.6. Measurement of total mitochondrial protein and it's activity by using Janus green

Total mitochondrial protein (TMP) was spectroscopically observed. TMP levels decreased significantly ($p<0.001$) in the dox treated group when compared to the control group, as shown in Figure 6(A), whereas TMP levels increased significantly ($p<0.001$) in the Dox+DMM group when compared to the dox group. TMP levels were also significantly greater in the Telmi+ Nebi group than in the dox group.

After determination of TMP value of each group, Janus green assay was performed. The dox group had the lowest absorption value. Total mitochondrial activity decreases in the dox-treated group compared to the control group, as seen in Figure 6(B). The absorbance value in the DMM and Telmi+Nebi groups was significantly greater than in the dox group. This suggests increased mitochondrial activity.

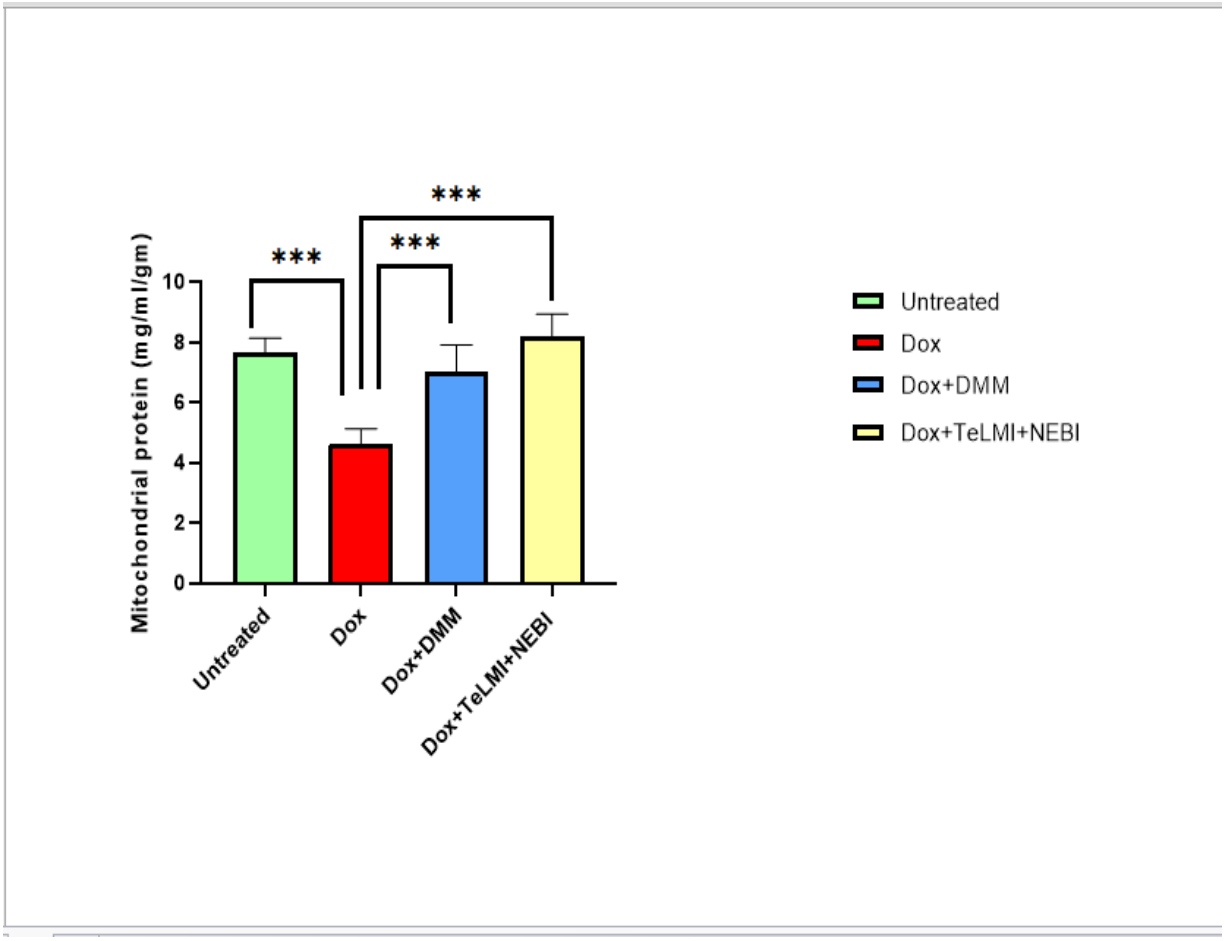


Figure 6(A) - Represents mean ± SD of Total mitochondrial protein from the hearts of Control, Dox, DMM, Telmi+Nebi group's rat. (*p< 0.001)**

