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CARDIOPROTECTIVE EFFECT OF FULVIC ACID ON DOXORUBICIN INDUCED CARDIAC OXIDATIVE STRESS IN RATS

Tabassum S. Shikalgar * and Nilofar S. Naikwade

Department of Pharmacology, Appasaheb Birnale College of Pharmacy, Sangli - 416416, Maharashtra, India.

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Cardioprotective, Doxorubicin, Fulvic acid, Antioxidant, Cardiotoxicity

Correspondence to Author:
Tabassum S. Shikalgar

Assistant Professor, Department of Pharmacology, Appasaheb Birnale College of Pharmacy, Sangli - 416416, Maharashtra, India.

E-mail: tabbu_shikalgar@rediffmail.com

ABSTRACT: The aim of study was to evaluate cardioprotective activity of fulvic acid in doxorubicin induced cardiac toxicity. In current investigation, female/male wistar rats were divided into five groups, normal, control, fulvic acid 100 mg/kg, fulvic acid 200 mg/kg, and fulvic acid 300 mg/kg. Doxorubicin 15 mg/kg was administered in six divided doses to all except normal group for 2 weeks. Fulvic acid was administered to respective groups once daily for total 21 days, on the last day of study, the animals were anesthetized to record ECG and BP (by cannulating carotid artery), blood was collected from carotid artery and SGOT, LDH and CK-MB were estimated, animals were sacrificed to isolate heart and preparation of tissue homogenate. The antioxidant status is analyzed by measuring MDA content, SOD, CAT, GSH activity. The tissue sample is also preserved for histological studies. Doxorubicin cause cardiac damage which was manifested by alteration in serum cardiac markers, antioxidant markers, ECG and hemodynamic and histological changes. Which were assuaged due to treatment with fulvic acid 300 mg/kg. With data obtained in study it have been concluded that fulvic acid treatment for 3 weeks shield the heart of rat from cardiotoxicity.

INTRODUCTION: Doxorubicin, also known as Adriamycin® or Rubex®, is an anthracycline antibiotic that was discovered from a mutated strain of *Streptomyces peucetius* [1]. Doxorubicin is useful anticancer drug because it's used with other chemotherapeutic drug in treatment of acute leukemia, Hodgkin's and non-Hodgkin's lymphomas, bone and soft tissue sarcoma, Wilms cancer and many other malignant neoplasm [2].

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However, it also carries a risk of adverse effects that might leave an unfavourable damage to cancer patients. Chemotherapy-induced cardiotoxicity is a serious complication that limits the clinical use of chemotherapeutic agents, particularly the anthracyclines, since it could eventually culminate in the development of life-threatening cardiomyopathy [3].

The acute toxicities are myelosuppression, nausea, vomiting and arrhythmias are reversible while the chronic toxicities such as cardiomyopathy and heart failure are irreversible and unmanageable. The exact mechanism is still not known but the possible mechanisms have been postulated by the various researchers such as generation of oxidative stress, induction of apoptosis, activation of renin-angiotensin system (RAAS), oxidative stress

induced DNA damage, lipid peroxidation and impairment of enzyme activity of creatinine kinase [4]. The heart is particularly susceptible to free radical injury, as it contains less free radical detoxifying enzymes (superoxide dismutase, glutathione and catalases) than the other metabolic organs such as liver or kidney. Generation of reactive oxygen species (ROS) is the main side effect of doxorubicin. At cellular level, quinone form of doxorubicin gets reduced to semiquinone by interacting with NADH or NADPH oxidases. Semiquinone undergoes autooxidation in presence of oxygen and generates superoxide anion and then free radicals [5].

Many of naturally occurring herbs and antioxidant substances has produced protective action against doxorubicin induced cardiac stress. Many of naturally occurring substances may prove their cardioprotective potential because of their antioxidant ability in problems like ischemia reperfusion and doxorubicin induced cardiotoxicity. Polyherbal formulation/ antioxidant compounds have shown protective effect in doxorubicin induced cardiotoxicity without reducing their therapeutic efficacy. Moreover there is a growing interest in the usage of natural antioxidants as a protective strategy against cardiovascular related problems in experiments such as ischemia reperfusion and doxorubicin induced cardiotoxicity [6]. Even the drugs like probucol also had proved their cardio protective action in this model [7].

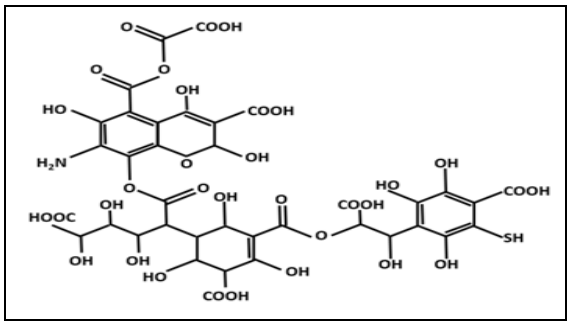

FIG. 1: ON AVERAGE CHEMICAL FORMULA AND STRUCTURE OF FULVIC ACID [8]