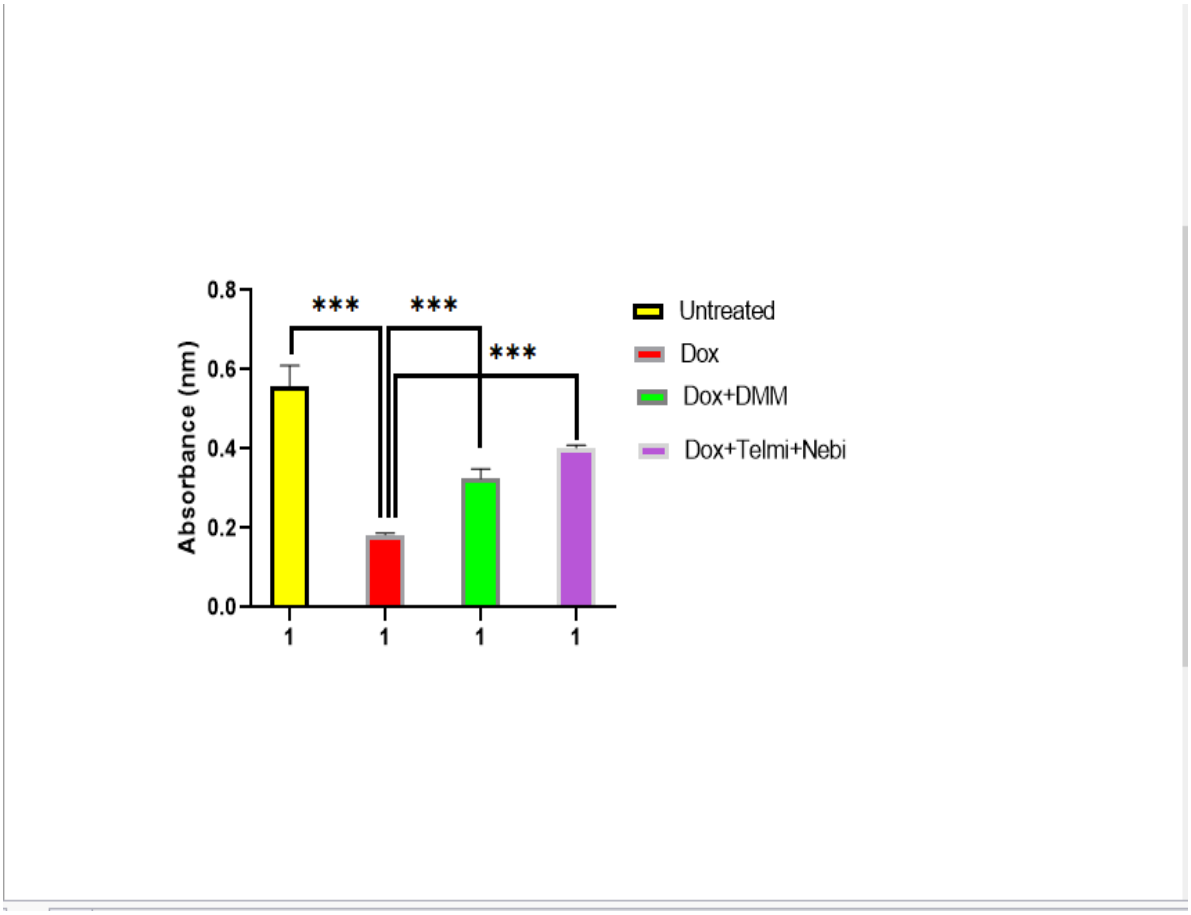


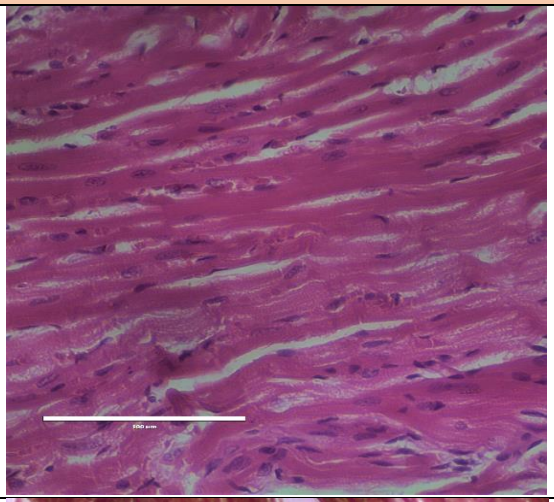
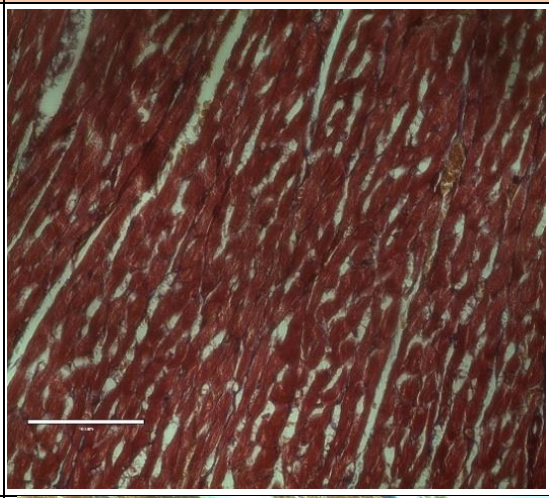
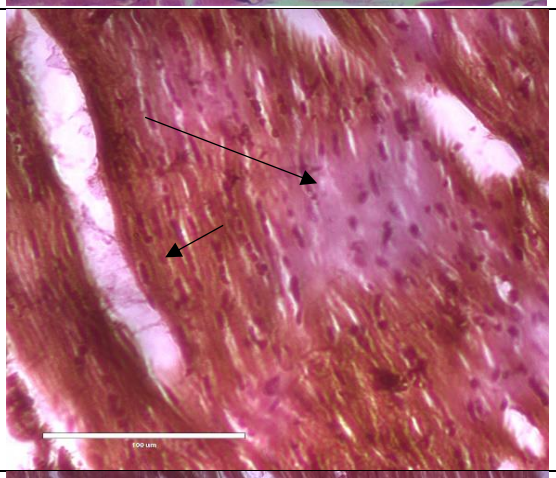
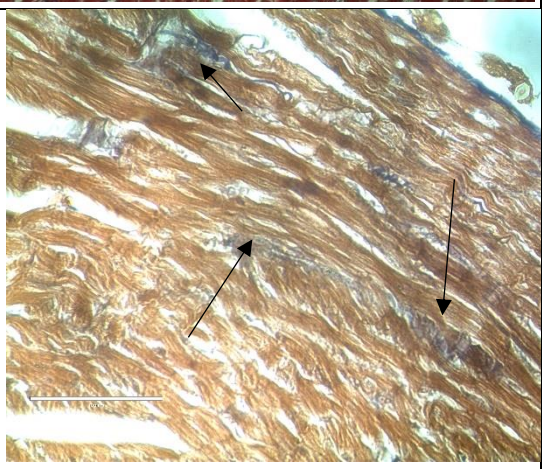