Its average Chemical formula is $C_{37}H_{33}NSO_{33}$ [8]. Fulvic acid (FA) is a class of compound including humic substances together with humic acid and humin. It is formed through the degradation of organic substances by chemical and biological process. FA consists of a mixture of closely related complex aromatic polymers with the presence of aromatic rings, phenolic hydroxyl, ketone carbonyl, quinone carbonyl, and carboxyl and alkoxyl groups. The possible application of fulvic acid has reported for antimicrobial and anti-inflammatory property, actually, it is used as a soil supplement in agriculture and as a human nutritional supplement, fulvic acid has been screened for antioxidant activity. As it have antioxidant properties, FA partially support the health beneficial properties therefore, will become a good candidate to be used in pharmaceutical or food industries as an source of natural antioxidants [9].

Fulvic acid is stated as a natural antioxidant by many nutritional specialists. The various basis have been proposed which include that fulvic acids greatly enhance the bioavailability of important trace minerals. It regenerate and prolong the residence time of essential nutrients in the cells. It also modify the damage or toxic compounds such as heavy metals and free radicals, enhance the permeability for digestive, circulatory, and cell membranes. Fulvic acid minerals are thought, by leading natural health experts, to be one of the most important "missing links" in the modern food chain. Medical and agricultural research continues to conclusively point to one fact: fulvic acid minerals either directly or indirectly may hold the keys and solutions to many of the world's health problem.

Fulvic mineral complexes are the world's finest electrolyte, which improves energy function, increases assimilation, stimulates metabolism, restores electrochemical balance, reduces high blood pressure, enhances nutrients, and helps rebuild the immune system. Fulvic acid is yet not well known or understood by medical community regarding its health benefits to heart. It has extremely complex nature. Various claims of encouraging health benefits by the public have been simply remarkable. Many of these health assertions have also shown that they could be disease preventative in nature and may dramatically increase longevity. Yet until now, fulvic acid has been entirely overlooked by the majority of alternative health concerns as well [10]. Fulvic acid is one of the ingredients present in humus and have been well documented for having antithyroid and immunomodulating activity [11].

Humic substances also have been reported for having anti-inflammatory as well as immuno-modulatory activity and it is safe upto 1.8 g/day [12].

In China, humic and fulvic acids had been used in hospitals and among the general population for the treating of a wide range of diseases with success and where it is referred to as 'gold medicine' or 'Wu Jin San' [13].

Fulvic acid benefits are described tremendously in many literatures but scientific studies are still lacking to elaborate its protective action in animal models, hence we have made an attempt to evaluate its aptitude for being a cardio protective aspirant.

MATERIALS AND METHODS:

Drugs and Chemicals: Fulvic acid was purchased from Mana life (Wanek Medical Center, Greensboro NC). Doxorubicin was a gift sample from Cipla Goa. Urethane (Himedia India), DTNB, heparin, *n*-butanol, Pyridine, Sodium dodecyl sulphate, Conc. HCL, Hydrogen peroxide, Ammoium acetate, Potassium hydrogen phosphate, Formalin, Potassium dihydrogen phosphate, Trichoro Acetic Acid (all from Research-lab Fine Chem Industries Mumbai), Pyrogallol (Sigma Aldrich, Pvt. Ltd., Bangalore) Thiobarbituric acid (Loba chemicals (Mumbai). All chemicals were of analytical grade, the diagnostic kits from Corals clinical systems were used for serum SGOT, LDH and CK-MB analysis.

Experimental Animals: Wistar albino rats of either sex weighing 200 ± 30 gm were randomly selected from animal house of Apppassheb Birnale College of Pharmacy, Sangli. They were housed in air conditioned room in polypropylene cages lined with rice husk. The animals were fed on conventional diets and had free access to water. They were maintained under standard conditions of humidity (45% - 55%), temperature (25 ± 2 ºC) and light/dark cycle (12 h: 12 h).

The experiments was carried after approval and clearance from Institutional Animal Ethics Committee constituted in accordance of CPCSEA, India (Protocol no. ABCP/IAEC/ 03/2012-13)

Preparation of Drug Solution: Fulvic acid is dissolved in chlorine free water, Doxorubicin in prepared in normal saline.

Experimental Design and Protocol:

Animal Grouping and Procedure: A total no. of 30 animals were divided into five groups contain six animals in each.

Group I: Served as normal, receive water 5 ml/kg of body weight p.o. and

Group II: Control animal were treated with doxorubicin 2.5 mg/kg of body weight i.p. for six times in 2 weeks on alternate days (Water up to 7 days + Doxorubicin *i.e*. DXR of 2.5 mg/kg i.p. Injected on 8^{th}, 10^{th}, 14^{th}, 16^{th}, 18^{th} and 21^{st} day to reach total cumulative dose of 15 mg/kg).

Group III: Received fulvic acid 100 mg/kg up to 21 days + Doxorubicin *i.e*. DXR of 2.5 mg/kg i.p. Injected on 8^{th}, 10^{th}, 13^{th}, 16^{th}, 18^{th} and 21^{st} day to reach total cumulative dose of 15 mg/kg).

Group IV: Received fulvic acid 200 mg/kg up to 21 days + Doxorubicin *i.e*. DXR of 2.5 mg/kg i.p. Injected on 8^{th}, 10^{th}, 13^{th}, 16^{th}, 18^{th} and 21^{st} day to reach total cumulative dose of 15 mg/kg).

Group V: Received 300 mg/kg up to 21 days + Doxorubicin *i.e*. DXR of 2.5 mg/kg i.p. Injected on 8^{th}, 10^{th}, 13^{th}, 16^{th}, 18^{th} and 21^{st} day to reach total cumulative dose of 15 mg/kg). All animals were observed for whole study period for appearance, behaviour, occurrence of necrosis at the site of administration, and mortality. Before and after completion of experimental period body weights were recorded. Heart/body weight ratio was also calculated using formula heart weight/body weight X1000.

Electrocardiogram: 24 h after last dose of doxorubicin, animals were anesthetized by injecting urethane 1.25 mg /kg. i.p. and then taken for ECG recording using lead II. This recording are done using digital, 4 channel data acquisition system Biopac MP35. Santa Barbara California USA. The ECG records include QT interval, ST elevation, and QRS complex duration.

Hemodynamic Parameters: 24 h after last dose of doxorubicin, animals were anesthetized by injecting urethane 1.25 mg /kg. i.p. The animals are dissected further and left carotid cannualation is performed using PE 50 cannula. The cannula is filled with heparinised saline (100 IU/ml) and

connection is made to pressure transducer to measure the systolic BP, diastolic BP and heart rate parameters. This recording are done using digital, 4 channel data acquisition system Biopac MP35. Santa Barbara California USA.

Serum Parameters: Blood was collected from carotid cannualation in plane tubes. Serum was separated after centrifuging the samples at 3000 rpm for 20 min. The serum samples were analyzed for determination of levels of SGOT, CK-MB; LDH using standard kit according to manufactures instructions using semiautoanalyser (Mispa Plus).

Antioxidant Parameters: Animals were euthanized and hearts tissue were quickly dissected out and washed in ice cold phosphate buffer, dried on filter paper and quickly weighed. A 10% w/v tissue homogenate is prepared in ice cold 0.05 M phosphate buffer using tissue homogenizer. The chilled tissue homogenate was used for estimation of level of MDA, SOD, CAT, Glutathione, and total protein.

Measurement of Lipid Peroxidation: The thio-barbituric acid reactive substance (malondialdehyde) was measured as a marker of lipid peroxidation by method of Okhawa. In a mixture of 0.4 ml of 10% tissue homogenate 1.5 ml 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8 % TBA solution were added. The mixture was heated at 95 ^{0}C for 60 min. the solution is then cooled to room temperature. After cooling 5 ml of *n*-butanol-pyridine (15:1) was added. The mixture is vortexed thoroughly and allow to stand until the organic and aqueous layers get separated. Further absorbance of organic layer was measured at 532 nm on UV/Visible spectro-photometer (Jasco V- 550). The MDA level was calculated using molar extinction coefficient of 1.56×10^{5} M^{-1} cm^{-1} [16].

Measurement of Superoxide Dismutase: SOD activity was measured by determining the ability of sample to inhibit autooxidation of pyrogallol by Improved Pyrogallol Autoxidation Method. One unit of SOD activity one unit of SOD activity is defined as amount of enzyme required to inhibit the rate of pyrogallol autooxidation by 50%. At pH 7.4, 50 µL of sample solution was mixed with 2950 µL of Tris-HCl buffer (0.05 M, pH 7.4, 37 °C) containing 1 mM Na_2EDTA and 50 µL of pyrogallol (60 mM in 1 mM HCl, 37 °C) and then rapidly shaken by hand at 37 °C.

The absorbance was measured against the Tris-HCl buffer every 30 s for 5 min at 325 nm using UV visible double beam spectrophotometer (Jasco V-550). The oxygen radical scavenging ability was calculated as

$$\{\frac{\Delta A \text{ (control)}- \Delta A \text{ (test)}}{T}\} \Big/ \frac{\Delta A \text{ (control)} \times 100}{T}$$

Here, ΔA (control) is the increase in Absorbance at 325 nm of the mixture without the sample and ΔA (sample) is that for the mixture with the sample; T = 5 min [14].

Measurement of Catalase Activity: Catalase enzyme degrades hydrogen peroxide (H_2O_2) into oxygen and water. Ultraviolet absorption of H_2O_2 can be measured at 240 nm. In the presence of catalase, absorption decreases due to degradation of H_2O_2. 0.1 ml of tissue homogenate is mixed with 1.0 ml freshly prepared hydrogen peroxide and 1.9 ml phosphate buffer into cuvette. Blank were similarly prepared without tissue homogenate. Absorption of test was measured at 240 nm against blank. On using UV-visible double beam spectrophotometer (Jasco V- 550). For at least 3 min at 240 nm. Activity of CAT was expressed in unit/mg of protein and calculated using molar extinction coefficient 43.6 M^{-1} cm^{-1} [15, 16].