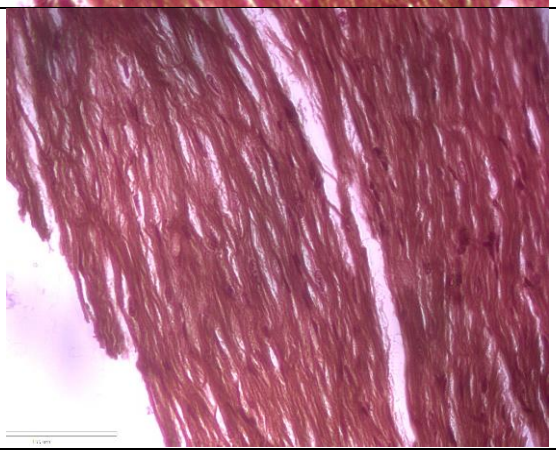
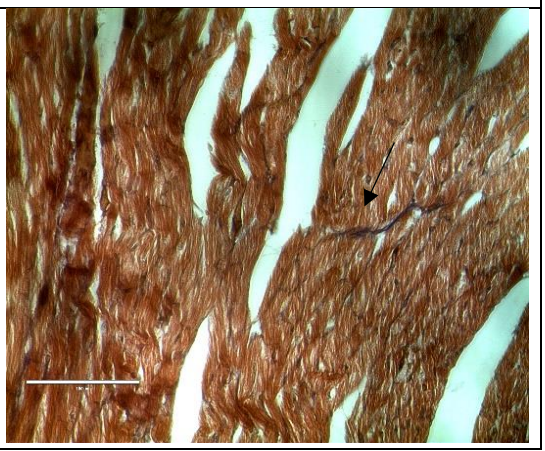
Figure 6(B) - Represents mean ± SD of the absorbance value of Janus green reduction of Control, Dox, DMM, Telmi+Nebi group's rat. (*p< 0.001)**