Measurement of Glutathione Activity: GSH reacts with Ellman's reagent (5, 5-dithio bis Nitrobenzoic acid or DTNB) to produce a chromophore Thio Nitrobenzoic acid (TNB) that give maximal absorbance at 412 nm. Absorbance value can give the estimation of enzyme value. 1 ml of 10% tissue homogenate is mixed with 1 ml 20% trichloroacetic acid (TCA) containing 1mM EDTA and mixture is centrifuged for 10 min at speed 1000 rpm. In 1 ml supernantant 0.5 ml DTNB solution and 3 ml Phosphate Buffer were added. In 0.2 ml of supernantant is added to new set of test tubes containing 1.8 ml of Ellmans reagent (0.1 mM DTNB (5, 5'-dithio bis-2-nitro-benzoic acid) prepared in 0.3 M Phosphate buffer containing sodium citrate). Mixed well. Absorbance is measured the at 412 nm using UV visible double beam spectrophotometer (Jasco V- 550) against blank.

The amount of glutathione is Calculate using the molar extinction coefficient 13600 M^{-1} cm^{-1} [17, 16].

Total Protein: Total protein is estimated using method of lawry. 0.5 ml of tissue homogenate is mixed with 0.5 ml of 10% TCA and centrifuged for 10 min. Precipitate is dissolved in 0.1N NaOH. 0.1 ml of aliquot from above solution was mixed with 5 ml alkaline copper reagent and allowed to stand at room temperature for 10 min. 0.5 ml of Folin's phenol reagent was added and kept for 20 min to develop blue colour. Absorbance was read at 640 nm. The protein concentration was determined by using standard curve which prepared by using standard sample of bovine serum albumin [18].

Histology of Heart: The heart tissues were excised and placed overnight in 10% buffered formalin. Dehydration and further impregnation is carried out in paraffin wax. Specimen were cut into thin sections and stained with hematoxyline and eocin. The sections were mounted by diestrene phthalate xylene. The slides were prepared by professional technically expert person. The heart sections were further assessed for infiltration of inflammatory cells, necrosis and other inflammatory changes. The slide micrographs were taken using Saglo soft microimaging software from Saglo research equipments Pvt. Ltd., India.

Statistical Analysis: The data was expressed as mean ± SEM. Statistical analysis was performed by one way analysis of variance followed by Sidak's multiple comparision test Using graph pad prism 7 software for windows (GraphPad software, San Diego CA, USA). P value < 0.05 was considered statistically significant.

RESULTS: Chronic administration of doxorubicin induced cardiac toxicity and effects of fulvic acid administration was demonstrated analyzing levels of cardiac biomarker enzymes and endogenous antioxidant. The results are supported by additional finding related to histology and hemodynamic studies. The general appearance of all groups of animals was recorded during the study period.

Heart Weight to Body Weight Ratio: Table 1 shows effect of treatment with fulvic acid on doxorubicin- induced changes in body weight, heart weight, and % mortality. The control group rats showed a significant (p<0.05) alteration in heart to body weight ratio as compares to normal group. On treatment with fulvic acid 200 mg/kg and 300 mg/kg for a period of 21 days significantly increases heart to body weight ratio when compared to doxorubicin control group, while treatment with fulvic acid 100 mg/kg produces non significant increase in ratio when compared to doxorubicin group.

TABLE 1: EFFECT OF TREATMENT WITH FULVIC ACID ON DOXORUBICIN - INDUCED CHANGES IN BODY WEIGHT, HEART WEIGHT AND % MORTALITY

Groups	Body weight	Heart weight	Heart/Body weight %	Mortality %
Normal	208 ± 1.99	0.745 ± 0.0165	3.52 ± 0.103	0
Control	227 ± 2.63##	0.635 ± 0.0449#	2.94 ± 0.15##	17
Fulvic acid 100 mg/kg	232 ± 6.1	0.72 ± 0.0239ns	3.11 ± 0.126	0
Fulvic Acid 200 mg/Kg	232 ± 2.86	0.75 ± 0.0171*	3.33 ± 0.0945*	0
Fulvic Acid 300 mg/Kg	233 ± 2.7	0.773±0. 0161**	3.38 ± 0.0333*	0

Data are expressed as mean ± SEM (n=6) and analyzed by one way ANNOVA followed by Sidak's multiple comparision test,* P <0.05, **P<0.01, ***p<0.001, ****p<0.0001 compared with positive control group, # P <0.05, ##P<0.01, ###p<0.001, ####p<0.0001 compared with normal group.

Elecrocardiographic Changes and Hemo-dynamic Changes: Table 3 shows the effect of fulvic acid on electrocardiographic changes in different groups. The control group showed significant increase in ST voltage, QRS duration and QT interval as compared to normal. Treatment with fulvic acid 300 mg/kg produced significant modification in value.

The **Fig. 2 (E)** also reflect the restoration of deflections due to treatment with fulvic acid 300 mg/kg, Treatment with fulvic acid 200 mg/kg and 100 mg/kg produced no significant modifications in the values when compared to control. But **Fig. 2 (C)** and **2(D)** reflect mild changes in ST elevation due to treatment.