6.7. Histopathological changes

HE (Haematoxylin and Eosin assay) and MT (Masson's trichrome assay)

As per HE staining, myofibrils in the DOX group were thickened, crowded and swollen, indicating that they were more hypertrophic than myofibrils in normal heart tissue. In Dox+DMM and Telmi+Nebi groups, myofibrils' hypertrophic features are lower than in the Dox group.

MT staining of cardiac tissue shows more amount of blue area (representing fibre and collagen production) in dox-treated group. However, the blue area reduces in the Dox+DMM and Telmi+Nebi groups.

Group	HE staining of cardiac tissue	MT staining of cardiac tissue
Control		
Dox		
Dox + DMM		

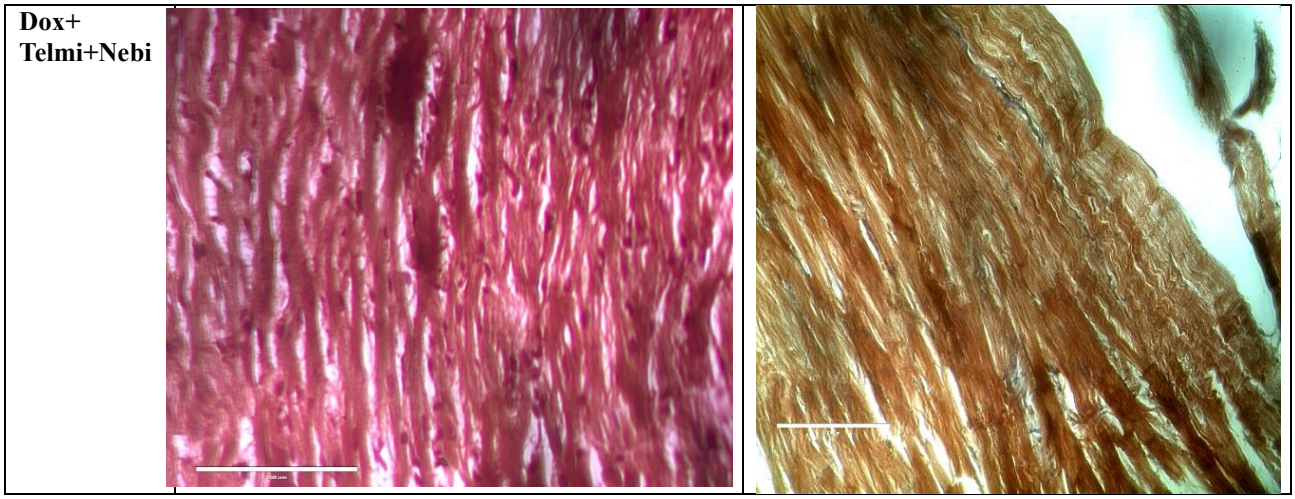


Figure 6 – HE (Haematoxylin and Eosin assay) and MT (Masson's trichrome assay) staining of cardiac tissue