The doxorubicin treated animals showed significant reduction in systolic BP (P<0.001) and non significant reduction in diastolic BP compared to vehicle control group **Table 2**. The treatment with fulvic acid 300 mg/kg, significantly (p<0.05) raise level of systolic and non-significant increase in diastolic BP. While other group treated with 200 mg/kg and 100 mg/kg are also cause non significant increased level of systolic and diastolic BP.

Heart rate in control group is reduced significantly ($p<0.05$) (**Table 2**) in contrast to normal group, the group treated with fulvic acid 100 mg/kg produced non-significant increase in heart rate, while group 200 mg/kg and 300 mg/kg produced significant increase ($P<0.05$ and $P<0.001$ respectively) in heart rate.

Evaluation of Serum Parameters: Table 3 shows the effect of fulvic acid on cardiac markers, control group showed significant alteration ($p<0.0001$) in level of serum SGOT, LDH and CK-MB when compared to normal group. The oral treatment with fulvic acid 200 mg/kg and 300 mg/kg significantly reduces ($P<0.001$) elevated level of these marker enzyme. While fulvic acid 100 mg/kg also significantly ($p<0.05$) reduces level of cardiac markers but with comparatively less intense than fulvic acid 200 mg/kg and 300 mg/kg treated groups.

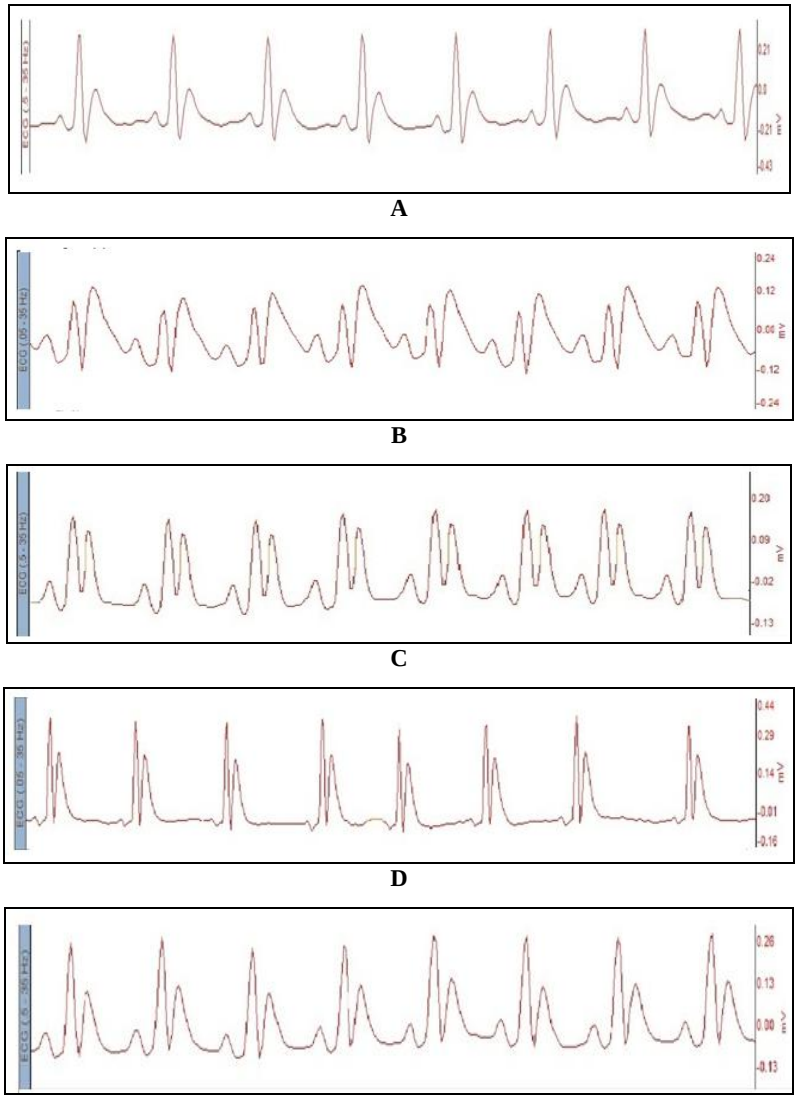

FIG. 2: SHOWS ECG ALTERATION OF NORMAL, CONTROL AND FULVIC ACID TREATED GROUPS

(A) ECG of Normal, (B) ECG of control, (C) ECG of animal treated with fulvic acid 100 mg/kg, ECG of Group treated with fulvic acid 200 mg/kg, ECG of animal treated with fulvic acid 300 mg/kg.