7. Discussion

This discussion analyses the Cardioprotective effect of an unani medicine, DMM (Dawa-ul-Misk Motadil), against DOX-induced cardiac hypertrophy and impaired heart function. The following lines of evidence can be emphasised from the present study. Dawa-ul-Misk Motadil Sada is an Unani herbal tonic. It is said to strengthen the heart and possess cardioprotective effects [17]. The present study aims to investigate the cardioprotective effects of oral administration of DMM against Dox-induced cardiotoxicity Doxorubicin, a widely used anticancer drug, has shown remarkable efficiency in treating various cancers. However, it has potential adverse effects on the heart. Reactive oxygen species (ROS), mitochondrial dysfunction, angiotensin II, and calcium dysregulations play major roles in promoting doxorubicin-induced cardiac hypertrophy. Doxorubicin 2.5 mg/kg body weight i.p. in 6 equal injections alternatively for two weeks to make a total cumulative dose of 15 mg/kg body is sufficient to produce cardiotoxicity in albino rats. Telmisartan and Nebivolol are widely used in the treatment of hypertension. Also, these two drugs have been explored as a potential cardioprotective strategy in doxorubicin-induced cardiac hypertrophy models. Telmisartan is an angiotensin II receptor blocker (ARB), while Nebivolol is a beta-blocker. Both drugs have antioxidant property also [18]. This study uses a combination of these drugs as standard drugs. The cardioprotective effects of DMM have been compared with the standard drugs. In the Dox-

treated group, the animal fur became scruffy, and necrosis was observed at the site of dox injection. Changes in body weight are a sign of the overall protective effect of a drug. The change in body weight is the most intuitive, effective and basic indicator to evaluate the damage induced by chemotherapy drugs. The result obtained from the experiment indicates a significant decrease ($p<0.001$) in body weight in the doxorubicin (dox)-treated group of rats. However, when the doxorubicin treatment was combined with DMM, the reduction in body weight was lower compared to the doxorubicin group alone ($p<0.05$). In contrast, the standard group showed no significant changes in body weight. The decrease in body weight observed in the doxorubicin-treated group may be due to oxidative damage and induced cardiotoxicity [19], leading to muscle wasting and weight loss. The finding that the reduction in body weight was lower in the dox+ DMM group than in the doxorubicin group alone suggests that the DMM may have potential protective effects against doxorubicin-induced weight loss. Unani medicine, an ancient traditional system of medicine, utilises a combination of herbal, mineral, and animal-based formulations. These formulations often possess antioxidant, anti-inflammatory, and immunomodulatory properties that may counteract the adverse effects of doxorubicin and protect against weight loss. The presented results demonstrate the potential inhibitory effect of DMM on doxorubicin-induced changes in systolic blood pressure (SBP) in rats. The control group maintained a relatively stable SBP level, hovering around 135 mm Hg throughout the treatment period. This data suggests that the experimental conditions did not significantly affect the baseline blood pressure of the rats. In contrast, the doxorubicin-treated group exhibited a significant drop in SBP at day 28 compared to the control group ($p<0.001$). The reduction in SBP might be attributed to the cardiotoxic effects of doxorubicin on the heart, leading to impaired contractility and cardiac dysfunction. Interestingly, the DMM-treated group showed a non-significant decrease in SBP compared to the doxorubicin-treated group (ns). This finding suggests a potential trend towards mitigating the doxorubicin-induced drop in blood pressure. The Telmisartan and nebivolol treatment groups exhibited a significant decrease in SBP ($p<0.001$) compared to the doxorubicin-treated group. Telmisartan and Nebivolol are well-known anti-hypertensive medications that act through different mechanisms, including blocking angiotensin and beta-adrenergic receptors. The significant reduction in SBP observed in these treatment groups further supports the potential of these drugs in counteracting the detrimental effects of doxorubicin on blood pressure regulation. However, it is important to note that direct comparisons between the DMM and these standard anti-hypertensive agents were not performed in this study, limiting our ability to draw definitive conclusions regarding their relative efficacy. Doxorubicin damages cardiac cells via oxidative