TABLE 2: EFFECT OF FULVIC ACID ON ECG PARAMETERS AND BP

Groups	ST elevation (mv)	QRS complex (ms)	QT interval	Systolic BP	Diastolic BP	Heart rate
Normal	0.0808 ± 0.00788	33.7 ± 0.422	75.5 ± 0.342	132 ± 0.253	95.4 ± 1.98	356 ± 0.401
Control	0.129 ± 0.0.00413###	39.3 ± 1.67#	96.7 ± 2.11###	117 ± 2.373###	89.2 ± 2.21 ns	323 ± 3.36#
Fulvic acid 100 mg/kg	0.108 ± 0.0114ns	35 ± 1.29ns	94.2 ± 3.27ns	121 ± 2.38ns	91.4 ± 2.16 ns	327 ± 3.36ns
Fulvic acid 200 mg/Kg	0.113 ± 0.00558ns	33.3 ± 1.67*	91.8 ± 3.77ns	125 ± 2.97ns	90.8 ± 0.63 ns	333 ± 4.98*
Fulvic acid 300 mg/Kg	0.0995 ± 0.00655*	30.08 ± 0.833***	77.2 ± 2.46****	126 ± 1.78*	94.8 ± 2.08 ns	345 ± 2.68***

Data are expressed as mean ± SEM (n=6) and analyzed by one way ANNOVA followed by Sidak's multiple comparison test,* P <0.05, **P<0.01, ***p<0.001, ****p<0.0001 compared with positive control group$^{\#}$ P <0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001 compared with Normal group.

TABLE 3: EFFECT OF FULVIC ACID ON SERUM BIOCHEMICAL PARAMETERS

Groups	SGOT µ/L	LDH µ/L	CK-MB µ/L
Normal	91.5 ± 5.79	421 ± 7.27	273 ± 10.2
Positive	177.8 ±	571 ±	363 ±
Control	13.86####	19.4####	5.58####
Fulvic acid	97.09 +	476 ±	333 ±
100 mg/kg	2.98****	17.5***	15.7*
Fulvic acid	96.01 +	454 ±	300 ±
200 mg/Kg	7.057****	15.7****	10.3***
Fulvic acid	101.4 +	435 ±	296 ±
300 mg/Kg	5.879****	14.7****	5.13***

Data are expressed as mean ± SEM (n=6) and analyzed by one way ANNOVA followed by Sidak's multiple comparision test,* P <0.05, **P<0.01, ***p<0.001, ****p<0.0001 compared with positive control group $^{\#}$P <0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001 compared with Normal group.

Evaluation of Antioxidant Parameters:

Malondialdehyde (MDA): There was increase in the lipid peroxidation in doxorubicin group which results in significant (p<0.0001) increase in Malondialdehyde levels (MDA). Treatment with fulvic acid 300 mg/kg, 200 mg/kg, 100 mg/kg significantly reduced (p<0.0001) this enhanced level of MDA.

Glutathion Reductase: Table 4 There was a significant decrease (P<0.0001) in the glutathione levels in doxorubicin treated control group when compared to normal group. However treatment with fulvic acid 300 mg/kg, 200 mg/kg, 100 mg/kg significantly (****p<0.0001, **P<0.01, **P<0.01 respectively) enhance these levels.

Superoxide Dismutase: There was significant hike level of superoxide dismutase due to doxorubicin treatment. The non significant increase in SOD levels were found in groups treated with 100 mg/kg and 200 mg/kg, while the group treated with fulvic acid 300 mg/kg has shown significant increase (p<0.001) in level of SOD as compared to control.

Catalase: The control group shows significant decrease in level of catalase due to doxorubicin treatment. However treatment with fulvic acid 300mg/kg significantly enhances reduced level of enzyme while groups treated with 200 mg/kg and 100 mg/kg showed no significant increase in level of catalase.

TABLE 4: EFFECT OF FULVIC ACID ON TISSUE MALONDIALDEHYDE, GLUTATIONE REDUCTASE, CATALASE, SUPEROXIDE DISMUTASE

Groups	Malondialdehyde (µMol MDA/g of tissue)	Glutatione (nMol/g of tissue)	Catalase (units /mg of protein)	Superoxide dismutase (units /mg of protein)
Normal	30.3 ± 1.22	36.5 ± 0.987	34.9 ± 4.08	245 ± 14.2
Control	88.6 ± 4.74$^{####}$	17.2 ± 1.62 ####	9.37 ± 1.2####	170 ± 9.83###
Fulvic acid 100 mg/kg	52.9 ± 2.85****	23.3 ± 1.65 **	14 ± 2ns	173 ± 4.81ns
Fulvic acid 200 mg/kg	42.3 ± 3.86****	28.4 ± 0.846**	21.2 ± 1.87ns	191 ± 6.46 ns
Fulvic acid 300 mg/kg	41 ± 1.45****	28.7 ± 1.58**	29.9 ± 5.38***	237 ± 11.9***

Data are expressed as mean ± SEM (n=6) and analyzed by one way ANNOVA followed by Sidak's multiple comparision test, *P <0.05, **P<0.01, ***p<0.001, ****p<0.0001 compared with positive control group, $^{\#}$ P <0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001compared with normal group

DISCUSSION: The present investigation reveals the effect of fulvic acid in doxorubicin induced cardiac stress. Fulvic acid is natural products derived from the humidification process of plant materials. The fulvic used in current investigation is a peat derived which was reported and proposed to have variety of medical applications. Fulvic acid is called as miracle molecule in different literatures, it was considered as one of missing link of life [19].

A article by Prof. Dr. Renate Klocking and *et al.*, summaries the different medical application of fulvic acid where they mentioned about its antiviral, bone generating capabilities [20]. Furthermore its *in-vitro* antioxidant activity has been also reported [9].

Doxorubicin has reported as a drug causing cardiotoxicity. The cardiomyocyte damage and oxidative stress has been confirmed by many investigators. The control group was given doxorubicin within 2 weeks in six divided doses as 2.5 mg/kg i.p. The treatment with doxorubicin resulted in various physiological, biochemical and histological alterations.

Reduction of body weight, associated with multiple, long administration of DOX in experimental animals, are considered multifactorial. Doxorubicin induced cardiotoxicity and it has been shown that these animals have scruffy fur as well as significantly decreased body weight, heart weight, heart/body weight ratio.

It may be attributed to reduced food intake, DOX also caused a significant decrease in heart weight and heart/body weight ratio, which may be due to the loss of myofibrils and myocardial necrosis [21]. The cardiac markers levels were investigated which were used in clinical practice for diagnosis of cardiac damage.

The doxorubicin induced cardiomyocyte damage was manifested by elevated levels SGOT, LDH, CK-MB, which reflects cardiac damage due to doxorubicin. Which was consistent with earlier findings made by. V. S. Warpe *et al*., [22]. The treatment with fulvic acid 100 mg/kg, and 200 mg and 300 mg/kg significantly prevented increase in levels of enzymes. Which indicate possible ability of fulvic acid to reduce leakage of enzyme from cardiomyocyte. The highest restoration achieved by group treated with fulvic acid 300 mg/kg.

Doxorubicin is a know anticancer drug which produces cardiotoxicity. Doxorubicin operates on several levels by different molecular mechanisms including an interaction with iron, upsetting calcium homeostasis, altering the activity of intracellular or intra-mitochondrial oxidant enzymes, and binding to topoisomerases promoting their dysfunction. The major pathway of cell toxicity is doxorubicin induced increases in intracellular radial oxygen species. The early-onset cardiomyopathy seen after treatment with doxorubicin is thought in part to be induced by oxidative stress and mitochondrial dysfunction in cardiomyocytes [1].

With this orientation it was considered that oxidative stress is basis in DOX related cardiotoxicity, and therefore also investigated the level of different antioxidant markers like MDA content (to measure extend of lipid peroxidation), SOD, CAT and GSH levels from heart tissue homogenate. **Table 4** showed that control group cause significant in increase level of MDA while decrease in SOD, CAT, GSH which is in correlation with previous studies reported by Chakraborty Manodeep and many others [23] .

These finding suggest that DOX is responsible for produce increase oxidative stress and cause cell damage. Data presented in **Table 4** clearly indicate improvement in 'endogenous antioxidant reserve' as fulvic acid treatment increased SOD, CAT, GSH activity while reduced MDA content. The activities are highest at dose 300 mg/kg which indicate that that fulvic acid is capable to prevent free radical mediated damage to cardiac myocytes being a antioxidants.

ECG alterations were monitored in present study as it is one of the important part of investigation. The changes in ECG are parallel to knows clinical cardiotoxicity. We observe elevation in ST, prolongation in QT interval and QRS complex in contrast to normal animals. The similar alterations were also reported by Naiya *et al*.,[24]. The observed changes reflecting defects in ventricular depolarization and repolarisation.

The fulvic acid treatment with 300 mg/kg restored these ECG alteration to significant level which we can observe in **Fig. 2E**, the calculated value are also much closer to normal, while FA 200 mg/kg and FA 100 mg/kg are not totally prevent this alterations (**Fig. 2C**, **D**). The BP changes are also documented; Doxorubicin treatment in control group produces significant decrease in systolic BP and heart rate and non-significant decrease in Diastolic BP. The decrease in heart rate, systolic and diastolic BP may be due to disturbances in calcium homeostasis which reduced excitability of pacemakers and other cells of cardiac conducting system.

It has been documented that these ECG changes are associated with the prolongation of action potential duration and DOX could strongly affect the recovery phase of the transmembrane action potential, influencing preferentially Ca^{2+} movements across the cellular membrane. In addition, it has been reported that DOX alters calcium homeostasis in fact, several previous studies demonstrated the role of Ca^{2+} disturbances in DOX-induced cardiotoxicity *in-vivo* and *in-vitro* [25].

Histological Findings: The histology of heart of normal animal showed absence of infiltration of inflammatory cells, and myocytolysis, the control animals due to doxorubicin showed cellular infiltration of inflammatory change as well as necrosis in most of regions and at certain level we also observe hyaline change, hyperemia in control slide.

The group treated with 100 mg/kg and 200 mg/kg showed less occurrence inflammatory changes and necrosis when observed visually. The fulvic acid 300 mg/kg has reduced the myocytolysis and inflammatory cell infiltration, at most side normal cellular structure is preserve even though exposed to cardiotoxic agent doxorubicin.