damage, which decreases the strength and efficiency of the heart's contraction, reducing SBP. Or dox may affect endothelial cells lining the blood vessels, disrupting their function and impairing bp regulation. DMM may improve these conditions while inhibiting dox-induced pressure drop. The administration of doxorubicin has been shown to induce significant QTc prolongation ($p<0.001$, fig 3C) and was significantly attenuated by both DMM and Telmi+Nebi treated groups ($p<0.01$). Doxorubicin acutely lengthened the QT interval by selectively inhibiting IKs potassium channels; it can also block hERG (human ether-a-go-go) potassium channels, but the effect is less significant[20]. Blocking the potassium channel prolongs repolarisation, which leads to Qt prolongation. According to Bazett's formula. Qtc will also extend if Qt prolongs. We know from prior experiments that DMM increases K+ levels [21]; thus, we can hypothesise that boosting potassium levels of DMM may reverse the doxorubicin effect on QTc. The result of our study revealed a significant increase ($p<0.001$) in the heart weight/ tail length ratio (HW/TL) in the dox-treated group. These findings indicate that doxorubicin administration is associated with cardiac hypertrophy or enlargement of the heart due to fibrosis, collagen deposition or cardiomyocyte enlargement. In contrast, the Significant decrease ($p<0.001$) in HW/TL ratio in the Dox+DMM group Suggests a potential protective effect of DMM against dox-induced cardiotoxicity. The Telmi+Nebi treated group also shows a significant decrease in the ratio ($p<0.001$). Dox-induced cardiac fibrosis may happen by several mechanisms like oxidative stress, the release of inflammatory cytokines, mitochondrial dysfunction etc. All these can activate various pro-fibrotic factors and their associated pathway, such as TGF-β/ Smad pathway. As an unani medicine, DMM has potent antioxidative and anti-inflammatory potential; it may also have a protective effect on mitochondria that helps irrigate dox-induced cardiotoxicity. The level of MDA, an indicator of oxidative stress, was also significantly increased in the DOX group ($p<0.001$) as compared to the control group (ns- non-significant) and was effectively decreased by treatment with DMM. In the Dox+Dmm group, this rise in MDA level is significantly lower ($p<0.01$) than in the Dox group. Through in Telmi+Nebi group, MDA level reduction is significantly lower than the Dox+Dmm group ($p<0.05$).

MDA has been recognised as a relevant lipid peroxidation marker and an important biological marker of oxidative stress. However, oxidative damage is the primary mechanism of DOX toxicity. The level of MDA may reflect the degree of damage caused by DOX. The MDA levels measured in the present study confirmed that DMM effectively inhibited the oxidative stress induced by DOX. Doxorubicin generates ROS that may cause oxidative damage to mitochondrial proteins, leading to their degeneration via activating several proteolytic enzymes

like proteosomes and caspases. Dox can also damage mitochondrial DNA that may damage mitochondrial DNA (mtDNA). This damaged mtDNA can interfere with the transcription and translation process necessary for the mitochondrial protein. Mitochondrial proteins are primarily synthesised in the cytosol and then transported in the mitochondria through specific protein transporter. Doxorubicin may associate with disturbance in the protein transport mechanism, proteins left in the cytosol and degraded by the proteasome system. Doxorubicin also damages mitochondrial structure, therefore reducing its activity. As per the result of the study, total mitochondrial protein (TMP) declined in the dox-treated group compared to the control group. In the Dox+Dmm group, the TMP level increases significantly ($p<0.001$). Therefore, we can assume that Dmm may have mitochondrial protective activity. Mitochondrial dehydrogenases can convert Jeuns green (JG-B) to diethyl safranine. A better reduction value indicates better mitochondrial activity. As per histological data, cardiomyocytes appear as enlarged, swollen, irregular nucleus in Dox-treated group, whether in normal control group, the cardiac muscle fibres were found to be of uniform size, shape. The histopathological changes observed in the Dox -treated rats were similar to those previously reported, cardiac tissue consists of numerous fibrous tissues, that is attenuated in Dox+Dmm group along with Telmi+Nebi group.

8. Conclusion

This experiment highlights the potential cardioprotective effects of the Unani formulation Dawa-ul-Misk Motadil (DMM) against doxorubicin-induced cardiac hypertrophy and impaired heart function in laboratory animals exerted through its antifibrotic effect, restoration of histoarchitecture of heart and mitochondrial function. DMM may play significant role on the potassium channel, indicated by the attenuation of the QTc lengthening. Doxorubicin-induced QTc interval lengthening is of special concern since it can cause a deadly polymorphic ventricular tachycardia called torsade de pointes (TdP). We believe that DMM may also address torsade de pointes (TdP) generated by doxorubicin toxicity. In combination with doxorubicin, DMM has therapeutic promise and could be used as an adjuvant therapy.

9. References

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