Histological observation (**Fig. 3B**) of control group showed infiltration of inflammatory cells, myo-cytolysis (M), and congestion of blood vessels, this findings are in accordance with the finding reported by Vikas Warpe *et al*., [25]. Fulvic acid 300 mg/kg (**Fig. 3E**) treatment decreases mocytolysis and overall reduction in inflammatory cell infiltration. Which suggest its cardioprotective potential because of which it has reduced cellular necrosis and protect myocardium from damage due to doxorubicin. Mechanism of this cardioprotective action of fulvic acid is possibly involve preservation of antioxidants glutathione, superoxide dismutase as well as catalase. The inhibition in oxidative stress reduces lipid peroxidation and leakage of various cardiac markers from cardiomyocytes. The prevention of cardiac cell damage also restored functional and histological alteration occurred due to doxorubicin.

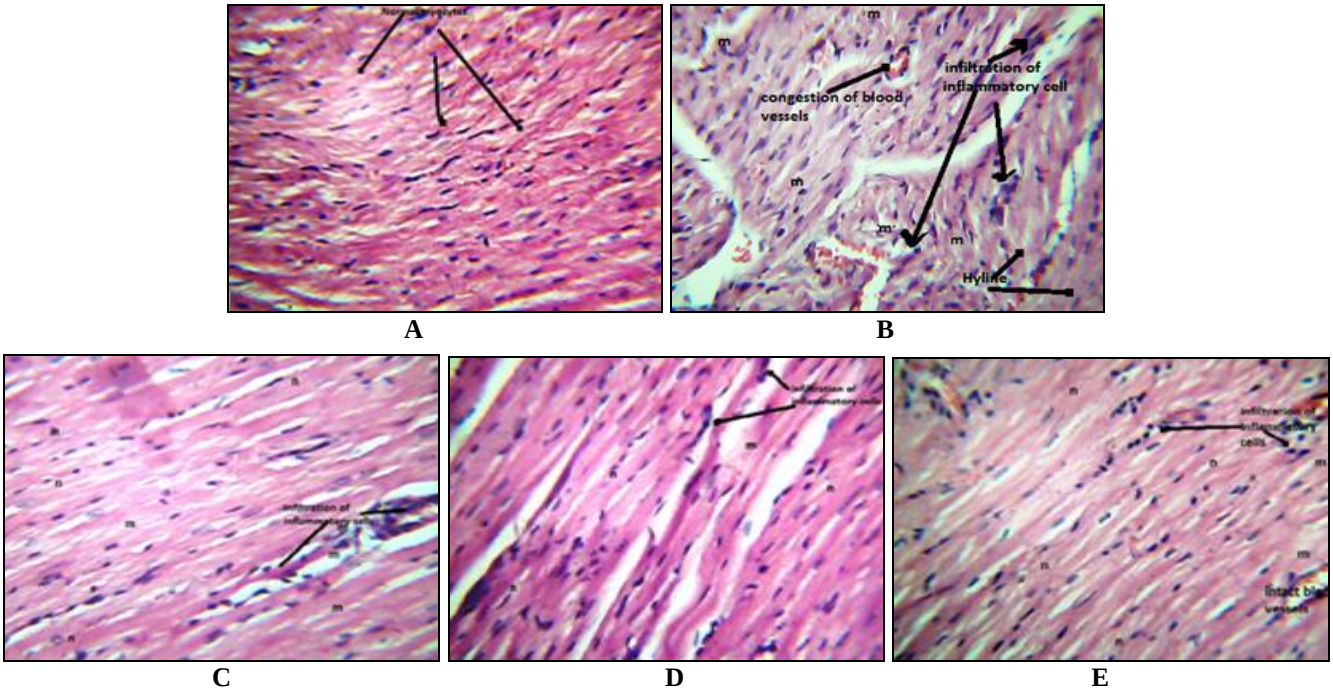

FIG. 3: ILLUSTRATE THE HISTOLOGICAL FINDINGS IN NORMAL, CONTROL, AND TREATED GROUPS

A light microscopic photograph of cardiac muscle tissue stained with H and E, Original magnification of 100X. (A) (n) shows normal cardiac muscle structure, (B) shows control cardiac muscle structure, (m) myocytosis, hyaline change, necrosis, (C) shows cardiac muscle structure in group treated with fulvic acid 100 mg/kg, (D) shows cardiac muscle structure in group treated with 200 mg/kg, (E) shows cardiac muscle structure in group treated with 300 mg/kg.

CONCLUSION: The outcome of study summaries that fulvic acid at dose 300 mg/kg, have restored pathological alteration which cause elevation in cardiac biomarkers (SGOT, LDH, CK-MB), reduction in antioxidant status of tissue (Glutathione, SOD and CAT activity) electro-cardiographic changes (ST elevation, QRS interval, QT interval), BP, heart rate and histopathology modification caused by doxorubicin.

These findings of present study reflect cardioprotective effect of fulvic acid in doxorubicin induced cardiotoxicity.

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